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RESEARCH ARTICLE

Ambient temperature affects multiple drivers of physiology and behaviour: adaptation for timely departure of obligate spring migrants

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ABSTRACT

We investigated the role of ambient temperature in departure from wintering areas of migratory black-headed buntings in spring. Birds transferred at 22 and 35°C to long days were compared with one another and with controls held on short days for indices of readiness to migrate (Zugunruhe, fattening, mass gain), levels of testosterone and gonadal recrudescence. Temperature affected the development of migratory behaviour and physiology: buntings under long days at 35°C, compared with those at 22°C, showed altered migratory behaviour (daily activity and Zugunruhe onset), and enhanced muscle growth and plasma testosterone levels, but showed no effect on testis growth. Temperature was perceived at both peripheral and central levels, and affected multiple molecular drivers culminating into the migratory phenotype. This was evidenced by post-mortem comparison of the expression of 13 genes with known functions in the skin (temperature-sensitive TRP channels: *trpv4* and *trpm8*), hypothalamus and/or midbrain (migration-linked genes: *th*, *ddc*, *adcyap1* and *vps13a*) and flight muscles (muscle growth associated genes: *ar*, *srd5a3*, *pvalb*, *mtor*, *myod*, *mstn* and *hif1a*). In photostimulated birds, the expression of *trpv4* in skin, *th* in the hypothalamus and midbrain, and *srd5a3*, *ar, pvalb* and *mtor* in flight muscle, in parallel with testosterone levels, was greater at 35°C than at 22°C. These results demonstrate the role of ambient temperature in development of the spring migration phenotype, and suggest that transcriptional responsiveness to temperature is a component of the overall adaptive strategy in latitudinal songbird migrants for timely departure from wintering areas in spring.

KEY WORDS: Gene expression, Hypothalamus, Muscle, Photoperiod, Skin, *Emberiza melanocephala*

INTRODUCTION

The pervasiveness of the ability to assess and use annual photoperiodic cycle in regulation of annual life-history states (LHSs) has been identified as a central component of the metabolic and reproductive fitness across broad taxa including birds (Bradshaw and Holzapfel, 2007; Cassone and Yoshimura, 2015). Photoperiod change is closely accompanied with temperature change both during the 24-h day as well as across seasons of the year. Daytime is warmer than the nighttime, and the spring/summer period is warmer than the autumn/winter period. Increasing evidence has shown that photoperiodic birds use temperature in the development of robust system-level behaviour associated with seasonal migration and reproduction, with species and sex variations (Berchtold et al., 2017; Helm et al., 2017; Sur et al., 2019; Trivedi et al., 2019). For example, high temperature advanced regression, but not recrudescence, of the testes in photostimulated mountain white-crowned sparrows (*Zonotrichia leucophrys oriantha*; Wingfield et al., 2003), European starlings (*Sturnus vulgaris*; Dawson, 2005) and great tits (*Parus major*; Silverin et al., 2008). A recent study using within-individual experimental approach further showed that warmer conditions in climate-controlled aviaries advanced egg-laying timing in great tits (Verhagen et al., 2020). By contrast, photostimulated gonadal maturation in migratory white-crowned sparrows (*Z. l. gambelli*) was resistant to temperature extremes, except a negative temperature effect in female, but not male, sparrows (Wingfield et al., 1996).

The latitudinal avian migrants present an elegant example of biological adaptation to both photoperiod and temperature (Helm et al., 2017). They are exposed to widely different photoperiods and temperatures between breeding and wintering areas, and to consistently varying photoperiods and temperatures while migrating across latitudes twice a year (Karagicheva et al., 2016, 2018). Experiments have shown that ambient temperature affected the photoperiodic induction of body fattening and mass gain, Zugunruhe (nocturnal migratory restlessness in captive birds; Wagner, 1930; Berthold and Querner, 1988), and gonadal maturation in migratory species (Ramenofsky and Wingfield, 2007; Kumar et al., 2010). For example, reduced body fattening and mass gain, delayed Zugunruhe onset, and slowed testis maturation was shown in migratory black-headed buntings (*Emberiza melanocephala*) exposed to stimulatory long days at 40°C compared with those at 27°C (Singh et al., 2012). Similarly, photostimulated white-throated sparrows (*Zonotrichia albicollis*) showed increased intensity of Zugunruhe at 14°C, compared with 24°C (Berchtold et al., 2017). In addition, previous studies showed reduced tissue-specific fat deposition and overall mass gain, and accelerated testis growth in migratory red-headed buntings (*Emberiza bruniceps*) exposed to long day at 38°C compared with those at 22°C (Sur et al., 2019; Trivedi et al., 2019).

Studies from the field support the results obtained from captive birds subjected to experimental manipulations. In both great tits and blue tits (*Cyanistes caeruleus*), for example, the ambient temperature influenced egg-laying dates through its effects on the availability of insects as food (Visser et al., 2009). Similarly, decades-long observations showed advancement of the egg-laying dates in migratory pied flycatchers (*Ficedula hypoleuca*; Both and Visser,

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2001). Similarly, a number of field studies over the years have reported an early arrival to the breeding ground of many migratory bird species, perhaps in response to global warming (Tryjanowski et al., 2002; Jonzén et al., 2006; Palm et al., 2009; Kullberg et al., 2015). Further, the phylogenetic meta-analysis of 413 bird species concluded the advancement of spring departure dates by 1.2 days per 1°C rise in ambient temperature (Usui et al., 2017).

Although many studies have shown the influence of temperature in the development of migratory behaviour, it remains inconclusive whether temperature is a direct causal factor or whether it acts by affecting the energetic cost and food availability (Newton, 2008). There is also some debate as to whether an early expression of migratory behaviour in response to warm temperature in captivity consistently relates to the early arrival at the breeding grounds as reported by many field studies. Much of the experimental evidence is consistent with idea that migratory birds use temperature and respond to it independent of the other cues, such as the photoperiod (Berchtold et al., 2017).

We hypothesized that migratory songbirds use ambient temperature in behavioural decisions of when to depart from the wintering areas in spring. The prediction was that a warmer temperature prior to the onset of spring migration would accelerate the underlying molecular processes, and consequently increase the probability and intensity of Zugunruhe; however, a sudden high rise in temperature might activate thermoregulation and, in turn, adversely affect migratory behaviour. To test this hypothesis, we found an ideal experimental system in migratory black-headed buntings. The bunting is an obligate Palearctic–Indian songbird migrant with predictable yearly migrations between same breeding (west Asia and southeast Europe) and wintering (mainly India) areas (Ali and Ripley, 1999). It arrives in India during October/November, overwinters, and begin to return to breeding grounds (spring or vernal migration) during very late in March or early April, when the length of the natural light period (sunrise to sunset) is ∼12.5 h and the average ambient daytime temperature is ∼35°C (Ali and Ripley, 1999). Both the development of spring migratory phenotype (body fattening and mass gain, and Zugunruhe) and departure timing can be reproduced if black-headed buntings are held in captivity under semi-natural photoperiod and temperature conditions, and so they were disallowed from migrating (Gupta and Kumar, 2013). Similar photostimulated spring migratory and breeding (testicular growth) states are induced in buntings exposed to artificial stimulatory photoperiod (Misra et al., 2004; Rani et al., 2006; Singh et al., 2015), but ambient temperature affects both timing and magnitude of the photoperiodic response (Singh et al., 2012).

Here, black-headed buntings transferred from short days to long days at 22 and 35°C were compared with one another and with controls held at both these temperatures on short days for the indices of readiness to migrate (Zugunruhe, fattening, mass gain), levels of testosterone and gonadal recrudescence. Post-mortem, they were compared for the expression of 13 candidate genes in the skin (peripheral temperature perception), hypothalamus and/or midbrain (the regulatory site of photoneuroendocrine effects), and flight (skeletal) muscles (the site of intense aerobic exercise when in flight). These genes with known functions were chosen based *a priori* on three postulations that we made at the beginning of the study.

