Terminal Reproductive Investment, Physiological Trade-offs and Pleiotropic Effects: Their effects produce complex immune/reproductive interactions in the cricket *Gryllus texensis*

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Summary

1. Should females increase or decrease reproduction when attacked by pathogens? Two hypotheses provide opposite predictions. Terminal reproductive investment theory predicts an increase in reproduction, but hypothesized physiological trade-offs between reproduction and immune function might be expected to produce a decrease. There is evidence for both hypotheses. What determines the choice between the two responses remains unclear. We examine the effect of age on the reproductive response to immune challenge in long-wing females of the Texas field cricket, *Gryllus texensis*, when fed an ecologically valid (limited) diet.

2. The limited diet reduced reproductive output. However, immune challenge had no effect on their reproductive output either in young or middle-aged crickets, which is contrary to either prediction.
3. Flight muscle maintenance correlated negatively with reproductive output, suggesting a physiological trade-off between flight muscle maintenance and reproduction. Within the long-wing variant there was considerable variability in flight muscle maintenance. This variability may mask physiological trade-offs between immunity and reproduction.
4. Middle-aged crickets had higher total phenoloxidase (PO) activity in their hemolymph, compared to young females, which is contrary to the terminal investment theory. Given that PO is involved in both immunity and reproduction, the increased PO may reflect simultaneous investment in both functions.
5. We identified four proPO transcripts in a published RNA-seq dataset (transcriptome). Three of the proPO genes were expressed either in the fat body or the ovaries (supporting the hypothesis that PO is bifunctional); however, the two organs expressed different subsets. The possible bifunctionality of PO suggests that it may not be an appropriate immune measure for immune/reproductive trade-offs in some species.
6. Increasing age may not cue terminal reproductive investment prior to senescence.

Key-words

Life history theory, Ecoimmunology, phenoloxidase, Physiological trade-off, Terminal investment

Introduction

Resources are finite in animals. The partitioning (i.e. allocation) of those resources among resource-intensive traits such as immunity and reproduction can lead to physiological trade-offs, resulting in negative correlations between them (e.g. in insects, Zera & Harshman 2001; Lawniczak *et al.* 2007; Schwenke, Lazzaro & Wolfner 2015; in vertebrates, French et al. 2007, McCallum and Trauth 2007; Kalbe et al. 2009; Nordling et al. 1998; and Mills et al. 2009). Consistent with this argument, activation of an immune response leads to reduced reproductive effort in a range of species (e.g. insects, see Schwenke *et al.* 2016). However, under some conditions, an immune challenge leads to increased reproductive output, which is usually interpreted as a type of fecundity compensation (Duffield *et al.* 2017). An immune challenge signals a risk of early death from infection, and, therefore, a decline in an animal's residual reproductive value. In response, the animal shifts investment away from somatic maintenance to fuel a final bout of reproduction. This strategy is called terminal reproductive investment (Clutton-Brock 1984). Whether an individual should increase or decrease reproduction when infected depends on a range of poorly understood factors (e.g. age, Duffield *et al.* 2017).

Insects make good model systems for these types of questions, in part because their reproductive output is easy to quantify, and their immune systems are simpler than those of vertebrates (Adamo 2017). Moreover, insects like the cricket *Gryllus texensis* are large enough to measure multiple immune components simultaneously (e.g. key insect immune components such as phenoloxidase (PO) activity (Cerenius, Lee & Söderhäll 2008)).

Age is expected to reduce the fitness benefit gained from increasing immune function and decreasing reproduction (i.e. a physiological trade-off) when infected (see Duffield *et al.,* 2017). Cricket residual reproductive value declines with age (Shoemaker et al. 2006), and, therefore, the fitness pay off for reducing current reproduction to preserve future reproduction should decline over time. Furthermore, if declining immune function due to age (i.e. during senescence) reduces the chance of recovery (e.g. (Adamo, Jensen & Younger 2001)), then it may be adaptive to prioritize reproduction during an immune challenge in older animals. Therefore, age should increase the likelihood that terminal reproductive investment will be activated by an immune challenge (Duffield et al., 2017). Supporting this, interactions between age, immune challenge and reproductive investment have been observed in several male insect models such as *Gryllodes sigillatus* (Duffield *et al.* 2018), *Drosophila nigrospiracula* (Polak & Starmer 1998), and *Allonemobius socius* (Copeland & Fedorka 2012). However, evidence in female insects is relatively scarce, even though reproductive output in females is often easier to quantify. Immune challenge has a variable effect on female crickets (Table 1); possibly female age may help explain this variability. In *G. texensis*, females retain high immunocompetence throughout their adult stage, while in males it declines (Adamo, Jensen & Younger 2001). These results suggest that females may have a reproductive resource allocation strategy that is different from males (also see Rapkin *et al.* (2018) for sex-specific effects of macronutrient intake on trade-offs between reproduction and immunity in *G. sigillatus*). In this study, we examine the effect of age on the female reproductive response to infection.

Table 1. Effect of immune challenge on reproduction in female crickets

Species	Dosage	Age (post adult) at the start of treatment	Duration of immune challenge	Effect on reproduction	Reference
Acheta domesticus	100 µg/cricket of *Serratia marcescens* LPS 5 x 10^4 live cells of *S. marcescens*	2 weeks 2 or 5 weeks	Acute	+ (positive) +	(Adamo 1999)
Acheta domesticus	1 or 2 nylon pieces/cricket (implantation)	18 days	Chronic (3 weeks)	- (negative)	(Bascuñán-García *et al.*, 2009)
Gryllus texensis	8.75 x 10^3 live cells of *S. marcescens* 1 x 10^5 live cells of *S. marcescens* 1.2 x 10^5 live cells of *S. marcescens*	11 to 19 days 11 to 19 days 2 weeks	Acute	+ 0 (no effect) +	(Shoemaker *et al.* 2006)
Gryllus texensis	LD_{01} of *S. marcescens* live cells LD_{01} of *Bacillus cereus* live cells	2 weeks 2 weeks	Acute	0 -	(Adamo & Lovett 2011)
Gryllus texensis	20 µg/cricket of *S. marcescens* LPS 100 µg/cricket of *S. marcescens* LPS	13 to 19 days	Chronic (every three days for 12 days)	0 0	(Shoemaker & Adamo 2007)
Gryllus texensis	1 x 10^4 heat-killed *S. marcescens*	1 day	Chronic (every three days for 17 days)	-	(Stahlschmidt *et al.* 2013)
Hemideina crassidens	100 µg *S. marcescens* LPS 500 µg *S. marcescens* LPS	Uncontrolled (field collection)	Chronic (every four days for 17 days)	- -	(Kelly 2011)
Gryllodes sigillatus	Nylon implantation (encapsulation response)	14 day	Chronic	0	Rapkin et al. 2018

In this study, we chose two age classes to assess the effect of immune challenge in this study: young (11 days as an adult) and middle-aged (21 days old). At 11 days of age (i.e. young crickets), females have mated and begun to produce eggs, but their reproductive activity (oviposition rate) is less than maximal in this cricket (Shoemaker, Parsons & Adamo 2006). By 21 days of age, females are fully mature, with high oviposition rates (Shoemaker *et al.* 2006). However, they are still within the typical age for females found in the field (Murray & Cade

1995), thus they are not old in an ecological sense. We predicted that young female crickets would respond to an immune challenge with decreased reproduction, but an enhancement of immune function (e.g. increased lysozyme-like function). Middle-aged females, on the other hand, would increase oviposition, but would show a more modest activation of their immune response compared with younger females.

One technical difficulty in determining whether there has been a physiological trade-off between immunity and reproduction is assessing immunity. Immune function is made up of multiple components, which can sometimes be traded-off for each other (Adamo 2004b). Immune systems can also reconfigure their molecular network pathways, and therefore a reduction in a single immune component may be mistaken for a reduction in investment, as opposed to a reconfiguration (Adamo *et al.* 2016). Finally, the primacy of different immune pathways can shift depending on the physiological context (Armitage & Boomsma 2010; Piñera *et al.* 2013; Adamo 2014; Adamo *et al.* 2016). Therefore, to monitor immunological investments in crickets, it is important to measure multiple aspects of immune function on each animal. We measured PO, glutathione (GSH, which helps buffer the self-damage caused by PO (Clark, Lu & Strand 2010)), and lysozyme-like activity. PO and lysozyme-like activity respond differently to immune challenges; lysozyme-like activity is inducible in response to pathogen challenge in insects while PO may form a constitutive immune defense (Adamo 2004a).

