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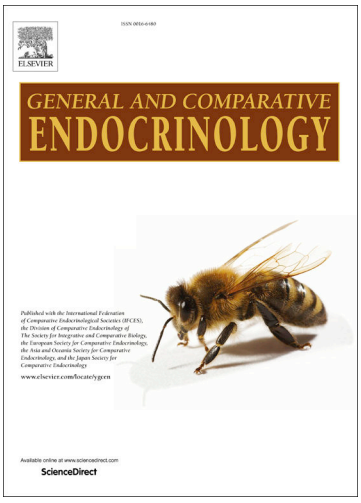

Research paper

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Relationships between avian malaria resilience and corticosterone, testosterone and prolactin in a Hawaiian songbird

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Abstract

Glucocorticoids, androgens, and prolactin regulate metabolism and reproduction, but they also play critical roles in immunomodulation. Since the introduction of avian malaria to Hawaii a century ago, low elevation populations of the Hawaii Amakihi (*Chlorodrepanis virens*) that have experienced strong selection by avian malaria have evolved increased resilience (the ability to recover from infection), while high elevation populations that have undergone weak selection remain less resilient. We investigated how variation in malaria selection has affected corticosterone, testosterone, and prolactin hormone levels in Amakihi during the breeding season. We predicted that baseline corticosterone and testosterone (which have immunosuppressive functions) would be reduced in low elevation and malaria-infected birds, while stress-induced corticosterone and prolactin (which have immunostimulatory functions) would be greater in low elevation and malaria-infected birds. As predicted, prolactin was significantly higher in malaria-infected than uninfected females (although more robust sample sizes would help to confirm this relationship), while testosterone trended higher in malaria-infected than uninfected males and, surprisingly, neither baseline nor stress-induced CORT varied with malaria infection. Contrary to our predictions, stress-induced corticosterone was significantly lower in low than high elevation birds while testosterone in males and prolactin in females did not vary by elevation, suggesting that Amakihi hormone modulation across elevation is determined by variables other than disease selection (e.g., timing of breeding, energetic challenges). Our results shed new light on relationships between introduced disease and hormone modulation, and they raise new questions that could be explored in experimental settings.

Keywords

Avian malaria, Hawaii Amakihi, immunomodulation, corticosterone, testosterone, prolactin

1. Introduction

The vertebrate immune system is highly dynamic. Changes in immunity occur over an individual's life, but also at shorter annual, seasonal, daily, and even hourly intervals. Previous research suggests that variation in immune function is the result of trade-offs with other energetically expensive processes, such as reproduction and growth (Martin et al., 2008). Vertebrate immune defenses are modulated by multiple internal and external variables (e.g., temperature, diet, social cues, reproductive state), many of which are integrated by and act through hormonal mechanisms (Demas and Nelson, 2012). Hormones are particularly effective regulators of immune function because their effects can be both direct (through hormone receptors on immune cells) and indirect (e.g., through energy allocation, aromatization; Koutsos and Klasing, 2014; Owen-Ashley et al., 2004).

Glucocorticoids have several fundamental functions, which include regulation of metabolism and energy mobilization, the stress response, development, osmoregulation, behavior, as well as immunity (Sapolsky et al., 2000). Corticosterone (CORT), the primary glucocorticoid in birds, reptiles, adult amphibians, and many rodents (Comendant et al., 2003; Gong et al., 2015; Narayan et al., 2013; Romero et al., 1998), can modulate antibody and inflammatory responses, regulate the expression of sickness behaviors, influence the growth, mortality, and gene expression of bacteria, and affect the size, cell numbers, and gene expression of immune organs (Koutsos and Klasing, 2014). Based on the magnitude and/or duration of CORT levels, immunity can either be suppressed or stimulated in birds (Martin, 2009). Over short periods of time (minutes to hours), high CORT levels generally prepare the immune system for enhanced activity through immunostimulation or immunoredistribution, but chronically high glucocorticoids (days to weeks) can suppress immunity (Koutsos and Klasing, 2014). For

example, bacterial killing increased significantly in red knots (*Calidris canutus*) experiencing an acute increase in CORT (Buehler et al., 2008), while spleen and bursa weights decreased significantly in chickens treated with elevated CORT for several days (Shini et al., 2010).

Androgens regulate the male reproductive axis, but they also affect the metabolism, neuronal growth, development, and immunity of male and female vertebrates (Duffy et al., 2000; Staub and De Beer, 1997; Trigunaite et al., 2015). Immunosuppression by testosterone is documented in mammals, reptiles, and birds (e.g., Derting and Virk, 2005; Klukowski and Nelson, 2001; Peters, 2000), effects including decreased antibody response, impaired macrophage activity, disrupted development of immune organs, increased susceptibility to immune challenges, and reduced cell-mediated immune responses (Davison, 2014; Fargallo et al., 2007; Koutsos and Klasing, 2014; Martin et al., 2008; Mase and Oishi, 1991). For example, testosterone treatment caused a decrease in T cell-mediated responses in male spiny-footed lizards (*Acanthodactylus erythrurus*; Belliure et al., 2004) and a decrease in antibody responses in superb fairy-wrens (*Malurus cyaneus*; Peters, 2000). However, while some studies link testosterone to lower immunity, other do not (e.g., Buchanan et al., 2003; O'Brien et al., 2018), and two meta-analyses found that the relationship between testosterone and immunity can vary by the study taxa involved, the indices of immunocompetence measured, and the study type (experimental versus correlational; Foo et al., 2017; Roberts et al., 2004).

Prolactin plays a key role in regulating parental care but also has over 300 described functions related to metabolism, osmoregulation, the onset of molt, and immunity (Angelier et al., 2016; Bole-Feysot et al., 1998; Martin et al., 2008; Scanes, 2015). Prolactin can affect immune organs (e.g., thymus, lymph nodes, spleen), stimulate proliferative responses of T cells, B cells, and natural killer cells, improve macrophage function, modulate inflammatory

responses, and reduce tumoricidal activity of natural killer cells (Martin et al., 2008; Skwarło-Sońta, 1992; Yu-Lee, 2002). For example, bacterial killing was positively correlated with prolactin in superb starlings (*Lamprotornis superbus*; Rubenstein et al., 2008), and prolactin increased the expression of pathogen-identifying receptors in rainbow trout (*Oncorhynchus mykiss*) cells *in vitro* (Peña et al., 2016).

Since the introduction of avian malaria to Hawaii a century ago, the disease has contributed to population collapses of the endemic avifauna, particularly the Hawaiian honeycreepers (Drepanidinae). Declines have been extensive at low elevation, where avian malaria and its mosquito vector (*Culex quinquefasciatus*) are prevalent (Atkinson et al., 2013). Fortunately, in recent years, stable populations of the native Hawaii Amakihi (*Chlorodrepanis virens*, hereafter referred to as Amakihi), a non-migratory, monogamous honeycreeper that provides biparental care, have emerged at low elevations (Spiegel et al., 2006; Woodworth et al., 2005). When first infected with avian malaria, honeycreepers experience an acute phase of infection, when parasite load increases rapidly to a peak. Mortality is very high in most species of honeycreepers during the acute phase. In individuals that survive, the acute phase is followed by a sharp drop in parasitemia, but low parasite loads then persist for life in a prolonged chronic phase of infection (LaPointe et al. 2012). A study on the windward side of Hawaii Island found that 85% of low elevation Amakihi were infected with avian malaria, but that mortality due to infection was significantly reduced in low compared to high elevation Amakihi populations (Samuel et al., 2015). Experimental infections revealed that while parasite load during the acute phase did not differ between high and low elevation Amakihi, mortality was significantly lower for low than high elevation birds, and body mass and food consumption during infection were significantly higher in low than high elevation birds (Atkinson et al., 2013). Since low elevation

Amakihi maintain parasite loads similar to high elevation birds during the acute phase but do not allow parasitemia to remain high permanently, these findings suggest that low elevation Amakihi have evolved increased resilience, defined as the set of properties that allow a host to recover its original health state following infection (Schneider, 2011), rather than increased resistance (the host's ability to limit pathogen burden; Schneider and Ayres, 2008) or tolerance (the host's ability to support high parasite load without enduring severe illness or death; Richardson, 2016). However, the mechanisms and consequences of Amakihi resilience remain largely unexplored.

