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RESEARCH ARTICLE

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Associations between testosterone and immune activity in alligators depend on bacteria species and temperature

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Abstract

1. The immunocompetence handicap hypothesis (ICHH) postulates that testosterone supports the development of secondary sexual traits while simultaneously suppressing immune function, creating a trade-off between trait quality and pathogen vulnerability.
2. The nature of interactions between testosterone and immunity is complex. Conflicting patterns from the literature suggest that testosterone–immunity relationships are variable across immune measures and may be modified by factors both intrinsic and extrinsic to the organism.
3. In this study, we tested the ICHH in free-ranging American alligators *Alligator mississippiensis* and examined how both intrinsic (steroid hormone levels) and extrinsic (temperature) factors modulate the relationship between testosterone and immunity. Specifically, we quantified the simultaneous effects of testosterone and dehydroepiandrosterone (DHEA) on microbial killing capacity of three bacteria species (*Escherichia coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae*) at two challenge temperatures (15°C and 30°C).
4. We found that accounting for circulating levels of DHEA was important for predicting testosterone-mediated effects on microbial killing capacity. We also found that testosterone-mediated immunosuppression was dependent on temperature and bacteria species, with negative effects of testosterone present only for *S. typhimurium* at 15°C.
5. Our results highlight the context dependency of interactions between testosterone and immunity, and illustrate the importance of evaluating the ICHH in natural systems to identify key intrinsic and extrinsic factors modulating testosterone-immunity trade-offs.

KEYWORDS

dehydroepiandrosterone, immunity, immunocompetence handicap hypothesis, microbial killing capacity, temperature, testosterone

1 | INTRODUCTION

Males often use elaborate secondary sexual traits (e.g. ornamental plumage, weapons) to enhance reproduction, but they simultaneously experience trade-offs between investment in the development of these traits and other physiological needs (Houslay et al., 2017). One widely hypothesized physiological mediator of the trade-offs associated with secondary sexual traits is testosterone.

Testosterone positively affects the development of secondary sexual traits, but can simultaneously suppress immune function, creating a trade-off between sexual signalling and vulnerability to pathogen infection (Folstad & Karter, 1991; Greives et al., 2006; Mougeot et al., 2004). This idea, formalized by the immunocompetence handicap hypothesis (ICHH; Folstad & Karter, 1991), implicates testosterone-mediated immunosuppression as a key mechanism facilitating honest signalling in males. However, current evidence suggests that interactions between testosterone and immunity are complex.

The complexity of testosterone-immunity relationships is evidenced by conflicting (i.e. both enhancing and suppressing) patterns reported from studies examining relationships between non-manipulated testosterone levels and components of the immune response (e.g. Ezenwa et al., 2011; Trumble et al., 2016). Indeed, a recent meta-analysis of 52 species spanning from fish to mammals found support for the ICHH for a subset of studies that manipulated testosterone, but there was no significant link between testosterone and immunity for non-manipulative studies (Foo et al., 2017). These findings suggest that while testosterone can indeed have suppressive effects on components of immune function, natural variation in testosterone is not always linked to clear changes in immunity. Therefore, identifying factors that potentially modulate the relationship between testosterone and immunity is central to understanding the relevance of the ICHH in natural systems.

A large number of factors that are both intrinsic and extrinsic to an animal, such as the activity of other hormones or abiotic factors linked to seasonality (e.g. temperature), may exert a strong influence on the relationship between testosterone and immunity. For example, dehydroepiandrosterone (DHEA) is a steroid hormone that serves as a precursor to testosterone that may play an important role in mitigating the immunosuppressive effects of its derivative. DHEA has been described as a 'low-cost' substitute for testosterone because of its ability to maintain a subset of testosterone-associated functions (e.g. aggression; Boonstra et al., 2008; Soma & Wingfield, 2001), without compromising immunity (Wingfield et al., 2001). In humans and laboratory mice, DHEA has been shown to stimulate certain components of immune function such as cytokine secretion (e.g. interleukin 2) and lymphocyte function (Hazeldine et al., 2010; Regelson et al., 1994); and more generally, DHEA treatment conferred protection against a range of viral, bacterial and protozoan infections in mice (Loria & Ben-Nathan, 2011). Consequently, it has been suggested that the immune-enhancing effects of DHEA may compensate for the immune costs of testosterone when both hormones are co-circulating (Owen-Ashley et al., 2004; Prall et al., 2015). Alongside the effects of intrinsic factors such as DHEA, extrinsic factors such as temperature may also influence associations between testosterone and immunity. Hormone synthesis, secretion and metabolism all depend on temperature (Van der Kraak & Pankhurst, 1996). Furthermore, thermal sensitivity in immune performance is well described in both ectotherms and endotherms (Butler et al., 2013). With temperature potentially acting on both hormone activity and immune performance, testosterone–immunity interactions may be strongly modified by seasonal variation in temperature.