First, ambient temperature is perceived at both peripheral (skin) and central (hypothalamus) levels. This involves member genes of the subfamilies V and M of the transient receptor potential (TRP) cation channels, which are sensitive to warm (∼27–37°C; *trpv4* gene) and cold (∼8–25°C; *trpm8* gene) temperatures (Bautista et al., 2007; Wang and Siemens, 2015). TRP channels of skin keratinocytes sense the temperature, and convey it to the brain via sensory neurons (Yang et al., 2017). Hypothalamic TRP channels also play a key role in the temperature perception (Bautista et al., 2007; Wang and Siemens, 2015), with thermosensitive neuron clusters (30% warm temperature sensitive, 5% cold temperature sensitive) housed in the pre-optic area (Boulant, 2000). Consistent with this, differential *trpm8* expression was found in the hypothalamus of photostimulated redheaded buntings exposed to two temperatures 16°C apart (Trivedi et al., 2019).

Second, ambient temperature influences the expression of migration-linked genes, namely the adenylate cyclase activating polypeptide 1 (*adcyap1*) and vacuolar protein sorting 13a (*vps13a*) genes, and genes coding for enzymes of the dopamine biosynthesis pathway (the rate limiting tyrosine hydroxylase, *th*; and dopamine decarboxylase, *ddc*; Daubner et al., 2011). The size of *adcyap1* alleles was found to be associated with Zugunruhe in migratory European blackcaps (*Sylvia atricapilla*; Mueller et al., 2011), but not in migratory juncos (*Junco hyemalis*: Peterson et al., 2013). Similarly, a single nucleotide polymorphism of the *vps13a* gene was found to be associated with migration directionality of very close genetically related *Vermivora* warbler species with wintering sites located in Central America (golden-winged warbler, *V. chrysoptera*) and South America (blue-winged warbler, *V. cyanoptera*; Toews et al., 2019). Further, the departure from the wintering area is closely related to the timely arrival at breeding grounds of migrating birds; males, in particular, tend to have a stronger drive (sexual motivation) during the spring migration (Tryjanowski and Yosef, 2002). A role of dopamine was, therefore, envisaged in the spring migration of black-headed buntings, as suggested by increased hypothalamic *th* expression in captive red-headed buntings during photostimulated spring migratory, compared with the autumn migratory state (Sharma et al., 2018). Likewise, the association of the *drd4* gene (coding for the dopamine receptor D4) with exploratory behaviour found in great tits suggested a role of the dopaminergic pathway in the overall migratory movements (Fidler et al., 2007). Furthermore, temporally spaced daily injections of L-DOPA (L-dihydroxyphenylalanine, a dopamine precursor) and 5-HTP (5 hydroxytryptophan, a serotonin precursor) also mimicked photostimulated gonadal response in red-headed buntings (Bhatt and Chaturvedi, 1992).

Third, ambient temperature affects extensive physiological changes that occur in the flight muscle in anticipation of the migratory travel. In the pre-migratory period, the pectoralis muscles grow in size in many migratory birds including dunlins, *Calidris alpina*, sanderlings, *Calidris alba*, semi-palmated sandpipers, *Calidris pusilla*, red knots, *Calidris canutus canutus*, and lesser knots, *Calidris canutus rogersi* (Battley and Piersma, 1997; Dietz et al., 1999; Driedzic et al., 1993; Evans et al., 1992). In red knots, there was a 2-fold seasonal variation in the ratio of upstroke to downstroke flight muscles, suggesting an adaptation for savings on overall energy expenditure by reduced escape flight ability when the birds have lower body (pectoral muscle) mass (Piersma and Dietz, 2007; Piersma et al., 2007). Therefore, as an anatomic measure of the muscle growth, we measured the width of flight muscle fibres of black-headed buntings exhibiting photostimulated Zugunruhe. To assess further whether photostimulated muscle hypertrophy was the result of molecular remodelling (Pradhan et al., 2019), we measured muscular expression of seven genes including testosterone-signalling (T-signalling) pathway genes, and those involved in the muscle growth and metabolism (*ar*, *srd5a3*, *pvalb*, *myod*, *mstn*, *mtor* and *hif1a*). Both T-signalling pathway genes (*ar* and *srd5a3*) showed a significantly increased expression during the

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pre-migratory period in hypertrophied pectoralis muscle of the white-crowned sparrows (Pradhan et al., 2019). The *srd5a3* gene encodes for steroid 5alpha-reductase, mediating the conversion of testosterone into 5α-DHT (5α-dihydrotestosterone), which binds to androgen receptors (ARs) (Fuxjager et al., 2016) and influences muscle growth (Dubois et al., 2014; Morton et al., 2018). Testosterone-induced activation of ARs in skeletal muscles involves *pvalb* (parvalbumin) and *igf1* (insulin growth factor 1) genes, as reported by a study in golden-collared manakins (*Manacus vitellinus*; Fuxjager et al., 2012). Similarly, the expression of the *mstn* (myostatin) gene was increased in photostimulated captive, but not wild, white-throated sparrows (Price et al., 2011). In photostimulated migratory red-headed buntings, temperature affected the expression of myogenic differentiation 1 (*myod1*), which acts as a prime regulator of the muscle growth and metabolism (Sur et al., 2019). Importantly, intensively exercising flight muscles require excessive oxygen while migrants are in flight. Therefore, we also measured the expression of genes coding for mechanistic target of the rapamycin kinase (*mtor*) and hypoxia inducible factor 1 (*hif1a*), which are crucial enzymes for the myocellular adaptation of skeletal muscles to both exercise and hypoxia by maintaining the oxygen delivery and augmented oxygen diffusion capacity (Scott, 2011; Song et al., 2017).

MATERIALS AND METHODS

Animals and maintenance

This study was carried out as per approval of the Institutional Animal Ethics Committee of University of Lucknow, India (protocol no. LU/ZOOL/IAEC/5/16/01/2A). Adult male black-headed buntings (*Emberiza melanocephala* Scopoli 1769) were procured from overwintering flock in late February, and acclimated for 1 week to captive conditions in an outdoor aviary (size=3×2.5×2.5 m) under semi-natural conditions (photoperiod, sunrise to sunset=∼11.3 h; average daytime temperature 21–25°C). Thereafter, birds were moved indoors and kept in an aviary (size=2.2×1.8×2.8 m) under artificial short days [SD: 8 h:16 h light: dark (8L:16D); L=∼200 lux, D<0.1 lux; Müller SC88 timer controlled light and dark periods] and 22±1°C temperature. Under SD, buntings maintain an unstimulated photosensitive state, i.e. they remain responsive to stimulatory effects of long days (Misra et al., 2004; Singh et al., 2015). Food and water were provided *ad libitum*.

Experiment

Fig. 1 describes a schematic outline of the hypothesis conceptualized and protocol used for the present experiment. Photosensitive birds in four groups of five each were individually housed in activity cages (size=60×35×45 cm; 1 bird per cage), and placed in separate wooden photoperiodic boxes (size=75×50×70 cm; 1 bird per cage per box) providing 8L:16D at 22±1°C, as before. After 1 week, two groups were retained on SD, while the other two groups were exposed to 13 h long-day conditions (LD: 13L:11D; L=200 lux, D<0.1 lux) by 5 h delayed lights-off time each day. Then, under both SD and LD, one group of birds was maintained at 22±1°C, as before; this temperature was close to average daytime temperature that buntings usually experience at this time of the year. In the other groups under both SD and LD, temperature was raised to 35±1°C; this was close to the average daytime temperature that buntings experience during the period of late March/early April, during which they depart from the wintering areas (∼25°N). Here, the two experimental groups are called low- and high-temperature groups. After 3 weeks under SD or after a bird under LD had shown seven consecutive nights of Zugunruhe (hence the LD exposure period ranged from 14 to 37 days

