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Author for correspondence:
Thamyrys Bezerra de Souza,
Email: biotbs@gmail.com

Egg development and viability in three species of *Cyclocephala* (Coleoptera: Scarabaeidae: Dynastinae)

Thamyrys Bezerra de Souza[1], Larissa Simões Corrêa de Albuquerque[2], Luciana Iannuzzi[3], Fábio Correia Costa[2], Marc Gibernau[4] and Artur Campos Dália Maia[2,5]

[1]National Institute of the Atlantic Forest (INMA), 29650-000 Santa Teresa, Brazil; [2]Graduate Program in Animal Biology, Federal University of Pernambuco, 50670-420 Recife, Brazil; [3]Department of Zoology, Federal University of Pernambuco, Recife 50670-420, Brazil; [4]Laboratory of Sciences for the Environment, University of Corsica, UMR 6134 SPE, Ajaccio, France and [5]Graduate Program in Biological Sciences, Federal University of Paraíba, 58051-900 João Pessoa, Brazil

Abstract

Different species of *Cyclocephala* scarab beetles (Scarabaeidae, Dynastinae) perform key functional roles in both natural and agricultural systems, such as the cycling of organic matter and pollination, while also being known as destructive pests both as immatures and adults. Therefore, the identification of biological parameters is crucial for defining strategies for their conservation and efficient pest management. In a forest fragment within the Brazilian Atlantic Forest biodiversity hotspot, we field-captured adult individuals of *Cyclocephala cearae*, *C. celata*, and *C. paraguayensis* then reared and bred them under controlled temperature and humidity conditions. On a daily basis, we individually weighted eggs of all three species, from oviposition until hatching, and monitored egg development parameters (i.e., incubation duration, viability, and egg weight increase). Our findings provide novel empirical evidence showing (i) a positive correlation between egg weight and incubation duration, (ii) idiosyncratic characteristics on egg development, and (iii) a negative (involuntary) effect of manipulation on egg development and viability. Thus, the successful breeding and rearing of *Cyclocephala* spp. is correlated with egg integrity and the targeted species. Our analyses present a quantitative understanding of the egg phase and can assist in refining strategies for ovicidal activity and pest management of *Cyclocephala* spp. in agriculture systems. Moreover, they can provide a basis for new studies related to captivity breeding, pollinator management, and developmental biology for biodiversity conservation.

Introduction

Cyclocephaline scarabs (Scarabaeidae, Dynastinae, Cyclocephalini) are a diverse group of medium-sized beetles (ca. 10–35 mm) predominantly distributed in South and Central Americas (Endrödi, 1985). With over 350 species, many of which yet undescribed (Moore *et al.*, 2018), *Cyclocephala* encompasses more than 85% of the tribe's diversity and is one of the most speciose genera among all of the Scarabaeoidea (Lawrence *et al.*, 1999; Moore *et al.*, 2018).

Many species of *Cyclocephala* (i.e., masked chafers) have long gained attention for being lawn, reforestation, and agricultural pests, mainly during the larval stage (i.e., white grubs). Larvae of several species are associated with different cultures, such as blue agave (*Agave tequilana* F.A.C. Weber, Agavaceae; Garcia *et al.*, 2009), maize (*Zea mays* L., Poaceae; Gassen, 1993), Welsh onion (*Allium fistulosum* L., Alliaceae; Villegas *et al.*, 2008), soy (*Glycine max* (L.) Merr., Fabaceae; Santos and Ávila, 2007), sugarcane (*Saccharum officinarum* L., Poaceae; Cherry, 1985), among others (Ritcher, 1966; Salvadori *et al.*, 2004; Pardo-Locarno *et al.*, 2005; Diez-Rodríguez *et al.*, 2015). Different countries in the Americas (e.g., USA, Brazil, Mexico, Colombia) experience moderate to severe difficulties related to damage promoted by white grubs (Ritcher, 1966; Santos and Ávila, 2007; Villegas *et al.*, 2008; Garcia *et al.*, 2009).

On the other hand, adult *Cyclocephala* scarabs are often associated with flowers (anthophilous), although their role as specialized pollinators has been historically overlooked (Buchmann and Nabham, 1996). Schatz (1990) predicted that at least 900 Neotropical night-blooming angiosperm species belonging to nine extant families would rely on these insects for their reproductive success, a testimony of their relevance in natural ecosystems. Moreover, some commercially explored crops (e.g., soursop, *Annona muricata* L.) are often so dependent on these specialized pollinators that their decline leads to considerable fruit yield losses

(Cavalcante, 2000; Paulino-Neto and Oliveira, 2006). Several factors such as scarce or excessive rainfall, low food availability, unfavorable conditions for oviposition, and subsequent development of the soil-dwelling larvae may negatively affect the abundance of *Cyclocephala* species (Gonçalves *et al.*, 2020).