In this study, we tested the ICHH in free-ranging American alligators *Alligator mississippiensis* and examined how both intrinsic (DHEA) and extrinsic (temperature) factors modulate the relationship between testosterone and immunity. Alligators are highly sexually dimorphic (Chabreck & Joanen, 1979; Reber et al., 2017; Vliet, 1989). During the breeding season males compete aggressively for access to females (Garrick & Lang, 1977; Vliet, 1989), and correspondingly, testosterone levels of adult males peak during this period (Hamlin et al., 2011). When compared to other crocodilians, alligators show potent innate immune defences against many bacterial species (Merchant et al., 2003, 2006; Zimmerman et al., 2013), but whether these defences are compromised by testosterone is unknown. Interestingly, in the only study to examine DHEA concentrations in free-ranging alligators, DHEA levels in males cycled seasonally in parallel with testosterone, but concentrations were consistently higher than testosterone except during the breeding season when levels of both hormones were similar (Hamlin et al., 2011; see Figure S1). Thus, given seasonal fluctuations in testosterone, robust innate immune responses to bacteria, and potentially compensatory DHEA levels, alligators represent an excellent model for testing the ICHH and the role DHEA may play in mitigating the immunosuppressive effects of testosterone. Furthermore, the fact that alligators are ectotherms whose physiological functions, including immune function (Butler et al., 2013; Merchant et al., 2003), are strongly dependent on environmental conditions provides a unique opportunity to examine how temperature affects associations between testosterone, DHEA and immune function.

To explore these questions, we quantified the simultaneous effects of testosterone, DHEA and temperature on the microbial killing capacity of alligator blood. We focused on three bacterial pathogens—*Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella typhimurium*—that range from commonly to rarely encountered by alligators in their natural environments (Johnston et al., 2009; Keenan & Elsey, 2015; Scott & Foster, 1997). Furthermore, we selected two temperatures at which to assess alligator microbial killing capacity that corresponded to the average minimum and optimal maximum body temperatures experienced by free-ranging alligators across a majority of their geographical range (15°C and 30°C; Lang, 1987; Seebacher et al., 2003). We predicted that: (a) the concentration of DHEA relative to testosterone would be a better predictor of immune performance than testosterone alone; (b) testosterone would correlate negatively with immune function; and (c) temperature would affect immunity in ways that modify correlations between testosterone, DHEA and immune function. To test these predictions, we compared suites of models with different combinations of testosterone and DHEA included as predictors of microbial killing. The model that best explained variation in killing ability for each pathogen was then used to assess relationships between testosterone, DHEA and immune function.

2 | MATERIALS AND METHODS

2.1 | Animals and sampling

Adult male alligators were captured from Merritt Island, Florida, between 2006 and 2010 with intense monthly sampling occurring in 2008–2009. Individuals were identified using numbered metal and passive internal transponder tags to identify recaptures (Hamlin et al., 2011). Blood samples and morphometric data, including snout-to-vent length (SVL), were collected for each individual at capture (Hamlin et al., 2011). Blood was drawn from the postcranial supravertebral sinus into heparinized vacutainer tubes. Plasma was isolated by centrifugation, stored at −20°C until hormone assays were performed, and then archived at −80°C prior to immunological assays. Only hormone and immune data from an individual's first capture were used in this study.

2.2 | Hormone assays

Testosterone and DHEA concentrations in alligator plasma samples were quantified using solid-phase radioimmunoassays as described in Hamlin et al. (2011). Briefly, plasma samples were extracted twice using diethyl ether and reconstituted in phosphate-buffered saline (PBS) with an average extraction efficiency of 94%–96%. Testosterone- (Fitzgerald Industries, REF# 20-1672) and DHEA- (REF# 20-1422) specific antibodies were used to coat the wells of a 96-well plate and then incubated at room temperature for 2 and 8 hr respectively. All wells then received 100 μl of the sample or standard and 12,000 cpm of ^{3}H-labelled steroid, followed by a 3-hr incubation at room temperature. Samples used to quantify inter-assay variation were prepared by pooling plasma from five adult male alligators. All standards and samples were run in duplicate. Plates were counted using a Microbeta 1450 Trilux counter and concentrations were extrapolated from standard curves as the percentage of bound versus log^{10} concentration. The average intra-assay coefficients of variation (CVs) was 3.8 for testosterone and 4.9 for DHEA, and the average inter-assay CVs for the two hormones were 8.68 and 9.84.

