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Exogenous leptin promotes reproductive behavior during aphagia in red-sided garter snakes (*Thamnophis sirtalis parietalis*)

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ABSTRACT

Despite the established dichotomy between investment in either reproduction or self-maintenance, a hormonal mechanism that influences an organism's decision to prioritize these behaviors remains elusive. The protein hormone leptin is a likely candidate because it is secreted from adipocytes in proportion to the amount of stored fat in numerous species. Although the majority of studies suggest that leptin stimulates reproduction, the actions of leptin can be context-dependent. Leptin increases sexual behavior in fed individuals, but inhibits sexual behavior in food-restricted individuals. We investigated if exogenous leptin influences sexual behavior in red-sided garter snakes (*Thamnophis sirtalis parietalis*) experiencing a predictable bout of aphagia during the mating season. We tested two doses of recombinant murine leptin injected for three days. Males were subjected to three mating trials, one on each day of injections, while females were subjected to one mating trial on the last day of injections. Leptin affects male and female snakes similarly by increasing both appetitive (i.e., mating behavior score) and consummatory (i.e., number of copulations, proportion of individuals copulated) sex behavior. We found no evidence to suggest that leptin influenced latency to copulate or duration of copulation. Because leptin promotes reproductive behavior in non-feeding garter snakes, these findings do not align with research on food-restricted mammals. Further investigations into how leptin affects sexual behavior in snakes exposed to food-restriction manipulations would clarify if the role of leptin is evolutionarily divergent.

1. Introduction

All organisms must decide to invest in either reproductive or self-maintenance (e.g., feeding or foraging) activities, as organisms often cannot perform these activities simultaneously. Despite the thoroughly established tradeoffs associated with investment in either reproduction or self-maintenance, a physiological mechanism that influences an organism's decision to prioritize one behavior over another remains elusive. A multitude of evidence suggests that energy substrates, specifically fat stores, positively relate to reproduction (Aubret et al., 2002; Doughty and Shine, 1998; Harlow et al., 2002; Lu et al., 2012; Smith and Moore, 2003; Wapstra and Swain, 2001). Further, insufficient energy stores can prematurely end reproductive activities (Groscolas et al., 2008). Because reproduction is highly energetically expensive, an energy-related signal likely exists to indicate when it is appropriate to prioritize reproductive over other self-maintenance activities such as foraging and feeding behaviors.

A likely candidate to signal the appropriate time to perform reproductive activities is the protein hormone leptin, as it is secreted from adipocytes in proportion to the amount of fat in numerous mammalian species (Delavaud et al., 2000; Frederich et al., 1995; Fuglei et al., 2004). However, during the prehibernation period in little brown bats (*Myotis lucifugus*), leptin secretion is dissociated from body fat percentage (Kronfeld-Schor et al., 2000). Such a dissociation may be necessary to ensure the accumulation of sufficient energy stores for survival during life-history stages such as hibernation or migration. Despite this dissociation in some species, leptin decreases with fasting and increases with refeeding in a few fish species (Garcia-Suarez et al., 2018; Volkoff, 2015; Won et al., 2012) and a wide range of mammals (Chelikani et al., 2004; Daniel et al., 2002; Delavaud et al., 2000; Walker et al., 2005; Weigle et al., 1997; Widmaier et al., 1997).

While comparatively little research has focused on the function of

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leptin in ectotherms other than fishes, leptin has been identified in various tissues of a few reptiles. Preliminary results identified the leptin gene and its receptor in the Green anole (*Anolis carolinensis*; reviewed in Denver et al., 2011). The leptin protein was identified in several lizard tissues including plasma, whole blood, brain, and stomach (Spanovich et al., 2006; Muruzábal et al., 2002; Paolucci et al., 2001; Niewiarowski et al., 2000). To date, only one study has examined the leptin protein in snakes, and it was found in the stomach of natricine water snakes (*Natrix maura*; Muruzábal et al., 2002). In some non-mammalian species including fishes, leptin mRNA is expressed in tissues other than adipose (e.g., liver; Denver et al., 2011; Muruzábal et al., 2002). Further, leptin does not accurately signal stored energy substrates in fishes, and therefore species-specific validations are necessary to determine what energetically-relevant information leptin conveys (Londraville et al., 2014).

Regardless of the tissue that produces and secretes leptin, numerous lines of evidence from an array of taxa (e.g., mammals, reptiles, fishes) suggest that leptin promotes reproductive behavior and physiology (Finn et al., 1998; Peyon et al., 2001; Putti et al., 2009; Schneider et al., 2007; Weil et al., 2003). For example, whole blood leptin varies seasonally and between populations in eastern fence lizards (*Sceloporus undulatus*; Spanovich et al., 2006), and plasma leptin is highest at the start of the reproductive season in female Italian wall lizards (*Podarcis sicula*; Paolucci et al., 2001). However, the role of leptin can be context-dependent, as leptin causes the initiation of a second clutch in one study on great tits (*Parus major*), but not in another using identical methods (Lõhmus and Björkŏund, 2009; te Marvelde and Visser, 2012; but see Friedman-Einat and Seroussi, 2019, 2014; Londraville et al., 2014; and Prokop et al., 2014 for further dicussion on avian leptin). Further highlighting the context-dependent role of leptin is the finding that leptin promotes reproductive behavior in fed female Syrian hamsters (*Mesocricetus auratus*) but inhibits reproductive behavior in food-deprived females (Wade et al., 1997). It is possible that leptin's ability to exert its effects [e.g., promote reproduction, increase body temperature and metabolic rate (e.g., Mistry et al., 1997; Niewiarowski et al., 2000)] depends on its ability to mobilize energy substrates. Indeed, when administered in conjunction with treatment that blocks the oxidization of metabolic fuels, leptin cannot reverse fasting-induced anestrus in Syrian hamsters (Schneider et al., 1998). To further explore how leptin interacts with energy metabolism to influence reproduction, we aimed to determine the influence of leptin on reproductive behavior in a species that is aphagic prior to and during the mating season.