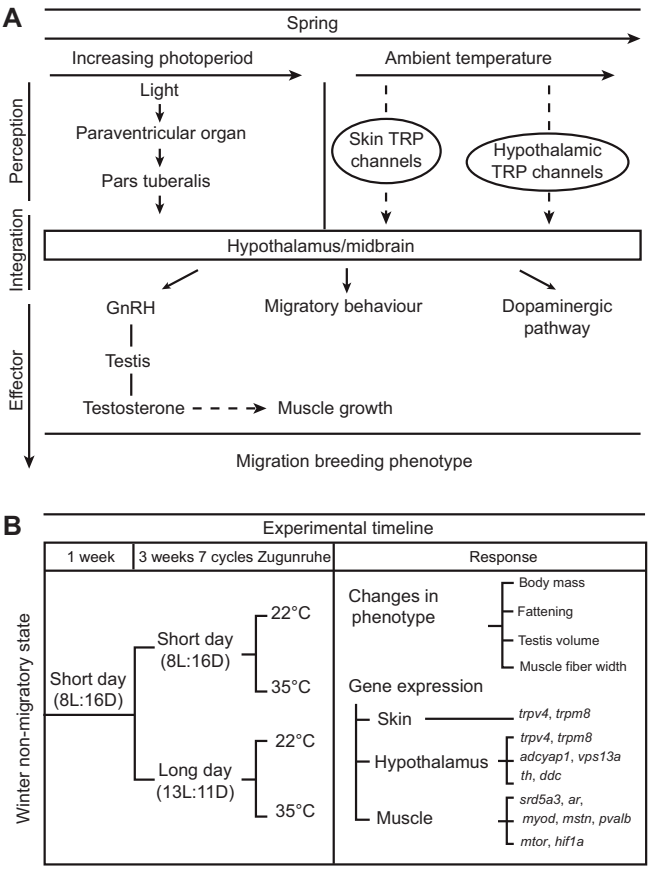

Fig. 1. A schematic outline of the conceptualized hypothesis and experimental protocol used for the present study. Broken line indicates a suggested pathway.

among 10 birds at two temperatures), birds were killed by decapitation at hour 19 (hour 0=lights on) of the day. Decapitation was preferred to anaesthesia usage to preclude its probable effects on mRNA expression of genes in different tissues (Hamaya et al., 2000; Staib-Lasarzik et al., 2014). Further, we chose hour 19 as the sampling time because it coincided with period of intense nocturnal Zugunruhe, and with the predicted time of increased mRNA expression of genes involved in the photoperiodic response (Nakao et al., 2008; Majumdar et al., 2014).

Monitoring of the activity behaviour

We monitored the 24-h activity–rest pattern as a behavioural response of the photostimulated transition from the non-migratory to the migratory LHS. For this, each activity cage was furnished with two perches at unequal heights to facilitate perching activity, and mounted with a passive infrared motion sensor (DSC, LC100 PI Digital PIR detector, Canada) to detect movement of the bird in its cage. We used 'The Chronobiology Kit' software program from Stanford Software Systems (Stanford, CA, USA) to collect movements in 5-min bins into designated channels of the computerized data-logging system, and to collate and analyze daily activity pattern at the end of the experiment. A 7-day segment of activity record (actogram) was used to statistically analyze differences in activity pattern between treatment groups. For this, we first averaged activity for a defined time over 7 days for each individual, and from this group mean (±s.e.m.) activity was calculated. This has been published in several previous publications (e.g. Singh et al., 2010; Sharma et al., 2018).

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Measurement of surface body temperature, body fattening and mass gain, and testes size

At weekly intervals, the surface body temperature and body fattening and mass gain were recorded as indicators of the overall metabolic and migratory state, respectively. The size of left testis was measured, however, at the beginning by unilateral laparotomy (under general anaesthesia) and end of the experiment after the bird was sacrificed. These are routine measurements in our laboratory (e.g. Kumar et al., 1991; Singh et al., 2018).

For laparotomy, birds were administered with a mixture of ketamine/xylazine solution (67.5 mg ketamine+7.5 mg xylazine per kg body mass). The testes were located through a small incision on the left flank between the last two ribs, and the dimensions of the left testis were measured to an accuracy of 0.5 mm. From these values, testis volume was calculated using the formula $4/3\pi ab^2$ (where a is half of length and b is half of width). The incision was sutured, and treated with an antibacterial skin cream (Soframycin, Aventis Pharma Ltd). The bird was then returned in its cage to a warm pad for approximately 30 min (resumption of normal perch hopping in ∼1 h indicated full recovery from the anaesthesia effect). We presented change in testis size as function of time (initial versus final) as testis growth rate (k), which was calculated using the formula $k=[(\log V_t - \log V_0)/t]$, where V_0 is initial testis volume and V_t is volume after time t in days (Farner and Wilson, 1957).

As a marker of the homeostatic response, the surface body temperature was recorded at hour 4 of the day (hour 0=lights-on) by using a touch-free thermoscan thermometer (Quick Shot Infra-red thermometer; model Exp-01B; temperature range: 32–42°C) placed at a distance of ∼2 cm from exposed keel region of the bird. Each time, we averaged four consecutive readings for each individual.

The body fattening (fat depots in the furcular, scapular and abdominal areas) was assessed using a subjective criterion on a scale of 0 to 5, where 0=no visible fat depot, and 5=heavy fat bulging all over the abdomen, as per the scheme of scoring the subcutaneous fat outlined based on previous studies in passerine migrants (Helms and Drury, 1960; Wingfield and Farner, 1978; Biebach et al., 1986), and routinely used in our laboratory for scoring photostimulated fat deposition in migratory buntings (e.g. see Malik et al., 2004; Budki et al., 2009). Briefly, the scoring ran as follows: 0=no visible subcutaneous fat depot; 1=light fat depots overlying musculature, but musculature remains clearly visible; 2=heavier fat deposits overlying musculature (vasculature still visible), 3=fat deposits overlie the entire region; 4=areas filled with whitish, bulging fat deposits; and 5=copious fat deposits all over three regions (Budki et al., 2009). For body mass, each bird was weighed using a tabletop balance to an accuracy of 0.1 g.

The assessment of muscle growth

We measured from each bird the width of muscle fibres from the pectoral muscle as an anatomic measure of muscle growth in response to Zugunruhe, which mimicked migratory activity in the caged situation. For this, a piece of muscle tissue from each bird was sectioned in 10 µm thick cross-sections sing Leica 1850 cryostat. Five sections from each bird were stained with hematoxylin and eosin (H&Ea. Images were captured at 40× magnification (10× ocular×4× objective) using a Nikon DS-Fi2 microscope. In each image, we measured the fibre width at eight random sites (hence per bird, 40 measurements; 5 sections×8 sites) using the Nikon NIS-elements BR software program (version 2.3), as described by Chaves et al. (2018).

Measurement of plasma testosterone levels

In order to assess the effects at peripheral levels, we measured plasma testosterone, which also plays a role in muscle growth. For this, 150–200 µl blood samples collected from each bird at the end of the experiment were centrifuged at 845 ***g*** for 10 min and stored at −20°C. Testosterone levels were measured by ELISA using an immunoassay kit from Enzo Life Sciences (Ann Arbor, MI, USA; Cat. no. ADI-900-065), as per the manufacturer's protocol. Briefly, 100 µl plasma at 1:20 dilution (10 µl plasma+10 µl of 1% steroid displacement buffer+180 µl assay buffer) was aliquoted in a 96-well ELISA plate. Then, 50 µl of antibody was added to each well, except those assigned to the testing of no activity (blank), total activity (TA) and non-specific binding (NSB). After 1 h incubation in an orbital shaker (500 rpm) at room temperature, 50 µl conjugate (alkaline phosphate conjugated with testosterone) was added to each well, except to the blank and TA wells. After re-incubation at 500 rpm for 1 h at room temperature, we performed three buffer washes and first added 5 µl conjugate to the TA wells and then 200 µl of p-nitrophenyl phosphate in buffer (pNpp) to every other well. The plate was kept at 37°C for 1 h, and then 50 µl of stop-solution was added to stop the reaction. The plate was read at 405 nm on SpectraMax M2e microplate reader (Molecular Devices, USA). The sensitivity and intra-assay variability of the assay were 0.08 ng ml^{-1} and 5.7%, respectively.

Measurement of mRNA expression of candidate genes

We used qPCR to measure mRNA expression of candidate genes in the skin, muscle and hypothalamus/midbrain (excised out from the brain), as per methods standardized and published earlier (Mishra et al., 2018; Sur et al., 2019; Trivedi et al., 2019). To begin with, we measured mRNA expression of temperature-sensitive *trpv4* and *trpm8* genes in the skin and hypothalamus to assess their role in relay of the temperature information. We then measured hypothalamic expression of genes implicated in the development of migratory behaviour (*adcyap1*, *vps13a*), and genes of the dopamine biosynthesis pathway (*ddc*, *th*) to examine whether spring migration is linked to sexual motivation (Sharma et al., 2018). Further, we measured in muscle the mRNA expression of a total of seven genes (*srd5a3*, *ar*, *myod1*, *pvalb*, *mstn*, *mtor* and *hifl1a*), which could be associated with muscle growth and excessive oxygen requirement.

