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Infertile egg production in the lady beetle *Eriopis connexa*

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Abstract

Oogenesis and egg development are metabolic process with a high energy cost for females. This suggests that most developing eggs should yield viable offspring. Females of *Eriopis connexa* (Germar) (Coleoptera Coccinellidae) lay infertile eggs even when continuously paired with fertile males and have access to an abundance of food. We studied the production of infertile eggs and the role of egg consumption by unrelated females in this species, based on the hypothesis that production of infertile eggs varies as a function of pesticide resistance in *E. connexa* females given the reproductive costs imposed by the resistance trait. Further, we also hypothesize that prey shortage provides a cue to produce more infertile eggs as food for newly hatched larvae. Under these conditions, egg cannibalism and egg intraguild predation should improve the reproductive performance of *E. connexa* females. Contrary to our hypothesis, the egg infertility rates of *E. connexa* were similar between susceptible- and insecticide-resistance phenotypes. The number of infertile eggs per egg batch correlated with the number of eggs laid, with percentages of infertile eggs ranging from 21.8 and 34.8%, irrespective of egg batch size. Periods of prey deprivation significantly reduced *E. connexa* fecundity but did not stimulate females to produce more infertile eggs as food for newly hatched larvae. Moreover, cannibalism and intraguild predation did not enhance reproduction. However, life-history traits were improved when larvae and adults engaged in egg cannibalism or intraguild predation if combined with a standard prey diet. In conclusion, we rejected the proposed hypotheses on the role of infertile egg production by *E. connexa*, even though egg cannibalism and intraguild predation were significant food sources for *E. connexa*.

Key words: life history, fertility, maternal behaviour, trophic eggs, food shortage.

Introduction

Egg production is an energetically costly process in many insects. Therefore, the expectation that many of the eggs within a batch would yield viable offspring would seem logical (Wheeler, 1996; Chapman, 1998). Offspring production in lady beetles (Coccinellidae) is a trait that is routinely considered when evaluating the impacts of biotic and abiotic factors on female reproductive success, which is correlated with high egg fertility (Omkar and Pathak, 2006; Ferreira *et al*., 2013; Lira *et al*., 2016). However, evolutionary outcomes can diverge from this expected result in some cases, e.g., when females use infertile eggs to nourish themselves (Santi and Maini, 2007) or their offspring (Perry and Roitberg, 2005). The observation that some lady beetles lay infertile eggs mixed with fertile ones in the same batch has been interpreted as a female's strategy to provide the infertile eggs as food for the newly hatched larvae (Polis, 1981; Perry and Roitberg, 2005; 2006). Newly hatched larvae commonly remain next to egg remnants and consume infertile and unhatched eggs (Banks, 1956; Osawa, 1989). In *Eriopis connexa* (Germar) (Coleoptera Coccinellidae), females lay infertile eggs even after single or multiple mating bouts with mature young males (Colares *et al*., 2015; supplemental material table S1).

In environments with food scarcity or intermittent food availability, gravid females may lay unfertilized rather than fertilized eggs, therefore, sacrificing some potential progeny. Under such conditions, egg predation (cannibalism) occurs in both parents and offspring (Polis, 1981; Perry and Roitberg, 2006). This behaviour provides newly emerged larvae some nourishment before they locate their first prey (Brown, 1972). The egg itself is a highly nutritious food. About 90% or more of the egg content is yolk, which is composed mostly of lipids and proteins in equal proportions (Chapman, 1998; Slogett and Lorenz, 2008). The consumption of a single egg permits the survival of a newly hatched coccinellid larvae for at least four days in *Chilomenes sexmaculatus* (F.), *Eocaria muiri* (Timberlake), and *Harmonia quadripunctata* (Pontoppidan), and for up to 6 days in *Coccinula sinensis* (Weise) (Elnagdy *et al*., 2011). Furthermore, the survival of larvae produced by adults fed a cannibalistic diet increased, even though the larvae did not engage in cannibalism (Bayoumy *et al*., 2016). Thus, cannibalism can result in energetic and nutritional benefits for cannibals. Such benefits include higher rates of development, growth, and survival, in addition to increased body size and better reproductive outputs (Polis, 1981). Individuals engaging in cannibalism have a higher probability of survival (Eickwort, 1973).

From a biological control standpoint, consumption of conspecifics (cannibalism) or heterospecific guild members (intraguild predation) would reduce the biological control value of the species (Honek and Evans, 2012; Lucas, 2012). The objective of biocontrol is to avoid or minimize economic losses caused by insects. However, predators cannot avoid all the damage, since their relationships with prey tend to evolve for the survival of both (Cruz, 2002). For predator population establishment and suppression of the pest population, both need to co-exist in certain level (Stehr, 1982). However, the indiscriminate use of insecticides can lead to prey or host shortage, and if cannibalism and intraguild predation enhance individual survival during periods of prey shortage, they could be considered as positive factors for biological control. As mentioned before, if the risk of death by starving is imminent for both the cannibal and its victim, cannibalism is potentially a net positive outcome (Eickwort, 1973). In such cases, cannibalism could benefit biological control by maintaining energy flow in the food web and supporting the predator population during periods of prey scarcity (van den Bosch and Gabriel, 1997; Ohlberger *et al*., 2012). Nutrients obtained through cannibalism can help sustain older, healthier individuals in a natural enemy population (Polis, 1981; Perry and Roitberg, 2006). Similarly, intraguild predation allows the most abundant predator species to serve as intraguild prey (intraguild prey) when the preferential extraguild prey is rare or absent in the area (Lucas and Rosenheim, 2011). It would increase the persistence of predator populations in environments characterized by large, unpredictable fluctuations of aphid densities commonly faced by aphidophagous predators, including lady beetles.

Resistance to pyrethroids entails costs, possibly due to enhanced enzymatic activity that increases the insecticide detoxification capacity of the resistant *E. connexa* phenotype. This mechanism seems to be associated with energy-use trade-offs between resistance and reproduction, mainly in support of lower female fecundity in the pyrethroid-resistant population (Ferreira *et al*., 2013). Previous studies have evaluated the reproductive performance of *E. connexa*, mainly in terms of fecundity and survival, under different environmental conditions (Ferreira *et al*., 2013; Colares *et al*., 2015; Lira *et al*., 2016; D'Ávila *et al*., 2018; Nascimento *et al*., 2021). However, there is a lack of data concerning the production of infertile eggs by *E. connexa*. Therefore, we hypothesized (1) that resistant and susceptible *E. connexa* females may differ in their rate of production of infertile eggs and the ratio of infertile eggs is correlated to clutch size. Furthermore, considering the extra nutritional gain acquired by the consumption of eggs, we hypothesized (2) that the consumption of conspecific or heterospecific eggs by *E. connexa* adults would enhance the performance of those individuals. Finally, we proposed (3) that under conditions of food scarcity, females experiencing food shortages should use this experience as a cue to lay more infertile eggs to nourish their offspring. Therefore, in this study, we evaluated the production of infertile eggs by females from two *E. connexa* populations (pyrethroid susceptible and resistant) and the impact of egg cannibalism and intraguild predation on predator development and reproductive performance.

