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Seasonal sex steroids indicate reproductive costs associated with snake fungal disease

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Abstract

Emergent diseases may result in population declines by inducing mortality directly or through sublethal effects on host reproduction. Snake fungal disease (SFD) is an emerging threat to biodiversity, but the sublethal impacts of disease on host fitness are poorly characterized. The cryptic nature of most snakes makes direct assessment of the fitness consequences of SFD challenging. In such contexts, measurement of sex steroids that correlate positively with seasonal reproductive investment may be useful in inferring the scope of disease impacts. To test the hypothesis that SFD is associated with reproductive suppression, we measured testosterone and estradiol in free-ranging pygmy rattlesnakes with varying clinical signs of SFD. We also used real-time PCR to validate the relationship between clinical signs and *Ophidiomyces ophiodiicola* (Oo) DNA presence on the skin. Infected males had lower testosterone compared to uninfected males during summer spermatogenesis and the fall breeding season. Infected females were less likely to have elevated estradiol compared to uninfected females during spring vitellogenesis. Approximately 85% of individuals with clinical signs were positive for Oo DNA. Our findings are consistent with the hypothesis that coping with SFD comes at a cost to the reproductive success of afflicted individuals, and that seasonal sex steroids may be valuable early indicators of sublethal effects.

Introduction

Emergent diseases are one of many threats to biodiversity (Sutherland *et al.*, 2014). While direct effects of pathogens on host survival are appreciated (Berger *et al.*, 1998; Blehert *et al.*, 2009), less is understood about their sublethal impacts on host fitness and reproduction. For cryptic species, in particular, effects of infection on fitness are rarely determined on timescales that facilitate timely conservation initiatives. In such contexts, physiological proxies for reproductive investment, coupled with knowledge of host reproductive ecology, can aid in characterizing the scope of the threat that a pathogen poses to populations.

Conservation endocrinology attempts to use hormones as indicators of ecosystem disturbances and to demonstrate the physiological mechanisms that underlie population declines (Cockrem, 2005; Tubbs *et al.*, 2014). Toward these goals, much focus in the field has been placed on adrenal glucocorticoids and their role in the vertebrate stress response (Busch & Hayward, 2009). While glucocorticoids may be good indicators under many circumstances, the relationship between glucocorticoids and reproductive fitness may vary among populations and across environmental contexts, making the fitness consequences of glucocorticoid suppression or elevation difficult to infer (Romero, 2002; Bonier *et al.*, 2009). Gonadal 'sex' steroids, on the other hand, correlate directly with reproductive investment in many vertebrates, including Viperid snakes (Emerson & Hess, 2001; Yoccoz *et al.*, 2002; Lind & Beaupre, 2015; Smith, Schuett & Amarello, 2015), and may provide a more proximate mechanistic indicator of the reproductive costs associated with an emergent disease.

Disease may be associated with suppression of the vertebrate reproductive axis (Spratt *et al.*, 1993; Kindermann, Narayan & Hero, 2017). In reptiles, reproduction is mediated by the hypothalamo-pituitary-gonadal (HPG) axis and its downstream steroid products. Testosterone (T) mediates spermatogenesis and mating behaviors in males (Moore & Lindzey, 1992; Kahn and Rai 2004). Estradiol (E2) stimulates vitellogenesis and pheromone production in females (Ho *et al.*, 1982; Mendonça & Crews, 1996). Investment in gametogenesis and mating occurs during discrete seasons and is associated with elevated mean sex steroid concentrations in most cases (Reviewed in: Aldridge & Duvall, 2002; Aldridge *et al.*, 2009; Taylor & Denardo, 2011). Coping with pathogens may require

physiological and behavioral responses that constrain individual energy budgets and alter resource allocation strategies (Zuk & Stoehr, 2002). Seasonal tradeoffs between host defense, reproductive physiology, and behavior may therefore underlie seasonal dynamics and population-level responses to disease. Sublethal effects on reproduction are likely most evident during periods of seasonal reproductive investment in males and females, and knowledge of the reproductive ecology of hosts may be critical to investigations aimed at assessing disease impacts.