To investigate relationships among avian malaria selection, infection and hormones with immunomodulatory functions, we measured circulating CORT, testosterone, and prolactin in 349 free-living Amakihi captured at three low elevation sites (< 700 m above sea level, asl), where avian malaria selection is strong and infection is high, and three high elevation sites (> 1500 m asl), where selection is weak and infection is low, on Hawaii Island from February-April (during the Amakihi breeding season) in 2017 and 2018. The Amakihi presents an excellent study system to examine relationships between disease selection and hormones because of the variation in avian malaria selection and the consistency in life-history strategy across elevation. We explored two non-mutually-exclusive hypotheses. First, our Immunosuppression Hypothesis states that the immunological benefits of low levels of hormones with immunosuppressive actions outweigh the reproductive and other survival benefits of high hormone levels in populations that have undergone selection by avian malaria. We predicted that low elevation Amakihi would have lower circulating baseline CORT and testosterone compared to high elevation Amakihi. In addition, we predicted that infected Amakihi would have lower baseline CORT and testosterone than uninfected Amakihi. Second, our Immunostimulation Hypothesis states that the immunological benefits of high levels of hormones with immunostimulatory

133 actions are greatest in populations that have undergone selection by avian malaria. We predicted
134 that low elevation Amakihi would have higher circulating prolactin and acute increase in CORT
135 (during the stress response) compared to high elevation Amakihi, and that infected Amakihi
136 would have higher prolactin and stress-induced CORT than uninfected Amakihi. By exploring
137 relationships between hormones and avian malaria in the field, our study sheds new light on the
138 immunomodulatory functions of CORT, testosterone, and prolactin in the context of introduced
139 disease and paves the way for future experimental investigations of these multi-function
140 hormones in Hawaiian songbirds.

141 ## 2. Materials and Methods

142 *2.1 Study species and sites*

143 The Amakihi is a small passerine nested within a clade of cardueline finches (Sibley and
144 Ahlquist, 1982; Eggert et al., 2008). Amakihi are nectarivorous (diet supplemented with insects,
145 particularly during breeding), relatively sedentary, and inhabit a wide range of habitats, from
146 native shrublands and rainforests to heavily modified housing subdivisions (Baldwin, 1953;
147 Lindsey et al., 1998). The Amakihi has a protracted breeding season that extends from
148 November to July. For high and low elevation birds, most breeding occurs in the winter and
149 spring months, but peak breeding can vary across years and may coincide with high nectar
150 availability (Ralph and Fancy, 1994; Samuel et al., 2015; van Riper III, 1987; Wingfield, 1993).
151 Amakihi are monogamous and can retain the same mate for multiple seasons, and pairs defend
152 territories during the breeding season. Only females incubate and brood, but both parents feed
153 young (van Riper III, 1987). Yearling (age 1 year) and adult (age 2+ years) birds can breed, but
154 yearling birds are less successful breeders (Lindsey et al., 1998; van Riper III, 1987). Nearly all
155 data on Amakihi breeding has been collected from three high elevation sites (> 1600 m asl) on

Hawaii Island so these patterns may differ in other habitats, and our brood patch data suggest that breeding may be later and/or less synchronous in low elevation birds (details below).

Samples were collected from Amakihi captured at three high elevation (1510-2080 m asl) and three low elevation (30-690 m asl) parks and reserves on the windward and leeward sides of Hawaii Island (Fig. S1, Table S1), as well as at several private properties. Sites were separated by a minimum of 9 km and an average of 60 ± 29 (s.d.) km, and genetic and natural history data suggest that birds from these sites are likely distinct from each other (Baldwin 1953; Eggert et al. 2008). Avian malaria infection (and likely selection) is highly dependent on temperature and rainfall (Samuel et al., 2011), but studies of avian malaria on Hawaii Island have been conducted almost exclusively on the windward side of the island, where annual mean rainfall is much higher than the leeward side of the island (Giambelluca et al., 2014). We sampled Amakihi on both the windward and leeward side of the island to obtain a more robust dataset.

2.2 Capture and blood sampling

Birds were captured using 3 m-high Japanese mist nets between 07:00 and 14:00. 349 blood samples were obtained from 342 individuals for hormone quantification (Table 1). Blood samples were taken by pricking the alar vein with a sterile 26G needle and collecting the blood into heparinized microhematocrit tubes. Baseline CORT samples (N = 133) were collected within 3 min of capture to obtain baseline or near baseline CORT levels (Wingfield and Romero, 2010). Additional CORT samples to measure stress responsiveness (hereafter referred to as stress-induced CORT) were collected at 20 and 60 min after capture from a subset (N = 74) of the birds measured for baseline CORT (Romero and Wingfield, 2015). Testosterone (N = 88) and prolactin (N = 128) samples were collected within 10 min of capture to minimize changes in response to capture (Angelier and Chastel, 2009; Deviche et al., 2012). Following Institutional

Animal Care and Use Committee (IACUC) blood collection policies (< 1% of body mass) and given the size of captured Amakihi (mean mass: 12.58 ± 0.061 g [s.e.m]), we were generally limited to measuring one hormone per blood sample. As we rarely recaptured birds (2017: N = 12/205, 2018: N = 19/215) and never sampled an individual more than twice, we did not have the statistical power to study individual variation, so samples for hormone analysis obtained from recaptured birds (N = 7) were assigned to different hormone assays to avoid individual duplicates within a hormone dataset. Blood samples were stored on ice for no more than 6 h before being centrifuged for 5 min at 13,000 g (Unico Powerspin MH Centrifuge, Dayton, NJ, USA). Plasma and packed cells were separated and stored at – 30 °C for a maximum of 20 months before use in hormone and malaria diagnostic assays, respectively. Following sample collection, each bird received a numbered aluminum U.S. Geological Survey leg-band. Wing chord and cloacal protuberance length (abdomen to cloacal tip) were measured using calipers (to the nearest 0.1 mm), mass was measured using a 30 g Pesola Scale (to the nearest 0.5 g), and fat stores (furcular and abdominal) were scored on a scale of 0 (lean) to 5 (fat, Kaiser, 1993). Sex and age (yearling, adult) were determined by visual inspection of plumage and presence of a brood patch (bare and vascularized region on the breast and abdomen of birds incubating eggs and brooding small young), which develop only in female Amakihi (Samuel et al. 2015). All capture, handling, and sampling was approved by the University of California IACUC (protocol no. 19297), the State of Hawaii Department of Land and Natural Resources (permit no. WL17-11, WL19-08, and Natural Area Reserves System and Forest Reserve access permits issued to GRN), and the U.S. National Park Service (permit no. HAVO-2016-SCI-0027, HAVO-2018-SCI-0001).

