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Melatonin, immunity and cost of reproductive state in male European starlings

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The effects of reproductive condition and exogenous melatonin on immune function were investigated in castrated European starlings, *Sturnus vulgaris*. Photorefractory and photostimulated starlings exposed to long days were implanted with melatonin or with blank capsules. Photostimulated starlings with blank capsules exhibited reduced splenocyte proliferation in response to the T-cell mitogen, concanavalin A, compared with the other long-day birds. Exogenous melatonin prevented the suppression of immune function by photostimulation. Photorefractory starlings, with or without melatonin implants, exhibited enhanced immune function compared with photostimulated starlings implanted with blanks. This enhancement was not mediated by endogenous melatonin, but appeared to be related to changes in reproductive state. In addition to the traditional costs of reproduction in birds (e.g. raising of young), there may be a cost of the reproductive state of starlings (i.e. whether they are photorefractory or photostimulated). These data are, we believe, the first to indicate a direct effect of reproductive state on immune function that is independent of both photoperiod (i.e. changes in the duration of melatonin secretion) and gonadal steroids.

Keywords: endocrine–immune interactions; photoperiod; photorefractoriness; seasonal breeding; splenocyte proliferation

1. INTRODUCTION

Seasonal breeding of European starlings, *Sturnus vulgaris*, is controlled by day length (Rowan 1926; Burger 1947; and see Nicholls *et al*. (1988) for a review). Lengthening photoperiods during the spring are gonado-stimulatory and cause full reproductive maturation, but prolonged exposure to long days eventually causes the reproductive system to switch off (Hamner 1971; Dawson & Goldsmith 1983; Dawson *et al*. 1985*a*). The reproductive system then remains in a quiescent state. During this period, starlings are reproductively refractory to long days (including further increases in day length), and are therefore said to be in a photorefractory condition. Photorefractoriness in starlings entails a mutually inclusive series of events that occurs 4–6 weeks after exposure to long day lengths (Nicholls *et al*. 1988). The gonads markedly regress as gonadotrophin-releasing hormone (GnRH) content in the hypothalamus wanes to undetectable concentrations, as shown by radioimmunoassay and by immunocytochemical staining of GnRH cell bodies in starlings (Dawson *et al*. 1985*a*; Foster *et al*. 1987). The gonadotrophins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) also decrease to undetectable concentrations in response to the termination of GnRH release. In addition, there is a peak in plasma prolactin that is coincident with the onset of moult. None of these events occurs in the absence of the others, and all are associated with the termination of the breeding season. Photorefractoriness can only be dissipated by a long period of short days (<11.5 h light). The dissipation of photorefractoriness by short days causes the hypothalamo–pituitary–gonadal axis of starlings to enter a state of photosensitivity. Photosensitivity primes the starling reproductive system so that it can mature in response to long day lengths the following spring (i.e. photostimulation occurs), and breeding thus occurs at the appropriate time of year (for reviews, see Farner *et al*. (1983), Nicholls *et al*. (1988) and Wilson & Donham (1988)). The neural and pituitary responses to changing photoperiod occur in exactly the same time-course in gonadectomized starlings as in intact individuals, indicating that the hypothalamo–pituitary interactions in response to changes in day length are independent of gonadal steroids (Dawson & Goldsmith 1984; Dawson *et al*. 1985*b*).

Unlike mammals, birds do not use the duration of nocturnal melatonin secretion to mediate photoperiodically induced reproductive activity (Chakraborty 1995). Indeed, the function of the nightly melatonin signal from the avian pineal gland remains controversial, although melatonin mediates entrainment of circadian activity rhythms in some birds (Menaker & Keatts 1968; Wilson 1991; Juss *et al*. 1993). In birds and mammals, melatonin is secreted from the pineal gland into the circulation during the night, and thus there is a longer period of melatonin release during the winter months than during any other season. The short-day pattern of melatonin release generally enhances immune function in mammals (see Nelson & Demas (1996) for a review).

