1 TITLE: The influence of androgens on hibernation phenology of free-living male arctic
2 ground squirrels

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22 ABSTRACT

23 Free-living ground squirrel species are sexually dimorphic in hibernation phenology. The
24 underlying causes of these differences are not yet known. Androgens, testosterone (T)
25 in particular, inhibit hibernation. To determine the influence of endogenous androgens on
26 annual timing of hibernation we first measured circulating levels of T and
27 dehydroepiandrosterone (DHEA), an adrenal androgen implicated in non-mating season
28 aggression in other species, in free-living male arctic ground squirrels (*Urocitellus parryi*,
29 AGS). We also manipulated endogenous androgen levels by surgical castration, and
30 consequently compared body temperature records from intact (n = 24) and castrated (n
31 = 9) males to elucidate the influence of endogenous androgens on annual body
32 temperature cycles. The highest T levels (0.53 ± 0.10 ng/mL) were in reproductively
33 mature male AGS in spring; whereas, both immature males in spring and all males in
34 late summer had T levels an order of magnitude lower (0.07 ± 0.01 and 0.06 ± 0.00
35 ng/mL, respectively). DHEA levels were higher in males during the late summer
36 compared to reproductively mature males in spring (120.6 ± 18.9 and 35.9 ± 2.3 pg/mL,
37 respectively). Eliminating gonadal androgens via castration resulted in males delaying
38 euthermia by extending heterothermy significantly in spring (Apr 22 ± 2.9) than
39 reproductive males (Mar 28 ± 3.9) but did not change the timing of hibernation onset
40 (castrate: Oct 12 ± 1.0 vs. intact: Oct 3 ± 3.1). We conclude that while androgens play a
41 significant role in spring hibernation phenology of males, their role in fall hibernation
42 onset is unclear.

43 Key words: hibernation phenology; androgen; arctic ground squirrel

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46 INTRODUCTION

47 Arctic ground squirrels (*Urocitellus parryii*, AGS) are the northernmost hibernator,
48 experience the most extreme winter conditions, and have a very short active season in
49 which to mate, rear offspring, and prepare for the following winter (Buck and Barnes
50 1999b, Carl 1971). In early spring to prepare for the mating season, adult males end
51 heterothermy (period of hibernation when animals undergo torpor-arousal cycles) and
52 remain sequestered below ground (Williams et al. 2012b). This pre-emergent period is
53 characterized by 10-27 days of euthermic but arrhythmic body temperature (T_b ; Williams
54 et al. 2012a) and is required for testicular recrudescence and spermatogenesis (Barnes
55 et al. 1987); all the while, animals draw from a food cache to regain body mass lost
56 across their heterothermic season (Buck and Barnes 1999a). In addition to males that
57 engage in the mating season, a varying proportion of males (25-60%) forgo mating
58 opportunities, continue hibernating through the mating season (Bronson 1979; Buck and
59 Barnes 1999b; Buck et al. 2008; Schwagmeyer and Brown 1983; Sheriff et al. 2011;
60 Slade and Balph 1974), and neither undergo testicular maturation nor have a significant
61 pre-emergent euthermic period (Williams et al. 2012b). Unlike the differences found in
62 timing of spring emergence, timing of entrance into hibernation is nearly synchronous
63 among males (Sheriff et al. 2011). In contrast, female AGS begin hibernation
64 significantly earlier, emerge from hibernation significantly later than reproductive males,
65 and exhibit no pre-emergent euthermic period (Buck and Barnes 1999a; Sheriff et al.
66 2011; Williams et al. 2012b). The modulator(s) of sex differences in hibernation
67 phenology is as yet unknown.

In the early spring, male AGS have high levels of circulating androgens (Boonstra et al. 2001; Buck and Barnes 2003) coincident with reproductive development and behaviors, male-male aggression and mate guarding (Carl 1971; Buck and Barnes 2003). At the conclusion of the breeding season, testes atrophy (Barnes and York 1990; Buck and Barnes 1999a), mating season aggression ends and circulating androgen levels decline (Buck and Barnes 2003). In the late summer, males accumulate a cache to be utilized the following spring; this cache, and the surrounding territory, is defended against other males in the weeks prior to the start of hibernation (Carl 1971; Buck and Barnes 1999a; Buck and Barnes, 2003). The hormonal correlate of this second period of male-male aggression, associated with the defense of territory and caches, has yet to be determined.

