Aspects of the life cycle of the avian parasite *Philornis torquans* (Diptera: Muscidae) under laboratory rearing conditions

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Abstract—The life cycle of the avian parasitic flies *Philornis* Meinert (Diptera: Muscidae) is poorly known, limiting the understanding of the ecology of these flies, including interactions with their hosts. We provide data on length and survival of pupal and adult stages and the duration of the pre-oviposition period of *Philornis torquans* Nielsen. Specimens were collected at larval and pupal stages from infested broods. The pupal stage lasted on average 10.5 days and adults lived up to 100 days in the laboratory. At least 90.2% of larvae pupated and 85.7% of the latter emerged as adults. For individuals collected as larvae, pupal mortality was 3.5 times higher than for those collected as pupae. Females laid from 1–8 clutches in their lifetime and deposited, on average, 41 eggs per female (range: 1–148). Females collected as pupae were larger and had shorter pre-oviposition periods and lifespans than females collected as larvae, but there were no differences in the total eggs laid by these females. This is the first information on reproductive parameters of a subcutaneous species of *Philornis*, and forms the basis for studies on conditions required for reproduction of this species in captivity.

Introduction

Philornis Meinert (Diptera: Muscidae), is a genus of Neotropical muscids represented by about 50 species (Couri 1999). While adults are free-living, larvae are associated with nestlings of many altricial or semi-altricial bird (Aves) species (Skidmore 1985; Teixeira 1999). Larval feeding behaviour is known for 22 of the described species, most of them (82%) being obligatory subcutaneous parasites (Dudaniec and Kleindorfer 2006). Subcutaneous larvae burrow into the host integument and develop between the dermis and body musculature, resulting in the formation of individual cysts (Teixeira 1999; Spalding *et al.* 2002). There, they feed on tissue, blood, and serous fluids (Dudaniec and Kleindorfer 2006).

Parasitism by subcutaneous *Philornis* significantly reduces nestling growth (Arendt 1985a; Antoniazzi *et al.* 2011; Quiroga and Reboreda 2012) and survival (Arendt 1985b; Delannoy and Cruz 1991; Antoniazzi *et al.* 2011; Segura and Reboreda 2011). Over 200 species of birds have been documented as hosts of subcutaneous *Philornis* (Teixeira 1999; Salvador and Bodrati 2013), some of which are of conservation concern (Arendt 2000; Guedes *et al.* 2000; Dominguez *et al.* 2014; Woolaver *et al.* 2015).

Subcutaneous *Philornis* and their hosts are useful systems to study the ecology of host-parasite interactions in nature (Manzoli *et al.* 2013). Nonetheless, a limitation for its use as a model system resides in the paucity of data on their life cycles. This information is also essential to minimise the impact of these parasites on endangered species of birds. The only species in the genus with considerable available data about its life cycle, including information about reproductive biology, is *Philornis downsi*

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Dodge and Aitken (Fessl *et al.* 2006; Lincango and Causton 2008; Dudaniec *et al.* 2010; O'Connor *et al.* 2010a). This fly was accidentally introduced to the Galapagos Islands, Ecuador (Fessl *et al.* 2001) and is considered the main threat for conservation of Darwin finches (Causton *et al.* 2006, 2013; O'Connor *et al.* 2010b; Kleindorfer *et al.* 2014; Kleindorfer and Dudaniec 2016). Contrary to most *Philornis* species, *P. downsi* larvae are semi-haematophagous and live freely within nesting material (Dudaniec and Kleindorfer 2006) making comparisons with subcutaneous species tentative.

Most literature referring to subcutaneous *Philornis* occurring in southern South America is taxonomical (*i.e.*, Skidmore 1985; Couri 1999; Couri and Carvalho 2003; Patitucci *et al.* 2017) or to interactions between *Philornis* and their hosts (Nores 1995; Rabuffetti and Reboreda 2007; Quiroga and Reboreda 2012; Manzoli *et al.* 2013). The only available information on the life cycle of a subcutaneous *Philornis* is for *P. seguyi* García on duration and survival of larval, pupal, and adult stages (Quiroga and Reboreda 2013).

