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A potential mate influences reproductive development in female, but not male, pine siskins

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Abstract

The role of photoperiod in avian reproductive timing has been well studied, and we are increasingly recognizing the roles of other environmental cues such as social cues. However, few studies have evaluated the extent to which males and females of the same species respond similarly to the same type of cue. Moreover, previous studies have rarely examined how variation in the quality or nature of a given social cue might modulate its effect. Here, we examine the sensitivity of male and female pine siskins (*Spinus pinus*) to a potential mate as a stimulatory cue for gonadal recrudescence, and we investigate whether variation in the relationship between a bird and its potential mate modulates the effect of that potential mate. Birds were initially housed without opposite sex birds on a 12L:12D photoperiod with ad libitum food. After gonadal recrudescence had begun males and females were randomly paired with an opposite sex bird or housed alone. An additional group of males was paired with estradiol-implanted females. In males, these social treatments had no effect on testis length, cloacal protuberance length, luteinizing hormone (LH) levels, or testosterone levels. In females, presence of a potential mate had a significant and positive effect on ovary score, defeathering of the brood patch, and LH levels. Among paired birds, the degree of affiliation within a pair corresponded to the extent of reproductive development in females, but not males. Thus, reproductive timing in females appears to be sensitive to both the presence of a potential mate and her relationship with him.

Keywords

affiliation; birds; breeding; environmental cues; luteinizing hormone; pair formation; reproductive timing; sex differences; social cues

Introduction

Understanding how environmental factors affect physiology and behavior in order to time important life history events, such as reproduction and migration, has been an area of long-

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standing interest in biology. Moreover, it is an area of growing interest as we seek to anticipate and potentially mitigate the consequences of rapidly occurring global environmental change (Bronson, 2009; Visser et al., 2004; Wingfield, 2008). For example, the ability to time reproduction such that it coincides with favorable environmental conditions is critical to an organism's reproductive success (reviewed in MacDougall-Shackleton et al., 2015). Temperate-zone seasonally breeding species, especially birds, have been the focus of much research aimed at understanding reproductive timing mechanisms. From this work, we know that in seasonally breeding birds increasing photoperiods typically stimulate the hypothalamic-pituitary-gonadal (HPG) axis, bringing about changes in physiology and behavior in preparation for breeding. However, additional environmental cues, such as food availability, temperature, or social information and interactions may also be important in fine-tuning the timing of breeding (Wingfield, 1983). Although these non-photic cues have received less attention, we are increasingly realizing their importance in determining reproductive timing in temperate-zone and seasonally breeding species (Schaper et al., 2012; Wingfield et al., 2003), as well as in tropical species (Hau, 2001) and more flexibly or opportunistically breeding species (Hahn, 1995; Ligon, 1974; Perfito et al., 2008).

The role of social cues in reproductive timing has been a topic of interest across taxonomic groups, and broadly, both inter- and intra-sexual cues have been found to up- and down-regulate reproductive functions (Bronson, 1989; Crews, 1980; Helm et al., 2006; Wingfield et al., 1994). Previous studies of birds have found that cues from opposite sex individuals can stimulate gonadal recrudescence and advance the onset of breeding in both males and females (Brockway, 1965; Hinde and Steel, 1978; Lehrman, 1965; Morton et al., 1985; Perfito et al., 2015; and references below). Most studies of females have examined the effects of male displays, particularly vocal displays (e.g., Bentley et al., 2000; Friedman, 1977; Kroodsma, 1976; Waas et al., 2005), whereas most studies of males have examined the effect of the presence of a female (e.g., Burger, 1953; Haase et al., 1976). Rarely, have studies examined the effect of a particular type of social cue on both males and females. Also, studies of males and females have tended to use different systems. Studies of females have focused mostly on domesticated and captive-bred animals belonging to a few species such as the ring dove, *Streptopelia risoria*, and the canary, *Serinus canaria* (e.g., Bentley et al., 2000; Friedman, 1977; Kroodsma, 1976). On the other hand, studies of males have more often focused on wild-caught birds representing a different set of species (e.g., Burger, 1953; Dufty and Wingfield, 1986; Hahn et al., 1995). Consequently, our understanding of sex differences in responses to social cues (or other environmental cues for that matter) is extremely limited (Ball and Ketterson, 2008).

Additionally, studies examining the effect of social cues on reproductive timing have often ignored potential qualitative differences in the nature of a given cue. In most cases, a generalized form of a particular social cue (e.g. a vocalization) is considered sufficient to induce a change in the HPG axis, or experiments are designed specifically to minimize variation in the quality of the cue. Yet, with respect to the effect of intersexual signals on females, there is a large literature documenting the effects of male phenotype (e.g., song quality, ornamentation) on various aspects of female reproductive behavior (e.g., mate choice, frequency of extra-pair copulations, expression of proceptive behaviors) and parental

investment, as well as acute effects on circulating hormone levels (Baker et al., 1986; Gil et al., 2004; Hasselquist et al., 1996; Kingma et al., 2009; Marshall et al., 2005; Safran et al., 2005; Vallet and Kreutzer, 1995; Zuk et al., 1992). Thus, it seems likely that variation in cues from potential mates might also have differing effects on reproductive development. Indeed, two studies provide important evidence that not all forms of a given cue are equally stimulatory with respect to reproductive physiology. First, Kroodsma (1976) found that the size of song repertoire influenced the degree to which male song served as a stimulatory cue for female canaries, with larger song repertoires being a more potent stimulus. Second, Bluhm (1985) found that female canvasback ducks (*Aythya valisineria*) only advanced to egg-laying with their chosen mate, not a mate who was force-paired, even though males exhibited intense courtship behavior in both circumstances. Thus, variation in the ‘quality’ of any given social cue may influence its potency.