2.3 Hormone assays

CORT and testosterone were quantified using the radioimmunoassay described in Krause et al. (2015) at the University of California Davis. Plasma volumes of 15 µL for 3 min CORT, 10 µL for 20 min and 60 min CORT, and 35 µL for testosterone were used in extractions and to determine recoveries. Reconstituted steroids were assayed in 200 µL duplicates by adding 100 µL (~10^4 CPM) of tritiated CORT or testosterone (Perkin Elmer NET399250UC or NET370250UC, respectively, Waltham, MA, USA) and 100 µL of antibody (anti-CORT: MP Biomedicals 07120016, lot 3R3-PB, Solon, OH, USA; anti-testosterone: Fitzgerald 20R-TR018w, lot 01916, Acton, MA, USA). Assay samples and 100 µL aliquots of reconstituted steroids were combined with scintillation fluid (Perkin Elmer Ultima Gold 6013329, Waltham, MA, USA) and counted for 5 min or within 2% accuracy on a Beckman Coulter 6500 LS counter (Brea, CA, USA). Results were averaged across duplicates and corrected for individual sample recoveries. For CORT, mean recoveries were 87.74% ± 4.20 (s.d.), intra-assay variation (calculated using C.V. between duplicates) and inter-assay variation (calculated using C.V. among assay standards) were 5.12% and 6.75%, respectively, the limit of detection was 9.38 ± 0.61 (s.d.) pg per tube, and the mean bound to free ratio was 0.35. For testosterone, mean recoveries were 82.00 ± 7.44 (s.d.), intra-assay and inter-assay variation were 7.90% and 0.41%, respectively, the limit of detection was 2.42 ± 0.56 (s.d.) pg per tube, and the mean bound to free ratio was 0.37. We validated this assay for the Amakihi by plotting the antibody percent bound vs. hormone added for a pooled Amakihi sample and for the standard curve (Fig. S2A,B) and plotting the hormone added vs. hormone measured of the pooled Amakihi sample (Fig. S2C,D) for CORT and testosterone.

Prolactin was measured using a heterologous radioimmunoassay described in Angelier et al. (2006) at the Centre d'Etudes Biologiques de Chizé. 50 µL of plasma per sample (25 µL in

duplicate) was used in assays. Pooled Amakihi plasma samples produced a dose-response curve that paralleled chicken prolactin standard curves, validating the assay for Amakihi (Fig. S3). Samples were run in two assays and the intra- and inter-assay variations were 2.56% and 3.54%, respectively. The limit of detection was 0.45 ng mL^{-1}, and samples that fell below this limit (N = 1/128) were excluded from data analysis.

2.4 Malaria diagnostics

To determine the malaria infection status of individuals, we used a modified version of the nested polymerase chain reaction (PCR) method in Fallon et al. (2003). Purified DNA for PCR analysis was extracted from approximately 10 μL of packed red blood cells using the Zymo Quick-DNA Miniprep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's protocols. The first amplification was run using 5 μL purified DNA, and the second amplification was run using 1 μL of template from the first reaction. Amplifications were run in 25 μL volumes using Promega GoTaq®G2 polymerase (Promega North America, Madison, WI, USA) and using identical concentrations of all reagents as in Fallon et al. (2003), except 0.5 μM of each primer and 0.25 units of Taq polymerase were used in the second amplification. Cycling conditions were identical to those described in Fallon et al. (2003) except 20 cycles were run per amplification. Products from the second amplification were observed on 1.8% agarose gels. All reactions were run with a positive and negative control (infected Amakihi DNA and water substituted for DNA, respectively). All samples were run twice, and samples with different results for the two runs (N = 2/349) were run a third time to determine results. The sensitivity of this assay is estimated at one parasite per 10^5 red blood cells (Fallon et al., 2003).

2.5 Statistical analyses

Data were analyzed in R version 3.5.0 (R Core Team, 2019) using linear models (LM, lm function, base R) and linear mixed models (LME, lmer function, lme4 package). CORT and testosterone data were log transformed and prolactin data square root transformed to obtain normality of model residuals. The corrected Akaike's information criterion (AICc, AICc function, MuMIn package) and Akaike weights (ω_i, a measure of the relative likelihood of a given model, Weights function, MuMIn package) were utilized to compare the series of possible models and the null model (intercepts only) and to choose the best-fit models among them (Burnham et al., 2011). We considered all models within ΔAICc < 2 of the lowest scoring model to be statistically supported (Richards, 2005). Coefficient estimates (the change in response variable associated with a one-unit change in the predictor [continuous predictors] or compared to the reference intercept [distinct predictors]), standard errors, and 95% confidence intervals (CI) for parameters included in the best-fit models are reported (summary and confint functions, base R). Parameter estimates with CIs that did not include zero were considered statistically significant predictors of hormones levels. Post-hoc analyses were conducted using Tukey's Honestly Significant Difference test (emmeans function, emmeans package).

To investigate relationships between malaria selection and hormones or malaria infection and hormones, respectively, we tested for the effect of elevation (high, low) or malaria infection status (infected, uninfected) on response variables of baseline and stress-induced CORT, testosterone, and prolactin. Since malaria infections can alter hormone levels (Dunlap and Schall, 1995; Hanley and Stamps, 2002), we restricted analyses testing the effect of elevation to only uninfected individuals. Due to collinearity between malaria infection status and elevation, we restricted analyses testing the effect of malaria infection status to only low elevation individuals. When pertinent, based on established physiological relationships as well as data collection and

exploration, we included predictor variables of side-of-island (leeward, windward), age (yearling, adult) in testosterone and male prolactin analyses, body condition (Hayward and Wingfield, 2004) in CORT analyses, brood patch (presence, absence; Angelier et al., 2016) in female prolactin analyses, sex (Astheimer et al., 1994) in CORT analyses, year (2017, 2018) in CORT analyses, and biologically relevant interactions between age, brood patch, elevation, malaria infection, sex, and side-of-island. Models of stress-induced CORT also included bird identity as a random effect, sample collection time point (3 min, 20 min, 60 min), and interactions between time point and elevation, malaria infection, and sex. In addition, we excluded side-of-island because we were able to measure only one low elevation, windward bird. CORT data points from two sampled birds were excluded from analyses because their hormone levels were considered outliers by the median and interquartile deviation method and because they were potentially affected by acute noise exposure (dog barking), which can affect CORT levels, during the 20 min preceding sampling (Chloupek et al., 2009). Since prolactin and testosterone can vary significantly over the breeding season in passerines (Angelier et al., 2016; Wingfield et al., 1990), brood patch and cloacal protuberance size can be used as proxies for breeding status of females and males, respectively (Bailey, 1952; Wolfson, 1952). Brood patch presence is a reliable indicator of female Amakihi breeding status because they develop a brood patch in preparation for incubation and brooding (Lindsey et al., 1998). However, variation in cloacal protuberance size across breeding sub-stages can differ between and within species (Bears et al., 2009; Li et al., 2017; Morton et al., 1990; Sax and Hoi, 1998), and cloacal protuberance size can be a poor predictor of reproductive condition (Quay, 1986; Schultz et al., 2017). Due to the absence of data on relationships among cloacal protuberance size, testis size, and breeding status in Hawaiian honeycreepers, we chose not to include cloacal protuberance

length in analyses of male testosterone and prolactin, but these data are included in publicly available datasets. Since we were able to control for breeding status of females but not males, prolactin analyses were run separately for each sex. Very few females captured at low elevation had a brood patch and none of these could be included in prolactin analyses, so we were unable to assess variation in prolactin by brood patch presence in low elevation females. Due to collinearity between malaria infection and side-of-island, side-of-island was not included in analyses involving malaria infection status as a predictor variable. All results are presented as means ± s.e.m.