Snake fungal disease (SFD) is caused by the fungus *Ophidiomyces ophiodiicola* (Oo) and infects phylogenetically and ecologically diverse snake taxa (Allender *et al.*, 2015; Lorch *et al.*, 2015; Burbrink, Lorch & Lips, 2017). The fungus invades the epidermis resulting in lesions, nodules, and crusts which may occlude sensory organs and impair successful foraging (Lorch *et al.*, 2016). Emergence of SFD has been linked to decline of several snake populations in the Eastern United States (Allender *et al.*, 2011; Clark *et al.*, 2011; Lorch *et al.*, 2016). Recent work has documented the broad distribution of the disease in North America and Europe (McBride *et al.*, 2015; Allender *et al.*, 2016a,b; Lorch *et al.*, 2016; Franklinos *et al.*, 2017) and has described negative impacts on individual survival in clinical settings (Allender *et al.*, 2011). Still, very little is known regarding the effects of SFD on the physiology or behavior of free-ranging individuals (but see Allender *et al.*, 2016a,b; McCoy, Lind & Farrell, 2017; Tetzlaff *et al.*, 2017; Lind *et al.*, 2018a), and a framework for demonstrating potential costs of disease emergence is lacking in the literature.

Pygmy rattlesnakes, *Sistrurus miliarius* (Linneaus, 1766: Reptilia:Viperidae), in central Florida are afflicted with SFD, and are easily accessible for sampling throughout the year (May *et al.*, 1996; Cheatwood *et al.*, 2003; McCoy *et al.*, 2017). The phenology of reproduction in *S. miliarius* has been well described in previous ecological studies (Farrell, May & Pilgrim, 1995; May *et al.*, 1996; Rowe, Farrell & May, 2002; Lind *et al.*, 2018b), making populations in central Florida an ideal model for investigating disease impacts on reproduction. Central Florida populations exhibit a single fall mating season. Seasonal steroid profiles suggest that spermatogenesis occurs throughout the summer and peaks at the onset of the breeding season (Lind *et al.*, 2018b). Females have palpable follicles indicative of vitellogenesis in the spring (Rowe *et al.*, 2002). In *S. miliarius*, the severity of clinical signs of SFD varies with season (McCoy *et al.*, 2017). Individuals with clinical signs have increased glucocorticoids, and females with severe clinical signs are less likely to enter reproductive bouts (Lind *et al.*, 2018a). To test the hypotheses that SFD suppresses the HPG axis, and that suppression is coincident with seasonal periods of reproductive investment, we measured T (males) and E2 (females) in field active snakes with varying degrees of SFD clinical sign severity across all seasons. We also examined the association of clinical signs with the presence of Oo DNA using real-time PCR. We predicted lower circulating sex steroids in infected individuals compared to uninfected individuals sampled during the seasons when males and females actively invest in reproduction.

Materials and methods

Field sampling

Blood samples were drawn from the caudal vein of pygmy rattlesnakes captured at Lake Woodruff National Wildlife Refuge from January 2015 to July 2016. In total, 255 blood samples were taken from 113 individual adult males, and 254 samples were taken from 139 adult females. Snakes were sampled opportunistically, and repeated sampling of individuals in all seasons was not logistically feasible. Samples were drawn with a 27 gauge needle and stored on ice in 1.5 mL microcentrifuge tubes with EDTA. Plasma was removed after centrifugation and stored at −80°C. Samples were shipped on dry ice to Virginia Tech (Blacksburg, VA, USA) for radioimmunoassay. SFD severity was assessed visually on a 0–3 point scale using methods described in McCoy *et al.* (2017). Any snake with one lesion consistent with SFD was assigned a score of one. Scores greater than one were assigned based on the number and location of lesions. Snout to vent length was measured using a squeeze box and mass was measured in the field using a spring scale (Pesola®, Schindellegi, Switzerland). Snakes were individually marked by injection of a PIT tag (Avid®, Norco, CA, USA).

