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Correlation between levels of sex hormones (progesterone, testosterone, and estrogen) and ecophysiological-behavior stages in two species of desert snails (*Sphincterochila zonata* and *Sphincterochila prophetarum*) in the Northern Negev Desert

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Abstract

The steroidal hormonal profiles of two sympatric species of desert snails, *Sphincterochila zonata* and *Sphincterochila prophetarium* were determined at three ecophysiological-behavior stages, i.e., aestivation, cryptobiosis, and active-feeding phases. Live snails were collected in their natural habitat every month for 13 months, the corpi removed and extracted with organic solvents and the progesterone, testosterone, and estrogen concentrations determined by radioimmunoassay. In both these hermaphroditic species during aestivation, a peak of testosterone followed by a peak of estrogen was observed. During the brief active intervals, minor peaks of estrogen were also observed but these were much lower than seen during aestivation. Although the two species have different microhabitats during aestivation, there was little difference in the hormonal profile, although *S. prophetarum* had about two fold higher progesterone concentration than *S. zonata*.

Keywords: Snails; Progesterone; Testosterone; Estrogen; Aestivation; Cryptobiosis

1. Introduction

The terrestrial snails (order Stylommatophora) *Sphincterochila zonata* and *Sphincterochila prophetarum* are known as sympatric populations sharing similar biotope, inhabiting different microhabitats and acknowledged as well to be the most common snail populations in the Northern Negev Desert (Hodgson and Shachak, 1991), where their activity is drastically driven by the environment (Evenari, 1981). The snails are active for brief periods during the rainy season after each rain (about 20 days/year). In between rains, the snails can be found in a cryptobiotic state. Cryptobiosis is differentiated from the active state by the presence of a semi-transparent calciferous membrane (epiphragma). This membrane is much thinner that the thick membrane characteristic of aestivation (Arad et al., 1989). In the remainder of the year during the dry season (240–280 days/year) the snails aestivate (Steinberger et al., 1982; Hodgson and Shachak, 1991). In the short activity period, the two snail species share the same niche and fulfill all life functions including feeding, growing, mating, and laying eggs. However, during aestivation, the snails occupy different microhabitats—remaining on the soil surface or under stones, for *S. zonata* and *S. prophetarum*, respectively (Shachak et al., 1976; Steinberger et al., 1981; Yom-Tov, 1971).

Both species of snails are simultaneous hermaphrodites, an advantage in an unpredictable activity environment where finding a mate is difficult considering the snails low mobility (Heller, 1993). As described in Flari and Edwards (2003), there are major gaps in our understanding of the

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reproductive endocrinology of terrestrial pulmonate gastropods. Nonetheless, *in vitro* experiments have shown that some stylommatophoran snails are capable of producing sex hormones such as: progesterone in *Helix pomatia* (Krush et al., 1979), in *H. aspersa* (Le Guellec et al., 1987), and in *Ariolimax californicus* (Lehoux and Sandor, 1970); testosterone in *H. aspersa* (Le Guellec et al., 1987) and in *Arion ater rufus* (Gottfried and Lusis, 1966); estrogen in *H. aspersa* (Le Guellec et al., 1987) and in *A. ater rufus* (Gottfried et al., 1967).

The mechanism of action of "mammalian" steroids in invertebrates is not known. The reproductive organs of marine and land snails are known to respond to testosterone, estrone, and estradiol (LeBlanc et al., 2005; Casaba and Birbauer, 1979; Takayangi and Takeda, 1985; Bettin et al., 1996) and marine snails do respond to synthetic steroids (ethinyloestradiol and methyltestosterone; Schulte-Oehlmann et al., 2004). However, the mechanism of action is unlikely to be similar to that in mammals as the primordial steroid receptor is not functional (Keay et al., 2006) nor is there any evidence that non-genomic transmembrane receptors (Zhu et al., 2003) are functional in invertebrates. However, the lack of estrogen receptor has not been demonstrated using invertebrate cells.

Hodgson and Shachak (1991) investigated the spermatogenic cycle of *S. zonata* and *S. prophetarum* and found that the male gametes mature first and afterwards female gametes. The sperm is stored in the hermaphrodite duct till mating at winter time. Although the precise information is lacking, it appears that the steroid hormones are important in spermatocyte formation and testicular steroidogenesis as well as oogenesis in the snail (Casaba and Birbauer, 1979) and testosterone rises with the onset of laying in *Pomacea paludica* and at the beginning of the reproductive season in both male and female in *Ilyanassa obsoleta*. (LeBlanc et al., 2005). The effect of the steroids is mediated in part by actions of the hormones on the optic gland (see Flari and Edwards, 2003 for review). However, there have been no studies variations in hormone levels in desert land snails which undergo aestivation.