For each gene expression assay, we extracted total RNA from the tissue using trizol solution (Ambion, 15596-018), and checked its quality using a Nanodrop 2000c; the ratio of 260/280 close 2.0 was accepted as pure RNA. A 1 µg RNA aliquot treated with RNase free DNase (Promega, M610A) was reverse transcribed to synthesize cDNA using the Revert Aid First Strand cDNA synthesis kit (Thermo Scientific, K1622). We used primer sequences of *trpv4*, *trpm8*, *adcyap1*, *th*, *myod1* and *ar* genes as previously published by Sur et al. (2019) and Trivedi et al. (2019). However, we used degenerate primers and cloned the partial sequence of *vps13a*, *ddc*, *srd5a3*, *pvalb*, *mstn*, *mtor* and *hif1a* genes. The amplified cDNA products were commercially sequenced (Eurofins, Bangalore, India), and subjected to NCBI database BLAST search to ascertain the gene identity. An identified gene sequence was submitted to NCBI GenBank for the accession number (Table S1).

Thereafter, we performed qPCR measurement using gene-specific primers, which were designed using online Primer Quest (https://eu.idtdna.com/PrimerQuest). qPCR assays were performed in duplicate using SYBR Green chemistry (Applied Biosystems, Life Technology, 4367659) run on a ViiA7 real-time PCR system (Applied Biosystems, Foster City, CA, USA). In each PCR plate, we included *beta-actin* as the reference control gene, which was found to be most stable among three potential reference genes (*beta-actin*, *hprt1* and *ppia*) that we had tested in an earlier study (Sharma et al., 2018). The relative mRNA expression of each candidate gene was

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determined by the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001), as published in several previous bunting studies (Sharma et al., 2018; Sur et al., 2019; Trivedi et al., 2019). The value of the cycle threshold (C_t) determined by the fluorescence exceeding the background (noise) gave the ΔC_t value [C_t(gene of interest)–C_t(reference gene)]. C_t values thus obtained were normalized against the C_t value of pooled cDNA from all samples; this gave the $\Delta\Delta C_t$ value. The negative value of $\Delta\Delta C_t$ powered to 2 ($2^{-\Delta\Delta Ct}$) was plotted.

Statistics

IBM SPSS Statistics software (version 20) was used for statistical analyses. Because our data passed the normality test (Shapiro–Wilk's normality test), we employed the parametric general linear model (GLM) to test. For activity pattern, body mass and surface temperature, we used three-way repeated-measures general linear mixed model (GLMM), with temperature as factor 1, LHS as factor 2 and the duration of experiment or time of day as factor 3. However, we used a two-way test for testes (k values), testosterone levels and gene expression, with temperature and LHS as factors 1 and 2, respectively. We calculated the effect size (partial eta squared, η_p^2) to show the magnitude of the increased response as a result of the photoperiod/temperature treatment. We also performed non-linear Gaussian fit analysis on the 24-h activity distribution, and used an F-test to determine differences in the mesor (mean 24 h mRNA expression value) and amplitude (maximum change in mRNA expression levels relative to the mesor) of daily (24 h) rhythm in activity behaviour. Data on fat score were analysed using the ordinal logistics regression. Student's unpaired t-test was also used to test differences between two groups at one time point. Further, Pearson's correlation coefficient was calculated to show the relationship, if any, between the measures of interest. For significance, alpha was set at 0.05.

RESULTS

Effect of temperature on photostimulated changes in the behaviour and physiology

Daily pattern of activity–rest behaviour

At the beginning of the experiment, irrespective of the photoperiod (SD or LD) and ambient temperature (22 or 35°C), all birds showed

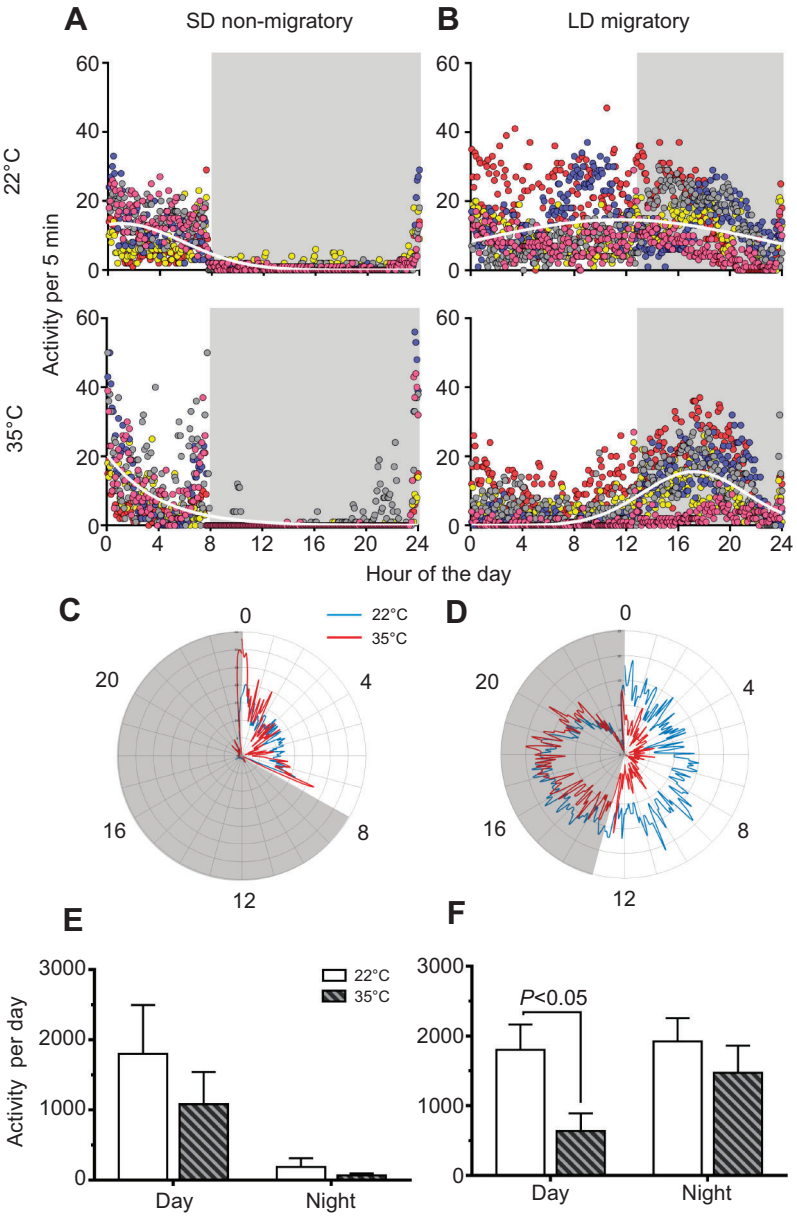

Fig. 2. Daily activity pattern of black-headed buntings (*Emberiza melanocephala*) in photoperiod-induced non-migratory (under short days, SD) and migratory (under long days, LD) life-history stages (LHSs) at 22°C and 35°C. (A,B) Mean activity levels of individual birds (n=5 birds per group, shown by different colours) for seven consecutive days in 5 min bins over 24 h. The white line is a non-linear Gaussian curve fit to 24-h activity distribution. (C,D) Distribution in a polar plot of mean activity over 24-h in each LHS at both temperatures. (E,F) Mean (±s.e.m.) total activity during the day and night in each LHS at both temperatures.