Despite recent advances in studies demonstrating the importance of *Cyclocephala* spp. to agriculture and natural systems, the knowledge about the biological parameters of these insects is surprisingly limited. Published data on the life cycle of *Cyclocephala* is restricted to less than 3% of the currently described species (Gavotto, 1964; Potter, 1983; Cherry, 1985; Mondino *et al.*, 1997; Morón, 1997, 2004; Santos and Ávila, 2007; Rodrigues *et al.*, 2010; Stechauner-Rohringer and Pardo-Locarno, 2010; Coutinho *et al.*, 2011; Nogueira *et al.*, 2013; Sisne Luis *et al.*, 2013; Albuquerque *et al.*, 2014; Souza *et al.*, 2014, 2015; Saldanha *et al.*, 2020). Some of these authors provide a few insights into egg development in species of *Cyclocephala*, which include the observation of significant weight gain from oviposition until hatching (Gavotto, 1964; Cherry, 1985; Souza *et al.*, 2014). Potter (1998) reports that the eggs of several soil pest species of Scarabaeiodea engorge up to threefold in weight (and volume) during embryogenesis.

This phenomenon is attributed to water absorption, which is quite common among species that lay their eggs in moist or aquatic environments. Structures in the serosal layer of the eggshell, called hydropyles, promote active uptake of liquid water when the embryo is undergoing rapid growth (Hinton, 1981). Another aspect of egg development in *Cyclocephala* spp. is their susceptibility to desiccation and shell rupture during the earliest stages (Potter and Gordon, 1984), which would render them sensitive to experimental manipulation. According to the aforementioned authors, the ability of eggs to survive periods of heat and water stress depends upon egg developmental stage, stress duration, and temperature. Nevertheless, a thorough and comparative assessment of egg development parameters in *Cyclocephala* spp. is still lacking and long overdue.

A more comprehensive understanding of egg development within the genus *Cyclocephala* can be applied to (i) refine strategies directed to ovicidal activity and pest management (Canela *et al.*, 2000; Abou-Taleb, 2010); (ii) improve pollinator management plans (Allen-Wardell *et al.*, 1998; Peña *et al.*, 2002); (iii) elucidate characters for systematic and phylogenetic studies (Hinton, 1981; Hansen, 2000); and (iv) improve current knowledge on population biology (Fox and Czesak, 2000) and life-history traits within the genus (Fox *et al.*, 1997; McIntyre and Gooding, 2000). We tackled this topic by monitoring key parameters of egg development (e.g., incubation duration, viability, and egg weight increase) in three anthophilous species of the genus which are naturally found in forest remnants of the northeastern coastal Atlantic Forest of Brazil: *Cyclocephala cearae* Höhne, 1923, *C. celata* Dechambre, 1980, and *C. paraguayensis* Arrow, 1913.

Both *C. cearae* and *C. celata* are implicated with the pollination of night-blooming Araceae (Maia and Schlidwein, 2006; Maia *et al.*, 2010, 2013). Additionally, *C. celata* are among the main pollinators of soursop orchards in northeastern Brazil (Parizotto and Grossi, 2019). Adult *C. paraguayensis*, on the other hand, are florivores associated with numerous plant hosts in the Amaryllidaceae, Annonaceae, Apocynaceae, Cactaceae, Calophyllaceae, and Solanaceae (Moore and Jameson, 2013; Favaris *et al.*, 2020; ACD Maia, pers. comm.). Among the three investigated species, *C. paraguayensis* exhibits the broadest distribution range and is found from Honduras across most of the extension of non-Andean South America, from Colombia until Uruguay (Endrödi, 1985). It has also been recently documented as the most ubiquitous species of *Cyclocephala* recovered in light traps the majority of Brazilian biomes (Gonçalves *et al.*, 2020), where its larvae are likely associated with different monocultures (e.g., sugarcane, maize, soy; PC Grossi, pers. comm.).

