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MELATONIN, AGGRESSION, AND SOCIAL DOMINANCE IN BLACK-CAPPED CHICKADEES (POECILE ATRICAPILLUS)

Buddhamas Kriengwatana

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MELATONIN, AGGRESSION, AND SOCIAL DOMINANCE IN BLACK-CAPPED CHICKADEES (POECILE ATRICAPILLUS)

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Buddhamas Kriengwatana

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London, Ontario, Canada

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CERTIFICATE OF EXAMINATION

Supervisor	Examiners
Dr. S. MacDougall-Shackleton	Dr. E. Hampson
Supervisory Committee	Dr. W. Roberts
Dr. E. Hampson	Dr. L. Zanette

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ABSTRACT

Non-breeding black-capped chickadees form linear dominance hierarchies in the winter. Dominants establish and maintain status by repeatedly directing aggression towards subordinates despite having regressed testes and likely also basal levels of testosterone. Melatonin has been shown to facilitate aggression in some animals under non-breeding conditions. The first aim of this study was to investigate whether melatonin was associated with aggressive black-capped chickadee dominant-subordinate interactions during the establishment of dominance status. The second aim of this study was to determine whether exogenous melatonin affected aggressive vocalizations more than non-aggressive vocalizations. Plasma melatonin was not significantly correlated to aggression. Moreover, exogenous melatonin treatment had no effect on aggressive or non-aggressive vocalizations. Results from this study therefore indicate that there is no relationship between melatonin and aggression and also no relationship between melatonin and dominance status in black-capped chickadees.

KEYWORDS: melatonin, aggression, dominance hierarchy, black-capped chickadee

CO-AUTHORSHIP

This thesis incorporates material that is result of joint research by Buddhamas Kriengwatana, Yong Seok An, Amy E. Newman, Elizabeth A. MacDougall Shackleton, and Scott A. MacDougall-Shackleton. Buddhamas Kriengwatana conceptualized the experimental design, performed analysis on all samples, interpreted data, wrote the manuscript, and acted as corresponding author. In chapter 2, Yong Seok An provided essential aid in data collection, Amy E. Newman provided crucial advice regarding assay validation and evaluation of statistical tests performed on data, and Elizabeth A. MacDougall-Shackleton performed experimental procedures required to determine sex of subjects. Scott A. MacDougall-Shackleton supervised development of work and helped in data interpretation and manuscript evaluation.

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CHAPTER 1

GENERAL INTRODUCTION

Dominance hierarchies are common in social animals. Hierarchies help determine which individuals have priority access to resources. As a result, an individual's rank in a dominance hierarchy can have immediate and long-term consequences on survival and reproduction. Many animals, including black-capped chickadees, use aggression to establish and maintain rank (Ekman, 1989; Forkman & Haskell, 2004; Ratcliffe et al., 2007). However, mechanisms supporting dominance-related aggression in black-capped chickadees are currently unclear. In this chapter I will first describe the nature of dominance hierarchies in black-capped chickadees and the role aggression plays in shaping and maintaining hierarchy rank. Second, I will discuss mechanisms of aggression and the potential influence of the hormone melatonin on these mechanisms of aggressive behaviour. Lastly, I will present the experimental hypotheses and objectives.

1.1 SOCIAL DOMINANCE IN BLACK-CAPPED CHICKADEES

Black-capped chickadees (*Poecile atricapillus*) are small songbirds belonging to the family Paridae that inhabit almost two-thirds of North America (Smith, 1997). They are seasonally breeding, non-migratory birds that form monogamous breeding pairs in the spring and summer, but live in non-breeding flocks of about four to twelve birds during the fall and winter (Smith, 1991). Black-capped chickadee flocks consist of local adults (usually mated pairs) that have survived the breeding season and unrelated juveniles that have dispersed from their natal territory (Smith, 1991). Flocks forage and defend their territories of roughly 6.5 ha to 14.6 ha through vocalizations and other aggressive behaviors (Smith, 1993).Within flocks, chickadees establish a stable, linear dominance hierarchy where every member has its own position or rank relative to other members (Smith, 1991). These hierarchies are described as linear because one bird maintains the most dominant position at the top the hierarchy with all other flock members subordinate to that bird, while a second bird is subordinate to the top bird but is dominant over all remaining flock members, and so on (Smith, 1976). Evidence suggests that there is considerable competition for positions in dominance hierarchies (Ekman, 1989), which is not surprising as rank in the flock dominance hierarchy can have important consequences on a chickadee's behaviour, survival, and reproduction (Smith, 1991; Ekman, 1989; Ficken et al., 1990; Zanette & Ratcliffe, 1994; Otter et al., 1999).

Animals in social groups often establish dominance hierarchies to reduce potentially costly conflict between other members (Lockie, 1956). Status and rank in dominance hierarchies enable members to more reliably predict the outcome of agonistic encounters against other group members and use this information to help decide whether to partake in or withdraw from a fight (Drews, 1993). One very important risk of fighting is the potential for the fight to escalate, as escalated fights increase costs for both sides. Many social birds avoid escalated fights by establishing a "peck order" dominance hierarchy, where the dominant bird is always aggressive towards the subordinate bird but the subordinate never reciprocates the aggression (Schejelderupp-Ebbe, 1922 in Drews, 1993). In other words, the subordinate always submits to the dominant, thus eliminating escalation. Therefore, dominance hierarchies provide a useful way for animals to assess whether advantages of behaving aggressively, such as gaining priority access to resources, outweighs the risks of behaving aggressively, such as needlessly using energy.

While individuals in social groups benefit from increased predator protection and foraging (see Ekman, 1989), individuals that achieve high rank in the dominance hierarchy gain additional advantages. In black-capped chickadees, high status appears to be correlated with factors that increase both winter and lifetime fitness. In the winter, dominants have priority access to food resources and safer foraging sites. They also demonstrate more predator-avoidance behaviours, which altogether helps to explain why higher-ranked birds are more likely to survive winter than lower-ranked birds (Ekman, 1989; Desrochers, 1989; Ficken et al., 1990; Zanette & Ratcliffe, 1994). Additionally males that ranked high in the dominance hierarchy during the previous non-breeding season are more attractive to females, are more likely to have extrapair copulations, and produce nestlings that are more likely to survive and fledge in the breeding season (Otter & Ratcliffe, 1996; Smith, 1991; Otter et al 1994; Otter et al., 1999). While there are costs associated with acquiring and maintaining high social rank (see Hogstad, 1987), the advantages of being dominant appear to outweigh such costs (Ekman, 1989). Thus, dominant members of a dominance hierarchy derive more benefit overall compared to subordinate members.