Radioimmunoassay

Testosterone data from the uninfected group were reported at finer seasonal scales as part of another study (Lind *et al.*, 2018a,b). Plasma T and E2 were each measured in a single radioimmunoassay as described in Lind *et al.* (2010). Samples were extracted in dichloromethane, then dried in a 40°C water bath under nitrogen gas. Samples were incubated overnight in antiserum (Esoterix Endocrinology, Calabasas Hills, CA, USA) and tritiated steroid. Unbound steroid was separated from bound steroid using dextran-coated charcoal. A liquid scintillation counter was used to count bound steroid in samples, and final concentrations were corrected for extraction efficiency. Serial dilutions for the standard curves were performed in duplicate (curve range = 1–500 pg). In the T assay, the limit of detection was 0.7 ng mL^{-1}, and the intra-assay coefficient of variation (CV) was 8.9%. In the E2 assay, the limit of detection was 0.18 ng mL^{-1}, and the intra-assay CV was 9.1%.

PCR validation

A separate group of snakes was swabbed dermally to test for the presence of Oo DNA using real-time PCR. A sterile swab (Medical Wire product #MW113, Corsham, UK) was used to sample the skin as described in Lind *et al.* (2018a,b). From October 2016 to January 2018, the skin surface of 131 adult snakes was sampled. Samples were shipped to the US Geological Survey – National Wildlife Health Center (Madison, WI, USA) for real-time PCR analysis. The estimated number of copies of PCR target in the reactions used to generate the standard curve were a series of 10 dilutions ranging from 1.16×10^2 to 1.16×10^6. Samples were considered positive

for Oo if they crossed the threshold at or below 40 replication cycles (Bohuski *et al.*, 2015). Individuals with equivocal clinical signs ($n = 13$) or evidence of DNA amplification above 40 cycles ($n = 2$) were discarded. The rate of potentially false positives based on clinical signs (clinical signs but Oo negative by PCR) and the rate of potentially false negatives (no clinical signs but Oo positive by PCR) was calculated on a final sample of 116 individuals.

Calculations

To examine the effects of meteorological season and SFD status on square root-transformed T levels, a repeated measures model was fit in SAS proc mixed. Any snake with clinical signs consistent with SFD (i.e. had at least one grossly visible nodule or lesion and scored ≥1 on the clinical sign scale) was considered positive. Body condition index (BCI) was calculated as the residual of the regression of log-transformed mass on log-transformed SVL and included as a continuous covariate in the model. To test whether the main effect of SFD was consistent across seasons, a SFD*Season interaction term was included. If an individual was sampled more than once per season, only the first sample was included, leaving a total of 180 samples on 111 individuals in the final model. Post-hoc Tukey tests were used to identify pairwise differences.

Estradiol production is pulsatile and released at condition thresholds in vipers (Aubret *et al.*, 2002). Therefore, E2 concentrations could not be analyzed by traditional parametric statistics. Female E2 was instead converted into a binary response variable. Females with <3 ng mL^{-1} E2 were categorized as baseline, and females with >10 ng mL^{-1} E2 were categorized as elevated. Females with E2 levels between three and 10 (six out of 253 total females) were discarded from the analysis. The effect of SFD status on the probability of having elevated E2 was analyzed within each season by Chi square likelihood ratio tests.