Unveiling aspects of the life cycle of these parasites is crucial to achieve a better understanding of the ecology of *Philornis*, which would enhance its use to study host-parasite interactions in nature and would provide crucial information where there is need to predict and minimise its impact. Here we provide data on survival of pupal and adult *P. torquans* Nielsen and on the duration of the pre-oviposition period.

Materials and methods

Collection of immature stages and development to adults

The study was conducted during the breeding seasons (September to April) of 2013–2014, 2014–2015, and 2015–2016. Third instars of *P. torquans* were collected from infested nestlings in a native forest from Santa Fe Province, Argentina (60°55'0"W, 31°23'08"S). Molecular studies have shown that *P. torquans* appears to be the only species present (Monje *et al.* 2013).

Larvae were taken to the laboratory and reared under controlled conditions (temperature: 26 ± 1 °C; humidity: $65 \pm 15\%$). Larvae were kept individually in 25 cm^3 plastic containers with pieces of tissue paper until pupation and were examined daily to record adult emergence. Individuals were weighed

three to four days before and after pupariation using a precision scale ± 0.001 g. Mortality was documented for each stage.

In addition, 71 pupae found in the nests of hosts were collected and exposed to the same conditions described above to obtain more adults. Larvae and pupae collected in the 2015–2016 breeding season were subjected to different temperature and humidity regimes and therefore are not included in the analysis of data on the pupal stage.

Adult maintenance and egg collection

Adults obtained during the three breeding seasons were sexed following Couri (1999). Pairs consisting of one female and one male coming from different host broods were placed in 250-mL plastic containers to study their reproductive biology under pre-mentioned room conditions. Adults were fed once a day with a diet consisting of water (17 mL), orange juice (45 mL), white sugar (5.7 g), and hydrolysed protein (2 g) (diet adapted from Lincango and Causton 2008). We inspected adults daily to record survival and reproductive activity. Dead individuals were preserved in 70% ethanol.

Eggs laid were removed with a moistened fine brush and placed in a 5 cm in diameter glass Petri dishes. Each Petri dish housed all the eggs laid by one female. Dates, number of eggs, and the identification of the female were recorded. Eggs were maintained under the same temperature and humidity used for adults and inspected daily looking for newly hatched larvae. Eggs that did not hatch within 12 days were considered infertile and preserved in 70% ethanol.

After their natural death, a subset of females was dissected and their spermathecae examined under a stereomicroscope (20–40×) to determine their mating status (13 females that laid eggs and 24 females that did not lay eggs). Voucher specimens were deposited in the Laboratorio de Ecología de Enfermedades, Santa Fe, Argentina and in the Museo Argentino de Ciencias Naturales "Bernardino Rivadavia", Buenos Aires, Argentina.

Developmental time, survival, and reproductive parameters

We examined the fate of each individual to determine percentage pupation for the cohort and, for pupating individuals, the duration of pupariation and pupation. Following Fraenkel and Bhaskaran (1973), the pupariation period was estimated as the

time elapsed from the retreatment of the larvae and the beginning of cocoon secretion to the darkening of the cuticle (brown colour). The pupation period was estimated as the time elapsed between the darkened puparium and the emergence of the adult. Similarly, adult lifespan was calculated as the time elapsed from emergence from the puparium until death.

We determined survival of each stage by calculating the number of survivors at the end of the stage divided by the number of individuals at the beginning of that stage. Differences in survival between male and females were tested by a non-parametric Wilcoxon rank-sum test.

Sex ratio is the ratio between the total number of females and the total number of males. We use a χ^2 test to determine if sex ratio was significantly different from expected (1:1).

The date the first egg was laid and the number of eggs and clutches laid were recorded for each female. The pre-oviposition period was estimated as the number of days elapsed from the emergence of the female and the oviposition of the first egg; the oviposition period was assessed as the number of days during which a female laid eggs. Differences in duration of pre-oviposition period and in total eggs laid were tested by a non-parametric Wilcoxon rank-sum test.

