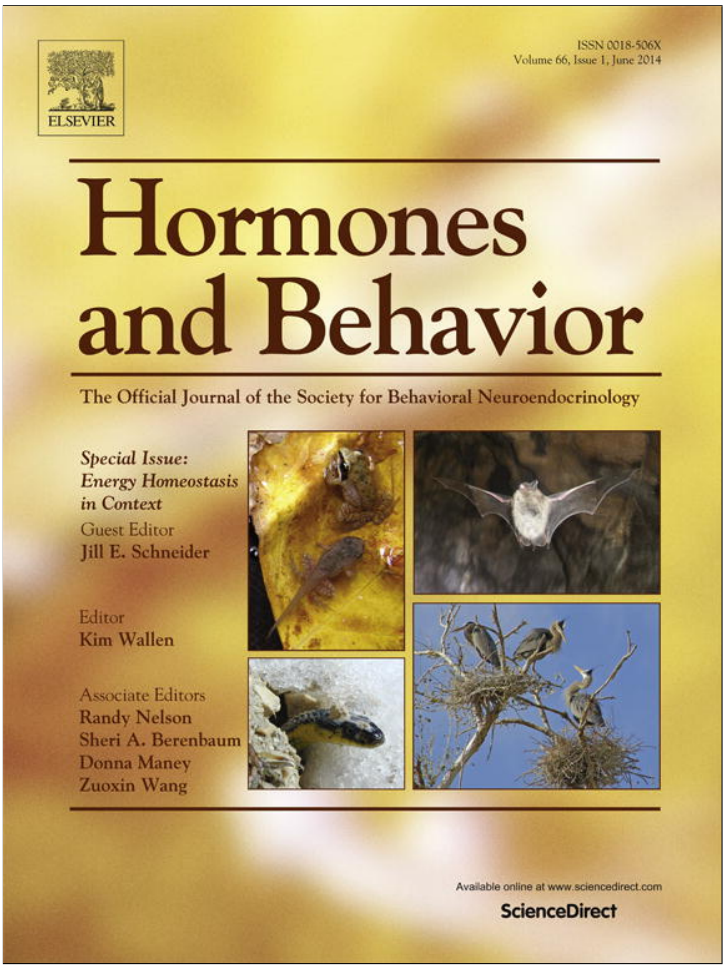

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Regulatory mechanisms for the development of the migratory phenotype: Roles for photoperiod and the gonad

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ABSTRACT

This article is part of a Special Issue "Energy Balance".

Male white-crowned sparrows, *Zonotrichia leucophrys gambelii*, were studied to investigate roles of natural day length and the testes in regulating development and expression of the vernal migration phenotype. Previous work suggested that a pulse of androgen during winter months followed by the vernal increase in photoperiod promotes fueling (fat deposition) to support long distance flight; however, other traits required for successful migration remain untested. To investigate these points, birds were captured on their wintering grounds and castrated prior to winter solstice following Mattocks (1976). A subset of the castrates received 8 mm Silastic implants of testosterone (T-castrates) and others blank implants (Blank-castrates) for 16 days in February. Shams were surgical controls. Migratory traits measured were as follows: 24 h locomotor activity, prenuptial molt, body mass, fat score, flight muscle profile, cloacal protuberance (CPL) and plasma androgens measured over 28 weeks divided into 3 experimental periods (pre-implant, implant, and post-implant). Under short day lengths, castration increased diurnal locomotor activity over Shams. Testosterone implants temporarily enhanced CPL, plasma androgens and flight muscle enlargement, but failed to induce migratory restlessness. Whereas all groups exhibited seasonal increases in mass, fat score and muscle profile, only Shams showed timely onset and completion of prenuptial molt and migratory restlessness. Thus, for castrated males exposed to naturally increasing day lengths, the organizational effects of a transient testosterone surge were not sufficient to actuate a timely spring molt and migratory behavior. A fully functional testis that can organize central processes is required for the entire expression of the spring migratory phenotype.

Introduction

For many migrant birds, regulatory processes that coordinate preparation, execution and ultimately termination of vernal (spring or prebreeding) migration begin on the wintering grounds and progress from late autumn through the early months of spring (Farner, 1955; Gwinner, 1990; Lofts and Marshall, 1961; Ramenofsky and Wingfield, 2007; Tonra et al., 2013; Weise, 1967; Wingfield et al., 1990). The environmental cue or initial predictive factor that is prerequisite for the development of spring migration and breeding in many species is photoperiod — the annual increase in day length beyond the spring equinox of 12L:12D (Hahn et al., 1997; Holberton and Dufty, 2005; Lofts and Murton, 1968; Rowan, 1925; Wang et al., 2013; Wingfield, 2008; Wolfson, 1942). In response to the lengthening days, migrants increase food intake (hyperphagia), body mass, fat deposits, flight muscle size for enhanced energy, power and endurance for long-distance flight and commence expression of nocturnal specific behaviors called migratory restlessness or *Zugunruhe* (Agatsuma and Ramenofsky, 2006; Guglielmo et al., 1998; Jenni-Eiermann and Jenni, 1992; King and Farner, 1963; Marsh, 1984; Owen et al., 2014; Ramenofsky, 1990). Photostimulation also activates the hypothalamic-pituitary-gonad axis initiating preliminary stages of gonadal recrudescence, steroidogenesis and gametogenesis (Bauchinger et al., 2007, 2008; Blanchard, 1941; Blanchard and Erickson, 1949; Bullough, 1941; Lofts and Marshall, 1960, 1961). Thus, the key to understanding the mechanisms regulating the behavioral and physiological traits of vernal migration has been to focus upon both variables of increasing day length and gonad development. Yet, to date, the hormonal regulation of most of the vernal traits remains poorly understood.

Whereas photostimulation and gonadal functions are involved in seasonal changes in the energy balance of migrants, questions remain regarding 1) the duration and timing of testicular action necessary for the expression of the full migratory sequence and 2) the effects of testicular secretions under natural photoperiod. For example, a small but significant pulse of androgen has been measured in some migratory species during the winter but the exact onset and duration of the

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pulse are unknown (Ramenofsky et al., 1992; Wingfield and Farner, 1978). It has been suggested that this winter pulse may serve to organize neuroganglia of the hypothalamic appetite centers of the arcuate nucleus (Boswell et al., 1995b). Once in place, spring photoinduction stimulates the release of orexigenic neuropeptides as neuropeptide Y (NPY), agouti-related protein (AGRP), vasoactive intestinal peptide (VIP) and prolactin (PRL) that bind to the neuroganglia inducing hyperphagia leading to body mass increase and fattening, which coincide with the preparation for spring migration (Boswell et al., 1995b; Cornelius et al., 2013; Deviche, 1995; Farner, 1955; Kuenzel et al., 1999).

Castration completed before but not after the winter solstice prevents the spring day length induced increases in body weight and fat deposition in some (Schwabl and Farner, 1989a; Schwabl et al., 1988; Stetson and Erickson, 1972; Tonra et al., 2011; Weise, 1967) but not all species (Boswell et al., 1993, 1995a; Thapliyal et al., 1983). Whereas these studies document effects of androgens on energy-balancing traits, most of these studies failed to examine the other traits that make up the full migratory sequence. In particular, it is important to determine whether, castration delays migratory restlessness or the energetically costly prenuptial molt, an event that precedes migration in the field and may constrain timing of departure in spring (Bauchinger and Biebach, 2006; Pyle, 1997). Finally, most investigations of seasonal traits exposed birds to abrupt changes in day length, a stimulus that differs from the gradual increase in day length that transpires in nature (Lofts and Marshall, 1961; Morton and Mewaldt, 1962; Schwabl and Farner, 1989b; Tonra et al., 2011; Wagner, 1961; Weise, 1967). Abrupt changes in photoperiod might have a telescoping effect on the individual traits leading up to migration, thereby obscuring the actual temporal sequence of endocrine and behavioral events. By observing birds housed under natural photoperiod, the individual events occur in a more protracted time frame, thereby allowing for detection of plausible cause-and-effect relations among endocrine and behavioral changes (Weise, 1967).