Dominant individuals can usually be distinguished morphologically and behaviourally from subordinate individuals. In black-capped chickadees, dominance is strongly correlated with age, sex, condition, plumage, vocalizations, and the social rank of their partner (Ratcliffe et al., 2007). More specifically, dominant birds tend to be older males and superior singers compared to subordinate birds (superior singers are better able to maintain song stereotypy) (Smith, 1991; Otter et al., 1999; Glase, 1973; Christie et al., 2004). Dominant birds also tend to have darker black caps with more distinct plumage contrast and less body fat than subordinate birds (Ratcliffe et al., 2007; Schubert et al., 2006). Furthermore, Smith (1991) suggested that the most important factor in determining a female's rank is the rank of her partner, and that juveniles paired with adults can also elevate their rank. In other words, a female or juvenile chickadee's partner's social rank may help to determine its own.

In black-capped chickadee dominance hierarchies, escalated fights are uncommon and aggression is reduced, but not completely eliminated. This is because aggressive displays and rituals, more often than aggressive physical fights, are used to determine and maintain hierarchy rank (Smith, 1991; Ratcliffe et al., 2007). Research by Ratcliffe and colleagues (2007) suggest that black-capped chickadees form and maintain dominance hierarchies consistent with the predictions made by the suppression hypothesis (see Forkman & Haskell, 2004). This hypothesis predicts that aggressive interactions should occur more often between individuals that hold adjacent ranks, but that the cost of any one fight should be low because status is determined by the cumulative outcome of fights. Moreover, dominant individuals will repeatedly attack lower ranking individuals in order to prevent the subordinates from attempting to challenge their status. In accordance with the predictions, dominant chickadees repeatedly instigate low-cost, aggressive actions towards subordinate flock members (Ratcliffe et al., 2007). Such actions include displacing or chasing the subordinate bird from a feeder, or eliciting a submissive posture. The researchers consider these interactions low cost when compared to aggressive behaviours observed in the breeding season, such as physical fights that sometimes result in injury. Often the consequence of low cost encounters in the non-breeding season is simply that the subordinate has to wait until the dominant bird has left before it can have access to the feeder (Ratcliffe et al., 2007). Thus, aggression clearly plays an important role in black-capped chickadee winter flocks, whereby dominant birds exhibit aggression towards subordinate birds in order to establish and maintain their social rank in the dominance hierarchy.

1.2 SEASONAL MECHANISMS OF AGGRESSION

Aggression is a complex set of social behaviours that are controlled by multiple endocrine systems that respond to various environmental stimuli. Androgenic steroid hormones synthesized primarily by the gonads (and to a lesser extent by tissue such as the adrenal glands and the brain; Soma et al., 2008) such as testosterone are thought to play a central role in regulating aggression. This is because testosterone and aggression are often highly correlated, especially in male-male breeding aggression. Specifically, high levels of testosterone are often associated with increased aggressive behaviour (e.g. Wingfield, 1984). However, the relationship between androgens and aggression is not simply a positive correlation between levels of circulating testosterone and aggressive behavior (e.g. Wingfield & Hahn, 1994). For example, many effects of testosterone on aggression are actually due to its metabolism into estradiol in the brain as well as its interactions with other steroidal and non-steroidal hormones (Soma et al., 2008; McCarthy, 1995). Non-steroidal hormones involved in the regulation of aggression include serotonin, catecholamines, oxytocin, arginine vasotocin (mammalian arginine vasopressin), glucocorticoids, and neurotransmitters such as GABA and dopamine (Kravitz, 2000; Haller et al., 1998a, 1998b; Campbell, 2008; Cadwell et al., 2008; Soma et al., 2008).

In a number of animal studies, castration does not decrease aggression (e.g.

Wingfield & Hahn, 1994; Jasnow et al., 2001; Fleming et al., 1988). In these cases, activation of aggression is probably not highly dependent on gonadal steroids. Black-capped chickadee non-breeding aggression is also likely regulated by gonadal steroid-independent mechanisms because chickadees have regressed testes in the winter (Phillmore et al., 2006). Song sparrows (*Melospiza melodia morphna)* that live along the west coast also display high levels of aggression in the non-breeding season when their testes are regressed and levels of circulating testosterone are non-detectable (Wingfield & Hahn, 1994). These birds are non-migratory and defend their territories and sing year-round. Studies on the male sparrows indicate that their territorial aggression in the breeding season is activated by gonadal steroids, whereas their territorial aggression in the non-breeding season is activated by non-gonadal steroids, even though the quantity and quality of their aggression is consistent between the two seasons (Wingfield & Soma, 2002, Soma, 2008). Mechanisms that maintain non-breeding aggressive behaviour that do not rely on high levels of circulating gonadal testosterone are likely adaptive. For black-capped chickadees, aggression in the non-breeding season helps them to secure rank and access to resources. However, sustaining functioning gonads that synthesize high levels of circulating testosterone during the winter would be costly because elevated concentrations of testosterone often result in increased metabolic rate, decreased body mass and fat stores, lowered immunocompetence (Soma & Wingfield, 2001; Wingfield, 1984; Ketterson et al., 1991, Owen-Ashley et al., 2004), and maintenance of breeding condition at an inappropriate time of year. Activating gonad-independent mechanisms of aggression in the non-breeding season is thus a mechanism by which black-capped chickadees could avoid the energetic costs associated with high testosterone levels while still maintaining aggression required to preserve rank in the flock dominance hierarchy.

Assuming that black-capped chickadees rely on gonad-independent mechanisms to sustain aggression in the non-breeding season, these mechanisms of aggression should be seasonally activated. In addition to research on song sparrows, there is some evidence in mammals to support this hypothesis. Male Siberian hamsters (*Phodopus sungorus)* housed on short days that mimic non-breeding, winter photoperiod display increased aggression in a resident-intruder test despite gonadal regression or gonadectomy (Jasnow et al., 2000). Female golden hamsters (*Mesocricetus auratus*) held on short days also exhibit higher levels of aggression than those held on long days, and ovariectomized animals held on long days do not show the same level of aggression as animals held on short days (Fleming et al., 1988). Overall, these studies suggest that photoperiod-dependent increases in aggression in hamsters are likely mediated by non-gonad hormones.

