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Non-photoperiodic regulation of reproductive physiology in the flexibly breeding pine siskin (*Spinus pinus*)

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Abstract

In order to time reproduction to coincide with favorable conditions, animals use environmental cues to up- and down-regulate the reproductive axis appropriately. Although photoperiodic cues are one of the best studied of such environmental cues, animals also attend to others such as temperature, food availability, rainfall and social cues. Such non-photic cues are expected to be particularly important for tropical species and temperate-zone species that exhibit flexible or opportunistic breeding schedules. In this study, we investigate the use of non-photic cues, specifically food availability and social cues, to time the initiation of reproductive development in the pine siskin (*Spinus pinus*), a temperate-zone songbird with a flexible breeding schedule. Following winter solstice, males were housed on a 12L:12D photoperiod with either access to a preferred food, a potential mate (social cue), or both. Control birds received only maintenance diet and no mate. Access to a preferred food had a significant positive effect on testis size and circulating luteinizing hormone (LH). However, we found no effect of social treatment on reproductive development. The effect of the food treatment on reproductive development did not appear to result from effects on body mass or fat, as neither measure differed across treatments. The food treatment influenced not only reproductive physiology, but also reproductive behavior in this species, as access to seeds had a positive effect on affiliation of pairs. This study demonstrates that food is a potent stimulus for the initiation of reproductive development in pine siskins.

Keywords

reproduction; luteinizing hormone; testosterone; gonad; seasonality; supplementary cue

1. Introduction

A major challenge facing most organisms is to appropriately time reproduction to coincide with favorable conditions in order to maximize reproductive success. To achieve this timing, many animals use environmental cues (e.g. photoperiod, rainfall, food availability) to up- and down-regulate the hypothalamic-pituitary-gonadal (HPG) axis. The best studied of such environmental cues is photoperiod, the use of which has been particularly well documented in birds. Birds are excellent subjects for these studies because they typically exhibit discrete

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breeding and non-breeding life cycle stages that are characterized by marked changes in physiology, behavior and morphology. Notably, individuals exhibit repeated, usually annual, cycles of gonadal recrudescence in preparation for breeding and gonadal regression as breeding terminates.

Typically, increasing day lengths stimulate the activation of the HPG axis in preparation for reproduction [6]. Thus, photoperiod is considered to be an ‘initial predictive cue’ to initiate reproductive development [35]. In some species photoperiod may be sufficient to induce complete reproductive development. But in most species, additional cues (‘supplementary cues’ and ‘synchronizing and integrating cues’) such as food availability, rainfall, temperature, or social interactions are used to fine-tune the timing of completion of reproductive development and onset of breeding [35]. Indeed, temperate-zone and high-latitude species that exhibit highly seasonal breeding schedules (which have been the subjects of the majority of studies), rely very heavily on photoperiod for reproductive timing [1, 6, 11, 29]. By contrast, tropical species and temperate-zone species that exhibit more flexible or opportunistic breeding schedules are expected to rely on non-photic cues to a greater extent than more seasonal and temperate-zone species [11, 12, 24, 26, 36]. Although, such non-photic cues have received less attention, a growing number of empirical studies indicate their importance for reproductive timing in tropical species [16, 17, 23] and in species with opportunistic or flexible breeding schedules [10, 17-19, 24, 32].

In this study we investigate the use of non-photic cues to time reproduction in the pine siskin (*Spinus pinus*). Pine siskins are temperate-zone songbirds with flexible breeding schedules. Egg-laying can occur from March to August-September and its timing varies inter-annually [7, 14]. Moreover, pine siskins are irruptive nomadic migrants, and consequently an individual may breed across a range of latitudes within its lifetime [5, 7]. Therefore, although pine siskins are sensitive to photoperiodic cues [14, 20], they are also likely to rely heavily on non-photoperiodic cues to appropriately time reproduction.