Thus, it is suggested that behavioral and physiological traits of vernal migration involve a relationship of the incremental increase in natural photoperiod and the neuroendocrine system. To determine the regulatory mechanisms that may apply to migratory species as a whole, we focus on Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*, GWCS), a common overland, long-distance migrant with an extensive history in migration research (King and Farner, 1963; Ramenofsky, 2011; Wingfield and Farner, 1978). We hypothesize that a pulse of androgen on short, post-solstice day lengths influences the behavioral and physiological characteristics of the vernal migratory phenotype under naturally increasing photoperiods. We predict that the androgen pulse acts in an organizational manner to establish hyperphagia that leads to fattening, alterations in flight muscle and migratory restlessness once day length extends beyond spring equinox of 12L:12D. As a test of these ideas, we have followed the methods presented previously for GWCS (Mattocks, 1976). In short, birds were held on natural photoperiods allowing for development and expression of the vernal migratory condition and gonadal function to proceed naturally in gradual fashion (Weise, 1967). To follow events throughout, the study was divided into 3 distinct experimental periods: photosensitive birds were castrated prior to winter solstice (pre-implant period) with the prediction that birds would be in the wintering stage without expression of migratory features. To test for the potentiating effect of a winter pulse of testosterone, steroid is administered from February 1 to 16 to castrates, 3 weeks prior to spring equinox on March 20 (implant period). Prior to 12L:12D photoperiod, it is predicted that birds would show no migratory features but exhibit the anabolic effects of androgen therapy. Following this, the study extended through the end of May (post-implant period), a time when natural populations have arrived on territory, terminated vernal migration and commenced to breed (Wingfield and Farner, 1978). The prediction for this period is that all migratory features of fueling, muscle hypertrophy, migratory restlessness, and molt should be apparent in Shams and testosterone treated-castrates. We propose that taking this experimental approach will help elucidate the regulatory mechanisms of the natural progression of events of vernal migration.

Methods

Subjects and housing

Twenty-three adult male GWCS were captured in Yolo County, California (38° 32″N, 121° 4611″W) in late Nov, 2010, held in flight aviaries during the 1.5 weeks quarantine and then transferred to individual registration cages (35 cm (w) × 40 cm (l), 45 cm (h)) under the local photoperiod. Birds were fed ad libitum with commercial seed-mix (millet, cracked corn and sunflower seeds), Mazuri Small Bird Maintenance Diet (#56A6) (www.mazuri.com) and Health Blue Grit (Seed Factory, Ceres, CA). Fresh drinking and bath water were available daily. Room temperature ranged between 18 °C and 23 °C daily. GWCS do not express MR in total darkness (Agatsuma and Ramenofsky, 2006) therefore a single low intensity night light (Limelite Nightlight, Austin Innovations, TX) measured <1 lx at the source by a Greenlee Digital Light Meter (93-172; Rockford, IL) was positioned centrally in the room and visible to all cages.

Each registration cage was equipped with a photodetector (Alarm Entry Device, RadioShack Corporation; Forth Worth, TX) that emitted an infrared beam 0–10 cm above a central perch positioned 34 cm above the bottom of the cage. As birds pass through the beam, an electric signal was transmitted to a Mini Mitter Acquisition System — Vital View (Sun River, OR). Twenty-four hour locomotor activity was recorded in 15 min bins and analyzed in hourly intervals per week for each bird over the entire length of the 6 month study.

Behavioral criteria for migratory restlessness

While seasonal nocturnal locomotor activity of captive birds is historically synonymous with migratory restlessness or *Zugunruhe*, more complete analyses of the 24 h locomotor cycles of various migrant species including GWCS identified characteristic components of this behavior. These include expression of intense nocturnal activity greater than daytime levels and presence of a quiescent phase or period of complete inactivity at the end of the daytime or photophase followed by onset of intense activity at the outset of the night or scotophase (Agatsuma and Ramenofsky, 2006; Coverdill et al., 2011; Farner et al., 1954; Palmgren, 1949; Wagner, 1961). Herein, we utilize the 3 criteria for representations of migratory restlessness: (1) nocturnal activity that exceeds daytime, (2) presence of a quiescent phase and (3) followed by elevated activity at the outset of the scotophase.

Surgery and testosterone replacement

On 10 December 2010, 16 individuals were castrated bilaterally under anesthesia with isoflurane (Attane, Baxter Co) while 8 individuals were sham operated — treated identically but gonads were manipulated only and left intact. On 1 February 2011, the 16 castrated individuals were implanted subcutaneously with one Silastic medical grade tube or implant (Dow Corning) (1.47 i.d. × 1.95 o.d., ×10 mm in length) that was sealed with Silastic glue. Eight testosterone or T-castrates received implants packed with 8 mm of crystalline testosterone (Sigma Chemical) and eight blank or Blank-castrates received empty implants for the vehicle control. All implants were removed on 17 February 2011 and incision was closed with Vetbond (3M Co). Effectiveness of the castrations was determined once birds had regained photosensitivity. Birds were photostimulated with 18L:6D for 2 weeks and autopsied for the presence of testicular fragments. In 8 cases testicular tissue was found and subjects (regenerates) were removed from the study. In addition, 3 birds were removed from the Sham group as 2 were found to be females and 1 died late in the study. The final group sample sizes

were as follows: Blank-castrate = 5, T-castrate = 3, and Sham = 5. All handling procedures were approved by the Institutional Animal Use Committee of the University of California Davis (#15522).

Energetic condition, CPL, and plasma androgens

Measurements were collected from each bird at weekly intervals by the same observer. Body mass was determined to the nearest 0.1 g with a Pesola scale. Fat score was assessed using the sum of values observed from the presence of adipose tissue located in the abdominal cavity and chorico-clavicular fossa and ranging from 0 or no visible fat to 5 with bulging fat deposits (Landys et al., 2004; Ramenofsky et al., 1999). Cloacal protuberance, an androgen dependent organ in breeding birds, is the site of sperm storage in the seminal glomerula and used as an intromittent organ. Organ length (CPL) was assessed by calipers (to the nearest 0.01 mm). Five blood samples were collected throughout the six months study for plasma androgens — testosterone (T) and 5α-dihydrotestosterone (DHT). Samples were collected from each bird within 10 min of disturbance.

A visual scoring system was used to estimate flight muscle profile following Bairlein (1995). The area was accessed by blowing the ventral feathers aside to expose the breast muscles. Body of the flight muscle was viewed on the midline at the attachment site along the sternum (Bauchinger et al., 2011). In profile, muscle that is extremely concave from the point of attachment and extending bilaterally is scored as a 0 and is typical of emaciated birds. As the muscle expands becoming more convex a maximal score of 3 indicates that fibers lie horizontally from the attachment site then bulge laterally.

Molt

Prenuptial body molt was scored on three body regions: crown, back (including nape, back and rump) and abdomen (including throat, breast, abdomen and flanks). Each region was given a score between 0 and 3 depending on the extent of molting feathers: 0–no molt, 1 light molt (1–15% area), 2 moderate molt (16–50% area), and 3 heavy molt (51–100% area). Scores of each region were summed to generate a total molt score for each week of the study period.

