Title: Age-dependent variation in the terminal investment threshold in male crickets

Running head: Age-dependent variation in terminal investment

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Abstract

The terminal investment hypothesis proposes that decreased expectation of future reproduction (e.g., arising from a threat to survival) should precipitate increased investment in current reproduction. The level at which a cue of decreased survival is sufficient to trigger terminal investment (i.e., the *terminal investment threshold*) may vary according to other factors that influence expectation for future reproduction. We test whether the terminal investment threshold varies with age in male crickets, using heat-killed bacteria to simulate an immune-inducing infection. We measured calling effort (a behavior essential for mating) and hemolymph antimicrobial activity in young and old males across a gradient of increasing infection cue intensity. There was a significant interaction between the infection cue and age in their effect on calling effort, confirming the existence of a *dynamic terminal investment threshold*: young males reduced effort at all infection levels, whereas old males increased effort at the highest levels relative to naïve individuals. A lack of a corresponding decrease in

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antibacterial activity suggests that altered reproductive effort is not traded against investment in this component of immunity. Collectively, these results support the existence of a dynamic terminal investment threshold, perhaps accounting for some of the conflicting evidence in support of terminal investment.

Keywords: Age-dependent reproductive effort, fecundity compensation, *Gryllodes sigillatus*, residual reproductive value, trade-offs

Introduction

Trade-offs among life history traits are widespread (Stearns 1989, 1992; Schluter et al. 1991; Roff 1992; Zera and Harshman 2001). Of particular interest in the evolution of life history strategies are trade-offs between defense and reproductive effort (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Harshman and Zera 2007). Because of allocation and physiological trade-offs between these important fitness-related components, the conventional view has been that individuals faced with a threat to survival (e.g., injury or infectious disease), should shift investment away from reproduction and towards defense or recovery to ensure their continued survival (Festa-Bianchet 1988; Gustafsson et al. 1994; Norris et al. 1994; Svensson et al. 1998; Adamo 1999; Jacot et al. 2004; Ahtiainen et al. 2005; Stahlschmidt et al. 2013). However, a growing body of evidence suggests that, at least in some species, individuals increase investment in current reproductive effort upon perceiving a threat to survival. While this result might seem counter-intuitive, it can be explained within the bounds of life-history theory via the *terminal investment hypothesis* (Clutton-Brock 1984).

The terminal investment hypothesis posits that as an individual's expectation for future offspring (i.e., residual reproductive value) decreases, investment in current reproduction should increase to maximize reproductive output and decrease the fitness costs associated with a shortened reproductive lifespan (Williams 1966). The stimulus that prompts a terminal investment response can be regarded as the *terminal investment trigger*. Empirical evidence for terminal investment following an actual or simulated threat to survival acting as the terminal investment trigger has been documented across multiple taxa and for various components of reproductive effort (Poveda et al. 2003; Derting and Virk 2005; Weil et al. 2006; González-Tokman et al. 2013; Poisot et al. 2013; Leventhal et al. 2014; Brannelly et al. 2016), including attractiveness of sexual signals (Sadd et al. 2006; Velando et al. 2006; Kivleniece et al. 2010; Copeland and Fedorka 2012; Thanda Win et al. 2013; Duffield et al. 2015; Roznik et al. 2015; An and Waldman 2016), offspring production (Minchella and Loverde 1981; Adamo 1999; Bonneaud et al. 2004; Chadwick and Little 2005; Blair and Webster 2007; Altincicek et al. 2008; Schwanz 2008; Heinze and Schrempf 2012; Giehr et al. 2017), and parental care (Pugesek 1981; Festa-Bianchet 1988; Hanssen 2006; Descamps et al. 2007; Creighton et al. 2009; Bowers et al. 2015). Although not always considered, these increases in reproductive effort should be accompanied by decreases in investment into somatic maintenance, such as immune defense, due to trade-offs between reproduction and immunity (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Faivre et al. 2003; Harshman and Zera 2007; Lawniczak et al. 2007; Schwenke et al. 2016; Durso and French 2017).

The terminal investment hypothesis usually is regarded as a static strategy, with the switch from investment towards reproduction occurring when an individual encounters a specific threat to longevity or future reproduction. The level or intensity of a threat that

precipitates a life history shift to terminal investment can be regarded as the *terminal investment threshold* (dashed line, Fig. 1). However, the strategy of terminal investment and the intensity of a threat that is sufficient to elicit a reallocation of resources towards current reproduction may depend on context. Specifically, any extrinsic or intrinsic factors further influencing residual reproductive value beyond the potential terminal investment trigger itself may alter an individual's assessment of the threat posed, and subsequently the propensity to terminally invest (reviewed in Duffield et al. 2017). For example, an individual's age, which is directly correlated with residual reproductive value (Williams 1966; Pianka and Parker 1975) and may itself elicit terminal investment (e.g., Lafaille et al. 2010; Ligout et al. 2012), is likely an important intrinsic factor that influences the propensity to terminally invest upon experiencing an unrelated cue of reduced survival (e.g. infection). As individuals age and move closer towards the end of their lifespan, the prospect of future reproductive opportunities diminishes and the context established by such intrinsic variation in residual reproductive value may alter the optimal terminal investment threshold as determined by selection (Duffield et al. 2017). Thus, age may determine the severity of an infection cue that is required to elicit terminal investment. Due to the intrinsic differences in residual reproductive value between them, old individuals are expected to be more likely to respond to cues of impending mortality than young individuals by increasing current reproductive effort. Consequently, old individuals should terminally invest at lower levels of a threat, and thus exhibit a lower terminal investment threshold, than young individuals (Fig. 1).