Focusing on the three aforementioned *Cyclocephala* species, we asked the following questions: (i) What is the egg weight increase during the incubation phase? (ii) How does egg development vary among species? and (iii) Does experimental manipulation in the laboratory negatively influence egg development? And to which extent? We expect to find a positive correlation between egg weight increase and incubation duration due to the water absorption capacity of the eggs (Potter, 1983). We also predict idiosyncratic responses among species, since studies suggest that the development stages of embryogenesis have specific durations (Campos-Ortega and Hartenstein, 1985; French, 1988). We also anticipate a negative effect of experimental manipulation (in the laboratory) on all tested parameters of egg development.

Methods

Insect sampling

Male and female adults of three species of *Cyclocephala* were collected in their natural habitat: *Cyclocephala celata* Dechambre, 1980; *C. cearae* Höhne, 1923; and *C. paraguayensis* Arrow, 1913. Field expeditions were conducted in April and May 2008 (*C. celata*), June 2008 (*C. cearae*), and March 2009 (*C. paraguayensis*) to a private Atlantic Forest reserve on the grounds of the Usina São José S/A sugarcane company (USJ) in the municipality of Igarassu, Pernambuco, Northeastern Brazil (7°49′S; 35° 02′W; approx. 110 m a.s.l.), with mean annual temperature and rainfall of 25°C and ca. 2000 mm, respectively (data from 2008 to 2010; Lamepe/Itep, 2012). Although months/years of field expeditions have been different, the fieldwork date was due to the abundance of individuals in the field and during these dates the temperature and humidity ranged only 1.5°C and 10%, respectively (IMEP, 2022).

The Usina São José S/A sugarcane company (USJ) covers the municipalities of Igarassu (where beetles were collected), Abreu e Lima, Araçoiaba, Goiana, Itapissuma, and Itaquitinga (Trindade *et al.*, 2008). The Usina São José is located within of Atlantic Forest and their vegetation is classified as seasonal semideciduous forest – low lands (Veloso *et al.*, 1991; IBGE, 2012) and their fragments are embedded mainly within a mosaic of sugarcane monoculture matrix (Trindade *et al.*, 2008).

Within the private Atlantic Forest we actively searched inside inflorescences of *Philodendron acutatum* Schott, *Caladium bicolor* (Aiton) Vent., and *Taccarum ulei* Engl. & K. Krause (Araceae), known hosts of flower-visiting *C. celata* and *C. cearae* (Maia and Schlindwein, 2006; Maia *et al.*, 2010). Since most species of cyclocephaline scarabs are attracted to light sources at night (Ratcliffe and Cave, 2009; Albuquerque *et al.*, 2016), light traps were also installed during early evening hours, from 17h30 to 21h00. A 250 W mercury vapor light bulb was disposed in front of a $2.5 \times 2.0\ m^2$ sheet of white cloth, stretched along the border of wooded areas. Settling beetles are then manually recovered (Janzen, 1983). Individuals of *C. paraguayensis* are frequently sampled with this method (Albuquerque *et al.*, 2014, 2016). Beetles were identified to species through the Neotropical Scarabaeidae specialists Dr Paschoal Coelho Grossi and Dr

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Fernando Zagury Vaz-de-Mello. Voucher specimens were deposited in the Coleção Entomológica – CEUFPE, Universidade Federal de Pernambuco (UFPE), Brazil.

Captivity breeding and rearing

Collected male and female beetles were accommodated in transparent plastic containers with perforated lids ($45 \times 45 \times 30\ cm^3$) and a layer of ca. 15 cm of topsoil extracted from the collection sites. We distributed 20–50 beetles per container (1:1 gender ratio) and provided them with a diet of fresh sliced apples and plantains, substituted every 2 days.

The rearing containers were kept under permanent shade inside a greenhouse where temperatures oscillated roughly between 22 and 30°C. On a daily basis we examined the soil substrate for newly oviposited eggs, which were recovered and transferred individually to new containers. The methodology of 'manipulated' and 'unmanipulated' eggs follows Albuquerque *et al.* (2014). The oviposition substrate (soil) was kept moist daily with the aid of a hand water sprayer, thus avoiding desiccation of the eggs.

Egg weight measurement

The bioassays were started immediately after laying of the females. Eggs from *C. cearae*, *C. celata*, and *C. paraguayensis* were individually weighted on a daily basis, from oviposition until hatching (eggs were weighted up to 1 day before hatching). This procedure refers only to the 'manipulated' eggs and was carried out with a 4-digit electronic scale (AE260 DeltaRange®, Mettler-Toledo, USA) and a precision scale of 0.0001 g.