Materials and methods

Insects

We used *E. connexa* and *Coleomegilla maculata* De Geer (Coleoptera Coccinellidae) for the intraguild predation bioassays. These species can be found in many crop ecosystems and share the same habitat (Lixa *et al*., 2010; Harterreiten-Souza *et al*., 2012; Rodrigues *et al*., 2013a). Both species lay eggs in clutches on plant foliage, placing them at risk for conspecific and heterospecific larval and adult predation. Colonies of the two lady beetle species were maintained in the Biological Control Laboratory of Rural Federal University of Pernambuco, Brazil to provide insects for experiments. For *E. connexa*, two populations were used in bioassays: pyrethroid-resistant and susceptible populations. The pyrethroid-resistant population has been maintained under insecticide pressure for approximately 100 generations in the laboratory and receives eventually resistant individuals, which had been identified through testing for insecticide susceptibility, from several locations in Brazil (Costa *et al*., 2018). The susceptible population was established from individuals collected from cotton fields in Frei Miguelinho County, Pernambuco State. It has been supplemented annually with susceptible individuals, after being tested for susceptibility, collected from cotton fields in the same area. Meanwhile, the colony of *C. maculata* was initiated with adults collected in cotton and coriander fields located at Frei Miguelinho and Chã Grande Counties, respectively, both in Pernambuco (PE), Brazil.

Colonies of *E. connexa* were reared at 25 ± 2 °C and photoperiod of 12:12 (L:D), whereas *C. maculata* was reared at 22 ± 1 °C and photoperiod of 16:8 (L:D). Larvae from both species were reared at a rate of 2-3 larvae per rearing container (80 mL) and fed *ad libitum* eggs of *Ephestia kuehniella* (Zeller) (Lepidoptera Pyralidae). Adults of both species were reared in groups of approximately 100 individuals in Plexiglas® cages (30 × 45 × 50 cm) as per Rodrigues *et al.* (2013b). Crumpled paper towels were placed inside adult rearing cages to provide oviposition sites. As food for adults, *E. kuehniella* eggs *ad libitum*, a paste of 50:50% honey: yeast, and aphids were provided. The diet was smeared over pieces of scotch tape fixed to the inner cage wall, accessible to the adults. Also, *C. maculata* adults were provided with distilled water in a moist sponge inside the cage. Larvae were reared individually in separate transparent plastic containers (80 mL) to avoid cannibalism; they were fed *E. kuehniella* eggs. For larvae, water was provided as droplets on the underside of the container lids.

In bioassays with a preferred extraguild prey (aphids), we used the turnip aphid, *Lipaphis pseudobrassicae* (Davis) (Hemiptera Aphididae), which we obtained from collard plants cultivated in the Horticulture area of the "Departamento de Agronomia" of UFRPE.

Frequency of infertile egg production by *E. connexa*

In this test, we conducted two experiments. The first experiment (Exp. #1.1) measured the frequency of infertile eggs in randomly collected egg batches produced by different females from the laboratory rearing colony of *E. connexa.* Egg batches (> 5 eggs) were randomly collected from the rearing colonies of pyrethroid-resistant (n = 50) and susceptible (n = 47) every other day for 30 days, covering the period of major oviposition activity for *E. connexa* female lifetime (Ferreira *et al*., 2013). These eggs were incubated in small Petri dishes at 25 ± 1 °C and 60-70% RH. Three days after being laid, the embryonic development of each egg was evaluated with the aid of a stereomicroscope 4-10X (Opton^{TM}, Anatomic do Brazil S.A., Cotia, SP) to determine its fertility status.

For all subsequent assays, we only used egg batches from pyrethroid-resistant females, since both phenotypes laid similar rate of egg fertility, and the natural enemy resistance trait is of more interest for biological control purposes.

In Exp. #1.2, we expected that the earlier egg batches would represent the maximum investment of females in their reproductive output following a successful mating. In this assay, we monitored the embryonic development of 2nd (n = 86) and 3rd (n = 85) egg batches with more than 5 eggs from pyrethroid-resistant females that had been isolated at adult emergence. The number of fertile and infertile eggs within each egg batch was determined following the same procedures as previously described. Since the rates of infertility from 2nd and the 3rd egg batches did not result in any statistical differences, 2nd and 3rd egg batches were pooled.

The number of eggs per egg batch, the number of infertile eggs, and the percentage of infertile eggs in the egg batch were compared between pyrethroid-resistant and susceptible populations for both egg batch experiments (#1.1 and #1.2) by the Student's t-test, using the PROC TTEST of SAS (SAS Institute, 2002). This analysis indicated a lack of population effect ($P > 0.05$) on these characteristics, subsequently the following analysis disregarded the population effect. Thus, the relationship of the number of infertile eggs produced as a function of the total number of eggs per batch was calculated using a regression analysis performed by the PROC REG of SAS (SAS Institute, 2002). The selected model yielded an equation with significant parameters ($P < 0.05$) with the greatest adjusted coefficient of correlation (r) for the egg batch experiment.

Development of *E. connexa* larvae that engaged in egg cannibalism or intraguild predation and resulting level of egg production in subsequent adults

In this experiment, we investigated the influence of egg cannibalism or intraguild predation on larval developmental and adult reproductive output of *E. connexa.* We used 3-day-old larvae in this and subsequent experiments because newly emerged larvae had higher mortality rates if isolated immediately. Until reaching 3-days old they remained grouped and fed *E. kuehniella*. Thus, larvae (aged from 3 days-old to time of pupation) were reared under each of the following diets: (1) *E. kuehniella* eggs only, as control; (2) *E. connexa* eggs only, representing the pure cannibalism treatment; (3) both *E. kuehniella* eggs and *E. connexa* eggs; (4) *C. maculata* eggs only, as the intraguild predation treatment; and (5) *E. kuehniella* eggs and *C. maculata* eggs. For all treatments, *E. connexa* eggs (cannibalism) or *C. maculata* eggs (intraguild predation), respectively, were offered *ad libitum* during a 24h period when the standard food (*E. kuehniella* eggs) was withheld.

The diets were not made available continuously, but in three feeding events set as 3, 7, and 12 days old because finding a conspecific (cannibalism) or heterospecific (intraguild predation) prey item in the field is random. To avoid cannibalism from larvae on other larvae or pupae, larvae were reared individually to the adult stage. Data collected were the time required for total development until pupation, the rate of adult emergence (%), and the adult fresh body weight at emergence (<12h old). We evaluated 30, 31, 30, 39, and 38 larvae in treatments 1 through 5, respectively.

Newly emerged adults were fed the same prey regime used during the larval stage with the first cannibalism (treatments 2 and 3) and intraguild predation (treatments 4 and 5) feeding options being presented on day 4 of adulthood. Male and female adult pairing occurred at day 5 after emergence. Matings occurred during the photophase period from 8 to 18h. After verifying the occurrence of mating, males were discarded, then females were monitored, individually, for egg production for 20 days. Cannibalism and intraguild predation feedings run on day 4 of adult stage were repeated with these females on days 12 and 24 of adulthood. Oviposition was recorded for 13, 13, 13, 15, and 15 females for treatments 1 to 5, respectively. The number of egg batches produced were counted and the number of fertile and infertile eggs per egg batch recorded.

The duration period of larval and pupal stages, the period from larval emergence to adult and fresh body weight at adult eclosion did not fit the ANOVA assumptions despite data transformation. Thus, these data were analysed with the Kruskal-Wallis test for multiple comparisons ($\alpha = 0.05$), followed by Mann-Whitney-Wilcoxon Rank Sum test for more than two means, using 'R' and the package 'agricolae' (R Core Team, 2013). Fecundity data were subjected to tests for normality (Shapiro-Wilk) and homogeneity of variance (Bartlett) and subjected to one-way ANOVA and Tukey HSD's test ($\alpha = 0.05$) for mean separation. The percentage of infertile eggs did not fit the ANOVA assumptions, despite data transformation, and were analysed with the Kruskal-Wallis test for multiple comparisons ($\alpha = 0.05$), followed by Mann-Whitney-Wilcoxon Rank Sum test for more than two groups, using 'R' and the package 'agricolae' (R Core Team, 2013). The survival of the larval and adult stages was calculated using the Kaplan–Meier method; survival means were compared with the LogRank's test using PROC LIFETEST of SAS (SAS Institute, 2002).