Results

During the breeding seasons of 2013–2014 and 2014–2015, a total of 194 third instars were recovered from infested nestlings. Of these, 90.2% ($n = 175$) pupated within 24–48 hours (pupariation process duration) after recovery, whereas 9.8% died. Pupae were surrounded by a cocoon (white spongy secretion) in 87% of the cases (Fig. 1). A total of 150 (85.7%) pupae successfully produced adults. Out of the 71 individuals collected as pupae from nests, 68 (95.8%) emerged as adults. The duration of pupal and adult stages and the relative survival of each stage are shown in Tables 1 and 2, respectively. Females survived significantly longer than males ($W = 22944$; $P = 0.002$; Table 1).

The mean larval and pupal weights were 0.11 ± 0.04 g ($n = 125$) and 0.05 ± 0.02 g ($n = 125$), respectively, equating to a mean weight loss during metamorphosis of 54.5%.

The 512 individuals that develop into adults comprised 259 males (50.6%) and 253 females

Fig. 1. A, Spongy secretion produced by a third instars of *Philornis torquans* before pupariation. **B**, Larva totally enclosed in a cocoon.
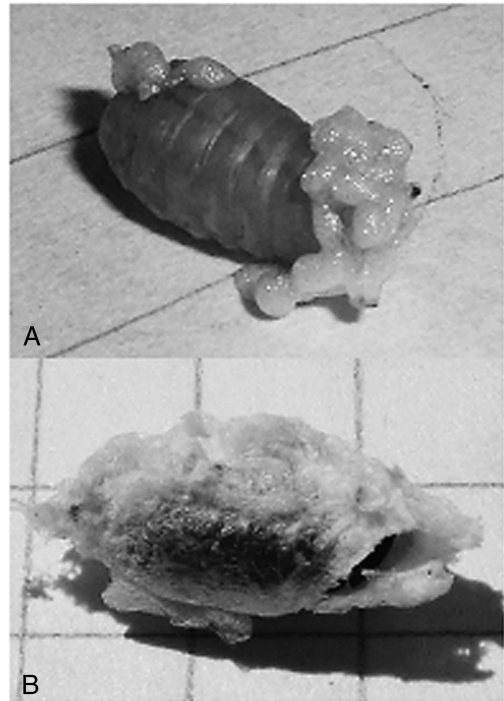
Table 1. Duration of pupal, and adult stages of *Philornis torquans*, as well as pre-oviposition and oviposition periods under laboratory rearing conditions.

Stage/period	Duration (days)	
	Range	Median/mean ± SD (*n*)
Pupa	8–15	10/10.5 ± 1.5 (150)
Adults: male	1–85	14/17.0 ± 14.5 (206*)
Adults: female	1–100	17/24.1 ± 22.4 (191*)
Pre-oviposition: females from larvae	6–100	30/35.3 ± 20.4 (29)
Pre-oviposition: females from pupae	13–19	16/16.1 ± 2.5 (12)
Oviposition	2–33	15/16.7 ± 10.0 (23[†])

* Total males and females obtained along the three breeding seasons with date of death known (date of death could not be determined for 115 adults).

[†] Females with more than one clutch.

SD, standard deviation; *n*, simple size.

(49.4%). The sex ratio was not significantly different from the expected 1:1 ratio ($\chi^2 = 0.07$; $P = 0.79$).

Eggs of *P. torquans* were obtained for the first time in captivity during the first breeding season

Table 2. Relative survival of each stage of *Philornis torquans* life cycle.