3. Results

3.1 Corticosterone

Overall, mean Amakihi baseline CORT was 3.46 ± 0.19 ng mL^{-1}. In analyses involving uninfected birds (N = 119), to investigate relationships between malaria selection and baseline CORT, elevation was excluded from the best-fit model, which included the main effects of body condition, sex, and year (ΔAICc = 1.8). Baseline CORT was significantly higher in males than females (estimate: 0.20 ± 0.093, CI: 0.020 to 0.39) and in 2018 than 2017 (estimate: 0.36 ± 0.092, CI: 0.18 to 0.54), but was not significantly affected by body condition (estimate: -0.091 ± 0.054, CI: -0.20 to 0.016). In analyses involving low elevation birds (N = 50), to investigate relationships between malaria infection status and baseline CORT, malaria infection status was not included in the best-fit model (ΔAICc = 2.6). Like above, baseline CORT was significantly higher in males than females and in 2018 than 2017 (Table 2). In addition, body condition was a significant predictor of baseline CORT (estimate: -0.31 ± 0.11, CI: -0.53 to -0.089).

Elevation, time point, and their interaction were included in the best-fit model of stress-induced CORT in uninfected birds (N = 67 per time point, ΔAICc = 3.1). CORT increased

315 significantly between capture restraint time points for birds from high elevation (Tukey's test, 3
316 to 20 min – estimate: -2.22 ± 0.086, CI: -2.43 to -2.02, 20 to 60 min – estimate: -0.32 ± 0.086,
317 CI: -0.53 to -0.12) and low elevation (Tukey's test, 3 to 20 min – estimate: -1.80 ± 0.10, CI: -
318 2.04 to -1.57, 20 to 60 min – estimate: -0.29 ± 0.10, CI: -0.53 to -0.058, Fig. 1). High elevation
319 birds had higher CORT than low elevation birds at the 20 min (Tukey's test, estimate: 0.37 ±
320 0.14, CI: 0.091 to 0.64) and 60 min (Tukey's test, estimate: 0.39 ± 0.14, CI: 0.12 to 0.67) time
321 points, but not at the 3 min time point (Tukey's test, estimate: -0.055 ± 0.14, CI: -0.33 to 0.22,
322 Fig. 1). Similar to baseline CORT results, males had higher stress-induced CORT than females
323 (estimate: 0.33 ± 0.10, CI: 0.13 to 0.53, Fig. 1) and body condition was negatively correlated
324 with stress-induced CORT (estimate: -0.18 ± 0.073, CI: -0.32 to -0.041). In analyses involving
325 low elevation birds (N = 31 per time point), malaria infection status was not a good predictor of
326 stress-induced CORT, and relationships between stress-induced CORT and the predictors in the
327 best-fit model (time point, sex, body condition, ΔAICc = 2.2) were similar to those in uninfected
328 birds (Table 2).

329 Integrated CORT during capture restraint is a measure of total CORT exposure during the
330 capture restraint protocol and calculated as area under the capture restraint curve (Romero and
331 Wingfield, 2015). Elevation, sex, and their interaction, as well as body condition, were included
332 in the best-fit model of integrated CORT in uninfected birds (N = 67, ΔAICc = 0.2). Integrated
333 CORT was higher in high than low elevation males (estimate: 0.50 ± 0.15, CI: 0.20 to 0.79) but
334 no differences were observed for females (estimate: 0.15 ± 0.19, CI: -0.23 to 0.52). Males also
335 had higher integrated CORT than females (estimate: 0.53 ± 0.15, CI: 0.23 to 0.83), and higher
336 condition birds had lower integrated CORT (estimate: -0.16 ± 0.078, CI: -0.32 to -0.0036).

Malaria infection status was not included in either of the supported models of integrated CORT in low elevation birds (N = 31, Table S2).

3.2 Testosterone

Mean male Amakihi testosterone was 1.73 ± 0.18 ng mL^{-1}. Age, and not elevation, was included in the best-fit model of testosterone in uninfected males (N = 67, ΔAICc = 0.8). Adult males had significantly higher testosterone than yearling males (estimate: 0.57 ± 0.27, CI: 0.028 to 1.12). The models including age and side-of-island as well as age and elevation were also supported by AICc (Table S3), but neither was a significant predictor of testosterone (windward vs. leeward – estimate: 0.33 ± 0.27, CI: -0.22 to 0.87, high vs. low – estimate: 0.28 ± 0.29, CI: -0.31 to 0.87). Age was included in the best-fit model of testosterone in low elevation birds (N = 37, ΔAICc = 1.6, Table 3), but malaria infection status and the interaction between age and malaria infection status were also included in an AICc supported model (Table S3). Post-hoc tests of the interaction between infection and age revealed that in adult males, testosterone trended higher in infected than uninfected individuals (Tukey's test, estimate: 0.54 ± 0.35, CI: -0.17 to 1.25), while in yearling males, testosterone was lower (but not significantly) in infected than uninfected birds (Tukey's test, estimate: -0.52 ± 0.53, CI: -1.61 to 0.56, Fig. 2).

3.3 Prolactin

Mean Amakihi prolactin was 40.28 ± 4.22 ng mL^{-1} in females and 22.81 ± 1.67 ng mL^{-1} in males. Elevation was excluded from the best-fit model of prolactin in uninfected females (N = 42), and brood patch was included as a main effect (ΔAICc = 1.8). Females with a brood patch had significantly higher prolactin than those without (estimate: 3.42 ± 0.48, CI: 2.46 to 4.39, Fig. 3A). By contrast, elevation was included in the best-fit model of prolactin in uninfected males (N = 63), as was age (ΔAICc = 1.8). Males from high elevation had significantly higher prolactin

than males from low elevation (estimate: 1.10 ± 0.44, CI: 0.33 to 1.65) and adult males had significantly higher prolactin than yearling males (estimate: 1.22 ± 0.40, CI: 0.48 to 1.95, Fig. 3B). In low elevation females (N = 12), malaria infection status was included in the best-fit model of prolactin (ΔAICc = 3.4). Infected females had significantly higher prolactin than uninfected females (estimate: 2.71 ± 0.96, CI: 0.57 to 4.85, Fig. 3C). In contrast, malaria infection status was excluded from the best-fit model of prolactin in low elevation males (N = 31, Table S4, Table 4).

4. Discussion

The goal of this study was to examine relationships between avian malaria (selection and infection) and three hormones with immunomodulatory functions (CORT, testosterone, and prolactin) to broaden our understanding of interactions between disease selection and hormone modulation, a topic that has received minimal attention. While testosterone did not vary significantly by elevation or malaria infection, relationships between stress-induced CORT and elevation as well as between prolactin and elevation and malaria infection emerged from our analyses. Our correlational study is the first to describe honeycreeper hormone levels across a disease gradient and provides insight into relationships between disease and hormones while raising new questions that will help to guide future investigations.