Radioimmunoassay

Blood samples (100 μl) were collected from the alar vein into a heparinized microhematocrit tube (Landys et al., 2004). Plasma was separated by centrifugation and stored at −20 °C until assayed. A 2000 CPM dose of both labeled steroids for internal standards was added to each paired sample and later adjusted for the percent recovery. Next, lipids were extracted with ethyl ether after which samples were added to Diatomaceous Earth/glycol, reconstituted in 10% ethyl acetate in isooctane then transferred to Diatomaceous Earth/glycol microcolumns for partial purification. Samples were then run in two specific radioimmunoassays using two standard curves that ranged from 3.8–1000 pg (Ramenofsky et al., 1999). The range of recoveries for each steroid was T: 53–70% and DHT: 50–69%. Final concentrations were calculated in units of ng/ml of plasma after correcting for plasma volume and recovery counts calculated for each sample. Average detection limit (as determined by water blanks) and intra-assay variation for T were 0.19 ng/ml and 2.3%, and for DHT 0.28 ng/ml and 2.4%, respectively. Inter-assay variation for T was 4.5% and for DHT 7.6%. Both testosterone [1,2,6,7,16,17-^{3}H(N)] and 5α-dihydrotestosterone (5α androstan-17β-OL-3-One) [1,2,4,5,6,7-^{3}H(N)] were purchased from PerkinElmer Inc. (Waltham, MA) and Rabbit Anti-Testosterone-3 from Fitzgerald Industries International (Acton, MA).

Period divisions

The study was run over a total of 28 weeks and divided into 3 periods each characterized by distinct experimental states: pre-implant (Dec 14–Jan 31), implant (Feb 1–Feb 16) and post-implant (Feb 17–Jun 20) periods and discussed results accordingly (Table 1).

Statistics

Linear mixed models (LMM) were used to assess the effects of fixed factors: treatment (with 2 levels in the pre-implant period: Castrates and Shams, and with 3 levels in the implant and post-implant periods: Blank-castrates, T-castrates, Shams), and week of study (i.e., gradually increasing photoperiod) and their interaction on the dependent variables (locomotor activity, body mass, fat stores, muscle profile, reproductive hormones, CPL and molt score) separately in each experimental period. Individual bird ID was included as a random factor and week was entered as a repeated variable. We used first-order autoregressive covariance structure because we assumed that adjacent observations on the same individual would have errors that are more similar than errors for observations farther apart. We report F-tests for fixed effects and parameter estimates (i.e., intercepts, slopes) for linear models whenever significant effects are found.

To test whether temporary T-replacement and/or castration had an effect on the termination of the migratory phenotype, we conducted within-group comparisons of energetic condition between two time points: peak migration vs. end of the experiment (approaching summer solstice). Peak migration was identified as the middle two of the 4 weeks period when (1) Shams showed migratory restlessness and (2) the local field populations of GWCS were migrating (M. Ramenofsky and Z. Németh, unpublished data). Based on these criteria, we identified weeks 21–22 (April 26–May 9) as peak migration time, averaged data for these and the final two weeks of the experiment (weeks 27–28, June 7–20), and combined the three variables related to energetic condition (fat, body mass, muscle profile) with principal component analysis (PCA). We compared the resulting PC1 scores (which explained 64.4% of variability in the peak migration period and 70.2% of variability at the final period) within treatment groups by using paired t-tests. Finally, we separately calculated the difference in energetic condition (PC1) across treatment groups in the final two weeks of the experiment by a one-way ANOVA. Effect size measures (Cohen's d, partial eta squared [η_p^2], or effect-size correlation coefficient [r]) are reported for

Table 1
Descriptions and explanations of parameters measured during the three experimental periods throughout the 28 weeks of the study.

Experimental period	Date (week of experiment)	Range of the photophase (h)	Parameters measured
Pre-implant	Dec 14–Jan 31 (2–8)	9.5–10.1	- Day-/night-time activity - Body mass - Fat score - Muscle profile - Plasma androgens
Implant	Feb 1–16 (9–11)	10.3–10.8	- Day-/night-time activity - Body mass - Fat score - Muscle profile - Plasma androgens - Cloacal protuberance
Post-implant	Feb 17–Jun 20 (11–28)	11.0–14.8	- Day-/night-time activity - Body mass - Fat score - Muscle profile - Plasma androgens - Cloacal protuberance - Molt

significant effects for all tests except linear mixed-models. All analyses were conducted with SPSS 21.0 (SPSS Inc., 2012).

Results

Pre-implant (Dec 7–Jan 31) (weeks: 1–8)

Effects of castration

Diel activity. The 24 h locomotor activity records obtained from recently captured birds in late November showed that nocturnal activity associated with autumn migration had ceased; thus all experimental birds were categorized in the wintering stage (Fig. 1B). Castration at this time had a dramatic effect on diurnal pattern of activity. All Castrates (n = 8) exhibited significantly more intense daytime activity than Shams (n = 5) (LMM, $F_{1, 96.1} = 8.992$, P = 0.003); whereas no difference in nighttime activity across the groups was observed (LMM, $F_{1, 14.2} = 0.742$, P = 0.403) (Figs. 1A, B). Overall, nighttime ($F_{1, 14.7} = 5.441$, P = 0.034) but not daytime activity ($F_{1, 96.1} = 2.087$, P = 0.152) increased over time; however, no interaction between treatment and week for either daytime ($F_{1, 96.1} = 1.495$, P = 0.224) or nighttime activity ($F_{1, 14.9} = 0.27$, P = 0.871) was identified. Daytime activity remained significantly greater than nighttime activity in Castrates (RM Wilcoxon signed rank test, P = 0.05, r = 0.49) but was not different in the Shams (RM Wilcoxon signed rank test, P = 0.5, r = 0.21) by the end of the pre-implant period (week of Jan 25–31).

Energetic condition (body mass, fat score and muscle size). Castration had a rapid effect on mass between weeks 2 and 3, as birds increased within one week of the surgery (RM Wilcoxon signed rank test, P = 0.012, r = 0.63, Fig. 2A). Beyond this there was no effect of either treatment or week on mass (LMM, $F_{1, 9.8} = 2.743$, P = 0.129; $F_{1, 30.9} = 2.11$, P = 0.156, respectively) and no interaction was observed ($F_{1, 30.9} = 0.11$, P = 0.742). Both Castrates and Shams increased fat deposits over time ($F_{1, 31.4} = 7.52$, P = 0.01) but fat scores did not differ across groups ($F_{1, 10.9} = 0.712$, P = 0.417) nor changed depending on treatment groups ($F_{1, 31.4} = 0.396$, P = 0.534) (Fig. 2C). Muscle profile was not influenced by either week ($F_{1, 10.286} = 1.060$, P = 0.327) or treatment ($F_{1, 19.842} = 0.460$, P = 0.505) and the interaction term ($F_{1, 10.286} = 1.060$, P = 0.327) was not significant (Fig. 2E).

Implant period (Feb 1–16) (weeks 9–11)

Diel activity

Treatment had no effect on either daytime (LMM, $F_{2, 5.28} = 1.154$, P = 0.384) or nighttime activity (LMM, $F_{2, 10} = 0.626$, P = 0.554) and neither did week (daytime: $F_{1, 6.28} = 0.991$, P = 0.356; nighttime activity: $F_{1, 10} = 0.401$, P = 0.541) (Figs. 3A, B). There was no significant interaction between the two fixed factors for either daytime ($F_{2, 6.28} = 1.008$, P = 0.417) or nighttime activity ($F_{2, 10} = 0.489$, P = 0.627).

Energetic condition (body mass, fat score and muscle size) and reproductive measures

Both week and treatment were significant predictors of body mass (LMM, week: $F_{1, 19.2} = 74.086$, P < 0.001; treatment: $F_{2, 10.85} = 5.39$, P = 0.024) but the interaction term was not significant ($F_{2, 19.2} = 2.45$, P = 0.112) (Fig. 2B). Intercept estimates of the linear model for T-castrates were significantly lower than those of Shams (t = −3.205, P = 0.009). A marginally significant interaction of treatment and week was found with the slope increasing by 0.99 for T-castrates compared to Shams (t = 2.064, P = 0.053). All other relationships were not significant.