Northern populations of red-sided garter snakes (*Thamnophis sirtalis parietalis*) exhibit an annual life-history cycle that allows the investigation of leptin's role in reproduction in the absence of feeding behavior. Snakes overwinter for approximately 8 months in underground hibernacula and upon spring emergence engage in an intense and short mating period all while being aphagic (Gregory and Stewart, 1975). During the spring mating season, red-sided garter snakes exhibit robust and stereotyped mating behaviors (e.g., Crews et al., 1984). After varying amounts of time spent at the den performing mating behaviors, snakes migrate upwards of 17 km to summer feeding grounds to replenish energy stores (Gregory, 1977; Gregory and Stewart, 1975). Snakes leave the summer feeding grounds to migrate back to overwintering sites in the fall and once again become aphagic. Therefore, investigating if leptin promotes or inhibits reproduction in an organism experiencing predictable bouts of aphagia will provide insight into whether the role of leptin differs with an animal's ecology.

While research into serpentine leptin is extremely limited, preliminary analyses indicate that leptin is present in fat bodies and plasma of red-sided garter snakes (Wilson and Lutterschmidt, unpublished data). Although our preliminary data suggest leptin does vary seasonally, whether or not leptin influences feeding behavior in red-sided garter snakes is not clear. Surprisingly and contrary to the mammalian data, the one study investigating the effect of leptin on food consumption in reptiles reports leptin increases food consumption in vitellogenic tree lizards (French et al., 2011). In addition to leptin promoting reproduction in various other species, several studies suggest that fat stores promote reproductive behavior and physiology in snakes (Aldridge et al., 2003; Aubret et al., 2002; Bonnet et al., 1994; Scott et al., 1995). For instance, gravid eastern cottonmouth snakes (*Agkistrodon piscivorus*) have higher total lipid content compared to nongravid conspecifics (Scott et al., 1995). Fat mass of male diamondback watersnakes (*Nerodia rhombifier*) positively and significantly relates to seminiferous tubule diameter and the sexual segment of the kidney (Aldridge et al., 2003). Further, leading up to the spring mating season in red-sided garter snakes, elevated temperatures during hibernation deplete fat stores of male snakes to a greater extent than low-temperature dormancy conditions (Wilson, 2020). Therefore, examining how fat-related metrics influence reproduction would provide insight into how depletion of energy stores during hibernation alter reproductive effort, which may ultimately influence fitness in this and other species.

In the present study, we aimed to determine how leptin influences both appetitive and consummatory sex behaviors in red-sided garter snakes. Appetitive sexual behavior describes an individual's motivation to gain access to sexual partners. In contrast, consummatory sexual behavior concerns behaviors associated with the act of copulation and often includes measurements such as the number of mounts, intromissions, and ejaculations (Beach, 1956). Because different regions of the brain control appetitive and consummatory sexual behaviors (Balthazart and Ball, 2007), we wanted to investigate the effect of leptin on both, as prior evidence suggests that leptin can differentially affect consummatory versus appetitive sexual behaviors in female rats (García-Juárez et al., 2011). In red-sided garter snakes, we measured appetitive sex behavior by quantifying reproductive behavior using ethograms. In male snakes, we measured the following consummatory sexual behaviors: number of copulations, duration of copulation, and copulatory plug mass. In females, we measured the proportion of females that copulated and duration of copulation. We hypothesized that leptin treatment affects both appetitive and consummatory sexual behaviors in red-sided garter snakes, and predicted that leptin would increase reproductive behavior.

2. Methods

Experiments were conducted on field-caught red-sided garter snakes (*Thamnophis sirtalis parietalis*) from Inwood, Manitoba, Canada. Female snakes were studied over two consecutive years during the May 2016 (n = 29) and 2017 (n = 90) spring mating seasons. Male snakes (n = 72) were collected during May 2017. The Portland State University Institutional Animal Care and Use Committee (protocol numbers 40 and 54) and Manitoba Department of Sustainable Development (collecting permit WB18801) approved all experimental procedures. For all snakes, we measured mass and snout-vent length (SVL) to ensure these variables did not differ among treatment groups. After completion of the experiments, we released snakes at their initial capture site.

2.1. Experimental design

2.1.1. Leptin injections

In 2016, female snakes were assigned to one of two treatment groups: vehicle or high leptin dose. Snakes collected in 2017 were randomly assigned to one of three treatment groups: vehicle, low leptin dose, or high leptin dose. Recombinant murine leptin was purchased from the National Hormone and Pituitary Program at Harbor-UCLA Medical Center, Torrance CA. Similar to French et al. (2011), we used doses of 0, 7, or 70 μg leptin for females; males were injected with 0, 3, or 30 μg leptin. Injection volume was 0.1 mL of solution regardless of leptin treatment, which allowed us to efficiently treat a large number snakes each day within a relatively short time period. Ampoules of recombinant leptin were reconstituted with 0.1 M phosphate buffer following manufacturer instructions, which we also used as vehicle

injections. Snakes were injected intraperitoneally once a day between 0900 and 1000 h for three consecutive days.