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a diurnal pattern in activity–rest behaviour, with activity consolidated during the lights-on period. Whereas buntings under SD continued showing for the next 3 weeks almost similar diel activity–rest pattern (akin to activity behaviour during the winter non-migratory state), those exposed to LD gradually developed nocturnal Zugunruhe (migratory state) albeit with individual differences in time (days) taken to show its first onset (Fig. S1). Thus, there were differences in daily activity behaviour at two levels. First, as revealed by three-way GLMM, there was a significant effect on 24-h activity distribution of LHS (F=5.43, P<0.05, η_p^2=0.237), temperature (F=4.33, P<0.05, η_p^2=0.216), time of day (F=10.03, P<0.005, η_p^2=0.313) and the LHS×time of day interaction (F=21.19, P<0.005, η_p^2=0.598) (Fig. 2A–D). In particular, we found a significant difference in activity during the light, but not dark, period, with total activity levels lower at 35°C than at 22°C (Student's t-test, P<0.05; Fig. 2F). There was also temperature-dependent 24-h distribution of activity, as shown by significant differences in the mesor and amplitude values from Gaussian best-fit curves under LD (22°C: amplitude=14.7±0.3; mesor=11.6±0.3 h versus 35°C: amplitude=15.6±0.4; mesor=17.2±0.1 h; P<0.0001), but not under SD (22°C: amplitude=13.5±0.5, mesor=1.0±0.6 h versus 35°C: amplitude=13.4±6.8, mesor=1.5±1.0 h). Second, temperature affected the number of days taken to show the photostimulated Zugunruhe; it was significantly advanced at 35°C compared with at 22°C (Student's t-test, P<0.05, η_p^2=0.627; Fig. 3A).

Surface body temperature, fat deposition and mass gain

Surface body temperature was significantly affected by ambient temperature (F=279.05, P<0.0001, η_p^2=0.822) and experiment duration (F=11.31, P<0.005, η_p^2=0.411) and the experiment duration×temperature interaction (F=20.73, P<0.005, η_p^2=0.627) as well as LHS (F=3.60, P<0.05, η_p^2=0.218, GLMM; Fig. 3B). Similarly, measured as indices of the photostimulated phenotypic characteristics of migratory state, weekly changes in both fat score and body mass showed significant differences between SD and LD LHSs. In particular, there was a significant effect of LHS (χ^2=14.97, P<0.005, η_p^2=0.372) and experiment duration (χ^2=14.61, P<0.005, η_p^2=0.294) on fat score, and of LHS (F=18.48, P<0.005, η_p^2=0.447), experiment duration (F=9.10, P<0.005, η_p^2=0.303) and the LHS×experiment duration interaction (F=15.28, P<0.005, η_p^2=0.359) on body mass of black-headed buntings (GLMM; Fig. S2). However, there was no difference in the photostimulated body fattening and mass gain between the two temperature groups (Fig. S2).

Testes, plasma testosterone and muscle growth

At both temperatures, testes recrudesced during LD, but not SD, exposure. There was a significant effect of the LHS (F=27.29, P<0.005, η_p^2=0.725; Fig. 3C) on testicular growth rate (k), and of both LHS (F=56.49, P<0.0001, η_p^2=0.899, GLM) and temperature (F=12.24, P<0.005, η_p^2=0.690) on plasma testosterone levels (GLM; Fig. 3D). We found a significant effect on muscle fibre width of temperature (F=7.35, P<0.05, η_p^2=0.276), LHS (F=96.56, P<0.0001, η_p^2=0.852) and the temperature×LHS interaction (F=8.90, P<0.05, η_p^2=0.395) on muscle growth (GLM; Fig. 4).

Effect of temperature on photostimulated changes in gene expression

Temperature responsive genes

We measured mRNA expression of *trpv4* and *trpm8* genes to assess temperature effects at both body surface (skin) and central (hypothalamus) levels. These genes differentially responded to photoperiod and temperature treatments (Fig. 5). In skin, we found a significant effect of temperature and LHS on *trpv4* (F=8.06, P<0.05, η_p^2=0.381) and *trpm8* (F=6.38, P<0.05, η_p^2=0.352) mRNA expression, respectively (GLM; Fig. 5A,B). In the hypothalamus, in contrast, there was a significant effect of LHS (F=12.37, P<0.005, η_p^2=0.452) and the LHS×temperature interaction (F=6.63, P<0.05, η_p^2=0.307) on *trpv4*, and of LHS alone (F=6.10, P<0.05, η_p^2=0.306) on *trpm8* mRNA expression (GLM; Fig. 5C,D).

Migration-linked genes

We measured mRNA expression of *adcyap1* and *vps13a* genes to examine whether they were associated with the development of Zugunruhe. There was no significant effect of temperature, LHS or the temperature×LHS interaction on hypothalamic expression of either the *adcyap1* or *vps13a* genes (Fig. 6A,B). In both the hypothalamus and midbrain regions, we found a significant effect of

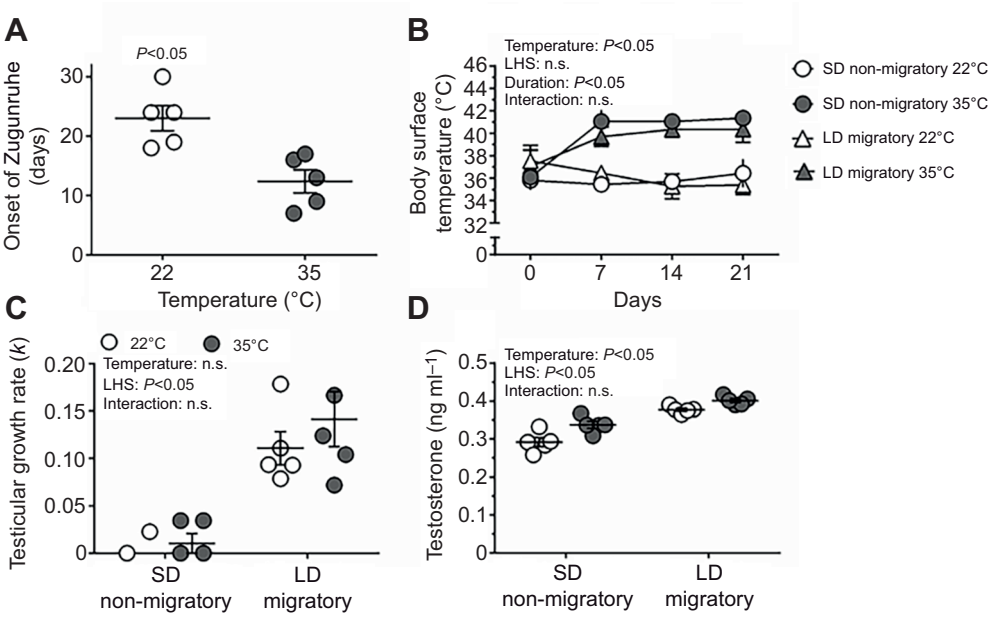

Fig. 3. Changes in physiology of black-headed buntings (n=5) during photoperiod-induced non-migratory (under short days, SD) and migratory (under long days, LD) life-history stages (LHSs) at 22°C and 35°C. (A) Number of days to show the first night of Zugunruhe; (B) weekly record of body surface temperature; (C) the rate of testicular growth (k); and (D) plasma testosterone (T) levels. Each point is an individual value in A, C and D (mean± s.e.m. are indicated by horizontal and vertical lines). In B, each point is the mean, and vertical lines (if they extend beyond the limit of the symbol) represent the standard error. For statistical significance, alpha was set at 0.05.

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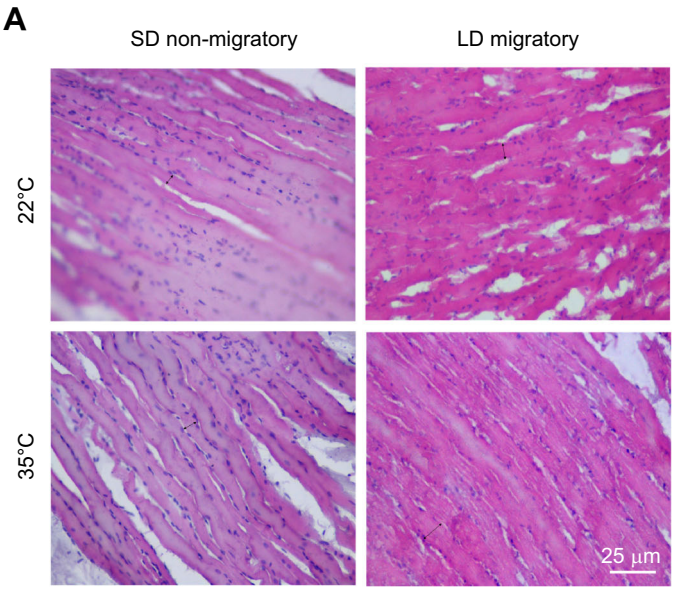

Fig. 4. Changes in the skeletal (flight) muscles of black-headed buntings (*n*=5) in photoperiod-induced non-migratory (under short days, SD) and migratory (under long days, LD) life-history stages (LHSs) at 22°C and 35°C. (A) Photomicrograph of H&E stained muscle fibres at 400× magnification (10× eye piece×40× objective lens); scale bar=25 µm. (B) Mean (±s.e.m.) muscle fibre width in each LHS at both temperatures. For statistical significance, alpha was set at 0.05.