Egg cannibalism and intraguild predation by *E. connexa* larvae

In this experiment, we investigated the predatory behaviour of *E. connexa* larvae concerning cannibalism and intraguild predation. To better understand the benefits of predation on conspecific or heterospecific eggs, a series of three bioassays measured the effects of cannibalism or intraguild predation by *E. connexa* larvae on their development. For choice tests, 3-day-old larvae were allowed to choose among conspecific eggs (cannibalism), heterospecific eggs (intraguild prey, *C. maculata* eggs), or the turnip aphid (extraguild prey), the field prey (Nascimento *et al*., 2021). We tested *E. connexa* larvae, rather than adults, because larvae are more prone to suffer prey shortage; therefore, cannibalism and intraguild predation are likely to be more common in larval stages. Adults can move to new areas by flight to escape prey shortages.

Egg batches produced by both lady beetle species were harvested daily to provide eggs for this study. Papers bearing egg batches were cut into pieces, sealed inside plastic pots, and stored at −5 °C in a refrigerator until used. Preliminary tests showed that larval preference and consumption of eggs did not vary between frozen and fresh eggs.

Three tests (Exp. #3.1, #3.2, and #3.3), including no-choice and choice tests, assessed cannibalism and intraguild predation by *E. connexa* larvae with and without the extraguild prey, the turnip aphid. In each test, the same number of prey items of each type was used (e.g., 20 eggs of each lady beetle species, and the turnip aphid). An equal number of lady beetle eggs for each species was obtained by discarding any excess eggs in a batch or by combining several egg batches. To differentiate the species of each egg batch in the choice tests, papers bearing the eggs from one lady beetle species were identified by a red dot. Before tests, larvae were starved for 24h to equalize hunger levels. Prey and predator interactions were observed in Petri dishes (1.5 cm height × 9 cm diameter). Each dish was lined with a collard leaf disc of the same diameter cut from a fresh collard leaf. On the leaf disc, egg batches or aphids were offered as prey.

For Exp. #3.1 (the no-choice test), a single prey type was offered in each dish (i.e., only eggs of *E. connexa*, eggs of *C. maculata*, or aphids) (n = 20 prey per larvae). The aphids were obtained from infested collard leaves harvested from the field. Leaves were left to wither for 4-5 h in a white plastic tray. Under this condition, most of the aphids abandoned the leaf and started to walk away in the tray, which avoided any damage to their mouthparts and facilitated their collection and transfer to leaf discs inside Petri dishes. Third instar nymphs of the turnip aphid were transferred to the leaf discs for 1h (to allow them time to acclimate in the dish) prior to releasing *E. connexa* larvae.

In Exp. #3.2 (the choice test), three comparisons were offered together to individual *E. connexa* larvae: (1) *E. connexa* vs. *C. maculata* eggs (cannibalism vs. intraguild predation) (n = 16 larvae); *E. connexa* eggs vs. turnip aphids (cannibalism vs. extraguild prey) (n = 19 larvae); and (3) *C. maculata* eggs vs. turnip aphids (intraguild prey vs. extraguild prey) (n = 20 larvae). The arena and prey availability were set up similarly to the non-choice test with 20 prey items of each type.

In Exp. #3.3, we used a three-way choice test, in which all three prey were offered simultaneously. There were two subsets of this experiment, in which aphid density was changed, being either 20 aphids (high) or 5 aphids (low). The control for Exp. #3.3 was to offer the low aphid densities alone (no other prey) (n = 16 larvae) to measure the aphid predation rate without any influence of other foods. For each part of Exp. #3.3, we recorded the number of each prey consumed by the larva during 24h from the time of release into the dish.

For a better explanation of consumption rates of *E. connexa* larvae, we calculated egg volume for each of 20 freshly laid eggs by *E. connexa* and *C. maculata*. The egg volume (V) of both species was calculated using the formula $V = \pi LW^2/6$, where L and W represent length and width (Takakura, 2004) adapted from the ellipsoid volume formula $[V = 4/3\pi a(b^2)]$ where "a" and "b" represent radius of the length and width. To obtain these measurements, eggs (<24h) were aligned in a Petri dish, lined with filter paper, and photographed with a digital camera attached to a stereomicroscope. Images were imported into AutoCAD® software (Autodesk, 2020) and adjusted to 1:1 scale, using the SCALE command. The measure of L and W were obtained by applying the command COTA (adapted from Damasceno *et al.*, 2015).

Data on predation in the non-choice test, conducted with three prey types separately (Exp. #3.1) regarding egg cannibalism and intraguild predation, or when caged with high and low aphid density (in the experiment #3.3) were square root (x + 0.5) transformed to fit ANOVA assumptions; then analysed by one-way ANOVA followed by mean comparisons using Tukey's HSD test ($\alpha = 0.05$). Alternatively, the data were submitted to PROC TTEST for two prey under the pooled method for equality of variance. Otherwise, the data from choice tests were compared under the statistical hypothesis of equal 50:50% output between two prey items (Exp. #3.2) or 33:33:33% output across three prey items (Exp. #3.3). The comparisons were performed using the PROC FREQ of SAS (SAS Institute, 2002) and interpreted through a Chi-square test ($\alpha = 0.05$). The egg volume data were square root ($x + 0.5$) transformed to satisfy the assumptions of normality and homoscedasticity, which were verified using the PROC UNIVARIATE from SAS. In addition, differences in egg volume were determined with Student *t*-tests using PROC TTEST with the Satterthwaite method for equality of variance (SAS Institute, 2002).

The risk of food shortage and infertile egg production in *E. connexa*

If infertile eggs are produced to improve food availability for newly emerged larvae, experiencing a food shortage could lead to increased production of infertile eggs. Thus, in this experiment (#4), we tested the hypothesis that prey shortage and cannibalism play a role in a female's decision to lay infertile eggs to guarantee nourishment for newly emerged larvae. We measured the impact of food deprivation by larvae or adults on the level of production of infertile eggs by females when adults were also allowed to cannibalize their own eggs, compared to larvae and adults fed *ad libitum*. Thus, we allowed larvae to experience food shortage and cannibalism. Then, the adults were kept on the same diet and starvation regime as their larval group to determine if these environmental conditions increased infertile egg production by adult females. Larvae (3 days-old) were reared until pupation and then as adults under a dietary regime of inconstancy of food availability. The prey deprivation condition was set as 48h without any type of food followed by 24h of *ad libitum* feeding.

For this experiment, we compared four treatments including the control with larvae and adults fed continuously on *E. kuehniella* eggs (1). In treatments (2-4 below), larvae and adults were fed regimes that incorporated some degree of prey deprivation, followed by opportunities for cannibalism or intraguild predation. This form of prey deprivation was applied to three treatments in which the diet varied: (2) larvae and adults fed only the standard rearing prey *E. kuehniella* eggs, (3) larvae and adults fed only *E. connexa* eggs (i.e., cannibalism), and (4) larvae and adults that were alternately fed *E. connexa* eggs and *E. kuehniella* eggs (i.e., predation and cannibalism). All larvae were reared individually across all treatments to avoid cannibalism from larvae upon larvae or upon pupae.