1.3 AGGRESSION AND MELATONIN

If different mechanisms for black-capped chickadee breeding and non-breeding aggression exist, then a physiological trigger is needed to activate and maintain these mechanisms at the appropriate season. Black-capped chickadees show seasonal changes in behavior and morphology that vary with photoperiod (MacDougall-Shackleton et al., 2003), which suggests that they use photoperiod as a cue to current or changing seasons. Changes in photoperiod involve a change in the ratio of hours of light (photophase) to hours of darkness (scotophase). Because melatonin release is primarily during the scotophase, many animals use this hormone as a measure of photoperiod. Melatonin is produced by the pineal gland and synthesis can be inhibited by light (e.g. Yamada et al., 1988). In birds, the pineal gland responds to photic stimuli, thus melatonin release is sensitive to current environmental conditions. As a result, melatonin levels fluctuate daily, and release is synchronized with night length such that levels are low during the day and high at night. Winter, non-breeding conditions extend the duration of nighttime melatonin release due to the shorter days and longer nights in the winter. In this manner, pattern of melatonin release can function as an indicator of seasons, and melatonin levels have been found to vary seasonally. In the Indian Jungle Bush Quail (*Perdicula asiatica*), nighttime concentrations of melatonin increase gradually in August and peak in December, after which levels slowly decline and remain low from March to June (Singh & Haldar, 2007). Duration of melatonin release can also affect processes that undergo seasonal changes in European starlings (*Sturnus vulgaris*), such as immune function and neuroplasticity of the song control system (Bentley, 2001). Therefore, melatonin may cue and support the action of alternative mechanisms to support aggression in the non-breeding season when gonad-dependent mechanisms of aggression are not available.

In mammals, increasing melatonin may facilitate aggression by interacting with adrenal hormones (e.g. Paterson & Vickers, 1981; Demas et al., 2004). Exogenous melatonin treatment increases territorial aggression (Paterson & Vickers, 1981; Jasnow et al., 2002) and pinealectomy eliminates this aggression in mice (Fleming et al., 1988). Additionally, injecting adrenalectomized mice with melatonin makes them less aggressive, whereas injecting intact mice with melatonin makes them more aggressive (Paterson & Vickers, 1981). Melatonin also seems to be affected by agonistic disputes over dominance status. Melatonin concentrations increase immediately after losing an aggressive interaction, and repeated agonistic encounters induce a temporal delay in nighttime rise of melatonin levels in male Mongolian gerbils (*Meriones unguiculatus*) (Heinzeller et al., 1988). Similar results were found by Fuchs & Shumacher (1990) in tree shrews (*Tupaia belangeri*): subordinates had elevated levels whereas dominants had reduced levels of a melatonin metabolite in their morning urine after 10 days of social interaction. In rainbow trout (*Oncorhynchus mykiss*), subordinates tend to exhibit increases in nighttime melatonin levels whereas dominants tend to show no effect (Larson et al., 2004). Overall, these results suggest that increasing melatonin can facilitate aggression and that a subordinate status is associated with higher nighttime melatonin levels.

Melatonin may also regulate aggressive behaviour by activating mechanisms of aggression that are advantageous in only a particular season in birds. To the best of my knowledge, the song sparrow is the only avian species in which the relationship between melatonin and aggression has been studied. Similar to what was found in rodents, exogenous melatonin increases territorial aggression in male, non-breeding song sparrows (B. Kriengwatana, unpublished data). Wild, male song sparrows were given either empty or melatonin-filled silastic implants, and their response to a simulated territorial intrusion was observed approximately one week later. Interestingly, birds given melatonin were faster to respond and sing aggressively to a conspecific intruder than controls. This finding is particularly interesting because it suggests that melatonin may increase specific agonistic behaviours, namely vocalizations, as opposed to increasing overall aggression. This idea is supported by studies demonstrating that melatonin treatment can affect volume of regions of the avian song control system, where melatonin receptors have been identified (Bentley et al., 1999; Gahr & Kosar, 1996). Further work is necessary to determine the role of melatonin in mediating aggression, territoriality, and singing behaviour.

1.4 EXPERIMENTAL HYPOTHESES & OBJECTIVES

Based on the findings reviewed above, I propose that melatonin affects aggressive behaviour of black-capped chickadees in the context of forming and maintaining flock dominance hierarchies in the non-breeding season. This hypothesis makes the following assumptions:

I. There are seasonal differences in levels of melatonin in black-capped chickadees.

II. Gonad-independent mechanisms of aggression mediate dominance-related aggressive behaviour in the non-breeding season, but not aggressive behaviours in the breeding season.

III. Melatonin affects aggressive songs vocalizations more than other aggressive behaviors.

IV. Dominant and subordinate individuals have different levels of melatonin.

Therefore the hypothesis makes the following predictions:

i. Melatonin levels in the non-breeding season are higher than in the breeding season.

ii. Phillmore et al. (2006) have demonstrated that chickadees have regressed testes in the winter, thus agonistic behaviour is displayed in the non-breeding season despite non-functioning gonads.

iii. Individuals with higher levels of melatonin are expected to sing more or show the winter, thus agonistic behaviour is displayed in the non-breeding season despite non-functioning gonads.

iii. Individuals with higher levels of melatonin are expected to sing more or show more change in song characteristics or vocalizations.

iv. Dominant birds have higher levels of melatonin during the day, but subordinates have higher levels of melatonin at night.

v. Individuals with higher levels of melatonin during the day are more aggressive than individuals showing high levels of melatonin at night.

In Chapter 2, I will quantify daily and seasonal fluctuations in plasma melatonin in order to test the prediction that melatonin levels in the non-breeding season are higher than in the breeding season. I will also examine the effect of paired interactions on melatonin in order to investigate whether melatonin is associated with aggression and formation of dominance status. In Chapter 3, I will discuss the effect of artificially extending the duration of elevated plasma melatonin levels on black-capped chickadee aggressive vocalizations. Last, in Chapter 4, I will provide a general discussion of the role of melatonin in black-capped chickadee aggression and social dominance based on the findings described in the previous chapters.

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CHAPTER 2

HOW DOES MELATONIN CHANGE DAILY, SEASONALLY, AND IN AGGRESSIVE ENCOUNTERS THAT ESTABLISH DOMINANCE STATUS IN BLACK-CAPPED CHICKADEES (*POECILE ATRICAPILLUS*)?

