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Season- and context-dependent sex differences in melatonin receptor activity in a forebrain song control nucleus

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ABSTRACT

There are dense populations of melatonin receptors in large areas of the songbird brain, in particular in the visual system and the song control system. Melatonin has therefore been implicated in neuroplasticity of the song control system. Previously we demonstrated large changes in activity of melatonin receptor in Area X, a forebrain song control nucleus involved in song learning and production. In a laboratory environment, melatonin receptor activity was down-regulated in male and female European starlings during photostimulation (a simulated breeding season). The functional significance of this large change in Area X is unclear, so we sought to elucidate it by tracking melatonin receptor activity in male and female starlings housed in a semi-natural environment and permitted to breed. Males and females all exhibited high melatonin receptor activity in Area X during short days at the start of the breeding season, and maintained this high activity during photostimulation until females laid eggs. At this point the females down-regulated melatonin receptor activity in Area X, whereas the males maintained high activity until later on in the breeding season. Mel 1b was the most abundantly expressed of the 3 known melatonin receptor subtypes in Area X. There was a positive correlation between the expression of Mel 1b and the transcription factor ZENK, indicating that high melatonin receptor expression is correlated with high activity of Area X. Overall, we observed a gradual termination of activity in Area X as the breeding season progressed, but the timing of termination was different between the sexes.

Introduction

The hormone melatonin in all vertebrates studied is secreted from the pineal gland into the peripheral circulation at night. The duration of the night dictates the duration of melatonin secretion. Thus, the melatonin signal provides organisms with a very accurate measurement of the length of the day. In addition, if it is able to make reference to prior days, an organism can tell whether the day lengths are increasing (spring) or decreasing (fall). Thus, many seasonally-breeding mammals use the nightly melatonin signal to coordinate season-appropriate changes in their reproductive physiology and behavior. The action of melatonin in photoperiodic mammals differs depending on whether the animal breeds during short days, as in winter, or long days, as in late spring. Examples of short-day breeders are sheep, goats and deer (Lincoln and Ebling, 1985), and many photoperiodic rodents such as hamsters and several vole species are long-day breeders (Prendergast et al., 2001). Removal of the pineal gland or administration of melatonin in these species can severely disrupt the timing of reproduction relative to changes in ambient day length (Grosse et al., 1993; Maywood et al., 1993). Thus, it is clear that in these mammalian species, the timing of release of melatonin into the circulation is critical for accurate timing of seasonal gonadal growth and reproductive behaviors.

Seasonal breeding in photoperiodic bird species does not appear to be as impacted by melatonin manipulation as in photoperiodic mammals. Highly photoperiodic birds in temperate zones, such as European starlings (*Sturnus vulgaris*) exhibit gonadal growth in response to lengthening days of spring and become *photostimulated*. After several weeks of photostimulation, the reproductive system regresses, reproductive behaviors cease and birds molt. In this condition, the birds are in what is termed a *photorefractory* condition. It is important to note that species such as European starlings become photostimulated and subsequently photorefractory even while day lengths are still increasing, and thus while the duration of the nocturnal melatonin signal is still decreasing. These birds will not be able to become photostimulated again until they become *photosensitive* as a result of experiencing short day lengths again for a number of weeks. Clearly, changes in day length are important for the timing of reproduction in these species, but the role of melatonin is less obvious.

In one study on American tree sparrows (*Spizella arborea*), removal of the pineal gland and eyes, which are both sources of melatonin in birds, did not influence the timing of a photoperiodically-induced

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cycle of gonadal growth and regression (Wilson, 1991). In another study on Japanese quail (*Coturnix japonica*), melatonin administration did not prevent gonadal growth during long days (Juss et al., 1993). Despite the apparent lack of effect of melatonin on the avian photoperiodic response in these studies, other studies indicate that melatonin might play an important role in modulating reproductive timing. Melatonin induces synthesis and release of gonadotropin-inhibitory hormone (GnIH) (Chowdhury et al., 2010; Ubuka et al., 2005), a neuropeptide that acts at the levels of the hypothalamus, pituitary gland and gonads to reduce synthesis and release of pituitary gonadotropins and gonadal steroids (McGuire and Bentley, 2010; McGuire et al., 2011; Ubuka et al., 2006). Melatonin can even act directly on the gonads to induce gonadal GnIH synthesis and reduce gonadal steroid production (McGuire et al., 2011). In a more recent study on wild great tits, *Parus major*, melatonin administration significantly delayed, but did not prevent, the onset of egg-laying in spring (Greives et al., 2012).