Although PO activity is commonly used as a proxy for immune function in ecoimmunology, PO is also involved in egg production in insects, which potentially complicates the interpretation of PO levels in female insects. PO is involved in processes such as the tanning of the egg chorion (Li & Christensen 1993; Li 1994) and/or the eggs' antimicrobial defense (Rizki & Rizki 1990; Abdel-latief & Hilker 2008). In *G. texensis*, PO activity in eggs has also

116 been reported (Stahlschmidt *et al.* 2013). There appears to be several sources of PO in insects,
117 and this may depend on the species. Hemocytes have been viewed as a major source of PO
118 (Cerenius *et al.* 2008; Kanost & Gorman 2008; Lu *et al.* 2014); however, in some insects, the fat
119 body and the ovaries also express POs (e.g. mosquitoes, see Fig. 5 of Cui, Luckhart & Rosenberg
120 2000). Little is known in insects about how these POs are trafficked between organs, thus it
121 remains unclear whether the hemolymph PO level reflects either immune investment or
122 reproductive investment, or both. To fulfill the knowledge gap on molecular information about
123 PO production in crickets, we assess PO gene expression in both fat body and ovaries.
124

125 # Materials and Methods

126 ## Animals

127 Female *G. texensis* crickets were originally obtained from San Antonio, Texas, USA, and have
128 been maintained in the laboratory for approximately 8 generations. The colony was maintained at
129 26ºC on a 12/12 hour light/dark cycle, supplied with food and water *ad libitum*. Long-winged
130 adult females were weighed and isolated from the colony within 48 hour after the final molt (the
131 day which we call 'day 1' in this study). We did not use the short-wing morphs in this study. Each
132 of the isolated females was isolated in a plastic container and supplied with a shelter and water
133 bottle. Food was placed in the individual containers for 3 hours every 3 days. During those 3
134 hours, crickets could feed *ad libitum*. This diet has been shown to produce females with the same
135 fat content as females collected in the field (Adamo *et al.* 2012). On days 7 and 8 (female adult
136 age), each female was provided with three different males. Each male was placed in the female's
137 container for about 8 hours. After each mating, males were switched so as to ensure that each

138 female was exposed to three different males. All experiments were approved by the Animal
139 Care Committee of Dalhousie University (# I-11-025) and are in accordance with the Canadian
140 Council on Animal Care.
141

142 Treatments

143 Female crickets were randomly assorted by weight, and assigned one of the following eight
144 treatments on the day they were isolated from the colony. Days were counted from the day of
145 isolation (see timeline in Fig. 1).

146 *Early Controls (Control (E))*. Crickets were handled on day 11, and hemolymph samples
147 were collected on day 12 and day 36.

148 *Late Controls (Control (L))*. Crickets were handled on day 21, and hemolymph samples
149 were collected on day 22 and day 36.

150 *Early Immune Challenge (IC (E))*. On day 11, crickets were injected with 2 µL of a
151 mixture of heat-killed pathogen cells *(Serratia marcescens*, *Bacillus cereus* and
152 *Beauveria bassiana*.) The dose of each pathogen was approximately 1/10 of the LD50
153 dose prior to heat inactivation. Hemolymph samples were collected on day 12 and day
154 36.

155 *Late Immune Challenge (IC (L))*. Crickets were injected on day 21 with 2 µL of the same
156 heat-killed pathogen mixture described above. Hemolymph samples were collected on
157 day 22 and day 36.

158 *Early Sham (Sham (E))*. On day 11, crickets were poked by an empty injection needle, but
159 not injected with any sample. Hemolymph samples were collected on day 12 and 36.

Late Sham (Sham (L)). On day 21, crickets were poked by an empty injection needle, but not injected with any sample. The hemolymph samples were collected on day 22 and 36.

No Treatment Control (NTC). Hemolymph samples were collected on day 36.

No treatment Control (ad lib feeding) (NTC (ad lib)). Hemolymph samples were collected on day 36 and crickets were fed *ad libitum*.

Figure 1. Experimental Schedules

The crickets used in this study (except for ones for the ovarian gene expression experiment) went through one of the eight experimental timelines shown in the chart. Details are described in Materials and Methods.

Reproductive Output

We monitored the number of eggs laid in the cotton balls twice a week. Five eggs were subsampled from each cotton ball and placed separately in centrifuge tubes (1.5mL) with a small piece of cotton and 500 µL of water. In cases where the number of eggs laid in the cotton ball was less than five, all eggs were sampled. These eggs were then kept at 26ºC and monitored for 35 days. Hatch date, hatchling survival (daily), and hatchling body mass at 35 days after the hatch day were monitored for the sampled eggs. Eggs were censored and assumed not viable if they had not hatched within 35 days. The reproductive value (RV), a proxy for fitness, was calculated as a product of the number of eggs and the hatch ratio. For example, if a female laid 50 eggs and 3 out of the 5 subsampled eggs hatched, then the reproductive value would be 30.

Dissection

If still viable, crickets were dissected on day 36. For each cricket, we: 1) measured body mass, 2) collected fat body tissues for gene expression analyses (described below), 3) counted eggs in the lateral oviducts, and 4) observed flight muscle state (functional/histolysed as described by Zera 2003). We also measured length of the hind leg femurs and recorded the average of the two legs. For gene expression analysis in the ovaries, we dissected a group of females on day 15 that were independent of the rest of the study and collected both the fat body and the ovaries. These crickets were also given the intermittent diet during adulthood.

Hemolymph Collection

We collected hemolymph samples by poking the membrane under the pronotum plate with an ice-cold pipette tip (to retard coagulation), and the hemolymph was collected as it exited the wound. We collected 8 µL of hemolymph which was mixed with 55 µL of ice-cold MilliQ water in a 1.5 mL centrifuge tube. Samples were then split into three fractions (20 µL for the PO and Bradford assays, 23 µL for the GSH assay, and the rest (20µL) for the Lysozyme assay). Immediately after the sample collection, we spun the hemolymph sample for the GSH assay (23 µL) at 18,800 g for 10 min at 4 ºC, and 20 µL of the supernatant was immediately mixed with 20 µL of 100 mg/mL meta-phosphoric acid. After 5-minute incubation at room temperature, we spun the samples at 2,900 g for 3 min at room temperature. 35 µL of the deproteinated supernatant was collected in a new 1.5 mL centrifuge tube. All the samples for PO, Bradford, GSH, or Lysozyme assays were stored at -80ºC until use.

Hemolymph Assays

Total PO activity and total protein concentration were measured as described previously (Adamo 2004a). GSH concentration was measured as described previously (McMillan, Miller & Adamo 2017). A detailed information for the assays is described in the Supplementary file. Briefly, for lysozyme-like activity, hemolymph samples were collected as described above. Samples were thawed and spun at 12,000 g for 3 min at 4 ºC. 5 µL of the supernatant was mixed with 45 µL *Micrococcus luteus* cell (Sigma #M3770) suspension (10 mg/20 mL Phosphate-buffered Saline (PBS), pH = 7) in a 96-well (flat bottom) plate. The mixture was incubated at 30 ºC, and we measured OD_{450} every 30 seconds for 50 minutes. Lysozyme derived from chicken egg white was used to produce a standard curve (Sigma-Aldrich, #62971-10G-F). A blank (PBS) were run concurrently. The mean value from triplicate technical replicates was used for each sample.

Gene Identification

To identify the gene transcripts in the cricket, we first constructed a transcriptome database based on a raw sequence of RNA reads available online (National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). The accession number of the bioproject is PRJNA429132 (submitted by Natural History Museum, Berlin, Germany). We then set up a searching pipeline (written in Python programming language, the code is available at the author's GitHub repository at https://github.com/atmiyashita/CricketGeneFinder2018/). The code: 1) fetches cDNA sequences in arthropods from NCBI Nucleotide database that are associated with the target protein name (i.e. 'vitellogenin', 'phenoloxidase' etc.), 2) runs BLAST locally using the fetched sequence as a query and the transcriptome (of *G. texensis*) as a database, 3) outputs the result in xml format, and 4) returns a summary. The hit sequences were then confirmed by blastx at

https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome to confirm its homology at amino acid sequence level (i.e. primary structure). For vitellogenin, we further performed a physiological validation in this study, because the sequence similarity was relatively low (compared to proPO, see figure S1).

Gene Expression Analysis

The primers used in this study are listed in Table S1. We followed the MIQE guideline (Bustin *et al.* 2009; Taylor *et al.* 2010) for the qPCR experiments. The fat body (the speckled white tissues found in the abdominal cavity) was collected carefully to minimize collecting other tissues such as the trachea. The ovaries were collected carefully so as not to contaminate the sample with fat body. We washed ovaries once with PBS to further avoid potential contamination of the sample with hemocytes. The tissues were stored in 300 μL of RNAlater (Thermo Fisher Scientific, #AM7020) in 1.5mL centrifuge tubes and frozen at -80ºC until further use. Detailed information for RNA extraction, cDNA synthesis, and quantification is described in the Supplementary information.

Data Analysis

In this study, we isolated 240 female adult crickets assigned across 8 treatment groups. 8 out of the 240 crickets did not mate (i.e. 232 crickets contained spermatheca filled with sperm when dissected). These 8 were excluded from the analysis. 66 crickets were also excluded from the analysis because some data were lost (e.g. due to death, Fig. S2). Thus, we acquired a complete dataset on 166 female crickets that: 1) mated, 2) survived for 36 days. We noted some inter-trial variation in the baseline level of immune factors, so we treated the trial numbers as a random

248 factor in the linear mixed models. Unless stated otherwise, we used packages 'lme4' and
249 'lmerTest' running on R (version 'Short Summer' (3.4.2)) for the linear mixed models. The details
250 for each analysis is described in the figure legends. As a measurement of condition, the Scaled
251 Mass Index (SMI) (Kelly, Tawes & Worthington 2014) was calculated for each cricket.