In light of these gaps in our understanding of the role of social cues in reproductive timing, here we examine the sensitivity of both males and females to a potential mate as a stimulatory cue for gonadal recrudescence in a single species, the pine siskin (*Spinus pinus*; Experiment 1). Furthermore, we investigate whether variation in the relationship between a bird and its potential mate modulates the effect of that potential mate (Experiment 2). 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 (Dawson, 1997; Hahn et al., 2004). Although pine siskins are sensitive to photoperiodic cues (Hahn et al., 2004; MacDougall-Shackleton et al., 2006), they are also likely to rely heavily on non-photoc cues to time reproduction. We have previously found that both male and female pine siskins are sensitive to food availability for the initiation of reproductive development (Watts and Hahn, 2012). In this study, we focus on the effect of a potential mate during the later period of reproductive development, as an individual approaches full mature capabilities, rather than the period in which development is initiated. It is during this later period that females are expected to be most sensitive to social cues (reviewed in Ball and Ketterson, 2008; Perfito et al., 2015). And, we have previously found no effect of the presence of a female on the earlier period of initiation of reproductive development in male pine siskins (Watts and Hahn, 2012).

Methods

Ethics

Experimental procedures were approved by the Institutional Animal Care and Use Committees at University of California Davis (Experiment 1) and Loyola Marymount University (Experiment 2) and were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Birds were captured under permits from the U.S. Fish and Wildlife Service, Oregon Department of Fish and Wildlife, and Wyoming Game and Fish Department.

Experiment 1: Effects of a potential mate on reproductive development

Animals—Birds were captured in Jackson WY (43° 28' N, 100° 48' W) in August and September of 2009 and in Mt Ashland OR (42° 4' N 122° 43' W) in December 2009; two

additional females were captured in Jackson WY in October 2008. Birds were transported to facilities at the University of California, Davis where they were housed in large indoor flight cages on photoperiods that simulated natural changes in photoperiod at a latitude of 38°N until winter solstice (21 December 2009), after which time they were held on winter solstice day length (9.7L:14.3D) until 13 January 2010 when they were put on a 12L:12D photoperiod. This 12L:12D photoperiod is permissive for breeding, but not highly stimulatory. Throughout the study birds were fed a diet of Roudybush Small Bird Maintenance Diet (Woodland, CA) and a mixture of seeds (black oil sunflower seeds, sunflower hearts and thistle seeds). Water and fine grit were also provided *ad libitum*. Birds were sexed either by laparotomy or genetic sex determination (Zoogen, Inc., Davis, CA).

Experimental design—The experiment included 3 treatment groups for males and 2 treatment groups for females. Both males and females were housed either alone (control, n = 8 males, n = 7 females) or with an opposite sex partner (a potential mate, n = 9 males and females, female subjects served as stimulus females for male subjects and vice versa). Additionally, a third group of males was paired with females that received estradiol implants (n = 9 males). These estradiol-implanted females were expected to behave similarly to reproductively mature females and thus be maximally stimulatory to males. Birds were randomly assigned to treatment groups, with birds captured at different locations balanced across groups. Male-female pairings were randomly assigned except that no bird was paired with an individual with which they were captured. One pair each from the unmanipulated female group and the estradiol-implanted group were removed partway through the study because one member of the pair died unexpectedly. Data from these pairs was included for those sampling points for which it was available.

Prior to winter solstice, males and females were separated into different cages and were visually isolated from opposite-sex birds. For the experiment, birds were housed in ‘individual’ cages that contained either a single bird or pair of birds. Cages were placed in 12 acoustic isolation chambers (Industrial Acoustics Company, Bronx, NY), 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. Birds assigned to each treatment group were divided among 2–4 chambers. Chambers were arranged within a single room, in alternating order. The positions of cages on shelves within a chamber were rotated weekly throughout the experiment.

The experiment began on 20 February 2010 (Day 0), when birds were moved into acoustic chambers either alone in a cage or in a cage with a randomly assigned partner of the opposite sex. At this point, birds had been on a permissive photoperiod for 38 days and had begun gonadal recrudescence (Figs. 1 & 2).

Estradiol implants—Females receiving estradiol implants were implanted two days prior to the start of the experimental manipulation (18 February 2010, Day -2). Estradiol implants were created using silastic tubing (1.96mm outer diameter) filled with crystalline 17 β -estradiol (Sigma E-8875). Implants were 7mm in length and sealed at both ends with silicon adhesive to create an implant with 5mm of packed hormone. Implants were soaked in sterile saline overnight before implanting. Following application of a topical anesthetic, implants

were inserted subcutaneously on the flank through a small incision, which was sealed with veterinary adhesive. All females defeathered the brood patch after receiving an implant, consistent with elevation of estradiol levels.

Because we previously had experienced problems using estradiol implants in another species of cardueline finch (house finch, *Haemorhous mexicanus*), we performed a pilot study testing tolerance of females for estradiol doses. In that test, no females exhibited adverse effects (lethargy or excessive lipid in the blood) to 7mm (5mm packed hormone) implants over the course of 28 days. One female showed an elevation of lipids in the blood, but was otherwise normal. Therefore, this dose was selected for the full study. Nevertheless we closely monitored implanted females throughout the experiment, and after 1-2 months post-implantation, several females (n = 4) showed signs of the estradiol dose being too high (lethargy or excessive lipid in the blood). When these symptoms occurred in a female, the original implant was removed and replaced with a lower dose implant. Because females were monitored closely and responded quickly to the removal of the original implant, we were able to resolve the symptoms within a few days. To make 'low dose' implants we mixed 2mg of crystalline 17 β -estradiol per mL of silicon adhesive and extruded an implant that was ~2mm diameter and 10 or 7mm in length. The 7mm length implants were used for females for which the dose from the 10mm extruded implant was still too high. The silastic implants were monitored visually to ensure that there was still remaining hormone in the implants throughout the study. Since this was not possible for the low dose implants, these were replaced with new ones after approximately 2 months.

Measures of reproductive physiology—Measures of reproductive condition were collected repeatedly throughout the experiment. Gonadal condition was measured via laparotomy under general anesthesia (Isoflurane, Abbott Laboratories, Abbott Park, IL) prior to the start of the experiment (day -4 in males and day -2 in females) and repeatedly during the experiment on days 36, 73 and 120 in males and days 38, 74 and 120 in females. This was done by making a small incision on the left flank of the bird and exposing the left testis or ovary. The length of the left testis was measured by positioning the tips of forceps at each end of the testis, pressing the tips into clay, and measuring the distance between the impressions to the nearest 0.1 mm with dial calipers. Ovarian condition was scored on a scale from 1 to 6 as: 1, smooth, entirely regressed; 2, slightly granular appearance, some follicles may be visible; 3, small distinct follicles visible, but no follicular hierarchy evident; 4, follicles obvious, hierarchy evident but none yolky; 5, large, yolky follicles evident; 6, yolky follicle ready for ovulation and/or egg in oviduct (Hahn, 1998; Stevenson et al., 2008). Intermediate scores (± 0.5) were also given where appropriate. Most notably, cases in which yolk was visible in one or more small follicles, but there were no large yolky follicles were scored as 4.5. Testis length and ovarian condition were also measured at the end of the experiment on days 134-137 after the birds were euthanized and the gonads dissected out.