In addition to modulating the activity of the neuropeptide GnIH in birds via its receptor subtype Mel 1c (Ubuka et al., 2005), melatonin can influence the song control system. The song control system is a network of interconnected brain nuclei that is involved in the learning and production of song (Brenowitz et al., 1997). In seasonally-breeding songbirds, specific nuclei in the song control system grow and recruit new neurons in response to increases in circulating gonadal steroids during photostimulation (Smith et al., 1997; Tramontin et al., 2000). The song control nucleus HVC, Area X and the robust nucleus of the arcopallium (RA) have been particularly well-studied in this regard. These same nuclei express melatonin receptors (Bentley, 2003; Bentley and Ball, 2000; Gahr and Kosar, 1996; Whitfield-Rucker and Cassone, 1996). Administration of melatonin to European starlings and house sparrows (*Passer domesticus*) reduces the volumes of HVC and Area X (Bentley et al., 1999; Cassone et al., 2008), and can disrupt song output from zebra finches the day after administration (Jansen et al., 2005).

Under laboratory conditions, melatonin receptor activity in Area X (and in no other song control nucleus) changed markedly in male starlings according to photoperiod-induced changes in reproductive status (Bentley, 2003; Bentley and Ball, 2000). When photosensitive, binding of radioactively-labeled melatonin (^{125}Iodomelatonin) was high throughout Area X. When birds were transferred to long days and photostimulated, there was a huge downregulation of melatonin receptor binding capability such that there was almost no ^{125}Iodomelatonin binding. When held on those same long days for an extended period of time, birds became photorefractory and there was a subsequent upregulation of ^{125}Iodomelatonin binding (a return to short day, photosensitive levels) even in the absence of any further change in day length (Bentley, 2003; Bentley and Ball, 2000). These changes in melatonin receptor activity are independent of changes in concentrations of circulating gonadal steroids and melatonin. The functional significance of such a large change in melatonin receptor activity in a brain area that is involved in song learning and production is as yet unclear. We previously concluded that the data indicated an association with centrally-mediated reproductive status and melatonin receptor activity. As such, there is a short time-window, when birds are photostimulated, in which melatonin is unable to bind to Area X.

Area X is involved in processing auditory feedback that is required for song learning and maintenance and, in zebra finches (*Taeniopygia guttata*), neurons in Area X respond strongly to a bird's own song in addition to the song of its tutor (Kojima and Doupe, 2007). Starlings are open-ended learners, which means that they can add new songs to their repertoire every year. It is not known exactly when during the annual cycle adult starlings add new songs to their repertoire, although young male starlings can learn songs in the first few months of their lives (Chaiken et al., 1993). Female starlings seem to prefer to associate with males that have longer, and more complex songs (Gentner and Hulse, 2000). Melatonin appears to have an inhibitory action on the song control system (Bentley et al., 1999; Cassone et al., 2008; Jansen et al., 2005). Thus our working hypothesis was that the downregulation of melatonin binding in Area X during photostimulation represented a release of inhibition of some processes during this time-window when breeding would occur in the wild (Bentley, 2003; Bentley and Ball, 2000). During the breeding season, song is a critical component of starling reproductive behavior and is used for mate attraction, nest box defense, and pair-bonding. We proposed that this might be a time when adult male starlings add new songs to their repertoires.

Motivated by the question of the functional significance of changes in melatonin receptor activity in Area X, and by the fundamental question of whether data from laboratory experiments agree with data collected from the wild (Calisi and Bentley, 2009), we performed the current study. We predicted that in a semi-natural housing environment (instead of an indoor laboratory environment), starlings would show a similar pattern of changes in melatonin receptor activity in Area X, that the downregulation of melatonin receptor binding would be most prominent during the period of mate/nestbox acquisition, and that the timing of changes in melatonin receptor activity in Area X would be the same in males and females. Further, we measured expression of the transcription factor ZENK, an immediate-early gene that is associated with changes in the activity of Area X. We then compared ZENK expression with melatonin receptor expression to determine if high melatonin receptor activity equates to high cellular activity within this forebrain nucleus.

