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

Sarah N. Waksmonski a, Justin M. Huffman a, Carolyn G. Mahan a,*, Michael A. Steele b

a Division of Mathematics and Natural Sciences, 3000 Ivyside, The Pennsylvania State University, Altoona, PA 16601, USA
b Department of Biology, Wilkes University, 84 West South St., Wilkes-Barre, PA 18766, USA

ARTICLE INFO

Article history:
Received 18 February 2017
Received in revised form
20 May 2017
Accepted 23 May 2017

Keywords:
Flying squirrels
Immu-no-competence hypothesis
Parasites
Strongyloides robustus
Testosterone
Fecal extraction
High-performance liquid chromatography-ultraviolet spectroscopy

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.

© 2017 The Authors. Published by Elsevier Ltd on behalf of Australian Society for Parasitology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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 medium) 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 <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 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% Na3PO4, 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 Aporient complex 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 to 12342.2 ng/g (X = 5066.8 ng/g ± 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 [X = 1468.1 ng/g ± 637.3 SE] than in male northern flying squirrels (3965.4 ng/g ± 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](image)

| Fecal testosterone level (ng/g) for ten (10) adult, male northern flying squirrels (Glaucomys sabrinus) captured in Pennsylvania and species of endoparasite detected, 2002–2004. |
|---|
| Fecal testosterone level (ng/g) | 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](image)

| 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) | 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 | August 2002 |
| 230.1 | Citellinema bifurcatum | July 2003 |
| 407.1 | Citellinema bifurcatum | October 2004 |
| 1920.4 | Citellinema bifurcatum | June 2004 |
| 2324.6 | None | December 2004 |
| 3257.6 | None | December 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

We thank the Pennsylvania State University-Altoona College for funding for this study and Laura Palmer and Greg Turner for laboratory and field assistance, respectively.

References

Abu el Maaty, M.A., Hanafi, R.S., Aboul-Enein, H.Y., Gad, M.Z., 2014. Design-of-Experiment approach for HPLC analysis of 25-hydroxyvitamin D: a Comparative assay with ELISA. J. Chromatogr. Sci. 1–7.

Billirri, J.E., Lasley, B.L., Wilson, R.W., 1998. Development and validation of a fecal testosterone biomarker in Mus musculus and Peromyscus maniculatus. Biol. Reprod. 59, 1023–1028.

Ezenwa, V.O., Ekernas, L.S., Creel, S., 2011. Unraveling complex associations between testosterone and parasite infection in the wild. Funct. Ecol. 26, 123–133.

Fishwick, L., Isaza, N., Greiner, E.C., 2007. Fecal flotation studies comparison of fecal flotation media and methods. Vet. Tech. 28, 442.

Folstad, I., Karter, A.J., 1992. Bright males and the immunocompetence handicap. Amer Nat. 139, 603–622.

Gorrell, J.C., 2006. Individual Variation in Parasitism, Testosterone and Indices of Health in North American Red Squirrels (Tamiasciurus hudsonicus). PhD thesis, Ecology. University of Alberta, Edmonton, Alberta, Canada, 121 pp.

Gorrell, J.C., Schulte-Hostedde, A.I. 2008. Patterns of parasitism and body size in red squirrels (Tamiasciurus hudsonicus). Can. J. Zool. 86, 99–107.

Krichbaum, K., Mahan, C.G., Steele, M.A., Turner, G., Hudson, P.J., 2010. The potential role of Strongyloides robustus on parasite-mediated competition between two species of flying squirrels (Glaucomys). J. Wildl. Dis. 46, 229–235.

Lupica, S.J., Turner, J.W., 2009. Validation of enzyme-linked immunosorbent assay for measurement of faecal cortisol in fish. Aquac. Res. 40, 437–441.

Mahan, C.G., Bishop, J.A., Steele, M.A., Turner, G., Meyers, W.L., 2010. Habitat characteristics and revised gap landscape analysis for the northern flying squirrel (Glaucomys sabrinus), a state endangered species in Pennsylvania. Amer Midl. Nat. 164, 283–295.

Perez-Orella, C., Schulte-Hostedde, A.I., 2005. Effects of sex and body size on ectoparasite loads in the northern flying squirrel (Glaucomys sabrinus). Can. J. Zool. 83, 1381–1385.

Sheather, A.L., 1923. The detection of intestinal protozoa and mange parasites by a flotation technique. J. Comp Par. Ther. 36, 266–275.

Sheriff, M.J., Danzer, B., Belehrady, B., Palme, R., Boonstra, R., 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 166, 869–887.

Touma, C., Palme, R., 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. Ann. N. Y. Acad. Sci. 1046, 54–74.

Weigl, P.D., 2007. The northern flying squirrel (Glaucomys sabrinus): a conservation challenge. J. Mammal. 88, 897–907.