Results

Effect of food availability on overall reproductive output

Controls that were food-limited (NTC) produced fewer eggs than controls fed *ad libitum*. (i.e. (NTC (*ad lib*)) (Fig. 2A). *Ad lib* fed crickets also laid more eggs, and had more eggs in the lateral oviducts on day 36 than did food-limited crickets (Fig. 2B and 2C). The ratio of eggs laid by day 36 over total eggs produced, was similar between the two feeding conditions (Fig. 2D). The relative number of eggs laid compared with eggs held in reserve in the lateral oviduct did not vary with diet (Student's t-test; $t = 0.87$, $df = 29$, $p = 0.39$). Also, 3 out of 20 females had pink (functional) flight muscles on day 36 in NTC group, compared to 3 out of 19 females in NTC (*ad lib*) group, suggesting that the feeding condition did not affect the likelihood of flight muscle histolysis by day 36 (Chi-squared test; $\chi^2 = 1.3e\text{-}31$, $df = 1$, $p = 1$).

Figure 2. Effect of feeding condition on reproductive output

The feeding condition (intermittent vs. *ad libitum*) significantly affected the reproductive output of the crickets. (A) The total number of eggs produced by day 36 (a sum of the laid eggs and the eggs found in the lateral oviducts) was significantly lower in the NTC than NTC (*ad lib*). (B,C) The number of eggs laid by day 36 (B) and the number of eggs found in the lateral oviducts (C) were also significantly lower in the intermittent feeding condition. The p-values shown in the figure is calculated by Welch Two Sample t-test. (D) The portion of laid eggs (i.e. laid:total ratio) was comparable between the two feeding conditions.

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275

276 Treatment effects on immune function and survival

277 IC (E) and IC (L) groups did not show any differences in PO level, GSH level, and PO:GSH
278 ratio in the hemolymph 24 hours after the immune challenge (i.e. day 12 and 22) compared with
279 controls (Fig. S4). The hemolymph protein concentration in the IC (E) group increased relative
280 to controls 24 h after the immune challenge (Fig. S4). IC (L) showed higher lysozyme-like
281 activity relative to controls 24 hours after the challenge (Fig. S4). Only one group produced
282 effects that lasted until day 36, the IC (L) on GSH (Fig. S4). The overall survival rate on day 12,
283 22, and 36 was 100% (210/210), 99% (207/210), and 79% (166/210) as shown in Fig. S2, and
284 there was no difference in survival across the seven groups (Fig. S2B; $\chi^2 = 9.8$, df = 6, p = 0.13).

285

286 No effect of immune challenges on reproductive output

287 The total number of eggs produced, the number of eggs laid, and the number of eggs in the
288 lateral oviducts, and the ratio of laid eggs to the total number of eggs on day 36 was comparable
289 across all seven treatment groups (Fig. 3). No effect of treatment was observed at any time point
290 (Fig. S5).

291

292 **Figure 3. No effect of immune challenge on reproductive output**

293 There was no significant effect of treatments on overall (36-day) reproductive outputs measured
294 as (A) number of eggs laid, (B) number of total eggs produced, (C) number of eggs found in the

295 lateral oviducts, and (D) ratio of laid:total number of eggs. Also, there was no acute effect on the
296 reproductive output immediately after the treatments (E and F). Detailed time course is also
297 shown in Fig. S5, which corroborates the lack of treatment effect on reproductive output.
298
299

300 Association between dispersion capability and reproductive output.

301 Most crickets had white (histolysed) flight muscle on day 36 (130 of 150 observations), which
302 was observed equally across the eight groups ($\chi^2 = 3.9$, df = 7, p = 0.79). The crickets that still
303 retained pink (functional) flight muscles on day 36 produced and laid fewer eggs than the
304 crickets with histolysed flight muscle (Fig. 4A, B). The number of eggs found in the lateral
305 oviduct on day 36 was not significantly different between the two morphs (Fig. 4C), but the
306 laid:total ratio was significantly lower in pink-muscle crickets (Fig. 4D). The body condition
307 measure, SMI, was comparable between the two morphs on day 1, but was higher in the white-
308 muscle morph on day 36 (Fig. 4E). Also, an increase in SMI over time was observed in the
309 white-muscle morph, but not in the pink-muscle morph (Fig. 4E). The body mass on day 1 was
310 significantly higher in pink-muscle crickets, but that on day 36 was comparable between the two
311 morphs (Fig. 4F). Increase in body mass over time was only observed in the white-muscle morph
312 (Fig. 4F).
313

314 **Figure 4. Flight capability is negatively correlated with reproductive output**

315 Total number of produced eggs (A), number of laid eggs (B), and the laid:total ratio (D) was
316 higher in the crickets that had histolysed (white) flight muscle on day 36. The number of eggs

found in the lateral oviducts showed a trend toward significance (C). SMI represents Scaled Mass Index (E). d01 and d36 represents day 1 and day 36 (E and F).

Age-dependent changes in immune measures

Phenoloxidase activity was higher in 22 day old and 36 day old adult females than in 12 day old adult females (Fig. 5A), Other parameters we measured in the hemolymph (GSH, PO:GSH ratio, lysozyme-like activity, and protein level) did not show age-dependent differences (Fig. 5B-E). The PO level showed a modest positive correlation with reproductive output (r-squared = 0.11, p=1.03e-07) (Fig. S6).

Figure 5. Age-dependent increase of PO level

Age-dependent change in hemolymph parameters. The PO level (µg (tyrosinase equivalent)/mL), the GSH level (µM), the PO:GSH ratio, the lysozyme-like activity (µg/mL), and the protein level (µg/mL) are log10-transformed and shown in the chart. The numbers below each chart represent the ages at which the blood was collected. For days 12, 22, and 36, results from Control (E/L) and NTC are plotted. Each dot represents an individual cricket. The treatment effects were examined using Mixed Linear Models (using 'lme4' package in R), considering cohort identity (i.e. experimental date) as a random factor in the model. Only PO showed a significant increase over age (A), while other parameters showed no trend (B-E).

337 Expression of proPOs in the ovaries

338 We detected three proPO transcripts in the ovary and the fat body. proPO1 was consistently
339 expressed in the fat body and the ovaries at comparable levels (Fig. 6A). proPO2 was expressed
340 specifically in the ovaries (Fig. 6B), while proPO3 was expressed specifically in the fat body
341 (Fig. 6C). Vitellogenin was expressed specifically in the fat body (Fig. 6D).

342

343

344 **Figure 6. Ovaries produce phenoloxidase (PO) in the cricket**

345 Gene expression levels of proPOs (A-C) and Vitellogenin (D) were measured in the fat body and
346 the ovaries. Experimental procedures are described in Materials and Methods. Translated
347 sequences of each transcript is shown in Figure S1. The values were normalized by two reference
348 genes, and the relative expression levels (arbitrary units), where the expression level for the
349 reference is set to be 1.0.

Discussion

Food limitation reduced the total number of eggs produced, corroborating earlier studies that this diet reduces reproduction (e.g. Adamo *et al.* 2012). We had assumed that under low food availability, females would have proportionately fewer eggs in reserve in the lateral oviducts than did *ad lib* controls. In other words, we expected that food-limited females would lay proportionately more of their eggs in order to maintain egg output even as egg production fell. However, females maintained the same proportion of eggs in reserve when food-limited as when resources were abundant. Possibly females reduce their risk of low offspring survival by laying eggs in different places at different times. This strategy of oviposition site diversification would explain why females are found with eggs in their lateral oviducts even in the field (Adamo 1999).

Despite the evidence that the food-limited diet reduced the resources needed for reproduction (i.e. because food-limited crickets had fewer total eggs), we found no evidence of a physiological trade-off between immunity and reproduction in either young or middle-aged adult females in response to an immune challenge. This result corroborates other studies in crickets that did not find a reduction in reproduction after a single immune challenge or repeated immune challenges (Table 1). Previous studies on this species have demonstrated that the immune challenge we used induces a robust immune response in female crickets (Adamo 2004a; 2010). Moreover, in this study, we found an increase in lysozyme-like activity 24 h later in older (middle-aged) females, suggesting that the minimal effect was not due to a lack of immune response. However, it is possible that the heat-killed challenge did not induce an enough effect to trigger a physiological trade-off, although the immune challenge does trigger sickness behaviours in this cricket (Sullivan, Fairn & Adamo 2016). It is also possible that the decrease in

373 reproduction was small, which was not noticeable given the large variability in egg number and
374 egg-laying behaviour. Assuming the same effect size and variability as found in our data set (for
375 example, the effect size (Cohen's d) was 0.05 between the numbers of eggs laid in NTC and IC
376 (E) groups), we would need more than 780 crickets/group to potentially find a positive effect.
377 Such a small effect is inconsistent with most studies on immune/reproductive trade-offs (e.g.
378 Stahlschmidt *et al.* 2013).