Results

There were significant effects of season ($F_{3,61} = 6.65$, $P < 0.001$), SFD ($F_{1,20} = 15.38$, $P < 0.001$), BCI ($F_{1,61} = 6.65$, $P < 0.001$) and the interaction term (SFD*Season; $F_{3,61} = 5.14$, $P = 0.003$) on testosterone levels. Tukey's tests revealed significantly lower T in SFD-positive compared to SFD-negative snakes sampled in the summer and fall but not in the winter or spring (Fig. 1). Negative males had higher T in the summer compared to negative males in the winter ($P < 0.001$) and spring ($P < 0.001$). Mean T did not vary by season in SFD-positive snakes ($P > 0.5$ in all comparisons).

Snake fungal disease-positive females were significantly less likely to have elevated E2 in the spring ($\chi^2 = 7.87$, $P = 0.005$; Fig. 2). Although the percentage of individuals with elevated E2 appeared lower in positive compared to negative snakes in fall ($\chi^2 = 2.81$, $P = 0.09$) and winter ($\chi^2 = 3.67$, $P = 0.055$), the differences were not statistically significant. No effect of SFD status was observed on E2 in the summer ($\chi^2 = 0.23$, $P = 0.63$).

In the PCR validation, 28 (84.8%) of the 33 individuals with clinical signs were Oo positive. Seventy-one (85.5%) of 83 snakes with no clinical signs were Oo negative.

Discussion

Our results support the hypothesis that SFD is associated with suppression of the HPG axis in males and females, particularly when infection coincides with seasonal bouts of reproductive investment. SFD-positive males had lower T during summer spermatogenesis and during the fall mating season. Females were less likely to have elevated E2 during the spring vitellogenic period. Seasonal monitoring of sex steroids, coupled with sound methods of disease detection, may provide an effective tool for indirectly identifying the reproductive costs of SFD in afflicted populations.

Males afflicted with SFD during summer spermatogenesis have T concentrations comparable to individuals sampled during the non-reproductive seasons (winter and spring) and significantly lower T compared to uninfected males in the same season (Fig. 1). The direction of causality linking SFD and T was not definitively established. Infection in males may elevate corticosterone levels (Kindermann *et al.*, 2017; Lind *et al.*, 2018a), and such elevations are negatively associated with sex steroid levels in *S. miliarius* (Lind *et al.*, 2018b) and many other vertebrates (reviewed in Moore & Jessop, 2003). Alternatively, low quality or energetically compromised males may have lower T and be more susceptible to disease. However, the consistency of the positive relationship between T and BCI across seasons and SFD status supports the hypothesis that SFD results in HPG suppression even in high quality (i.e. high BCI) males. Circulating T concentrations are directly related to male reproductive investment (Lind & Beaupre, 2015) and mating success (Smith *et al.*, 2015) in Viperid snakes and other vertebrates (Emerson, 2001; Yoccoz *et al.*, 2002). Breeding season males with SFD and circulating T concentrations comparable to the non-breeding seasons likely invest less, or not at all, in testosterone-mediated physiological processes and behaviors that promote annual reproductive success.

In the current study, females with SFD were less likely to have E2 concentrations indicative of vitellogenesis when sampled in the spring. Estradiol stimulates vitellogenin production by the liver (Ho *et al.*, 1982), and vitellogenesis is likely the most energetically costly component of seasonal reproduction (Van Dyke & Beaupre, 2011). Infected females appear to forgo reproductive bouts, suggesting impacts on individual fitness, especially in *S. miliarius* populations where females reproduce, on average, biennially (Rowe *et al.*, 2002). This result is bolstered by previous work on the population that demonstrated a low incidence of infection in pregnant females (McCoy *et al.*, 2017) and that females with severe clinical signs are less likely to enter annual reproductive bouts (Lind *et al.*, 2018b). In addition, infected females tended to be less likely to have elevated E2 compared to uninfected females during the fall and winter, the seasons that include the mating period (Rowe *et al.*, 2002; Lind *et al.*, 2018a), although the differences were not statistically significant. Estradiol is likely elevated during the fall breeding season to stimulate production of lipid