Fat scores were significantly influenced by week (LMM, $F_{1, 36.96} = 25.35$, P < 0.001) but not treatment ($F_{2, 44.24} = 0.444$, P = 0.645) or their interaction ($F_{2, 36.96} = 0.062$, P = 0.940) (Fig. 2D). However, muscle size was influenced both by week ($F_{1, 10.86} = 18.77$, P = 0.001) and treatment ($F_{2, 12.048} = 5.88$, P = 0.016) as well as their interaction ($F_{2, 10.86} = 6.79$, P = 0.012) (Fig. 2F). Although, the intercept estimates for T-castrates were significantly lower than those of Shams (P = 0.009), T-castrates showed a significantly (P = 0.007) higher increase in muscle score, with a slope of 0.55, compared to Shams. Furthermore, treatment had a significant effect on the CPL (one-way ANOVA, $F_2 = 6.831$, P = 0.013, $\eta_p^2 = 0.58$) with T-castrates exceeding those of the Blank-castrates (LSD post hoc: P = 0.005, d = 2.3) and Shams (P = 0.016, d = 2.7) (Fig. 5). Shams and Blank-castrates did not differ (P = 0.426, d = 0.52).

Plasma androgens

Testosterone administration resulted in increased plasma levels of DHT in the T-castrates compared to Blank-castrates and Shams (LMM, treatment: $F_{2, 11.53} = 0.531$, P = 0.602, week: $F_{1, 11.85} = 25.40$, P < 0.001, treatment by week interaction: $F_{2, 11.85} = 24.48$, P < 0.001). Treatment by week interaction for T-castrates was significantly greater

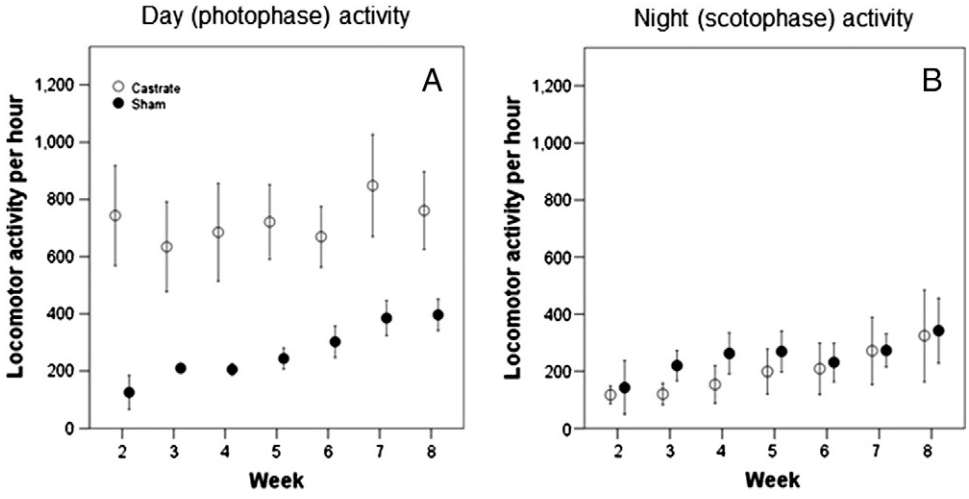

Fig. 1. Locomotor activity (average electronic beam breaks/h) recorded throughout the pre-implant period during photophase (A) and scotophase (B) in Shams (n = 5) and Castrates (n = 8). Each symbol represents mean ± SEM.

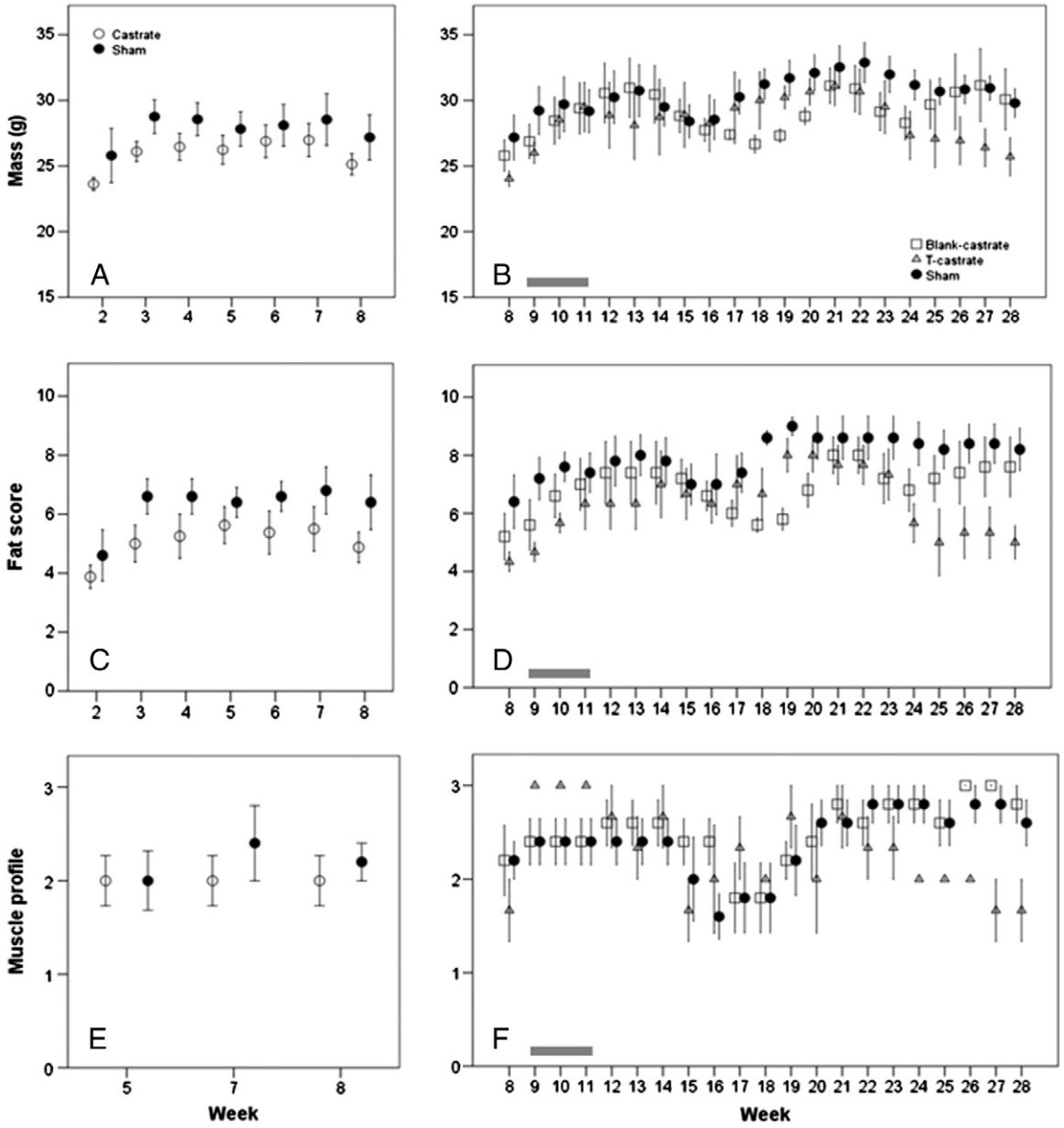

Fig. 2. Weekly changes in the pre-implant period (left panel, weeks 2–8) in Shams (n = 5) and Castrates (n = 8) and implant and post-implant periods (right panel, weeks 9–28) in Shams (n = 5), Blank-castrates (n = 5) and T-castrates (n = 3) of body mass (A, B), total fat score (C, D) and flight muscle profile (E, F). Each symbol represents mean ± SEM and gray-filled horizontal bar designates the implant period.

than the Shams with a slope of 1.1 ($P < 0.001$) (Fig. 4A). Similarly, plasma levels of T were elevated (treatment: $F_{2, 10} = 0.542$, $P = 0.598$, week: $F_{1, 10} = 15.570$, $P = 0.003$ with a treatment by week interaction: $F_{2, 10} = 14.713$, $P = 0.001$). Treatment by week interaction in the T-castrates was significantly greater than in Shams with a slope of 5.83 ($P = 0.001$) (Fig. 4B).