We expect that both food and social cues may be important non-photoperiodic cues for reproductive timing in pine siskins. Like some other Cardueline finches and irruptive nomadic migrants, pine siskin breeding likely corresponds to fluctuating food availability [22]. Furthermore, social cues have been found to influence reproductive physiology in the red crossbill, a close relative of the pine siskin as well as an opportunistic breeder and irruptive nomadic migrant [15]. Therefore, this study examines the effects of food availability, specifically food type, and social cues, specifically the presence of a potential mate, on the initiation of reproductive development in pine siskins. To our knowledge, no studies have simultaneously investigated the effects of multiple non-photoperiodic cues on reproductive physiology. Here, we employed a full factorial design in order to evaluate possible interaction among these cues. Finally, we were also interested in the relationship between environmental cues, reproductive physiology and behavior of our paired birds. Specifically, we assessed whether the food treatment influenced affiliative behaviors and whether degree of affiliation of the pair was related to gonadal development.

2. Materials and methods

2.1 Animals

Pine siskins were caught in September and October of 2008 near Mt. Hood, OR (45°23’N, 121°50’W) and Jackson, WY (43°28’N, 100°48’W). Birds were transported to facilities at the University of California, Davis where they were housed in large indoor flight cages on photoperiods that simulated the natural decline in photoperiod. Throughout the study water, Roudybush Small Bird Maintenance Diet (Woodland, CA), and fine grit were provided *ad libitum*. Birds were also provided with a mixture of seeds (black oil sunflower seeds,

sunflower hearts and thistle seeds) as they acclimated to captivity, but no seeds were given for approximately 2 months prior to the start of the experiment.

Blood samples were collected from each bird either at capture or once housed at UC Davis and submitted to Zoogen, Inc (Davis, CA) for genetic sex determination. On November 17–18, 2008 males and females were separated and housed in different rooms until the start of the experiment. Following winter solstice, birds were held on winter solstice day length (9.7L: 14.3D) until the start of the experiment (approximately 1 month). All experimental procedures were approved by the UC Davis Institutional Animal Care and Use Committee. Birds were captured with the necessary permits from the U.S. Fish and Wildlife Service, Oregon Department of Fish and Wildlife, and Wyoming Game and Fish Department.

2.2 Experimental design

The experiment included a food treatment (presence or absence of *ad libitum* access to the seed mixture described above, in addition to the maintenance diet) and a social treatment (presence or absence of a female as a potential mate), creating four treatment groups. Thus, males were assigned to either: receive seeds and be housed alone (seeds + no mate, $n = 8$), receive only maintenance diet and be housed with a female (no seeds + mate, $n = 9$), receive both seeds and a female (seeds + mate, $n = 9$), or receive only maintenance diet and be housed alone (no seeds + no mate, $n = 8$). Males captured in WY and OR were balanced across groups; otherwise males were randomly assigned to treatments groups. Each male receiving a female partner was randomly assigned a female from the location at which he was not captured.

Prior to the start of the experiment males were moved into individual cages and into 12 acoustic isolation chambers (Industrial Acoustics Company, Bronx, NY). Birds for each treatment were divided among 2-4 chambers. Chambers were arranged within a single room, in alternating order. Within chambers, cages were arranged on multiple shelves such that each cage was visually isolated from the other cages in the chamber, but birds could hear other birds belonging to the same treatment group. The positions of cages on shelves within a chamber were rotated weekly throughout the experiment. On January 21, 2009 all males were transferred from a winter solstice photoperiod to 12L:12D and treatments began. This photoperiod was selected to be “permissive” for breeding, but not highly stimulatory.

2.3 Data collection

Physiological and behavioral measures were collected repeatedly during the experiment.

2.3.1 Measures of body condition and blood collection—Body mass and subcutaneous fat were measured, and blood collected 6 days prior to, and 13 and 30 days following the start of the treatments. These measurements occurred at the same time of day on each sampling day. Body mass was measured to the nearest 0.1 g using a Pesola spring scale. Subcutaneous fat was scored visually on a scale from 0 (no fat) to 5 (bulging fat) for the furcular and abdominal regions, and the values added. Blood was collected from the alar vein into heparinized microhematocrit tubes and stored on ice until centrifugation to separate plasma. Plasma was harvested and kept at -20°C until assayed for luteinizing hormone and testosterone.