In other animal species, non-mating season aggression can be influenced by an adrenal androgen, dehydroepiandrosterone (DHEA). Among many songbirds, red squirrels (*Tamiasciurus hudsonicus*), and dwarf hamsters (*Phodopus sungorus*), non-mating season aggression coincides with increased concentrations of circulating DHEA, rather than testosterone (T) (reviewed in Soma et al. 2008; Boonstra et al. 2008; Scotti et al. 2008). It is possible that in male AGS DHEA facilitates aggression during late summer when their testes are regressed and quiescent (Barnes and York 1990) and that its actions may contribute to the late hibernation onset observed in males compared to females.

The differences in hibernation patterns observed between reproductively mature males, immature males, and females combined with the known inhibitory effects of T on hibernation (Darrow et al. 1988; Goldman et al. 1986; Lee et al. 1990; Smit-Vis 1972; Vitale et al. 1985) strongly imply an important phenological role for endogenous androgens. We hypothesized that only intact males in spring would have high levels of

T, and that T levels influence the timing of spring euthermia and emergence. We also hypothesized that males would have higher levels of DHEA in the late summer/fall compared to females or spring males to facilitate late season aggression and delay hibernation onset in the fall. To elucidate the role of androgens on circannual timing, we castrated males to remove the main source of endogenous T, and compared T_b patterns between intact and manipulated males. We predicted that castrated males would exhibit similar hibernation seasons to reproductively immature males, with late entrance and exit from heterothermy thus supporting the role of endogenous T in control of spring phenology. We also measured plasma T and DHEA concentrations in samples collected from females, intact males and castrate males across the active season.

METHODS AND MATERIALS

Animals and Blood Sampling:

Free-living adult and juvenile AGS were live-trapped using carrot-baited, Tomahawk traps (Tomahawk Live Trap, Tomahawk, WI, USA) north of the Brooks Range, AK near the Atigun River (68°27'N, 149°21'W, elevation 812m). Animals were trapped during the active season (April to September) between 2008 and 2013 and transported by truck to the nearby Toolik Field Station where they were anesthetized by a 3-5 min exposure to isoflurane vapors. On first capture, animals were uniquely tagged (Monel no. 1 ear tags, National Brand & Tag Company, New Port, KY, USA and AVID MUSICC passive integrated transponder [PIT] tags, Norco, CA, USA), weighed, and assessed for sex. Males were scored for reproductive state (spring captures only, score of 0-3; 0: no palpable testes; 3: fully enlarged, scrotal testes), and sampled for blood (1-2 mL) via cardiac puncture. The blood sample was collected ~1-8h following capture. The latency

between capture and blood sampling has been shown to not obscure seasonal patterns of hormones in AGS (Buck and Barnes 2003) and another sciurid species (Place and Kenagy, 2000). Blood samples were immediately added to vials containing EDTA, centrifuged and the plasma drawn off and frozen at -80°C until time of assay. Animals were held overnight in the laboratory, provided rodent chow *ad libitum* (Mazuri Rodent Chow, Brentwood, MO, USA) and fresh carrots, and released at site of capture the following morning.

Hormone Assays:

Plasma T was measured in randomized batches between 2010 and 2012. Samples were analyzed in duplicate using a commercially available RIA kit (ImmunoChem™ Double Antibody RIA cat. No. 07189102; MP Biomedicals, Santa Ana, CA, USA). The antibody used in this kit is highly specific (highest cross-reactivity with 5 α -dihydrotestosterone at 3.40%) and the assay has a reported sensitivity of 0.03 ng/mL. We followed the manufacturer's recommended protocol with the following modifications: all volumes were halved and we added an additional standard at 0.05 ng/mL. This protocol provided inter- and intra-assay coefficient of variations (CV's) of 13.6% and 14.1 $\pm$ 8.8%, respectively. We measured inter-assay CV's using a pooled sample quantified in each assay; results of this same pooled sample assayed at both the beginning and end of each assay were used to calculate intra-assay CV's. The assay was validated with both an analysis of standard addition and a test of parallelism. Samples with measured T levels below the lower detectable limit for this assay were assumed at the level of detectability (0.05 ng/mL; 241 from intact males, 31 from castrated males, 43 from females). Two out of the 419 samples from intact males were determined to be statistical outliers, based on a box-plot (higher than the upper quartile + 3*inner quartile range), and therefore omitted

from analysis and presentation; none of the 44 samples collected from female animals were considered outliers.