Post-implant period (Feb 17–Jun 20)

Diel activity

Across group comparisons. Overall, treatment and week were not significant factors in determining the post-implant period nighttime activity (LMM, treatment: $F_{2, 19.24} = 0.635$, $P = 0.540$; week: $F_{1, 32.65} = 2.38$, $P = 0.132$). However, parameter estimates of the linear model show a significant positive linear relationship of week with nighttime activity for Shams (slope: 64.72, $t = 2.324$, $P = 0.027$) only (Fig. 3B). Treatment by week interaction did not reach significance either in the omnibus F test ($F_{2, 32.65} = 1.21$, $P = 0.310$). By contrast, week was a significant predictor of daytime activity (LMM, $F_{1, 17.88} = 5.58$, $P = 0.03$) while treatment was only a marginally significant factor ($F_{2, 17.63} = 3.001$, $P = 0.076$), similarly to the treatment by week interaction ($F_{2, 17.88} = 2.67$, $P = 0.097$) (Fig. 3A). Intercept estimates of the linear model for daytime activity in Blank-castrates were significantly higher than those of Shams ($t = 2.273$, $P = 0.036$), whereas intercept estimates for T-castrates did not differ from those of Shams ($t = 0.161$, $P = 0.874$).

Within group comparisons of activity. Once nocturnal locomotor activity was detected (week 20), we verified whether this represented migratory restlessness by using the two criteria established previously for GWCS (1) intensity of nocturnal activity exceeded that of the day and (2) onset of intense nocturnal activity is preceded by a quiescent phase observed at the end of the day or photophase (Fig. 7). Pair-wise comparisons of photo- and scotophase activities within each treatment

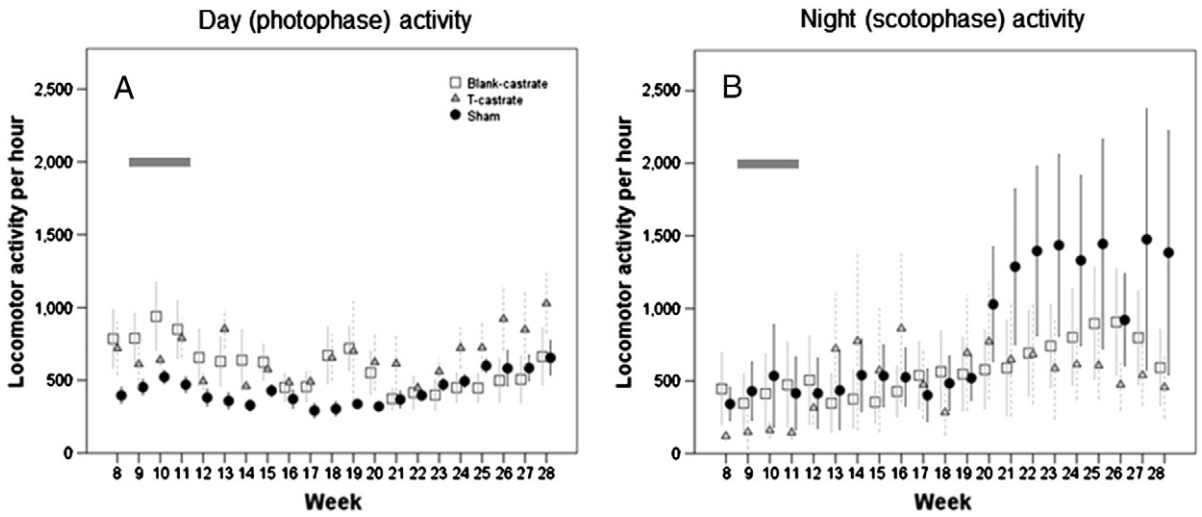

Fig. 3. Locomotor activity (average electronic beam breaks/h) recorded throughout the implant and post-implant periods during the photophase (A) and scotophase (B) in Shams (n = 5), Blank-castrates (n = 5), and T-castrates (n = 3). Each symbol represents mean ± SEM and gray-filled horizontal bar designates the implant period.

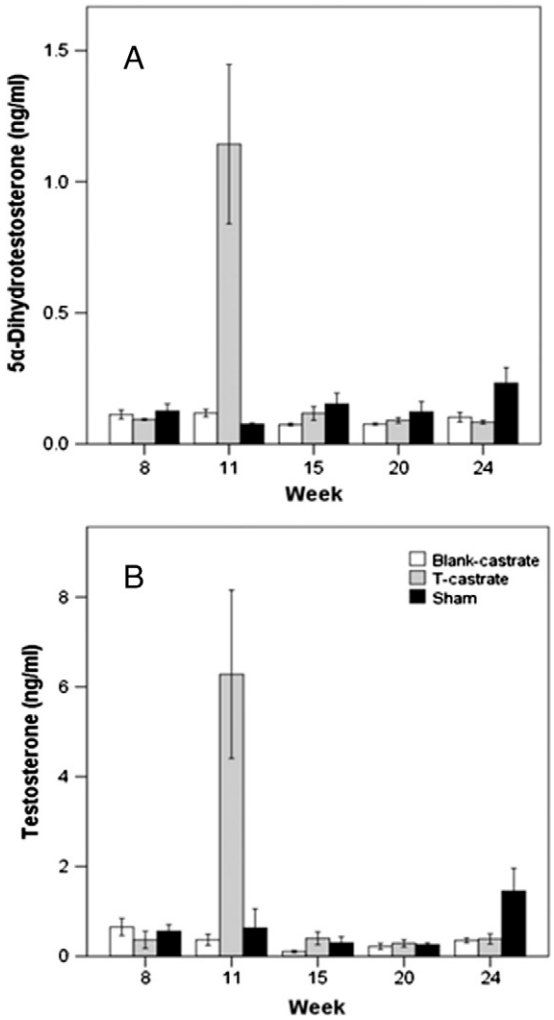

Fig. 4. Plasma levels of (A) 5α-dihydrotestosterone (ng/ml) and (B) testosterone (ng/ml) measured in the three groups at the outset and end of the implant period (weeks 8–11) and throughout the post-implant period weeks 15–24 in Shams (n = 5), Blank-castrates (5), and T-castrates (3). Bars represent mean ± SEM for each group.

group identified a two-week period for the Shams (weeks 20–21, April 19–May 2) when nighttime activity was significantly elevated over daytime activity (RM Wilcoxon signed rank test, $P = 0.043$, $r = 0.64$). This peak of migratory restlessness corresponded with migratory departure of the field population of white-crowned sparrows at our field sites in Yolo County, CA (M. Ramenofsky and Z. Németh, unpublished data). Furthermore, in pair-wise comparisons of activity between the last hour of the photophase and the first hour of the scotophase identified a quiescent phase as described previously (Agatsuma and Ramenofsky, 2006; Ramenofsky et al., 2003). For weeks 22–24, Shams exhibited basal activity at the conclusion of the photophase (quiescent phase) with enhanced intensity in the first hour of scotophase (onset of migratory restlessness) (RM Wilcoxon signed rank test, $P = 0.043$, $r = 0.64$) (Fig. 7). Castrates never exhibited the quiescent phase. Quite the contrary, throughout weeks 13–14 and 18–20 Blank-castrates had elevated activity in the last hour of the photophase with significantly lower activity in the first hour of night (RM Wilcoxon signed rank test, $P = 0.043$, $r = 0.64$); and for the remaining weeks, as well as for T-castrates, no change between the two phases was recorded ($P > 0.05$).