Female doses were higher than males because of a sexual size dimorphism in red-sided garter snakes where females are larger and heavier than their male counterparts. We utilized the mean mass of snakes from prior experiments to approximate leptin doses a priori. Because we measured mass for each snake in this study, we report here the mean (± s.e.m) mass-specific doses (μg leptin/g of body mass) as calculated post-hoc: the low leptin dose in females and males was 0.11 ± 0.00 and 0.10 ± 0.00 μg per g of body mass, respectively; the high leptin dose was 1.09 ± 0.03 and 1.06 ± 0.03 μg per g of body mass for females and males, respectively.

2.1.2. Female reproductive behavior

One hour following the third leptin injection, we subjected female snakes to a single mating trial, as females become unattractive after mating (Garstka et al., 1982; O'Donnell et al., 2004). Mating trials occurred in 1 × 1 × 1 m^3 cloth arenas where we provided females with the opportunity to mate for 60 min in the presence of 25 males in 2016 or 15 males in 2017. We developed an ethogram (Table 1) to score the behavior of each female once every 20 min during the 60-min mating trial or until copulation occurred for a maximum of three scores. To score behavior, observers spent approximately 1 min to classify a female's behavior. All mating behavior scores received prior to mating during the trial were then used to calculate a mean score for each individual; these mean scores are a measure of appetitive reproductive behavior. When a mating occurred, we recorded latency to copulate and then carefully removed the copulating pair to a separate arena to measure duration of copulation. In some cases (n = 29), females mated before we could score their mating behavior, and these females were not included in analysis of behavior scores (i.e., appetitive behavior). Equal numbers of females from each treatment group mated prior to receiving a behavior score: Control = 10, Low leptin = 10, High leptin = 9.

2.1.3. Male reproductive behavior

To determine if leptin affects male reproductive behavior, we conducted a three-hour long mating trial in 1 × 1 × 1 m^3 cloth arenas on each day of injections. Trials were initiated 1 h after injections. Equal numbers of males from each treatment group (n = 4 per group for a total of 12 males per arena) were introduced to a single, sexually attractive female red-sided garter snake. We deemed females attractive if they elicited courtship from at least 5 males.

During mating trials, we measured courtship scores once every 20 min using a previously described ethogram (Table 1; Crews, 1976; Lutterschmidt et al., 2004). Because there were 12 males to score in each arena, observers spent approximately 5 min to determine the males'' behavior at the end of each 20-min period. All mating behavior scores received prior to mating during the trial were then used to calculate a mean courtship score for each individual (i.e., a measure of appetitive behavior). When a mating occurred, we recorded latency to copulate and the copulating pair was carefully removed from the mating arena and placed in a separate arena to measure duration of copulation. At cessation of copulation, we placed the mated female in a cloth bag until we could remove and weigh copulatory plugs later that day to determine if exogenous leptin affects copulatory plug mass; the mated male was moved to his home arena. After removal of a copulating pair, we introduced a new female into the arena along with a new, non-experimental male to maintain an equal number of males in all arenas. We taped the cloaca of the non-experimental male with medical adhesive tape to prevent him from copulating. This procedure limited the number of copulations a male could perform to 1 per day. In the event a mating did not take place in an arena within 60 min, we removed the unreceptive female (e.g., Dayger et al., 2013) and introduced a new, sexually attractive female into the mating arena.

Table 1
Ethograms of sex behavior of female and male red-sided garter snakes (*Thamnophis sirtalis parietalis*).

Score	Female behavior description	Male behavior description
0	Female is moving rapidly away from courting males	No reproductive behavior
1	Female is moving slowly	Male investigates female, increased tongue-flick rate
2	Female's body, including head and tail, is flattened to ground	Male chin rubs female with rapid tongue flicks
3	Stationary female allows males to manipulate her limp tail	Male aligns body with female
4	Stationary female actively elevates tail, responds to male manipulation of tail	Male actively tail searches and attempts cloacal apposition and copulation with female; possible caudocephalic waves
5	Female rolls her body slightly to the side and gapes her cloaca	Male copulates with female
6	Female copulates	–

The female ethogram was developed for this experiment, while male behavior descriptions are as in Lutterschmidt et al. (2004) (modified from Crews et al., 1984 and Moore et al., 2000). Note that the above ethograms are independent of one another and are specific to the focal animal (i.e., male or female) rather than a copulating pair.

2.2. Statistics

Where necessary, data were either log- or square root-transformed to meet the assumptions of parametric tests. If data could not be transformed to adhere to assumptions, we used non-parametric tests. All statistics were run in Sigma Stat 12.5 (Systat Software, Inc.). We report effect sizes as η^2 (SS_{factor}/SS_{total}) for analysis of variance (ANOVA), r for Mann-Whitney *U* tests, and Cramer's V for Chi squared analyses.

To verify that our ethogram of female mating behavior (Table 1) accurately assesses a female's interest in copulation, we used a *t*-test to determine if the mating behavior score differed between females that mated and those that did not mate during behavior trials. We excluded any scores of 6 (i.e., female copulates) in this analysis, as only females that mated could achieve this score. To ensure no differences existed in female body morphometrics between years and among treatment groups, we ran a two-way ANOVA on both mass and SVL.

We used a one-way ANOVA to determine if treatment with leptin affected average mating behavior score; we did not include year in this analysis because we developed the female behavior ethogram in 2017 and therefore there were no mating behavior scores from 2016. To determine if leptin affected the proportion of females that mated, we used a 3 × 3 Chi-square test. Because we observed the same mating proportions across years (from a Chi-square test on controls between 2016 and 2017: $X^2 = 0.11$, p = 0.746; and a Fisher's exact test on high leptin-dosed females between 2016 and 2017: p = 1.00), we combined data for analysis to increase sample size. As post-hoc tests, we used 2 × 2 Chi-square tests to determine which treatment groups significantly differed. In those females that mated (i.e., were receptive), we determined if exogenous leptin affects latency to copulate (i.e., proceptivity; Dayger et al., 2018) and the duration of copulation using a two-way ANOVA with treatment and year as between-subjects factors. An unbalanced experimental design, with 2 leptin treatments in 2016 and 3 leptin treatments in 2017, precluded us from running an interaction between year and treatment group for these analyses.