Data were collected for *E. connexa* for each of the treatments and the control for the time for pupation, rate of adult emergence (%), fresh body weight at adult emergence (<12h old) for mixed sexes (adult *E. connexa* do not exhibit any sex morphological dimorphism). For each treatment, we initiated the experiment with 46 larvae, and each larva was considered as one replicate.

Newly emerged adults were reared on the same food regime (i.e., 48h without any type of food followed by 24h of *ad libitum* feeding) as in the larval stage. Adults were paired at 5 days-old. After verification of mating, males were discarded to avoid male cannibalism on eggs. Females were monitored daily for egg production and egg batches were removed daily. For each egg batch, we recorded the number of fertile and infertile eggs determined as previously described for Exp. #1. Females reared under food shortage did not produce enough eggs to enlarge their abdomens when reaching sexual maturity precluding male and female distinction like those reared with abundant food. As we often observed in this species male-male trying to "mate", at the end of this experiment we dissected those females that did not lay eggs to confirm the female gender. Ultimately, data were obtained on 17, 15, 11, and 17 females for the treatment control (*E. kuehniella* eggs *ad libitum*), prey shortage when fed *E. kuehniella* eggs, prey shortage when fed *E. connexa* eggs (i.e., cannibalism treatment), and prey shortage when alternately fed *E. connexa* eggs (cannibalism) and *E. kuehniella* eggs.

None of the parameters evaluated fitted the ANOVA assumptions despite data transformation. Thus, these data were analysed with Kruskal-Wallis's test followed by Mann-Whitney-Wilcoxon Rank Sum test for more than two means, using 'R' and the package 'agricolae' (R Core Team, 2013).

Results

Frequency of infertile egg production by *E. connexa*

There was no significant difference in the frequency of production of infertile eggs between pyrethroid resistant and susceptible *E. connexa* females (Exp. #1.1). Similarly, there was no significant difference between the rates of infertile eggs in a female's 2nd vs 3rd egg batches per lifetime (Exp. #.2) (table 1). Furthermore, the mean number of eggs and infertile eggs per batch and the proportion of infertile eggs per batch were also similar between the susceptible and resistant populations and egg batches (table 1). Thus, these data were pooled to infer the production of infertile eggs by *E. connexa* females.

The number of infertile eggs per batch is proportional to the number of eggs per batch in both Exp. #1.1 and #1.2 (figure 1A and 1B), because females maintained a similar percentage of infertile eggs laid per egg batch (figure 1A' and 1B'). On average there were 34.8 and 21.8% infertile eggs for batches randomly collected from the rearing colony and for batches produced as the 2nd or 3rd ones in a female's lifetime, irrespective of batch size.

Development of *E. connexa* larvae that engaged in egg cannibalism or intraguild predation and resulting level of egg production in subsequent adults

Prey treatment significantly affected *E. connexa* larval developmental time (H =137.67, df = 4, P < 0.0001) and adult fresh body weight at emergence (H = 101.78, df = 3, P < 0.0001) (table 2). The duration of larval development varied from 10.7 to 14.1 days, with larvae fed *C. maculata* eggs alone or combined with *E. kuehniella* eggs developing faster, whereas *E. connexa* eggs resulted in approximately 4 days of development delay, followed by the combination of *E. kuehniella* and *E. connexa* eggs with approximately 3 days of development delay. Nonetheless, faster development of larvae fed *C. maculata* eggs alone resulted in smaller adults compared to adults originating from larvae fed *E. kuehniella* and *E. connexa* eggs or larvae fed *E. kuehniella* eggs alone (table 2).

Females fed *C. maculata* eggs, *C. maculata* + *E. kuehniella* eggs or *E. connexa* + *E. kuehniella* eggs had higher fecundity than females fed *E. connexa* eggs only or *E. kuehniella* eggs only (table 2). In contrast, egg fertility and survival rates were unaltered by these treatments. Females fed *E. kuehniella* eggs had lower fecundity for the 20-day evaluation period compared to females whose diet was supplemented with *E. connexa* eggs or *C. maculata* eggs (table 2).

Table 1. Egg batch size and egg infertility rates by *E. connexa* (A) from a pyrethroid-resistant (EcRes) or a pesticide-susceptible (EcSus) population from the laboratory rearing colonies or (B) from 2nd- vs. 3rd-egg batches produced in a female's lifespan. Note: mean values with minimum and maximum values within bracts.

Egg batches	No. of egg batches analysed	Mean eggs per batch (min - max)	Mean number of infertile eggs/batch	% infertile eggs
A. Rearing colony				
EcRes	50	20.7 (8 - 36)	6.8 (1 -19)	34.6 (4.5 - 93.3)
EcSus	47	17.7 (6 - 46)	7.4 (1 - 29)	42.7 (5.8 - 93.5)
Mean		19.2 (6 - 46)	7.1 (1 - 29)	34.8 (4.5 - 93.5)
t-test $_{df = 95}$		$1.95^{0.056}$	$-0.31^{0.753}$	$-1.73^{0.086}$
B. Selected egg batches				
2nd egg batch	78	21.8 (6 - 50)	5.3 (1 - 18)	22.8 (2.8 - 69.2)
3rd egg batch	79	24.2 (6 - 57)	4.9 (1 - 19)	20.9 (2.9 - 64.7)
Mean		23.4 (6 - 57)	5.1 (1 - 19)	21.8 (2.8 - 69.8)
t-test $_{df = 155}$		$1.20^{0.239}$	$0.69^{0.496}$	$0.80^{0.423}$