Stage	Initial number of individuals	Final number of individuals	Relative survival (%)
Larva–pupa	194	175	90.2
Pupa–adult	246*	218	88.6
Larva–adult	194	150	77.3

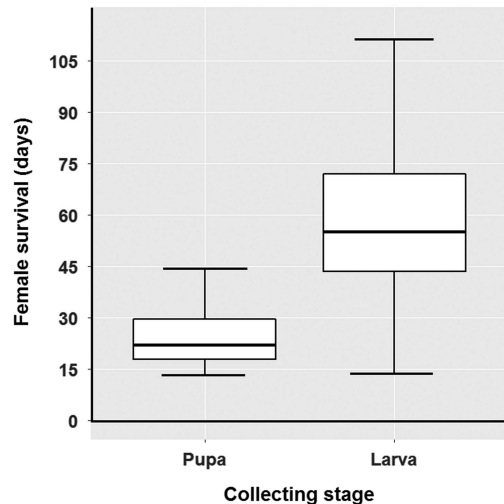*Pupae obtained from larvae and pupae collected from nest material.

Table 3. Total eggs laid by female *Philornis torquans* in relation to the final quantity of clutches.

Total clutches laid*	Eggs laid	
	Range	Median/mean ± SD (n)
1	1–53	21/23.6 ± 18.4 (17)
2	7–96	50.5/54.2 ± 32.1 (6)
3	33–69	53.5/52.3 ± 18.0 (4)
4	29–69	49.0/49.0 ± 13.2 (7)
5	48–148	77.0/88.8 ± 43.1 (5)

*As we only registered one female each laying seven and eight clutches, they were excluded from the analyses. No females laid six clutches.

SD, standard deviation; *n*, simple size.

Fig. 2. Survival time of *Philornis torquans* females obtained under laboratory rearing, by stages of collection (only females that laid eggs were considered). Females collected in pupal stage: $n = 10$; females collected in larval stage, $n = 27$.

(2013–2014). Out of 115 females that were exposed to males, 41 (35.7%) laid eggs. In addition, five females that were not exposed to males laid a total of 112 eggs and oviposited one or two clutches. Those eggs were found on food, on eppendorf tubes where food was provided, and on the walls of plastic containers. Considering all three breeding seasons together, 1843 eggs were collected. The mean number of eggs laid per female was 41.0 ± 31.1 eggs (range = 1–148). Females laid 1–8 clutches in their lifetime with one clutch being the mode, occurring in 41.5% (17 of 41) of the cases. Of the remaining females, 14.6% laid two clutches, 9.8% three clutches, and 34.2% four or more clutches (Table 3). There was a positive correlation between number of clutches and median number of eggs laid per clutch ($\rho = 1$, $P = 0.017$). When females laid more than one clutch, the mean duration of the period between first and final clutches was 16.7 ± 10.0 days (Table 1), and the mean interval between clutches was variable: 4.9 ± 3.2 days. Females began to lay eggs at least six days after emerging from the puparium. There was no significant difference in the pre-oviposition period between females that

laid only one clutch and females that laid two or more clutches ($W = 217.5$; $P = 0.73$). Nonetheless, females that were collected as pupae had a median pre-oviposition period 53% shorter than females that were collected in the larval stage (Table 1; $W = 30.5$; $P < 0.001$). There were no significant differences in the total number of eggs laid by these females ($W = 172.5$, $P = 0.98$).

At the time to oviposition, the body size of females collected as pupae was longer and wider (length: range of 7.4–9.0 mm, median 8.0 mm; width: range of 3.0–3.7 mm; median 3.3 mm; $n = 12$) that of females collected as larvae (length: range of 6.4–8.3 mm, median 7.3 mm; width: range of 2.4–3.4 mm, median 2.9 mm; $n = 29$) (length: $W = 176$; $P < 0.001$; width: $W = 176$; $P < 0.001$). Differences in the time of survival of these two groups were also detected (Fig. 2); females collected as pupae survived significantly fewer days than females collected as larvae ($W = 25$; $P < 0.001$).

Less than 1% ($n = 15$ of 1843) of eggs hatched. Two females laid these eggs and the first instars were detected four days after they were laid. Unfortunately the larvae were already dead when found.

Seven of the 37 females that were dissected contained sperm in their spermathecae. Only

two of this seven females laid eggs. A female without sperm in the spermathecae laid eggs that hatched.

Discussion

Studies on *Philornis* life cycle are crucial to increase the knowledge about these parasitic flies and how they interact with their hosts. We provide data on pupal and adult stages and reproductive parameters of *P. torquans* under laboratory rearing conditions. Also, these are the first data on the reproductive biology of any subcutaneous species of *Philornis*.