Secondary sexual traits were also measured as indicators of reproductive condition. In males, cloacal protuberance (CP) length, an androgen-dependent trait (Deviche, 1992; Schwabl and Farner, 1989; Schwabl and Kriner, 1991), was measured to the nearest 0.5mm using dial calipers. In females, defeathering of the brood batch (BP), an estrogen-dependent

trait (Jones, 1971), was scored visually by estimating the percentage of the chest that was defeathered to the nearest 5%. BP and CP were measured every 12-24 days.

Blood samples were collected to measure circulating hormone levels. Blood was collected from the alar vein into heparinized microhematocrit tubes and stored on ice until it was centrifuged to separate plasma. Plasma was collected and stored at -20°C until assayed for luteinizing hormone (LH) and testosterone (T). LH was measured on days -5, 13, 53, 68, and 116 in males and on days 4, 19, 32, 70, and 117 in females. T was measured in males on days -5, 30 and 92.

Experiment 2: Pair affiliation and reproductive development

The goal of this experiment was to investigate whether the relationship of a pair influenced the effect of a potential mate on reproductive development. We used data from the paired treatment group from Experiment 1 (females that did not receive hormone implants, $n = 8$ pairs with complete data available) and supplemented these data with a second experimental group that was designed to mimic the paired treatment group from Experiment 1 as closely as possible.

For this additional experimental group, birds were captured in Jackson WY in August 2011 and transported to facilities at Loyola Marymount University in Los Angeles CA. There they were housed in small groups on day lengths that simulated natural changes in photoperiod at a latitude of 38°N until winter solstice (21 December 2011), after which time they were held on winter solstice day length (9.7L:14.3D) until 19 January 2012 when they were put on a 12L:12D photoperiod. As in Experiment 1, birds were fed a diet of Roudybush Small Bird Maintenance Diet and a mixture of seeds, with water and fine grit provided *ad libitum*. Birds were sexed based on the appearance of secondary sexual traits at the time of capture or by genetic sex determination (Zoogen, Inc., Davis, CA).

Prior to winter solstice, males and females were housed separately until the start of the experiment. For the experiment, birds were housed as pairs in 'individual' cages within a single room ($n = 11$ male-female pairs). Cages were arranged on shelves such that each cage was visually isolated from all other cages, but birds could hear the other birds. The positions of cages on shelves were rotated regularly throughout the experiment. As in Experiment 1, male-female pairs were randomly assigned except that no bird was paired with an individual with which they were captured. The experiment began on 27 February 2012 (Day 0), when birds were paired. As in Experiment 1, birds had been on a permissive photoperiod for 40 days at this point and had begun gonadal recrudescence.

Gonadal condition was measured via laparotomy as described for Experiment 1 on days -3 (males) or -2 (females) and on 138 (females) or 139 (males), which closely match sample days from Experiment 1. BP defeathering in females was measured as described above near the end of the experiment, on day 135. This provided a comparable measure to the one taken on day 131 in Experiment 1. Blood samples were collected as described above in order to measure plasma levels of very-low-density lipoprotein (VLDL, a yolk precursor). Blood samples were collected on days 53 and 93. Additionally, blood samples collected from

females in Experiment 1 on days 54 and 93 were used to measure plasma VLDL in those birds.

LH, T, and VLDL assays

Plasma LH was measured with a doubleantibody, post-precipitation radioimmunoassay (Wingfield et al., 1991), which has been used previously with this species (Hahn et al., 2004; Watts and Hahn, 2012). Duplicate 20 μ L plasma samples were run in a single assay. The intra-assay coefficient of variation was 4.74%. Assay sensitivity was 0.148 ng/mL; samples below this limit were assigned this sensitivity value as a concentration.

Plasma testosterone (T) was measured using an enzyme immunoassay kit from Enzo Life Sciences (ADI-901-065) as previously described (Watts and Hahn, 2012). 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 2 plates. The intra-assay coefficient of variation was 3.75% and the inter-plate coefficient of variation was 6.29%. Average assay sensitivity was 0.165 ng/mL. Samples below the limit for assay sensitivity were assigned a concentration of the sensitivity value for the plate on which they were run.

We measured triglyceride-rich yolk targeted very-low-density lipoprotein (VLDL) in plasma as an indicator of ovarian development (Mitchell and Carlisle, 1991; Vanderkist et al., 2000). Plasma VLDL was quantified using an analytical assay for total triglycerides and free glycerol (Sigma-Aldrich) with VLDL measured as triglyceride levels by subtracting glycerol from total triglycerides (Caro et al., 2009). Samples were run in duplicate. Intra-assay variation was 10.2%, and inter-assay variation was 2.5% for samples from Experiment 2. Samples from Experiment 1 were run in a single assay. We do not have a direct estimate of inter-assay variation between experiments, but we accounted for this statistically by including year of the experiment as a factor in models for statistical analysis (described below).

Behavioral data collection

To measure affiliative behavior, paired birds in both experiments were video recorded for 10 min every 2-7 days for 1 month after pairing ($n = 5$ 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. Bill touching is an affiliative social behavior and is a component of courtship in pine siskins (Mundinger, 1970). As we have done previously (Watts and Hahn, 2012), we used mean measures of bill touching and perching for each pair to calculate a single composite affiliation score for each pair: affiliation score = [(mean bill touching for pair/mean bill touching for all pairs)+(mean perching for pair/mean perching for all pairs)]/2. This is a measure of the degree of affiliation of a pair relative to all other pairs in a given experimental year, with larger values indicating greater affiliation.