As in females, we ensured that no significant differences existed in male body morphometrics among groups using separate one-way ANOVAs. We utilized a two-way repeated measure ANOVA with treatment as the between-subjects factor and day as the within-subjects factor to determine if leptin affects average courtship score. To determine if exogenous leptin affected the number of matings a male achieved, we utilized a one-way ANOVA. Within males that mated, we also utilized one-way ANOVAs to determine if leptin affected latency to copulate, duration of copulation, and copulatory plug mass. We analyzed these response variables only for the first mating a male

performed regardless of the day that the mating occurred because the low sample sizes of males that performed multiple matings (second matings: n = 4, 5, 9; third matings: n = 0, 1, 2 for control, low leptin, and high leptin individuals, respectively) may not provide statistically meaningful results.

3. Results

3.1. Female reproductive behavior

In females, neither mass nor SVL differed significantly by year (Mass: $F_{1,115} = 2.54$, $p = 0.114$, $\eta^2 = 0.021$; SVL: $F_{1,115} = 0.26$, $p = 0.615$, $\eta^2 = 0.002$) or treatment group (Mass: $F_{2,115} = 0.56$, $p = 0.570$, $\eta^2 = 0.010$; SVL: $F_{2,115} = 0.14$, $p = 0.873$, $\eta^2 = 0.002$). Females that went on to mate had a significantly higher average mating behavior score prior to mating (i.e., prior to achieving a score of 6) than females that did not mate (Fig. 1, $U = 222.5$, $p = 0.001$, $r = 0.45$), verifying that our female ethogram (Table 1) accurately assesses a female's interest in mating. Note that we excluded from this analysis all scores of 6 (i.e., mating). This analysis therefore compares the appetitive aspects of female mating behavior prior to the occurrence of mating.

All females received similar levels of courtship from males regardless of leptin treatment (data not shown). The average mating behavior score, a measure of appetitive reproductive behavior, was influenced by leptin treatment (from a nonparametric one-way ANOVA on ranks; Table 2; Fig. 2). Females injected with a low dose of leptin had significantly higher average mating scores compared to control individuals (Fig. 2). We also found that exogenous leptin significantly increased the proportion of female snakes that mated (Table 2, Fig. 3A). Two-way Chi-squared tests for pairwise comparisons between treatments revealed that only the control and high leptin treatment groups differed significantly in the proportion of females mating (Fig. 3A; $X^2 = 7.17$, $p = 0.007$, $r = 0.29$). Of those females that mated, neither treatment nor year influenced latency to copulate (Table 2, Fig. 3B). There was no effect of leptin treatment on duration of copulation (Table 2, Fig. 3B), but females tested in 2017 had a longer duration of copulation compared to females in 2016 (Table 2).

Because the differences in copulation duration observed between years may reflect variation in environmental conditions and/or resource availability, we wanted to examine potential factors that could explain the difference in copulation duration between years. Body condition index can be an integrative proxy for environmental conditions and resource availability (McKinnon et al., 2015). As such, we wanted to determine if female body condition index differed between years and/or influenced responses to exogenous leptin. We calculated body condition index as the residual from a regression of log-transformed body mass on log-transformed SVL in females collected in each of 2016 and 2017. We converted each numerical body condition index into categorical data by assigning females with a residual below 0 as negative and above 0 as positive body condition (Dayger et al., 2013). After running a two-way ANOVA, we found that the mean body condition index of females in 2017 (mean 0.005 ± 0.005 SE) was significantly higher than that in 2016 (−0.019 ± 0.010; Table 2). Female body condition index did not differ significantly among treatment groups (Table 2). We then re-

Table 2
Statistical results from analyses of the effects of exogenous leptin on female reproductive behavior in red-sided garter snakes (*Thamnophis sirtalis parietalis*).

Response variable	Factors	Test statistic	p-Value	Effect sizes
Mating behavior score	Leptin treatment	$H = 7.34$	0.025	$r = 0.30$
Proportion of mated females	Leptin treatment	$X^2 = 8.51$	**0.014**	$V = 0.269$
Latency to copulate	Leptin treatment	$F_{2,72} = 2.26$	0.112	$\eta^2 = 0.059$
	Year	$F_{1,72} = 0.14$	0.711	$\eta^2 = 0.002$
Duration of copulation	Leptin treatment	$F_{2,72} = 1.20$	0.307	$\eta^2 = 0.030$
	Year	$F_{1,72} = 7.06$	**0.010**	$\eta^2 = 0.088$
Body condition index	Leptin treatment	$F_{2,115} = 0.72$	0.491	$\eta^2 = 0.012$
	Year	$F_{1,115} = 4.98$	**0.028**	$\eta^2 = 0.041$
Duration of copulation	Leptin treatment	$H_{2,70} = 1.41$	0.486	$r = 0.09$
	Body condition	$H_{1,70} = 0.27$	0.606	$r = 0.10$
	Leptin * Body condition	$H_{2,70} = 1.54$	0.462	$r = 0.08$

Because of the significant difference in duration of copulation between years, we used body condition index as a proxy for environmental conditions and re-analyzed the data (indented Response variables).
Bold font indicates $p < 0.05$.