Plasma DHEA was measured in randomized batches in 2013 and 2016. We measured the active form of DHEA. 100 μ L samples were assayed in duplicate using a commercially available RIA kit (DSL8900, BeckmanCoulter, Pasadena, CA, USA). The antibody used is specific to DHEA with low cross-reactivity (highest with isoandrosterone at 0.733%) and the reported sensitivity of the assay is 9.0 pg/mL. We used the manufacturer's protocol with the following modification: standards were created at 26, 53, 105, 210, 325, 650 and 1650 pg/mL by diluting the provided calibrators with 0 ng/mL calibrator. Inter-assay CV, measured using a single pooled sample run in each assay, was 3.6%, and intra-assay CV, calculated using the pooled sample assayed at the start compared to end of each assay, was $15.7 \pm 7.8\%$. Prior to analysis, samples were extracted using methylene chloride dried under nitrogen gas and re-suspended in phosphate-buffered saline. Other extraction methods using additional solvents were tested, but this method gave the most reproducible results. Extraction efficiencies were calculated for each sample using a hot spike-recovery and resulted in a mean extraction efficiency of $33.1 \pm 0.01\%$. Values are presented without correction as there was little variation in extraction efficiencies and a large number of samples were found to be at or below the detectable limit of the assay. These methods were validated for use in this species using both an analysis of standard addition and a test of parallelism. Samples found to be below the lower detectable limit for this assay (25.5 pg/mL; 62 from intact males, 3 from castrates, 47 from females) were listed at the lower limit for presentation and analysis. One sample out of the 224 analyzed was determined to be a statistical outlier, based on a box-plot, and therefore omitted from analysis and presentation.

Body temperature (T_b) measurement:

One of three different temperature loggers (modified TidBit Stowaway model TBICU32-05+44, (14g, $\pm 0.3^{\circ}\text{C}$), Onset Computer Corp, Bourne MA, USA; iButton DS1922L and DS1921G (both 3g, $\pm 0.5^{\circ}\text{C}$), Maxim Integrated, San Jose, CA, USA), programmed to recorded T_b every 20-120 min (interval dependent on type of logger), was implanted in animals (total of 24 males, 17 females) for semi-continuous measurement of T_b and subsequent analysis of hibernation phenology. Prior to implantation, loggers were calibrated, coated in Elvax (DuPont, Wilmington, DE, USA), and gas-sterilized. Briefly, animals were anesthetized using isoflurane, a 3-5 cm incision was made along the animal's midline through the linea alba and the logger was placed inside the peritoneal cavity. The muscle and subcutaneous layers were closed using chromic gut and polydioxanone sutures (Ethicon, Somerville, NJ, USA) respectively, and the skin subsequently glued (Vetbond, 3M, St. Paul, MN, USA). After surgery animals were returned to their traps and provided with fresh carrots and rodent chow, and held overnight in the laboratory before being released at site of capture the following morning. Another group of 14 males underwent all the surgical procedures described above, and, additionally, were castrated. Castration was accomplished via exposure of the gonadal fat pad, isolation of the testis, ligation of the vas deferens and blood supply with chromic gut suture, followed by gonadal excision. Sham-castrates included all of the intact males implanted with a data logger. Nine of the 14 castrates were subsequently recovered and had their temperature loggers downloaded. The animals without recovered loggers were all recaptured at least once after surgery and were likely lost to predation or dispersal. All surgeries occurred either in the early spring, within a month of emergence, or in the late summer/fall, prior to hibernation entrance.

Characteristics of T_b regulation across hibernation were analyzed as described in Buck et al. 2008. Heterothermy start date was defined as the first day T_b decreased below

30°C and heterothermy end date was defined as the last time T_b increased above 30°C. Mean torpor bout length was calculated for the duration that T_b was $\leq 30^\circ\text{C}$ for each bout of torpor. The length of the pre-emergent euthermic period was determined as the duration prior to the resumption of strong diel rhythms in T_b indicative of aboveground activity (Williams et al. 2012b). We use hibernation to refer to the period that animals remained below ground, including the pre-emergent euthermic period, and heterothermy to refer to the period of time when animals were undergoing cycles of torpor and arousal.