Material and methods

Birds — Phase 1 of the study

A total of 22 male and 17 female European starlings were used in the receptor autoradiography study. All birds were caught locally as juveniles during the previous fall. Juvenile starlings can be identified by their brown plumage for a few months post-fledging, making it easy to age them. Birds were housed in large ($12 \times 6 \times 3.5$ m), outdoor aviaries at the UC Berkeley Field Station for the Study of Behavior, Ecology and Reproduction. In these aviaries, the birds experienced natural light, weather, food sources (along with supplied chicken layer pellets and water ad libitum) and were able to interact socially. Under these conditions, European starlings engage in a full range of natural breeding behaviors, including singing, copulation solicitation, nest construction and defense, egg-laying, incubation and care of hatchlings.

Experimental groups — Phase 1

Males and females were housed together in three separate but essentially identical aviaries. Group size varies slightly because of birds escaping during the experiment as a result of unidentified animals creating holes in the aviaries. *Group 1*, *February* (photosensitive), 7 males and 3 females, was sampled at the beginning of the breeding season (February 18; sunrise at 6.55 a.m. and sunset at 5.51 p.m. = day length of 11 h 56 min). *Group 2*, *April* (photostimulated), 7 males and 10 females, was sampled during what we termed the middle of the breeding season (24 April; sunrise at 6.22 a.m. and sunset at 7.53 p.m. = day length of 13 h 31 min). *Group 3*, *September* (photorefractory), 8 males and 4 females, was sampled at the beginning of the non-breeding season (24 September; sunrise at 6.58 a.m. and sunset at 7.03 p.m. = day length of 12 h 05 min). Sunrise and sunset were determined from the US Naval Observatory Astronomical Applications Department website.

Brain, blood and gonad sampling — Phase 1

Birds were collected using mist nets and hand nets and decapitated following rapid terminal anesthesia using isoflurane. Immediately following decapitation, brains were extracted and frozen on dry ice

and then stored at −80 °C until sectioning. Trunk blood was collected for radioimmunoassay of testosterone. Approximately 1–2 ml of blood was collected and refrigerated immediately. Blood was centrifuged within an hour of collection to separate plasma and solid blood fractions. The plasma was drawn off with a pipette and then frozen for later analysis. Gonads were dissected out and measured to the nearest 0.1 mm using a pair of calipers. Testis volumes (left testis) were calculated using the formula $V = 4/3\pi a^2 b$, where a is half of the width and b is half of the length (long axis). The volume of the largest ovarian follicle was calculated using the formula $V = 4/3\pi a^3$, where a is half of the diameter of the follicle.

All procedures were approved by and in compliance with the University of California Office of Lab Animal Care and federal regulations.

Localization of melatonin receptor

In order to assess the status of melatonin receptor activity in the brain, we localized melatonin binding using ^{125}Iodomelatonin (IMEL) receptor autoradiography as described in Gahr and Kosar (1996). Briefly, slide-mounted tissue sections (20 μm) were incubated for 1 h at room temperature in 20 pM IMEL (Perkin Elmer; SA 2,000 Ci/mmol) in 50 mM Tris–HCl buffer (pH 7.4) with 4 mM $CaCl_2$ in either the presence (nonspecific binding) or the absence (total binding) of 1 μM melatonin (Sigma). No IMEL binding was found in any brain area in the presence of 1 μM melatonin. Slides were rinsed in ice-cold Tris–HCl buffer (once for 2 min and twice rapidly), dried on a hot plate and apposed to X-ray film at room temperature for 7 days. The films were developed in a standard developer.

Analysis of autoradiograms

Digitized images of the films were analyzed using the program NIH Image and an image analysis system interfaced with an Apple Macintosh computer. Binding data were determined from the film density in areas of interest relative to non-specific binding (background). Background binding values were taken from the same area but in adjacent sections that had also been incubated with 1 μM non-labeled melatonin. A sum of these values and values for film background density were subtracted from the areas with specific binding to provide a more accurate measure of specific binding.

Testosterone radioimmunoassay

Plasma testosterone was measured using the methods of Wingfield and Farner (1975), and modified by Ball and Wingfield (1987). Samples were assessed in duplicate and measured in a single RIA to avoid inter-assay variation.