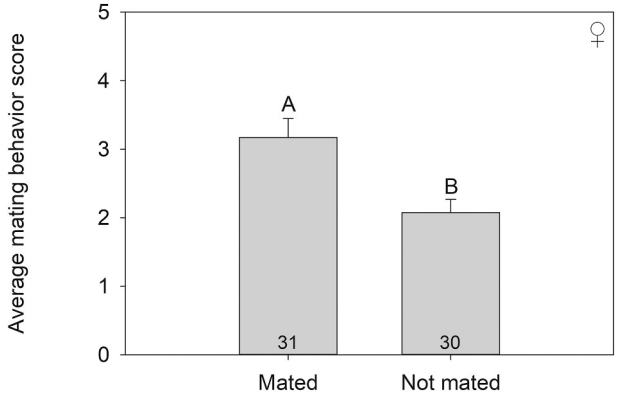

Fig. 1. Verification that the mating behavior ethogram for female red-sided garter snakes accurately assesses a female's interest in copulation. Females that went on to mate displayed significantly higher average mating scores prior to mating than females that did not go on to mate during a 60 min mating trial. Scores were compared prior to mating, and therefore these data did not include any scores of 6 (i.e., mating). Data are mean + 1 s.e.m. Letters indicate significant differences between treatment groups.

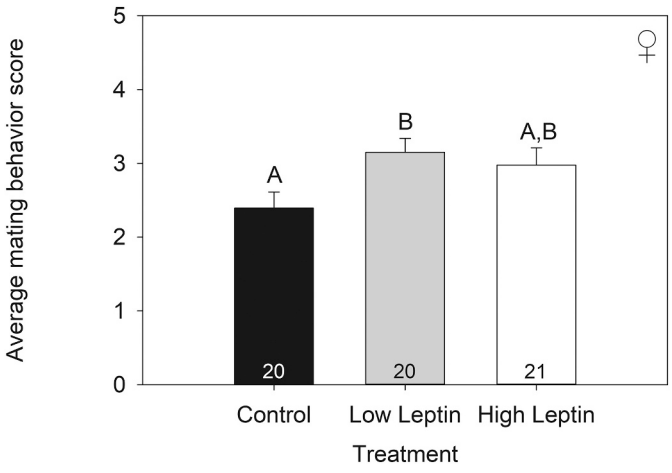

Fig. 2. Exogenous leptin affects average mating behavior score of female red-sided garter snakes. Data shown are the mean + 1 s.e.m. of all mating behavior scores received prior to mating; these data do not include any scores of 6 (i.e., mating) and therefore represent the snakes' appetitive reproductive behavior. Snakes were injected with 0, 7, or 70 μg of mouse recombinant leptin (Control, Low leptin, or High leptin, respectively) for 3 days prior to mating trials. Numbers along the x-axis represent the sample size in each group. Letters indicate significant differences among treatment groups.

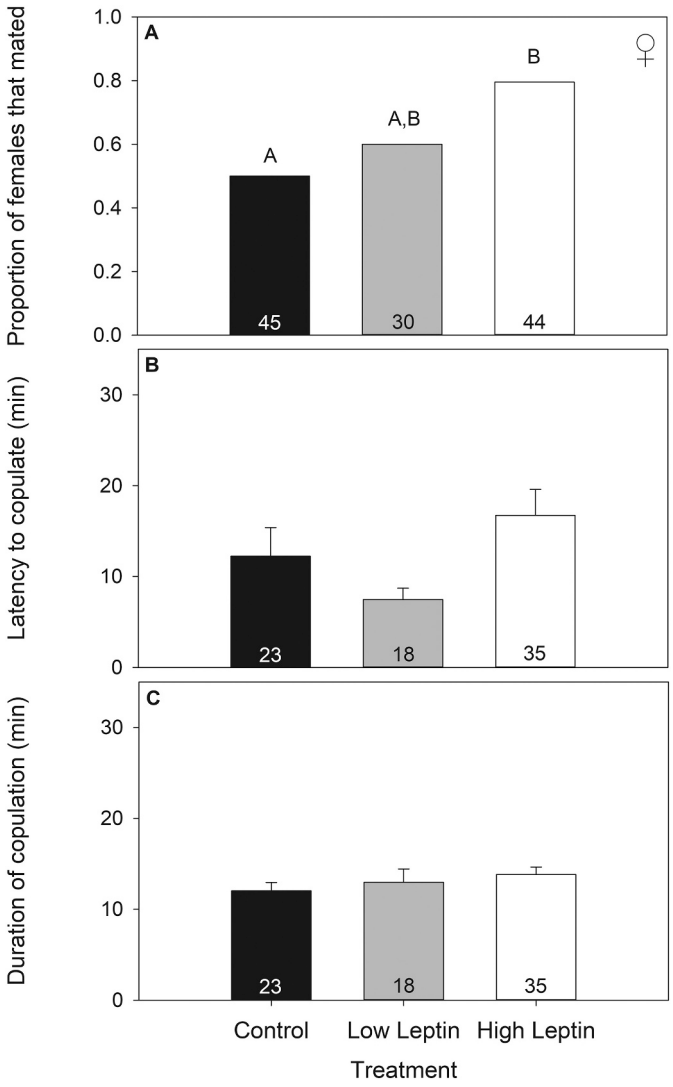

Fig. 3. The effect of exogenous leptin on (A) the proportion of females that mated, (B) latency to copulate, and (C) duration of copulation in female red-sided garter snakes. Snakes were injected daily with 0, 7, or 70 μg of mouse recombinant leptin (Control, Low leptin, or High leptin, respectively) for 3 days prior to mating trials. Only females that copulated were included in panels B and C. Numbers along the x-axes represent sample sizes in each group. Letters indicate statistically significant differences among treatment groups.

analyzed the duration of copulation data with body condition index instead of year. Leptin treatment, body condition index, and the interaction between treatment and body condition index did not significantly affect duration of copulation (Table 2).