In sexually reproducing organisms, male fitness is primarily limited by lifetime mating success (Bateman 1948; Trivers 1972). Therefore, any assessment of male reproductive effort should focus on investment in traits that are necessary for successfully acquiring mates. Male orthopterans (i.e., grasshoppers, locusts, crickets, and katydids) are ideal study subjects for quantifying reproductive effort because many species produce readily quantifiable, acoustic signals that are essential for attracting receptive females. Previous studies have documented significant sexual selection on both song structure (Ower et al. 2013) and male calling effort, measured as the time invested in signaling (Gray and Cade 1999; Hunt et al. 2004). Although critical to male mating success, calling is associated with numerous costs including increased metabolic demands (Hoback and Wagner 1997; Houslay et al. 2017), increased attraction of parasitoids (Cade 1975), increased risk of predation (Sakaluk and Belwood 1984), decreased immune function (Adamo et al. 2001), and accelerated senescence (Hunt et al. 2004). Because of these costs, selection should act on males to adopt an optimized pattern of calling over the course of their reproductive lifetime.

Here, we test the hypothesis that male decorated crickets (*Gryllodes sigillatus*) exhibit terminal investment in calling effort depending on the strength of an infection threat, but that the terminal investment threshold, and thus the intensity of cue required for males to employ a terminal investment strategy, is age-dependent. We examined male reproductive effort in the form of calling effort following manipulation of perceived residual reproductive value of males using varying levels of a simulated extrinsic infection cue, heat-killed *Escherichia coli*, across two age classes. To further investigate how changes in calling effort impact immune function, as expected within the context of any reproduction-immunity trade-off, we also quantified the antibacterial capacity of hemolymph in a second group of crickets treated identically to those in which calling effort was measured.

Methods

1. Study animals

Experimental males were randomly selected from outbred populations initiated with crickets collected from wild populations in Las Cruces, New Mexico (2001), Phoenix, Arizona (2010), and Riverside, California (2014); population of origin was included as a random effect in subsequent analyses. Juvenile crickets were reared in 55L plastic storage bins filled with egg carton to increase rearing surface area, and provisioned with food (Harlan 2018CM Teklad Certified Global 18% protein rodent diet meal) and water (40mL water vials plugged with cotton rolls and small plastic containers filled with moistened peat moss) *ad libitum*. When sex differences became apparent (4^{th} or 5^{th} instar), juvenile males were removed from stock colonies and housed in small (450mL) individual plastic containers and provisioned with dry cat food pellets (Purina®) and water *ad libitum*. A small section of egg carton was also provided as a refuge. All individuals were housed in an environmental chamber at 32°C on a 16h:8h light:dark cycle. Males were checked twice weekly to determine the date of final molt to adulthood. Upon eclosion, males were randomly assigned to one of two age classes, hereafter termed "young" or "old". Males assigned to the young age group were scheduled for infection cue treatment one week (± 2 days) following their adult eclosion, whereas males assigned to the old age group were scheduled for infection cue treatment three weeks (± 2 days) following their adult eclosion. As adult *G. sigillatus* live on average 3-4 weeks under field conditions (Sakaluk et al. 2002), this two-week age difference is biologically relevant. Adult male G. sigillatus begin mating 4.5 days on average after eclosion (Burpee and Sakaluk 1993b); because calling is essential for mating, it follows that males also begin calling at that time. Body mass for each male was measured immediately prior to infection cue administration using an analytical balance (Mettler Toledo AG245).

2. Infection cue

The triggering of an immune response acts to simulate an infection that may signal reduced residual reproductive value to the host, and the use of non-pathogenic immune elicitors is a preferred approach when investigating shifts in host life history strategies, as this technique eliminates the confounding effects of parasite proliferation, pathogenicity, or parasite manipulation. Thus, this approach ensured that calling effort investment was related to the host's strategy, and not the result of alternative causes related to a live infection. Earlier, we demonstrated that heat-killed *E. coli* invokes a terminal investment response in another form of reproductive effort in *G. sigillatus*, the composition of males' nuptial food gifts (Duffield et al. 2015); thus, a similar protocol was implemented here. Males from both age classes were randomly assigned to one of five treatments within an increasing spectrum of an infection cue: i) "naïve" (unmanipulated control), ii) "sham-control" (injection of 2µL ringer saline), iii) "low dose" infection cue (injection of $5x10^5$/mL heat-killed *E. coli* in 2µL ringer saline), iv) "moderate dose" infection cue (injection of $5x10^7$/mL heat-killed *E. coli* in 2µL ringer saline), or v) "high dose" infection cue (injection of $5x10^8$/mL heat-killed *E. coli* in 2µL ringer saline). Here we consider the sham-control treatment not only as a control for the effect of injection, but also as a low-level mortality threat because injection causes cuticle damage and induces insect immunity that potentially signals a mortality threat to the individual (Gillespie and Khachatourians 1992; Wigby et al. 2008; Ardia et al. 2012). Injections were performed using a 5µl syringe with a 1mm compression fitting (Hamilton® brand) within which a hollow-tipped heat-pulled glass capillary tube was inserted. Crickets were injected between the 6^{th} and 7^{th} pleurite of the thorax. Pulled capillaries were cleaned in

70% ethanol and rinsed with nanopure water between each injection. Treatments were always applied at the same time (0900 hours ±1h) throughout the experiment.

The *E. coli* strain (DH5α) used to create our low, moderate, and high infection cues was obtained from the American Type Culture Collection (Manassas, VA). The bacteria were cultured at 30°C in medium (10g bacto-tryptone, 5g yeast extract, 10g NaCl in 1000mL of distilled water, pH 7.2). To prepare bacterial suspensions for challenge injections, 1mL of an overnight culture was centrifuged (3000 rpm, 4°C, 10 minutes), the supernatant discarded, and replaced with sterile ringer saline. This procedure was repeated three times, and the concentration of bacterial cells was adjusted to the concentrations described earlier for each infection cue dose. The bacteria were then heat-killed (90°C, 5 minutes). Efficiency of the heat-killing was confirmed by plating out samples of the suspension on media agar.