Phase 2 of the study

A second phase of the study was carried out in the following year to allow for a molecular analysis of Area X. 28 female and 26 male starlings were captured and housed as described for Phase 1. As in Phase 1, birds were collected when they were in different reproductive conditions. Sample sizes were different from those in Phase 1, as these birds were also used for part of a separate study. There were 6 photosensitive birds (4 females, 2 males), 40 photostimulated birds (21 females, 19 males) and 8 photorefractory birds (3 females, 5 males). We measured ZENK expression to compare it with melatonin receptor subtype expression and generate insight as to whether changes in melatonin receptor expression are indicative of overall changes in Area X activity.

qRT-PCR on Area X tissue to measure melatonin receptor subtype and ZENK expression

Brains were collected and sectioned as in Phase 1 of this study, but in this case Area X was punched out unilaterally for total RNA extraction and measurement of the expression for melatonin receptor subtypes Mel 1a, Mel 1b and Mel 1c, along with expression of the immediate-early gene *ZENK* using qRT-PCR. Tissue punches were immediately added to 1 ml PureZol reagent (BioRad), homogenized and stored at −80 °C until extraction. Total RNA was extracted according to the manufacturer's protocol, and DNase treated (Ambion® DNA free, Invitrogen), and 1 μg of treated RNA was reverse transcribed (iScript cDNA synthesis kit, BioRad). qRT-PCR was performed in a manner similar to that of Perfito et al. (2012) on cDNA diluted 1:10 using SsoADV SYBR Green (BioRad) in a duplicate 15 μl reaction volume for 40 cycles using the manufacturer's PCR protocol. Primers were designed based on the three *S. vulgaris* melatonin receptor subtype sequences and the ZENK sequence (GenBank Accession #s DQ470808 for Mel 1a, DQ470809 for Mel 1b, DQ470810 for Mel 1c, and EF568327 for ZENK). Published *Gallus* sequences were used to design primers for control house-keeping genes: hypoxanthine phosphoribosyltransferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All primers were used in 5 μM concentrations. Non-template controls were included for each primer pair to check for the formation of primer-dimers. These samples always resulted in difference of at least 10 cycles of the Ct values compared to samples containing template. The specificity of each primer pair was confirmed using a melt curve analysis. The raw fluorescent data were analyzed using the RT-PCR Miner program (Zhao and Fernald, 2005). The PCR efficiency and fractional cycle threshold number were used for gene quantification. Expression values were calculated as $1/(1+E)^{Ct}$, where E is the average PCR efficiency and Ct is the cycle threshold. Two stable internal reference genes (HPRT and GAPDH) were used to normalize mRNA levels among samples. We used GeNorm (Vandesompele et al., 2002) to determine which reference genes were suitable and calculated a normalization factor for their expression. We then normalized the gene of interest expression by dividing expression values by the normalization factor for the controls.

Data analysis

In general, data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Linear regression analysis was used for the analysis of ZENK/Mel 1b correlation.

Results

Melatonin receptor binding in Area X

As shown in Fig. 1, the pattern of melatonin binding was overall very different from that seen under laboratory conditions. In photosensitive males (February), birds exhibited high IMEL binding in Area X, as observed in the laboratory under short day lengths. The same was true for females at this time point (Bentley, 2003; Bentley and Ball, 2000). During photostimulation in April, males differed from those sampled under laboratory conditions in that they maintained high IMEL binding in Area X, whereas females at this time point exhibited the dramatic down-regulation observed during photostimulation in previous laboratory experiments. During September, when all birds had become photorefractory, males and females all exhibited a large down-regulation in IMEL binding — this is opposite to that seen in previous laboratory experiments, when male starlings exhibited an up-regulation in IMEL binding when they were photorefractory (one-way ANOVA: $F = 26.35$ (5,33), $P < 0.0001$. Tukey's posthoc analysis: Feb male vs. Apr fem, $P < 0.001$; Feb male vs. Sept male,

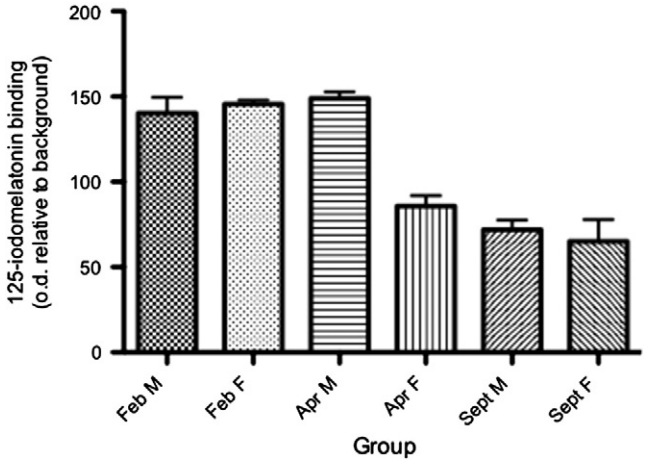

Fig. 1. ^{125}Iodomelatonin binding in Area X of male and female starlings at different times of year. Note that the only time that males and females differ from one another in binding activity is in April, when females started laying eggs. Overall, there is a gradual down-regulation of ^{125}Iodomelatonin binding in Area X in both sexes as the year progresses. This is very different from data collected in a laboratory experiment (see Discussion and Fig. 5).