Reproduction involves trade-offs where benefits, in terms of reproductive success, and costs, as a result of

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investing time and energy towards reproduction, are weighed against one another. The costs of reproduction can be manifest in many ways, but they generally lead to reduced fitness of the parents in terms of future reproductive success and survival (Williams 1966). For this reason, most birds and mammals tend to synchronize reproductive effort with a time of year when environmental conditions are optimal for reproductive success (Nicholls *et al*. 1988; Bronson 1989). Although the evolutionary trade-offs that birds and mammals face during reproduction have been extensively studied (e.g. Hamilton & Zuk 1982; Lindén & Møller 1989), the physiological mechanisms behind evolutionary trade-offs are unclear.

One cost of reproduction is a reduction in an individual's ability to prevent parasitism (Applegate & Beaudoin 1970; Hamilton & Zuk 1982; Møller 1993; Deerenberg *et al*. 1997). Increased susceptibility to parasitic infection has been correlated with a reduction in the immune response to antigenic challenge (Deerenberg *et al*. 1997). Immune response in some species is higher during winter months (i.e. non-breeding periods) than in summer months (breeding periods). The proximate factor driving this seasonal variation in the ability of the immune system to ward off infectious agents appears to be changing photoperiod. For example, immune function in rodents is often boosted during short day lengths (i.e. winter, or non-breeding period) compared with long day lengths (i.e. summer breeding season) (Demas *et al*. 1996; Demas & Nelson 1996; Nelson & Demas 1996). It is not known if similar seasonal fluctuations in immune function occur in birds, although some field studies are consistent with this possibility (Silverin 1981; Gustafsson *et al*. 1994; for a review, see John (1994)).

As already stated, mammals exhibit a seasonal trade-off between energetic investment in reproductive function and immune capability that is mediated by photoperiod (Demas *et al*. 1996; Demas & Nelson 1996; Nelson & Demas 1996). The present study was conducted to determine whether or not immune function in starlings is compromised by sexual maturation as a result of exposure to long photoperiods and, if so, the extent to which it can be enhanced by melatonin administration. Because long days induce sexual maturation followed by sexual quiescence (photostimulation and photorefractoriness, respectively), the effect of different reproductive states during exposure to the same long-day photoperiods was investigated. In mammals, immune function is lowered by high plasma testosterone and is enhanced by castration (Schuur & Verheul 1990; Nelson & Demas 1996). In birds, several indicators suggest that immune function is depressed by testosterone (Kirkpatrick & Andrews 1944; Loose *et al*. 1973; for a review, see John (1994)). Thus, all of the birds used in this study were castrated to remove any confounding effects that testosterone might have on immune function.

2. METHODS

(a) *Birds and treatments*

Twenty-four photorefractory male starlings (held on an 18L:6D cycle, i.e. 18 h light and 6 h darkness per day) were castrated as described below and randomly allocated to one of four groups ($n=6$ per group). They were housed in cages (49 cm × 95 cm × 51 cm; $n=6$ per cage) and supplied with food (turkey starter crumbs) and water *ad libitum*. All groups were held in cages at equivalent positions in separate cage racks. Groups 1 (photorefractory birds to be implanted with melatonin capsules, hence termed PRMEL) and 2 (photorefractory birds to be implanted with blank capsules, hence PRBLANK) remained on 18L:6D for 58 d, during which time groups 3 (eventually to be photostimulated and implanted with melatonin capsules, hence PstimMEL) and 4 (eventually to be photostimulated and implanted with blank capsules, hence PstimBLANK) were transferred to short days (8L:16D) to regain photosensitivity. Once all the birds were in the correct reproductive state for this experiment, the two MEL groups were implanted with silastic capsules containing melatonin. The two BLANK groups were implanted with empty silastic capsules. Each group was transferred to its respective photoperiod on the day of implantation. Groups PRMEL and PRBLANK were maintained on 18L:6D; PstimMEL and PstimBLANK were transferred from short days to 18L:6D in order to photostimulate them. The birds remained on their respective photoperiods for 24 d, at which point they were decapitated and the spleens collected aseptically to assess splenocyte proliferation. A period of 24 d was chosen so that the photostimulated groups had time to become fully photostimulated, but were not exposed to long days for a sufficient period of time to become photorefractory.