3.2. Male reproductive behavior

Differences in male body morphometrics were statistically non-significant among treatment groups (Mass: $F = 0.24$, $p = 0.786$, $\eta^2 = 0.007$; SVL: $H = 2.09$, $p = 0.352$, $r = 0.03$). Leptin treatment and day significantly affected the average courtship score (i.e., appetitive reproductive behavior) of males (from a two-way repeated measures ANOVA; Table 3, Fig. 4). The interaction between treatment and day was statistically non-significant (Table 3). Pairwise comparisons from a Student-Newman-Keuls test revealed males treated with the low and high leptin dose had significantly higher average courtship scores compared to controls (Fig. 4). Average courtship scores significantly decreased after day 1 for males dosed with vehicle and high leptin, but

Table 3
Statistical results from analyses of the effects of exogenous leptin on male reproduction in red-sided garter snakes (*Thamnophis sirtalis parietalis*).

Response variable	Factors	Test statistic	p-Value	Effect sizes
Courtship score	Leptin treatment	$F_{2,138} = 3.82$	**0.027**	$\eta^2 = 0.055$
	Day	$F_{2,138} = 18.297$	**<0.001**	$\eta^2 = 0.088$
	Leptin * Day	$F_{4,138} = 2.25$	0.070	$\eta^2 = 0.022$
Mean no. of copulations	Leptin treatment	$F = 4.09$	**0.022**	$\eta^2 = 0.115$
Latency to copulate	Leptin treatment	$F = 0.77$	0.469	$\eta^2 = 0.033$
Duration of copulation	Leptin treatment	$F = 0.06$	0.941	$\eta^2 = 0.003$
Copulatory plug mass	Leptin treatment	$H = 0.09$	0.958	$r = 0.20$

Bold font indicates $p < 0.05$. One male achieved intromission and immediately detached from the female; we therefore included him in the latency to copulate analysis but excluded him from duration of copulation and copulatory plug mass. Analyses for latency to copulate, duration of copulation, and copulatory plug mass are of first matings only.

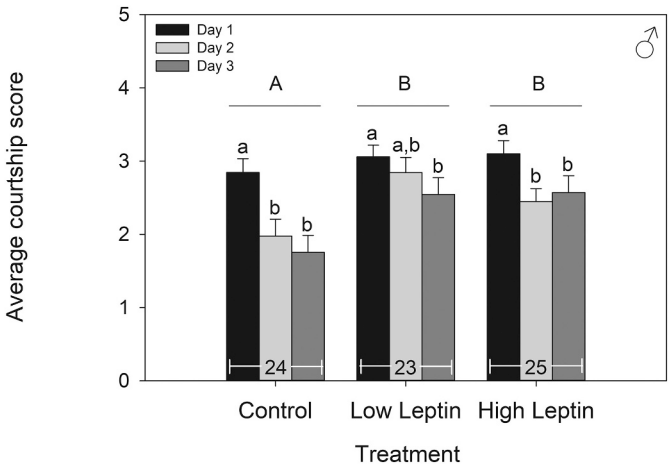

Fig. 4. The effect of exogenous leptin on average male courtship score in red-sided garter snakes. These data do not include any scores of 5 (i.e., mating) and therefore represent the snakes' appetitive reproductive behavior, Snakes were injected with 0, 3, or 30 μg of mouse recombinant leptin (Control, Low leptin, or High leptin, respectively) for 3 days. Males were subjected to a mating trial on each injection day for a total of three mating trials. Numbers along the x-axis represent sample sizes in each group. Statistically significant differences among treatment groups are indicated by capital letters above the horizontal lines. Lower case letters indicate significant differences among days within each treatment group.

remained elevated in low leptin-dosed individuals until the third day of mating trials (Fig. 4). The mean number of copulations achieved by male snakes significantly differed among treatment groups, with males receiving the high leptin dose performing significantly more copulations compared to controls (from a one-way ANOVA; Table 3, Fig. 5). Of the males that mated, the only snakes that performed 3 matings received injections with exogenous leptin (Low leptin: $n = 1$; high leptin: $n = 2$). There was no significant effect of leptin treatment on latency to copulate, duration of copulation, or copulatory plug mass (Table 3; Fig. 6A–C).

4. Discussion

We present evidence to suggest that leptin promotes reproductive

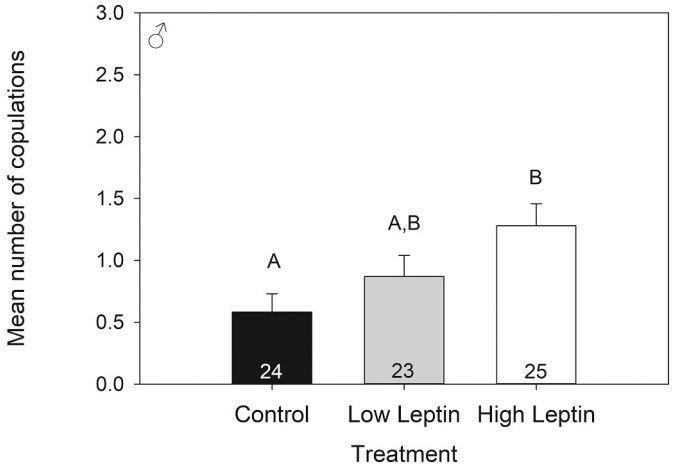

Fig. 5. The effect of exogenous leptin on the number of copulations a male red-sided garter snake achieved. Males were injected with 0, 3, or 30 μg of mouse recombinant leptin (Control, Low leptin, or High leptin, respectively) for 3 days. Males were subjected to a mating trial on each injection day; we limited males to one copulation per day. Numbers along the x-axis indicate sample sizes. Letters indicate statistically significant differences among treatment groups.