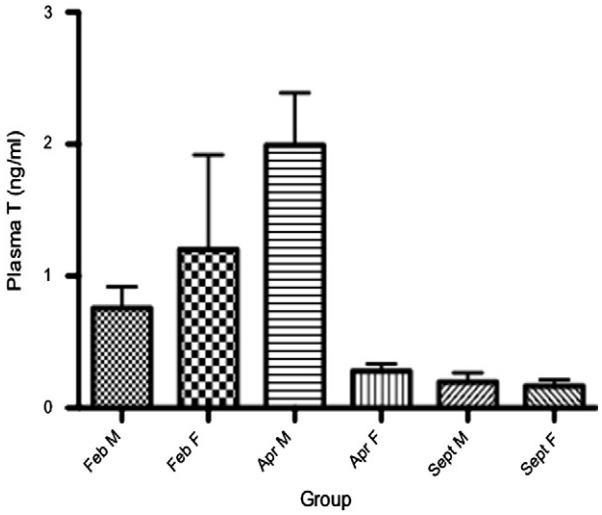

Fig. 3. Plasma testosterone in male and female starlings at the time of brain sampling. Note that changes in ^{125}Iodomelatonin binding in Area X are known to be independent of gonadal steroids when studied in a laboratory environment.

$P<0.001$; Feb male vs. Sept fem, $P<0.001$; Feb fem vs. Apr fem, $P<0.001$; Feb fem vs. Sept male, $P<0.001$; Feb fem vs. Sept fem, $P<0.001$; Apr male vs. Apr fem, $P<0.001$; Apr male vs Sept male, $P<0.001$; Apr male vs. Sept fem, $P<0.001$).

Gonad volumes

As shown in Fig. 2, males in April had larger testes than in either February or September (one-way ANOVA: $F=8.433$ (5,33), $P<0.0001$, Tukey's post-hoc analysis: $P<0.05$ and <0.001, respectively). Testicular volume did not differ significantly between February and September. Females did not differ in the volume of their largest follicle at any time. However, several females in April had yolking follicles in preparation for ovulation, and some had laid eggs. No females in February or September had yolking follicles or eggs.

Plasma testosterone

Recoveries were 52.02% and intra-assay variation was 1.36%, and the assay detection limit was ~0.1 ng/ml. The mean testosterone concentration per season and sex is shown in Fig. 3. Plasma testosterone did not differ across seasons in females (the high variance in February females is most likely a result of the small sample size and one high value here). Plasma testosterone was higher in April males than in the other two male groups. There was no difference in plasma testosterone between the other male groups (one-way ANOVA: $F=10.15$ (5,33), $P<0.0001$, Tukey's posthoc analysis, Feb male vs. Apr male $P<0.01$; April male vs Sept male, $P<0.001$).

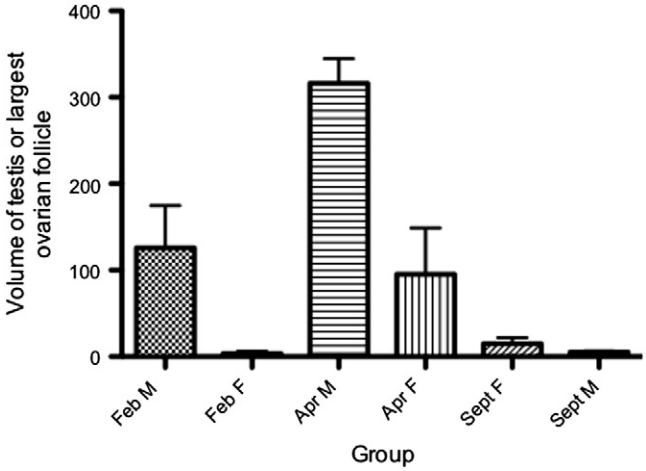

Fig. 2. Gonad volumes of male and female starlings housed in a semi-natural environment during the course of the experiment. Note that during April, females were laying eggs, so that the volume of the largest follicle would be changing in a dynamic fashion at the time of sampling (relative to the testes of males).