Our Immunostimulation Hypothesis predicted that prolactin and stress-induced CORT would be greater in low than high elevation Amakihi and greater in infected than uninfected Amakihi. While prolactin in females did not vary by elevation, prolactin was significantly higher in infected than uninfected low elevation females. These results could suggest that selection by avian malaria has resulted in the evolution of increased circulating prolactin, which can function as an immunostimulator, in infected individuals to facilitate increased immune function and

responses in birds actively combatting the disease. Alternatively, high prolactin in infected birds may be a physiological change caused by malaria parasites, as has been hypothesized in other malaria hosts (Dunlap and Schall, 1995). More robust sample sizes (particularly of infected females, N = 3), quantification of parasite loads in infected birds, and experimental manipulations of the causal effects of prolactin on Amakihi immunity would help to clarify these possibilities. Our results also revealed that Amakihi females with a brood patch had significantly higher prolactin than females without a brood patch, as would be expected given the critical function of prolactin in the development and maintenance of the brood patch and parental behavior (Angelier et al., 2016; Scanes, 2015).

In males, prolactin was significantly greater in high than low elevation individuals, which was opposite to the relationship we predicted with the Immunostimulation Hypothesis. However, this result must be treated with caution. One of the main weaknesses of our study was that we were unable to control for male breeding status in testosterone and prolactin analyses. We conducted our study during the Amakihi breeding season, but testosterone and prolactin can vary throughout the breeding season in passerines (Angelier et al., 2016; Wingfield et al., 1990), and while previous studies had detected similar breeding rates at high and low elevation in the months we sampled (Samuel et al., 2015), 35.71% (N = 35/98) of females we captured at high elevation had a brood patch compared to only 6.06% (N = 4/66) of females at low elevation. Since we also captured fewer recently hatched birds at low than high elevation (low: 1.15% [N = 2/174], high: 9.09% [N = 27/297]), our results may suggest that breeding is later or less synchronized at low elevation. As a result, differences in the timing of reproduction, rather than differences in selection by avian malaria, may explain some or all of the variation in male prolactin across elevation. Determining the breeding status of males (e.g., describing

406 relationships between cloacal protuberance size and testes mass or breeding sub-stage, observing
407 breeding behaviors) would help to clarify relationships between prolactin and elevation.
408 Contrary to our predictions, stress induced CORT was significantly greater in high than
409 low elevation birds and did not vary with avian malaria infection. Since acute increases in CORT
410 (e.g., minutes to hours, as observed in capture restraint) promote a variety of immunostimulatory
411 and immunomodulatory processes (Dhabhar and McEwen, 1999; Koutsos and Klasing, 2014),
412 we had instead expected stress-induced CORT to be greater in low elevation Amakihi
413 populations. Our results may therefore suggest that variation in the stress response by elevation
414 is the result of reproductive (rather than disease) trade-offs related to allostasis, the ability to
415 maintain or adjust homeostasis through environmental changes and across life-history stages.
416 When allostatic load (the costs necessary to maintain homeostasis) exceeds available energy, the
417 organism enters allostatic overload, an energetically costly state that can lead to death if
418 sustained. To ease the energetic deficit of allostatic overload, the hypothalamo-pituitary-adrenal
419 axis releases glucocorticoids (such as CORT), which can cause an individual to switch to an
420 Emergency Life History Stage (ELHS) that promotes survival and halts non-essential
421 physiological process and behaviors (McEwen and Wingfield, 2010). This response can be
422 adaptive, but sometimes it can be advantageous to modulate the tendency of switching to an
423 ELHS. For example, some seasonal breeders are less responsive to stressors during their
424 breeding than non-breeding life-history stage because entering an ELHS during breeding could
425 result in nest abandonment and a decrease in fitness (Holberton and Wingfield, 2003; Lendvai et
426 al., 2007), while CORT increases during breeding in some vertebrates with short breeding
427 seasons, potentially because breeding is more intense for those organisms (e.g., higher
428 competition for mates, territories) and higher CORT may be necessary to support the

429 accompanying high energy expenditures (Eikenaar et al., 2012; Hau et al., 2010). Brood patch
430 data from Samuel et al. (2015) and this study suggest that the breeding season may be shorter for
431 high than low elevation Amakihi, which could explain why higher CORT levels (necessary to
432 support the intense breeding behaviors of a shorter breeding season) were detected in high
433 elevation Amakihi. Alternatively, variation in Amakihi stress-induced CORT across elevation
434 may be related to differences in habitat conditions. Stress-induced CORT can be lower in
435 passerines living in habitats with more benign and stable conditions (Addis et al., 2011;
436 González-Gómez et al., 2018), potentially because energetic challenges are lower. Since
437 temperatures are substantially lower and are more variable at high than low elevation habitats in
438 Hawaii (Giambelluca et al., 2013), we would therefore expect stress-induced CORT to be greater
439 in high elevation Amakihi, as we observed. Measuring CORT levels in our study populations
440 outside the breeding season could help to tease apart these possible alternative explanations.
441 According to our Immunosuppression Hypothesis, we predicted that baseline CORT and
442 testosterone would be lower in low than high elevation Amakihi and in infected than uninfected
443 Amakihi because of the potential immunological benefits associated with low CORT and
444 testosterone. Baseline CORT did not vary with malaria infection. In some vertebrate species,
445 baseline CORT is higher in individuals infected with blood parasites than those that are
446 uninfected (Applegate, 1970; Hanley and Stamps, 2002), although an experimental manipulation
447 of malaria parasite load in chronically infected red-winged blackbirds (*Agelaius phoeniceus*) also
448 found no relationship between CORT and parasitemia (Schoenle et al., 2017). Baseline CORT
449 also did not vary by elevation, but analyses of birds at low elevation revealed a significant,
450 negative relationship between baseline CORT and body condition that was not detected in
451 analyses involving high and low elevation birds. CORT plays a fundamental role in regulating

452 metabolism and energy mobilization so this relationship was not surprising (Sapolsky et al.,
453 2000), but it is interesting that it was observed among only low elevation birds. Since negative
454 correlations between baseline CORT and body condition may be indicative of poor foraging
455 quality of a habitat (Foltz et al., 2015), our results may suggest that low elevation habitats are of
456 poorer quality. Further exploration of relationships among baseline CORT, body condition, and
457 foraging behavior in Amakihi could shed light on the quality of native bird habitats across
458 Hawaii Island.

459 As has been observed in some (Covino et al., 2017; Deviche et al., 2001; Morton et al.
460 1990) but not all (Peters et al., 2006) passerines, testosterone was significantly higher in older
461 (adult) than younger (yearling) males. Adult male Amakihi are more successful breeders than
462 yearling males (Lindsey et al., 1998; van Riper III, 1987), so this variation may be related to
463 differences in reproductive status. Testosterone did not, however, vary significantly with either
464 elevation or malaria infection, which may indicate a lack of interaction between testosterone and
465 avian malaria selection and infection in Amakihi, but these results should be treated with caution
466 because we were unable to identify the breeding sub-stage of males (discussed above) and
467 because our study did not involve testosterone manipulations. While experimental treatment of
468 superb fairy-wren (*Malurus cyaneus*) males with testosterone caused immunosuppression, free-
469 living birds with high testosterone were actually more immunocompetent (Peters, 2000),
470 possibly because high quality males are able to maintain both high testosterone and high
471 immunocompetence. If only high-quality males survived selection by avian malaria, we could
472 expect low elevation Amakihi to be more immunocompetent and maintain high or normal
473 testosterone levels, but testosterone may still cause immunosuppression. Experimental

manipulation of testosterone will be needed to determine whether the patterns observed in superb fairy-wrens also occur in Amakihi.