(b) *Castrations and melatonin implants*

Photorefractory starlings have regressed testes with a limited blood supply, facilitating castration. Starlings were anaesthetized by intramuscular injection of 3.5 mg secobarbital (sodium salt; Sigma, product no. S-1378), and the testes removed through bilateral incisions between the last pair of ribs. Silastic tubing (a total of 60 mm per bird, 1.47 mm i.d. × 1.96 mm o.d.) containing melatonin (Sigma, product no. M-5250) or left blank was implanted intraperitoneally, using a technique similar to that described for castration. The amount of melatonin used was calculated to give a 'high' dose, as described in Fuchs (1983).

(c) *Splenocyte proliferation assay*

Splenocyte proliferation in response to the T-cell mitogen, concanavalin A (Con A), was determined using a colorimetric assay as described in Demas *et al*. (1996), based on the assay by Cory *et al*. (1991). Briefly, splenocytes were separated from tissue by compressing whole spleens between sterile frosted slides and separated cells were suspended in culture medium. Adjusted aliquots containing *ca*. 100 000 cells were added in duplicate to 96 well-culture plates and varying concentrations of Con A (0–40 μg ml^{-1}) were added. Plates were incubated for 48 h, at which time 20 μl of MTS/PMS (Promega) were added. The optical density of each well was determined with a microplate reader equipped with a 490 nm filter.

(d) *Blood sampling and hormone assay*

Blood samples were obtained 3 d before the termination of the experiment by pricking a superficial wing vein and collecting *ca*. 0.5 ml blood into heparinized glass capillary tubes. The blood was centrifuged at 1500 *g* for 10 min, the plasma separated and stored at −20 °C. Plasma was subsequently assayed for testosterone via radioimmunoassay, as previously described (Bernard & Ball 1997).

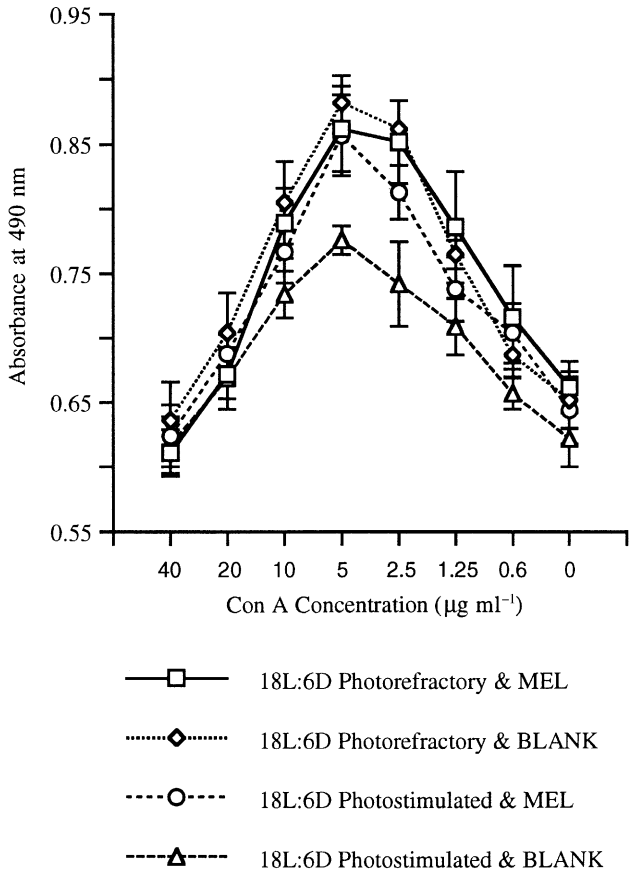

Figure 1. Dose–response curves of starling splenocyte proliferation (represented as absorbance units) in response to the T-cell mitogen, Con A. Birds were implanted with melatonin-filled (MEL) or empty (BLANK) capsules. Data points represent means ± s.e.m.