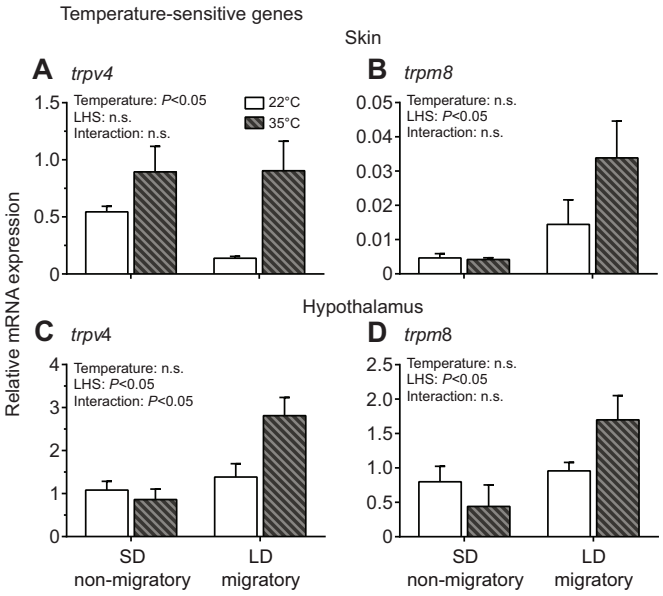

Fig. 5. mRNA expression of temperature-sensitive genes in skin and hypothalamus. Mean (±s.e.m.; *n*=5) mRNA expression of *trpv4* and *trpm8* genes in the skin (A,B) and hypothalamus (C,D) of black-headed buntings in photoperiod-induced non-migratory (under short days, SD) and migratory (under long days, LD) life history stages at 22°C and 35°C.

LHS (hypothalamus: F=23.47, P<0.005, η_p^2=0.610; midbrain: F=22.60, P<0.0001, η_p^2=0.601) and the temperature×LHS interaction (hypothalamus: F=7.38, P<0.05, η_p^2=0.330; midbrain: F=5.39, P<0.05, η_p^2=0.265) on *th*, but not *ddc*, mRNA expression (GLM; Fig. 6C–F).

Genes linked to muscle growth and oxygen supply

We found a significant effect of the LHS on *srd5a3* (F=39.79, P<0.0001, η_p^2=0.712, GLM; Fig. 7A), but of both LHS (F=45.04, P<0.0001, η_p^2=0.747, GLM) and temperature on *ar* gene expression (F=6.25, P<0.05, η_p^2=0.295, GLM; Fig. 7B). The mRNA levels of both *srd5a3* and *ar* were significantly higher in the LD migratory than in the SD non-migratory LHS (P<0.05, Student's *t*-test, Fig. 7A,B). We further assessed the effect of temperature on photostimulated expression of the *myod1*, *mstn*, *pvalb* and *mtor* genes involved in muscle growth, and of the *hif1a* gene involved in hypoxia-induced pathways. There was a significant effect of LHS on muscular expression of both *myod1* (F=20.48, P<0.005, η_p^2=0.575) and *mstn* (F=98.30, P<0.0001, η_p^2=0.860) genes (GLM; Fig. 7C,D). However, there was a significant effect of both LHS and temperature on *pvalb* (LHS: F=6.57, P<0.05, η_p^2=0.419; temperature: F=5.33, P<0.05, η_p^2=0.416), *mtor* (LHS: F=14.09, P<0.005, η_p^2=0.529; temperature: F=5.39, P<0.05, η_p^2=0.251) and *hif1a* (LHS: F=6.07, P<0.05, η_p^2=0.294; temperature: F=6.31, P<0.05, η_p^2=0.298) gene expression (GLM; Fig. 7E–G).

Then, we used Pearson's correlation to show the relationship of testosterone levels with mRNA expression of T-signalling pathway genes (*ar*, *srd5a3*), and of the latter (*ar*, *srd5a3*) with muscle growth genes (*pvalb*, *mtor*). There was a significant positive correlation of testosterone levels with *ar* (r=0.72, P=0.0003), *srd5a3* (r=0.81, P<0.0001), *pvalb* (r=0.45, P=0.04) and *mtor* (r=0.56, P=0.009) mRNA expression (Fig. 8A,B). Similarly, we found a positive correlation of both *ar* and *srd5a3* with *pvalb* (*ar*: r=0.60, P=0.004; *srd5a3*: r=0.44, P=0.04) and *mtor* (*ar*: r=0.74, P=0.0002; *srd5a3*: r=0.69, P=0.0006) gene expression (Fig. 8C,D).

DISCUSSION

Ambient temperature played a crucial role in the departure decision of spring migrants from wintering areas. This is suggested by temperature-dependent physiological and molecular effects on multiple target tissues (skin, hypothalamus and midbrain, and flight muscles) associated with migration in black-headed buntings. The ambient temperature seems to be perceived by both peripheral (skin) and central (hypothalamus) TRP channels (temperature sensors), and affect multiple transcriptional pathways regulating photoperiod-induced development of the spring migratory state in black-headed buntings.

Changes in migratory behaviour and physiology

There were differences in the overall 24-h activity behaviour in LD migratory state between 22 and 35°C temperature groups, with the advancement of Zugunruhe onset (akin to nocturnal flight) by ∼10 days at 35°C. We interpret that the overall dampened 24-h activity at 35°C, similar to previously reported decreases in activity at high temperature in this species and white-throated sparrows (Berchtold et al., 2017; Singh et al., 2012), was an adaptive behavioural response to a warmer climate; a reduced muscular activity will reduce body heat generation (Hafez, 1964; Chaffee and

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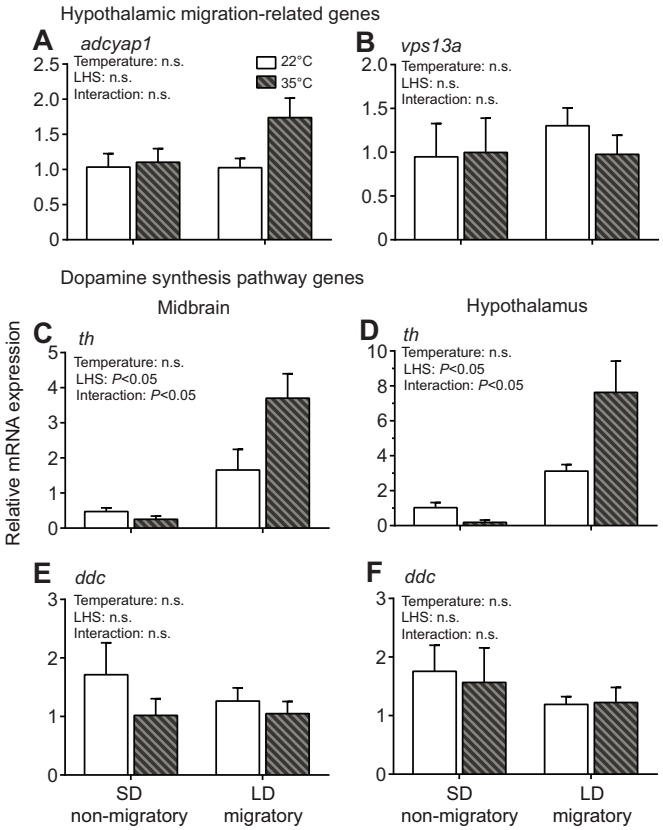

Fig. 6. mRNA expression of migration-related and dopamine synthesis pathway genes in hypothalamus and midbrain. Mean (±s.e.m.; *n*=5) mRNA expression of *adcyap1* (A) and *vps13a* (B) genes in the hypothalamus, and of *th* and *ddc* genes in the midbrain (C,E) and hypothalamus (D,F) of black-headed buntings in photoperiod-induced non-migratory (under short days, SD) and migratory (under long days, LD) life history stages at 22°C and 35°C.