2.3 | Immune assays

To quantify bacterial killing ability (BKA) of plasma, we followed a spectrophotometer-based protocol described by French and Neuman-Lee (2012) with minor modifications. By using plasma, we were evaluating the combined effects of various humoral innate immune components, such as complement proteins, acute phase proteins and natural antibodies, on bacteria killing (Demas et al., 2011). Assay conditions for alligator plasma, including bacterial concentrations, challenge temperatures and incubation times, were optimized for three bacteria: *E. coli* (EPower Microorganisms, Microbiologics, REF# 0483E7, ATCC# 8739), *S. typhimurium* (KwikStik, Microbiologics, REF# 0363P, ATCC# 14028) and *K. pneumoniae* (EPower Microorganisms, Microbiologics, REF# 0684E7, ATCC# 10031). All optimizations were performed on a subset ($n = 20$) of the samples used in the larger study. Bacteria were challenged at two different temperatures: 15°C and 30°C. These two temperatures were selected to reflect the optimal body temperature of alligators during summer (30°C; Lang, 1987) and the minimum body temperature during winter (15°C; Seebacher et al., 2003). A set of four assays were run at each challenge temperature using two challenge times (30 and 60 min) and two bacterial concentrations (10^5 and 10^3). A 1:5 plasma dilution, optimized in prior experiments, was used for all assays.

Assay conditions that yielded the closest to 50% average killing were selected for use at each challenge temperature (Table S1). For *E. coli*, samples were challenged for 60 and 30 min at 15°C and 30°C, respectively, with a 10^5 bacterial concentration. For *S. typhimurium*, samples were challenged for 30 min at both 15°C and 30°C, with a 10^3 bacterial concentration. For *K. pneumoniae*, samples were challenged for 60 min at both 15°C and 30°C, with a 10^3 bacterial concentration. As part of our optimization process, we verified that for *S. typhimurium* and *K. pneumoniae*, bacterial growth did not differ under the two temperature conditions. Likewise, although two different incubation times were used for the *E. coli* assays run at 15°C versus 30°C, bacterial growth was comparable across these two sets of conditions. Bacteria were prepared by creating a 10^8 solution from plated colonies using the BD BBL™ Prompt™ Inoculation System, followed by dilution with PBS to the appropriate concentration: *E. coli*: 10^5 (~1,500–2,000 CFUs) and *S. typhimurium*, *K. pneumoniae*: 10^3 (~400–500 CFUs).

Assays were performed by adding 4 μl of plasma, 4 μl of bacteria and 16 μl of PBS to a single well of a 96-well plate, and each sample was run in triplicate. Positive controls were made by adding 4 μl of bacteria to 20 μl of PBS and negative controls consisted of 24 μl of PBS. Each plate contained eight positive and eight negative controls. Samples were mixed by vortexing each plate and plates were then incubated under appropriate challenge conditions (see Table S1). After bacterial challenge, 125 μl of tryptic soy broth was added to all wells and an initial, background, absorbance reading was obtained for each well prior to bacterial growth. Plates were then incubated for 12 hr at 37°C. Following incubation and homogenization, sample absorbance was re-read. For *E coli* and *S typhimurium*, sample wells were homogenized by vortexing. For *K. pneumoniae*, which forms biofilms, sample wells were simultaneously vortexed and stirred to facilitate homogenization. Finally, to calculate sample BKA, the following equation was used:

$$\mathrm{BKA} = 1 - \frac{(\text{Sample mean absorbance})}{(\text{Positive control mean absorbance})}.$$

Background absorbance values were subtracted from all post-incubation absorbance values prior to averaging. Absorbance was read at 300 nm on a standard microplate reader. CVs were calculated for sample and positive control replicates for the post-incubation read. Samples with a CV > 3 were re-run. The average intra-assay CVs for

samples across the three bacteria were 0.68 for *E. coli*, 0.47 for *S. typhimurium* and 1.04 for *K. pneumoniae*. For positive controls, entire plates with any replicates with a CV > 3 were re-run. The average intra-assay CV for controls across the three bacteria were 0.66 for *E. coli*, 0.60 for *S. typhimurium* and 0.82 for *K. pneumoniae*.

Finally, because our samples were collected from 2006–2010, introducing a lag time varying from 9 to 13 years between sample collection and processing, we tested whether sample collection year influenced our estimates of bacteria killing. Sample collection year was significantly associated with plasma killing ability against *S. typhimurium* (GLMM: $n = 667$, $F_{4,309} = 3.89$, $p = 0.004$) and *K. pneumoniae* ($n = 622$, $F_{4,306} = 4.54$, $p = 0.001$), with a trend towards higher killing in more recently collected samples (see Figure S2a,b). For *E. coli*, there was a marginal association between sample year and killing ability ($n = 622$, $F_{4,306} = 2.36$, $p = 0.053$), and no clear directional trend in the data (see Figure S2c). To account for this variation in killing ability related to sampling, we included sample year as a random effect in all statistical models.