379 This discrepancy may be explained by unique aspects of cricket life history. Long-
380 winged *G. texensis* crickets histolyze their wing muscles at some point during their adult life
381 (authors' personal observation), which releases additional resources for reproduction in other
382 cricket species (Zera, Sall & Grudzinski 1997; Zera, Potts & Kobus 1998). Once the muscles are
383 histolyzed, they are no longer capable of flight (Zera & Denno 1997). The additional resources
384 provided by the wing muscles may reduce trade-offs between immunity and reproduction.
385 Mathematical models of trade-offs have demonstrated that trade-offs may be difficult to
386 demonstrate if the variance in resource acquisition is large compared with that in resource
387 allocation (van Noordwijk & de Jong 1986; Zera & Harshman 2001; Metcalf 2016). In this study
388 we observed large variability in the timing of flight muscle histolysis within each group,
389 suggesting that there is considerable individual difference in available resources at any particular
390 time point. Supporting our hypothesis that flight muscle histolysis may provide an important
391 boost in resources for reproduction, flight muscle histolysis showed a strong association with
392 reproductive output. Future studies should note whether wing muscles have been histolyzed
393 when studying physiological trade-offs in crickets.

394 There was also no evidence of terminal reproductive investment in either age class. We
395 expected that older (i.e. middle-aged) female crickets should increase reproduction when given

396 heat-killed bacteria, as has been observed previously (Shoemaker *et al.* 2006). However, there
397 was no evidence that females became more sensitive to an immune challenge with age. In
398 previous reports from our laboratory, female crickets showed terminal reproductive investment,
399 even at a young age (Adamo 1999; Shoemaker *et al.* 2006), but those immune challenges were
400 close to a lethal dose. The effect of a sub-lethal dose of bacteria on reproduction was only
401 observed in *G. texensis* when moist sand was used for egg-laying substrate, and was not
402 observed when moist-cotton was used, as in this study (Shoemaker *et al.* 2006). *G. texensis*
403 females prefer to oviposit in moist sand over moist cotton (Shoemaker *et al.* 2006), which may
404 have affected the terminal investment thresholds. This point needs to be validated in future
405 studies.

406 There is an alternative explanation for the lack of terminal investment with age in this
407 study: the threshold for terminal reproductive investment may not decrease with age until the
408 beginning of senescence. Although crickets can survive for more than 8 weeks in the laboratory,
409 they show signs of senescence after only 4 weeks (Shoemaker *et al.* 2006). This is consistent
410 with our study that showed an increase in mortality only at the last time point (i.e. 36 days, Fig.
411 S2). Crickets were not given an immune challenge at this time point, and, therefore, were not
412 tested for terminal reproductive investment at a time when mortality due to age was increasing.
413 Prior to senescence, the risk of death for female crickets may be the same each day, unless
414 predator or pathogen prevalence increases. Decreasing the threshold for terminal reproductive
415 investment prior to senescence may not be advantageous for female crickets when oviposition
416 site is important for offspring survival (Shoemaker et al., 2006) and optimal oviposition sites are
417 not available.

418 Instead, females may depress egg production and/or egg laying, even when infected, until
419 conditions are favourable for offspring development. In some females of this species, completing
420 a dispersal flight may also signal better oviposition opportunities. Flight is known to increase egg
421 production in this and other cricket species (Guerra & Pollack 2009; Zeng, Zhu & Zhao 2014),
422 although some long-winged females histolyze their flight muscles at a young age even without a
423 dispersal flight (Zera, Sall & Grudzinski 1997). The threshold for terminal reproductive
424 investment may be very high prior to wing muscle histolysis. Given that this event occurs at
425 different dates across individuals, the effect may be masked by the number of non-responders.
426 Contrary to the terminal investment theory, in which individuals are assumed to invest
427 less in somatic maintenance with age, we found an age-dependent increase in PO. This is
428 consistent with an earlier study that found an age-dependent increase in PO in female, but not in
429 male, *G. texensis* (Adamo *et al.* 2001). An increase in PO activity with age has been found in
430 females of other species, and this increase can lead to Malpighian tubule damage in old female
431 *Tenebrio molitor* (Khan, Agashe & Rolff, 2017). Whether PO-induced damage is involved with
432 the increase in mortality observed by us on day 36 is unknown. However, given that the PO:GSH
433 ratio remained constant across ages (GSH buffers the self-damaging toxicity of PO), the self-
434 damaging cost of the increased PO may be minor.
435 The increase in PO activity with age may not represent an increase in immune investment,
436 or be an example of immune dysregulation. The increase in PO may represent an increase in
437 reproductive investment. PO is needed for the tanning and defense of insect eggs (Rizki & Rizki
438 1990; Li & Christensen 1993; Li 1994; Abdel-latief & Hilker 2008), and in some insects, it
439 appears to be synthesized by sources outside of the ovary and transported to the ovaries through
440 the blood (e.g. mosquito (Kim *et al.* 2005)). Therefore, increases in PO hemolymph levels may

441 represent an increase in reproductive effort, which incidentally also increases the amount of PO
442 available for immunity, and may lead to immunopathology in old age (Khan, Agashe & Rolff
443 2017). We have 3 lines of evidence for this in our study. PO activity rises in middle-aged (day-
444 22) crickets, which is prior to senescence (Fig. S2). Middle-aged females have a high
445 reproductive output, consistent with an increased need for PO to maintain increasing egg
446 production (Fig. S5). The age-dependent increase in PO is observed only in female *G. texensis*
447 (Adamo *et al.* 2001), suggesting that the rise of PO is involved with female-specific life-history
448 traits such as egg production. Finally, we detected proPO gene expression in the fat body, and,
449 therefore, it could supply PO to both hemolymph and ovary. However, we also found that the
450 ovary expressed two subtypes of proPO genes, and, therefore it is uncertain to what extent the
451 ovary and fat body contribute to egg PO. We have not done an in-depth molecular analysis of
452 the proPOs expressed in the ovaries, but the differential expression of proPO subtypes between
453 the fat body (primarily an immune organ, but also involved in reproduction via vitellogenin (yolk
454 protein) production, Arrese & Soulages 2010)), and the ovary indicates the complexity and
455 pleiotropic nature of PO. Identifying the circulating PO subtype(s) in the hemolymph and in the
456 eggs would help answer this question. It is these types of mechanistic details that are needed to
457 understand trade-offs (e.g. Zera and Harshman 2001; Duffield *et al.* 2017). This complexity also
458 suggests that PO is not an ideal proxy for immune investment in immune/reproductive trade-off
459 studies in female insects.

460 The lack of effect of age on the terminal reproductive response to infection in female
461 crickets in this study reflects the generally weak effect of age on the reproductive response to
462 infection in other female animals (Duffield et al., 2017; Adamo 1999; Sanz et al. 2001; Cotter et

463 al. 2011). These results suggest that age has little effect on the threshold for terminal
464 reproductive investment in females during an infection prior to senescence.
465

Authors' Contribution

AM and SA conceived the ideas and designed methodology; RE established the qPCR methods; AM, ML, LM collected the data; AM and SA analyzed the data; AM wrote R and Python codes; AM and SA led the writing of the manuscript.

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Reference

1. Abdel-latief M, Hilker M. Innate immunity: Eggs of Manduca sexta are able to respond to parasitism by *Trichogramma evanescens*. *Insect Biochemistry and Molecular Biology*. 2008;38: 136–145. doi:10.1016/j.ibmb.2007.10.001
2. Adamo SA. The Effects of Stress Hormones on Immune Function May be Vital for the Adaptive Reconfiguration of the Immune System During Fight-or-Flight Behavior. *Integrative and Comparative Biology*. 2014;54: 419–426. doi:10.1093/icb/icu005
3. Adamo SA, Lovett MME. Some like it hot: the effects of climate change on reproduction, immune function and disease resistance in the cricket *Gryllus texensis*. *Journal of Experimental Biology*. 2011;214: 1997–2004. doi:10.1242/jeb.056531
4. Adamo SA. Why should an immune response activate the stress response? Insights from the insects (the cricket *Gryllus texensis*). *Brain, Behavior, and Immunity*. 2010;24: 194–200. doi:10.1016/j.bbi.2009.08.003
5. Adamo SA. Evidence for adaptive changes in egg laying in crickets exposed to bacteria and parasites. *Animal Behaviour*. 1999;57: 117–124. doi:10.1006/anbe.1998.0999
6. Adamo SA. Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. *Journal of Insect Physiology*. 2004;50: 209–216. doi:10.1016/j.jinsphys.2003.11.011
7. Adamo SA. How should behavioural ecologists interpret measurements of immunity? *Animal Behaviour*. 2004;68: 1443–1449. doi:10.1016/j.anbehav.2004.05.005
8. Adamo SA. The stress response and immune system share, borrow, and reconfigure their physiological network elements: Evidence from the insects. *Hormones and Behavior*. 2017;88: 25–30. doi:10.1016/j.yhbeh.2016.10.003
9. Adamo SA, Baker JL, Lovett MME, Wilson G. Climate Change and Temperate Zone Insects: The Tyranny of Thermodynamics Meets the World of Limited Resources. *Environmental Entomology*. 2012;41: 1644–1652. doi:10.1603/EN11188
10. Adamo SA, Easy RH, Kovalko I, MacDonald J, McKeen A, Swanburg T, et al. Predator stress-induced immunosuppression: trade-off, immune redistribution or immune reconfiguration? *The Journal of Experimental Biology*. 2016; jeb.153320. doi:10.1242/jeb.153320
11. Adamo SA, Jensen M, Younger M. Changes in lifetime immunocompetence in male and female *Gryllus texensis* (formerly *G. integer*): trade-offs between immunity and reproduction. *Animal Behaviour*. 2001;62: 417–425. doi:10.1006/anbe.2001.1786
12. Armitage SAO, Boomsma JJ. The effects of age and social interactions on innate immunity in a leaf-cutting ant. *Journal of Insect Physiology*. 2010;56: 780–787. doi:10.1016/j.jinsphys.2010.01.009
13. Arrese EL, Soulages JL. Insect Fat Body: Energy, Metabolism, and Regulation. *Annual Review of Entomology*. 2010;55: 207–225. doi:10.1146/annurev-ento-112408-085356