Statistical analyses

Eggs from each of the three *Cyclocephala* species were grouped according to the date of oviposition (assumed as the date of recovery from the substrate) and monitored daily to document the egg weight increase, incubation duration, and viability. We calculated egg weight increase as the ratio between final and initial egg weight – this procedure refers only to the 'manipulated' eggs –, and egg viability as the percentage ratio between the number of hatched larvae from the initial egg batches in each container. We used linear regression analyses to assess the relationship between egg weight increase and incubation duration. We preliminary assessed the distribution suitability and then model fit through residual analysis. Furthermore, to evaluate the normality of incubation durations, we applied the Shapiro–Wilk normality test for each studied species.

To evaluate if egg development parameters (i.e., initial and final egg weight, incubation duration, egg weight increase ratio, and viability) varies among species, we applied a comparative analysis using Kruskal–Wallis tests, because the data did not follow a normal distribution. When significant relationships ($P < 0.05$) were found we used a post hoc pairwise test for multiple comparisons of mean rank sums (Nemenyi test) to identify differences between species.

Finally, to assess the influence of laboratory manipulation on egg development parameters, we separated batches of eggs and compared 'manipulated' and 'unmanipulated' eggs from *C. celata* (manipulated $n = 255$; unmanipulated $n = 312$) to evaluated viability and eggs from *C. paraguayensis* (manipulated $n = 251$; unmanipulated $n = 237$) to access incubation duration. We value the least possible interference in unmanipulated eggs, therefore, destined a goal for each species. *C. cearae* did not have enough number to allow the test between manipulated and unmanipulated. For that aim, we performed a Mann–Whitney test.

We performed analyses in R software (R Core Team, 2020) using the following packages for computing: (1) Shapiro–Wilk, Kruskal–Wallis, Mann–Whitney, linear regression – stats; (2) Kruskal Nemenyi test – PMCMR; (3) figures – ggplot2.

Results

Linear regression analyses revealed strong support for the influence of time interval (days) – during incubation phase – on the gain of mass (mg) in eggs of *Cyclocephala* (fig. 1). We found a significant positive relationship between egg weight and time interval, for *C. cearae* (R^2 adjusted: 0.43; *P*-value: $<2.2 \times 10^{-16}$), *C. celata* (R^2 adjusted: 0.63; *P*-value: $<2.2 \times 10^{-16}$), and *C. paraguayensis* (R^2 adjusted: 0.12; *P*-value: 1.263×10^{-9}). The incubation durations of *C. celata* and *C. cearae* followed a normal distribution (*W*: 0.83; *P*-value: 0.06 and *W*: 0.93; *P*-value: 0.44, respectively) (fig. 2), implying that the embryonic development of this species is somewhat standardized. The highest hatching rate (median) of *C. celata* was on day 12 and *C. cearae* on day 22. On the other hand, *C. paraguayensis* did not follow a normal distribution in the incubation durations (*W*: 0.71; *P*-value: 0.001).

We found that different features of egg development are strongly species-specific (fig. 3, table 1). The initial and final egg weights were higher in *C. cearae* (Median – Mdn = 2.7 and 8.0 mg, respectively), followed by *C. celata* (1.7 and 5.0 mg) and *C. paraguayensis* (1.0 and 2.4 mg). During embryonic development, the eggs of all three studied species bloated and their shells became translucent. The eggs of *C. paraguayensis* presented a lower rate of mass gain along time (Mdn = 2.0) compared to *C. cearae* (Mdn = 2.9) and *C. celata* (Mdn = 3.1); however, no differences were observed between the latter. We also found that the period of egg incubation (days) was shorter in *C. celata* (Mdn = 12), while *C. cearae* (Mdn = 22) and *C. paraguayensis* (Mdn = 17) showed no significant differences between them. Finally, egg development viability in laboratory of *C. cearae* (38%) was higher when compared with *C. paraguayensis* eggs (14%).

Egg development was negatively affected by manipulation in the laboratory (fig. 4). We found that manipulated eggs had lower viability rate (*P*-value: 0.0003) and longer incubation durations (*P*-value: 5.063×10^{-6}) (25% and 18 days, respectively) than unmanipulated eggs (58% and 14 days).