2.4 | Statistical analysis

We examined the relationship between testosterone and immunity and the influence of two different factors on this relationship: DHEA and temperature. Analyses were performed separately for each bacterium. Killing ability scores for each bacterium were checked for normality using a Shapiro–Wilk test. BKA scores for both *S. typhimurium* and *K. pneumoniae* were approximately normal (*S. typhimurium*: $W = 0.980$, $p < 0.0001$; *K. pneumoniae*: $W = 0.947$, $p < 0.0001$), while scores for *E. coli* deviated substantially from normality ($W = 0.648$, $p < 0.0001$). As a consequence, we used a box cox transformation to normalize *E. coli* BKA scores ($W = 0.896$, $p < 0.0001$). We then used linear mixed models (LMMs) for all subsequent analyses and examined model residuals to assess model validity (Zuur et al., 2009).

As a first step, we tested for a possible role of DHEA in modulating relationships between testosterone and immune function by comparing four LMMs with different combinations of DHEA and testosterone as independent variables to identify which combination of these predictors best accounted for variation in killing ability for each bacteria. The models included either: (a) T only, (b) the ratio of T to DHEA [T/DHEA] only, (c) T + DHEA or (d) T + T/DHEA. In addition to these hormone-related predictors, the following covariates were also included in each model: challenge temperature, testosterone phase and SVL. Interactions between the hormone variables and each covariate were also included in all models. Testosterone phase was used to account for temporal variation in testosterone secretion. The phase designation (primary or secondary) identifies the two distinct testosterone peaks that occurred in the breeding and non-breeding seasons (Figure S1). The primary testosterone phase included samples collected during the first half of the year (January–June), which encompassed the build-up to the breeding season peak in April and subsequent decline; while the secondary phase included samples collected during the second half of the year (July–December), which encompassed the build-up to the non-breeding season peak in August and subsequent decline. SVL was used to account for variability in male size since larger males consistently have higher concentrations of testosterone than smaller males (Hamlin et al., 2011; Lance et al., 2015). Finally, sample ID and year were included as random effects in each model. Akaike's information criterion (AIC) was used to compare models. The model with the lowest AIC score was considered to be the best supported model, and models with a ΔAIC value ≤ 2 were considered to be of the same rank as the best model (Mazerolle, 2006). The top model was then used to interpret relationships between testosterone, DHEA, challenge temperature and immunity.

3 | RESULTS

3.1 | Hormone trends

Testosterone concentrations of male alligators in our study ranged from 0.01 to 1,000 pg/100 μl, while DHEA concentrations ranged from 13.9 to 623 pg/100 μl. Overall, there was a weak positive association between testosterone and DHEA (least squares regression, $n = 313$, $r^2 = 0.11$, $p < 0.001$; Figure 1), and this relationship was consistent during both the primary ($n = 178$, $r^2 = 0.10$, $p < 0.001$) and secondary ($n = 135$, $r^2 = 0.06$, $p = 0.002$) testosterone phases.

3.2 | Effect of hormones on immune function

Average BKA scores varied across bacteria (*E. coli*, *S. typhimurium* and *K. pneumoniae*) and challenge temperatures (Table 1). A comparison of

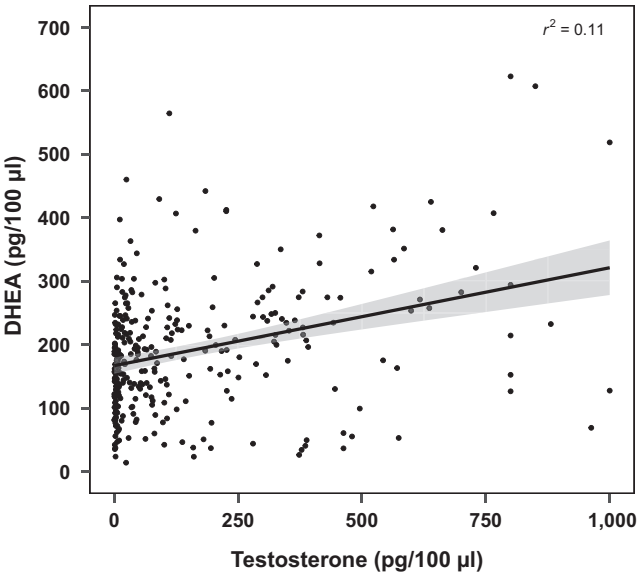

FIGURE 1 Relationship between testosterone and dehydroepiandrosterone (DHEA) across 313 male alligators

TABLE 1 Mean and range of bacteria killing scores for each temperature condition across all bacteria. Killing is represented on a scale of 0–1