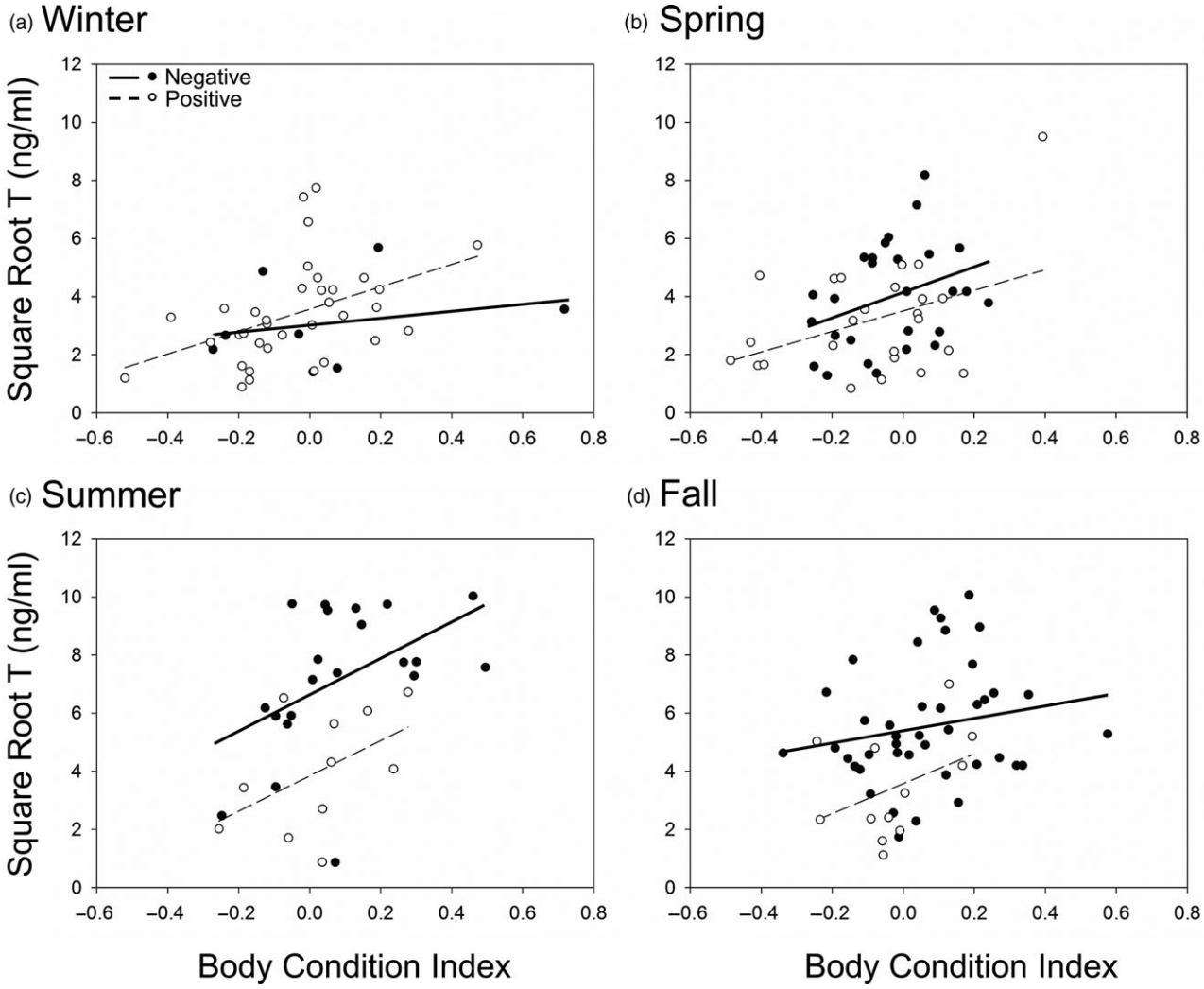

Figure 1 Scatterplots with linear regression lines depicting the relationship between body condition (BCI) and testosterone (T) in snake fungal disease (SFD)-positive and SFD-negative snakes within the four meteorological seasons. Significant effects of infection were observed during the summer and fall only.