2.3.2 Gonad measurements—Testis size was measured via laparotomy under general anesthesia (Isoflurane, Abbott Laboratories, Abbott Park, IL) 35-36 days prior to, and 31 days following the start of the treatments. A small incision was made on the left flank and the length of the left testis was exposed, and measured by positioning the tips of forceps at each end of the testis and then pressing the tips into clay and measuring the distance

between the impressions to the nearest 0.1mm with dial calipers. Final testis size was measured at the end of the experiment (day 38-42); following euthanasia and collection of brains for a separate study, testes were dissected out, the length of the left testis measured to the nearest 0.1 mm with dial calipers and the combined mass of the testes recorded.

2.3.3 Behavioral data collection—Paired birds were video recorded for 10 minutes every 3-4 days to measure affiliative behaviors ($n = 7$ recordings per pair). The rate of bill touching and the proportion of time that a pair spent perched together (within 1 body length) were quantified from videos using JWatcher v. 1.0 [4]. Bill touching is a social behavior in which two birds touch bill tips or one bird inserts its bill into the mouth of another bird. Although bill touching is not associated exclusively with reproduction, it is a component of siskin reproductive behavior [21]. Bill touching is considered a form of courtship feeding [21]; however, here we distinguish between cases in which food is transferred between birds (courtship feeding) and cases in which it is not (bill touching). Courtship feeding was not observed during this study.

Bill touching and perching together were used to calculate a single composite affiliation score for each pair for the entire period of experimental treatment. Following Silk et al. [31] the affiliation score was calculated as:

$$\frac{\left(\frac{B_{ij}}{\bar{B}} + \frac{P_{ij}}{\bar{P}}\right)}{2}$$

where B_{ij} and P_{ij} are the frequency of bill touching and the proportion of time spent perched together, respectively, for pair i, j , and \bar{B} and \bar{P} are the mean frequency of bill touching and the mean proportion of time spent perched together, respectively, for all pairs. Thus, the affiliation score is a measure of the degree of affiliation of a pair relative to all other pairs, with larger values indicating greater affiliation.

Additionally, we quantified the frequency of aggressive behavior for each pair from video recordings. Displays, supplanting attacks, and physical attacks, as described previously for Eurasian siskins [*Spinus spinus*; 27, 28], were scored as aggressive behaviors. Aggression behaviors exhibited by both the male and female of the pair were included in this measure

2.4 Hormone assays

Plasma luteinizing hormone (LH) was measured with a double-antibody, post-precipitation radioimmunoassay [8, 9, 30], which has been used previously with this species [e.g., 14]. Duplicate 20 μ L plasma samples were run in a single assay. The intra-assay coefficient of variation was 10.4%. Assay sensitivity was 0.113 ng/mL.

Plasma testosterone (T) was measured using an enzyme immunoassay kit from Enzo Life Sciences (ADI-901-065). Plasma dilution and steroid displacement buffer concentration were optimized for pine siskins using the methods of Wada et al. [33]. Based on the optimization, samples were run at a 1:20 dilution with 0.5% (of raw plasma volume) steroid displacement buffer. Samples were run in duplicate in a single assay using 3 plates. The intra-assay coefficient of variation was 7.8% and the inter-plate coefficient of variation was 9.5%. Average assay sensitivity was 0.146 ng/mL.

2.5 Data from females

Female pine siskins were used primarily as stimulus individuals in this study. Therefore, in order minimize disturbance to them, females were not subjected to blood collection or

laparotomy during the course of the experiment. However, data on female gonadal condition were collected at the end of the experiment in order to assess their response to the food treatment. As for the males, final gonadal condition was recorded for females following euthanasia. The ovary was dissected out, the diameter of the largest follicle was measured to the nearest 0.1 mm with dial calipers and the mass of the ovary was recorded. All females in the experiment were paired with a potential mate.