Bacteria	Range	Mean	Temperature-dependent range		Temperature-dependent mean	
			15	30	15	30
Escherichia coli (ATCC# 8739)	0–1	0.29 ± 0.30	0–1	0–1	0.24 ± 0.24	0.34 ± 0.35
Salmonella typhimurium (ATCC# 14028)	0–0.35	0.13 ± 0.07	0–0.35	0–0.34	0.15 ± 0.07	0.11 ± 0.06
Klebsiella pneumoniae (ATCC# 10031)	0–1	0.64 ± 0.27	0–1	0–1	0.67 ± 0.26	0.61 ± 0.28

TABLE 2 Comparison of models explaining variation in *E. coli*, *S. typhimurium*, and *K. pneumoniae* plasma killing ability. Parameters from the best fitting model for each bacteria are shown in bold. Covariates = challenge temperature, testosterone phase and snout-to-vent length. Random effects = sample year and sample ID. See Table S2 for all candidate models

Testosterone model	*Escherichia coli* (*n* = 622)			*Salmonella typhimurium* (*n* = 625)			*Klebsiella pneumoniae* (*n* = 622)		
	K	ΔAIC	*df*	*K*	ΔAIC	*df*	*K*	ΔAIC	*df*
T/DHEA + Covariates + Interactions + [Random Effects]	**10**	**0**	**12**	**10**	**0**	**12**	**10**	**0**	**12**
T + Covariates + Interactions + [Random Effects]	10	39.0	12	10	43.7	12	10	37.2	12
T + T/DHEA + Covariates + Interactions + [Random Effects]	14	78.7	16	14	88.7	16	14	74.8	16
T + DHEA + Covariates + Interactions + [Random Effects]	14	116.5	16	14	121.9	16	14	106.4	16

four models including the effects of T alone, T/DHEA, T + DHEA or T + T/DHEA ratio showed that accounting for DHEA (T/DHEA ratio model) best predicted variation in BKA for all three bacteria (Table 2). Given this, we used the T/DHEA ratio models to investigate the effects of testosterone on immunity.

We found that the effect of testosterone on immune function depended on bacteria species and was modulated by challenge temperature. Specifically, testosterone only emerged as a significant predictor of BKA for one out of three bacteria, and when it did, the effect depended on temperature. For *S. typhimurium*, there was no main effect of T/DHEA on killing ability, but killing was significantly higher at 15℃ compared to 30℃ (LMM, $n = 625$; temperature: estimate = −0.0383, $p < 0.001$; Figure 2a; Table S3). Importantly, temperature interacted with T/DHEA such that a negative effect of having higher testosterone was apparent only at the 15℃ challenge temperature (T/DHEA × temperature: estimate = 0.0048, $p = 0.0056$; Figure 2b; Table S3). Interestingly, in the T + DHEA model including the independent effects of both hormones, the interactions between challenge temperature and T and DHEA were both significant (Table S6). In this model, testosterone had a negative effect on killing at 15℃ and no effect at 30℃ (Figure S3a), while DHEA had a positive effect on killing at 15℃ and no effect at 30℃ (Figure S3b). This result corroborates the pattern seen in the T/DHEA model, while also highlighting the opposing effects of T and DHEA on microbial killing.

For *E. coli*, challenge temperature was the only significant predictor of BKA (Table S4), but in contrast to *S. typhimurium*, killing of *E. coli* was significantly higher at 30℃ compared to 15℃ (LMM, $n = 622$, estimate = 0.0305, $p = 0.0019$; Figure 2c). Neither T/DHEA nor its interaction with temperature had any effect on BKA (Figure 2d; Table S4). Likewise, there was no independent effect of either T or DHEA on *E. coli* BKA apparent in the T + DHEA model (Table S7).

For *K. pneumoniae*, challenge temperature and testosterone phase emerged as the only significant predictors of BKA (Table S5). Killing of *K. pneumoniae* was significantly higher at 15℃ (LMM, $n = 622$, estimate = −0.0756, $p = 0.0011$; Figure 2e) and during the secondary testosterone phase (phase: estimate = 0.0834, $p = 0.0148$; Table S5). Neither T/DHEA nor its interaction had any effect on BKA (Figure 2f; Table S5). Similarly, there was no independent effect of either T or DHEA on *K. pneumoniae* BKA in the T + DHEA model (Table S8).