pheromones (Mendonça & Crews, 1996). In viviparous snakes that primarily rely on capital to fuel reproduction, the decision to reproduce is largely dependent upon the ability to accrue a threshold of stored energy (Aubret *et al.*, 2002; Lourdais *et al.*, 2003; Lind & Beaupre, 2015), and it may be that coping costs associated with infection preclude attainment of stored energy thresholds in afflicted individuals. Previous descriptions of afflicted populations have demonstrated a strong negative correlation between infection severity and BCI, indicating potential energetic costs of coping (Allender *et al.*, 2016a,b; McCoy *et al.*, 2017). Future studies should attempt to directly quantify coping costs and establish causal mechanisms linking infection and reproductive suppression.

Real-time PCR results demonstrate that categorization of individuals as SFD positive by clinical signs was associated with the presence of Oo DNA in most cases, and that assignment of SFD status based on clinical signs is an imperfect, but close approximation of infection status in the population. However, the presence of potentially false positives (15.2%) and false negatives (14.5%) suggests that, when possible, establishing infection status is best accomplished using a combination of visual clinical signs and PCR. Specifically, clinical signs consistent with SFD in some individuals may be caused by etiologies other than Oo, and snakes without clinical signs may be carriers of the fungus. The frequency of Oo DNA negative samples in snakes with clinical signs was markedly lower compared to a recent study on a closely related species, *Sisturus catenatus*, which reported a false positive rate of 73% when snakes were swabbed with a single applicator (Hileman *et al.*, 2018). However, the false positive rate was reduced to <5% when 10 swabs were taken from each snake, suggesting that the actual false positive rate is also low in

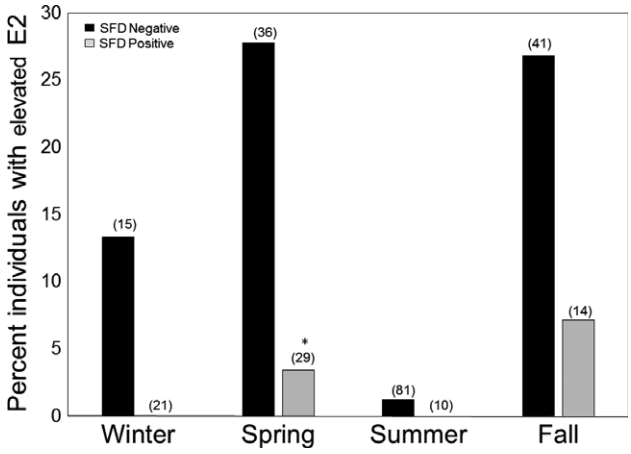

Figure 2 Percentage of female *Sistrurus miliarius* with elevated E2 (>10 ng mL^{-1}) in each season. Significant effects of snake fungal disease status within each season are indicated by an asterisk. The sample size within each group is indicated above bars. E2, estradiol.

S. catenatus. Reported differences in detection may result from seasonal or species differences in host clinical presentation or field and laboratory methodologies. Future studies should examine these variables in an attempt to infer potential sources of variation in detection probability with the goal of establishing a standard for SFD detection in the field.

Our results are consistent with the hypothesis that coping with SFD negatively impacts fitness. Suppression of the HPG in the fall may disrupt mate acquisition in infected males and females. Infection may reduce the likelihood of annual reproduction in females and investment in gamete production in males. The potential for SFD to seasonally impact reproductive processes and reduce individual fitness should be considered when predicting population level effects of SFD in at-risk species. Additionally, we present a framework for demonstrating the reproductive costs of SFD, and potentially other reptile pathogens, in the field through measurement of reproductive steroids paired with knowledge of the reproductive ecology of hosts and sound methods for establishing disease status.

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