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Egg development and viability in the laboratory of *Cyclocephala* species (Coleoptera: Melolonthidae: Dynastinae)

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Abstract

*Cyclocephala* species perform key functional roles in both natural and agricultural systems, such as the cycling of organic matter and pollination, whereas also being known as destructive pests. As such, the identification of biological parameters is crucial for defining strategies for their conservation and efficient pest management. We daily monitored egg parameters (e.g. initial - 1st day after oviposition - and final - last day before hatching - egg masses, incubation durations, egg mass increase, and viability) of three species of *Cyclocephala* native to the Brazilian Atlantic Forest – a biodiversity hotspot. Our findings provide novel empirical evidence showing i) a positive correlation between egg mass and incubation time, ii) idiosyncratic characteristics on egg development, and iii) a negative involuntary effect of manipulation on egg development and viability. Thus, for the successful breeding and rearing of *Cyclocephala* spp., one must be aware of the importance of egg conditions according to each focal 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* in agriculture systems. Moreover, they can provide a basis for new studies related to captivity breeding, pollinator management, and developmental biology for biodiversity conservation.

**Keywords:** breeding; Cyclocephalini; life cycle; masked chafer; rearing

**Introduction**

The cyclocephaline scarabs (Melolonthidae, Dynastinae, Cyclocephalini) comprise a fairly 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 to be described (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* have long gained special attention for being agricultural pests, mainly during the larval stage (i.e. white grubs). Larvae of several species have been associated with different cultures, such as agave (*Agave tequilana* var. Azul, Agavaceae; Garcia et al., 2009), corn (*Zea mays* L., Poaceae; Gassen, 1993), onion (*Allium fistulosum* L., Alliaceae; Villegas et al., 2008), soybean (*Glycine max* (L.) Merr., Fabaceae; Santos & Ávila, 2007), sugarcane (*Saccharum officinarum* L., Poaceae; Cherry, 1958) among others (Richter, 1966; Salvadori et al., 2004; Pardo-Locarno et al., 2005; Diez-Rodríguez et al., 2015). Furthermore, different countries (e.g. USA, Brazil, Mexico, Colombia) experience difficulties with the damage caused by the white grubs (Richter, 1966; Santos & Ávila, 2007; Villegas et al., 2008; Garcia et al., 2009).
On the other hand, adult *Cyclocephala* scarabs are often anthophilous, although their role as specialized pollinators has arguably been overlooked (Buchmann & Nabham, 1996). Schatz (1990) has predicted that at least 900 Neotropical night-blooming angiosperm species belonging to nine extant families 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 the presence of these specialized pollinators that their decline is reflected in a considerable decrease in fruit yield (Cavalcante, 2000; Paulino-Neto & 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 the studies demonstrating the importance of *Cyclocephala* to agriculture and natural systems, the knowledge about the biological parameters of these insects is surprisingly limited. Studies dealing with the life cycle of *Cyclocephala* species are restricted to less than 3% of the currently described species (Gavotto, 1964; Morón, 1977, 2004; Potter, 1983; Mondino et al., 1997; Santos & Ávila, 2007; Rodrigues et al., 2010; Stechauner-Rohringer & Pardo-Locarno, 2010; Coutinho et al., 2011; Nogueira et al., 2013; Albuquerque et al., 2014; Souza et al., 2014, 2015; Saldanha et al., 2020), and there is still no information about egg development along time nor on the influence of laboratory manipulation on their viability and duration.

The comprehension of egg development in *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 understanding on population biology (Fox & Czesak, 2000) and life-history traits within the genus, since the eggs are a direct product of female activity and provide the initial resources for the offspring (Fox et al., 1997; McIntyre & Gooding, 2000).

Here, we addressed these knowledge gaps about egg development in *Cyclocephala* by daily monitoring different parameters (e.g. egg mass, incubation durations, and egg viability) of three species of the genus. We ask the following questions: i) How does time influence egg mass gain? ii) Is the incubation duration variable follow a normal distribution pattern? iii) Does egg development vary among species? and iv) What influence does density and manipulation in the laboratory exert on egg development?