4 | DISCUSSION

Testosterone is often described as a 'double-edged' sword that facilitates the expression of secondary sexual characteristics while simultaneously suppressing immune function (Folstad & Karter, 1991). However, the magnitude of the trade-off between testosterone and immunity may depend on a range of intrinsic and extrinsic factors that modify the impact of testosterone on immune responsiveness. Here, we found that variation in BKA across three bacteria (*E. coli*, *S. typhimurium* and *K. pneumoniae*) was best explained when co-circulating levels of the hormone DHEA were simultaneously considered. This result suggests that DHEA may serve to modulate interactions between testosterone and immunity. We also found that testosterone–immunity relationships depended on both bacteria species and challenge temperature. Of the three bacteria we examined, *S. typhimurium* was the only one for which testosterone had a

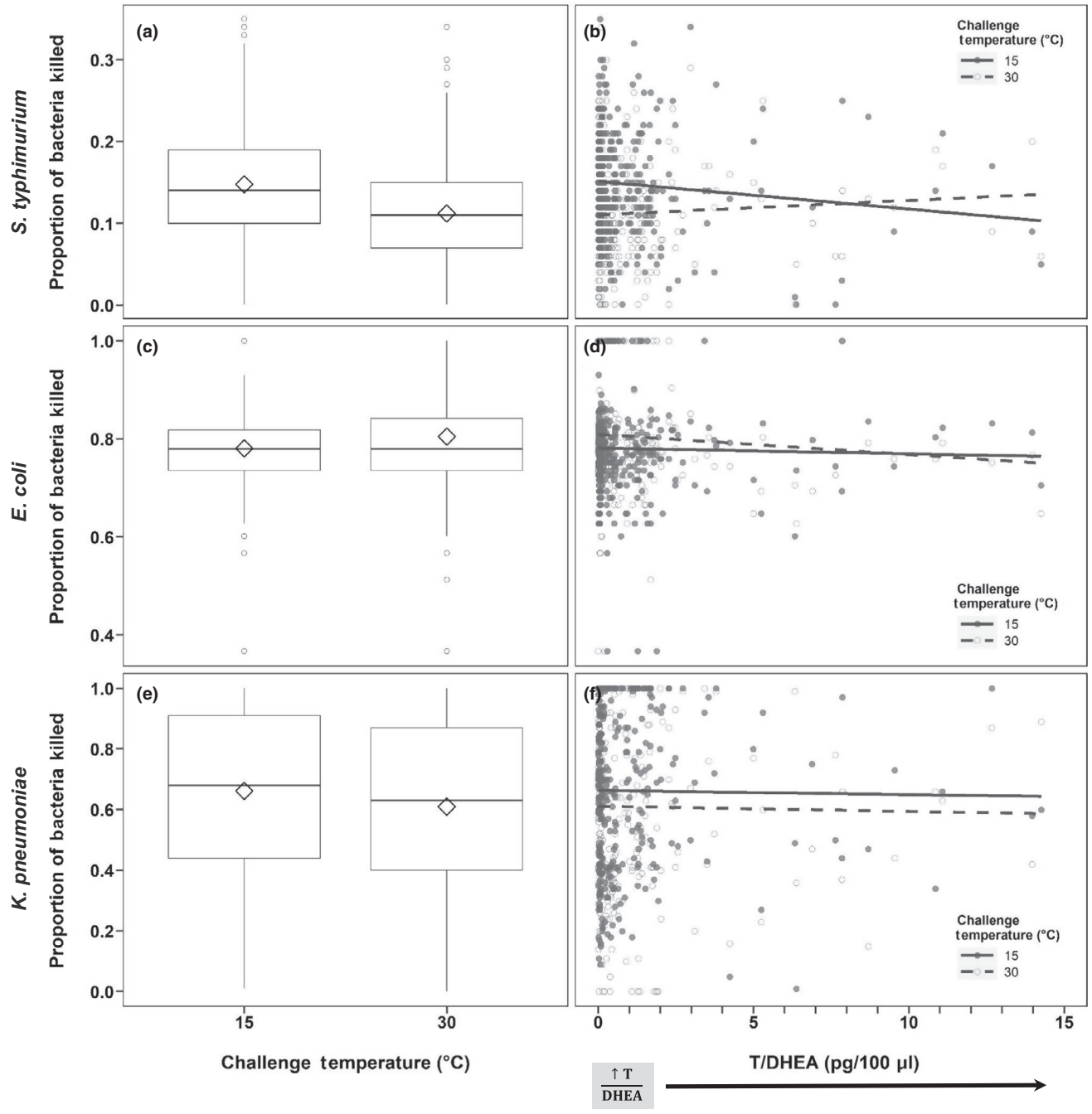

FIGURE 2 (a, c, e) A comparison of killing ability for three bacteria at two challenge temperatures (15°C and 30°C). (b, d, f) The relationship between bacterial killing and T/DHEA across both challenge temperatures (15°C: solid; 30°C: dashed). For *Escherichia coli*, the transformed BKA scores are shown. BKA, bacterial killing ability; DHEA, dehydroepiandrosterone

significant negative effect on plasma killing capacity. Moreover, this effect was only apparent at 15°C and not at 30°C, highlighting the role abiotic factors, like temperature, can play in shaping testosterone–immunity trade-offs.