Discussion

The monitoring of the laying activities of *Cyclocephala* species in the laboratory allowed us to access information on eggs development of three anthophilous species. We verified the gain mass over the incubation time in the three species, *C. paraguayensis* in smaller amounts, but the embryonic period was similar between species. Moreover, there is a pattern in the hatching time of *C. celata* and *C. cearae*. The handling of eggs contributed to disrupt the natural development of embryo (decreased viability and increased incubation time). Egg weight gain over time is a result of maintaining humidity in the laying chamber (Potter, 1983). Female *Cyclocephala* of the three studied species laid their eggs individually and inside an egg chambers, consisting of spherical clumps of loosely compacted soil. The construction of individualized soil chambers for the laid eggs is commonly

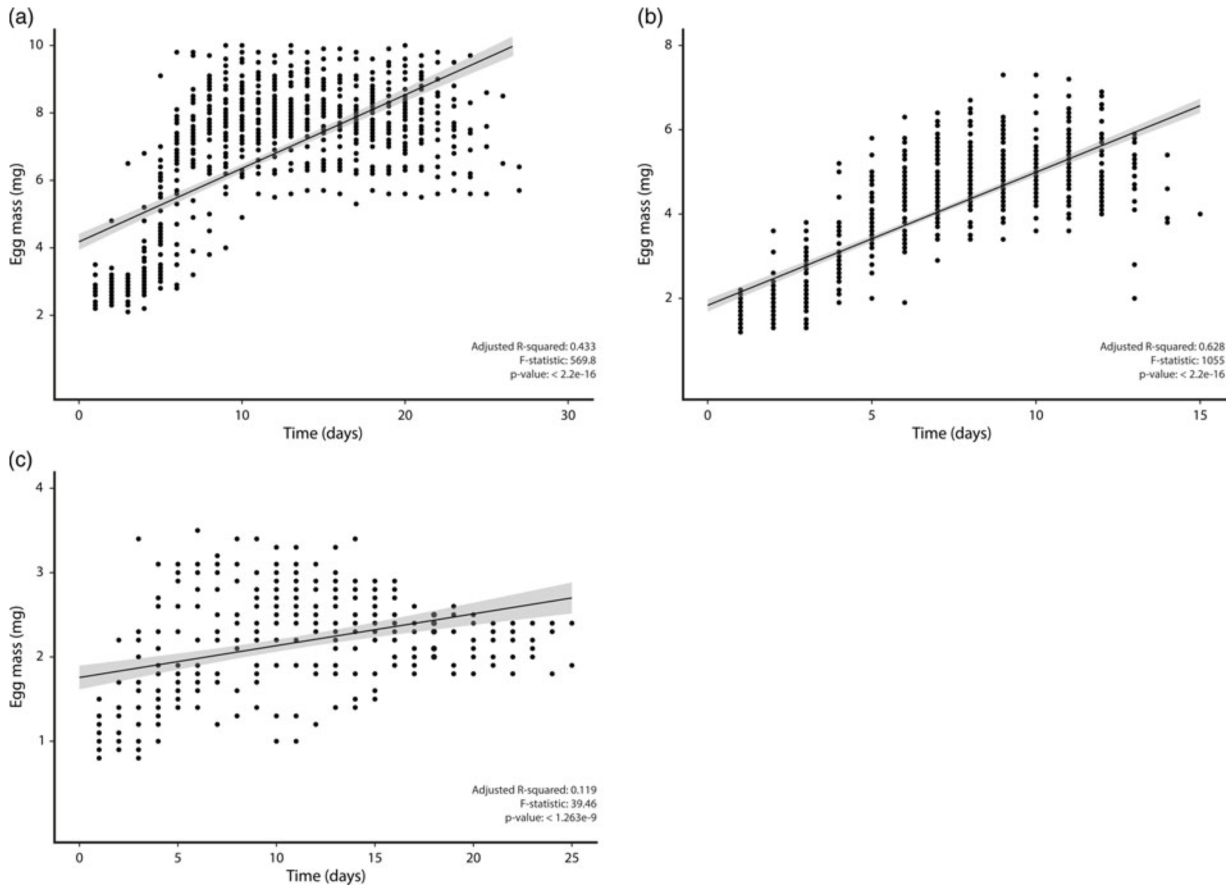

Figure 1. Influence of time interval (days) on egg weight (mg) for three *Cyclocephala* species: (a) *Cyclocephala cearae*, (b) *Cyclocephala celata*, and (c) *C. paraguayensis*. The black solid line is the linear regression of time interval vs. egg weight for all individuals measured (black circles).