We expect to find a positive correlation between egg mass and development time due to the water absorption capacity of eggs (Potter, 1983). We also predict a normal distribution pattern in egg development duration (hatching) and idiosyncratic responses, since studies suggest that the development stages of embryogenesis have a specific duration (Campos-Ortega & Hartenstein, 1985; French, 1988). We expect a negative effect of manipulations (in the laboratory) due to the highly sensible structure of eggs shell and their susceptibility to desiccation (Potter & Gordon, 1984).

Taken together, our analyses aims to provide a quantitative understanding on egg development of *Cyclocephala* in 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.
Methods

Insects 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 February-April 2008, January-May 2009 and February-April 2010 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).

The Brazilian Atlantic Forest is one of the world’s biodiversity hotspots (Myers et al., 2000), and has continuously experienced intense human disturbance and forest degradation ever since the early 1960s (Tabarelli et al., 2010; Melo et al., 2013; Filgueiras et al., 2016). Land use changes were particularly severe in large areas of previously continuous forests along the coast of the state of Pernambuco, in northeastern Brazil, which were converted to small fragments in different succession stages and 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 & Schindewein, 2006; Maia et al., 2010). Since most species of cyclocephaline scarabs are attracted to light sources at night (references), light traps were also installed during early evening hours, from 17h30 to 21h00. A 250W mercury vapor light bulb was disposed in front of a 2.5 x 2.0 m 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).

**Captivity breeding and rearing**

Collected male and female beetles were accommodated in transparent plastic containers with perforated lids (45 x 45 x 30 cm) 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 – 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 “non-manipulated” eggs follows Albuquerque et. al. (2014). The oviposition substrate (soil) was kept moist (what frequency?) with the aid of a hand water sprayer, thus avoiding desiccation of the eggs.

**Egg mass measurement**

Eggs from *C. cearae, C. celata*, and *C. paraguayensis* were individually weighted on a daily basis, from oviposition until hatching. This procedure was carried out with a 4-digit electronic scale (AE260 DeltaRange®, Mettler-Toledo, USA) and a precision scale of 0.0001g.

**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 initial and final egg masses, incubation duration, egg mass increase, and viability. We calculated egg mass increase as the ratio between final and initial egg mass, 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 mass 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 mass, incubation duration, egg mass 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.

To understand if oviposition density (number of oviposited eggs by area – 250 cm\(^3\)) influences egg development (i.e. initial and final egg mass, incubation duration, egg mass increase, and viability), we adopted a comparative analysis using ANOVA or Kruskal-Wallis tests according to the distribution of data for each species. Finally, to assess the influence of laboratory manipulation on egg development parameters (i.e. incubation duration and viability), we separated batches of eggs from *C. celata* (n = 312) and *C. paraguayensis* (n = 237) that were not handled and 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, ANOVA, linear regression – stats; (2) Kruskal Nemenyi test – PMCMR; (3) figures – ggplot2.
Results

In captivity, we observed that adult beetles of all studied species were most active during early evening hours, although neither feeding nor copulation was entirely restricted to this period. However, during most of the daytime, they preferably remained deeply buried in the soil layer, moving around over the surface only sporadically. Females of the three studied species always laid their eggs individually and enclosed them inside an egg chamber, formed by spherical clumps of soil. Freshly laid eggs of all species exhibited a milky white coloration and were oval-shaped, but each species presented idiosyncratic features in egg development.

Linear regression analyses revealed strong support for the influence of time (day) – during incubation phase – on the gain of mass (mg) in eggs of *Cyclocephala* (Fig. 1). We found a significant positive relationship between egg mass and time, for *C. cearae*, *C. celata*, and *C. paraguayensis*. According to the Shapiro-Wilk normality test, incubation durations of *C. paraguayensis* followed a normal distribution (Fig. 2), implying that the embryonic development of this species is somewhat standardized. The highest hatching rate of *C. paraguayensis* was on day 13. On the other hand, *C. cearae* and *C. celata* did not follow a normal distribution in the incubation durations (Fig. S1).