2.6 Statistical analysis

Statistical analyses were performed in STATISTICA (StatSoft, Tulsa, OK). Repeated measures analysis of variance (ANOVA) was used to test for differences in reproductive development (testis length, LH, testosterone) and body condition over time and across treatment groups. Tukey HSD tests were used for post hoc comparisons. For hormone analyses, samples that were below the detection limit of the assay were assigned the minimum sensitivity value. LH and testosterone values were log transformed to meet assumptions of repeated measures ANOVA. For the LH assay, there was a large number of samples below the detection limit in the first sampling time point (day -6), and data from this time point violated the homogeneity of variance assumption. Repeated measures ANOVA is quite robust to such a violation, therefore we present the results of this test. However, we also performed an additional analysis in which total LH secretion over the course of the experiment was quantified as area under the curve with respect to increase [AUC_i; 25]. Sample sizes for the testosterone assay were smaller than for other data types because of limited plasma volumes. In particular, sample size was small for day 13 and these data did not meet the assumption of homogeneity of variance. Therefore, we present results with and without this sample date included. Some variables (affiliation score and ovary mass) were log transformed to meet the assumptions of the statistical tests. To test for possible chamber effects, we performed mixed-model nested ANOVAs for final gonadal condition.

3. Results

3.1 Reproductive condition

Testis size increased in all birds over the course of the experiment, with a significant effect of food treatment, but not social treatment, on testis length (Fig. 1 and Table 1). Males receiving seeds grew their testes more rapidly than males receiving only maintenance diet. Similarly, there was a significant effect of food treatment (two-way ANOVA, $F_{1,30} = 20.67$, $p < 0.001$), but not social treatment ($F_{1,30} = 1.40$, $p = 0.25$; food x social interaction, $F_{1,30} = 0.47$, $p = 0.50$) on combined testes mass measured at the end of the experiment. We found no effect of chamber on final gonadal condition (testis length: treatment $p = 0.05$, chamber $p = 0.13$; testes mass: treatment $p = 0.03$, chamber $p = 0.23$; chamber means are presented in the supplementary material). Males had not reached full gonadal maturity at the end of the experiment. Fully mature gonads reach 7-8 mm in length in this species [14, 20], whereas males receiving seeds had a mean testis length of 4.4 mm.

3.2 Hormone levels

Circulating plasma LH levels increased over the experiment (Fig. 2 and Table 1). Males that received seeds had significantly higher LH levels than those that did not, but there was no effect of social treatment (Fig. 2 and Table 1; two-way ANOVA for AUC_i: food, $F_{1,25} = 4.57$, $p = 0.04$; social, $F_{1,25} = 0.44$, $p = 0.51$; food x social, $F_{1,25} = 0.13$, $p = 0.72$).

Circulating testosterone levels did not vary over the course of the experiment and were not influenced by either the food or social treatment (Fig. 3; day -6, 13 & 30: time, $F_{2,38} = 0.92$,

$p = 0.41$; food, $F_{1,19} = 0.27$, $p = 0.61$; social, $F_{1,19} = 0.21$, $p = 0.65$; day -6 & 30: time, $F_{1,24} = 1.18$, $p = 0.29$; food, $F_{1,24} = 0.87$, $p = 0.36$; social, $F_{1,24} = 1.75$, $p = 0.20$).

3.3 Body condition

Neither treatment nor time had a significant effect on body mass (Fig. 4 and Table 1). Subcutaneous fat declined after the start of the experiment, but there was no effect of treatment (Fig. 4 and Table 1).