2.1 INTRODUCTION

Black-capped chickadees are a non-migratory species that form non-breeding flocks in the winter. Flocks have a linear, stable dominance hierarchy. Achieving high rank in the hierarchy has important consequences for winter survival and lifetime fitness (Desrochers, 1989; Ficken et al., 1990) as higher-ranking birds often obtain priority access to resources such as food and safer foraging sites, which makes them more likely to survive winter than lower-ranking birds. Birds that were dominant in a previous winter also appear to be more successful at acquiring mates and raising offspring in the following breeding season (e.g. Otter & Ratcliffe, 1996; Smith, 1991).

Flock hierarchies are established and maintained through aggressive interactions, whereby dominants attempt to suppress subordinates' efforts to move up in the hierarchy (Ratcliffe et al., 2007). Dominant birds tend to be more aggressive than subordinate birds, and their aggression is always targeted towards individuals of lower rank (Ratcliffe et al., 2007). Clearly, sustaining aggression is necessary for wintering black-capped chickadees in order to preserve flock hierarchy rank.

Dominance-related aggression in non-breeding season is likely mediated by gonadal steroid-independent mechanisms because chickadees have regressed gonads in the winter (Phillmore et al., 2006). There is some evidence that animals may have distinct mechanisms for regulating aggression in the breeding versus non-breeding season. For example, photoperiod seems to have a greater influence on aggressive behaviour than gonad condition in hamsters (Jasnow et al., 2000; Fleming et al., 1988). Furthermore, activation of gonad-independent mechanisms during the non-breeding season may allow animals to reduce energetic and immunosuppressive costs associated with high levels of circulating gonadal steroids while still maintaining an appropriate amount of aggressive behaviour (Soma & Wingfield, 2001).

Black-capped chickadees show seasonal changes in behavior and morphology that vary with photoperiod (MacDougall-Shackleton et al., 2003), so activation and maintenance of gonadal steroid-independent mechanisms of aggression could be under the control of the hormone melatonin. Melatonin is produced primarily by the pineal gland in the absence of light (e.g. Yamada et al., 1988; Lewy et al., 1980), meaning that changes in the duration of melatonin release is photoperiod dependent. As a result, levels of circulating melatonin can be used as an indicator of seasons. Melatonin patterns associated with winter conditions could thus activate gonadal steroid-independent mechanisms of aggression during the non-breeding season.

Several studies demonstrate a link between melatonin and aggression, although the relationship seems to be complicated by time of day when measurements are taken. Exogenous melatonin also increases territorial aggression in mice and hamsters by interacting with adrenal hormones (Paterson & Vickers, 1981; Demas et al., 2004). In song sparrows, artificially increasing melatonin appears to increase specific type of aggressive behaviour – singing (B. Kriengwatana, unpublished data). These findings suggest that increased melatonin in the daytime is associated with elevated aggression, and may in this way be linked with a dominant status because dominant individuals are usually more aggressive. Interestingly, melatonin concentrations also rise immediately after male gerbils lose a territorial encounter (Heinzeller et al. 1988), suggesting that subordinates can also experience changes in diurnal melatonin in response to aggressive encounters. Nevertheless, changes in nocturnal melatonin appear to be more strongly associated with subordinate status (Larson et al., 2004; Heinzeller et al., 1988). As a result, altered nocturnal melatonin secretion may be associated with reduced aggression, as subordinate animals tend to be less aggressive. For example, in rainbow trout (*Oncorhynchus mykiss*) where only dominants display aggressive behavior towards subordinates, subordinate individuals tend to have higher levels of nighttime melatonin than dominant individuals after participating in a dominance interaction (Larson et al., 2004). Male gerbils that repeatedly lose territorial encounters also display a temporal delay in the rise of nocturnal melatonin when compared to controls (Heinzeller et al., 1988). Overall, whether higher melatonin is related to increased or decreased dominance status remains ambiguous, and the relationship between melatonin, aggression, and dominance status is further obscured by the effect of time of day which measurements are taken.

This study aims to describe daily and seasonal changes in plasma melatonin and to determine the relationship between melatonin and dominance status-related aggression in non-breeding black-capped chickadees. This experiment will be the first to quantify circulating levels of melatonin in black-capped chickadees and investigate the role of melatonin in shaping dominant-subordinate relationships in birds. I took blood samples at various times during the day and night and examined the effect of aggression during dominance status formation on melatonin levels in order to test the following two hypotheses and their predictions:

H1: There are seasonal differences in levels of melatonin.

Prediction: Melatonin levels in the non-breeding season are higher than in the breeding season.

H2: Dominant and subordinate individuals have different levels of melatonin.

Prediction: Dominant birds are more aggressive and have higher levels of melatonin during the day but subordinates have higher levels of melatonin at night.

2.2 METHODS

2.2.1 Subjects

Nine male and ten female black capped chickadees were captured from five different geographical sites in London, Ontario. All birds were captured using walk-in traps, between November and December 2007. Age was determined by examining the outer tail feathers (Meigs et al., 1983) to ensure that only birds hatched in 2007 were included in the studies in order to control for age as a factor in influencing dominance status. Subjects were then individually housed in 24 x 30 x 37 cm cages, given *ad libitum* food (mixture of sunflower seeds and Mazuri small bird maintenance diet) and water, and maintained on a 10L:14D light cycle. Cages were set up so that subjects could only see other members from the same flock and were thus visually isolated from birds that belonged to other flocks. Subjects were given at least two weeks to acclimate to laboratory conditions before any procedures were performed.

2.2.2. Blood sampling for daily and seasonal changes in melatonin

Blood samples were taken from the brachial vein at four different times throughout the day from all subjects: 1 hour after lights on (ON), midday (MD), 1 hour after lights off (OF), and midnight (MN). A sterile 26 ½ G needle was used to puncture the brachial vein and venous blood was collected in heparinized capillary tubes. Night sampling was done under weak blue light, achieved by covering a flashlight with a blue lens filter (Roscolux colour filter #74; Rosco Manufacturing, Glendale, CA). Short wavelengths have been suggested to be minimally photostimulatory (Kumar et al., 2000), and this blue filter effectively prevents wavelengths other than those of approximately 460 nm to penetrate. All subjects were bled within 30 minutes after entry into the colony room. Approximately 50 uL of blood was collected per session and subjects were given at least 10 days to recuperate in between each blood sampling session. Additionally, hematocrit levels were measured and recorded after every blood sampling session to monitor subjects' health. Blood samples were centrifuged immediately after collection and plasma was extracted and stored at -20° C for quantification of melatonin. The light cycle was then switched from 10L:14D to 15L:9D to simulate photic conditions during breeding season, and 5 weeks later the above blood sampling procedures were repeated at the same time of day (i.e. at ON, MD, OF, and MN).