5. Conclusion

Introduced diseases can have devastating consequences for wild animals, but relatively little is known about how selection by introduced diseases affects hormone modulation. This study is one of only a few to describe relationships between disease selection and hormones in free-living vertebrates. In Amakihi populations on Hawaii Island, avian malaria selection and infection are greater at low than high elevation. As predicted, prolactin, which has immunostimulatory functions, was higher in infected than uninfected females (although higher sample sizes will be necessary to confirm this relationship). Testosterone, which can be immunosuppressive, tended to be higher in infected than uninfected males, while no relationship between baseline or stress-induced CORT and malaria infection was detected. Contrary to our predictions, stress-induced CORT was higher in high than low elevation birds, suggesting that variation in CORT by elevation may be related to differences in the timing of breeding or energetic challenges rather than disease selection. Our results provide some initial evidence for an interaction between malaria infection and prolactin, while suggesting that selection by avian malaria has minimal effects on hormone modulation. These findings raise new questions regarding relationships between disease selection and hormones that will help to guide future manipulative experiments.

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CRediT Authorship Contributions

Gabrielle Names: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Visualization, Writing - Original draft, Visualization, Supervision, Project administration, Funding acquisition, **Jesse Krause**: Methodology, Software, Validation, Investigation, Writing - Review & Editing **Elizabeth Schultz**: Methodology, Software, Writing - Original draft **Frédéric Angelier**: Conceptualization, Methodology, Resources, Writing – Review & Editing, Funding acquisition **Charline Parenteau**: Methodology, Validation, Investigation, Writing - Review & Editing **Cécile Ribout**: Methodology, Validation, Writing - Review & Editing, Supervision **Thomas Hahn**: Conceptualization, Methodology, Resources, Writing - Original draft, Funding acquisition **John Wingfield**: Conceptualization, Methodology, Resources, Writing - Original draft, Funding acquisition

Declaration of Competing Interests

Declarations of interest: none.

Supplementary Materials

Supplementary materials for this article will be available online upon acceptance.

Data Availability

Datasets will be made publicly available upon acceptance.

Figure Legends

Figure 1: Effects of elevation (high, low), sex, and time point (3 min, 20 min, 60 min) on stress-induced CORT (ng mL^{-1}) in free-living high and low elevation Hawaii Amakihi (*Chlorodrepanis virens*). CORT was higher in males than females (estimate: 0.33 ± 0.10, CI: 0.13 to 0.53; asterisk representing statistical significance), higher at 20 min (estimate: 1.84 ± 0.099, CI: 1.65 to 2.03) and 60 min (estimate: 2.08 ± 0.099, CI: 1.89 to 2.27) than at 3 min (ampersand), and lower in low than high elevation birds at 20 min (estimate: -0.37 ± 0.14, CI: -0.64 to -0.91) and 60 min (estimate: -0.39 ± 0.14, CI: -0.67 to -0.12; pound sign). Sample sizes are indicated on the figure. Values are presented as mean ± s.e.m.

Figure 2: Effects of age class (yearling, adult) and malaria infection status (infected, uninfected) on testosterone (ng mL^{-1}) in free-living low elevation male Hawaii Amakihi (*Chlorodrepanis virens*). Testosterone was higher in adult than yearling males (estimate: 1.24 ± 0.28, CI: 0.67 to 0.82) in the best-fit LM. The interaction between malaria infection status and age class was included in another AICc supported LM, and post-hoc analyses revealed that testosterone trended higher in infected than uninfected adult males (estimate: 0.54 ± 0.35, CI: -0.17 to 1.25). Sample sizes are indicated on the figure. Values are presented as mean ± s.e.m.

Figure 3: Effects of brood patch (presence, absence), malaria infection status (infected, uninfected), elevation (high, low), and age (yearling, adult) on prolactin (ng mL^{-1}) in free-living Hawaii Amakihi (*Chlorodrepanis virens*). (A) In females from high and low elevation, prolactin

was higher in birds with than without a brood patch (estimate: 3.42 ± 0.48, CI: 2.46 to 4.39). (B) In males from high and low elevation, prolactin was higher in high than low elevation birds (estimate: 1.10 ± 0.44, CI: 0.22 to 1.98) and in adult than yearling birds (estimate: 1.22 ± 0.40, CI: 0.42 to 2.03). (C) In low elevation females, prolactin was higher in infected than uninfected birds (estimate: 2.71 ± 0.96, CI: 0.57 to 4.85). Sample sizes are indicated on each panel. Values are presented as mean ± s.e.m.

Tables

Table 1: Sample sizes corresponding to investigations of the relationships between elevation and hormones (involving uninfected individuals, on the left) and between avian malaria infection and hormones (involving low elevation individuals, on the right) in Hawaii Amakihi (*Chlorodrepanis virens*).

	Uninfected				Low elevation			
	High		Low		Infected		Uninfected	
Hormone	Female	Male	Female	Male	Female	Male	Female	Male
Baseline CORT	31	50	14	24	3	8	14	24
Stress Series CORT (per time point)	11	29	10	17	2	2	17	10
Testosterone	NA	47	NA	20	NA	17	NA	20
Prolactin	33	48	9	15	3	16	9	15

Variable abbreviations are elevation (high, low) and malaria (infection status: infected, uninfected),

Table 2: Coefficient estimates and 95% confidence intervals for the best-supported models predicting CORT (baseline, stress-induced, and area under the capture restraint curve [stress-

induced area]) in free-living malaria-uninfected or low elevation Hawaii Amakihi (*Chlorodrepanis virens*).

	Predictor variables	Estimate	S.E.M.	2.5%	97.5%
Baseline	Intercept	0.79	0.079	0.63	0.95
Malaria-uninfected	Condition	-0.091	0.054	-0.20	0.016
	Sex Male	**0.20**	**0.093**	**0.020**	**0.39**
	Year 2018	**0.36**	**0.092**	**0.18**	**0.54**
Baseline	Intercept	0.51	0.15	0.20	0.82
Low Elevation	**Condition**	**-0.31**	**0.11**	**-0.53**	**-0.089**
	Sex Male	0.29	0.15	-0.019	0.60
	Year 2018	**0.46**	**0.14**	**0.17**	**0.74**
Stress-induced	Intercept	0.84	0.14	0.58	1.10
Malaria-uninfected	Elevation Low	0.055	0.14	-0.21	0.32
	Time 20	**2.22**	**0.086**	**2.06**	**2.39**
	Time 60	**2.54**	**0.086**	**2.38**	**2.71**
	Sex Male	**0.33**	**0.10**	**0.13**	**0.53**
	Condition	**-0.18**	**0.073**	**-0.32**	**-0.041**
	Year 2018	0.17	0.11	-0.45	0.38
	Elevation Low*Time 20	**-0.42**	**0.13**	**-0.68**	**-0.17**
	Elevation Low*Time 60	**-0.45**	**0.13**	**-0.71**	**-0.19**
	Sigma	0.13	0.37	0.32	0.41
	Sigma Bird	0.10	0.31	0.22	0.38
Stress-induced	Intercept	0.95	0.19	0.58	1.31

Low Elevation	**Time 20**	**1.84**	**0.099**	**1.65**	**2.03**
	Time 60	**2.08**	**0.099**	**1.89**	**2.27**
	Sex Male	**0.29**	**0.16**	**0.0011**	**0.59**
	Condition	**-0.27**	**0.11**	**-0.48**	**-0.061**
	Year 2018	0.028	0.17	-0.30	0.35
	Sigma	0.15	0.39	0.32	0.46
	Sigma Bird	0.11	0.33	0.19	0.44
Stress-induced Area	Intercept	6.85	0.16	6.53	7.17
Malaria-uninfected	Elevation Low	-0.15	0.19	-0.52	0.23
	Sex Male	**0.53**	**0.15**	**0.23**	**0.83**
	Condition	**-0.16**	**0.078**	**-0.32**	**-0.0036**
	Year 2018	0.23	0.12	-0.0038	0.47
	Elevation Low*Sex Male	-0.35	0.22	-0.79	0.089
Stress-induced Area	Intercept	6.87	0.19	6.49	7.26
Low Elevation	Sex Male	0.26	0.156	-0.065	0.57
	Condition	-0.18	0.11	-0.41	0.045
	Year 2018	-0.047	0.17	-0.40	0.31

Baseline and stress area were analyzed using LMs, and stress series using LMEs (random effect of bird identity). Statistically significant parameters are bolded. Variable abbreviations are condition (body condition), elevation (high, low), time (capture restraint time point: 3 min, 20 min, 60 min), and bird (individual identity).