behavior in both male and female red-sided garter snakes. This is the first direct examination of whether leptin differentially affects appetitive and consummatory sex behaviors in males and females. The behaviors described within the mating behavior ethograms in Table 1 represent a sequence of events that occurs prior to copulation. However, in the scramble competition mating system of red-sided garter snakes, these behaviors do not always culminate in copulation. As such, these behaviors can be used to assess an individual's motivation to mate separately from that individual's ability to achieve a successful mating. These ethograms therefore represent a combination of both appetitive and consummatory sex behaviors. In the present study, we use the mean behavior scores received prior to mating as a measure of appetitive reproductive behavior, while the proportion of snakes mating and the number of matings achieved are indicative of consummatory sex behavior.

Our results do not indicate that leptin universally increases reproductive behavior, as we find no evidence to suggest that leptin influences latency to copulate or duration of copulation. Our results are consistent with research in fed mammals (García-Juárez et al., 2012, 2011; Schneider et al., 2007; Wade et al., 1997). Because these snakes are aphagic during the spring mating season (Crews et al., 1987; Gregory and Stewart, 1975; O'Donnell et al., 2004), our results do not align with research suggesting that leptin inhibits reproduction in organisms that are not feeding (Wade et al., 1997). For example, treatment with leptin in food-deprived female Syrian hamsters significantly decreased lordosis duration, a measure of sexual receptivity, compared to vehicle-treated and food-deprived female Syrian hamsters. This suggests that multiple signals convey an organism's energy status, and a mismatch in signals may inhibit reproduction. Because food restriction and aphagia are distinct phenomena and animals experiencing either of these nutritional stages likely exhibit vastly different hormone profiles, comparisons between aphagic and food-restriction studies must be made cautiously. Investigating the behavioral and physiological responses to aphagia and food restriction in a single species, such as garter snakes, would help clarify the relationship between leptin, sexual behavior, and feeding status.

We found that leptin promotes appetitive and consummatory sex behavior in both male and female snakes. For appetitive sex behavior in males, a low dose of leptin prolongs courtship intensity by 1 day. This is in contrast to vehicle and high leptin-treated males whose courtship

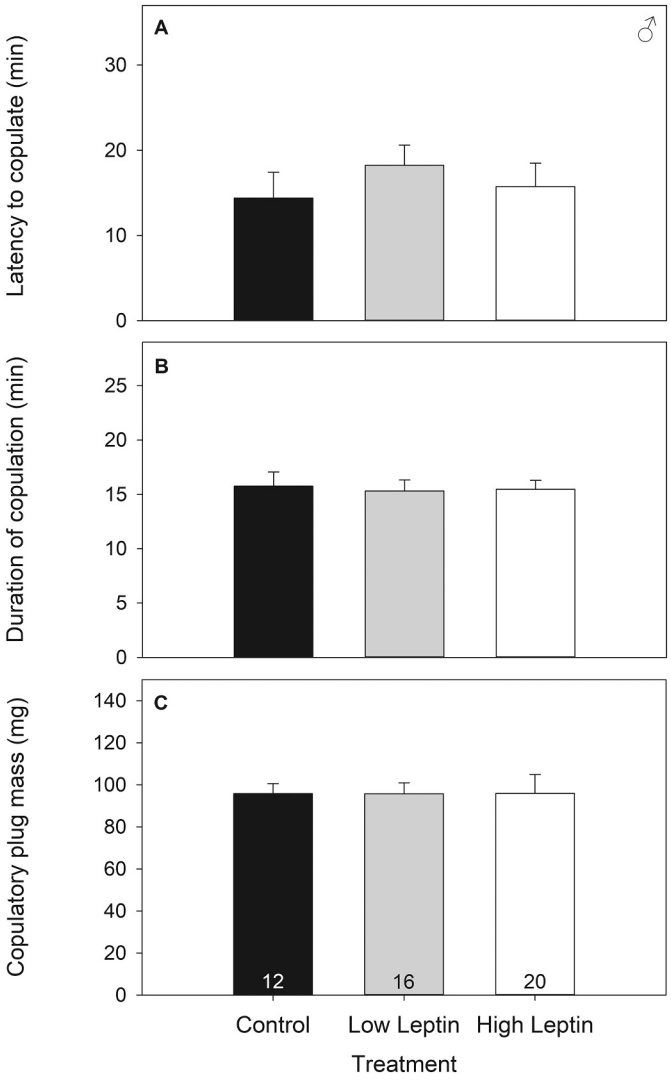

Fig. 6. The effect of exogenous leptin on (A) latency to copulate, (B) duration of copulation, and (C) copulatory plug mass in male red-sided garter snakes. Snakes were injected with 0, 3, or 30 μg of mouse recombinant leptin for 3 days (Control, Low, or High leptin treatment groups, respectively). Males were subjected to a mating trial on each injection day; data (mean + 1 s.e.m) presented here include only the first mating of those males that copulated regardless of what day the mating occurred on. Numbers along the x-axis in (C) indicate sample sizes for each treatment group in all panels.

score decreased from day 1 to 2. Because leptin appears to evoke different behavioral responses at low and high doses, it is possible that leptin does not elicit a linear dose-response curve for courtship score in these snakes as is the case for some mating behaviors in female rats (Fox et al., 2000; García-Juárez et al., 2011). For females, we found that a low dose of leptin significantly increased mating behavior score compared to controls. Although not significantly different from controls, a high dose of leptin also increased mating behavior score. It should be noted that it is possible that the average mating behavior scores were underestimated in this experiment, because some females mated very quickly, and therefore prior to recording a mating behavior score. This may have prevented us from observing a stronger effect of leptin on female mating score. Although we present significant effects of leptin on sex behavior in red-sided garter snakes, the mechanism as to how leptin exerts its influence remains unclear.