To confirm that the heat-killed bacteria stimulated an immune response in our male crickets, we collected 5μL hemolymph (see *Assessing immune function* methods below) from a separate subset of adult male crickets (approximately 2 weeks old) 4 hours after an injection of a high dose ($5x10^8$/mL cells in 2μL ringer saline) of heat-killed *E. coli* (n=6), 4 hours after an injection of 2μL ringer saline (n=6), or from unmanipulated (naïve) males (n=6). Immediately following hemolymph extraction, hemolymph was diluted (5μL of hemolymph in chilled Grace's insect saline at 20μL or 60μL, respectively, to make counting of cell populations of different densities feasible), and hemocytes were counted at 400x magnification under a phase-contrast microscope with a hemocytometer (Fast-Read 102® plastic counting chamber) to assess the circulating hemocyte population size as a proxy for immune stimulation (King and Hillyer 2013; Stoepler et al. 2013). Counting was performed blind to treatment.

3. Assessing reproductive effort

We quantified calling effort (i.e., the amount of time males spent calling) over the two consecutive nights following infection cue treatment. Calling effort was measured using a custom-built high-throughput sound monitoring array (Bertram and Johnson 1998) in which each male-containing individual box (250mL) was fitted with a lid-mounted microphone (C1163, Dick Smith Electronics) and was placed within a Styrofoam enclosure to prevent crosstalk between containers. Following administration of infection cues, males were isolated and given 7 hours (± 1h) to acclimate prior to the start of recording trials. Recording periods started at 1700 hours and ended at 0900 hours each night; this period was chosen to capture calling effort at a time most relevant to female mate attraction (Walker 1983; Sakaluk 1987; Burpee and Sakaluk 1993*a*). The sound monitor sampled each microphone throughout the night every 2 seconds, and based on the binary output resulting from this protocol, total calling time was calculated for each male each night (Hunt et al. 2004).

3a. Survival and post-mortem measurements

Following the end of the two-night calling trials, males were returned to their individual rearing boxes where they were provided water *ad libitum*. To make monitoring survival tractable, individuals were food-deprived during this time, as lab-reared *G. sigillatus* are known to live for 2-3 months in the lab when fed *ad libitum* (Sakaluk 1987; Burpee and Sakaluk 1993*b*; Ivy and Sakaluk 2005), far longer than their longevity under more natural field conditions (Sakaluk et al. 2002). Mortality was monitored and recorded daily. Upon their death, the pronotum width and right femur length of experimental subjects were measured

as proxies for structural size, using a stereomicroscope (Nikon SMZ800) equipped with a digital camera and imaging software (Nikon NIS-Elements Documentation v. 4.20). These two measures were highly correlated (r = 0.84624; P < 0.0001), and therefore pronotum width was used as a proxy in subsequent analyses, due to its greater repeatability.

3b. Statistical analyses

The distribution of male calls indicated that calling effort measurements were zero-inflated and over-dispersed. There were also a large number of very short calls; as our sound monitor system is susceptible to false positives from outside noise or sudden movements of crickets within boxes, we removed an arbitrary number of seconds (in this case, 5) from every measurement. Any negative measurements were rounded to zero. We then added each male's measurements to get his total investment in calling effort over the measurement period.

We analyzed calling effort data with Bayesian methods using the R package MCMCglmm (Hadfield 2010), fitting a zero-altered Poisson (ZAP) model in R (R 3.2.3, R Core Development Team 2015). The ZAP model includes a logistic regression for the zeroes in the data, and an over-dispersed Poisson regression for the zero-truncated counts. This type of model enabled us to answer two distinct questions within a single statistical structure: 1) what factors affect whether a male chooses to call, and 2) if a male calls, what factors affect the amount of calling (Houslay et al. 2015, 2017)? Our predictors for the model were treatment (naive, sham, low, moderate, and high infection cue doses), age class (young and old males), and the interaction between treatment and age. We used binary dummy variables to specify whether or not an individual belonged to each treatment group (0/1), with 'naïve' as our reference level. We specified young and old age classes as -0.5 and 0.5, giving a mean of 0 and difference of 1 between categories for this predictor. By setting up our variables in this way, overall treatment effects are shown as differences from the naïve level, and effect sizes can be compared across both treatments and ages. Model terms are considered significant if their 95% confidence intervals do not cross zero. We used an uninformative prior, and ran the model for 420,000 iterations (with a 'burn-in' period of 20,000, and saving every 100th iteration). Autocorrelation was low among consecutive thinned observations and fixed effects for both the Poisson and zero-altered parts of the model. The Gelman-Rubin diagnostic for 3 model runs was equivalent to 1 for each predictor and the model as a whole, confirming that multiple runs converged upon the same posterior distribution (Gelman and Rubin 1992). Model results were robust to different prior specifications. Male survival was analyzed using a Cox proportional hazards model (SAS v. 9.4), with male age class and infection cue included as main effects.

4. Assessing immune function

To quantify investment in immune function at similar time points as was established during the calling effort trials, bactericidal activity was quantified from hemolymph that had been collected either 7 hours ("day 1") or 31 hours ("day 2") after infection cue treatment had been applied, in a second group of males of the same age and infection cue regimen described earlier. Males were cold-anesthetized, and hemolymph was collected by piercing the membrane under the dorsal pronotum plate with a sterile 25G needle, and 5uL of outflowing hemolymph taken with a pre-chilled glass microcapillary tube positioned at the puncture site. Collected hemolymph was then expelled into 45uL of phosphate buffer saline

(PBS: 8.74 g NaCl; 1.78 g Na_2HPO_4, $2H_2O$; 1,000 mL nanopure water; pH 7.0) and snap-frozen in liquid nitrogen. Samples were stored at -80°C for later analysis.