Information on *Philornis* life cycle is scarce and mostly restricted to the pupal stage. Previous studies reported duration of pupal stage ranging from 5–18 days, depending on the *Philornis* species and the environmental conditions (Skidmore 1985; Spalding *et al.* 2002; Lincango and Causton 2008). The pupal stage in the life cycle of *P. seguyi* lasted nine days (Quiroga and Reboreda 2013), similar values to what we found for *P. torquans* (10.5 days on average).

The presence of a frothy cocoon in *P. torquans* was usually observed after the larvae started pupation. This structure is considered of taxonomic importance for *Philornis* (Couri *et al.* 2007), and it has not been previously observed in *P. torquans* (see Ferrar 1980; Couri *et al.* 2009). Ferrar (1980) and Teixeira (1999) suggested that the origin of a cocoon in Muscidae might be related to protection of the pupae against predation and parasitoids. This speculation is also shared by Bennett and Whitworth (1991) for *Protocalliphora* Hough species (Diptera: Calliphoridae), which also infest bird nestlings. The pupae of some *Philornis* that form frothy cocoons (Ferrar 1980; Couri 1999; Couri *et al.* 2006) are parasitised by species of *Brachymeria* Westwood and *Conura* Spinola (Hymenoptera: Chalcididae) (Couri *et al.* 2006; Bulgarella *et al.* 2015; Delvare *et al.* 2017). Although this observation supports the hypothesis of predator protection, the origin and function of the cocoon is still unknown for *Philornis*.

Mortality in pupae reared from larvae collected in the field was almost 15%, similar to reports for *P. seguyi* (Quiroga and Reboreda 2013). The mortality of pupae collected as larvae from nestlings was 3.5 times higher than the mortality of those collected as pupae from nests. Although it is possible that larvae were physically damaged during extraction, they were manipulated with the utmost caution to avoid detrimental effects. Because larvae were removed from the host before their natural detachment, they may not have had sufficient nutritional reserves to satisfy their physiological requirements to reach adulthood. Undernourished fly larvae in other studies pupated, but died before metamorphosis (Ullyett 1950; Kamal 1958). Although Quiroga and Reboreda (2013) found pupal mortality similar to ours, they collected *P. seguyi* larvae after they had naturally detached from nestlings. Further studies considering the stage at which samples are collected and the conditions for successful development in this and other *Philornis* species are needed to complement our findings.

Recovering individuals as larvae, rather than as pupae, also affected the pre-oviposition period, body size, and longevity of adult females. Females collected as pupae had a shorter pre-oviposition period, were larger and survived for less time than females collected as larvae. Females of species phylogenetically close to *Philornis*, such as *Muscina levida* Harris (Diptera: Muscidae) and *Synthesiomyia nudiseta* Van Der Wulp (Diptera: Muscidae) (Haseyama *et al.* 2015), laid eggs one or two weeks after they emerged from the puparia (Tirone *et al.* 1996; Aruna Devi *et al.* 2011). Female *P. torquans* collected as pupae had a pre-oviposition period similar to those in related species, whereas females collected as larvae had longer and more variable pre-oviposition periods.

Pre-oviposition periods provide information on the reproductive maturity rate of females; *i.e.*, the shorter the period, the faster in reaching maturity (Ghoneim *et al.* 2016). The prolonged pre-oviposition period of smaller female *P. torquans* collected as larvae may indicate delayed reproductive maturity. Large females of *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) produce more eggs soon after emerging and tend to copulate earlier than small females (Kaspi *et al.* 2002). The difference in adult size found by Kaspi *et al.* (2002) was associated with the diet quality at larval stage. Our results reinforce the importance of larval conditions on morphology and reproductive performance in adults.