Although the primary structure of vertebrate leptin is highly divergent, conserved secondary and tertiary structure points to murine leptin mimicking the effects of reptilian leptin (Denver et al., 2011). As

discussed earlier, identification of the leptin protein has occurred in several reptilian tissues: plasma, whole blood, brain, and stomach (Spanovich et al., 2006; Muruzábal et al., 2002; Paolucci et al., 2001; Niewiarowski et al., 2000). Although the mechanism by which leptin exerts its effects to induce the behavioral changes we observed are not clear, leptin likely binds to a receptor to evoke physiological changes (Myers et al., 2008). While the mammalian leptin receptor is present in an array of tissues [e.g., hypothalamus, pituitary, and gonads (Margetic et al., 2002)], investigations into the reptilian leptin receptor are sparse (Denver et al., 2011; Sciarrillo et al., 2005).

Numerous reviews describe the multitude of pathways that are activated when leptin binds to its receptor (e.g., Denver et al., 2011; Margetic et al., 2002; Zabeau et al., 2003; Villanueva and Myers, 2008), One likely pathway involves leptin removing the inhibitory effect of agouti-related peptide/neuropeptide Y (AgRP/NPY) neurons on gonadotropin-releasing hormone (GnRH) neurons (Egan et al., 2017; Landry et al., 2013). This mechanism may explain our findings of leptin increasing reproductive behavior, because exogenous NPY inhibits sexual behavior in male red-sided garter snakes (Morris and Crews, 1990). Alternatively, it cannot be ruled out that the effects of leptin in this study resulted from a non-receptor-mediated pathway. For example, leptin increases the number of phosphorylated (i.e., activated) nitric oxide synthase-immunoreactive neurons in the hypothalamus, thereby increasing the production of nitric oxide (Bellefontaine et al., 2014). Further, inhibitors of the nitric oxide pathway prevent the stimulatory effects of leptin on receptivity in female rats (García-Juárez et al., 2012). In red-sided garter snakes, initial studies indicate that nitric oxide synthase-immunoreactive neurons are present in areas of the brain associated with regulation of reproductive behavior (Krohmer and Lutterschmidt, 2011). Therefore, the positive effects of leptin on reproductive behavior we present here could be a product of leptin activating nitric oxide signaling pathways.

An unexpected, interesting finding in this study is the significant difference in the duration of copulation between females in 2016 and 2017, with females in 2016 having a shorter duration of copulation compared to females in 2017. We did not find evidence to suggest that body condition index explained this difference, even though females in 2017 had a significantly higher body condition index than females in 2016. Other potential factors that could influence copulation duration include male physiology regarding copulatory plug deposition, environmental conditions (e.g., ambient temperature), or population density. The large basal spines on the hemipenes of male red-sided garter snakes may preclude females from detaching from intromission prematurely. Indeed, ablating the basal spines significantly decreases the duration of copulation in red-sided garter snakes (Friesen et al., 2014). The finding that females under anesthesia mated longer than females not subjected to anesthesia further suggests that, given the opportunity, females will end intromission early (Friesen et al., 2014). Regardless of which sex is the primary driver of copulation duration in red-sided garter snakes, the question of why we observed this difference in copulation duration between years still persists.

Research into the effects of ambient temperature and density on copulation duration in reptiles is inconclusive (Olsson et al., 2011; Shetty and Shine, 2002; Shine et al., 2000) and lacking, respectively. One study that investigated the influence of male density on copulation duration may provide insight into our findings of a shorter copulation duration in 2016 when mating trials consisted of a higher density of males. This study found that male Australian Polydesmidan millipedes (*Gigantowales chisholmi*) exposed to a greater number of males prior to one-on-one mating trials copulate for a shorter duration compared to males kept in isolation (Holwell et al., 2016). Because the Australian Polydesmidan millipede and red-sided garter snake mating systems are both characterized by scramble competition (e.g., Duvall et al., 1993; Thornhill and Alcock, 1983), the findings of Holwell et al. (2016) are likely relevant to the red-sided garter snake system. Further investigations into how factors such as latency to copulate and duration of copulation are affected by density-dependency would provide a more comprehensive view of potentially more nuanced factors contributing to sexual conflict. As in most facets of biology, there is likely no one primary determinant influencing duration of copulation. More likely, there are culminating and interactive effects of multiple factors that influence copulation duration and other sexual processes.

The major findings of this study demonstrate that exogenous leptin increases reproductive behavior in red-sided garter snakes during a long-term bout of aphagia. Investigating the role of leptin on reproductive behavior in a species of garter snake that is phagic during the mating season, which would allow food-restriction manipulations, would provide further insight into how the role of leptin in reptiles compares to that in fed and fasted mammals. Exogenous leptin does not universally promote reproduction in red-sided garter snakes, as we did not observe differences in all measured variables (i.e., latency to copulate, duration of copulation, and copulatory plug mass). It is possible that leptin induces these behavioral changes through a variety of mechanisms after leptin binds to its receptor. Because neither leptin nor its receptor have been identified in snakes, identifying leptin and leptin receptor genes, along with characterizing the role of leptin in other reptilian species, would further clarify to what extent the role of leptin varies across species.

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