An examination of endoparasites and fecal testosterone levels in flying squirrels (*Glaucomys* spp.) using high performance liquid chromatography-ultra-violet (HPLC-UV)

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ABSTRACT

The immuno-competence hypothesis proposes that higher levels of testosterone increases the susceptibility to parasitism. Here we examined the testosterone levels in two species of flying squirrels (*Glaucomys*): one known to regularly host a nematode species (*Strongyloides robustus*) without ill effects (*G. volans*) and a closely related species that is considered negatively affected by the parasite. We quantified fecal testosterone levels in northern and southern flying squirrels (*G. sabrinus, G. volans*) with high-performance liquid chromatography-ultraviolet spectroscopy (HPLC-UV), and compared levels to endoparasites detected in individual squirrels. Qualitatively, we found highest levels of testosterone in male northern flying squirrels infected with *Strongyloides robustus*. This analytical approach represents an alternative and equally reliable method to using enzyme-linked immunosorbent assay (ELISA), for detecting and quantifying fecal testosterone levels.

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1. Introduction

In the eastern United States, the decline of northern flying squirrel (*Glaucomys sabrinus*) populations may be exacerbated by parasite-mediated competition due to the nematode *Strongyloides robustus* found in sympatric populations of southern flying squirrels (*Glaucomys volans*) (Weigl, 2007; Krichbaum et al., 2010). This parasite is suspected to have little negative effects on the southern flying squirrel but potentially lethal effects on northern flying squirrels. Susceptibility to parasite infection due to increased testosterone levels as proposed by the immuno-competence hypothesis may accentuate this parasite-mediated competition (Folstad and Karter, 1992; Ezenwa et al., 2011). Most studies that test the immuno-competence hypothesis in mammals, including squirrels, focused on increased ecto- and endo-parasitism rates and blood leukocyte density in males due to increased levels of testosterone (Perez-Orella and Schulte-Hostedde, 2005; Gorrell, 2006; Gorrell and Schulte-Hostedde, 2008). Here, we were specifically interested in the testosterone levels of flying squirrels infected with *Strongyloides robustus*.

To examine testosterone levels and parasites in flying squirrels, we first developed a method to extract and quantify testosterone levels in fecal samples collected from northern and southern flying squirrels. Second, we compared testosterone levels between northern and southern flying squirrels that varied in the number of endoparasite species detected. Our study is one of a few that quantitatively measures testosterone levels by using high performance liquid chromatography-ultra-violet (HPLC-UV) analysis and compares those levels to corresponding occurrences of endoparasitism. In general, the enzyme-linked immunosorbent assay (ELISA) is a more commonly used method of detection and quantification of fecal testosterone in wildlife (e.g., Sheriff et al., 2011), but HPLC-UV is an equally reliable method (Lupica and Turner, 2009; Abu el Maaty et al., 2014). Although fecal testosterone levels have been examined in cervids, carnivores, primates, and laboratory rodents, we could find no studies that documented testosterone levels extracted from sciurid fecal samples (Touma and Palme, 2005; Sheriff et al., 2011). Due to the increasing rarity of northern flying squirrels in our study areas, sample sizes in this study were

unavoidably small. In light of this, the methodology outlined in this study can be applied to larger sample sizes.

2. Materials and methods

As part of an on-going study, we monitored flying squirrel nest-boxes established at six study sites in northeastern Pennsylvania (Mahan et al., 2010). When captured, the northern or southern flying squirrel was transferred to a handling bag and sex, age, reproductive condition, and mass (g) determined. Fecal samples were collected from the bottom of the handling bag and immediately placed in vials containing 10% formalin solution. Fecal samples were examined for the presence of helminth parasite eggs by performing a Sheather's solution flotation test (Sheather, 1923). Fishwick et al. (2007) found Sheather's flotation test was as accurate as other standing (e.g., sodium nitrate as flotation media) and centrifugation with flotation methods at detecting the presence of a parasite infection but was less accurate at determining egg density. In addition, the rate of endoparasite egg shedding varies monthly. Therefore, we only used presence or absence when examining parasites in our samples (e.g., Gorrell and Schulte-Hostedde, 2008).