2.2.3 Paired interaction procedures

Sixteen black-capped chickadees (10 females, 6 males) used in the first study were assigned to pairs matched by sex and size. Sex was determined by PCR and size was determined by measuring the wing cord. To avoid the problem that a dominance status may already exist for any particular pair, all birds were paired with unfamiliar individuals from a different flock. Each bird was colour banded to facilitate identification, and two pairs were tested at the same time.

On the first day of testing, the focal pairs were transported from their individual cages into a separate testing room with vertically stacked cages measuring 76 x 45 x 45 cm, each containing one cup for food and one for water. Pairs of birds were tested such that each pair occupied one cage for the rest of the experiment. The subjects' first interaction was video recorded for 60 minutes immediately after subjects were released into the novel cage. After the 60 minute trial, 25uL of blood was collected from each subject from the brachial vein within 20 minutes. The following night, 30 hours after the first interaction, another 25uL of blood was collected from the brachial vein. Similar to the first study, blood sampling at night took place under a weak blue light. Subjects remained paired and approximately 50 uL of blood was collected once more during the day, 10 days after the night blood sample was taken. Following this, subjects were separated and housed individually for at least 7 days, and the experiment was repeated with different pairs of subjects (see Fig. 2.1 for timeline).

2.2.4 Coding and scoring paired-interactions

Four aggressive behaviours were recorded and scored: gargle, gape, supplant, and chase. The behaviours were categorized and defined based on descriptions by Popp et al. (1990), Smith (1991), and Otter et al. (1998). (A summary of the definitions for each of the behaviours are given in Table 2.1). Gargles were defined as vocal threat displays that are made up of a combination of 2 to 11 notes, with a short interval between each note (Ficken, 1981). Gape was scored if a bird had its bill open and was facing its opponent. Supplant was scored if a bird displaced its opponent by lunging or flying at it, and chase

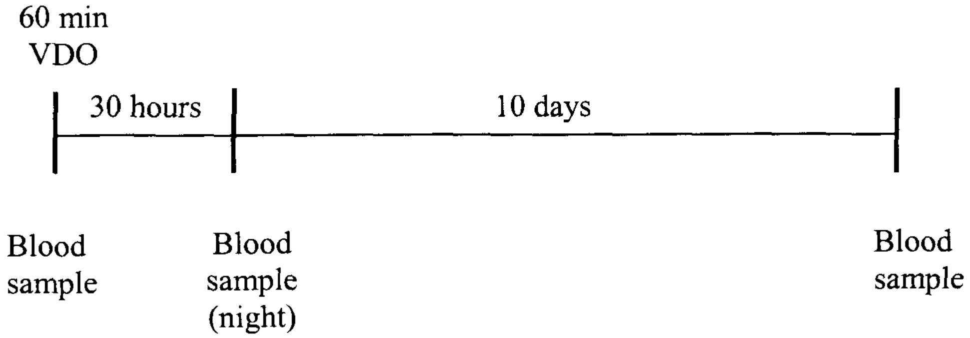

Fig. 2.1. Timeline for paired interaction study. This procedure was repeated with different pairs of individuals 7 days after the last blood sampling session.

Behavior	**Description**	**Reference**
Gargle	*A* gives gargle vocalization	Smith (1991) Popp et al. (1990)
Gape	*A* has bill open and pointed at *B*	Smith (1991) Popp et al. (1990)
Supplant	*A* displaces perched B by lunging or flying at *B*	Popp et al. (1990) Otter et al. (1998)
Chase	*A* flies in pursuit of *B*	Otter et al. (1998)
Immediate departure	*B* departs immediately from perch or food cup upon the arrival of *A* on the same perch or cup	Popp et al. (1990)

Table 2.1. Operational definitions used for coding and scoring paired interactions. For illustration purposes, the dominant pair member is represented by "*A*" and the subordinate member is represented by "*B*".

was scored if a bird flew in pursuit of its opponent.

Aggressive behaviours have been found to be positively correlated with dominance status (see Ratcliffe et al., 2007). Consequently a separate, non-aggressive behaviour was scored and used as an indication of status. Dominance status was determined by frequency of immediate departures from the feeder, whereby the bird exhibiting more immediate departures was defined as the subordinate member. The reason for choosing this behaviour as a signal of status is based on results from Popp et al. (1990), who found that lower ranking birds were more likely than higher ranking birds to depart immediately upon the arrival of another bird at the feeder. Immediate departures were scored for an individual if it left the perch or food cup immediately upon the arrival of the second bird on that perch or food cup. Although winners of dominance encounters in field studies are typically determined as the first bird able to obtain a seed from the feeder or the last bird to leave the feeder (e.g. Popp et al., 1990), this operational definition was not suitable for the current experiment. This is because subjects were tested in a small, enclosed space and were not food deprived prior to testing. Consequently, dominants may have not been motivated to gain first access to food, and in many cases, the pairs appeared to take turns feeding from the food cup (B. Kriengwatana, personal observation).

2.2.5 Enzyme immunoassay procedures and validation

Blood samples were assayed for melatonin using a commercial ELISA kit (Genway Biotech Inc., San Diego, CA), following the manufacturer's instructions. The assay procedure follows the basic principle of competitive ELISA whereby a biotinylated and non-biotinylated antigen compete for a fixed number of antibody binding sites.

Quantification of samples is achieved by comparing the enzymatic activity of samples with a response curve prepared by using known standards. Note that the volume of plasma samples collected was below the volume required by the kit, so each sample was diluted with assay buffer to meet the required volume and obtained melatonin values were individually adjusted according to the plasma volume assayed. Standard curves were fitted with 4-parameter logistic interpolation.

The ELISA kit used in this study was designed for quantification of melatonin in human plasma. There were two major potential problems with using an assay intended for use with human plasma to measure melatonin in avian plasma. First, nocturnal melatonin concentrations have been found to be considerably higher in birds than in humans (e.g. Bentley, 2003; Brandstatter, 2003; Benot et al., 1999). Second, the measured value may not reflect the actual concentration of melatonin present when using avian samples instead of human samples in the assay. This inaccuracy could be partially due to decreased antibody affinity and increased cross-reactivity that result from differences in human versus avian plasma. To determine whether the kit was suitable for measuring melatonin levels in avian samples, I conducted serial two-fold dilutions of pooled black-capped chickadee plasma samples, taken once during the day and once at night. Standards used for the validation ranged from 3, 10, 30, 100, 300 pg/mL. Each dilution was detectable but demonstrated decreases that were slightly above or below expected values (Fig. 2.2), which may have been due to the small quantity of plasma used in the assay (55 uL of plasma was used for the 1:1 dilution). The intra-assay coefficient of variation for the serial dilution was 9.0%.