Melatonin receptor subtype and ZENK expression in Area X

All 3 melatonin receptor subtypes were expressed in Area X, but Mel 1b was by far the most abundant receptor subtype (one-way ANOVA: $F=75.39$ (2,147), $P<0.0001$, Tukey's post-hoc analysis, Mel 1b vs. Mel 1a or Mel 1C $P<0.0001$). Mel 1a and Mel 1c did not differ from one another in their expression. These data are shown in Fig. 4A. Regression analysis between Mel 1b expression in all birds and ZENK expression in all birds shows a positive correlation: $R^2=0.31$, $P<0.001$(Fig. 4B). Note that this relationship was similar in males and females, so data for all the birds are shown in Fig. 4B.

Discussion

We did not investigate the regulation of IMEL binding in Area X in the lab and the semi-natural environment concurrently and with the direct aim of comparing results from the two environments to one another. Thus, one must bear in mind that there are numerous environmental variables that differ between the two experimental arenas and that might contribute, either separately or synergistically, to the results we observed. Nevertheless, it is clear that the results from our semi-natural environment are overall very different from those from the laboratory. Prior to breeding, IMEL binding in Area X is high in both sexes. This is similar to that seen in the laboratory (Bentley, 2003; Bentley and Ball, 2000; Bentley, unpublished data for female starlings). During the breeding season (within the coarse time-scale used in the present study), male starlings continue to exhibit high IMEL binding, which is the *opposite* of that seen in a laboratory environment. Female starlings that are breeding show the same pattern as in the laboratory: low IMEL binding in Area X. Later on in the year when breeding has terminated, both sexes exhibit low IMEL binding in Area X, which, again, is the opposite of that seen in the laboratory (see Fig. 5 for a diagrammatic representation of the differences observed in the lab and semi-natural ("field") environments). Overall, it appears that in a semi-natural environment,

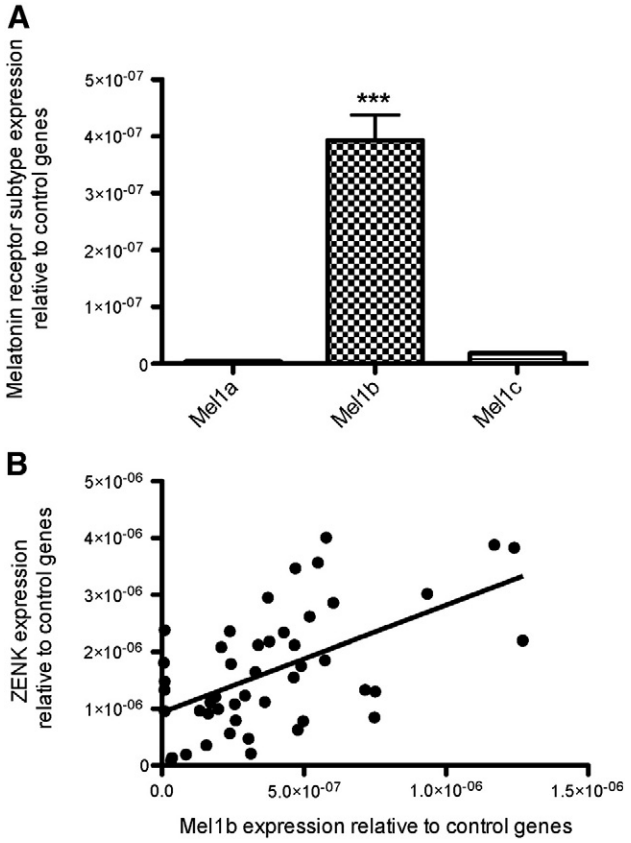

Fig. 4. A) Relative expression of the 3 melatonin receptor subtypes in Area X, Mel 1a, Mel 1b and Mel 1c. Mel 1b is expressed in much greater abundance than Mel 1a or Mel 1c. B) Correlation between expression of the immediate-early gene, ZENK, and Mel 1b in male and female starlings across the reproductive cycle.

starlings show a gradual termination of IMEL binding in Area X as the breeding season progresses, and that this termination occurs first in females as they are laying eggs. The functional significance of this is as yet unclear, and we are currently performing studies using a finer time-scale of sampling to address this. We have no evidence that the prominent sex difference of the size of Area X in this species is related to the sex difference in the timing of melatonin receptor activity.