Effects on energetic condition (body mass, fat score and muscle size), molt and CPL. Body mass over the post-implant period was not influenced by either main factor (LMM, treatment: $F_{2, 93.1} = 0.107$, $P = 0.898$; week: $F_{1, 11,575} = 0.874$, $P = 0.350$) or their interaction ($F_{2, 11,575} = 0.679$, $P = 0.507$) (Fig. 2B). Similarly, fat score was not influenced by either main factor (LMM, treatment: $F_{2, 32} = 0.219$, $P = 0.804$; week: $F_{1, 25.2} = 0.076$, $P = 0.785$) or their interaction ($F_{2, 25.2} = 1.371$, $P = 0.272$) (Fig. 2D). Neither treatment ($F_{2, 187.7} = 2.25$, $P = 0.109$) nor week ($F_{1, 572.6} = 0.352$, $P = 0.553$) had significant effects on muscle size (Fig. 2F). However, the interactions of these fixed factors were significant (LMM, treatment × week: $F_{2, 572.6} = 4.251$, $P = 0.015$). Specifically, T-castrates showed a significant negative relationship with week (slope: −0.08, $P = 0.006$) (Figs. 2B, D, F).

The within-group comparisons of energetic condition (PC1) revealed a significant decline in the T-castrate group (paired t-test, $t_{1, 2} = 7.227$, $P = 0.02$, $d = 4.17$) over the last 8 weeks of the study, as opposed to a marginally significant increase in the Blank-castrate ($t_{1, 4} = 2.58$, $P = 0.06$, $d = 1.15$) and no significant change in the Shams ($t_{1, 4} = 0.129$, $P = 0.9$, $d = 0.05$) (Fig. 8). As a result, treatment groups differed significantly by the conclusion of the experiment (one-way ANOVA, $F_{2, 10} = 4.386$, $P = 0.043$, $d = 0.77$) where the energetic condition of the T-castrates was lower than that of the Shams (Tukey

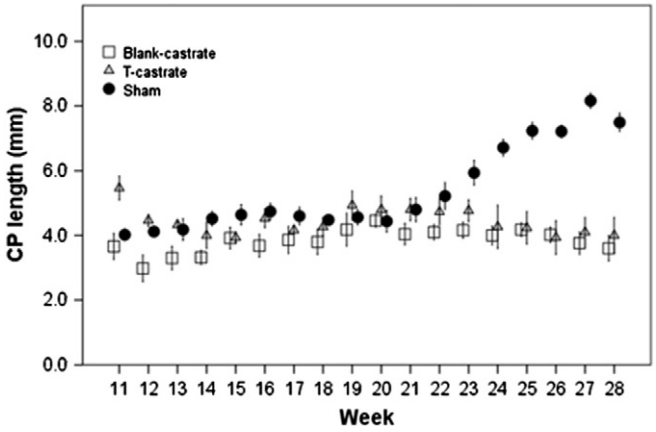

Fig. 5. Weekly changes in cloacal protuberance length (CPL) measured at the conclusion of the implant period (week 11) and throughout the post-implant period (weeks 12–28) in Shams, Blank-castrates, and T-castrates. Bars represent mean ± SEM for each group. Complete descriptions listed in Fig. 1.

HSD, $P = 0.05$, $d = 1.35$) but not significantly different from that of the castrates without T ($P > 0.05$).

CPL was influenced by both main factors (LMM, treatment: $F_{2,\ 18.2} = 7.838$, $P = 0.004$; week: $F_{1,\ 16.6} = 23.427$, $P < 0.001$) and their interactions ($F_{1,\ 16.6} = 19.761$, $P < 0.001$). The Shams showed significantly greater CPL than the other two groups ($P < 0.001$) (Fig. 5).

Prenuptial body molt was influenced by treatment (LMM, $F_{2,\ 42.3} = 7.497$, $P = 0.002$) and treatment by week interaction ($F_{2,\ 45.3} = 9.455$, $P < 0.001$) but not by week alone ($F_{2,\ 45.3} = 0.219$, $P = 0.642$). Initiation of molt was delayed in T-castrates and both castrate groups differed significantly from the Shams in how molt score varied by week (T-castrates: slope = 0.75, $P < 0.001$; Blank-castrates: slope = 0.4, $P = 0.011$) (Fig. 6).

Plasma androgens. Plasma levels of DHT were significantly influenced by only the interaction of treatment and week (LMM, $F_{2,\ 10} = 5.96$, $P = 0.02$) while week alone was only a marginally significant factor ($F_{1,\ 10} = 3.72$, $P = 0.082$) and treatment did not play a significant role ($F_{2,\ 10.46} = 0.468$, $P = 0.639$). Treatment by week interaction was significantly different from Shams for the T-castrates (slope: −0.05, $P = 0.006$) and marginally significant for the Blank-castrates (slope: −0.026, $P = 0.099$) (Fig. 4A). That is, DHT levels increased in Shams more than in the two castrate groups.

By contrast, plasma levels of testosterone were significantly influenced by both treatment and month (LMM, treatment: $F_{2,\ 15} = 4.7$, $P = 0.026$, month: $F_{1,\ 18.8} = 11.277$, $P = 0.003$) as well as their interaction ($F_{2,\ 18.8} = 6.86$, $P = 0.006$). Treatment by month interaction for both the T and Blank-castrates was significantly different from Shams with slopes of −0.58 ($P = 0.004$) and −0.45 ($P = 0.008$), respectively (Fig. 4B).

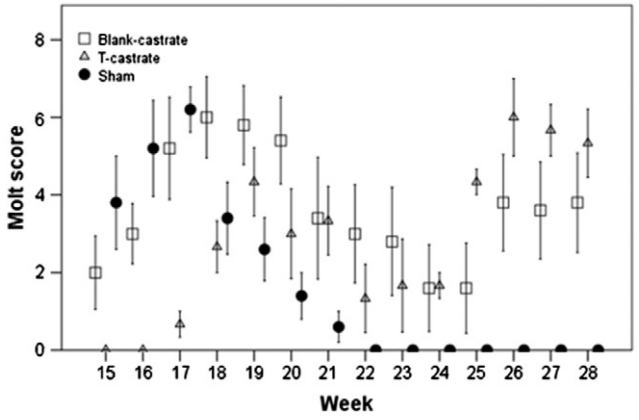

Fig. 6. Weekly measures of prenuptial molt throughout the post-implant period starting with its first appearance in week 15 and continuing through week 28 in Shams, Blank-castrates, and T-castrates. Complete descriptions listed in Fig. 1.