We found that different features of egg development are strongly species-specific (Fig. 3; Table S1). The initial and final egg masses were significantly different among the three studied species (F = ?, p ≤ 0.0023). The initial and final egg masses were higher in *C. cearae* (Median - Mdn = 2.7 and 8.0 mg, respectively), medium in *C. celata* (1.7 and 5.0 mg) and small in *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 (Mdn = 2.0) compared to *C. cearae* (Mdn = 2.9) and *C. celata* (Mdn = 2.4).
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 parameters (i.e., initial and final masses, incubation durations, mass increase, and viability) were not affected by oviposition density in the three species studied (Table 1). On the other hand, egg development was negatively affected by manipulation in the laboratory (Fig. 4). We found that manipulated eggs had lower viability rate and longer incubation durations (25% and 18 days, respectively) than non-manipulated eggs (58% and 14 days).

**Discussion**

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 recorded among studies with different groups of Scarabaeoidea, including *Cyclocephala* spp. (Morelli, 1991; McMonigle, 2006; Lai & 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). Moisture is a key factor during embryogenesis since it contributes to egg mass gain and development of the larva (Potter, 1983). Structures in the serosal layer of the eggshell, called *hydropyles*, promote active uptake of water when the embryo is undergoing rapid growth (Hinton, 1981).
Previous research has demonstrated that *Cyclocephala lurida* Bland, 1863 (=*Cyclocephala immaculata*) eggs absorbed water and gained mass more intensely during the first ten 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). Similar to the results obtained to *C. cearae*, *C. celata* and *C. paraguayensis*.

Although *C. paraguayensis* showed a normal distribution for the incubation duration, the lack of standards regarding the embryonic development period might indicate that this parameter is less strict for *C. cearae* and *C. celata*. It could also be interpreted as a delicate mechanism for sequential larvae eclosion for mated females that lay all their eggs at a single oviposition event. However, the rule among Melolonthidae females is to oviposit progressively over the course of a few days (McMonigle, 2006; Lai & 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 *Cyclocephala paraguayensis* (Albuquerque et al., 2014). Additionally, the destruction of the protective soil chamber might have disrupted normal embryonic development due to stress.

Egg development varies among different species of *Cyclocephala*. Previous studies suggest that embryonic development stages have a specific duration (Campos-Ortega & 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 and temperature (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).
Our results demonstrate that the investigated egg development parameters were not affected by oviposition density in any of the studied species. From natural conditions, the density of *Cyclocephala* eggs in the soil varies between 11.37 and 20.31 eggs/m$^2$, and the reduction in egg density over time is related to the hatching of the larvae (Mondino et al., 1997). From data obtained in laboratory conditions indicate that gravid females lay variable sets of eggs in one or more oviposition events (13-20 eggs per female; Gavotto, 1964; Mondino et al., 1997; Nogueira et al., 2013; Souza et al., 2015). If that observation is extrapolated to natural conditions, egg development in clusters clustered development of eggs together is common to *Cyclocephala* and this pattern may be related to abiotic factors since females prefer periods of greater rainfall to carry out oviposition and ensure soils with a higher moisture content (Mondino et al., 1997), favorable to the development of eggs (Potter, 1983).

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 & Johnson, 2011; Nogueira et al., 2013) and the negative influence of environmental stress on egg development (Potter & Gordon, 1984). The shells of freshly oviposited eggs are
thin and highly susceptible to desiccation (Potter & 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 & 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).

Conclusion

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 mass and incubation duration, and each species presented idiosyncratic traits in egg development. Efforts towards 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.

References


Figure 1. Influence of time interval (days) on egg weight (mg) for three Cyclocephala species: (a) Cyclocephala cearae, (b) Cyclocephala celata, and (c) C. para-guayensis. 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.
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. Influence of egg density (number by batch) on initial and final mass (mg), mass gain, incubation duration (days), and viability (%) for three species of *Cyclocephala*. 
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.