All samples obtained from an individual were assayed together on one plate when

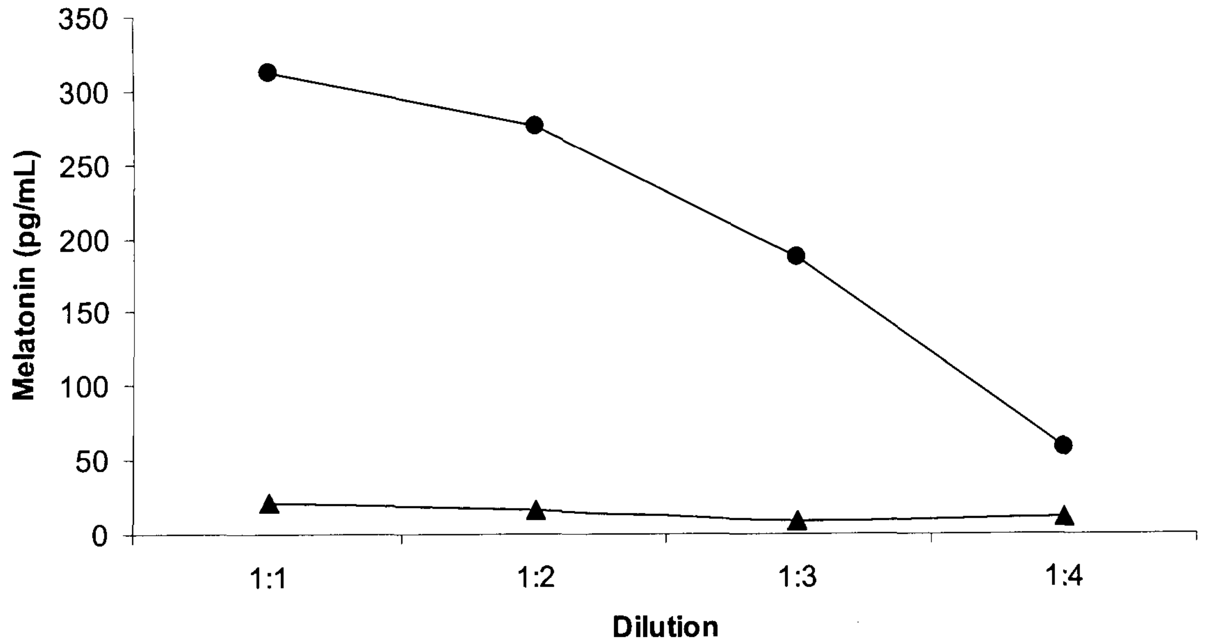

Fig. 2.2. Serial two-fold dilution of black-capped chickadee plasma to examine the linearity of the assay. Night sample values are represented with circles (●) and day sample values are represented with triangles (▲).

characterising profile of daily and seasonal changes in melatonin. The inter-assay coefficient of variation for melatonin profile assays was 45.49% and the intra-assay coefficient was below 18%. Falsely elevated between-subject differences may have resulted from the high inter-assay variability, but because each subject was run on the same plate, the variable of interest (within-subject variation in melatonin levels) was probably not greatly affected.

For the paired interaction study, the two rounds of interactions were assayed separately. Each pair in the interaction studies was assayed on the same plate, and the intra-assay coefficient for interaction assays was less than 28%.

2.2.6 Statistics

Statistical and graphical analyses were performed using SPSS version 16.0. In this study, non-parametric statistics were used because the distribution of the obtained melatonin values violated the assumption of normality, as indicated by the Shapiro-Wilk test, $W(120) = .48$, $p < .001$. The analysis of variance is robust in regards to violations of normality only if sample sizes from all groups are equal. However, this was not the case, as incidences of blood sampling difficulties and errors during assay procedures resulted in missing data points in this study. $p < .05$ was regarded as significant.

The Friedman test was used to determine whether different seasons and times during the day and night were associated with significantly different levels of melatonin. The Friedman test detects whether data points collected at different times vary significantly by rank-ordering measures of each subject or group of subjects and compares assigned rank values of each subject or group with each other. It is similar to the parametric repeated-measures analysis of variance except that it uses ranks instead of means and thus is unaffected by violations of normality. Subsequently, I used the Wilcoxon signed-rank test to determine which blood sampling sessions had melatonin concentrations that differed significantly from each other. The Wilcoxon signed-rank test is theoretically similar to Student's paired t-test, but like the Friedman test, it assigns ranks to data values instead of calculating the raw mean. Thus, the Wilcoxon signed-rank test also does not rely on the assumption of normality and is appropriate for this analysis.

To determine whether melatonin concentrations were associated with dominance status, I performed a Kruskal-Wallis one-way analysis of variance by ranks. The Kruskal-Wallis test is a non-parametric test used to compare three or more independent groups of data, and because it uses rank-ordered data instead of raw values to compute the statistic, it is robust even if the assumption of normality is not met. Following this, multiple pairwise comparisons were made using the Mann-Whitney U test. This test is similar to the one-sample t-test and can be used to test whether two independent samples come from the same population. The Friedman test was also used to examine differences between melatonin at the three different times sampled (i.e. 1 hour, 30 hours, and 1 week after initial interaction).

Spearman's rank correlation coefficient was used to assess whether melatonin was correlated with aggressive behaviour. This statistic is fundamentally similar to Pearson's product-moment coefficient, with the exception that data are first converted into ranks.

2.3 RESULTS

2.3.1 Melatonin levels are elevated at night and in the breeding season

Melatonin concentrations differed significantly between time of day and season, χ^2 (7, N=15) = 44.53, $p < .001$ (Fig. 2.3). Chickadees in non-breeding condition had melatonin concentrations that were higher at midnight ("MN", *Median* = 151.75) than at 1 hour after lights on ("ON", *Median* = 57.06), $Z = 2.27$, $p < .05$, and at midday ("MD", *Median* = 37.50), $Z = 3.24$, $p < .01$. Non-breeding chickadees also had higher melatonin levels at 1 hour after lights off ("OF", *Median* = 111.04) than at midday (*Median* = 37.50), $Z = 3.12$, $p < .01$. Levels at 1 hour after lights off also appeared to be higher than at 1 hour after lights on, although this difference did not reach significance, $Z = 1.93$, $p = .053$ (Fig. 2.4). A similar pattern was observed for chickadees in breeding condition (Fig. 2.5). Melatonin levels were higher at midnight (*Median* = 757.25) than at 1 hour after lights on (*Median* = 59.64), $Z = 2.39$, $p < .05$ and at midday (*Median* = 62.50), $Z = 2.95$, $p < .01$. Furthermore, concentrations at 1 hour after lights off (*Median* = 223.50) and at midday (*Median* = 62.50), were significantly different, $Z = 3.35$, $p < .01$. A seasonal difference was observed between melatonin levels at midnight, where birds in breeding condition (*Median* = 757.25) had significantly more melatonin than birds in non-breeding condition (*Median* = 151.75), $Z = 2.27$, $p < .05$ (Fig. 2.6).