14. Bascuñán-García AP, Lara C, Córdoba-Aguilar A. Immune investment impairs growth, female reproduction and survival in the house cricket, *Acheta domesticus*. *Journal of Insect Physiology*. 2010;56: 204–211. doi:10.1016/j.jinsphys.2009.10.005
15. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry*. 2009;55: 611–622. doi:10.1373/clinchem.2008.112797
16. Cerenius L, Lee BL, Söderhäll K. The proPO-system: pros and cons for its role in invertebrate immunity. *Trends in Immunology*. 2008;29: 263–271. doi:10.1016/j.it.2008.02.009
17. Clark KD, Lu Z, Strand MR. Regulation of melanization by glutathione in the moth *Pseudoplusia includens*. *Insect Biochemistry and Molecular Biology*. 2010;40: 460–467. doi:10.1016/j.ibmb.2010.04.005
18. Clutton-Brock TH. Reproductive Effort and Terminal Investment in Iteroparous Animals. *The American Naturalist.* 1984;123: 212–229. doi:10.1086/284198
19. Copeland EK, Fedorka KM. The influence of male age and simulated pathogenic infection on producing a dishonest sexual signal. *Proceedings of the Royal Society B: Biological Sciences.* 2012;279: 4740–4746. doi:10.1098/rspb.2012.1914
20. Cotter SC, Ward RJS, Kilner RM. Age-specific reproductive investment in female burying beetles: independent effects of state and risk of death: Age-specific reproductive investment. *Functional Ecology*. 2011;25: 652–660. doi:10.1111/j.1365-2435.2010.01819.x
21. Cui L, Luckhart S, Rosenberg R. Molecular characterization of a prophenoloxidase cDNA from the malaria mosquito *Anopheles stephensi*. *Insect Molecular Biology*. 2000;9: 127–137. doi:10.1046/j.1365-2583.2000.00169.x
22. Duffield KR, Bowers EK, Sakaluk SK, Sadd BM. A dynamic threshold model for terminal investment. *Behavioral Ecology and Sociobiology*. 2017;71: 185. doi:10.1007/s00265-017-2416-z
23. Duffield KR, Hampton KJ, Houslay TM, Hunt J, Rapkin J, Sakaluk SK, et al. Age-dependent variation in the terminal investment threshold in male crickets: AGE-DEPENDENT VARIATION IN TERMINAL INVESTMENT. *Evolution.* 2018;72: 578–589. doi:10.1111/evo.13443
24. French SS, DeNardo DF, Moore MC. Trade-Offs between the Reproductive and Immune Systems: Facultative Responses to Resources or Obligate Responses to Reproduction? *The American Naturalist*. 2007;170: 79–89.
25. Guerra PA, Pollack GS. Flight behaviour attenuates the trade-off between flight capability and reproduction in a wing polymorphic cricket. *Biology Letters*. 2009;5: 229–231. doi:10.1098/rsbl.2008.0570
26. Kalbe M, Eizaguirre C, Dankert I, Reusch TB., Sommerfeld RD, Wegner KM, et al. Lifetime reproductive success is maximized with optimal major histocompatibility complex diversity. *Proceedings of the Royal Society B: Biological Sciences*. 2009;276: 925–934. doi:10.1098/rspb.2008.1466
27. Kanost MR, Gorman MJ. PHENOLOXIDASES IN INSECT IMMUNITY. *Insect Immunology*. Elsevier; 2008. pp. 69–96. doi:10.1016/B978-012373976-6.50006-9
28. Kelly CD. Reproductive and physiological costs of repeated immune challenges in female Wellington tree weta (Orthoptera: Anostostomatidae): IMMUNE CHALLENGES IN FEMALE TREE WETA. *Biological Journal of the Linnean Society*. 2011;104: 38–46. doi:10.1111/j.1095-8312.2011.01714.x

29. Kelly CD, Tawes BR, Worthington AM. Evaluating indices of body condition in two cricket species. *Ecology and Evolution*. 2014;4: 4476–4487. doi:10.1002/ece3.1257
30. Khan I, Agashe D, Rolff J. Early-life inflammation, immune response and ageing. *Proceedings of the Royal Society B: Biological Sciences*. 2017;284: 20170125. doi:10.1098/rspb.2017.0125
31. Kim SR, Yao R, Han Q, Christensen BM, Li J. Identification and molecular characterization of a prophenoloxidase involved in *Aedes aegypti* chorion melanization. *Insect Molecular Biology*. 2005;14: 185–194. doi:10.1111/j.1365-2583.2004.00547.x
32. Lawniczak M, Barnes A, Linklater J, Boone J, Wigby S, Chapman T. Mating and immunity in invertebrates. *Trends in Ecology & Evolution*. 2007;22: 48–55. doi:10.1016/j.tree.2006.09.012
33. Li J. Egg chorion tanning in *Aedes aegypti* mosquito. C*omparative Biochemistry and Physiology Part A: Physiology*. 1994;109: 835–843. doi:10.1016/0300-9629(94)90231-3
34. Li J, Christensen BM. Involvement of l-tyrosine and phenol oxidase in the tanning of *Aedes aegypti* eggs. *Insect Biochemistry and Molecular Biology*. 1993;23: 739–748. doi:10.1016/0965-1748(93)90048-W
35. Lu A, Zhang Q, Zhang J, Yang B, Wu K, Xie W, et al. Insect prophenoloxidase: the view beyond immunity. *Frontiers in Physiology*. 2014;5. doi:10.3389/fphys.2014.00252
36. McCallum ML, Trauth SE. PHYSIOLOGICAL TRADE-OFFS BETWEEN IMMUNITY AND REPRODUCTION IN THE NORTHERN CRICKET FROG (*ACRIS CREPITANS*). *Herpetologica*. 2007;63: 269–274. doi:10.1655/0018-0831(2007)63[269:PTBIAR]2.0.CO;2
37. Metcalf CJE. Invisible Trade-offs: Van Noordwijk and de Jong and Life-History Evolution. *The American Naturalist*. 2016;187: iii–v. doi:10.1086/685487
38. Mills SC, Grapputo A, Jokinen I, Koskela E, Mappes T, Oksanen TA, et al. Testosterone-Mediated Effects on Fitness-Related Phenotypic Traits and Fitness. *The American Naturalist*. 2009;173: 475–487. doi:10.1086/597222
39. Murray A-M, Cade WH. Differences in age structure among field cricket populations (Orthoptera; Gryllidae): possible influence of a sex-biased parasitoid. *Canadian Journal of Zoology*. 1995;73: 1207–1213. doi:10.1139/z95-144
40. Nordling D, Andersson M, Zohari S, Lars G. Reproductive effort reduces specific immune response and parasite resistance. *Proceedings of the Royal Society B: Biological Sciences*. 1998;265: 1291–1298. doi:10.1098/rspb.1998.0432
41. Piñera AV, Charles HM, Dinh TA, Killian KA. Maturation of the immune system of the male house cricket, *Acheta domesticus*. *Journal of Insect Physiology*. 2013;59: 752–760. doi:10.1016/j.jinsphys.2013.05.008
42. Polak M, Starmer WT. Parasite-induced risk of mortality elevates reproductive effort in male *Drosophila*. *Proceedings of the Royal Society B: Biological Sciences*. 1998;265: 2197–2201. doi:10.1098/rspb.1998.0559
43. Rapkin J, Jensen K, Archer CR, House CM, Sakaluk SK, Castillo E del, et al. The Geometry of Nutrient Space–Based Life-History Trade-Offs: Sex-Specific Effects of Macronutrient Intake on the Trade-Off between Encapsulation Ability and Reproductive Effort in Decorated Crickets. *The American Naturalist*. 2018;191: 452–474. doi:10.1086/696147
44. Rizki RM, Rizki TM. Encapsulation of parasitoid eggs in phenoloxidase-deficient mutants of *Drosophila melanogaster*. *Journal of Insect Physiology*. 1990;36: 523–529. doi:10.1016/0022-1910(90)90104-N