Behavioral Observations:

Five free-living, intact adult male AGS were observed within a 4.2 hectare grid during the 2008 active season. The grid was separated into 5 equal sections (0.84 hectares) with each section observed, weather permitting, every 5 days. Observations were made from a fixed location on the grid each day at a distance of 5-10 m from the animal of interest using binoculars (10X x 42) when necessary. Behavioral observations were conducted from 09:30 – 17:00 from 4 July – 4 August, 2008. The four weeks of focal observations were pooled as follows: Week 1 = 4 – 12 July (Standardized day 106 – 114), Week 2 = 13 – 19 July (Standardized day 115 – 121), Week 3 = 20 – 26 July (Standardized day 122 – 128), and Week 4 = 27 July – 4 August (Standardized day 129 – 137). The weeks are unevenly divided due to inconsistencies with weather and the number of days animals were observed. When precipitation occurred for >50% of the day, animals showed very little activity (Williams et al. 2014a) and therefore these days were deemed inadequate for observation and omitted from analysis.

Behavior was tracked and recorded using a computer program (Behavior Tracker #158503, version 1.5). Behaviors monitored included foraging, aggressive interactions, grooming, alarm-calling, time below ground, and caching; however, only foraging,

aggressive interactions and caching are reported here since these are the behaviors we hypothesize are under the influence of late summer androgens. Behaviors were analyzed for total time, in addition to the number of times the behavior took place. All behaviors were standardized by the total amount of time each animal was observed as this substantially varied from animal to animal (0.2 – 4.0 h per week). The ratio of time spent engaged in each behavior/total time observed is presented.

All procedures used were designed to minimize any pain and discomfort to the animals, are in accordance with NIH standards, and were approved by the University of Alaska Fairbanks' and Anchorage's Institutional Animal Care and Use Committees (Protocol #'s: 340270-33, 148893-1, 130316-31, 160426-4).

Statistical Analysis:

All data presented are means $\pm$ SEM, unless otherwise noted. Comparisons were considered significant when $p < 0.05$. Timing of blood sampling, and thus reported hormone concentrations are standardized to the mean date of the end of heterothermy of reproductively competent males in the year the sample was collected. This method accounts for year-to-year variations in circannual timing such that Standardized Date 1 is the mean date reproductively mature males ended heterothermy and Standardized Date 50 is fifty days after. Each hormone was analyzed separately and grouped into Spring (Standardized Date 1 to 50), and Late Summer (Standardized Date 100 to 200) sample bins. Hormone data were analyzed using a One Way Analysis of Variance (ANOVA) with a Tukey multiple comparison post hoc test (SPSS Statistics, 17.0, IBM Armonk, NY, USA) and a linear regression in SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA, USA). Behavior data and associated DHEA values were analyzed using linear regressions; prior to analysis, percent time foraging data were arc-sin transformed. To

account for multiple samples from the same individual in the same bin (i.e., “Spring” or “Late Summer”), values were averaged and means were analyzed. Characteristics of hibernation phenology were analyzed for differences using a one-way ANOVA with LSD multiple comparisons correction. All statistical tests were performed using SPSS Statistics 17.0 (IBM Armonk, NY, USA) and differences considered significant at $p < 0.05$.

RESULTS:

Plasma androgen levels across the active season fluctuated as predicted (Fig 1 and 2). In the spring, reproductively mature males exhibited the highest levels of plasma T of all groups (Fig 2; 0.6 ± 0.09 ng/mL, $p < 0.05$). Plasma DHEA levels were highest in late summer males, but only significantly higher than those of reproductively mature spring males and females (120.6 ± 18.9 , 35.9 ± 2.1 , and 25.7 ± 0.2 pg/mL, respectively, $p < 0.05$). Using samples analyzed for both androgens and linear regression analysis we found no relationship between T and DHEA levels across the entire active season ($n = 66$, $p = 0.481$), in the spring ($n = 6$, $p = 0.349$), or in the late summer ($n = 57$, $p = 0.672$). Females had T levels below the detectable limit across the entire active season (<0.05 ng/mL, $n = 44$, Fig 2.) and the majority of samples assayed for DHEA were at or below the detectable levels of the assay (25.5 pg/mL, 41 of 47 analyzed) though more females had measurable levels of DHEA in the late summer than in the spring (5 of 18 samples).