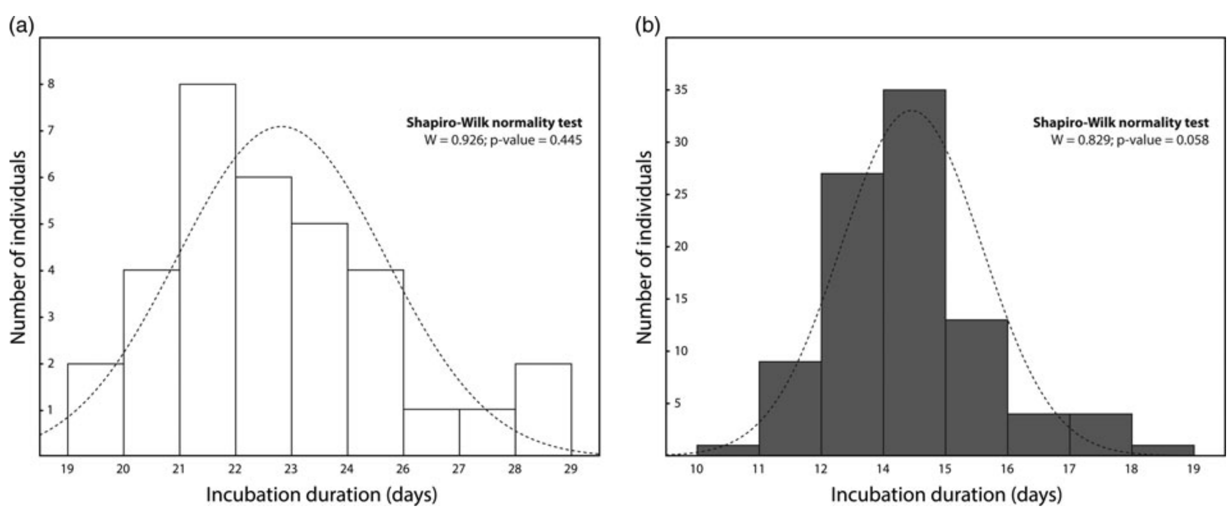

Figure 2. Histogram for the incubation duration of (a) *Cyclocephala cearae* and (b) *C. celata*. Black dotted line represents the curve of the normal function.

recorded among studies with different groups of Scarabaeoidea, including *Cyclocephala* spp. (Morelli, 1991; McMonigle, 2006; Lai and Hsin-Ping, 2008; Rodrigues *et al*., 2010; Nogueira *et al*., 2013; Souza *et al*., 2013, 2015, Albuquerque *et al*., 2014). These chambers protect the egg from desiccation and are likely built by the gravid females (Nogueira *et al*., 2013).

Previous research has demonstrated that *Cyclocephala lurida* Bland, 1863 (=*Cyclocephala immaculata*) eggs absorbed water and gained mass more intensely during the first 10 days of development (Potter, 1983). Eggs of some pest species of *Cyclocephala* enlarge significantly during embryogenesis, increasing in weight and volume up to threefold (Potter, 1998). Although there was a significant positive relationship between egg mass and time interval, for *C. cearae*, *C. celata*, and *C. paraguayensis* there was also a differential water uptake of the species with respect to time. Possibly, the differential water uptake among species may