Discussion

In general, our results are not consistent with the idea that a transient pulse of testosterone limited to a two week period between the winter solstice and the spring equinox is sufficient to actuate the full suite of migratory traits. Whereas a pulse of testosterone temporarily increased CPL and muscle hypertrophy, it failed to affect a continuous prenuptial molt and migratory restlessness. Thus, it is possible that 1) the transient pulse of testosterone is necessary but not sufficient for full development of the migratory phenotype, but that the testosterone pulse only primes central functions for further actions of both photoperiod and reproductive hormones that are secreted later in the spring or 2) the transient pulse of testosterone is neither sufficient nor necessary for the full migration phenotype and that continuous uninterrupted secretions from the gonad might be critical for the full expression to develop or 3) that timing of the organizational effects of T occurs earlier in the fall as suggested by Boswell et al. (1993) and Thapliyal et al. (1983). Previous studies have identified autumnal androgen in European quail (*Coturnix coturnix coturnix*), white-throated sparrows (*Zonotrichia albicollis*) and, Rouen duck (*Anas paltyrhynchos*) (Balthazart, 1983; Balthazart and Hendrick, 1976; Boswell et al., 1993, 1995a; Schlinger, 1987) and more recently in field studies on GWCS (M. Ramenofsky, Z. Németh, J. Krause, unpublished data). This seasonally earlier pulse could override the effects of our castrations in December. A possible source of autumnal androgen may be the gonad and/or adrenal gland (Balthazart, 1983; Boswell et al., 1995a). The mode of regulation is not well understood but suggestions have pointed to development of photosensitivity in late autumn as well as increased activity of the 3β-hydroxysteroid dehydrogenase converting dehydroepiandrosterone (DHEA) to androstenedione and testosterone in the adrenal gland (Balthazart and Hendrick, 1976; Boswell et al., 1995a; Matt, 1982; Schmidt et al., 2008). It is also possible that synthesis and effect of androgen may take place centrally without changes in the peripheral circulation according to Schlinger et al. (2001) and Soma et al. (1999). This could be tested with autumnal gonadectomies followed by antiandrogen therapy then recording of fattening and migratory restlessness thus helping to resolve the species distinctions. Thus, regulation of the spring events remains within the realm of photoperiodic and gonadal control; however, timing and location of the organizational cues exerted by androgen remain uncertain.

Effects of castration on short day lengths

In support of the prediction for the pre-implant period, minimal nocturnal locomotor activity was observed in all birds. However, castration at this juncture increased diurnal activity, as noted previously in domestic fowl, Japanese quail, white-throated sparrows, GWCS, and golden-crowned sparrows (*Zonotrichia atricapilla*) (Boswell et al., 1993; Morton and Mewaldt, 1962; Weise, 1967). Such findings have been correlated with elevated body temperature measured during the day in castrated Japanese quail over intact controls (Feuerbacher and Prinzinger, 1981; Hanssler and Prinzinger, 1979). Photostimulation and/or testosterone administration reversed both trends in the castrated domestic fowl and Japanese quail bringing activity commensurate with intact subjects (Feuerbacher and Prinzinger, 1981; Snapir et al., 1974). Thus, it is possible that the increase in daytime activity observed in the castrated GWCS may have contributed to or be influenced by elevated body temperature or changes in energy balance during the day. Even though plasma levels of androgen are basal at this time, the gonad or central production of androgen could exert effects on body

temperature or endogenous timing mechanisms influencing daily locomotor rhythms as observed in other migratory species (Gupta and Kumar, 2013; Gwinner, 1996; Rani et al., 2006). The causal relationships are not known but certainly of interest for migratory species throughout the wintering stage.

Short-term effects of T-replacement

Administration of T over the 16-day implant period when increasing day lengths were still less than 12L:12D achieved breeding levels of androgen overshadowing those of the Shams and Blank-castrates. As predicted, this had little effect on either daytime or nighttime activity, which is counter to results others have observed when T is administered along with continuous long photoperiods and suggests that exposure to androgen on short day lengths does not affect migratory restlessness. By contrast, when breeding levels of T are delivered to castrated GWCS, Japanese quail and dark-eyed Juncos under long continuous photoperiods, both reproductive and migratory functions are observed (Tonra et al., 2011; Wada, 1982; Wikelski et al., 1999). This highlights an interaction of photoperiod and androgen but does not elucidate the incremental progression of the spring events.

The elevated daytime activity observed previously in all Castrates over the Shams was no longer apparent during the implant period suggesting that increasing day lengths may have had a dampening effect on locomotor function or temperature regulation, the nature of which is uncertain. Although body mass and fat score in GWCS did not appear to be significantly affected by T administration, anabolic changes were apparent in flight muscle profile that enlarged within the first week of hormone implant but declined once the implants were removed. Androgen promotes protein synthesis in skeletal muscle through genomic pathways and signaling molecules that include protein kinase B (Akt), myostatin, insulin growth factor (IGF-I), and Notch, as well as non-genomic events that increase Ca^{++} uptake (Buas and Kadesch, 2010; Dubois et al., 2012). With muscle enlargement it is surprising that group distinctions in body mass were not observed; however, all groups had increasing mass and fat scores during this period, which was probably due to the incremental increase in day length (week) and/or conditions of captivity. By contrast, when T is administered to reproductive male song sparrows (*Melospiza melodia*), dark-eyed Junco males and domestic fowl (Lynn et al., 2000; Ketterson et al., 1991; Robinzon et al., 1987; Wingfield, 1984), body mass and fat scores were either diminished or unchanged suggesting a differential effect of androgen during the breeding stage. Furthermore, CPL was significantly lengthened with T administration as expected for an androgen-dependent organ. Therefore, in our hands, the two-week administration of T on natural day lengths in February induced flight muscle hypertrophy and breeding morphology all of which were reversed once androgen levels declined with implant removal. These results indicate the effectiveness of the treatment but appeared to induce more reproductive traits rather than those of migration.

Long-term effects of castration and temporary T-replacement

As expected, plasma levels of androgen among the T-castrates descended to values of the Shams once the implants were removed in the post-implant period. For the months of March and April (weeks 15, 20) (Figs. 4A, B), all groups had low plasma values that matched the level of detection of both androgen assays (<0.25 ng/ml) and were commensurate with those recorded for birds in the field at the time of spring departure (Wingfield and Farner, 1978) (M. Ramenofsky, Z. Németh, J. Krause, unpublished data). By week 24–May 20, the plasma levels of DHT and T were elevated in the Shams as well as their CPL (Fig. 5), which had increased significantly to 7–8 mm in length typical of territorial birds initiating breeding (Wingfield and Farner, 1978).

Nocturnal locomotor activity of all birds was variable and did not differ across groups until the appearance of a prominent elevation in Shams that occurred around week 20 (April 20), a time in the field when birds were departing the wintering grounds (Blanchard and Erickson, 1949; Wingfield and Farner, 1978) (M. Ramenofsky, Z. Németh, J. Krause, unpublished data) (Fig. 3B). Although not as evident, both castrate groups appeared to increase nocturnal activity but the intensity fell well below the Shams as reported by Gupta and Kumar (2013) and without an apparent diel pattern. The lower intensity of nocturnal activity suggested that this may not represent migratory restlessness. As a test, we applied the two behavioral criteria established for GWCS (Agatsuma and Ramenofsky, 2006; Coverdill et al., 2011). Both castrate groups showed neither a quiescent period at the conclusion of the photophase nor heightened activity at night suggesting that migratory restlessness was evident only in Shams (Fig. 7).

It is possible that our small sample sizes resulting in low statistical power could have contributed to our inability to detect migratory restlessness in the T-castrate group in the post-implant period, a time when we expected otherwise. However given that we employed multiple qualitative and quantitative criteria based on Agatsuma and Ramenofsky (2006) to determine migratory restlessness and none applied to either castrate groups, we feel our results suggest alternate explanations for a role of androgens in vernal migration.

At this juncture, our findings offer little support for the winter pulse of T acting to potentiate migratory restlessness once photoperiod extends beyond 12L:12D. However, there are indications that T may have a more immediate effect since administration of breeding levels of T to American redstarts (*Setophaga ruticilla*) correlated with early departure from the wintering grounds (Tonra et al., 2013). In our study, the 5 incomplete castrated subjects "regenerates" dropped from the analyses displayed earlier onset of nighttime activity with intensity commensurate with the Shams (data not shown) demonstrating a contribution of the gonad to the expression of migratory restlessness. Also spring departure for free-living GWCS occurs when plasma androgens are at or below detectability of our assay suggesting that elevated levels of plasma T may not be the regulatory component. Rather the gonad or other non-gonadal sources of androgen may be the sites of regulation for migratory flight behavior.