Statistical analysis

The effect of social treatment on measures of reproductive condition was examined using linear mixed models. Each measure of reproductive condition was modeled with day of experiment, treatment group, and the interaction between day and treatment as fixed effects

and individual identity as a random effect. Likelihood ratio tests were used to test for each main effect (by comparing model with all main effects to model without the effect of interest) and the interaction between day and treatment (by comparing the full model to the model with only the main effects). Visual inspection of residual plots was used to check for deviations from normality and homoscedasticity. Male LH and T data were log transformed to meet assumptions of the models. We were unable to fit a model for female plasma LH levels. Therefore, these data were analyzed by estimating total LH secretion as the AUC_i (area under the curve with respect to increase; Pruessner et al., 2003) for each female and comparing this measure among treatment groups using a t-test.

The effect of the degree of affiliation on reproductive physiology was examined using linear mixed models. The dependent variable was the measure of reproductive condition: final gonadal condition (testis length in males, ovary score in females), as well as final BP defeathering and VLDL levels in females. Affiliation score was a fixed effect in the model, and year of the experiment was included as a random effect. For the analyses of VLDL levels, day of experiment was also included as a fixed effect and individual identity was included as a random effect. VLDL data were transformed using a Box-Cox transformation ($\lambda = -0.9$). Likelihood ratio tests were used to test for a significant effect of affiliation.

To evaluate whether pair affiliation in the first month after pairing was influenced by gonadal condition of the birds at the time of pairing, a linear mixed model with affiliation score as the dependent variable was used. Gonadal condition measured immediately before pairing was included in the model as a fixed effect and year of the experiment was included as a random effect. Gonadal condition at the start of the experiment was compared between experimental years using a t-test and Mann-Whitney U-test.

Models were fit with random intercepts and random slopes for individuals with respect to treatment. In all cases, including random slopes did not improve the model, so only random intercepts were retained in final analyses. For linear mixed models, we report both marginal R^2 ($R^2_{\text{GLMM}(m)}$, a measure of variance explained by the fixed effects) and conditional R^2 ($R^2_{\text{GLMM}(c)}$, a measure of variance explained by the fixed and random effects) as measures of effect size for the models (Nakagawa and Schielzeth, 2013). To further evaluate effect sizes, we also report the estimates and standard errors for each fixed effect in these models. For t-tests, effect size was calculated as Cohen's d using mean and standard deviation values. For the Mann-Whitney U-test, effect size was estimated as $\hat{\rho}$ (Newcombe, 2006). Statistical analyses were performed in R (R Development Core Team, 2011) using the package lme4 (Bates et al., 2011) for linear mixed models and the package MuMIn (Barto , 2015) to calculate conditional and marginal R^2 values.

Results

Experiment 1: Effects of a potential mate on reproductive development

For males, there was a significant positive effect of day of the experiment on all measures of reproductive condition (Table 1, Fig. 1; estimate \pm SE: testis length, 0.008 ± 0.002 ; CP length, 0.007 ± 0.001 ; log-LH, 0.006 ± 0.001) except for plasma T for which this effect was only a trend (log-T, 0.003 ± 0.002). Most relevant to the focus of our study, there was no

significant effect of social treatment or significant day \times treatment interaction for any measure (Table 1, Fig. 1).

We also examined whether there were potential differences among males who were paired with females that were switched to a lower estradiol dose compared to those maintained on the higher dose. Testis length at the end of the experiment was similar between the two groups (mean \pm SE: high dose, 5.63 ± 0.37 mm; low dose, 5.50 ± 0.008 mm; $t = 0.28$, $df = 6$, $p = 0.79$, $d = 0.25$), as was affiliation score (mean \pm SE: high dose, 0.69 ± 0.59 ; low dose, 0.88 ± 0.24 ; $t = -0.32$, $df = 6$, $p = 0.76$, $d = 0.22$).

For females, ovary score and BP defeathering both increased significantly across time (Table 1, Fig. 2A, B; estimate \pm SE: ovary score, 0.01 ± 0.001 ; BP, 0.42 ± 0.05). There was also a significant effect of treatment on ovary score and BP (Table 1, Fig. 2A, B; estimate \pm SE: ovary score, 0.35 ± 0.17 ; BP, 21.8 ± 6.5) and a significant day \times treatment interaction for BP (Table 1; estimate \pm SE: 0.37 ± 0.09), but not ovary score. In 5 of 8 paired females we observed yolk deposition in follicles, compared with 1 of 7 unpaired females (Fisher's exact test, one-tailed, $p = 0.08$). LH concentrations in samples collected on days 4 and 19 were below the detection limit of the assay, so only samples collected on day 32 and later were included in analyses. Total LH secretion was higher in paired females than in unpaired females (Fig. 2C; AUCi: $t = -2.38$, $df = 13$, $p = 0.03$, $d = 1.23$).

Experiment 2: Pair affiliation and reproductive development

Gonadal condition at the start of the experiment did not differ between the two years of the experiment (female ovary score: $U = 29.5$, $p = 0.21$, $\hat{\theta} = 0.34$; male testis length: $t = -0.95$, $df = 17$, $p = 0.36$, $d = 0.45$). Degree of affiliation of pairs was not related to gonadal condition of either sex at the start of the experiment (female ovary score: $X^2 = 0.20$, $df = 1$, $p = 0.65$, $R^2_{\text{GLMM}(m)} = 0.003$, $R^2_{\text{GLMM}(c)} = 0.22$; male testis length: $X^2 = 0.54$, $df = 1$, $p = 0.46$, $R^2_{\text{GLMM}(m)} = 0.03$, $R^2_{\text{GLMM}(c)} = 0.31$).

There was a trend for females in more affiliative pairs to have more advanced ovary scores (Fig. 3; estimate \pm SE: 0.46 ± 0.21 , $X^2 = 3.48$, $p = 0.06$, $R^2_{\text{GLMM}(m)} = 0.13$, $R^2_{\text{GLMM}(c)} = 0.61$). Females in more affiliative pairs had significantly higher circulating VLDL levels (Fig. 4; estimate \pm SE: -0.06 ± 0.03 (this is for transformed variable, which reflects a positive effect of affiliation on raw VLDL levels), $X^2 = 3.99$, $p = 0.046$, $R^2_{\text{GLMM}(m)} = 0.10$, $R^2_{\text{GLMM}(c)} = 0.21$). There was no effect of affiliation on defeathering of the BP (estimate \pm SE: -10.68 ± 9.40 , $X^2 = 1.39$, $p = 0.24$, $R^2_{\text{GLMM}(m)} = 0.06$, $R^2_{\text{GLMM}(c)} = 0.07$). By the end of the experiment 58% of females (11 of 19) had completely defeathered the brood patch.