Experimental studies on trade-offs between reproduction and lifespan in *Drosophila melanogaster*

Meigen (Diptera: Drosophilidae) have found decreased early reproduction with increasing female lifespan (Luckinbill *et al*. 1984; Zwaan *et al*. 1995; Partridge *et al*. 1999). Our sample size was relatively small to analyse possible causes of differential lifespan between females collected as larvae and pupae, but it seems likely that early oviposition shortened lifespan in females collected as pupae. In addition, if there is delayed reproductive maturity (and late oviposition) in females collected as larvae this can in part explain the longer lifespan observed. However, further studies analysing gonada development of females at different ages are necessary to confirm these hypotheses.

Despite differences in life histories (subcutaneous versus semi-haematophagous), the adult lifespan observed here was similar to that of *P. downsi* (Lincango and Causton 2008). A shorter lifespan was reported for *P. seguyi* (5.46 days on average: Quiroga and Reboreda 2013), but rearing conditions (diet composition and size of rearing containers) were different from the ones used here and on *P. downsi*. Also, we detected sexual differences in adult longevity; *P. torquans* females lived longer than males, as reported for *P. downsi* (Lincango and Causton 2008). In many taxa, including species of Diptera, females live longer than males (Fletcher *et al*. 1990; Davies *et al*. 2005; Pinilla *et al*. 2013). Several processes are suggested to explain these differences in lifespan (asymmetric inheritance of sex chromosomes: Tower and Arbeitman 2009; maternal inheritance of mitochondrial DNA: Maklakov and Lummaa 2013; sex-specific selective pressures: Wolff and Gemmell 2013), which should be investigated for species of *Philornis*.

Almost half of *P. torquans* females exposed to males laid eggs. It is difficult to make direct comparisons between our results and those reported by Lincango and Causton (2008) for *P. downsi*, as they did not rear pairs individually. However, these authors did not find any relationship between the number of females per box and the total number of eggs recorded. In other parasitic flies reared under laboratory conditions (*Protocalliphora aenea* Shannon and Dobroscky (Diptera: Calliphoridae), *P. avium* Shannon and Dobroscky, *P. metallica* Townsend, *P. sialia* Shannon and Dobroscky: Bennett and Whitworth

1991; *Dermatobia hominis* Linnaeus (Diptera: Oestridae): Ribeiro *et al*. 1993), the proportion of females that failed to oviposit was high. During spermathecae examination, we found that the presence of sperm was not a good predictor of egg laying. Surprisingly, the spermathecae of the only female that produced larvae was empty. This suggests that the state of the spermathecae is not informative about mating status in our system. The causes for the failure in reproductive output have not been identified, neither the stage of the reproductive process where this failure occurs. However, this might be related to limited success in recreating the (unknown) conditions required for *Philornis* reproduction. In order to reach a better understanding of the reproductive process, studies focussing on the constraints of female and male reproductive and sexual maturity should be performed.

The number of eggs laid per female was highly variable, as was the number of clutches. Although most females laid only one clutch, they can oviposit up to eight. The number of clutches appeared to be positively correlated with the number of eggs laid per clutch. This great fecundity highlights the potential of *P. torquans* to harm bird populations, as one female could parasitise several nestlings and nests in her lifespan.

Lincango and Causton (2008) observed that female *P. downsi* began to oviposit at 12–20 days of age, but they found fully formed eggs when they dissected females six days old. This coincides with the age when *P. torquans* females began to oviposit, so the time for egg formation could be shorter in *P. torquans*.

Since less than 1% of the eggs collected hatched, our laboratory conditions probably were not sufficiently favourable for reproduction. First instars were detected four days after eggs were laid. Because they were already dead when found, we could not accurately determine the duration of the egg stage, but at least three days are necessary for eggs of *P. downsi* to hatch (Lincango and Causton 2008).

In conclusion, we showed that lifespan and survival in pupae and adults of *P. torquans* were similar to those reported for *P. seguyi* and *P. downsi*. Furthermore, we found that the stage (larvae, pupae) at which they were collected affected survival, body size and pre-oviposition period, but not the fecundity of females.

Additional studies are needed to know the optimal requirements for the reproduction of this species under laboratory conditions.

Acknowledgements

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