45. Sanz JJ, Arriero E, Moreno J, Merino S. Interactions between hemoparasite status and female age in the primary reproductive output of pied flycatchers. *Oecologia*. 2001;126: 339–344. doi:10.1007/s004420000530
46. Schwenke RA, Lazzaro BP, Wolfner MF. Reproduction–Immunity Trade-Offs in Insects. *Annual Review of Entomology*. 2016;61: 239–256. doi:10.1146/annurev-ento-010715-023924
47. Shoemaker KL. Interactions between immunity and reproduction in the cricket, *Gryllus texensis*. Dalhousie University. 2003.
48. Shoemaker KL, Parsons NM, Adamo SA. Egg-laying behaviour following infection in the cricket *Gryllus texensis*. *Canadian Journal of Zoology*. 2006;84: 412–418. doi:10.1139/z06-013
49. Shoemaker KL, Adamo SA. Adult female crickets, *Gryllus texensis,* maintain reproductive output after repeated immune challenges. *Physiological Entomology*. 2007;32: 113–120. doi:10.1111/j.1365-3032.2006.00552.x
50. Stahlschmidt ZR, Adamo SA. Warm and cozy: temperature and predation risk interactively affect oviposition site selection. *Animal Behaviour*. 2013;86: 553–558. doi:10.1016/j.anbehav.2013.06.009
51. Sullivan K, Fairn E, Adamo SA. Sickness behaviour in the cricket *Gryllus texensis* : Comparison with animals across phyla. *Behavioural Processes*. 2016;128: 134–143. doi:10.1016/j.beproc.2016.05.004
52. Taylor S, Wakem M, Dijkman G, Alsarraj M, Nguyen M. A practical approach to RT-qPCR—Publishing data that conform to the MIQE guidelines. *Methods*. 2010;50: S1–S5. doi:10.1016/j.ymeth.2010.01.005
53. van Noordwijk AJ, de Jong G. Acquisition and Allocation of Resources: Their Influence on Variation in Life History Tactics. *The American Naturalist*. 1986;128: 137–142. doi:10.1086/284547
54. Veenstra JA. Isolation and structure of corazonin, a cardioactive peptide from the American cockroach. *FEBS Letters*. 1989;250: 231–234. doi:10.1016/0014-5793(89)80727-6
55. Zeng Y, Zhu D-H, Zhao L-Q. Critical Flight Time for Switch from Flight to Reproduction in the Wing Dimorphic Cricket *Velarifictorus aspersus*. *Evolutionary Biology*. 2014;41: 397–403. doi:10.1007/s11692-014-9272-9
56. Zera AJ. The Endocrine Regulation of Wing Polymorphism in Insects: State of the Art, Recent Surprises, and Future Directions. *Integrative and Comparative Biology*. 2003;43: 607–616. doi:10.1093/icb/43.5.607
57. Zera AJ, Denno RF. PHYSIOLOGY AND ECOLOGY OF DISPERSAL POLYMORPHISM IN INSECTS. *Annual Review of Entomology*. 1997;42: 207–230. doi:10.1146/annurev.ento.42.1.207
58. Zera AJ, Harshman LG. The Physiology of Life History Trade-Offs in Animals. *Annual Review of Ecology and Systematics*. 2001;32: 95–126. doi:10.1146/annurev.ecolsys.32.081501.114006
59. Zera AJ, Potts J, Kobus K. The Physiology of Life-History Trade-offs: Experimental Analysis of a Hormonally Induced Life-History Trade-off in *Gryllus assimilis*. *The American Naturalist*. 1998;152: 7–23. doi:10.1086/286146
60. Zera AJ, Sall J, Grudzinski K. Flight-Muscle Polymorphism in the Cricket *Gryllus firmus:* Muscle Characteristics and Their Influence on the Evolution of Flightlessness. *Physiological Zoology*. 1997;70: 519–529. doi:10.1086/515865

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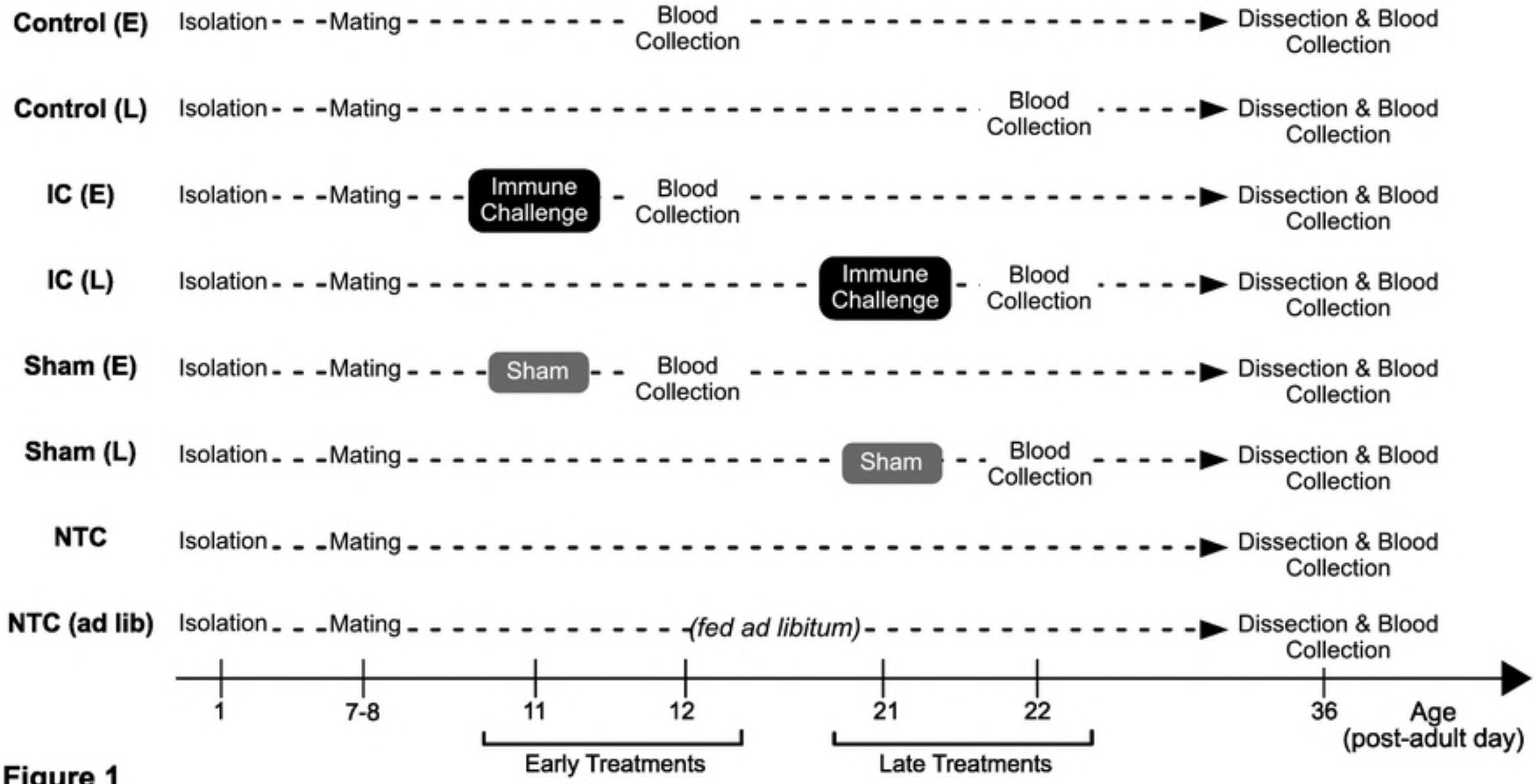