Our results suggest that factors that are intrinsic to an animal, such as circulating levels of other steroid hormones that may interact with immune function or testosterone, should be accounted for when evaluating relationships between natural variation in testosterone levels and immune function. We found that correcting testosterone levels for relative levels of DHEA improved the explanatory power of testosterone as a predictor of plasma killing ability across all bacteria. A low ranked *S. typhimurium* model including the independent effects of T and DHEA supported the presence of opposing effects of these two hormones on immune function (Figure S3; Table S6). Furthermore, the T/DHEA ratio model was consistently supported across bacteria suggesting that co-circulating levels of DHEA may modulate interactions between testosterone and immunity. For instance, many immune responses reported to be suppressed by

testosterone have also been reported to be enhanced by DHEA (e.g. T-cell production, immune cell cytotoxicity and natural killer cell activity; Hazeldine et al., 2010; Khorram et al., 1997; Suzuki et al., 1991). Particularly relevant to our results, testosterone has been shown to suppress levels of antibody production (e.g. in humans and birds; Furman et al., 2014; Greives et al., 2006; Kocar et al., 2000), while DHEA has the opposite effect (e.g. in humans and mice; Khorram et al., 1997; Prall & Muehlenbein, 2015; Sakakura et al., 2006; Zhang et al., 1999). Natural antibodies are non-specific immunoglobulins that can provide immediate defences against pathogens, contributing significantly to microbial killing in plasma (Baumgarth et al., 2005; Kohler et al., 2003; Ochsenbein et al., 1999). Thus, in our study, an opposing effect of T and DHEA on natural antibodies may help explain the effect we saw on plasma microbial killing. More generally, co-circulating DHEA may play an under-appreciated role in modifying the immunosuppressive effects of testosterone.

Another steroid hormone that has been widely proposed as a modulator of testosterone–immunity relationships is corticosterone, which is a stress-related hormone. Under the stress-linked immunocompetence handicap hypothesis (SL-ICHH), corticosterone is suggested to interact with testosterone to drive immunosuppressive effects (Evans et al., 2000; Poiani et al., 2000; Roberts et al., 2007). Interestingly, DHEA has been shown to counteract immunosuppressive effects of glucocorticoids (Hazeldine et al., 2010), suggesting an additional mechanism by which DHEA may lessen testosterone–immunity trade-offs. Non-steroid hormones are also potential players in testosterone–immunity trade-offs. For example, leptin, a hormone produced by adipose tissue that serves as an indicator of energy availability, has long been considered likely to mediate energy allocation between reproduction and immunity (Drazen et al., 2001). More recent discoveries also suggest that leptin may mediate testosterone–immunity trade-offs by attenuating the immunosuppressive effects of testosterone via its immuno-enhancing properties (Alonso-Alvarez et al., 2007; Ashley & Demas, 2017).

Scrutiny of the best fitting models for all three bacteria further revealed the key role of an extrinsic factor, in this case temperature, in shaping the outcome of testosterone–immunity relationships. We show that challenge temperature was a significant predictor of killing ability for all three bacteria we examined. Intriguingly, the temperature at which killing was highest differed among bacteria. The highest killing of *S. typhimurium* and *K. pneumoniae* occurred at 15℃, while highest killing of *E. coli* occurred at 30℃. Since our optimization process verified that bacterial growth in control samples was comparable across temperature treatments, these differences cannot be attributed to temperature-specific variation in bacterial growth. Host immune performance has been linked to temperature in a range of animal taxa (Hanson, 1997; Nikoskelainen et al., 2002; Rios & Zimmerman, 2001; Rollins-Smith & Woodhams, 2012). For instance, in alligators, complement activity, via the alternative pathway, declines at temperatures below 15℃ or above 30℃ (Merchant et al., 2005). Given that our three focal bacteria species elicit unique host immune responses (Broz et al., 2012; Lebeis et al., 2008; Paczosa & Mecsas, 2016), differential effects of temperature on these responses may explain variability in host immune performance at 15℃ versus 30℃ across the different bacteria.