In males, there was no effect of affiliation score on testis length (quantified as either maximum length observed: estimate \pm SE: 0.140 ± 0.19 , $X^2 = 0.593$, $p = 0.44$, $R^2_{\text{GLMM}(m)} = 0.03$, $R^2_{\text{GLMM}(c)} = 0.03$; or length on final sampling day: estimate \pm SE: 0.492 ± 0.38 , $X^2 = 2.14$, $p = 0.14$, $R^2_{\text{GLMM}(m)} = 0.07$, $R^2_{\text{GLMM}(c)} = 0.37$).

Discussion

We found that the presence of a potential mate stimulated reproductive development in female, but not male, pine siskins. This result is consistent with the suggestions that males are less sensitive than females to environmental cues that are supplementary to changes in photoperiod (Ball and Ketterson, 2008). However, in another study that directly compared male and female responses to a social cue, Runfeldt and Wingfield (1985) found males to be more sensitive than females to the physiological and behavioral state of their mate; this study focused on termination of reproduction, not reproductive development. Our finding of a sex difference in sensitivity to a social cue among pine siskins provides an interesting contrast to an earlier finding that both males and females are sensitive to food availability during reproductive development (Watts and Hahn, 2012). Thus, within a single species, we find differences among environmental cues in the extent to which the sexes vary in their responses.

Because of the challenges we encountered using estradiol implants in female pine siskins, the results from males paired with implanted females should be examined with caution. It is possible that not all females in this treatment group were similarly stimulatory to the male pine siskins. However, we found no evidence for differences in testis length or affiliation between males paired with high- and low-dose implanted females. This is not surprising given that taken together our results overwhelmingly suggest that male pine siskin reproductive development is not responsive to females.

Similar to pine siskins, in European starlings (*Sturnus vulgaris*) the presence of a male is a critical social cue for females to complete reproductive development (Perfito et al., 2015). In particular, Perfito et al. (2015) found that the presence of a male was associated with the progression to yolk deposition and final maturation of follicles. Our results suggest a similar pattern in female pine siskins. Paired females reached significantly more advanced stages of ovarian development than did unpaired females, and there was a trend for paired females to be more likely to advance to yolk deposition. Moreover, work in white crowned sparrows (*Zonotrichia leucophrys pugetensis*) has also shown the presence of a male to be a key cue for the progression to yolk deposition (Wingfield et al., 1997). Thus, findings from three songbird species are consistent with the hypothesis that females will initiate gonadal development when other environmental conditions, such as photoperiod, are suitable, but 'wait' for appropriate male cues before proceeding to the final gonadal maturation.

The mechanisms by which non-photoc cues, including social cues, alter functioning of the HPG axis in birds are not well understood. Several studies have found an increase in LH release concomitant with socially-stimulated gonadal development in males (Haase et al., 1976; Hahn et al., 1995) and females (Cheng et al., 1998; Hinde and Steel, 1978). These effects on LH are presumably mediated via effects on GnRH-I synthesis and/or release (Cheng et al. 1998). GnRH-I neurons in the preoptic area are sensitive to cues from a potential mate (Mantei et al., 2008; Stevenson and Ball, 2009), and recent evidence suggests that such cues may influence GnRH release via effects on expression of type 2 and type 3 deiodinases (Perfito et al., 2015). However, other data indicate that the extent to which social cues influence gonadal condition via effects on GnRH and gonadotropin release may be less

clear-cut (Bentley et al., 2000; Perfito et al., 2015; Stevenson et al., 2008). Here, we found that circulating LH levels were significantly elevated in paired females compared with unpaired females. However, the timing of this effect on LH appears to occur later than the emergence of the observed effects of our treatment on ovary condition and estradiol-dependent BP defeathering (see Fig. 2). Thus, these initial effects of a potential mate could be due to an early transient effect on LH secretion that we did not capture with our sampling, effects on follicle-stimulating hormone (FSH), and/or effects on tissue sensitivity to gonadotropins. In European starlings, the presence of a potential mate led to an increase in expression of the LH receptor in the ovary, at least in the short-term (Perfito et al., 2015).

We found that the effect of a potential mate on reproductive development in females was correlated with the degree of affiliation between a female and her partner. The greater the degree of affiliation among a pair, the more potent the stimulus was for the female. On the other hand, the pair affiliation was not related to male reproductive development. This is not surprising given that males did not respond to the presence/absence of a potential mate in Experiment 1. Because the relationship between pair affiliation and reproductive condition is correlational, we must be cautious in interpreting causality. However, several lines of evidence suggest that pair affiliation may be driving the effect on reproductive condition, rather than the reverse. Pair affiliation was measured in the first month of pairing, and was not correlated with gonadal condition at pairing. It was not until almost 2 months after pairing that our measures of reproductive condition correspond to pair affiliation. Thus, our results do not appear to derive from birds in more advanced reproductive states at the start of the experiment behaving more affiliatively. We should also note that while females may be responding directly to the quality of their relationships with males, it is also possible that it is variation in another male trait that females are responding to physiologically (reflected in reproductive condition) and behaviorally (reflected in affiliation). Thus, the degree of affiliation and the extent of female reproductive development may be two independent responses to mate attractiveness. Interestingly, in a study of budgerigars (*Melopsittacus undulatus*) in which females were experimentally paired with preferred and non-preferred males, preference for a male did not influence the latency to egg laying, but did influence maternal allocation of yolk and yolk androgens to eggs (Lahaye et al., 2015). The fact that a female's physiological response to a male may be sensitive to particular characteristics of the male or of the pair should not be surprising from an evolutionary point of view given that pair compatibility and pair bond strength can significantly influence reproductive success (Bluhm and Gowaty, 2004; Drickamer et al., 2000; McGraw and Hill, 2004; Ryan and Altmann, 2001; Spoon et al., 2006).