4a. Zone of inhibition assay

Although immune-challenged individuals in this study were injected with *E. coli*, preliminary assays resulted in no measurable antibacterial activity on plates seeded with *E. coli*. Therefore, antimicrobial activity was assayed from zones of inhibition induced by samples in petri dishes containing agar seeded with the Gram-positive bacterium, *Micrococcus luteus* (ATCC 4698) (see Sadd and Schmid-Hempel 2007 for methodological details). Briefly, *M. luteus* from a single colony on a streak plate were incubated overnight at 30°C in 7 mL of media (2.5g peptone and 1.5g meat extract in 500ml of nanopure water, pH 7.0). From this culture, bacteria were added to liquid media containing 1% agar held at 40°C to achieve a final density of $1.5x10^5$ cells/mL. Six milliliters of seeded medium were poured into a 100mm diameter petri dish to solidify. Sample wells were made using a Pasteur pipette (Volac D810) fitted with a ball pump, 2µL of sample solution thawed on ice were added to each well, and negative (PBS) control wells were included on each plate. Plates were inverted, incubated for 48h at 30°C, and then the diameter of inhibition zones was measured for each sample. The diameters of two inhibition zones, perpendicular to one another, were measured for each sample and averaged (measurements were performed blind to treatment). The area was then calculated from the averaged measurements.

4b. Statistical analysis

To analyze the antibacterial activity of hemolymph, we employed an analysis of covariance, with age class, infection cue, and their interaction as main effects, day as a covariate, and area of growth inhibition of *M. luteus* as the dependent variable, which was log-transformed to fit the assumptions of normality. Source population and body size measurements (body mass, pronotum width, and femur length) did not significantly affect zones of inhibition; thus, these effects were dropped from the final model.

Results

When accounting for differences due to variation in size as measured by pronotum width ($F_{1, 456}$ = 979.84, P < 0.0001), mass of young males, immediately prior to infection cue treatment, (Least-Squares mean ± SE = 229.97 ± 1.42) was significantly less than the mass of old males (Least-Squares mean ± SE = 235.88 ± 1.52) ($F_{1, 456}$ = 8.06, P = 0.0047). Injection with heat-killed *E. coli* stimulated an immune response in male crickets, as demonstrated by an increase in circulating hemocytes in hemolymph collected from individuals 4h post-injection (ANOVA: $F_{2, 15}$ = 12.11, P = 0.0013). Post-hoc comparisons (False discovery rate corrected for multiple comparisons) reveal that males injected with heat-killed *E. coli* had more circulating hemocytes (Least-Squares mean ± SE = 1.35×10^5 ± 1.34×10^4 hemocytes/µL hemolymph) than both naïve (Least-Squares mean ± SE = 5.96×10^4 ± 1.34×10^4 hemocytes/µL hemolymph; P = 0.0021) and sham-injected males (Least-Squares mean ± SE = 5.21×10^4 ± 1.33×10^4 hemocytes/µL hemolymph; P = 0.0026). Naïve and sham-injected males had similar levels of circulating hemocytes (P = 0.6938).

1. Reproductive effort

The calling effort of 508 males was measured over two consecutive nights following infection cue treatment (see Figure 2 for individual group sizes). Twenty-nine males died at some point during calling effort trials and were removed from further analysis; however, neither age (χ^2_1 = 0.390, P = 0.532) nor treatment (χ^2_4 = 1.049, P = 0.902) significantly affected survival through experimental trials. We found no significant correlation between the total amount of time spent calling and body size (femur length: r = 0.029, n = 454, P = 0.536, pronotum width: r = 0.020, n = 457, P = 0.676). However, we did find a small, but significant positive correlation between the total amount of time spent calling and body mass (r = 0.091, n = 508, P = 0.042); body mass was not significantly different across treatment groups ($F_{9,495}$ = 1.00, P = 0.439).

1a. Likelihood of calling (logistic regression for zeros)

Neither male age, infection cue dose, nor any of the interactions between these main effects significantly influenced the likelihood that a male would call during the calling period (Table 1A; Figure 2A). Any simplification of the logistic regression resulted in a poorer overall model fit, so all predictors were retained.

1b. Time spent calling (zero-truncated Poisson)

Male age and infection cue significantly altered the amount of time males spent calling over the call-recording period (Table 1B). Specifically, we found significant effects of the moderate infection cue dose (Table 1B), and interactions between age and both moderate and high treatments (Table 1B) on calling effort. Within the naïve treatment group, young males called significantly more than old males (Figure 2B). However, at moderate and high infection cue doses, old males called significantly more than young males (Figure 2B), diverging significantly from the pattern seen in unmanipulated young and old individuals.

1c. Survival post-calling trials

The interaction between pronotum width and body weight, potentially indicating condition, did not significantly affect survival (Wald χ^2_1 = 0.0002, P = 0.9902, interaction term removed from the final model). However, larger individuals, based on pronotum width, had decreased survival (Wald χ^2_1 = 7.7332, P = 0.0054) and heavier individuals had increased survival (Wald χ^2_1 = 45.1158, P <0.0001). The interaction between male age and infection cue did not significantly affect survival after calling trials (Wald χ^2_4 = 1.2085, P = 0.8767; n=440; interaction term removed from the final model). There was, however, a significant effect of age (Wald χ^2_1 = 14.2426, P = 0.0002; Figure 3A), in which the hazard ratio for mortality was 1.445 times greater in old males than in young males. Across both age classes, infection cue did not significantly influence male survival (Wald χ^2_4 = 6.9670, P = 0.1376; Figure 3B). The contrast between pooled naïve and sham treatment groups, and the 3 heat-bacteria treatments combined (low, moderate, and high), revealed a significant effect of the administration of heat-killed bacteria on survival (Wald χ^2_1 = 5.2374, P = 0.0221), where individuals injected with heat-killed bacteria died sooner than those receiving treatments without it (Fig. 3C).