Beyond temperature dependency of the host immune response, pathogens themselves use a variety of mechanisms to evade host immunity, some of which may be thermally sensitive. For instance, *K. pneumoniae* and *S. typhimurium* form protective biofilms when exposed to stressors triggered by host immunity (Tutar et al., 2015), but the process of biofilm production may be inhibited under low temperatures (Nguyen et al., 2014), leaving bacteria more vulnerable to immune attacks. Some pathogens also use temperature cues to regulate expression of virulence genes, often reducing pathogenic activity at temperatures outside those found in their preferred hosts (Lam et al., 2014). Such temperature-dependent virulence has been described in *S. typhimurium*, which shows reduced virulence at 25℃ compared to 37℃, likely due to its tight association with endothermic hosts (Duong et al., 2007). Both reduced pathogen defence and lower virulence may explain increased killing of *K. pneumoniae* and *S. typhimurium* at 15℃. Temperature can also influence the effectiveness of pathogen immune evasion. For instance, *E. coli* often use capsule formation to defend against serum bactericidal effects (Miajlovic & Smith, 2014). In alligators, phospholipase A_2, an enzyme that disrupts microbial membranes (Moreau et al., 2001) such as those composing the capsule, has reduced enzymatic activity at lower temperatures (i.e. 5–10℃; Merchant et al., 2009), which may explain our finding of lower killing of *E. coli* at 15℃.

Finally, temperature effects on host and pathogen physiology may interact to modify testosterone–immunity relationships. We found that the negative relationship between testosterone and immunity observed for *S. typhimurium* was only present at the 15℃ challenge temperature. The overall lower killing of *S. typhimurium* compared to the other two bacteria, and few records of *S. typhimurium* presence in reptiles (Pedersen et al., 2009; Scott & Foster, 1997), suggests that alligators are rarely exposed to these bacteria under natural conditions. Therefore, it is possible that alligators are generally less able to defend themselves against *S. typhimurium*. In addition, *S. typhimurium* might be more impaired in terms of both its defence mechanisms (Nguyen et al., 2014) and virulence activity (Duong et al., 2007) at 15℃ than at 30℃. Taken together, this may explain why individuals were consistently ineffective at killing *S. typhimurium* at 30℃, but not 15℃. Impaired pathogen activity at 15℃ may have allowed for a more effective host response against this pathogen. More generally, this result highlights the importance of accounting for abiotic factors, such as temperature, as well as pathogen species, and potential interactions between the two when assessing testosterone–immunity relationships in nature.

The temperature dependency of the testosterone–immunity trade-offs that we document in this study emphasizes the key role temperature can play in studies of the ICHH. Due to the sensitivity of immune function to temperature (Butler et al., 2013; Evans et al., 2016), especially in ectotherms like alligators (Merchant et al., 2003), using a single temperature to quantify immunity may

capture only a small fraction of the variation in immune performance an individual might exhibit in a natural context. For example, the challenge temperatures we used in this study represent the average minimum and optimal maximum temperatures that free-ranging alligators experience, and a comparison of immune performance across these two ecologically relevant temperatures showed that average immune performance differed by temperature for all three bacteria (Table 1). Thus, depending on how testosterone levels covary with immune performance in different environmental contexts, resulting interactions have the potential to mask or unmask testosterone–immunity trade-offs. Our findings are only a start to understanding the role temperature may play in the ICHH. Future studies are needed to understand how temperature-associated variation in immunity affects trade-offs between testosterone and immunity, and to evaluate whether seasonal variation in temperature can help explain natural variation in the strength of testosterone–immunity trade-offs.

Overall, our integrative approach to evaluating the ICHH suggests that context is key to understanding how the ICHH operates in natural populations. In particular, interactions between co-occurring physiological processes and seasonal drivers may serve as important modulators of testosterone–immunity trade-offs. This theme is particularly relevant in the light of the recent meta-analysis by Foo et al. (2017), which found consistent evidence for the ICHH only among studies that artificially manipulated testosterone levels. While artificial manipulation of testosterone in the absence of a biologically relevant context may consistently demonstrate immunosuppressive effects of testosterone, as documented by Foo and colleagues, our study suggests that fluctuations in intrinsic (e.g. reproductive activity, energy allocation, other hormones; Alonso-Alvarez & Tella, 2001; Houslay et al., 2017; Poiani et al., 2000) and extrinsic (e.g. temperature, photoperiod; Prendergast et al., 2003) factors that co-vary with testosterone levels in natural contexts could dampen testosterone–immunity trade-offs in some cases and enhance them in others. Future work identifying key contextual factors that modulate testosterone–immunity relationships in nature will help advance our understanding of the ICHH.

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AUTHORS' CONTRIBUTIONS

A.A.L., B.B.P. and V.O.E. conceived project ideas; A.A.L. and V.O.E. designed methodology; A.A.L., H.J.H. and R.H.L. collected data; A.A.L. and V.O.E. analysed the data; A.A.L. and V.O.E. wrote the manuscript. All authors provided feedback on the manuscript and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data are available in the Dryad Digital Repository at https://doi.org/10.5061/dryad.cjsxksn53 (LaVere et al., 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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