The results of the castrations are presented in Table 1. The start and end date of heterothermy, duration of hibernation, number of torpor bouts and mean torpor bout length were equivalent in castrates and intact, reproductively immature males (Table 1). The castrates only differed from intact, reproductively immature males in the duration of the penultimate arousal bout, which was significantly longer in castrates than any other

group (75.8 ± 11.4 h, $p \leq 0.033$). Castrated males had a pre-emergent euthermic period of 6.7 ± 2.4 days, which was intermediate between that of intact, reproductively immature males (3.2 ± 2.3 days) and reproductively mature males, which had significantly longer pre-emergent euthermic periods than any other group (16.6 ± 2.1 days, $p \leq 0.023$). Castrates (Apr 22 ± 2.9), intact, reproductively immature males (Apr 17 ± 6.0), and females (Apr 17 ± 1.4) all ended heterothermy on similar dates and significantly later than reproductively competent males (Mar 28 ± 3.9 , $p \leq 0.001$).

The results of a month-long observational period are summarized in Fig 3. We observed a trend towards a decline in the percentage of time males spent foraging ($74.5 \pm 13\%$ in Week 1 to $65.2 \pm 7.2\%$ in Week 4, however, this did not reach significance ($r^2 = 0.0784$, $p = 0.294$), possibly due to low power (0.178), Fig 3a). Additionally, males increased both the number of aggressive encounters per hour (Week 1: 8, Week 4: 26, $r^2 = 0.265$, $p = 0.041$; Fig 3b) and incidences of caching per hour (Week 1: 0, Week 4: 12, $r^2 = 0.399$, $p = 0.007$; Fig 3b) in the in the first 4 weeks of late summer. The increased instances of caching and aggression coincided with increasing levels of DHEA (one-way ANOVA, $F(6, 123) = 3.51$; $p = 0.027$). Unfortunately, behavioral observations were not conducted during the next 4 weeks when DHEA levels continued to rise.

DISCUSSION:

In many species of ground squirrels, adult males end heterothermy and emerge earlier than females in the spring (reviews: Michener 1984, Williams et al. 2014b); however, the endocrine drivers of sex differences in hibernation phenology are not known. We proposed that the differences may be driven by differences in patterns of circulating androgens. The objective of this study was to determine the role of androgens

in circannual timing of male AGS. We quantified active season concentrations of plasma T and DHEA using RIAs. We predicted that high levels plasma T would only be found in intact, reproductively mature males and that higher DHEA levels would be found only in males in the late summer to support their late season aggression and late hibernation onset. As we predicted, T levels were highest among reproductive males in the early spring coincident with the mating season (0.5 ± 0.01 ng/mL), and then quickly declined to baseline levels following breeding (females pregnant by 7 May (Standardized Date 40); Sheriff et al., 2011) and testicular regression. Males maintained low concentrations of T (0.06 ± 0.00 ng/mL) for the remainder of the active season, similar to the levels found in immatures, castrates and females, throughout the active season (Fig 1 and 2). We found that plasma DHEA levels remained low in mature males throughout the active season until late summer when males displayed a second aggressive period (Buck and Barnes 2003) and increased plasma DHEA levels (Fig 1 and 2). Using castration to eliminate the primary endogenous source of T, we established a link between T and the early end of the heterothermic season in male AGS (Table 1). The similarity between the timing of hibernation onset in the fall in intact and castrated male AGS suggests that gonadal androgens do not play a role in prolonging euthermia in males. The measureable levels of DHEA in some females (5 of 18 samples collected from late season females had measureable levels of DHEA) seemingly contradicts our hypothesis that late season DHEA is responsible for the delayed hibernation onset observed in male AGS because females enter hibernation at an earlier date.