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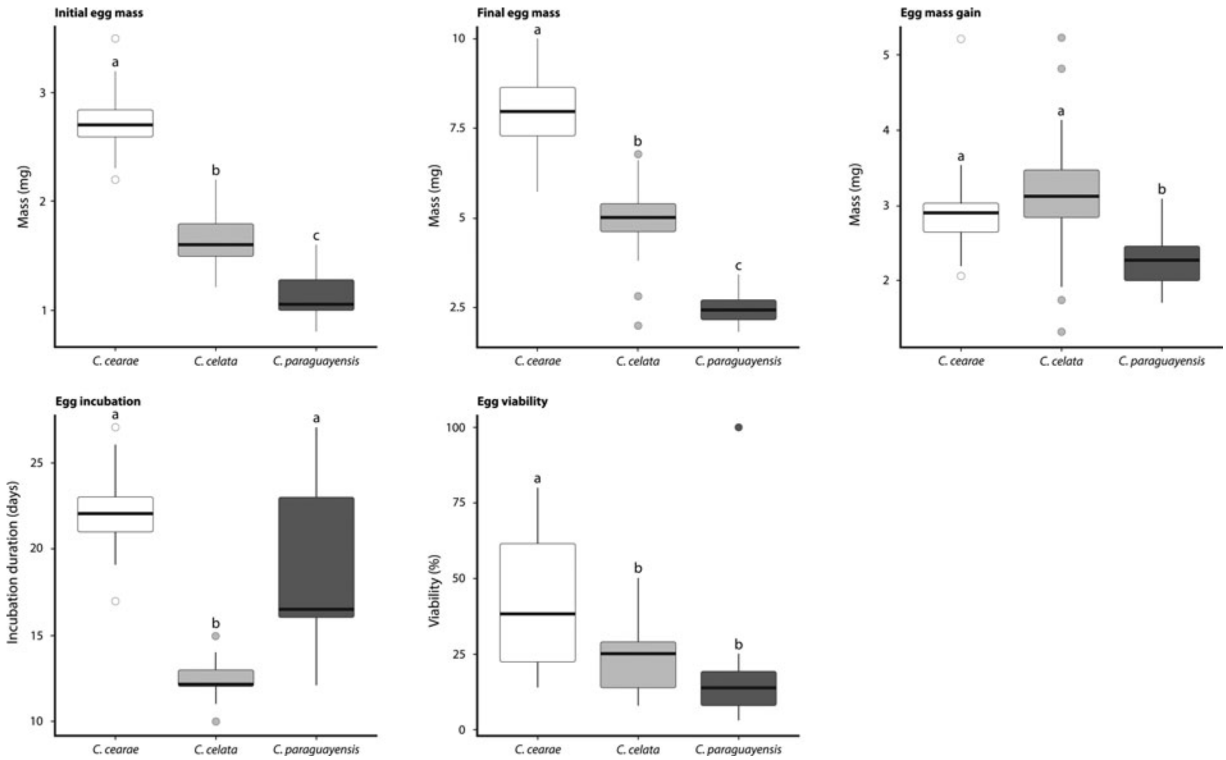

Figure 3. Boxplots of initial and final egg weights, egg weight gain, egg incubation duration, and egg viability for the three studied *Cyclocephala* species.

Table 1. *P*-value of initial and final egg weights, egg weight gain, egg incubation duration, and egg viability for the three studied *Cyclocephala* species

	P-value				
Species	Initial egg mass	Final egg mass	Egg mass gain	Egg incubation	Egg viability
C. cearae – *C. celata*	**7.1×10^{-11}**	**2.8×10^{-10}**	0.1172	**3.4×10^{-14}**	0.329
C. cearae – *C. paraguayensis*	**3.4×10^{-14}**	**3.0×10^{-14}**	**0.0036**	0.22	**0.023**
C. celata – *C. paraguayensis*	**0.0023**	**0.00023**	**2.2×10^{-6}**	**1.3×10^{-5}**	0.304

The numbers in bold represent a significant difference between species when applied to the Kruskal–Wallis test and the post hoc pairwise comparison Nemenyi test.

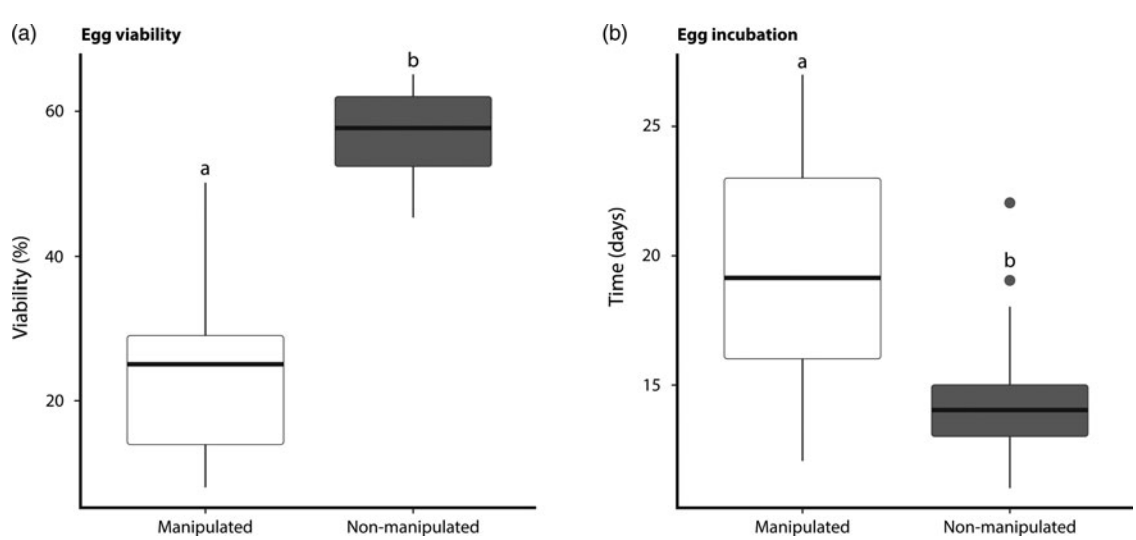

Figure 4. Boxplots showing the influence of manipulation in the laboratory on viability (%) and incubation (days) eggs of (a) *C. celata* and (b) *C. paraguayensis*. The letters represent a significant difference between individuals manipulated and unmanipulated.