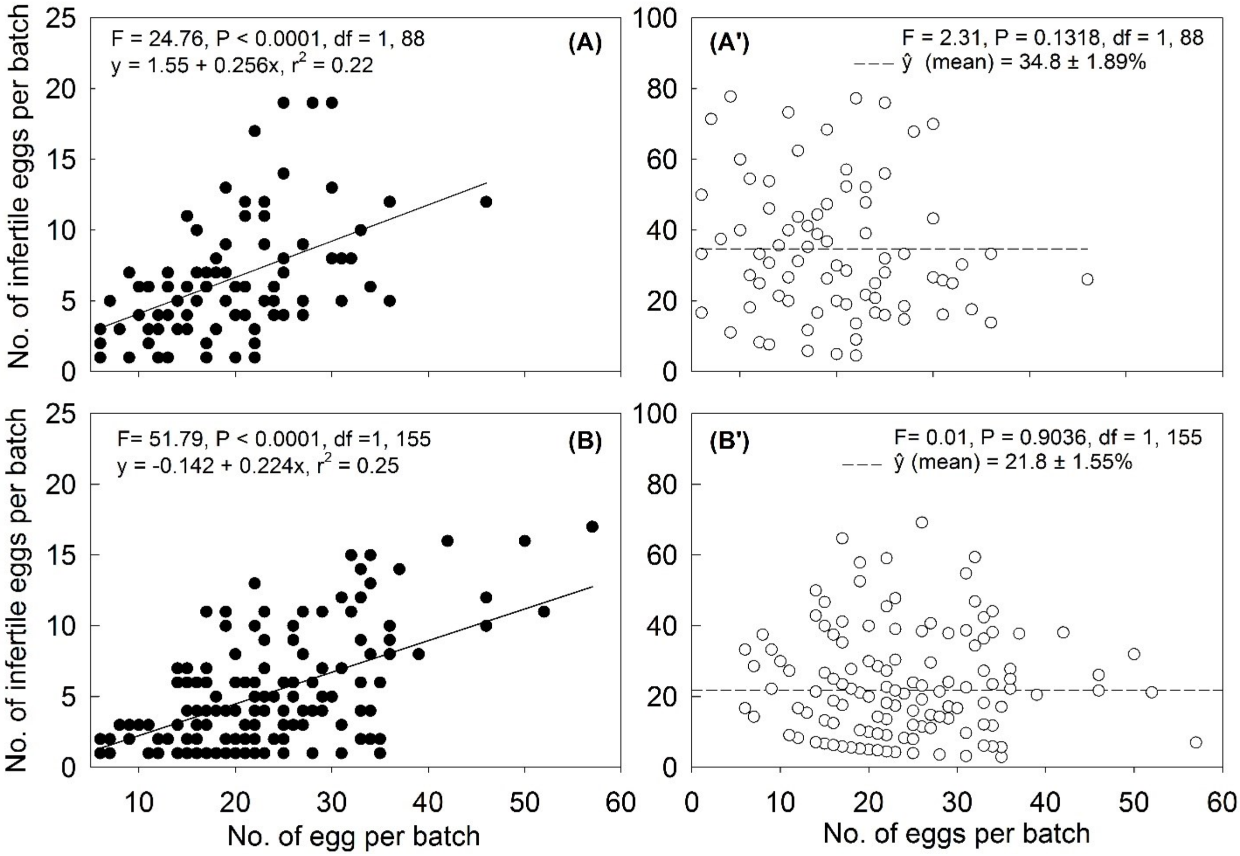

Figure 1. Number (A and B) or percentage (A' and B') of infertile eggs per egg batch for *E. connexa* in egg batches randomly collected from the rearing colony (A and A') or from the selected 2nd and 3rd produced egg batches per female (B and B').

Table 2. Life history characteristics (mean ± SE) of immature stages and adults of *E. connexa* fed the standard rearing prey *E. kuehniella* (*Ek*) eggs continuously, or *E. kuehniella* eggs plus occasional access to conspecific *E. connexa* (*Econ*), or heterospecific *C. maculata* (*Cmac*) eggs.

Characteristics[1]	Prey availability					Statistics		
	Ek eggs (n = 13)[2]	*Econ* eggs (n = 13)	*Ek* + *Econ* eggs (n = 13)	*Cmac* eggs (n = 15)	*Ek* + *Cmac* (n = 15)	*F* or *H*	*df*	*P*
Immature stages								
Duration of larval stage (days)**	12.7 ± 0.16 c	14.1 ± 0.25 a	13.3 ± 0.17 b	10.8 ± 0.09 d	10.8 ± 0.07 d	137.67	4	<0.0001
Duration of pupal stage (days)**	3.3 ± 0.10	3.5 ± 0.09	3.7 ± 0.10	3.6 ± 0.09	3.5 ± 0.08	8.38	4	0.079
Viability of larvae to adult (%)***	100	100	100	97.6 ± 2.41	97.5 ± 2.47	2.28	4	0.69
Adult fresh body weight (mg)**	11.7 ± 0.69 ab	10.9 ± 0.49 ab	12.1 ± 0.55 a	8.8 ± 0.24 c	9.6 ± 0.23 bc	22.13	4	<0.0001
Adult stage[2]								
No. of eggs/female*	171.9 ± 26.67 b	276.7 ± 32.72 b	309.1 ± 35.56 a	326.9 ± 32.04 a	327.6 ± 28.94 a	4.26	4	0.0041
Fertile eggs (%)**	67.6 ± 6.29	67.8 ± 3.81	62.1 ± 7.72	62.8 ± 7.33	54.8 ± 6.95	2.43	4	0.6585
Survival for 20 days (%)***	100	100	100	93.7 ± 6.05	100	3.37	4	0.4971

[1] Analysis by one-way ANOVA (*), or Kruskal-Wallis (**) or Log-Rank Test (***). Means within rows followed by different letters were significantly different (ANOVA: Tukey HSD's test; and Kruskal-Wallis: Wilcoxon pairwise test. α = 0.05). [2] Number of females monitored for 20 days observation period.

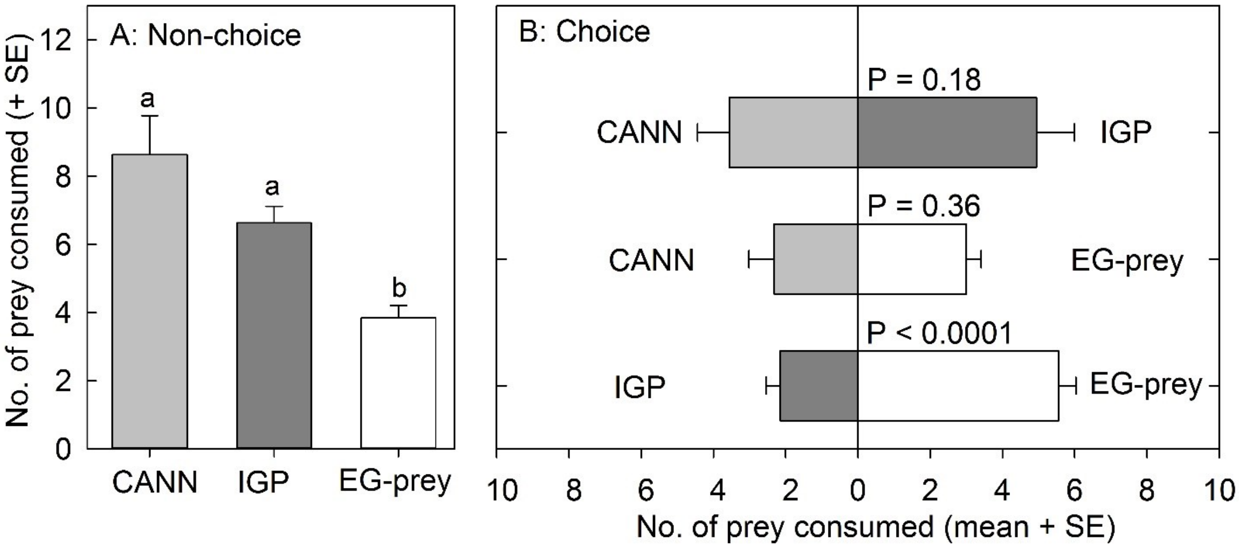

Figure 2. Predation by 3-day-old *E. connexa* larvae upon conspecific (cannibalism) eggs, heterospecific (*C. maculata*: intraguild predation) eggs, or the preferred extraguild prey (*L. pseudobrassicae*), in a non-choice test (A); or predation in a choice test comparing cannibalism vs intraguild prey (*E. connexa* vs. *C. maculata* eggs), cannibalism vs extraguild prey (*E. connexa* eggs vs. aphid) or intraguild vs extraguild prey (*C. maculata* eggs vs aphid). In (A), different letters indicate statistical differences by Tukey HSD's test ($\alpha = 0.05$), while p-values in (B) are based on Chi-square tests. (EG-prey: extraguild prey; CANN: cannibalism; IGP: intraguild predation).