2.3.2 Melatonin was not affected by dominance status

In the first round of paired interactions, melatonin concentrations of dominants and subordinates at the three different times sampled (1 hour, 30 hours, and 11 days after first interaction) differed more than expected by chance, χ^2 (5, N=7) = 29.53, $p < .001$. Specifically, subordinates had significantly higher levels of melatonin after 11 days of

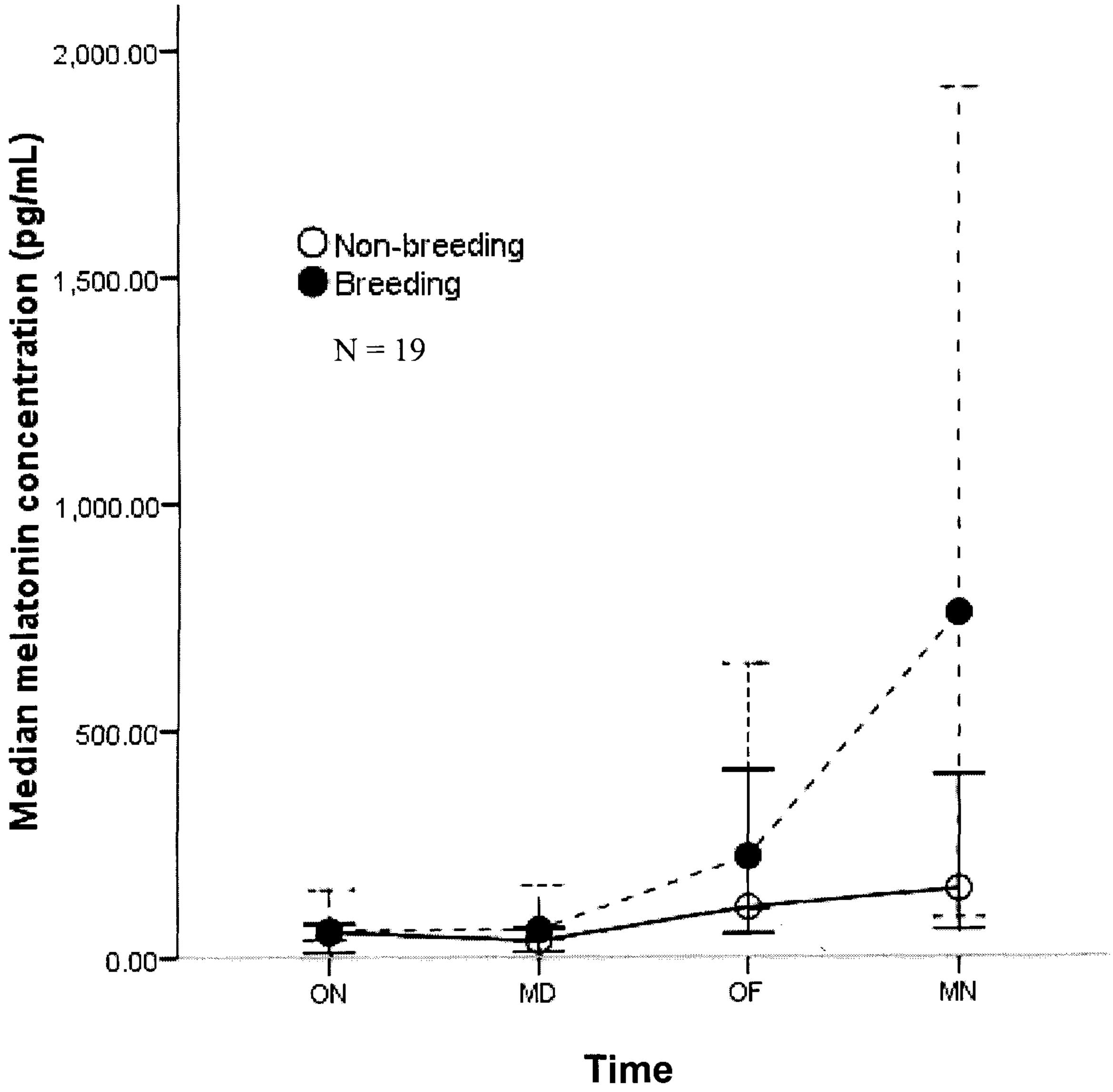

Fig. 2.3. Daily and seasonal variation of median melatonin levels in breeding and non-breeding chickadees. Blood sampling occurred at 1 hour after lights on (ON), midday (MD), 1 hour after lights off (OF), and midnight (MN). Error bars represent 95% confidence intervals.