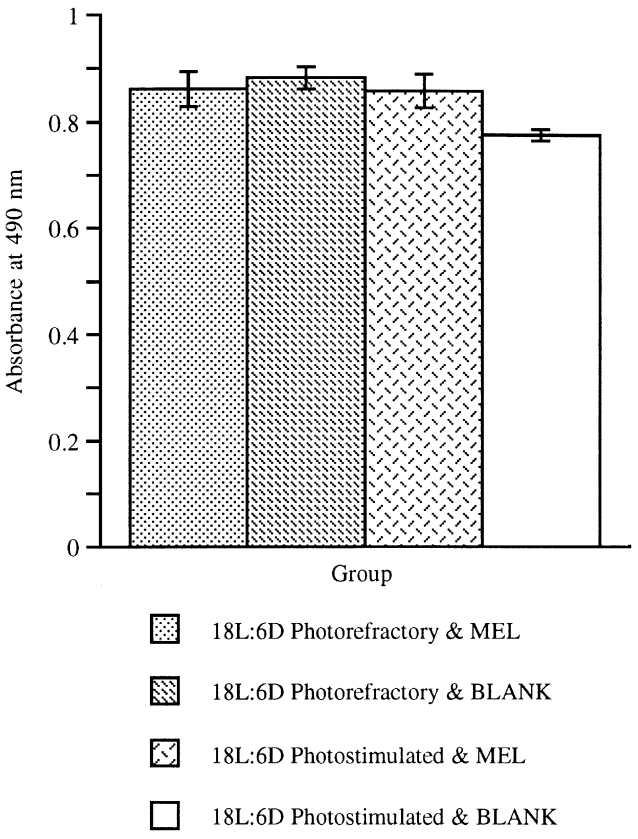

Figure 2. Maxima of the responses of splenocyte proliferation (represented as absorbance units) to the T-cell mitogen, Con A. Birds were implanted with melatonin-filled (MEL) or empty (BLANK) capsules. Data points represent means ±s.e.m.

(e) *Data analysis*

Data taken from the response to the optimal concentration of Con A ($5\,\mu g\,ml^{-1}$, see figure 1) were analysed using one-way analysis of variance (ANOVA), with Fisher's PLSD as the *post hoc* test. Differences between experimental group means were considered statistically significant if $p < 0.05$.

3. RESULTS

Photostimulated starlings exhibited depressed immune function compared with photorefractory starlings, and this depression of immune function was prevented by the administration of exogenous melatonin. As shown in figures 1 and 2, the photostimulated group with blank silastic implants (PstimBLANK) displayed lower splenocyte proliferation to Con A compared with the other groups exposed to long days (PRMEL, $p < 0.05$; PRBLANK, $p < 0.01$; PstimMEL, $p < 0.05$). Groups PRMEL, PRBLANK and PstimMEL displayed no statistical differences in the degree of their response to the T-cell mitogen.

The radioimmunoassay results confirmed that none of the starlings used in this experiment had detectable plasma testosterone concentrations. In addition, their beaks remained black (data not shown). Starling beaks change in colour from black to yellow in the presence of circulating testosterone (Witschi & Miller 1938; Ball & Wingfield 1987), and this is considered a sensitive assay for the long-term presence of low concentrations of testosterone. Thus, we can be confident that there were no confounding effects of testosterone on immune function.

4. DISCUSSION

We believe that our data are the first to show that change in reproductive state, and not simply change in photoperiod, has a significant bearing on the ability of the starling immune system to respond to a mitogenic challenge. In the absence of circulating testosterone, sexually mature (photostimulated) male starlings have compromised immune function compared with male starlings in the non-breeding condition (photorefractory). All groups of birds were housed under the same photoperiod and would have experienced similar cycles of endogenous melatonin release. Thus, the data presented in this paper are novel in that any effects of long days on immune function were not mediated by changes in the release of endogenous melatonin. As a result, we conclude that along with the other well-documented physiological changes that are associated with the termination of the breeding season of starlings, there is an elevation of immune function. As yet, the exact time-course of the onset of this change in immune function remains unspecified.