We do not yet know what specific signals from a potential mate influence reproductive development in female pine siskins. Studies in other species indicate a role for auditory signals (Bentley et al., 2000; Brockway, 1965; Kroodsma, 1976), which may have interactive or additive effects with visual signals (Friedman, 1977). But in other cases, such signals may not be sufficient in the absence of more direct interactions between males and females (Perfito et al., 2015). We know from detailed studies of ring doves that a female's response to a male, auditory and proprioceptive signals from her own vocal display, are important stimulatory cues for reproductive development (reviewed in Cheng, 1992). Given that pine

siskin pairs in this experiment were housed together, it is entirely possible that the effects we observed are a consequence of a suite of signals, including interactions of the pair.

Here we have focused on the effects of a potential mate on reproductive physiology. However, a wider array of social cues may be important in regulating reproductive timing. For example, signals, particularly auditory signals, from other pairs have been found to influence reproductive timing in captive and free-living birds (Mariette et al., 2015; Setiawan et al., 2007; Waas et al., 2005). The birds in our experiments could hear, but not see, conspecifics belonging to the same treatment group. Thus, auditory signals from other pairs could have contributed to the observed effects of pairing on females. However, the fact that pair affiliation influenced reproductive condition suggests that extra-pair signals alone are unlikely to explain the observed effects. Further, there appear to be no such effects of other pairs on male pine siskins.

Overall, we have found that female pine siskins are sensitive to the presence of a potential mate and the quality of her relationship with that potential mate during gonadal development. Thus, cues from males are important in female reproductive timing. On the other hand, we found no such effects of cues from females on male pine siskins. Thus, this study highlights the importance of considering sex differences when investigating the effect of environmental cues on reproductive timing. Moreover, we suggest that further examination of how variation in the quality of social cues influences their potency may further our understanding of the role of social cues in reproductive timing.

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Highlights

- We investigated the effect of a potential mate on gonadal recrudescence in pine siskins
- The presence of a mate enhanced gonadal recrudescence in females, but not males
- The degree of affiliation of a pair may modulate the effect of a mate in females

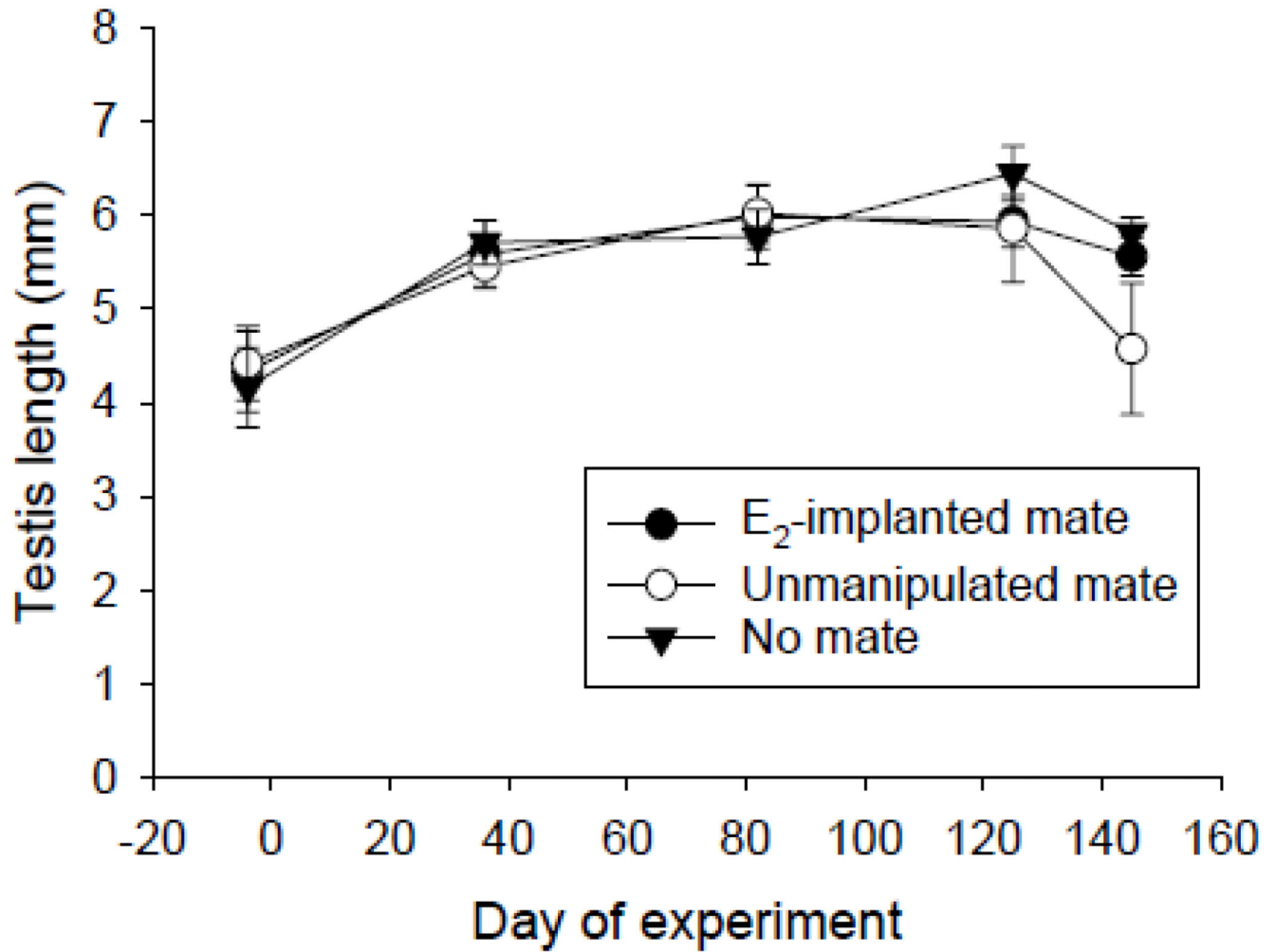


Fig. 1. Testis length (mm) of male pine siskins housed with an estradiol-implanted female (closed circle), an unmanipulated female (open circle), or without a female (filled triangle). Mean \pm 1SE is shown for each time point. There was no significant difference between treatment groups. In pine siskins, a completely regressed testis is typically 1mm in length, and a mature testis is typically 6-7mm in length (Hahn et al., 2004; MacDougall-Shackleton et al., 2006).