Fecal samples from adult male northern and southern flying squirrels were processed using a variation of the extraction procedure described by Billitti et al. (1998). We determined that the formalin would have no effect on the testosterone or its extraction because testosterone only is soluble in ethanol, chloroform, diethyl ether, ethyl oleate, acetone, dioxane, and fixed oils. Samples were dried in an oven at 95° C for 6 h to constant mass. The dried samples were crushed and $\leq$30 mg of sample was transferred into a 13×100-mm borosilicate tube. To extract testosterone from fecal samples, 50 μl of a 10% methanol solution was added to each sample as a wetting agent. Following a 24- hour incubation period at room temperature, 2 ml of 100% ethyl ether was added to the sample, which was then vortexed for 60 min. To this mixture, 250 μl of autoclaved distilled water was added and the sample placed in a dry ice/methanol bath for 20 s. The ethyl-ether layer was decanted from the frozen aqueous layer and placed into a second tube. A second, 2 ml portion of 100% ethyl ether was added to the remaining aqueous layer for a second extraction. The mixture was vortexed for 30 min and placed in the dry ice/methanol bath for 20 s. The second ethyl-ether layer was pooled with the first portion yielding 4 ml of total extract. The extract was placed in a water bath (37 °C) and evaporated to dryness (not a timed method). One ml of buffer (0.1% Na_3PO_4, pH 7.0, 0.87% NaCl, and 0.1% BSA) was added to the residue, followed by the addition of two, 4 mm glass beads, then vortexed for 60 min at medium speed. The samples were re-extracted with 2 ml of ethyl ether and vortexed for 5 min. The ethyl ether layer was separated from the aqueous layer and placed into a new tube and evaporated to dryness by placing it in a water bath (37 °C). The residue was reconstituted in 100 μl of anhydrous ethanol and prepared for high-pressure liquid chromatography (HPLC). Next, 10—20 μl of the extract were injected into an HPLC system (Infinity, model 1260; Agilent) with an Eclipse plus C18 column (100 mm by 4.6 mm [inner diameter]; 3.5 μm; Agilent). The mobile phases (MP) were water (MPA) and acetonitrile (MPB) with a gradient of 20—40% MPB in MPA in the first 5 min, 30—40% MPB in MPA from 5 to 10 min, 40—50% MPB in MPA from 10 to 15 min, 50—60% MPB in MPA from 15 to 20 min, 60—70% MPB in MPA from 20 to 25 min, and 70—75% MPB in MPA from 25 to 30 min. The flow rate was 1.0 ml/min. For quantitation of testosterone, the eluate from the column was monitored at 254 nm on a UV-visible detector (Infinity, model 1260; Agilent). Quantitation was performed relative to the content of testosterone and determined by peak integration (Agilent data analysis software) and reference to a calibration curve generated by standards that ran from 5×10^{-12}

to 50 ng testosterone which were produced using a purchased testosterone standard (1.0 mg/ml in dimethyl ether, Sigma-Aldrich).

3. Results and discussion

We examined fecal samples from eight male northern and 10 male southern flying squirrels captured at our study sites from 2002 to 2004 (Tables 1 and 2). No parasites were detected in the fecal samples from seven of the flying squirrels captured. *Citellinema bifurcatum* was detected in all the fecal samples that contained parasites (Tables 1 and 2). We also detected the Apicomplexan parasite, *Eimeria,* and the helminth, *Strongyloides robustus,* in our samples (Tables 1 and 2). The testosterone levels in the three northern flying squirrels that were infected with *Strongyloides robustus* ranged from 1991.9 ng/g — 12342.2 ng/g ($\overline{X}$ = 9066.8 ng/g $\pm$ 3540.7 standard error [SE])—the highest levels detected in our study (Table 1). We found only one southern flying squirrels infected with *Strongyloides robustus* and that individual had a testosterone level of 84.8 ng/g (Table 2). With one exception, squirrels infected with *Strongyloides* were concurrently infected with at least one other endoparasite. In general, lower testosterone levels were recorded in male southern flying squirrels ($\overline{X}$ = 1468.1 ng/g $\pm$ 637.3 SE) than in male northern flying squirrels (3965.4 ng/g $\pm$ 1927.0 SE) but we observed no pattern of testosterone level by season. In our study areas, male flying squirrels are in reproductive condition (scrotal) from January to early September. We found no published levels of testosterone levels in flying squirrels against which to compare our findings. However, in

Table 1
Fecal testosterone level (ng/g) for eight (8) adult, male northern flying squirrels (*Glaucomys sabrinus*) captured in Pennsylvania and species of endoparasite detected, 2002—2004.

Fecal testosterone level (ng/g fecal material)	Endoparasites detected	Date captured
122.9	None	October 2004
290.1	None	September 2004
607.5	*Citellinema bifurcatum*	January 2004
821.2	*Citellinema bifurcatum*	January 2004
1991.9	*Strongyloides robustus*	September 2004
6646.5	*Citellinema bifurcatum, Eimeria* spp.	September 2004
12866.3	*Citellinema bifurcatum, Strongyloides robustus*	February 2004
12342.4	*Citellinema bifurcatum, Strongyloides robustus*	January 2004

Table 2
Fecal testosterone level (ng/g) for ten (10) adult, male southern flying squirrels (*Glaucomys volans*) captured in Pennsylvania and species of endoparasite detected, 2002—2004.

Fecal testosterone level (ng/g fecal material)	Endoparasites detected	Date captured
47.7	*Citellinema bifurcatum*	July 2003
65.3	None	April 2003
84.8	*Citellinema bifurcatum, Strongyloides robustus, Eimeria* spp.	January 2004
219.2	None	July 2003
230.1	*Citellinema bifurcatum*	January 2004
407.1	*Citellinema bifurcatum*	August 2002
1920.4	*Citellinema bifurcatum*	July 2003
2244.6	None	October 2004
3257.6	None	June 2004
6203.6	None	December 2004

Mus musculus testosterone levels extracted from fecal pellets varied from 173 ng/g (basal testosterone level) - 510 ng/g (scrotal males). In the same study, *Mus musculus* males stimulated with HCG (human chorionic gonadotropin) demonstrated a maximum testosterone level of 1352 ng/g. Our small sample size makes it difficult to ascribe causation but quantitative methods of calculating testosterone levels may provide important information in ascertaining the effects of testosterone on parasitism rates in wildlife.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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