The temporal association of this change in immune function with the onset of photorefractoriness, and its significance, are unclear. However, it does appear that the attainment of sexual maturity in starlings incurs an energetic cost that is reflected in decreased immune function. There are many physiological and behavioural factors that could incur a cost of this type; increased GnRH and gonadotrophin secretion and higher rates of singing and aggressive male–male interactions could all contribute to the need for a reallocation of energetic resources away from immune function. Factors such as gonadal growth, changes in food supply and territory/mate defence can be excluded as all the birds were castrated and were housed in single-sex groups with free access to food and water.

As already stated, testosterone compromises immune function in birds and mammals (Kirkpatrick & Andrews 1944; Loose *et al*. 1973; Schuur & Verheul 1990; John 1994; Nelson & Demas 1996). Photostimulated starlings have high concentrations of circulating gonadotrophins, and photorefractory starlings do not (see Nicholls *et al*. (1988) for a review). Thus, it may be assumed that the depression of immune function in the photostimulated group would have been more extreme had these birds not been castrated, as they would have had mature testes producing high concentrations of circulating testosterone. It is difficult to hypothesize as to how debilitating the magnitude of suppression of immune function shown in this study might be to an individual. Obviously, the extent of debilitation experienced would depend on the pathogen involved, and the extent to which an individual is infected/infested. For example, suppressed T-cell proliferation would increase susceptibility to a viral infection more readily than to ectoparasite infection. Likewise, the extent of testosterone-induced reduction in immune function in birds is also likely to depend on the pathogen experienced and the immune response measured (Hillgarth & Wingfield 1997). The extent to which testosterone can suppress T-cell mitogenic activity in starlings is the subject of further study.

The results indicate that exogenous melatonin can boost immune function in photostimulated starlings, but not in photorefractory starlings. This is perhaps because immune function is operating at its maximal level in photorefractory starlings: a result of physiological changes associated with the onset of photorefractoriness. Alternatively, it is possible that the photostimulated (non-photorefractory) starlings had higher concentrations of circulating prolactin than the photorefractory groups (Dawson & Goldsmith 1983), and they may have been preparing for the postnuptial moult that is associated with the onset of photorefractoriness. Moult presumably incurs an energetic cost to an individual, and as such could affect immune function. However, starlings do not lose their first feather until 6–7 weeks after photostimulation. Even though preparation for moult (in terms of cell division in the feather follicles to produce new feathers) may have been imminent, the birds used in this study were probably sampled before this energetically draining process had been initiated.

Another possible confounding factor is that transfer from short to long days could be perceived by starlings as either an acute or a chronic stressor and thereby elevate circulating corticosterone. Stress and adrenal steroids are known to suppress immune responses (see McEwen *et al*. (1997) for a review). Thus, it is possible that the immunosuppressive effect of an acute elevation in circulating corticosterone could still be apparent at the time of sampling (24 days after transfer to long days). The degree of stress a starling experiences during an abrupt change in photoperiod, or how long the effects of stress on immune function are apparent in this species, is unknown. Paradoxically, if transfer to long days is perceived by starlings as a chronic stressor, then immune function could become enhanced (McEwen *et al*. 1997); there are also a small number of reports of specific conditions of acute stress that can enhance immune function (Dhabhar & McEwen 1996). It must also be noted that basal blood concentrations of corticosteroids are essential to antibody formation; they can also stimulate spleen differentiation in chickens (Betz & Nagler 1978; John 1994). Thus, there appears to be no consistent effect of stress on immune function, making it difficult to predict how an increase in photoperiod might affect immune function in starlings.

To summarize, maximal photostimulation of the starling reproductive axis was shown, in the absence of gonadal steroids, to suppress immune function. At some time point during or after the transition from a photostimulated to a photorefractory state, immune function in starlings is enhanced. This change in immune function is independent of changes in the duration of release of endogenous melatonin, as all birds were exposed to the same long-day photoperiod. Exogenous melatonin enhances immune function in photostimulated starlings, bringing it to levels similar to those in photorefractory starlings. The immune response of photorefractory starlings was not altered by melatonin treatment in this study. Reproductive state seems to have a strong bearing on how a starling's immune system will respond to antigenic challenge. The ways in which testosterone, prolactin, moult and stress might act to affect immune function in starlings require further study.

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