There are numerous functional implications of increased T in males in the spring. The most obvious function of high T in the spring is to initiate and maintain reproductive development and spermatogenesis. Another closely related function of high T in spring is to support the courtship behaviors and territoriality associated with the male-male

aggression observed in AGS during the spring mating season (Buck and Barnes 2003). The data presented here are unique in that they show that the specific androgen influencing these behaviors is gonadally derived T (Fig 1, Table 1). Because T is known to inhibit hibernation (Lee et al. 1990), an additional hypothesized function for elevated T in spring is to end heterothermy in males undergoing reproductive development (Barnes 1996). Support for this hypothesis comes from the increased circulating T and follicle stimulating hormone found in males just prior to the cessation of torpor-arousal cycles (Barnes et al. 1988) and spring-gonadectomized males re-entering torpor-arousal cycles (Dark et al. 1996). The data from the current study clearly demonstrate that free-living, castrate males end heterothermy and emerge significantly later in the spring compared to their intact and reproductively mature counterparts (Table 1).

The elevated levels of circulating DHEA found in late summer animals occurs over a longer period of time than the T peak found in reproductive spring males (Fig 1 and 2). We postulate two functions of this late summer androgen surge. One possible action of increased circulating DHEA is to prevent males from entering torpor at an earlier time point coincident with females (Table 1). Delayed entrance into hibernation by males does not appear to be driven entirely by energetics. Males attain peak lean body mass well before their immergence into hibernation and, in contrast to females, delay fattening and entrance into hibernation (Sheriff et al. 2013). Behaviors of males prior to hibernation are characterized by intense caching and aggression (Carl 1971; Buck and Barnes 2003). Thus, a second possible action of increased circulating DHEA is to facilitate these behaviors prior to the onset of hibernation. Non-mating season aggression has been linked to increased levels of DHEA in a number of mammalian and avian species such as red squirrels (*Tamiasciurus hudsonicus*): Boonstra et al. 2008; dwarf hamsters (*Phodopus sungoras*): Scotti et al. 2008; review: Soma et al. 2008,

2015). From observations made in the field we know that from the beginning of July through August, when the study ended, male AGS tended to decrease time above ground foraging (from $75.5 \pm 13.5\%$ of their time foraging in the first week of July to $65.2 \pm 7.2\%$ of their time foraging the first week of August). During this same period we also observed an increase in the number of aggressive interactions (from 8 to 26), and instances of caching (from 0 to 12). Additionally, we have observed aggressive encounters instigated by females during this same time period, which coincides with the measureable levels of DHEA (Fig 2).

Boonstra et al. (2011; 2014) have proposed an anabolic function and need for circulating androgens in AGS in late summer. They propose that elevated androgens and the localization of androgen receptors enable AGS to increase their lean mass in preparation for hibernation. This increased lean mass is subsequently drawn upon to support the mixed fuel metabolism of thermogenesis during hibernation (Buck and Barnes, 2000). Although compelling, in our population of free-living AGS, we find a temporal mismatch between the period of lean mass accretion (Sheriff et al. 2013) and elevated late summer DHEA concentration (Fig 1 and 2). Free-living AGS in this population complete lean mass growth by 12 August (Sheriff et al. 2013), well before the rise in DHEA, but after the high levels of T found in spring. Thus, in Arctic Alaska, the late summer increase in DHEA does not likely facilitate anabolism, but rather influences rates of aggression and caching behavior among males observed late in the active season.

CONCLUSIONS:

This contribution characterizes the pattern of secretion of two androgens, T and DHEA, in the plasma of free-living AGS (Fig 1 and 2) and links fluctuations of these hormones to

observed behaviors, caching and aggression (Buck and Barnes 2003, Fig 1, 2, 3).
Castrated male AGS displayed similar hibernation characteristics to reproductively
immature males, indicating a role for T in influencing the timing of the end of
heterothermy and pre-emergent euthermy, but not the timing of hibernation onset (Table
1). Further work is required to determine what role, if any, androgens play in influencing
the timing of hibernation onset. In particular, manipulating DHEA levels in both sexes to
determine its role in both hibernation phenology and late season aggression is of
particular interest.

FIGURE and TABLE LEGENDS:

Fig 1: Scatter-plot of individual plasma samples collected from free-living male AGS
from 2008 to 2013. Each point represents a single assayed sample. For illustrative
purposes, individual animals are represented from one to ten times, depending on the
number of times the animal was captured for subsequent sampling (total individuals for T
= 143, for DHEA = 129); statistical analyses utilize means for individuals sampled
multiple times during a sample bin (see Statistical Analysis section of Methods). Closed
circles represent plasma testosterone concentrations (ng/mL) and open squares
represent plasma DHEA concentrations (pg/mL). DHEA values were not corrected for
extraction efficiency due to the number that were at or below the detectable limit of the
assay. All samples that fell below the lower detectable limit of the assay have been
adjusted to the value of lower detectable limit. Sample dates have been standardized to
the mean date of euthermy for reproductive males in the year the sample was collected.
Total number of samples assayed for testosterone concentration is 406 and DHEA
concentration is 223.