3.4 Behavior

The degree of affiliation varied considerably among pairs. Pairs receiving seeds were significantly more affiliative than those not receiving seeds (Fig. 5; affiliation score: $t = -3.08$, $df = 16$, $p = 0.007$). In particular, rates of bill touching were higher among birds receiving seeds compared to those without access to seeds (bill touching, $U = 9.5$, $n_1 = n_2 = 9$, $p = 0.006$; perching together, $U = 28.0$, $n_1 = n_2 = 9$, $p = 0.27$). However, there was no correlation between gonadal condition at the end of the experiment and affiliative behavior (testis length: Pearson's $r = 0.04$, $n = 18$, $p = 0.87$; testes mass: Pearson's $r = 0.06$, $n = 18$, $p = 0.80$). There was no effect of food treatment on aggressive behavior ($U = 26.0$, $n_1 = n_2 = 9$, $p = 0.20$).

3.5 Response of females to food treatment

Females receiving seeds had significantly heavier ovaries at the end of the experiment than those not receiving seeds ($t = -2.84$, $df = 16$, $p = 0.01$), though follicle diameter did not differ significantly ($U = 23.0$, $n_1 = n_2 = 9$, $p = 0.12$). We found no effect of chamber on final gonadal condition (ovary mass: treatment $p = 0.07$, chamber $p = 0.16$; follicle diameter: treatment $p = 0.14$, chamber $p = 0.26$; chamber means are presented in the supplementary material).

4. Discussion

We found a significant effect of food type, but not the presence of potential mate, on the initiation of reproductive development in male pine siskins. Access to preferred food (seed mix) had a positive effect on testis size, circulating LH levels, and affiliative behavior with a potential mate. This effect of food was not influenced by the presence or absence of a potential mate. Moreover, access to seeds had a similar effect on female pine siskins, which had heavier ovaries compared to females without access to seeds.

The effect of food type on reproductive development in male pine siskins does not appear to be the result of effects on body mass or fat, as neither measure differed across treatments. This suggests that the influence of food type may be due to a perceptual effect rather than a nutritional effect. Such a perceptual effect of food on reproduction behavior has been nicely demonstrated by Hau et al. [16] who found that live, but not dead crickets, stimulated singing in male spotted antbirds. Moreover, such perceptual effects have the potential to be widespread, as studies from several other species have also found effects of food availability on gonadal condition in the absence of any effect on body condition [2, 10, 13, 24]. Further experiments are necessary to properly distinguish between nutritional and perceptual effects of seeds in pine siskins. For example, it is possible that our seed treatment may have provided particular nutrient(s), not reflected by body mass or subcutaneous fat, that stimulated reproductive development.

Our results documenting effects of food type on circulating LH levels in pine siskins are consistent with the hypothesis that this cue modulates reproductive physiology via the HPG axis. Previous studies that have investigated the effects of food cues on reproductive

development have generated mixed results as to whether this cue may be transduced to the gonads via the HPG axis. In the red crossbill, a close relative of the pine siskin, the effects of food availability on gonadal condition were accompanied by corresponding effects on circulating LH levels [10, 13]. Similar findings have also been reported in white-crowned sparrows (*Zonotrichia leucophrys gambelii*) [34]. On the other hand, studies of spotted antbirds (*Hylophylax naevioides*) and zebra finches (*Taeniopygia guttata*) have found effects of food availability on gonadal condition in the absence of corresponding effects on circulating LH levels [16, 24]. Although most studies have focused on circulating LH levels, the effects of environmental cues on gonadal development may also be dependent on follicle-stimulating hormone (FSH). Thus, the findings from the spotted antbird and zebra finch studies suggest that the effects of food cues were FSH-dependent or that separate systems were involved in transducing food cues to the gonads. Interestingly, investigation of the effects of temperature cues on LH, FSH, and gonadal development indicates that temperature cues appear to regulate reproductive physiology via a pathway other than the HPG axis [37].

In this experiment, food type influenced not only reproductive physiology, but also reproductive behavior. Access to seeds had a positive effect on affiliation of pairs. Similarly, in spotted antbirds, food cues have been shown to influence another component of reproductive behavior, singing [see also 3, 16]. Furthermore, in pine siskins, the effect of food type on affiliative behavior appears to be independent of the effect of food on gonadal condition, as there was no direct correlation between gonadal condition and affiliative behavior.