559

560 **Table 3:** Coefficient estimates and 95% confidence intervals for the best-supported models
561 predicting testosterone in free-living malaria-uninfected or only low elevation Hawaii Amakihi
562 (*Chlorodrepanis virens*).

	Predictor variables	Estimate	S.E.	2.5%	97.5%
Malaria-uninfected	Intercept	0.23	0.21	-0.19	0.66
	Age Adult	**0.57**	**0.27**	**0.028**	**1.12**
Low Elevation	Intercept	0.49	0.17	0.14	0.85
	Age Adult	**1.24**	**0.28**	**0.67**	**0.82**

Analyses were conducted using LMs. Statistically significant parameters are bolded. Variable abbreviations are age (yearling, adult).

563

564 **Table 4:** Coefficient estimates and 95% confidence intervals for the best-supported models
565 predicting prolactin in free-living high and low elevation or only low elevation female and male
566 Hawaii Amakihi (*Chlorodrepanis virens*).

	Predictor variables	Estimate	S.E.	2.5%	97.5%
Females	Intercept	4.43	0.31	3.79	5.06
Malaria-uninfected	**Brood Patch Present**	**3.42**	**0.48**	**2.46**	**4.39**
Females	Intercept	4.12	0.48	3.05	5.19
Low Elevation	**Malaria Infected**	**2.71**	**0.96**	**0.57**	**4.85**
Males	Intercept	5.23	0.25	4.72	5.73
Malaria-uninfected	**Age Adult**	**1.22**	**0.40**	**0.42**	**2.03**
	Elevation Low	**-1.10**	**0.44**	**-1.98**	**-0.22**
Males	Intercept	4.16	0.25	3.65	4.67
Low Elevation	Age Adult	0.88	0.62	-0.39	2.15

Analyses were conducted using LMs. Statistically significant parameters are bolded. Variable abbreviations are age (yearling, adult), brood patch (presence, absence), elevation (low, high), and malaria (infection status: infected, uninfected).

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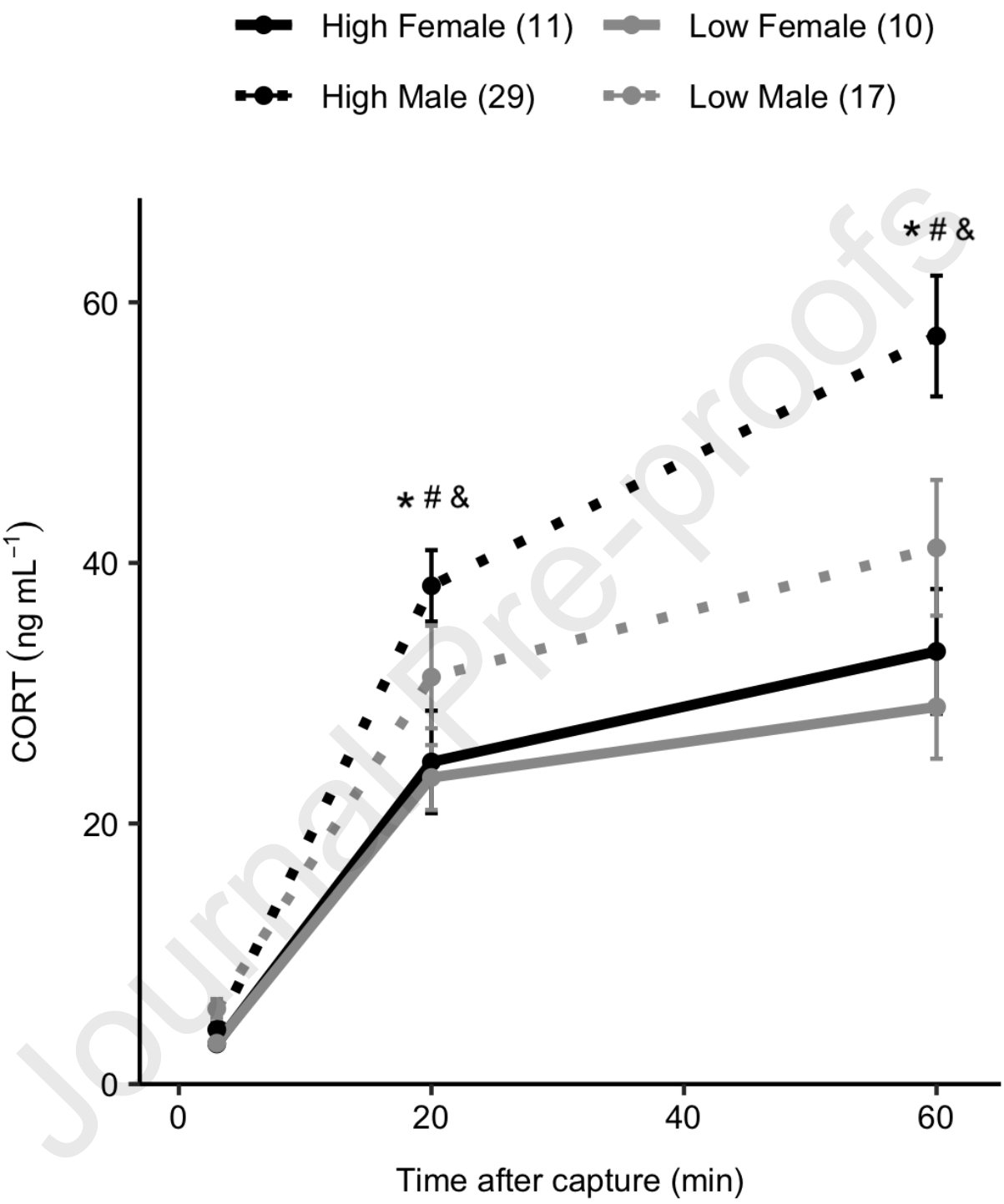
Highlights

- Avian malaria infection is greater in low than high elevation Amakihi populations.
- Stress-induced, but not baseline, CORT was greater in high than low elevation birds.
- Prolactin was higher in malaria-infected than uninfected females but not males.
- CORT and testosterone did not vary with malaria infection.