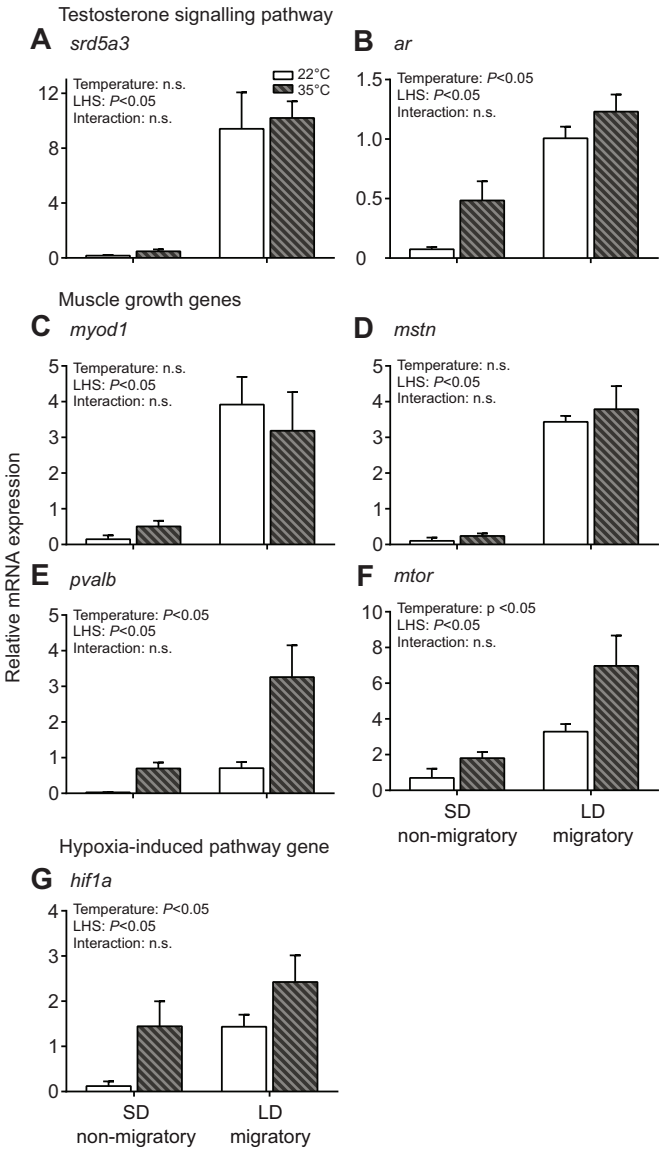

Fig. 7. mRNA expression of testosterone signalling, muscle growth and hypoxia-induced pathway genes in the flight muscle. Mean (±s.e.m.; *n*=5) mRNA expression of *srd5a3* (A), *ar* (B), *myod1* (C), *mstn* (D), *pvalb* (E), *mtor* (F) and *hif1a* (G) in the flight (skeletal) muscle of black-headed buntings in photoperiod-induced non-migratory (under short days, SD) and migratory (under long days, LD) life history stages at 22°C and 35°C.

Roberts, 1971). Perhaps the thermoregulation was activated in buntings at 35°C, as the surface body temperature of these birds was significantly higher than that of birds at 22°C. Intriguingly, although similar to other migratory species (Tryjanowski et al., 2002; Usui et al., 2017; Sur et al., 2019; Trivedi et al., 2019), an early Zugunruhe onset at 35°C is inconsistent with previously reported delayed photostimulated onset of Zugunruhe at 40°C, compared with 27°C, in black-headed buntings (Singh et al., 2012). We believe that the difference between the two studies is because of the difference in both 'low' and 'high' temperatures that were used. We speculate that in the study of Singh et al. (2012), 27°C was close to while 40°C was much above the favorable temperature for migratory departure of buntings from wintering areas. Notably, both field and laboratory (semi-natural environment) studies suggest that the start of spring migration of black-headed buntings occurs during the period from the last week of March to the first half of April, when daytime temperature at the overwintering sites (25–27°N) usually varies from 30 to 35°C (Ali and Ripley, 1999; Gupta and Kumar, 2013).

Photostimulated testicular growth in the LD migratory state reinforced the observations from field studies in several migratory species that gonads recrudesce and secrete hormones over the course of spring migration (Fry et al., 1972; Silverin, 1975; Bauchinger et al., 2005). However, as in white-crowned sparrows (Wingfield et al., 1997) and European starlings (Dawson, 2005), the lack of temperature effects on testicular growth in buntings is consistent with the idea that spring migratory and immediately following breeding state are separate, albeit overlapping, photoperiod-induced seasonal events (Misra et al., 2004; Kumar et al., 2006). Despite no effect on testes, plasma testosterone levels were significantly higher at 35°C in photostimulated buntings, which also showed early Zugunruhe onsets. We suggest that elevated testosterone levels facilitated an early departure of males from the wintering areas so that they were in an advanced breeding state when they arrived at the breeding ground and immediately initiated reproductive activities (Wingfield et al., 1990; 2001; Ketterson and Nolan, 1999; Tonra et al., 2011). Intriguingly, we also found elevated testosterone levels at 35°C in the SD non-migratory state, indicating probably an activated testosterone secretion at the temperature, which was close to favourable departure temperature. This suggests that testosterone facilitates Zugunruhe if birds are exposed to a stimulatory photoperiod. Differential roles of testes (possibly testosterone secretion) were

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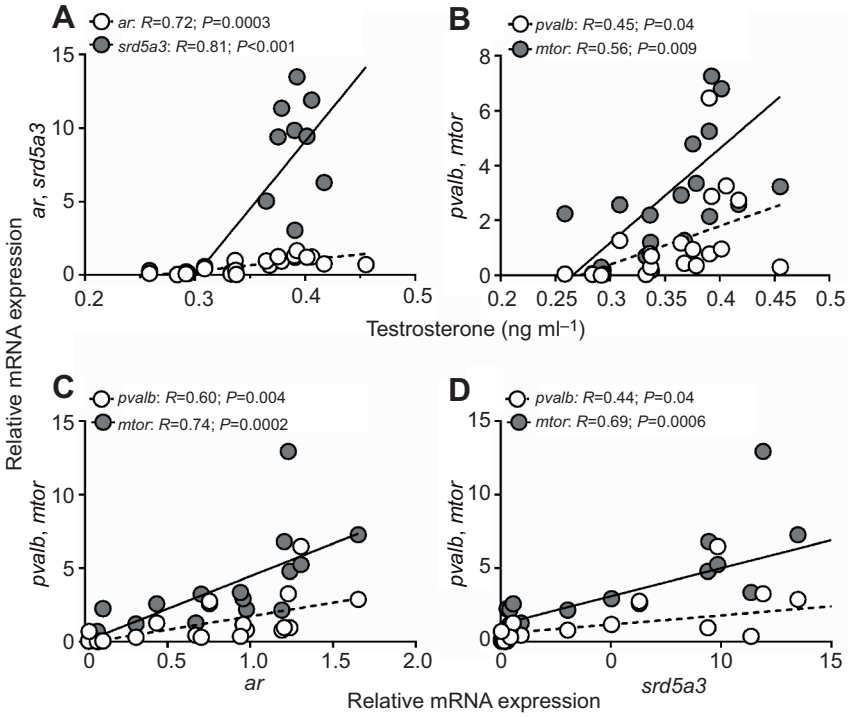

Fig. 8. Multiple correlations between plasma testosterone, mRNA levels of testosterone signalling and muscle growth genes. Scatter plots showing correlation of plasma testosterone levels with mRNA levels of *ar* and *srd5a3* genes (A), and *pvalb* and *mtor* (B). (C,D) Correlation of mRNA levels of *ar* (C) and *srd5a3* (D) genes with *pvalb* and *mtor* genes in the flight (skeletal) muscle of black-headed buntings in photoperiod-induced non-migratory (under short days, SD) and migratory (under long days, LD) life history stages at 22°C and 35°C. Lines in the scatter plot denote significant linear regression.

found in male red-headed buntings during the development of spring and autumn migratory states when birds showed recrudesced and regressed testes, respectively (Sharma et al., 2020).