Egg cannibalism and intraguild predation by *E. connexa* larvae

The number of conspecific (*E. connexa*) or heterospecific (*C. maculata*) eggs consumed by 3-day-old larvae during a 24h period under no choice conditions (Exp. #3.1) was greater than the number of turnip aphids (the preferred prey) consumed ($F_{2, 74} = 11.79$, $P < 0.0001$) (figure 2A). The average daily consumption was 8.6, 6.6, and 3.8 *E. connexa* eggs (cannibalism), *C. maculata* eggs (intraguild), or aphids (extraguild), respectively.

Under choice test conditions (Exp. #3.2), there was no difference between consumption of *E. connexa* eggs vs *C. maculata* eggs ($\chi^2 = 1.79$, $P = 0.18$), and *E. connexa* eggs vs aphids ($\chi^2 = 0.84$, $P = 0.36$) (figure 2B). However, 3-day-old larvae preferred to consume significantly more aphids than *C. maculata* eggs ($\chi^2 = 15.78$, $P < 0.0001$). Specifically, larvae consumed 2.1 *C. maculata* eggs and 5.5 aphids, in a 24h test period.

The density of aphids (Exp. #3.3) affected the number of other prey items (*E. connexa* eggs or *C. maculata* eggs) consumed (aphids at 5 vs. 20 individuals) (*t*-test = 6.71, df = 29, $P < 0.0001$) (figure 3). Increasing the number of aphids from 5 to 20 individuals resulted in nearly a 2-fold increase in aphid consumption at the higher aphid density (2.3 vs 5.7 aphids), but the percentage of aphids consumed declined, indicating that the higher extraguild prey density (ca. 20 aphids) resulted in a surplus of prey for larvae. Lower numbers of turnip aphids [which is one preferred extraguild prey (Lira *et al.*, 2019)] in this choice test resulted in a significant increase in cannibalism upon *E. connexa* eggs ($\chi^2 = 13.31$, $P = 0.0013$) (figure 3). In contrast, more aphids were consumed as more became available ($\chi^2 = 25.41$, $P < 0.0001$), with reduction of cannibalism. At low aphid density, consumption rate was 4.8 *E. connexa* eggs compared to 2.3 *C. maculata* eggs, and 1.7 aphids. However, when aphids were offered at a higher density (20 aphids), more aphids were consumed (average of 4.9 individuals) and that diet was supplemented with some others, but fewer, prey (1.5 and 1.1 *E. connexa* and *C. maculata* eggs, respectively).

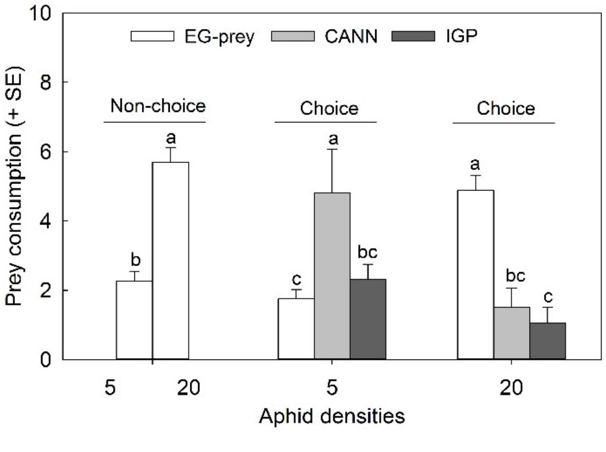

Figure 3. Predation by 3-day-old *E. connexa* larvae upon the turnip aphid, *L. pseudobrassicae* (extraguild prey) at two densities, and when simultaneous available with 20 conspecific (cannibalism, *E. connexa*) and heterospecific (intraguild predation, *C. maculata*) eggs. Bars bearing different letters indicate statistical differences by pairwise (non-choice test) or multiple pairwise Chi-square tests ($\alpha = 0.05$). (EG-prey: extraguild prey; CANN: cannibalism; IGP: intraguild predation).

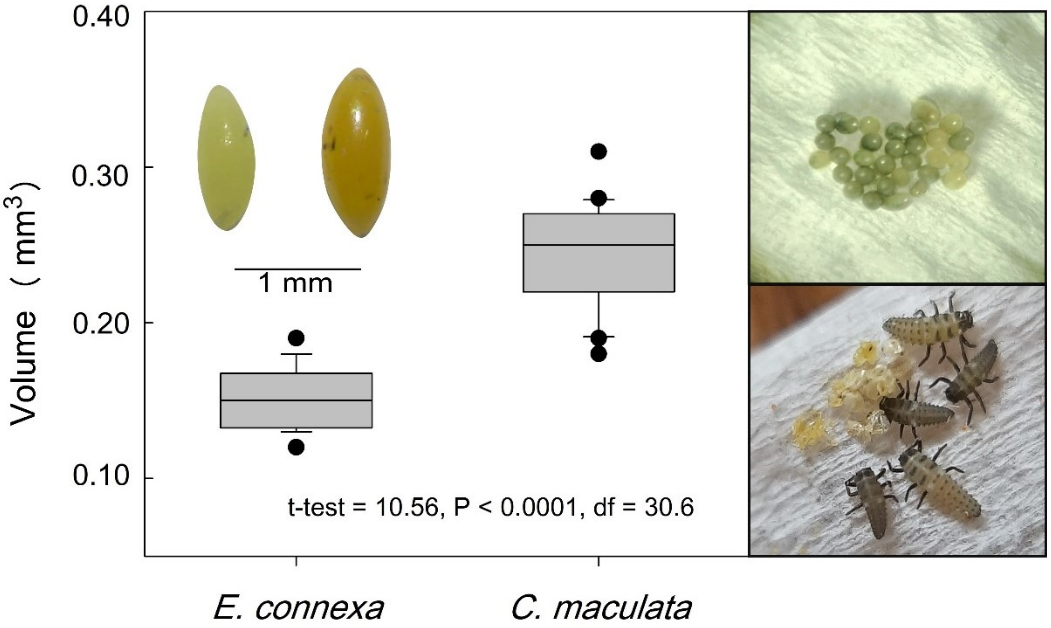

Figure 4. Estimated volume of *E. connexa* and *C. maculata* eggs (A), an *E. connexa* egg batch showing fertile (darker) and infertile (pale) eggs (B), and newly emerged larvae feeding on egg remains and infertile eggs (C).

Since prey varied in size, we also considered prey quantity as total volume consumed. The estimated volume of the lady beetle species indicated that *C. maculata* eggs were larger (t = 10.56, df = 30.6, P < 0.0001) than that of *E. connexa* eggs. The average volume of *C. maculata* eggs was 0.243 (± 0.007) mm^3 while the volume of *E. connexa* eggs was 0.152 (± 0.004) mm^3 (figure 4). Based on volume, a single *C. maculata* egg represents 37.4% more prey content than that of an *E. connexa* egg. Nonetheless, *E. connexa* larvae did not exhibit any difference between cannibalism and intraguild prey consumption in choice (χ^2 = 1.79, P = 0.1808) and non-choice tests (t = 1.33, df = 30, P = 0.1937).

The risk of food shortage and infertile egg production in *E. connexa*

Larva and adult evaluated characteristics of *E. connexa* were affected when subjected to 48h prey deprivation followed by 24h prey availability (table 3). Larvae fed continuously on *E. kuehniella* eggs exhibited shorter developmental time, greater adult fresh body weight at emergence compared to treatments with 48h prey deprivation in the larval stage. Furthermore, the option of cannibalism under prey deprivation (feeding *E. connexa* eggs) instead of improving performance caused a delay of 6 days in larval development and 5.8 days for complete development from larval to adult emergence. In addition, larvae under an exclusive cannibalistic diet exhibited reduced survival (71.7%) compared to 91.3 or 100% under the other diets.

