



Concurrent hypothalamic gene expression under acute and chronic long days: Implications for initiation and maintenance of photoperiodic response in migratory songbirds



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ABSTRACT

Hypothalamic expression of the thyroid hormone (TH) responsive gonadostimulatory (*eya3*, *cga*, *tshβ*, *dio2*, *dio3*, *gnrh*, *gnih*) and neurosteroid pathway genes (*androgen receptor [ar]*, *aromatase [cyp19]*, *estrogen receptor [er] α* and *β*) was examined in photosensitive redheaded buntings exposed to 2 (acute, experiment 1) or 12 (chronic, experiment 2) long days (16L:8D). Experiment 2 also included a photorefractory group. Acute long days caused a significant increase in *eya3*, *cga*, *tshβ*, *dio2* and *gnrh* and decrease in *dio3* mRNA levels. *eya3*, *cga* and *tshβ* expressions were unchanged after the chronic long days. We also found increased *cyp19*, *erα* and *erβ* mRNA levels after acute, and increased *cyp19* and decreased *erβ* levels after the chronic long-day exposure. Photorefractory buntings showed expression patterns similar to that in the photosensitive state, except for high *gnrh* and *gnih* and low *dio3* mRNA levels. Consistent with gene expression patterns, there were changes in fat deposition, body mass, testis size, and plasma levels of testosterone, tri-iodothyronine and thyroxine. These results show concurrent photostimulation of the TH-signalling and neurosteroid pathways, and extend the idea, based on differences in gene expression, that transitions in seasonal photoperiodic states are accomplished at the transcriptional levels in absolute photorefractory species.

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1. Introduction

In many vertebrates, day length (or photoperiod) regulates seasonal changes in reproductive phenotypes (Cassone and Yoshimura, 2015; Kumar et al., 2010). Following photostimulated gonadal growth phase, many birds undergo a period of photorefractoriness during which they cease to respond to the stimulatory effects of long days and exhibit gonadal regression (Dawson et al., 2001; Malik et al., 2014; Nicholls et al., 1988). These photorefractory birds remain in gonadally regressed state under long days, i.e. they become absolute photorefractory (Malik et al., 2014; Misra et al., 2004; Nicholls et al., 1988; Sansum and King, 1976). Some birds show partial (relative) photorefractoriness in the sense that their photoperiodic

responsiveness is reduced, not entirely lost, and gonadal maturation can be renewed in regressed individuals under a longer photoperiod (Dawson, 2015; Hahn et al., 2004). In contrast, many photoperiodic mammals exhibit spontaneous gonadal recrudescence under inhibitory short photoperiods to which they were initially refractory (Nicholls et al., 1988).

In birds, concomitant photoperiod-induced changes are found in the thyroid and steroid hormones (Goodson et al., 2005; Ramenofsky, 2011). Thyroid hormones (TH) are involved in the long-day photostimulation of GnRH response (Cassone and Yoshimura, 2015; Nicholls et al., 1988), and elevated circulating testosterone (T) levels are associated with the socio-sexual behaviours during reproductive state in many birds (Ball and Balthazart, 2002; Goodson et al., 2005). Both neural and peripheral steroids control changes associated with the gonadal growth-regression cycle, namely sexual behavior, aggression, territoriality, vocalization and other reproductive responses in birds, although with species, season and brain region-specific variations (Goodson et al., 2005; Ramenofsky, 2011). For example, brain androgen

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receptor and/or aromatase enzyme (coded by *cyp19* gene) activity regulates aggression during the non-breeding season (low T levels) in song sparrows (*Melospiza melodia*; Soma et al., 2000) and tropical spotted antbirds (*Hylophylax naevioides*; Hau et al., 2000). Further, the aromatase mRNA in preoptic area and ventromedial hypothalamus was found significantly higher and lower during the breeding and moulting states, respectively, in male song sparrows (Wacker et al., 2010). These differences are attributed to differences in the production, conversion to estrogenic metabolites, and/or dynamics of the androgen/estrogen receptor sensitivity (Canoine et al., 2007; Hau et al., 2000; Soma et al., 2000). In general, T-induced brain effects are produced when it binds directly to androgen receptor (ar) or, after conversion to 17 β -estradiol by aromatase enzyme to estrogen receptor alpha and beta (ers, Canoine et al., 2007; Goodson et al., 2005).

It is suggested that a highly conserved TH-responsive molecular mechanism mediates the photostimulation of gonadal growth and development. It begins with concurrent activation of the *eya3* (*eye absent 3*) and *tsh β* (thyroid stimulating hormone-beta) genes in pars tuberalis (PT) thyrotrophs (Cassone and Yoshimura, 2015; Nakane and Yoshimura, 2014; Majumdar et al., 2014; Nakao et al., 2008; Surbhi and Kumar, 2014). TSH (thyroid stimulating hormone) released from PT thyrotrophs activates and suppresses transcription of the genes coding for type 2 and 3 deiodinases (*dio2* and *dio3*), respectively, in tanyocytes. Dio2 and Dio3 mediate conversion of thyroxine (T4) into biologically active T3 (tri-iodothyronine) and inactive rT3 (reverse T3) forms, respectively. T3 regulates GnRH (gonadotropin releasing hormone) synthesis and/or release from the preoptic area into median eminence, and consequently the pituitary gonadotropins secretion (Cassone and Yoshimura, 2015; Nakane and Yoshimura, 2014). A role of hypothalamic GnIH (gonadotropin inhibiting hormone) in controlling GnRH activity has also been suggested in house sparrows, *Passer domesticus*, and European starlings, *Sturnus vulgaris* (Bentley et al., 2003; Ubuka et al., 2008). Increased *gnih* mRNA levels in hypothalamic explants from short days, compared to long days, paralleled significantly reduced plasma LH levels and gonadal size in Japanese quails, *Coturnix c. japonica* (Chowdhury et al., 2010). Possibly mediated through changes in brain estrogens, GnIH is involved in the regulation of reproduction-associated behavior, (Ubuka et al., 2014). GnIH may inhibit gonadotropins synthesis and release by also acting directly on the pituitary gland (Ubuka et al., 2016).