Although food is a potent stimulus for the initiation of reproductive development in pine siskins, it remains to be determined how this cue is integrated with other environmental cues. The presence of a potential mate did not modulate the effect of food type observed here. However, we expect that responsiveness to food type may be influenced by photoperiodic information. Birds in this experiment were held on a “permissive” photoperiod of 12L:12D. It is unknown whether birds on shorter or longer photoperiods would be similarly responsive. For example, a threshold day length may be necessary as an ‘initial predictive cue’ before birds become sensitive to food cues. This is the prevailing hierarchical view of how photoperiodic and non-photoperiodic cues are integrated [35]. But, opportunistically breeding red crossbills and zebra finches, as well as tropical spotted antbirds, are sensitive to food cues in the absence of photostimulation [13, 23, 24]. Furthermore, pine siskins held on constant short days grow their gonads very gradually to near-reproductive size [14], so these birds clearly do not require “long days” for reproductive development. Conversely, longer days may drive the HPG axis sufficiently strongly to overwhelm detection of any additional effect of supplementary cues [see 1, 13]. It will be important for future work to elucidate the extent to which food and photoperiodic cues are integrated hierarchically or non-hierarchically across species exhibiting a range of breeding schedules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- > We examined effects of food and social cues on initiation of reproductive development.
- > Access to a preferred food had a positive effect on testis size, plasma LH and affiliative behavior.
- > But, access to a potential mate had no effect on reproductive development.

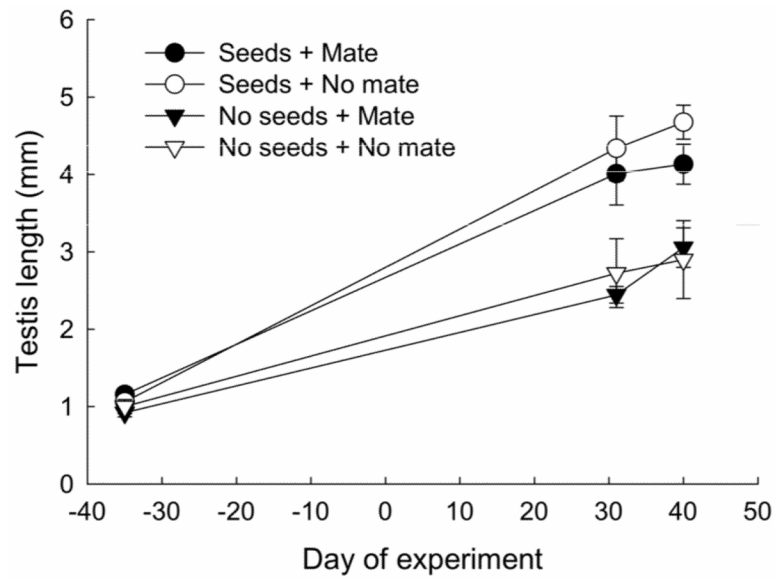


Figure 1. Testis length of male pine siskins receiving only maintenance diet (triangles) or maintenance diet supplemented with mixed seeds (circles), and housed singly in a cage (open symbols) or housed with a female (filled symbols). Mean \pm 1 standard error is plotted.