Adult females reared under prey deprivation had similar survival rates within the 20 days adulthood that were observed, with 100% survival across all treatments. Nevertheless, prey deprivation caused a drastic reduction in egg production for all prey combinations (table 3). Two females from the control treatment (females fed continuously with *E. kuehniella* eggs) were infertile; their data were excluded from the analysis of fecundity and egg fertility. Females reared exclusive under cannibalism exhibited the largest reduction in egg production. Females without prey deprivation (control) produced 3.5x, 4.7x, and 13.6x more eggs than females reared under 48h prey deprivation fed (1) *E. kuehniella* eggs, (2) alternating between cannibalism and *E. kuehniella* eggs, and (3) *E. connexa* eggs, respectively (table 3). Besides producing fewer eggs compared to females in the other treatments, females fed the cannibalistic diet also deposited more infertile eggs per egg batch, compared to females in the other treatments (table 3).

Discussion

The production of infertile eggs by *E. connexa* occurred at similar rates in both susceptible and pyrethroids resistant populations, regardless when the egg batch was laid within the female's lifetime and metabolic costs of the resistance. However, in all cases, the total number of infertile eggs increased with increasing the numbers of eggs per batch. This pattern of more infertile eggs in larger egg batches suggests that with larger egg batches, there would be higher costs to support food (egg) provisioning for newly emerged larvae. Irrespective of the size of the egg batches, ~21 to 34% of the eggs laid were infertile (figure 1). This high proportion of infertile eggs for *E. connexa* has been frequently noted previously. From other studies, infertility varies from 15.5% (Zazyckia *et al*., 2015) to 75.4% (Ferreira *et al*., 2013) (supplemental material table S1). Production of infertile eggs by *E. connexa* is not related to lack of sperm (Colares *et al*., 2015; Silva *et al*., 2021). However, the reason for this oviposition pattern remains unclear. Nevertheless, it is a common feature of the species, since only a small portion (5.8%) of egg batches in *E. connexa* (Exps. #1.1 and #1.2) exhibited 100% egg fertility.

Table 3. Life history characteristics (mean ± SE) of immatures and adults of *E. connexa* fed *E. kuehniella* (*Ek*) eggs or subjected to prey deprivation (48h, followed by 24h with abundant prey), in each of three diets: *Ek* eggs (control), conspecific eggs *E. connexa* (*Econ*), or alternation of *Ek*/*Econ* eggs.

Characteristics[1]	Standard prey (*Ek*) daily (n =17)[2]	48h of prey deprivation			Statistics		
		Standard prey (*Ek*) (n =15)	Conspecific eggs (*Econ*) (n =11)	*Ek* and *Econ* alternately (n =17)	*F*, *H* or χ^2	*df*	*P*
Immature stages							
Duration of larval stage (days)*	11.4 ± 0.09 c	14.9 ± 0.30 b	17.2 ± 0.41 a	14.5 ± 0.18 b	122.77	3	< 0.0001
Duration of pupal stage (days)*	3.6 ± 0.07 c	3.9 ± 0.08 ab	3.7 ± 0.08 bc	3.9 ± 0.05 a	10.03	3	0.0183
Time to reach adult stage (days)*	15.1 ± 0.07 c	18.8 ± 0.31 b	20.9 ± 0.39 a	18.4 ± 0.15 b	123.82	3	< 0.0001
% survival (larvae to adult)**	100 a	91.3 ± 4.15 b	71.7 ± 6.64 c	100 a	26.27	3	< 0.0001
Adult fresh body weight (mg)*	11.7 ± 0.38 a	5.4 ± 0.16 c	6.1 ± 0.28 b	6.4 ± 0.17 b	101.78	3	< 0.0001
Adult stage[2]							
No. of eggs/female*	463.2 ± 37.92 a	107.6 ± 11.95 b	37.7 ± 7.71 c	94.8 ± 9.47 b	44.17	3	< 0.0001
Fertile eggs (%)*	50.1 ± 6.92 a	64.8 ± 7.42 a	19.6 ± 10.15 b	59.0 ± 7.50 a	12.46	3	0.0059

[1]Analysis by Kruskal-Wallis (*) or Log-Rank Test (**). Means within rows followed by different letters were significantly different (Kruskal-Wallis: Wilcoxon pairwise test, α = 0.05, Kaplan-Meier Survival: Log-Rank). [2]Number of females monitored for 20 days and all ended the observation period alive (100%).

In phytophagous species held without food restriction, production of infertile eggs is usually low, with fertility being near 100% in most of the cases (Saeed *et al.*, 2010; Pacheco da Silva *et al.*, 2017), consistent with the fact that egg production is costly, and eggs should be converted efficiently into offspring. In the case studied here, of a resistant and a susceptible lady beetle population, low fertility is a negative influence for both given that the maintenance of the population requires sufficient offspring production. High levels of infertile eggs production works against the population growth, but it may enhance the probability of survival of newly emerged larvae justifying the level of consistence in infertile eggs production.

Prey type can affect the efficiency (Ingested Material Conversion Efficiency, IMCE) with which food is converted to eggs in lady beetles (Honek and Evans, 2012). In our system, larvae of *E. connexa* developed faster when fed *C. maculata* eggs (an intraguild prey) but did not when fed their own eggs (cannibalism). This reduction in larval developmental time when fed the intraguild predation (*C. maculata* eggs) cannot be generalized and deserve further studies using eggs of other lady beetle species to certify the benefits of intraguild predation over cannibalism, especially, due to difference on chemical compounds (nutrients and semiochemicals) of lady beetle eggs. Lady beetle eggs contain defense compounds against predation (Hemptinne *et al.*, 2000; Sloggett and Davis, 2010; Katsanis *et al.*, 2017), which likely vary by lady beetle species and the metabolism of these compounds may impose some costs. For instance, the lady beetle *Harmonia axyridis* (Pallas) possesses a specialized ability to metabolize alkaloids, which are found in most lady beetle species as a defensive compound (Sloggett and Davis, 2010). However, *C. maculata* eggs are toxic to *H. axyridis* (Sloggett *et al.*, 2009). In *E. connexa* larvae, it is probable that alkaloid metabolism obtained from *C. maculata* eggs diverted energy from adult biomass production, resulting in faster larval development, but resulting in smaller adults. Moreover, it may have occurred because *C. maculata* eggs are larger, which translates into more energy per prey item consumed compared to the conspecific eggs.

Interestingly cannibalism resulted in a delay in the development, contrary to *Hippodamia convergens* Guerin-Meneville, where cannibal individuals developed faster (Bayoumy and Michaud, 2015). However, *E. connexa* larvae fed their own species' eggs attained a final adult body fresh weight that was like the control group fed *E. kuehniella* eggs, possibly because any egg defence compounds would be like their own, and thus pose no important toxicological issues for larval development.

The nutritional requirements of insects vary depending on the energy demand of each life stage (i.e., development and reproduction), and adult females have a greater need of protein for oogenesis (Chapman, 1998). Egg consumption (as cannibalism or intraguild predation) increased the fecundity of *E. connexa* adults because eggs are an efficient source of protein for oogenesis. The nutritious value of foods, however, is variable. While the cannibalism of a single egg at a first larval stage may be sufficient to improve performance of *H. axyridis* larvae (Osawa, 2002), a diet of *E. kuehniella* eggs complemented with two eggs (cannibalism) did not improve either development or reproduction of *C. maculata*, perhaps due to fewer adaptations (behavioural or physiological) associated with egg cannibalism (Abdelwahab *et al.*, 2018). Indeed, fecundity even decreased when larvae of

Menochilus sexmaculatus F. were reared exclusively with eggs of their own species (Yadav *et al.,* 2020). Our results support the general view that both egg cannibalism and intraguild predation have benefits to *E. connexa,* but that these benefits are different between larvae and adults.