Considering the plasma testosterone data in Fig. 3 it is tempting to speculate that the changes in IMEL binding in Area X are driven by gonadal steroids, yet our prior study (Bentley and Ball, 2000) indicates that changes in IMEL binding occur independently from changes in gonadal steroids.

Overall, these data collected from starlings housed and allowed to breed in a semi-natural environment differ markedly from those collected from starlings housed in a laboratory environment. This is perhaps not surprising, given the number of lab and field studies that differ in their outcomes (Calisi and Bentley, 2009), and the environmental complexity of our semi-natural environment versus laboratory housing. As yet, the reasons for the differences in results from the two environments are unclear. Major differences in the two housing environments are as follows: exposure to natural light versus artificial light, natural changes in day length versus abrupt, overnight changes in day length (followed by a constant day length), housing in large, mixed-sex aviaries, versus housing in small, single sex cages (but with females in the same room), natural fluctuations in temperature and other climatic conditions versus a controlled and constant indoor climate, access to nest boxes versus no access, and access to a dirt floor for foraging versus a wire floor. Of course, there are also likely to be other, less tangible differences between the two environments. In addition, in the laboratory, birds were housed for almost 6 months on long days to induce and maintain photorefractoriness. In the present study, birds were photorefractory for less time, and experienced decreasing (albeit still long) day lengths. We do not know if a prolonged

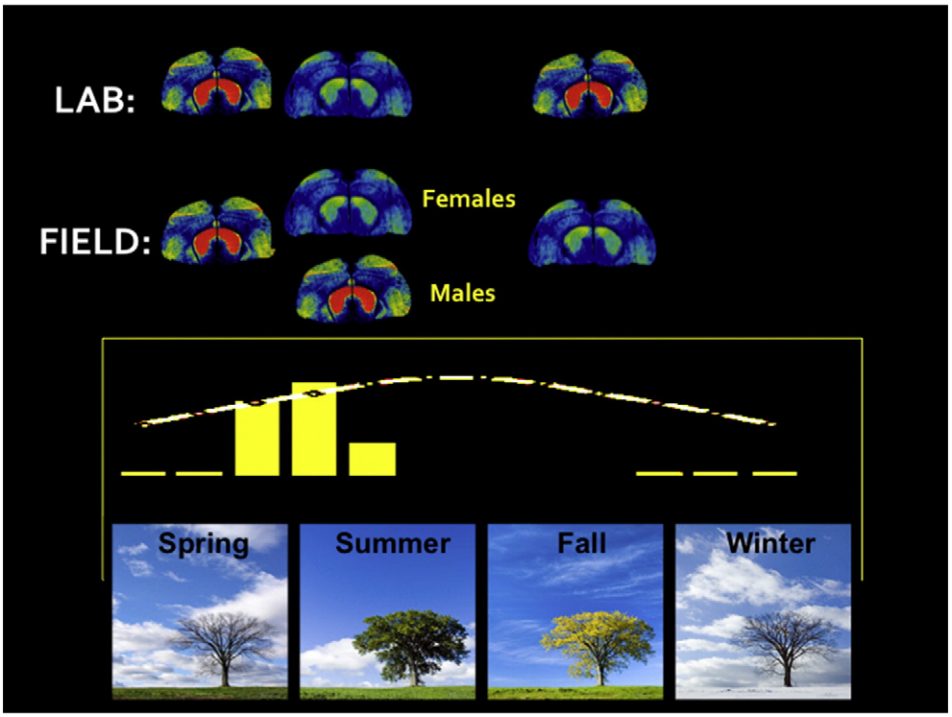

Fig. 5. Diagrammatic representation of a comparison of ^{125}Iodomelatonin binding in Area X in laboratory and "field" environments (here, "field" refers to our semi-natural environment). Autoradiograms are pseudocolor enhanced, and red and white indicated high ^{125}Iodomelatonin binding, with green and blue indicating low binding. Graph with yellow columns and dotted line indicate relative gonad size and annual change in day length. Colored stars indicate sampling dates in the semi-natural environment.

period of photorefractoriness during constant day length causes a change in melatonin receptor activity in Area X. Without performing follow-up controlled studies, it is impossible at this stage to determine which, if any, of these factors influence the results from the two environments.

It is tempting to infer that our data collected from birds housed in a semi-natural environment equate to what would be observed in starlings in the wild, but one cannot do so without additional studies. There is a continuum of environmental complexity from the lab to the field, with our semi-natural environment lying somewhere in between the two. Thus, Area X/IMEL data collected from starlings in the wild might differ from the data presented here. The present study makes it clear, though, that one should be wary of extrapolating from laboratory data to draw conclusions about "real-world" situations for wild species.