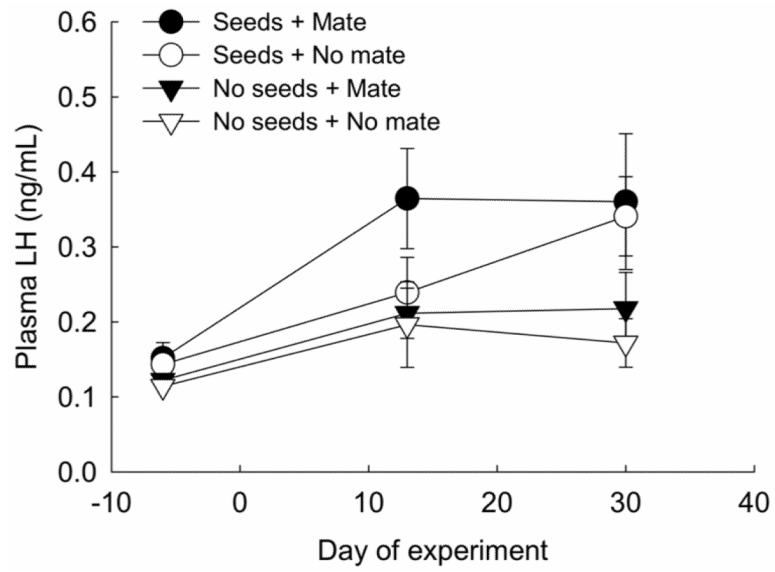


Figure 2. Plasma LH levels of male pine siskins receiving only maintenance diet (triangles) or maintenance diet supplemented with mixed seeds (circles), and housed singly in a cage (open symbols) or housed with a female (filled symbols). Mean \pm 1 standard error is plotted.

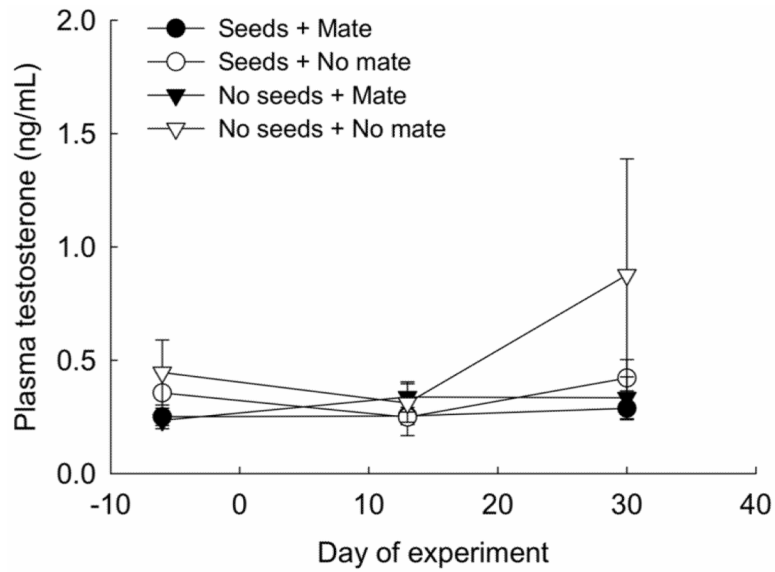


Figure 3. Plasma testosterone levels of male pine siskins receiving only maintenance diet (triangles) or maintenance diet supplemented with mixed seeds (circles), and housed singly in a cage (open symbols) or housed with a female (filled symbols). Mean \pm 1 standard error is plotted.

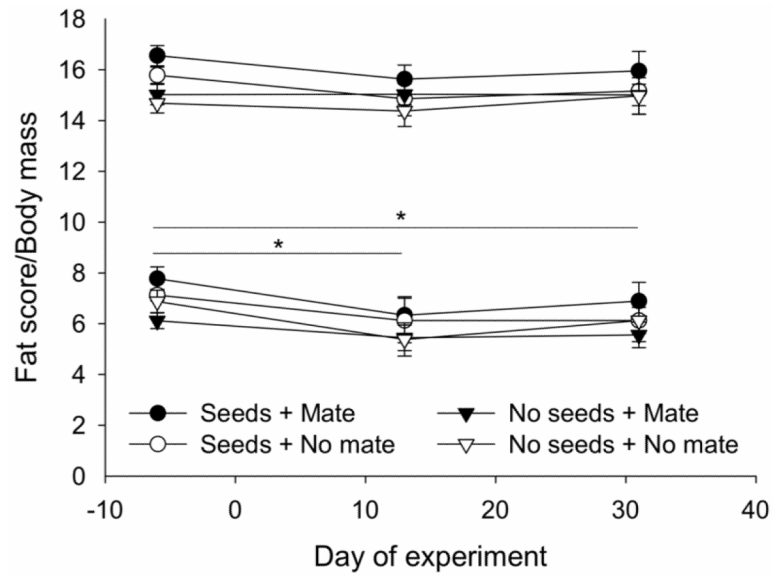


Figure 4.