E. connexa shares habitat and prey species in the crop ecosystems with other lady beetles (Lixa *et al*., 2010; Harterreiten-Souza *et al*., 2012; Rodrigues *et al*., 2013a). This overlap creates a wide range of potential intra- and inter-specific interactions, such as cannibalism and intraguild predation. Overall, both cannibalism and intraguild predation occur when larger (or older) individuals/species prey on smaller (or younger) individuals/species (Polis, 1981; Polis *et al*., 1989). In intraguild predation, the degree of trophic specialization is an important factor influencing the frequency and direction of the interaction (Polis *et al*., 1989). For cannibalism, prey abundance is a key factor with more cannibalism occurring when prey is scarce. For example, first instar larvae of *Tenuisvalvae notata* (Mulsant) and *Cryptolaemus montrouzieri* Mulsant show high rates of cannibalism, around 65% and 55%, respectively, under prey scarcity (Oliveira, 2020). This relationship acts as a buffer to limit the impact of variable food resources, adjusting the lady beetle population size to the existing resources (Polis, 1981). Intraguild predation is more complex but also increases with reducing availability of extraguild prey, driving the hungry predators to expand their diet to include intraguild prey (Polis *et al*., 1989). In *E. connexa* larvae, we found that in the absence of aphids, both cannibalism and intraguild predation increased, indicating that *E. connexa* larvae would use these foods to survive in the field during periods of scarcity of aphids. Even in the presence of aphids, *E. connexa* larvae engaged in egg cannibalism, showing its value as a source of extra or unique nutrients that were not obtained from eating only aphids. However, *E. connexa* demonstrated a preference for aphids over intraguild predation. This preference for aphids (extraguild prey) may be related to defensive compounds present in *C. maculata* eggs (Sloggett *et al*., 2009). The intense yellow colour from these eggs indicates large amounts of alkaloids, making these eggs less attractive or less palatable, since the more alkaloids in a species, more unpalatable it becomes (Pasteels *et al*., 1973). Secondly, *C. maculata* eggs are larger than either *E. connexa* eggs or turnip aphids; therefore, one *C. maculata* egg would promote satiety faster than consumption of the same number of prey items through cannibalism or aphid consumption. Regardless of prey availability, *E. connexa* consistently engages in egg cannibalism and intraguild predation, which implies that these food items are an important complement to aphid diet. This shows the aphidophagous feeding behaviour of *E. connexa* but also emphasizes the importance to this species of a mix prey diet (Lira *et al*., 2019; Nascimento *et al*., 2021) and of non-prey food (Santos *et al*., 2016). This behaviour can be further investigated testing field collected larvae and adults for conspecific and heterospecific lady beetle eggs as immunomarking in combination with anti-rabbit immunoglobulin enzyme-linked immunosorbent assays (ELISAs).

When *E. connexa* larvae were exposed simultaneously to extraguild prey in low density, intraguild prey, and cannibalism, their preference for cannibalism shows that conspecific eggs did not impose avoidance compared to the intraguild predation. However, larvae clearly showed a preference towards eating aphids (the extraguild prey) when they were abundant. Nevertheless, larvae continued to engage in cannibalism and intraguild predation (albeit at lower levels), what confirms the density-dependent nature of the influence of extraguild prey on levels of cannibalism and intraguild predation (Lucas and Brodeur, 1999). Therefore, it is expected that an increase in the abundance of an extraguild prey would result in more predation, but the lady beetle's response toward to the target pest (aphids, the extraguild prey) will depend on levels of other possible prey and food sources in the habitat.

In the field, aphidophagous lady beetles often face wide variation in prey density (Kindlmann *et al*., 2015), and in these situations they may turn to alternative foods (Giorgi *et al*., 2009; Lundgren, 2009) or cannibalism (Polis, 1981). To avoid starvation, coccinellids such as *E. connexa* often consume other non-prey foods (such as nectar, honeydew, pollen, sap, fruit, plants, and fungi (Castro-Guedes, 2013; Santos *et al*., 2016). In *E. connexa*, all individuals that experienced food deprivation had delayed development, but those reared only under cannibalism, a protein-rich diet, took longer to the standard prey to reach the adult stage and showed poor reproductive performance, highlighting the importance of a mixed diet (Castro-Guedes *et al*., 2013; Nascimento *et al*., 2021). Furthermore, individuals subjected to prey deprivation had reduced fecundity, and again, an exclusively cannibalistic diet gave the poorest result, in contradiction to our hypothesis that *E. connexa* females lay infertile eggs to nourish their offspring and cannibalism would enhance larval survival. Furthermore, larvae and adults of *E. connexa* have different nutritional requirements. Although both stages consume alternative food (both prey and non-prey items), not all these foods are nutritionally adequate for both development and reproduction (Giorgi *et al*., 2009; Lundgren, 2009; Lima *et al*., 2020). Despite the high lipid and protein content of eggs, they lack other nutrients (Chapman, 1998; Slogett and Lorenz, 2008), essential for metabolism (Lundgren, 2009).

Dissected females had malformation of the reproductive organs and poor development of the ovarioles. The poor reproductive performance of females reared exclusively on a cannibalistic diet indicates that diet did not provide sufficient energy for development of the reproductive organs. A similar result with *E. connexa* was found by Nascimento *et al*. (2021), who observed that females fed exclusively with larvae of diamond back moth, *Plutella xylostella* (L.), did not produce eggs, suggesting that failed to mature reproductively. However, these authors did not perform morphological studies, and they attributed the failure to produce normal numbers of eggs to a poor nutritional value of *P. xylostella* larvae.

Indeed, cannibalism in general should not be considered a reproductive strategy, but rather a survival strategy in times of prey scarcity (Polis, 1981; Perry and Roitberg, 2006) or, perhaps, a self-regulation mechanism acting to reduce the populations growth potential (Cushing, 1992). Thus, cannibalistic populations can self-regulate below the carrying capacity of the environment, before high density produces over exploitation and mass starvation

(Polis, 1981). In this context, cannibalism can be understood as a strategy to both sustain a population in the field and stabilize its density in the environment.

Conclusion

Our study found that the production of infertile eggs did not vary between the two *E. connexa* phenotypes studied (resistant and susceptible), but rather females from both phenotypes produced a stable proportion of infertile eggs in egg batches. Among the foods tested to preserve *E. connexa* during periods of starvation, intraguild predation reduced total developmental time, but egg cannibalism did not. Both cannibalism and intraguild predation, completing the standard diet, increased the adult fecundity but not change the rate of egg fertility. The density of the extraguild prey (turnip aphid) influenced both intra and interspecific interactions, and at high extraguild prey density, both cannibalism and intraguild predation declined (3.2 and 2.2 times, respectively), highlighting the fundamentally aphidophagous nature of *E. connexa*. Starvation did not stimulate females of *E. connexa* to produce more infertile eggs, and cannibalism alone did not enhance the fecundity. Considering these results, we could not explain why *E. connexa* produces infertile eggs at such high rates and to clarify the mechanism behind this phenomenon will require further research.

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