385

386 **Fig 2:** Effects of seasonal and reproductive status on plasma androgen concentrations.
387 Reproductive status was determined at time sample was collected. Star (*) and letters
388 (DHEA) indicate statistical significance between groups ($p < 0.05$) as determined by a
389 Tukey One Way Analysis of Variance. Values presented are means $\pm$ SE and
390 uncorrected for extraction efficiency. Number of samples per group for testosterone,
391 from left to right: 62, 15, 21, 10, 308, 15 and 26. Number of samples per group for
392 DHEA, from left to right: 93, 7, 35, 4, 158, 18 and 9.

393

394 **Fig 3:** Summary of behavior observations of free-living male AGS. Male AGS tended to
395 decrease the percentage of time spent foraging over the course of the observational
396 study (left panel). Male AGS also increased incidences of caching and aggression per
397 hour of observation (right panel). Week 1: 4 – 12 July (Standardized Date 106 – 114,
398 $n=4$), Week 2: 13 – 19 July (Standardized Date 115 – 121, $n=5$), Week 3: 20 – 26 July
399 (Standardized Date 122 – 128, $n=3$), and Week 4: 27 July – 4 August (Standardized
400 Date 129 – 137, $n=4$). The line presents plasma DHEA concentrations (means $\pm$ SEM;
401 pg/mL) during and after the observational study ($n= 0, 19, 9, 32, 35, 23, 10, 2$).

402

403 **Table 1:** Summary of characteristics of heterothermy of free-living AGS three
404 consecutive hibernation seasons (2009 to 2012), presented as means $\pm$ SEM.
405 Heterothermy start date = first day T_b decreased below 30°C and heterothermy end date
406 = last time T_b increased above 30°C. Mean torpor bout length = duration T_b remained
407 $\leq 30^\circ\text{C}$. The length of the pre-emergent euthermic period = the duration prior to the
408 resumption of above ground activity, as defined in Williams et al. 2012 by initiation of

pronounced 24 h rhythms of T_b . Differences were determined using a one-way ANOVA with LSD multiple comparison correction. Letters indicate groups that are statistically different ($p < 0.05$).

	Heterothermy Start	Heterothermy End	Heterothermy Duration (Days)	Number of Torpor Bouts	Mean Torpor Bout Length (Days)	Penultimate Arousal Bout Length (Hours)	Length of Pre-Emergent Euthermic Period (Days)
Repro (n=18)	Oct 3 $\pm$ 3.1 ^A	Mar 28 $\pm$ 3.9 ^A	174.8 $\pm$ 4.5 ^A	8.8 $\pm$ 0.6 ^A	14.1 $\pm$ 0.7 ^A	39.0 $\pm$ 7.7 ^A	16.6 $\pm$ 2.1 ^A
Non-Repro (n=6)	Sept 27 $\pm$ 5.7 ^A	Apr 17 $\pm$ 6.0 ^B	201.2 $\pm$ 7.4 ^B	11.2 $\pm$ 0.9 ^B	16.3 $\pm$ 0.7 ^B	34.4 $\pm$ 9.7 ^A	3.2 $\pm$ 2.3 ^{B,C}
Castrate (n=9)	Oct 12 $\pm$ 1.0 ^A	Apr 22 $\pm$ 2.9 ^B	191.3 $\pm$ 7.1 ^B	10.7 $\pm$ 0.8 ^{A,B}	14.9 $\pm$ 0.7 ^{A,B}	75.8 $\pm$ 11.4 ^B	6.7 $\pm$ 2.4 ^C
Adult Female (n=15)	Aug 18 $\pm$ 3.8 ^B (n=17)	Apr 17 $\pm$ 1.4 ^B	241.0 $\pm$ 3.5 ^C	14.5 $\pm$ 0.5 ^C	16.1 $\pm$ 0.5 ^B	27.2 $\pm$ 2.3 ^A	0.4 $\pm$ 0.2 ^B

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