Body mass and subcutaneous fat scores of male pine siskins receiving only maintenance diet (triangles) or maintenance diet supplemented with mixed seeds (circles), and housed singly in a cage (open symbols) or housed with a female (filled symbols). Means and standard errors are plotted. There were no effects of treatment on mass or fat. A significant difference ($p < 0.05$) between two time points is indicated by an asterisk.

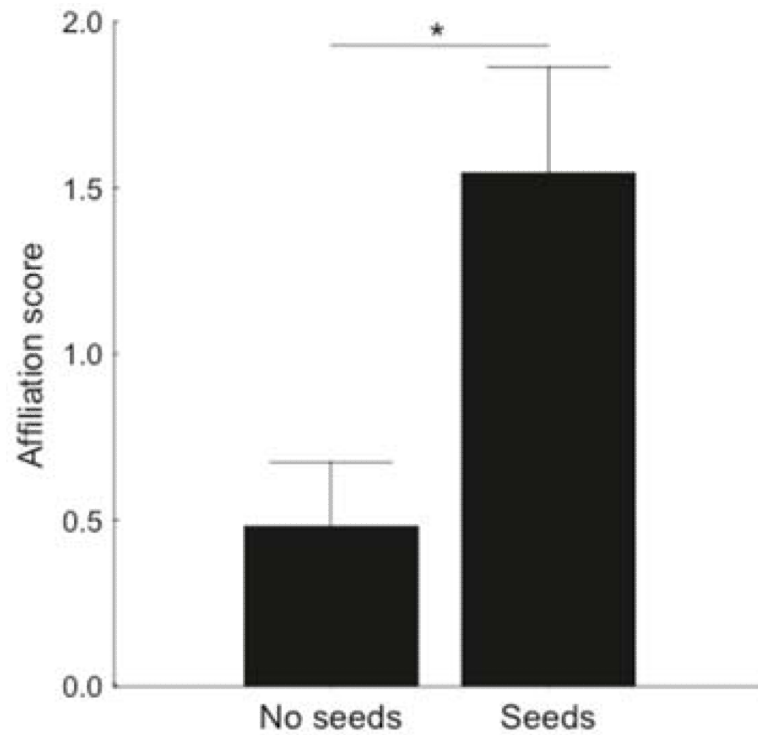


Figure 5. Mean (\pm standard error) affiliation score of paired pine siskins receiving maintenance diet only (open bar, $n = 9$ pairs) and maintenance diet supplemented with mixed seeds (grey bar, $n = 9$ pairs). An asterisk indicates a significant difference ($p < 0.05$) between treatment groups

Table 1

Effects of food treatment, social treatment, and time on measures of body condition and reproductive development in male pine siskins. Results are presented as $F(df_1, df_2)$.

	<u>Body condition</u>		<u>Reproductive development</u>	
	Mass	Fat score	Testis length	LH
Food	2.69 (1, 30)	2.73 (1,30)	23.37 (1, 30)**	10.95 (1, 30)*
Social	1.29 (1, 30)	0.01 (1, 30)	0.56 (1, 30)	1.04 (1, 30)
Food x Social	0.19 (1, 30)	0.95 (1, 30)	0.19 (1, 30)	0.01 (1, 30)
Time	2.32 (2, 60)	8.39 (2, 60)**	169.80 (2, 60)**	17.32 (2, 60)**
Time x Food	1.55 (2, 60)	0.13 (2, 60)	12.55 (2, 60)**	1.63 (2, 60)
Time x Social	0.18 (2, 60)	0.06 (2, 60)	0.50 (2, 60)	0.89 (2, 60)

* $p < 0.01$

** $p < 0.001$