However, it is largely unknown whether photostimulation of TH-signalling is linked with the neurosteroid pathway in a seasonally breeding species. Also, less is understood about the molecular processes underlying photo-maintenance of the gonadal response. We hypothesized that photostimulation would cause concurrent hypothalamic expressions of TH-responsive gonadostimulatory and neurosteroid pathway genes, and that these expressions would vary with photostimulation stages during initial weeks of the long-day exposure. To test this, we measured hypothalamic expressions of TH-responsive gonadostimulatory (*eya3*, *cga*, *tsh β* , *dio2*, *dio3*, *gnrh*, and *gnih*) and neurosteroid (*ar*, *cyp19*, *era* and *er β*) pathway genes, along with changes in the fat depots, body mass, testis size and plasma T3, T4 and T levels in male redheaded buntings (*Emberiza bruniceps*) exposed to short and long days. Buntings rapidly respond to long days, and they become absolute photorefractory following the gonadal growth and development (Majumdar et al., 2014, 2015; Malik et al., 2014; Rani et al., 2005). We predicted concurrent hypothalamic expressions of the gonadostimulatory and neurosteroid pathway genes, but with changes in expression patterns with the transition from sensitive to refractory photoperiodic state.

2. Materials and methods

2.1. Animals and maintenance

Housing, feeding, and overall care and maintenance of adult male redheaded buntings (*E. bruniceps*) are described in our earlier publications (Singh et al., 2010). Briefly, birds were captured from the overwintering flock in late February using mist nets, transported to the laboratory in temporary caging (60 \times 45 \times 45 cm, n = 4 birds/cage) and released into an outdoor aviary (size = 3 \times 2.5 \times 2.5 m) under natural day light (NDL), temperature and humidity conditions. During a week of acclimation under NDL with daylight period from sunrise to sunset of about 11.5 h/day, birds were given tetracycline (an antibiotic) and a multivitamin (Vimeral containing vitamin A, D₃, E, and B₁₂, marketed by Virbac Animal Health India Pvt. Ltd, Mumbai) in water for five days. At this time, buntings were unstimulated, with no fat deposition and with small, reproductively immature testes.

Acclimated birds were brought indoors and housed in an aviary (2.2 \times 1.8 \times 2.8 m) with 8 h light per day (8 h light: 16 h darkness, 8L:16D, SD; L = 300 \pm 5 lux, D = 0 lux) generated by compact fluorescent lamps (CFL, Phillips, 220–240 V) and maintained at 22 \pm 2 $^{\circ}$ C until use in experiments. Despite a decrease in the daily light period to 8L, buntings maintained physiological state equivalence to the time when they were caught in February. Under short days, buntings remain in the photosensitive unstimulated state, but under long days they spontaneously become insensitive to photostimulation following the photostimulated growth phase and maintain the regressed gonadal state, as evidence by repeated laparotomy (Malik et al., 2014; Rani et al., 2005; our unpublished obs.). All through the experiment, birds were fed with seeds of *Setaria italica*, and both food and water were given *ad libitum*.

2.2. Experiment

Experiments were performed on adult male redheaded buntings (*Emberiza bruniceps*), as per guidelines of the Institutional Animal Ethics Committee, and used photosensitive birds that have been maintained under short days (8L:16D). With such photosensitive birds, the experiments were carried out at the time of year when redheaded buntings in the wild are responsive to the photostimulation (November to May) to preclude any possible effect of the annual rhythms, if any, on photoperiodic responsiveness. Experiment 1 tested acute long-day effects (Fig. 1). Beginning in late April, 2 groups of short-day maintained photosensitive buntings (n = 5 each) with small testes (testis volume, TV = 0.33–0.52 mm³) were housed in cages (size = 46 \times 38 \times 36 cm; n = 2 or 3 per cage) and placed inside the photoperiodic boxes (size = 75 \times 75 \times 60 cm) programmed to provide short days, as before. After a week, one group remained on short days (SD sensitive, SDS), but the other group was exposed to long days for 2 days by extending the light off time by 8 h (16L: 8D, LD stimulatory, 2LDS). On day 3, both SDS and 2LDS groups were sampled during middle of the long photoperiod (zeitgeber time, ZT, 8; ZT 0 = light onset; Fig. 1).

Experiment 2 tested the chronic long-day effects, and used both photosensitive and photorefractory buntings. The experiment began in December when buntings in the wild are in sensitive photoperiodic states. At this time, both photosensitive and photorefractory birds had been exposed to short and long days, respectively, for about 40 weeks. Two groups of photosensitive birds (SDS, n = 4 each) were placed in separate photoperiodic boxes and received short days, as above. After a week, one group remained on short days (SDS), but the other group was exposed to long days (16L:8D) for 12 days (12LDS). Simultaneously, a group of photorefractory buntings under long days (LDR, n = 4) was placed in a

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