Given that an intact gonad is necessary for the complete behavioral repertoire in captive birds; the mechanisms may differ across traits. Previous studies using electrolytic lesions in GWCS and white-throated sparrows identified distinct neuroendocrine pathways linking the posterior median eminence of hypothalamus and anterior pituitary that inhibited both fattening, migratory restlessness and gonadal development (Kuenzel and Helms, 1967; Yokoyama et al., 1978). Administering testosterone reestablished fattening but had no effect on migratory restlessness. Lesions of the ventromedial hypothalamus (VMH) however

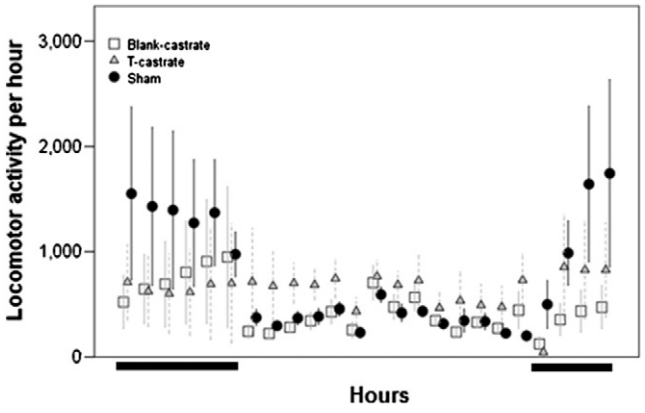

Fig. 7. Exemplar of the 24 h locomotor activity (average electronic beam break/h) measured in the three groups during week 21 illustrating expression of the Quiescent Phase and migratory restlessness apparent only in the Shams in comparison with both castrated groups that show neither. Each symbol represents a mean ± SEM and black-filled horizontal bars designate the scotophase.

selectively inhibited migratory restlessness (Kuenzel, 1964). Taken together these results suggest independent regulation of fattening and migratory restlessness since in the field fueling surely must be completed before birds initiate migratory flight.

Testosterone administration has been shown to delay onset and extend postnuptial molt in a variety of species (Boswell et al., 1993; Payne, 1972; Schleussner et al., 1985; Schwabl and Farner, 1989b). By contrast, androgen is known to induce bright prenuptial plumage of males in a limited number of species (Peters et al., 2000; Willow et al., 2009; Witschi, 1961). However, very few studies have investigated the effects of castration on prenuptial molt in migrants. Observations of prenuptial molt in Shams suggest that birds began to molt around week 12 and completed by week 22 coincident with free-living birds at our study site in Yolo County, CA (Fig. 6). Blank-castrates appeared to initiate molt at approximately the same time as Shams but T-castrates were delayed by approximately 4 to 5 weeks, which may have been inhibited by the declining levels of androgen following implant removal. Both castrate groups however decreased intensity of molt around weeks 24–25 only to commence again for another 3 weeks, that is 7 weeks after the Shams had finished thus prolonging the molt for both groups. The mechanisms for this are not clear but emphasize roles for both photoperiod and the gonad in the timely progression of one of the major phenotypic stages for vernal migration. Considering the delay in the completion of molt may offer an explanation for the lack of the complete expression of migratory restlessness in both castrate groups. Such findings could suggest a sequential linkage in terms of the necessity of molt completion before departure ensues from the wintering grounds. Furthermore, prenuptial replacement of body or contour feathers increases both energy and nutritional requirements and is nearly completed by the time birds depart for vernal migration allowing for temporal separation of these two mutually exclusive life history stages for GWCS (Kendeigh, 1949; King, 1980; King and Murphy, 1985).

Over the final 8 weeks of the study, energetic condition of the T-castrates declined in comparison with the other 2 groups (Fig. 8). Such changes in morphology suggest a precipitous conclusion of the migratory stage, which in captive migrants represents refractoriness that is more commonly associated with termination of the breeding stage when reproductive behavior and gonadal activities dissipate (Gwinner and Czeschlik, 1977; Schwabl and Farner, 1989b). Previous studies on GWCS indicated that castration may induce early onset of refractoriness (Wingfield et al., 1980) but without expression of true MR in our castrates, such conclusions are tentative. It is possible that the pulse of winter T in castrates altered the mechanisms that regulate the timing of the vernal events, which reiterates the roles for both photoperiod and gonadal development with the normal progression of the spring phenotype in migrants.

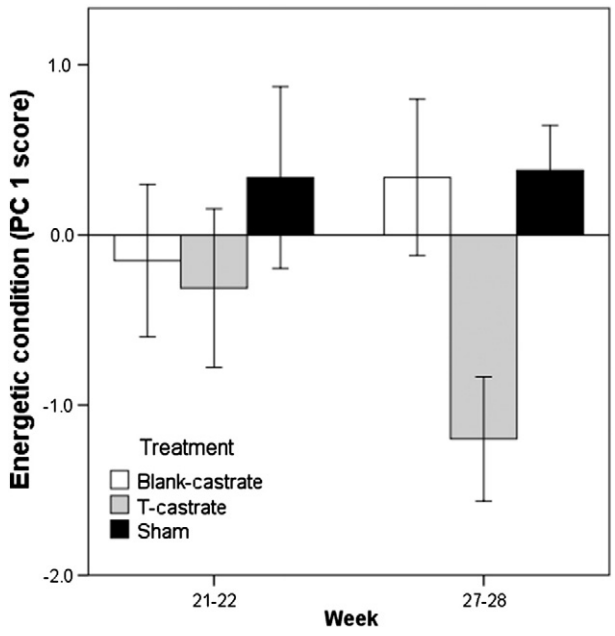

Fig. 8. Energetic condition measured at two time points: peak migration (weeks 21 & 22) and end of the experiment (weeks 27 & 28). Peak migration was identified as the middle two of the 4 week period that (1) Shams showed migratory restlessness and (2) local field populations of GWCS were migrating (M. Ramenofsky and Z. Németh, unpublished data). Bars represent mean ± SEM.

Conclusions

Herein we investigated the mechanisms regulating the behavioral and physiological traits of vernal migration in relation to the incremental increase in natural photoperiod and the neuroendocrine system. Our results failed to fully support our hypothesis that gonadal activity in terms of a winter pulse of testosterone contributes to the spring migratory phenotype once the stimulatory day lengths of spring are reached since both castrated groups increased their energetic condition but failed to express both timely and continuous prenuptial molt and migratory restlessness. Combining our results with previous studies (Kuenzel, 1964; Kuenzel and Helms, 1967; Stetson, 1971; Yokoyama, 1976) suggest that fueling in terms of fattening, mass and muscle hypertrophy may be regulated independently from expression of migratory restlessness since in the field fueling surely must be completed before birds initiate migratory flight. Thus, we emphasize that attention to the multiple characteristics or traits allows for a full interpretation of the preparation, expression and termination of the vernal migration stage. The gonadal activity in spring migrants has at least a two-fold effect, one acting possibly in autumn to establish hyperphagic patterns of food intake that can be expressed once spring day lengths are achieved. Two, in conjunction with daily increased photoperiod, gonadal activity mediates in some manner the timely progression of events culminating in departure with completed molt, sufficient fuel and muscle power for successful flight. Upon arrival at destination, steroidogenic and gametogenic activities of the gonad are fully achieved so that breeding may ensue.

Applying these results from captive studies on GWCS to migratory species as a whole, our findings emphasize the reliance of the gonad and natural photoperiod during the spring stage of migration, where a functioning gonad is necessary once birds arrive on the breeding grounds. By contrast, during autumn migration many of the features of molt, fueling, fattening, muscle hypertrophy and migratory restlessness are similar to those of vernal stage. Though speculative, it is hypothesized that gonadal activity may not play a major role during the autumn stage given the lack of necessity of complete gonadal function once birds reach the wintering grounds.

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