2. Immune function

Antibacterial activity (as measured by the zone of inhibition) of hemolymph against *M. luteus* was quantified for 230 individuals (see Figure 4 for individual group sizes). Age significantly affected the size of zones of inhibition (ANOVA: $F_{1,229}$ = 7.65, P < 0.001; Figure

4), but neither infection cue (ANOVA: $F_{4,229}$ = 0.63, P = 0.428), nor the interaction between age and infection cue (ANOVA: $F_{4,229}$ = 1.52, P = 0.198; Figure 4), significantly altered antimicrobial activity. Hemolymph collected from old males, regardless of infection cue treatment, produced larger zones of inhibition (Least-Squares mean = 22.30mm (upper SE, lower SE = 2.00mm, 1.83mm)) than hemolymph collected from young males (Least-Squares mean=15.85mm, (upper SE, lower SE = 1.47mm, 1.35mm)). The day on which hemolymph was sampled (either the same day or the day after treatment) did not influence antibacterial activity (ANOVA: $F_{1,222}$ = 1.10, P = 0.295).

Discussion

Terminal investment has been demonstrated in numerous taxa and has been shown to be evoked by a variety of cues that signal reduced residual reproductive value (Part et al. 1992; Bonneaud et al. 2004; Hanssen 2006; Sadd et al. 2006; Velando et al. 2006; Descamps et al. 2007; Copeland and Fedorka 2012; Heinze and Schrempf 2012; Vale and Little 2012; González-Tokman et al. 2013; Thanda Win et al. 2013; Leventhal et al. 2014; Duffield et al. 2015; An and Waldman 2016; Brannelly et al. 2016; Hendry et al. 2016; Giehr et al. 2017). However, in a number of cases, the results are equivocal or even conflicting (Festa-Bianchet 1988; Langley and Clutton-Brock 1998; Ilmonen et al. 2000; Ahmed et al. 2002; Fessler et al. 2005; Leman et al. 2009; Reaney and Knell 2010; Koenig et al. 2017), suggesting that the conventional framework within which terminal investment has been addressed may be lacking. Recently, we proposed that the propensity for an individual to terminally invest (i.e. their terminal investment threshold) might depend on the internal state of the organism, its current external environment, or both (Duffield et al., 2017). Here, we demonstrate that the terminal investment threshold (i.e., the intensity of a threat required to elicit terminal investment) in male *G. sigillatus* is contingent on age. Older males exhibited a strategy of terminal investment when exposed to moderate and high doses of an infection cue, calling more relative to naïve individuals, but young males did not exhibit a terminal investment phenotype at any of the doses imposed in our study. The greater risk of mortality in old males relative to young males is consistent with assumed intrinsic changes in residual reproductive value with age, i.e. as individuals age, the per capita mortality rate increases, and thus residual reproductive value decreases. In contrast, we did not find evidence of increased reproductive effort resulting in a trade-off with antibacterial immunity as measured here, although antibacterial activity was found to be greater in older males when compared with young males across all infection cue treatments.

Several studies have shown that the propensity to terminally invest can depend on numerous intrinsic and extrinsic factors affecting individual residual reproductive value. For example, Velando et al. (2006) found an interaction between age and treatment with a non-pathogenic infection cue (lipopolysaccharide derived from *E. coli*) in male blue footed boobies (*Sula nebouxii*). Specifically, old males produced significantly fewer offspring than young males in control treatments, but elevated their reproductive success to similar levels as young males after treatment (young males did not alter their reproductive effort across treatments). In addition to age (Sanz et al. 2001; Copeland and Fedorka 2012; González-Tokman et al. 2013), other studies provide support for context-dependent terminal investment through significant interactions between treatment levels of a terminal investment trigger (e.g., immune stimulation) with a number of other factors, including genotype (Chadwick and Little 2005; Vale and Little 2012; Leventhal et al. 2014), time of season (Billman and Belk 2014; Roznik et al. 2015), geographic population (Rebar and Greenfield

2017), clutch size (Podmokła et al. 2014), food availability (Krams et al. 2015), and paternity assurance (Benowitz et al. 2013). In our study, old males show terminal investment, but young males do not. There are two explanations as to why we did not find evidence of terminal investment in young males, both of which are consistent with our theoretical framework: 1) only older males terminally invest or 2) the strength of the infection cue was not sufficiently high to elicit terminal investment in the young age class.

Consistent with our findings that the propensity to terminally invest depends on the severity of the terminal investment trigger, several studies have found evidence that individuals alter reproductive investment in a dose-dependent manner. For example, Hendry et al. (2016) found that female pea aphids (*Acyrthosiphon pisum*) increase reproductive rate only after administration of relatively high doses of a live pathogen (*Pseudomonas syringae*). However, aphids treated with the highest doses showed marked declines in offspring production, due largely to the high mortality rate caused by infection (Hendry et al. 2016). Additionally, terminal investment in pheromone attractiveness by male mealworm beetles is dependent on the severity of the immune challenge presented by implanted nylon filaments (Kivleniece et al. 2010; Krams et al. 2011).

We found that unmanipulated old males call significantly less than unmanipulated young males, which corroborates other studies demonstrating that specific calling characteristics vary with age in crickets (Cade and Wyatt 1984; Simmons and Zuk 1992; Brown et al. 1996; Bertram 2000; Hunt et al. 2004; Jacot et al. 2007; Judge et al. 2008; Fitzsimmons and Bertram 2011; Verburgt et al. 2011; Houslay et al. 2015). A decline in calling energetics with age could be due to degradation of muscles associated with forewings (Baker 1976; Sohal 1985) and decreased metabolic rate (Hack 1997); however, we found that immune-challenged old males in our study were able to increase their calling effort, even above that of young unmanipulated males. As such, our findings are consistent with predictions from the terminal investment hypothesis in which old males exhibit reproductive restraint under normal conditions, but increase reproductive effort when prospects of future reproduction diminish. Despite increasing total time spent calling, it could be that terminally investing old males are energetically constrained with respect to the quality or amplitude of song produced. Due to the logistic constraints of our call monitoring equipment, we could not simultaneously quantify the total time spent calling and song quality of males. Because the time spent calling and song quality likely trade-off due to energetic constraints, future work is needed to investigate sound quality parameters (for example, see Verburgt et al. 2011) that are known to influence female choice. Nevertheless, total time spent calling is critical for acquiring females (Hunt et al. 2004); thus, the increase in calling effort in terminally investing males as documented in this study should positively correlate with reproductive success.