Figure 1

figure 1

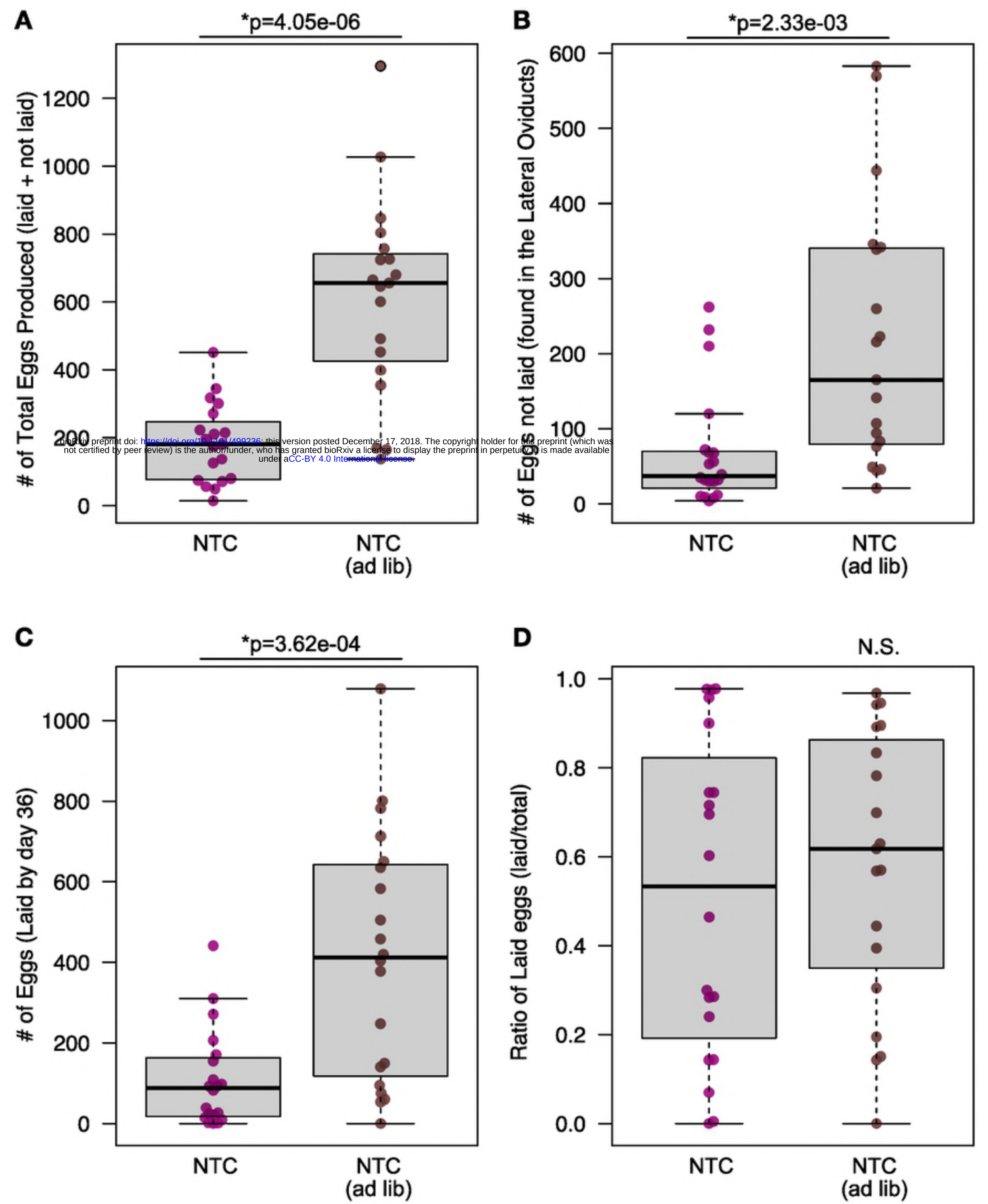

Figure 2

figure 2

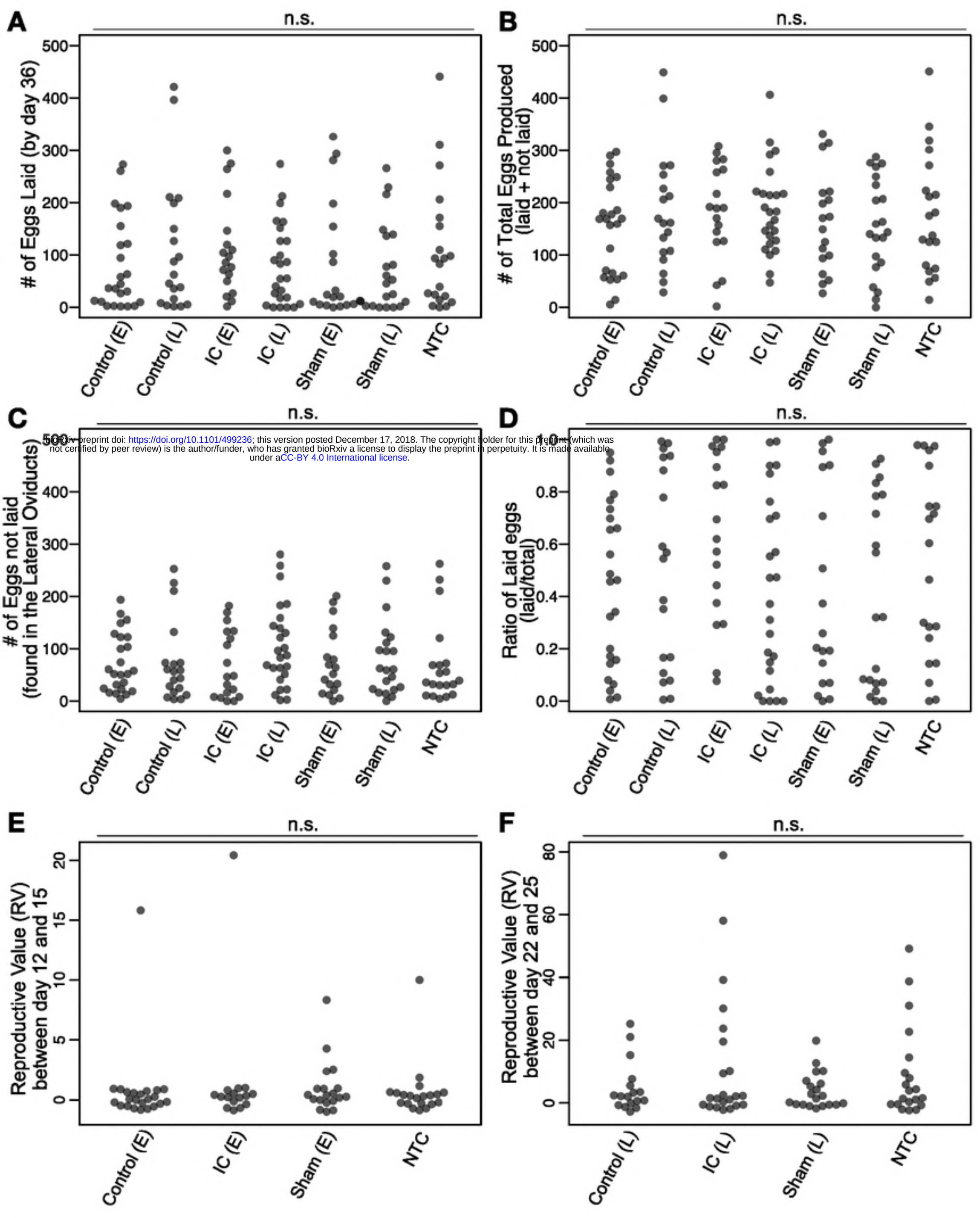

Figure 3

figure 3

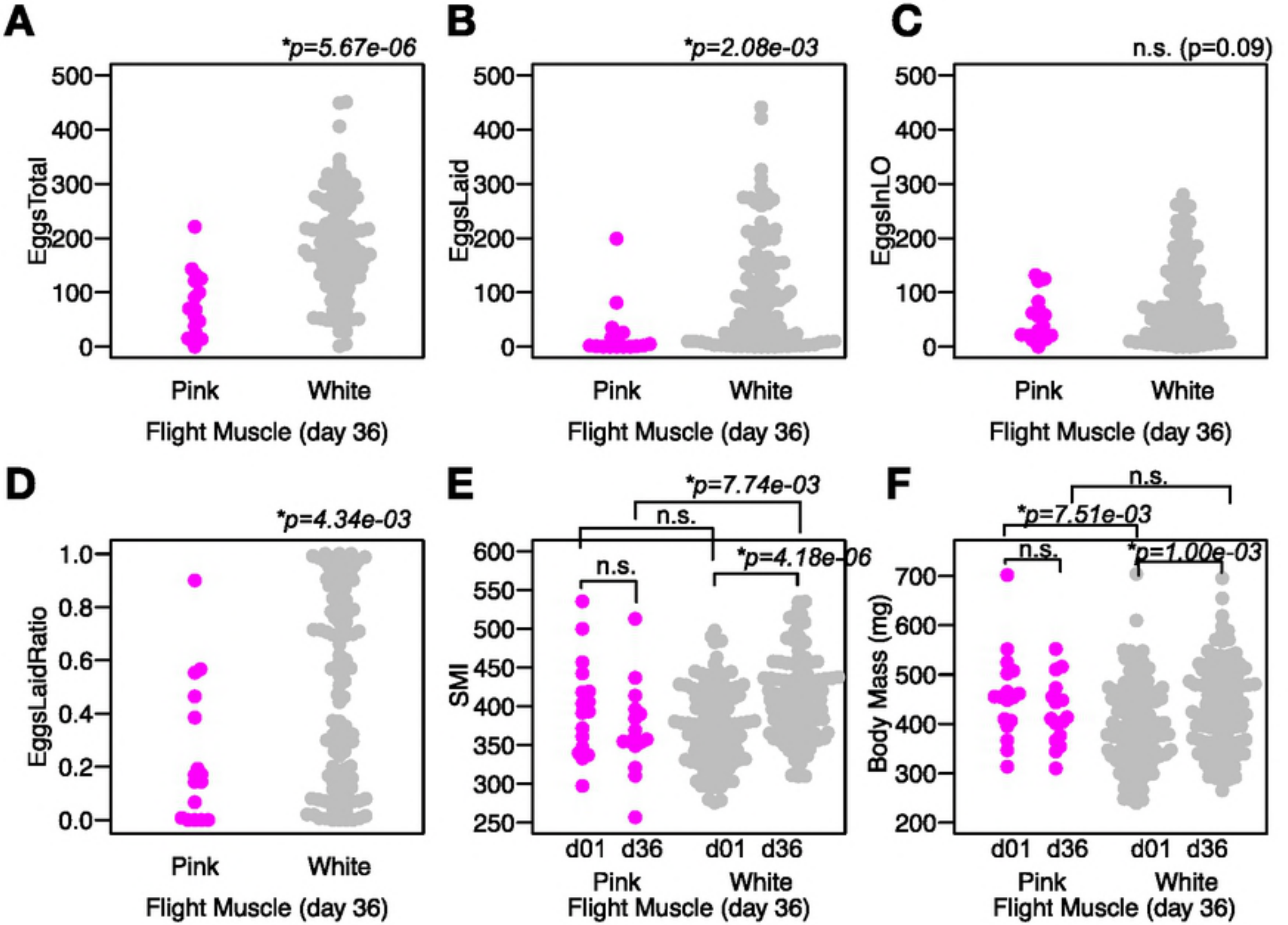