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be related to variations in the chorion thickness, fatty-acid water-impermeable layer, stretching of the membranes, or splitting of the chorion (Hinton, 1981; Potter, 1983). However, this subject is still little explored in *Cyclocephala* species (Potter, 1983). Thereby, we also support that further research should focus on the structure of eggs that are related to water absorption.

We also verified that egg weight varied between three species, increasing gradually from species with smaller bodies to species with larger bodies and *C. paraguayensis* had the lowest egg weights. This species is smaller (12 mm) than both *C. celata* (16 mm) and *C. cearae* (17 mm). Previous studies suggest that embryonic development stages have a specific duration (Campos-Ortega and Hartenstein, 1985; French, 1988) and theoretical models of ontogenetic growth indicate that the metabolic rate of individual organisms may be related to their body size (van der Meer, 2006). Also, other factors may also influence egg development, such as environmental fluctuation and genetic variation within a population (Johnson *et al*., 2007).

Cyclocephala cearae and *C. celata* showed a normal distribution for the incubation duration and a pattern in the hatching period. It could be interpreted as a delicate mechanism for sequential larvae eclosion for mated females that lay all their eggs at a single oviposition event. However, *C. paraguayensis* showed to follow the rule present among Scarabaeidae females is to oviposit progressively over the course of a few days (McMonigle, 2006; Lai and Hsin-Ping, 2008).

The manipulation of eggs at an early developmental stage may have influenced their incubation and viability, as already observed in a previous study with *C. paraguayensis* (Albuquerque *et al*., 2014). Additionally, the destruction of the protective soil chamber might have disrupted normal embryonic development due to stress. We found that egg viability and incubation duration were negatively affected by manipulation, reinforcing the importance of soil chambers built by gravid females to protect their eggs (Triplehorn and Johnson, 2011; Nogueira *et al*., 2013) and the negative influence of environmental stress on egg development (Potter and Gordon, 1984). The shells of freshly oviposited eggs are thin and highly susceptible to dehydration (Potter and Gordon, 1984) and the larvae hatched from eggs subjected to stress are significantly smaller in size and sometimes unable to disengage from the chorion membrane (Potter and Gordon, 1984). Thus, it is crucial to control egg conditions for the successful breeding and rearing of *Cyclocephala* spp. This information on egg susceptibility is particularly important because it can be applied to strategies on pest management during the initial ontogenic stage of targeted species (e.g., Burgess, 2009; Ferreira *et al*., 2011) or in reinforcing egg care for pollinating and/or threatened species (e.g., Klusener *et al*., 2018; Hanberry *et al*., 2020).

Final considerations

Our research assessed the egg development of three distinct *Cyclocephala* species from the Brazilian Atlantic forest hotspot (Myers *et al*., 2000). We found a positive correlation between egg weight and incubation duration, and each species presented idiosyncratic traits in egg development. Efforts toward the understanding of *Cyclocephala* embryogenesis already seem worthwhile from a developmental biology perspective and such investigations should become a standard on life cycle descriptions, helping for conservation and management strategies in agriculture or natural systems.

Taken together, our analyses aim to provide a quantitative understanding of egg development among different species of *Cyclocephala*, under controlled laboratory conditions. Such/our results contribute to refine strategies related to ovicidal activity and pest management, since the control of initial levels of infestation is economically advantageous and desirable in insect pest management (Dent, 2000). Moreover, this type of research can provide a basis for new studies related to breeding captive, pollinator management and developmental biology for biodiversity conservation.

Author contributions. Thamyrys B. Souza: methodology, formal analysis, writing – original draft, writing – review & editing, visualization, project administration. Larissa S. C. Albuquerque: methodology, writing – original draft. Luciana Iannuzzi: methodology, writing – original draft, writing – review & editing, project administration, supervision. Fábio C. Costa: methodology, writing – original draft. Marc Gibernau: methodology, writing – review & editing. Artur C. D. Maia: conceptualization, methodology, writing – original draft, writing – review & editing, project administration, supervision.

Conflict of interest. None.

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