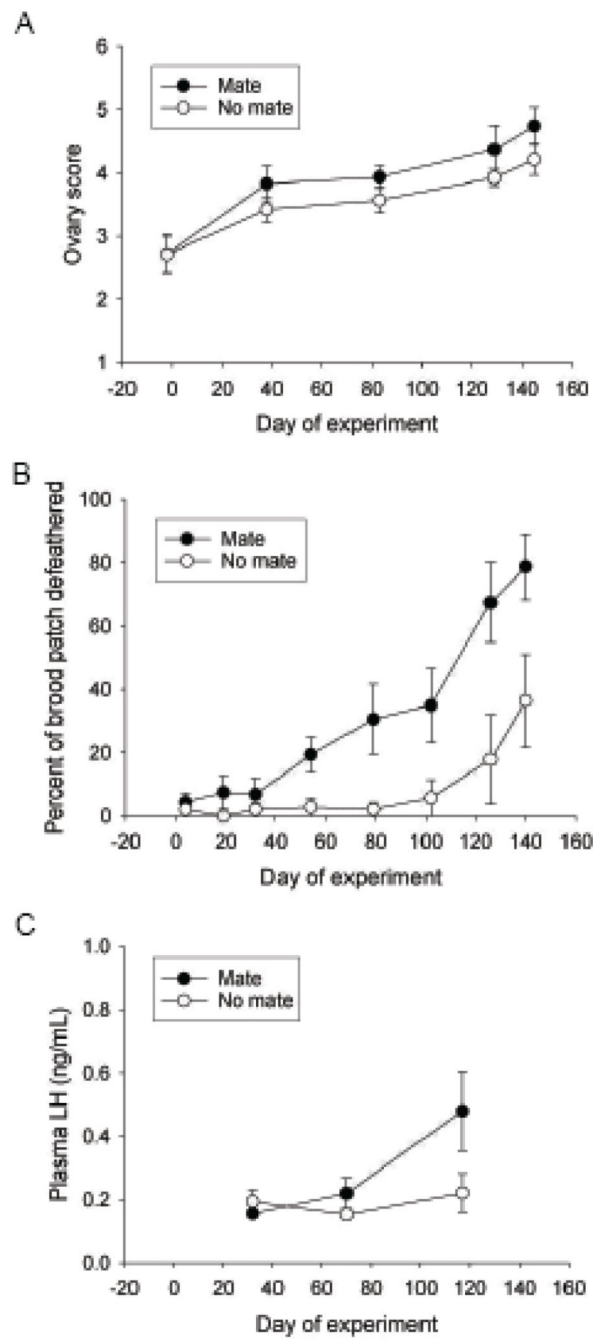


Fig. 2. Measures of reproductive condition in female pine siskins housed with a male (mate, closed circle) or without a male (no mate, open circle). (A) Ovary score, (B) percent of the brood patch defeathered, and (C) plasma LH differed significantly between treatment groups ($p < 0.05$). Mean \pm 1SE is shown for each time point. A completely regressed ovary would be given an ovary score of 1, and a reproductively mature ovary would receive a score of 5 or greater.

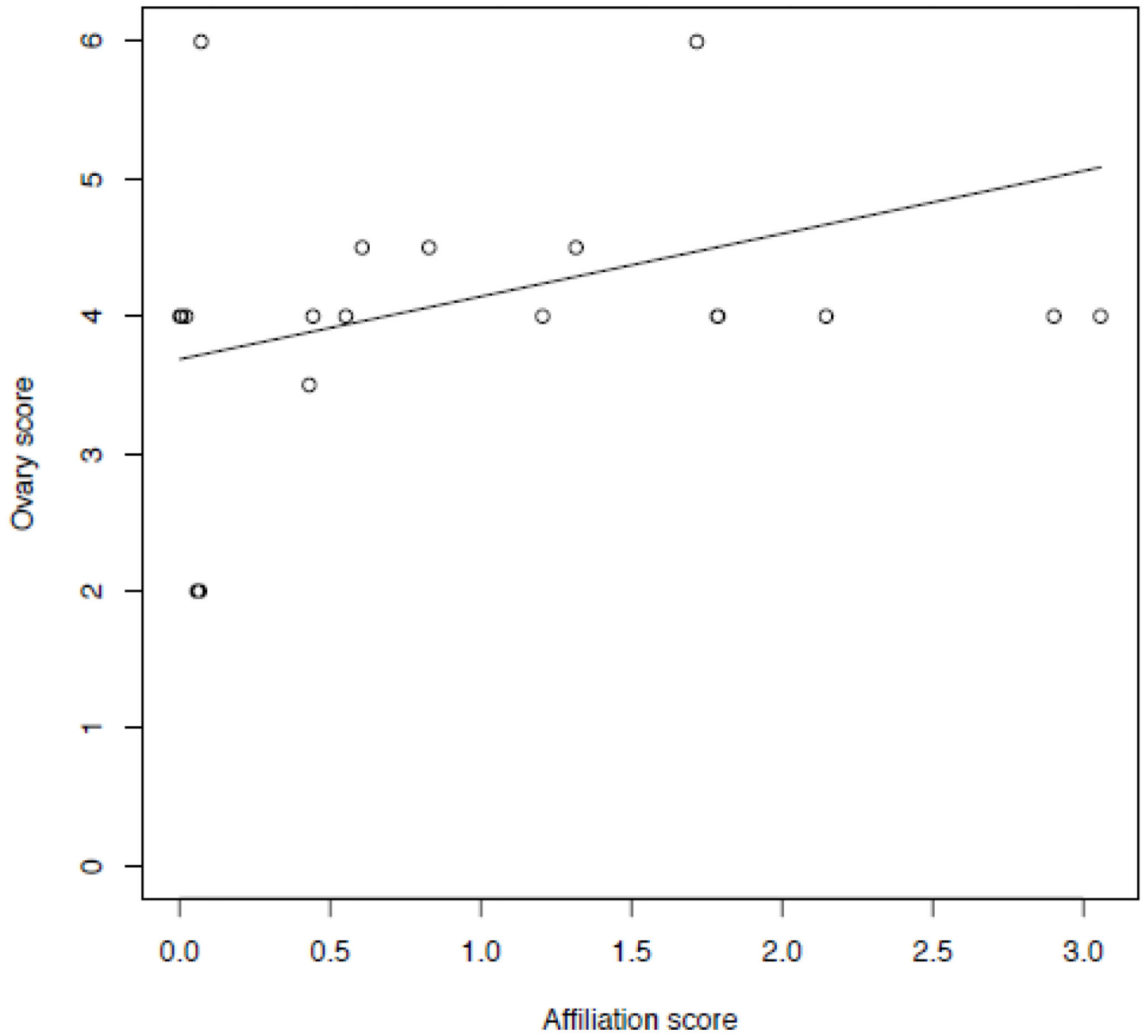


Fig. 3. Ovary scores for female pine siskins and the degree of affiliation with their mate. Circles indicate values for individual birds. The parameter estimate from the linear mixed model is represented by the line.