Figure 4

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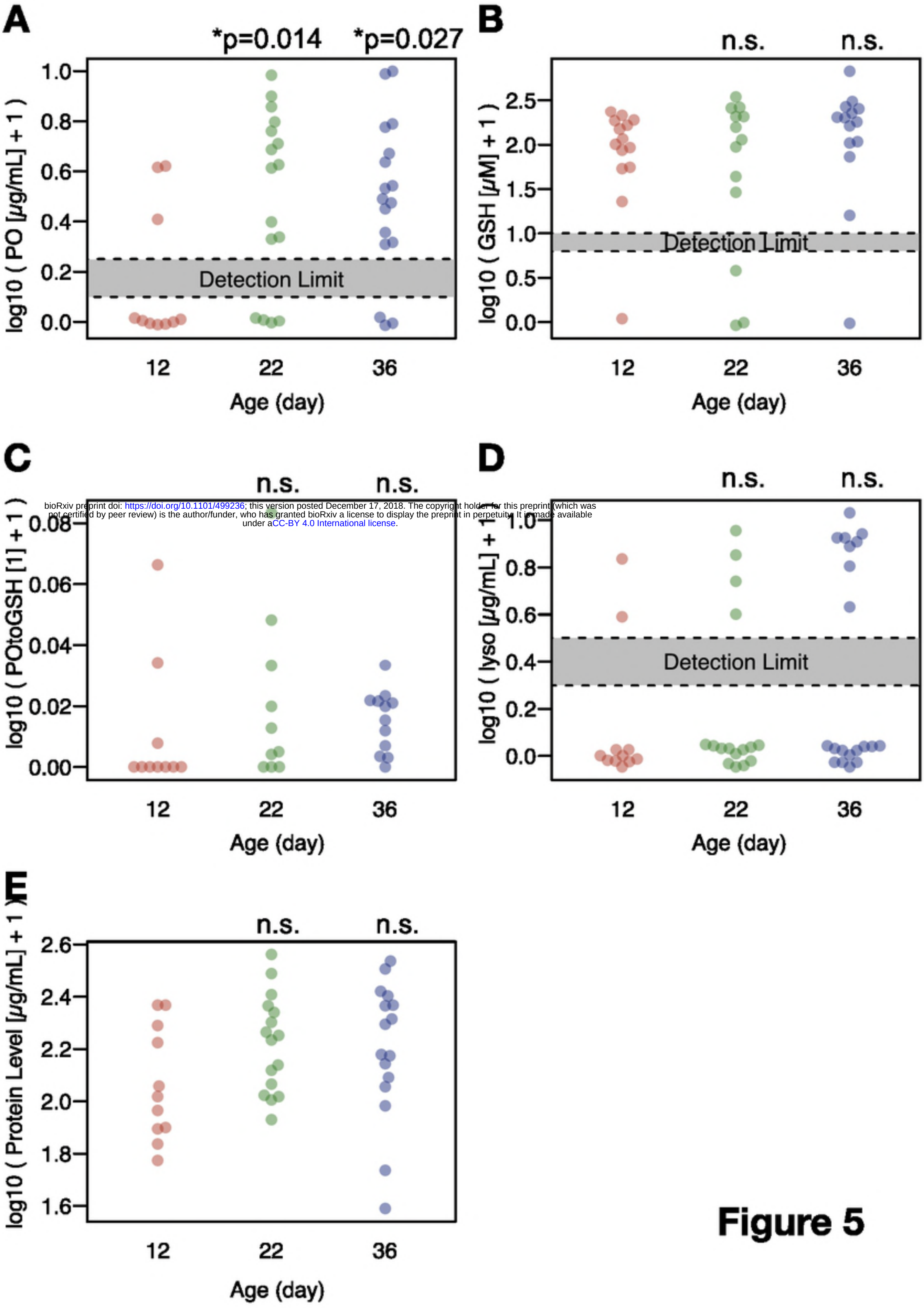

Figure 5

figure 5

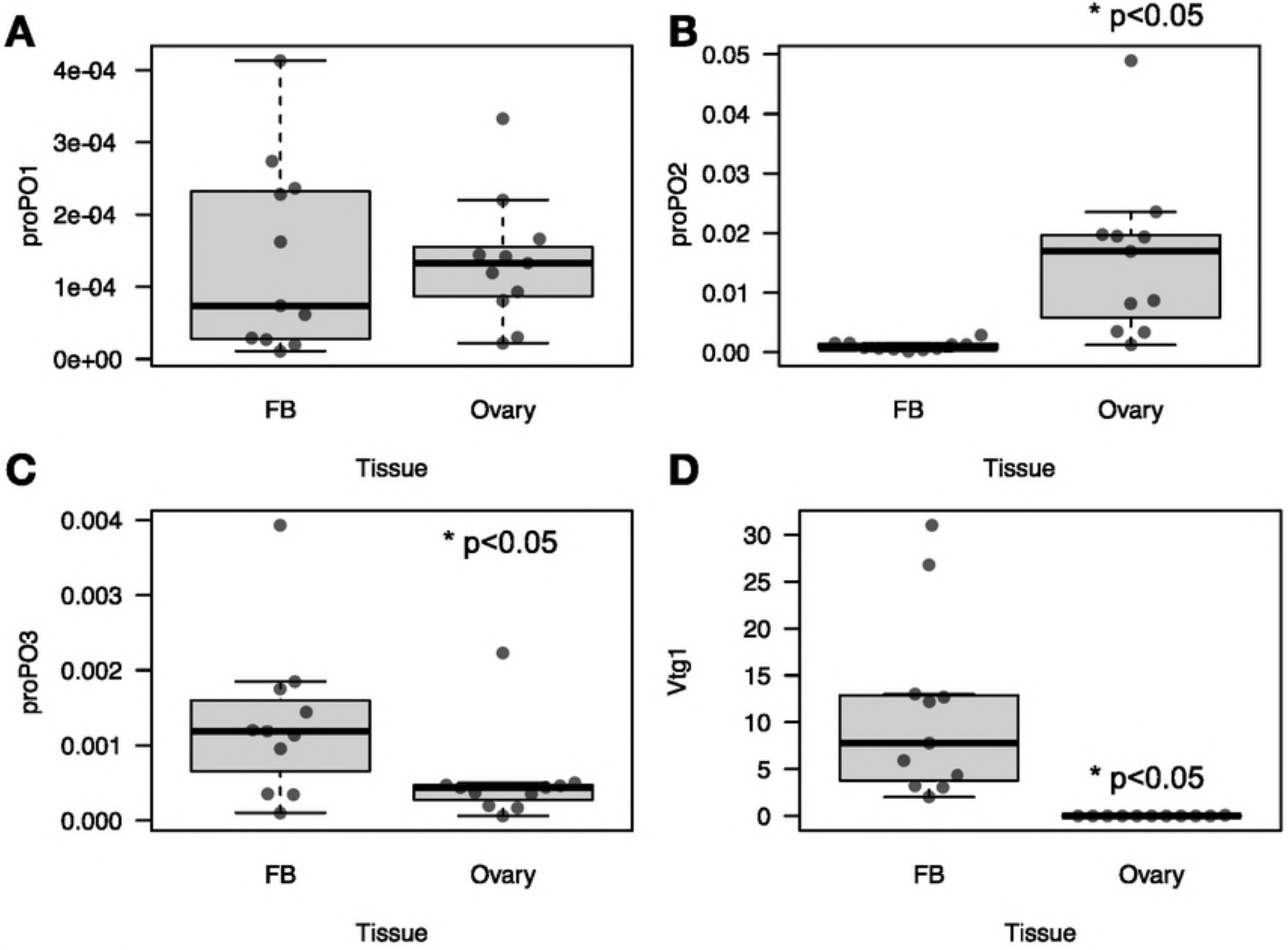

Figure 6

figure 6