It was hypothesized that steroidal hormones in the two sympatric snails will be different in concentrations during the three ecophysiological-behavior stages, i.e., aestivation, cryptobiosis, and active phases in accordance with physiological changes in the reproductive organs. Therefore, live *S. prophetarum* and *S. zonata* snails were collected during a period of 13 months in which all the three ecophysiological-behavior stages occurred and the steroid concentrations were determined.

2. Materials and methods

2.1. Study site

Both of the snails species were collected from the Northern Negev Desert (34°50′E/30°50′N), Israel. This site is approximately 500 m above sea level and characterized by cool winters (0–15 °C); mean multi-annual winter (December–April) rainfall of 90 mm, which is unpredictable in time, space, and quantity; and hot, dry summers (maximum air 46 °C, soil 60 °C) (Evenari, 1981; Buyanovsky et al., 1982; Yom-Tov, 1983).

2.2. Analysis of samples

Sphincterochila zonata and *S. prophetarum* were collected from December 2004 till March 2006 from Northern Negev Desert. The collections were throughout all ecophysiological-behavior stages of the snails: *aestivation* (May–November), *cryptobiosis* (December–April), and *active* (December–April). The snails collected at the study site were placed in a bin at −20 °C containing solid carbon dioxide, and transported to the laboratory. At the laboratory they were marked individually, weighed and preserved at −20 °C before steroid sex hormones determination. Cryptobiosis was differentiated from the active stage by the visible presence of a semi-transparent epiphragma during the winter months. Aestivation was defined as having a thick epiphragma during the summer, spring, and fall seasons.

Prior to determination of hormone levels, the soft tissue from each animal was removed from the shell and 0.5 g aliquots used for analysis. Due to the difference in size between the two species, the hormone levels were determined on individuals of *S. zonata*, while for *S. prophetarum* tissues were pooled from 2 to 4 individuals. Dry weights were determined using snails obtained at the same sampling as the fresh tissue used for extraction (average dry wt. for *S. zonata* 0.4 ± 0.1 g/individual, $N = 5$; vs. 0.1 ± 0.05 g for *S. prophetarum*, $N = 5$). The muscular foot was separated from the soft corpus.

The tissues were homogenized using a glass homogenizer, 2 ml distilled water added and extracted twice with five volumes of ethyl acetate. The organic solvent extract was then evaporated under air to dryness and redissolve in 1 ml of 100% methanol (Ultra-Resi-Analysed, J.T.Baker, Phillipsburg, NJ). Aliquots (100 μl) of the methanol extract were taken, evaporated and the residue redisolved in 100 μl of steroid assay buffer.

The steroid sex hormones (progesterone, testosterone, and estrogen) for each species ($N = 5$) were analyzed by using the RIA (radioimmunoassay) as described in Shemesh (1979) and Shore and Shemesh (1981, 1993). The antibody for testosterone cross-reacted with dihydrotestosterone (50%), the antibody for estradiol cross-reacted 50% with estrone (hereafter termed "estrogen") and the antibody for progesterone was specific for progesterone and reacted to a negligible extent with other steroids. Addition of the three steroids to preparations of podia or corpi indicated that the recovery (following subtraction of endogenous steroid) was similar for the three steroids regardless of species or tissue source ($64 \pm 20\%$, $N = 15$). Preliminary testing for androstenedione using a commercial ELISA kit, indicated that the levels of this compound were below the level of detection (<0.05 ng/g wet wt.).

Steroid concentrations are expressed as nanogram per gram dry wt. (means ± SD) for N preparations. Data were analyzed using Statistical Analysis System general linear models (SAS, 1985) for balanced ANOVA. Means were separated by a Waller-Duncan k-ratio t test. For the purpose of statistics and graphics, the value of 0.05 ng/g wet wt. was assigned to non-detectable. In the 106 preparations (79 individuals of *S. zonata* and 27 pools (2–4 individuals) of *S. prophetarum*) progesterone and testosterone were detectable. Estrogen was detectable in 70% of the preparations, equally distributed between the two species. The concentration of the three steroids was below the limit of detection in 20 preparations of the muscular foot (two snail each for *S. zonata* and 10 snails of *S. prophetarum*) taken from the same snails similarly assayed.

3. Results

3.1. Temporal changes in steroid concentrations

The fluctuations in levels of sex hormones during the study period reveal that, in both *S. prophetarum* (Fig. 1a) and *S. zonata* (Fig. 1b), there were distinct peaks in the levels of the different sex hormones. The first hormone in the highest concentration (in comparison to other sex