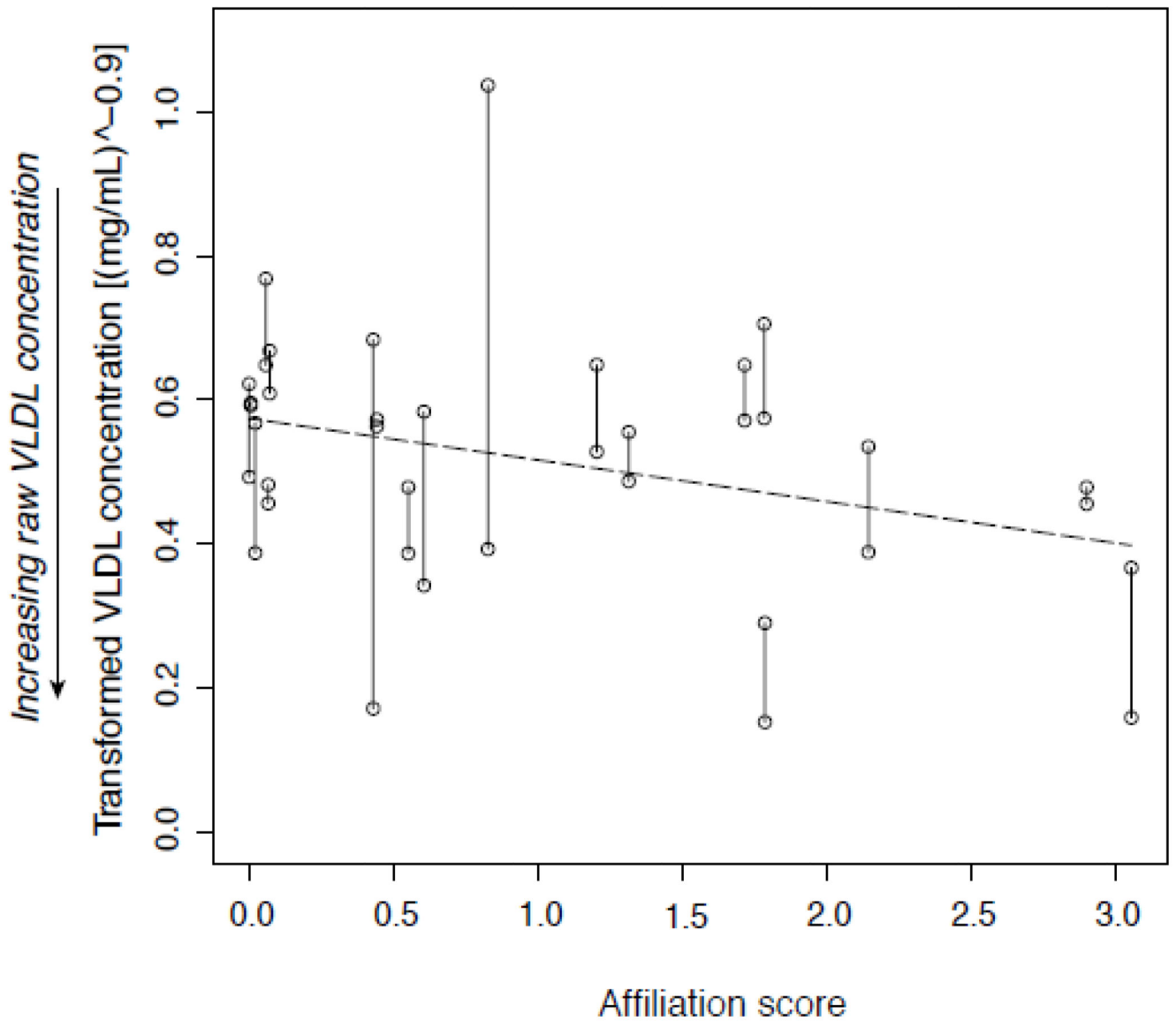


Fig 4. Plasma VLDL levels of female pine siskins and the degree of affiliation with their mate. Each female was sampled on two dates; the paired samples for each female are connected by a line. The dashed line represents the parameter estimate from the linear mixed model. Smaller values of the transformed VLDL variable reflect higher raw VLDL concentrations.

Table 1

Results of likelihood ratio tests for the effects of day of the experiment, treatment, and their interaction on measures of reproductive condition.

	Day		Treatment		Day × Treatment		$R^2_{GLMM(m)}$	$R^2_{GLMM(c)}$			
	χ^2	df	χ^2	df	χ^2	df					
<i>Males</i>											
Testis length	16.85	1	<0.001	1.06	2	0.59	3.72	2	0.16	0.14	0.29
CP length	73.82	1	<0.001	0.902	2	0.64	1.72	2	0.42	0.20	0.61
log-LH	37.54	1	<0.001	0.510	2	0.78	2.90	2	0.23	0.17	0.60
log-T	3.48	1	0.061	0.779	2	0.68	1.83	2	0.40	0.06	0.50
<i>Females</i>											
Ovary score	39.4	1	<0.001	4.47	1	0.035	0.956	1	0.33	0.41	0.43
BP	63.25	1	<0.001	9.26	1	0.002	17.76	1	<0.001	0.50	0.60

Significant p -values ($p < 0.05$) are highlighted in bold.

Marginal and conditional R^2 values are given for each model.