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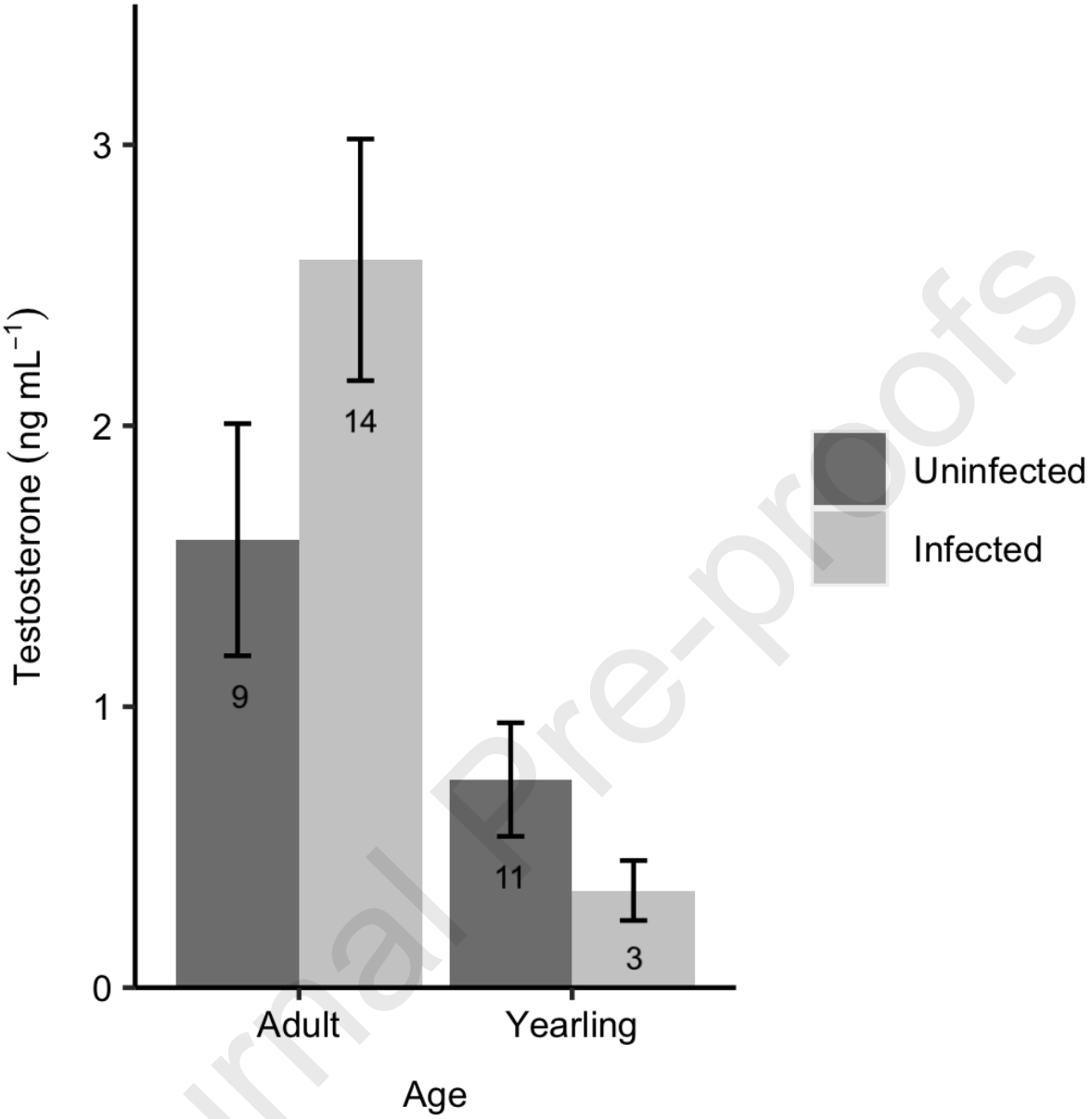

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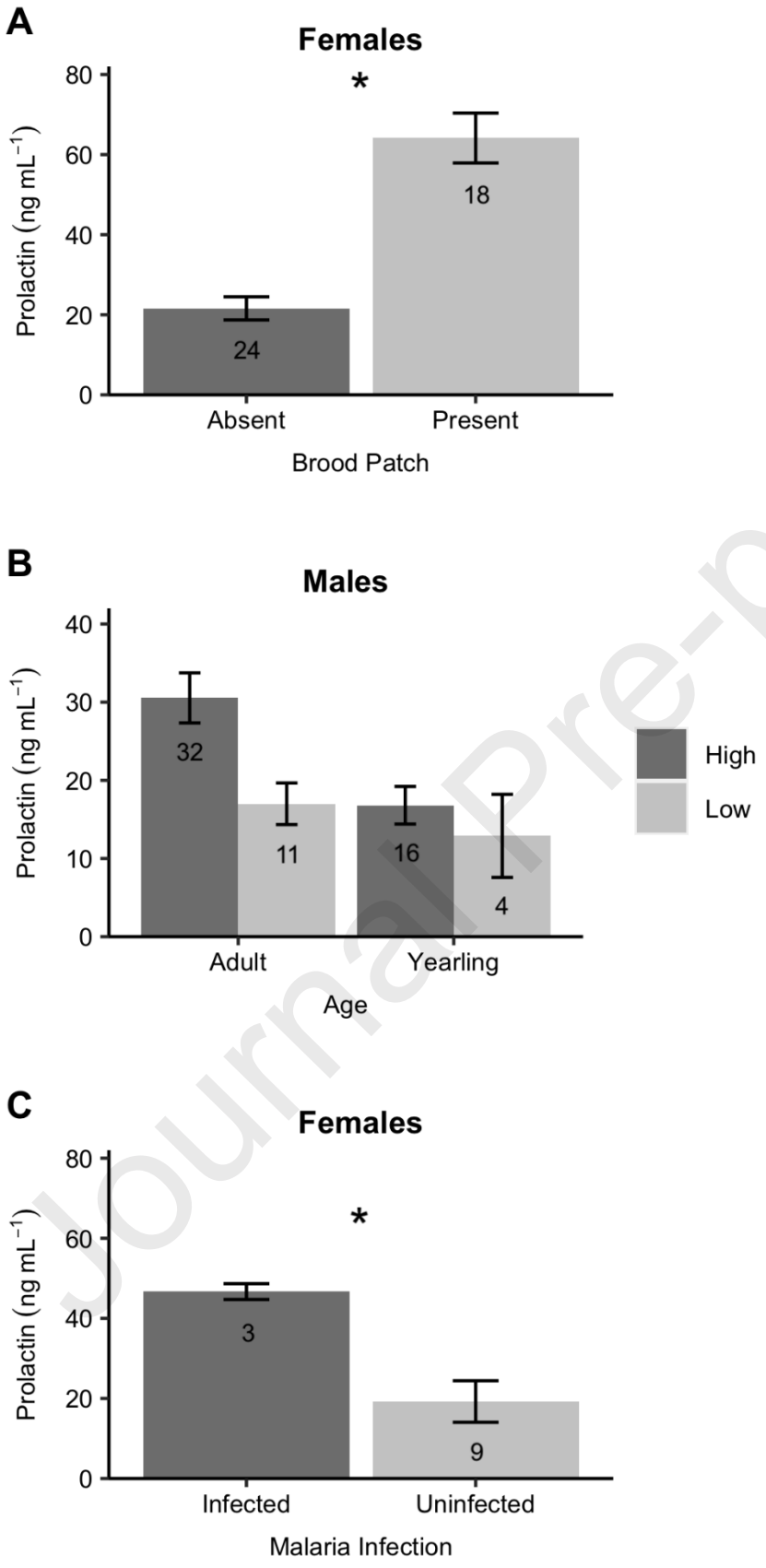

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