Contrary to our prediction that terminal investment should result in a decrease in immune function due to the pervasive trade-off between reproduction and immunity (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Harshman and Zera 2007), we found no evidence of a significant interaction between age and infection cue treatment in their effects on antibacterial activity of the hemolymph. These results suggest that changes in calling effort arising from the interaction of age and infection cue do not trade-off against this measure of immunity. As the invertebrate immune system is comprised of multiple, complex pathways that can trade-off with one another (reviewed in Rolff and Siva-Jothy 2003), it will be necessary to perform a more comprehensive assessment of immune function before general conclusions can be drawn about trade-offs between reproduction and immunity in

the context of terminal investment in this system. We did find an effect of age on antibacterial immunity, with older males having greater antibacterial activity than younger males. This is in contrast to the idea of immune senescence, which has been demonstrated for certain immune parameters measured in other insect species (Franceschi et al. 2000; Doums et al. 2002; Zerofsky et al. 2005). However, increases in immune function with age have also been demonstrated (Wilson-Rich et al. 2008), and varied patterns may be the result of disparate changes across different components of immunity (e.g. Doums et al. 2002). It is also feasible that a selection bias operating among age categories could be responsible for increases in immunity, with higher quality individuals, particularly those exhibiting greater antibacterial activity, more likely to attain old age and thus be assigned to the older age treatment. Alternatively, older males could be shifting towards a more pro-inflammatory state with age (e.g. see Khan et al. 2017).

In conclusion, our work supports the existence of a dynamic terminal investment threshold, whereby the propensity to switch to a terminal investment strategy is influenced not only by the potential terminal investment trigger, but is also dependent on other factors that independently influence residual reproductive value. It is likely that a number of other intrinsic and extrinsic factors could modulate terminal investment. Our results demonstrate that terminal investment is not simply a static strategy but is instead dynamic, helping to underscore the plasticity inherent to certain components of life history.

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Table 1: MCMCglmm zero-altered Poisson (ZAP) analysis of calling effort in young and old male crickets (*Gryllodes sigillatus*) across a gradient of increasing infection cue intensity (naïve, sham, low, moderate, or high doses). Overall treatment effects are shown as differences from the naïve level.

A) Likelihood of calling (logistic regression for zeros)

Predictor	Estimate	Lower 95% C.I.	Upper 95% C.I.	*P*
(Intercept)	-0.40	-1.04	0.24	0.2245
Age class	-0.01	-1.79	1.82	0.9800
Sham	-0.44	-1.73	0.78	0.5015
Low	-0.61	-1.88	0.64	0.3555
Moderate	-0.67	-1.86	0.61	0.2890
High	-0.86	-2.17	0.44	0.1965
Sham x Age class	0.50	-1.93	3.23	0.6865
Low x Age class	-1.22	-3.67	1.27	0.3400
Moderate x Age class	-0.35	-2.76	2.19	0.7590
High x Age class	-1.35	-3.86	1.40	0.3050

B) Time spent calling (zero-truncated Poisson)

Predictor	Estimate	Lower 95% C.I.	Upper 95% C.I.	*P*
(Intercept)	**4.18**	**3.57**	**4.82**	**<0.001**
Age class	**-1.28**	**-2.55**	**-0.03**	**0.0495**
Sham	0.33	-0.53	1.25	0.4505
Low	0.44	-0.46	1.31	0.3450
Moderate	**1.03**	**0.20**	**1.94**	**0.0190**
High	0.17	-0.77	1.10	0.7315
Sham x Age class	1.60	-0.24	3.32	0.0800
Low x Age class	1.67	-0.04	3.49	0.0625
Moderate x Age class	**2.09**	**0.32**	**3.72**	**0.0155**
High x Age class	**3.11**	**1.23**	**4.94**	**<0.001**

Figure 1: Predictions based on intrinsic residual reproductive value (RRV) from the *dynamic terminal investment threshold model* (adapted from Duffield et al. 2017). At low threat levels, individuals invest intermediately in reproduction to balance the reproduction-immunity trade-off. As a threat increases, investment in immunity increases to combat the threat. Thus, costs of immunity necessitate a decreased reproductive investment. At high threat levels, past where resistance is ineffective (*terminal investment threshold*, vertical dashed line), a terminal investment strategy of increased reproductive investment is predicted. Intrinsic RRV is expected to influence this threshold, with the threshold dropping as intrinsic RRV decreases.