In prior studies on melatonin and Area X, our working hypothesis had been that the down-regulation of melatonin binding during photostimulation represented a release of inhibition of some processes in Area X during the breeding season. Starlings do not breed in a laboratory environment, so the present study sought to utilize an environment that permits a full suite of breeding behaviors. The current data indicate that, although IMEL binding in Area X fluctuates in a seasonal manner (as in the laboratory), the pattern of fluctuation is different from that in the laboratory both within and between sexes. As such, our working hypothesis for the functional significance of changes in IMEL binding in Area X has to be revised. Analysis of expression of the immediate-early gene, *ZENK*, as a function of melatonin receptor 1b expression in Area X indicated a direct positive correlation between the two. Whether this means that ZENK expression is driving melatonin receptor expression, or vice-versa, or whether they are regulated independently but in parallel remains to be seen. However, as ZENK is a transcription factor, this correlation indicates that high IMEL binding probably demonstrates high activity of cellular processes within Area X. Thus, the gradual down-regulation of IMEL binding in both sexes as the season progresses likely indicates a gradual reduction in activity in Area X. It is possible that social context influences the activity of Area X in starlings, and thus influences melatonin receptor activity and ZENK expression. In male zebra finches, social context influences the electrophysiological activity of Area X (Hessler and Doupe, 1999), as well as immediate-early gene expression (Jarvis et al., 1998).

An alternative explanation is that subtle differences in singing behavior are driving changes in ZENK expression and IMEL binding. Starlings are open-ended learners and sing year-round, but it is clear that in a closed-ended learner the context of song can have dramatic effects upon the activity of Area X, as already mentioned (Jarvis et al., 1998). The laboratory and semi-natural environment studies discussed here were not conducted with subtle variations in song in mind, so we are unable to compare this between the two environments. It is possible that as social context changes from one season to the next in the semi-natural environment, so does melatonin receptor and ZENK expression. This is worthy of further study, as is the mechanism by which change in social context is perceived by the organism and transduced into an appropriate cellular output in Area X. Further, exactly which aspects of social context (mate presence, potential cuckoldry, territorial encounters, flocking behavior, to name a few that change over the seasons) that might influence Area X activity remain to be identified, but can be tested experimentally.

The fact that IMEL binding is reduced first in female starlings and then in males as the season progresses could result from one or more factors, and might be influenced by the seasonally-changing social environment, as described above. One factor might be as simple as the fact that females reduce their singing activity as they enter into the egg-laying/incubation stage, and males do not immediately do so. For example, males will sit on top or just outside a nest box to sing and display during the egg-laying/incubation stage, whereas song is subsequently greatly reduced during the period of raising young (Bentley, pers. obs). The second could be endocrine changes associated with the switch to a different life-history sub-stage (courtship to incubation). It appears from laboratory studies that melatonin receptor activity changes in Area X occur independently of gonadal steroids (Bentley and Ball, 2000), but there could be changes in neurosteroid activity at this time point that influence melatonin receptor activity. In addition, pituitary prolactin becomes elevated during incubation. It is also possible that social stimuli, such as a mate incubating eggs, could influence the activity of specific brain regions such as Area X. One thing that is particularly striking about regulation of melatonin receptor activity in this large brain area is that presumably the functional significance occurs at night, when melatonin is released into the circulation. It would be worthwhile comparing the behavior of the different sexes at night, when melatonin release and thus activation of its receptor are maximal. Thus, it is an enigmatic phenomenon, although there is some evidence that melatonin influences song organization in zebra finches the day after melatonin administration (Jansen et al., 2005), so a down-regulation of melatonin receptor activity at night in starlings might prevent effects on song organization the following day.

Clearly, mere photostimulation in the laboratory is not a simulation of the breeding season in a semi-natural environment and possibly the wild, as our data from this and our prior studies indicate. It has been known for a long time that female songbirds often will not ovulate/breed in captivity, let alone develop their ovaries to any significant degree, which is why most captive studies on the physiology of wild birds have focused on males (Caro, 2012). Our differing data on Area X from a laboratory and a semi-natural environment could result from a number of factors such as endocrine status, photoperiodic history, and social interactions — or combinations of these. Nonetheless, if we are to understand the true functional significance of physiological phenomena such as changes in cellular activity in Area X, we need to study them in both sexes and in as natural an environment as possible.

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