Change in functional gene expressions

Present results showed that the ambient temperature affected molecular underpinnings of the physiological pathways during photostimulated development of the spring migrant phenotype in black-headed buntings. Clearly, temperature was perceived at both peripheral and central levels, and influenced molecular processes at multiple levels culminating into the spring migratory phenotype.

Gene expression in skin and hypothalamus

Three main conclusions seem to have emerged. First, TRP channels were involved in relaying the temperature information. We suggest that skin TRPV4 channels acted as the peripheral sensor and conveyed temperature information to the brain, where it influenced the regulatory processes (Kobayashi, 2015). This is evidenced by an upregulated expression at 35°C of the warm-sensitive *trpv4* gene in the skin, irrespective of the LHS, but in the hypothalamus of birds exhibiting the LD-induced migratory phenotype. In contrast, *trpm8* expression showed an LHS-induced effect, as there were parallel changes (upregulated) in mRNA levels in both skin and hypothalamus of birds under LD at 35°C.

Second, the concurrent increased expression of genes coding for TRP channels and tyrosine hydroxylase (TH, rate limiting enzyme of dopamine synthesis) at 35°C in the LD migratory state suggested a functional interaction of the hypothalamic thermosensitive and dopaminergic neurons (Cronin and Baker, 1977; Heimovics and Riters, 2008; Miller and Lonstein, 2009; Wang and Siemens, 2015). The temperature effect on *th*, but not *ddc*, expression further suggested that from a functional viewpoint, TH-mediated step of dopamine biosynthesis was probably crucial for spring migration, which indeed is purposed with a strong sexual motivation (Tryjanowski and Yosef, 2002). The linkage of *th* mRNA expression with photostimulated spring migratory state has also been suggested in migratory red-headed buntings (Sharma et al., 2018).

Third, both *adcyap1* and *vps13a* genes were not involved in photoperiodic induction of the spring migratory state in black-headed buntings. This is not surprising because apart from species variations, the suggested role of *adcyap1* and *vps13a* genes in migration is in a different context. For example, it was the size of the 3′ untranslated region (UTR) of *adcyap1*, not its expression levels, which influenced migratory behaviour of European blackcaps (Mueller et al., 2011), but not of juncos (Peterson et al., 2013). Likewise, *vps13a* gene expression was found to be associated with directionality of migration, not with development of the migratory state, between golden-winged and blue-winged warblers preparing to migrate from their wintering areas in Central and South America, respectively (Toews et al., 2019). A seemingly higher *adcyap1* mRNA level under LD at 35°C, compared with 22°C, is perhaps consistent with the overall increased 24-h activity in these birds.

Gene expression in flight muscles

Temperature effects on migratory behaviour (24-h activity–rest pattern and nocturnal Zugunruhe) were accompanied with anatomical (=physiological) changes in the flight muscle. And, as expected with an early Zugunruhe onset, the muscle fibres were enlarged in photostimulated buntings at 35°C. This corroborated the changes as required in both physiological and molecular gears of muscles to meet ‘workloads’ of the migratory flight. Hypertrophied muscles show added strength (endurance) and enhanced molecular metabolic plasticity, which birds need during the migration (Bauchinger and Biebach, 2006; Price et al., 2011).

We suggest that activated T-signaling pathway was an adaptive physiological response of muscles to support more intense spring migratory flight. This was evidenced by upregulated expression of *srd5a3* and *ar* genes in parallel with circulating levels of testosterone in photostimulated buntings, consistent with findings in white-crowned sparrows prior to their migratory departure (Pradhan et al., 2019). Both plasma testosterone and hypothalamic *ar* mRNA levels

were higher at 35°C than at 22°C, corroborating the idea that elevated testosterone facilitated an earlier onset of Zugunruhe in buntings at 35°C. Apart from its effect on muscles via its own AR receptors, testosterone effects on muscle growth and strength involved the activation of the biosynthesis of PVALB and MTOR proteins encoded by *pvalb* and *mtor* genes, respectively. The positive correlations of both *pvalb* and *mtor* mRNA levels with plasma testosterone levels and with *ar* and *srd5a3* mRNA levels support this. A study on golden-collared manakins also showed testosterone effects on *pvalb* expression (Fuxjager et al., 2012). Perhaps Ca^{2+} binding PVALB protein hastened the relaxation, and hence enhanced contractility of the skeletal (flight) muscles (Fuxjager et al., 2012; Heizmann et al., 1982). We suggest a similar testosterone-induced effect on MTOR protein, which is involved in the muscular hypertrophy (Wu et al., 2010; Basualto-Alarcón et al., 2013).

Temperature did not affect the genes involved in muscle growth; expression of both *myod1* and *mstn* was upregulated in the photostimulated migratory state, irrespective of temperature. Increased *myod1* mRNA expression, in particular, suggested enhanced synthesis of the MYOD protein, which plays a crucial role in myogenesis (muscle growth) and muscle metabolism by affecting mitochondrial biogenesis, fatty acid oxidation and electron transport chain reaction (Shintaku et al., 2016). At the same time, although similar to white-throated sparrows (Price et al., 2011), elevated *mstn* mRNA expression in photostimulated buntings is inconsistent with the idea of enhanced muscle biogenesis, because MSTN is inhibitory to muscle growth. We interpret that MSTN levels counterbalance muscle growth during the period of intense muscular activity, when birds in flight need to maintain their body mass.

Further, muscular expression of *hif1a* was consistent with the overall physiological adaptation to support an enhanced aerobic exercise during the migration. Elevated *hif1a* mRNA levels at 35°C in buntings exhibiting an LD-induced migratory state evidence this. Perhaps *hif1a*-encoded HIF1 protein augments oxygen diffusion in the flight muscle (Scott, 2011). This, in turn, may facilitate more intense and longer migratory flights, when birds require enhanced oxygen supply but might be faced with a relatively lower atmospheric oxygen level while flying at higher altitudes.

We propose a functional interaction of the thermosensitive neurons with dopaminergic neurons in the hypothalamus. Downstream, the temperature-induced T-signalling pathway, in particular *ar* and *srd5a3* genes, affected both growth and activity of the flight muscle, which is an effector tissue directly involved with the migration. This pathway caused changes in the mRNA expression of a host of genes (e.g. *pvalb*, *mtor*, *myod1*, *mstn* and *hif1a*) and enhanced both muscle power and endurance in photostimulated birds. We suggest this as a significant component mechanism of the overall adaptive strategy in latitudinal avian migrants for timely departure from the wintering areas in spring.

Finally, our results support the hypothesis that one of the several factors in the environment songbirds use when making a behavioural decision to depart from wintering areas is temperature (Berchtold et al., 2017). This is, in particular, demonstrated by temperature effects on expression of genes that are related to the development of the photostimulated migratory phenotype. Although this cannot be determined from the present study, we speculate that songbirds use a temporal window of favourable temperature for departure from wintering areas during spring. A sudden rise above the favourable temperature range might adversely affect (delay) the departure time because birds need to become engaged more actively in thermoregulation. It might be an interesting idea for future investigations if there was indeed a critical narrow temperature range for migratory departure, similar to the widely demonstrated critical day length for the photoperiodic seasonal response in migratory buntings (e.g. body fattening and gonadal growth; Kumar, 1997; Misra et al., 2004; Gupta and Kumar, 2013; Majumdar et al., 2015).

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: V.K.; Methodology: S.S., K.C., S.M., S.R., V.K.; Validation: S.S., A.S.; Formal analysis: S.S., K.C., A.S., S.M.; Investigation: S.S., K.C., S.M., S.R., V.K.; Resources: S.R., V.K.; Data curation: S.S.; Writing - original draft: S.S., A.S., S.M., S.R., V.K.; Writing - review & editing: V.K.; Visualization: V.K.; Supervision: S.R., V.K.; Project administration: V.K.; Funding acquisition: S.R., V.K.

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Supplementary information
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