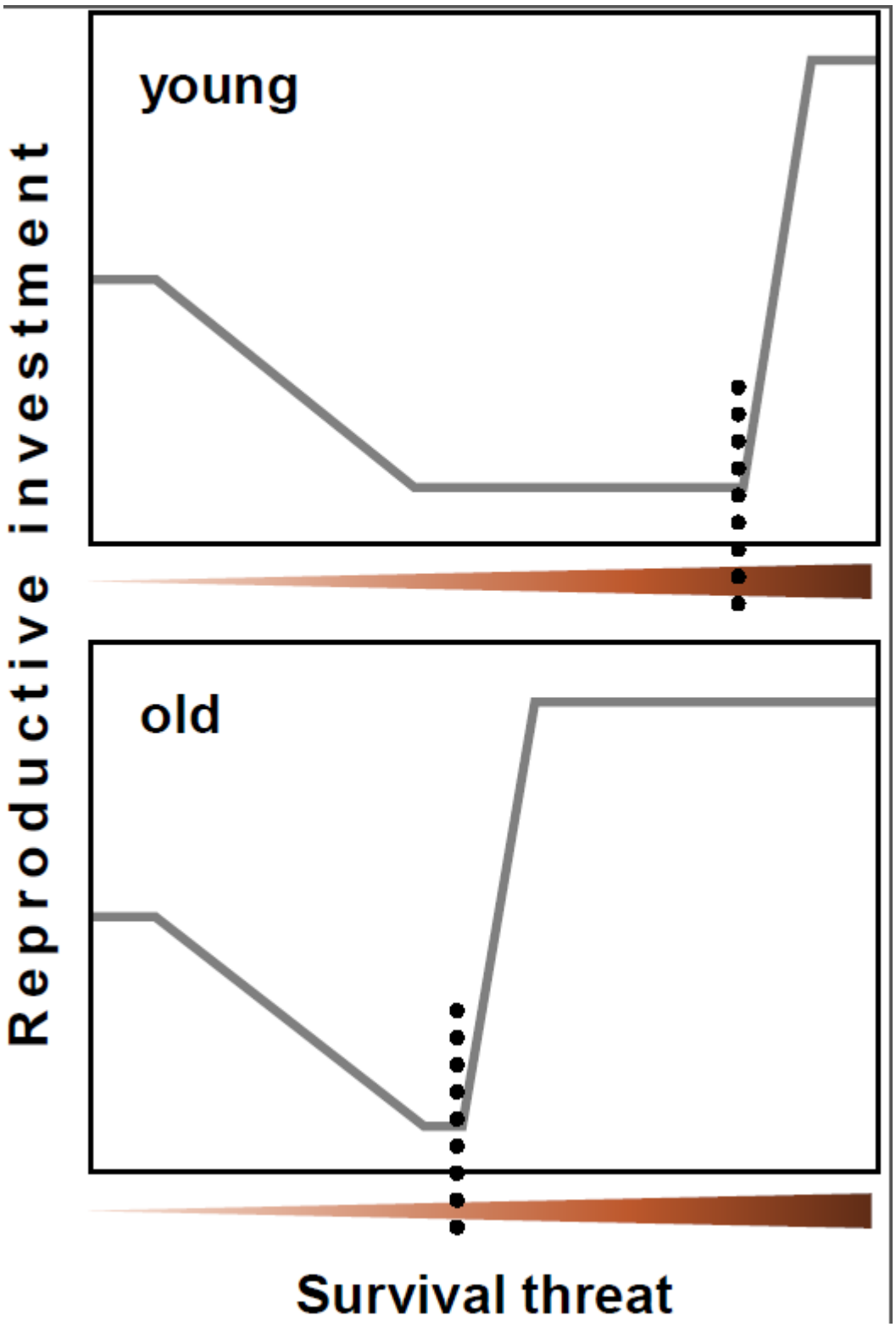

Figure 2: Model predicted effects of treatment (from a gradient of increasing infection cue intensity) and age class on the (A) likelihood of calling, and (B) time spent calling (given that a male did produce a call) in *Gryllodes sigillatus* crickets. Points show predicted effects with 95% confidence intervals, taken from MCMCglmm ZAP analysis.

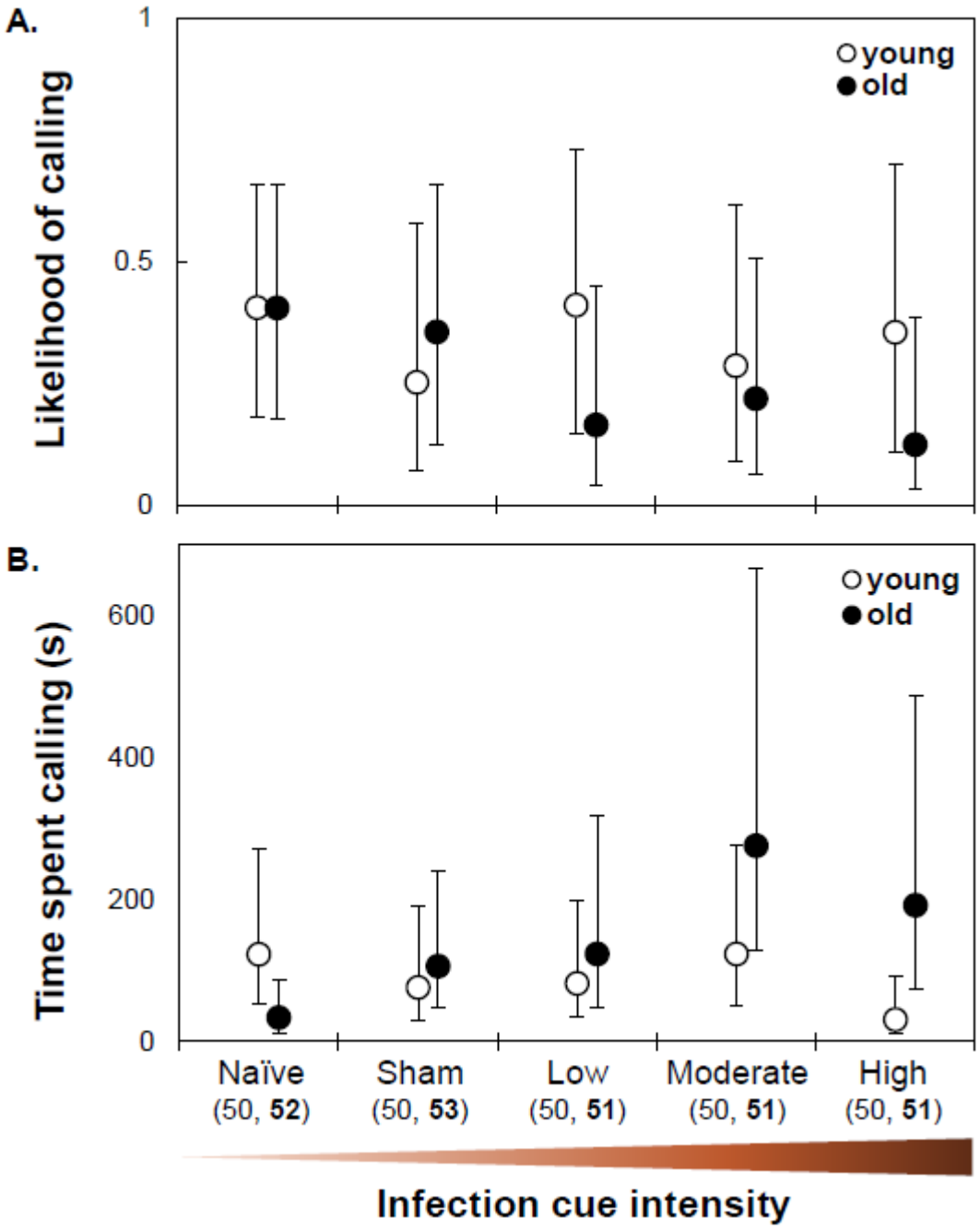

Figure 3: Survival of male crickets (*Gryllodes sigillatus*) across A) age categories, B) infection cue treatments, and C) between pooled treatments with (low, moderate, and high) and with no (naïve and sham) heat-killed bacteria. Asterisks (*) denote statistical significance ($P < 0.05$).

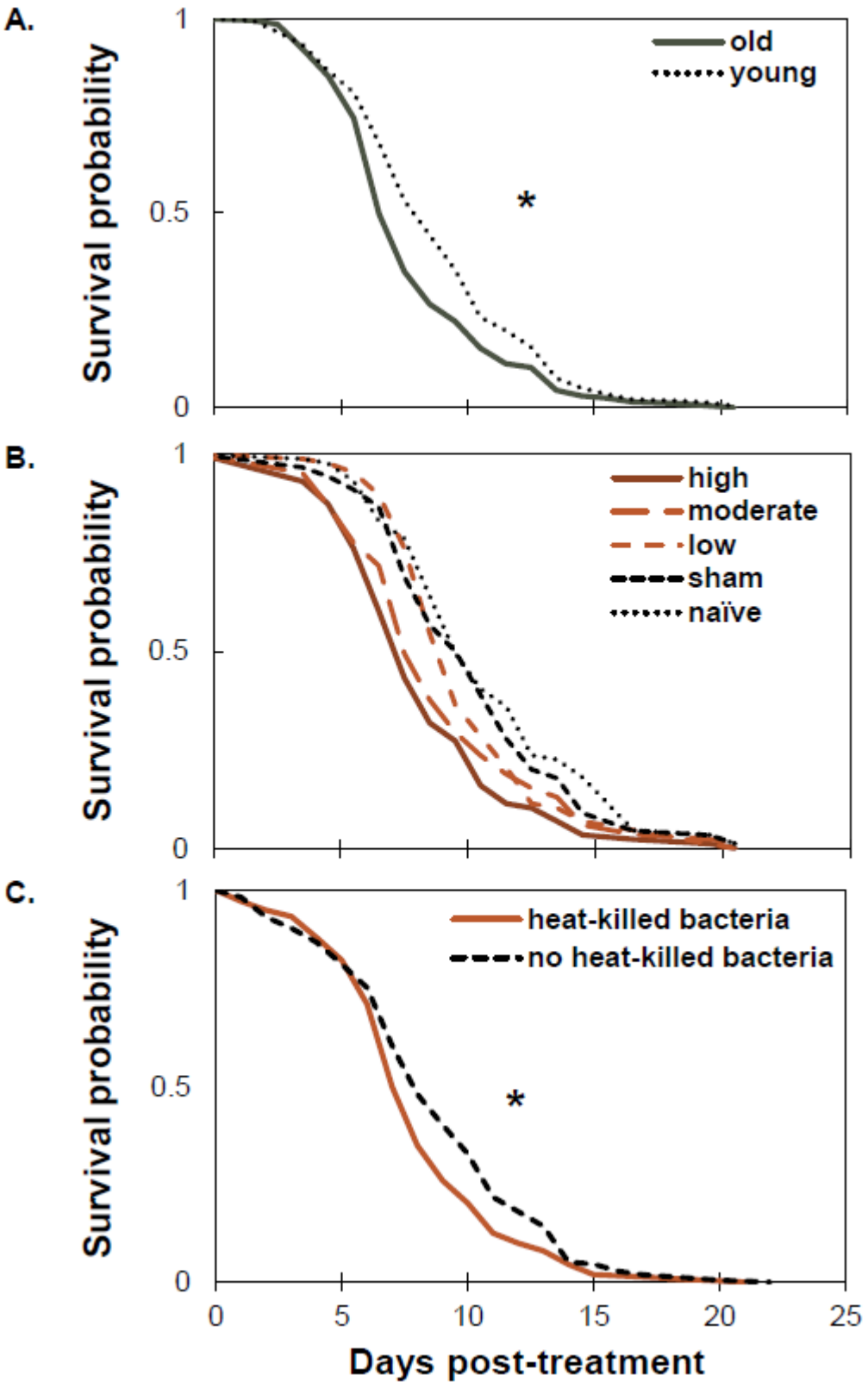

Figure 4: Hemolymph antibacterial activity against *Micrococcus luteus* (least-squares means ± standard error bars) across infection cue treatments for young and old male *Gryllodes sigillatus* crickets.