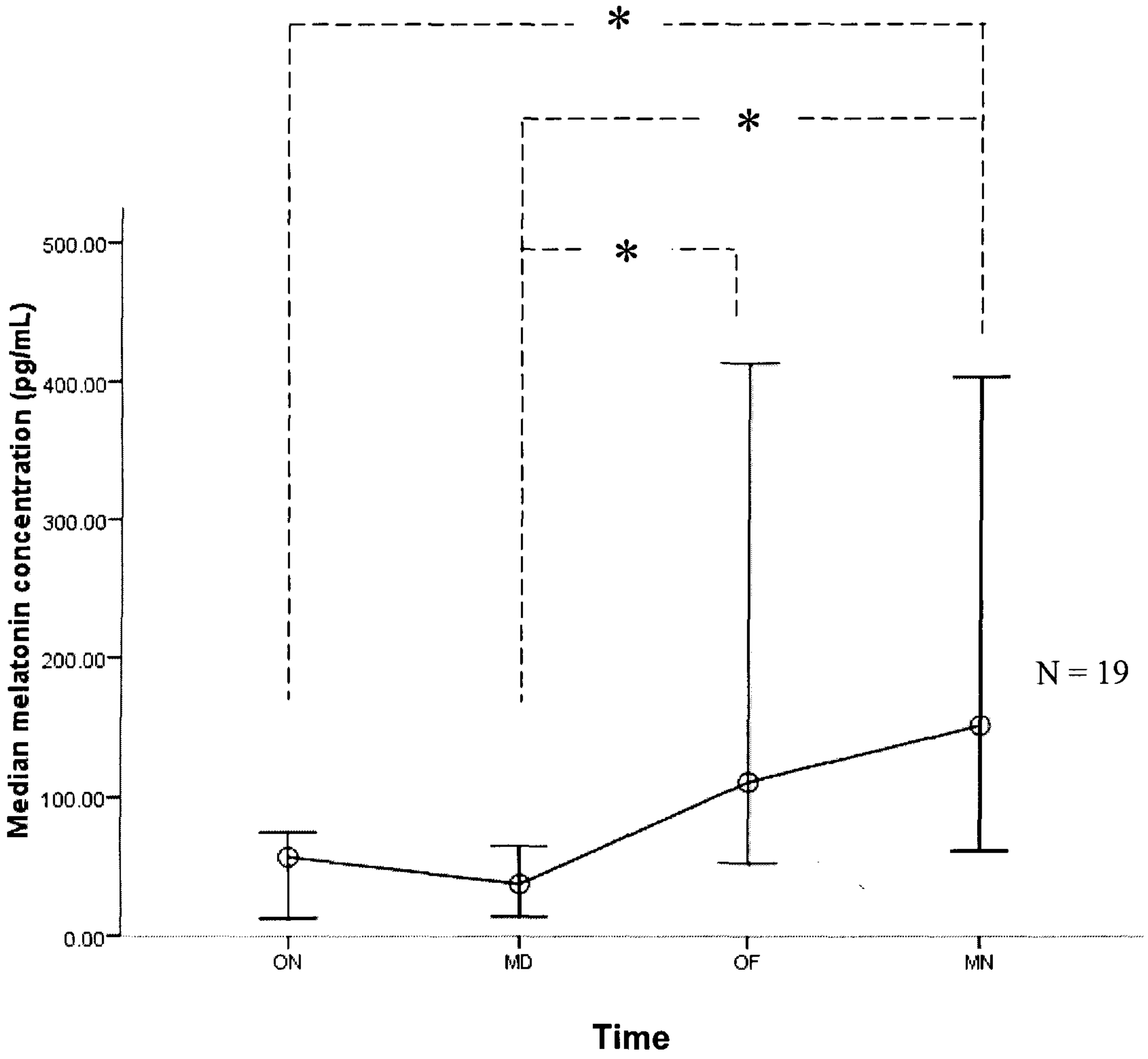

Fig. 2.4. Daily changes in median melatonin levels of non-breeding chickadees. Blood sampling occurred at 1 hour after lights on (ON), midday (MD), 1 hour after lights off (OF), and midnight (MN). Error bars represent 95% confidence intervals.

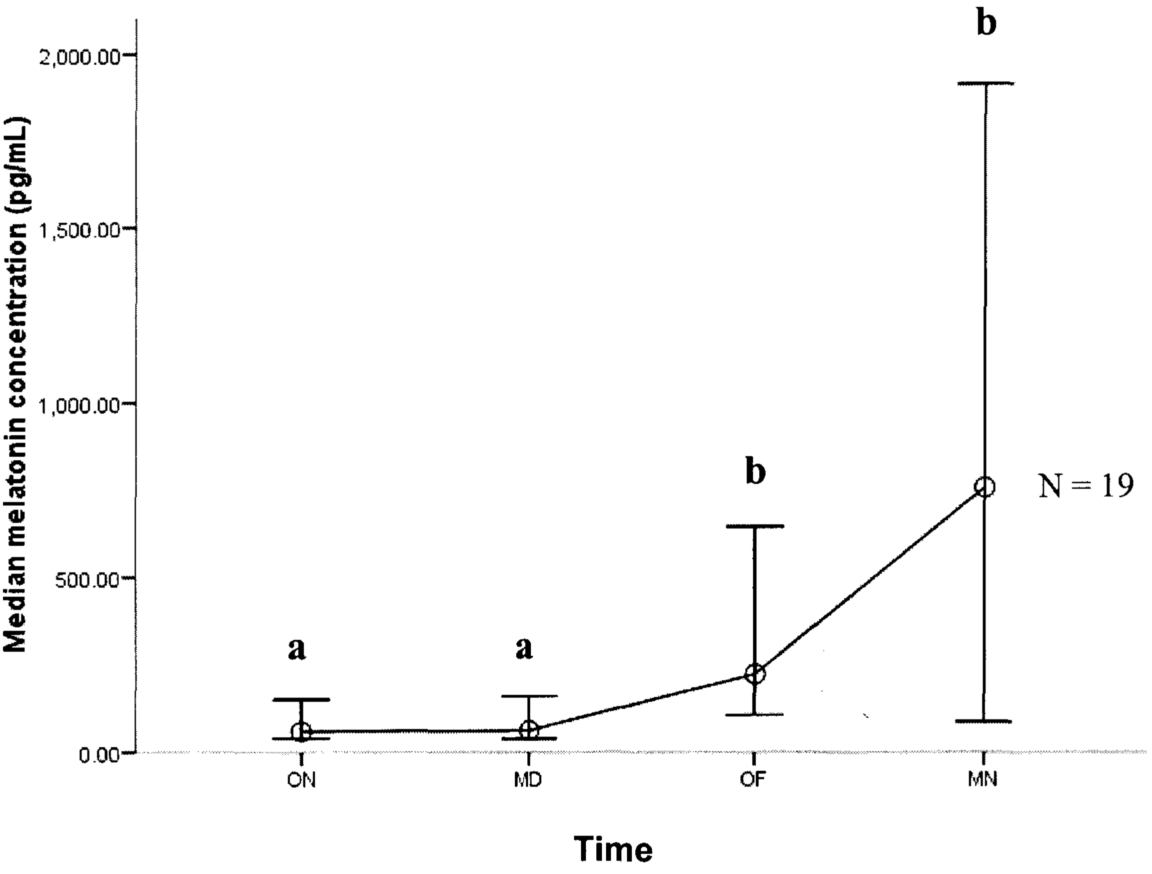

Fig. 2.5. Daily changes in median melatonin levels of breeding chickadees. Blood sampling occurred at 1 hour after lights on (ON), midday (MD), 1 hour after lights off (OF), and midnight (MN).). Error bars represent 95% confidence intervals.

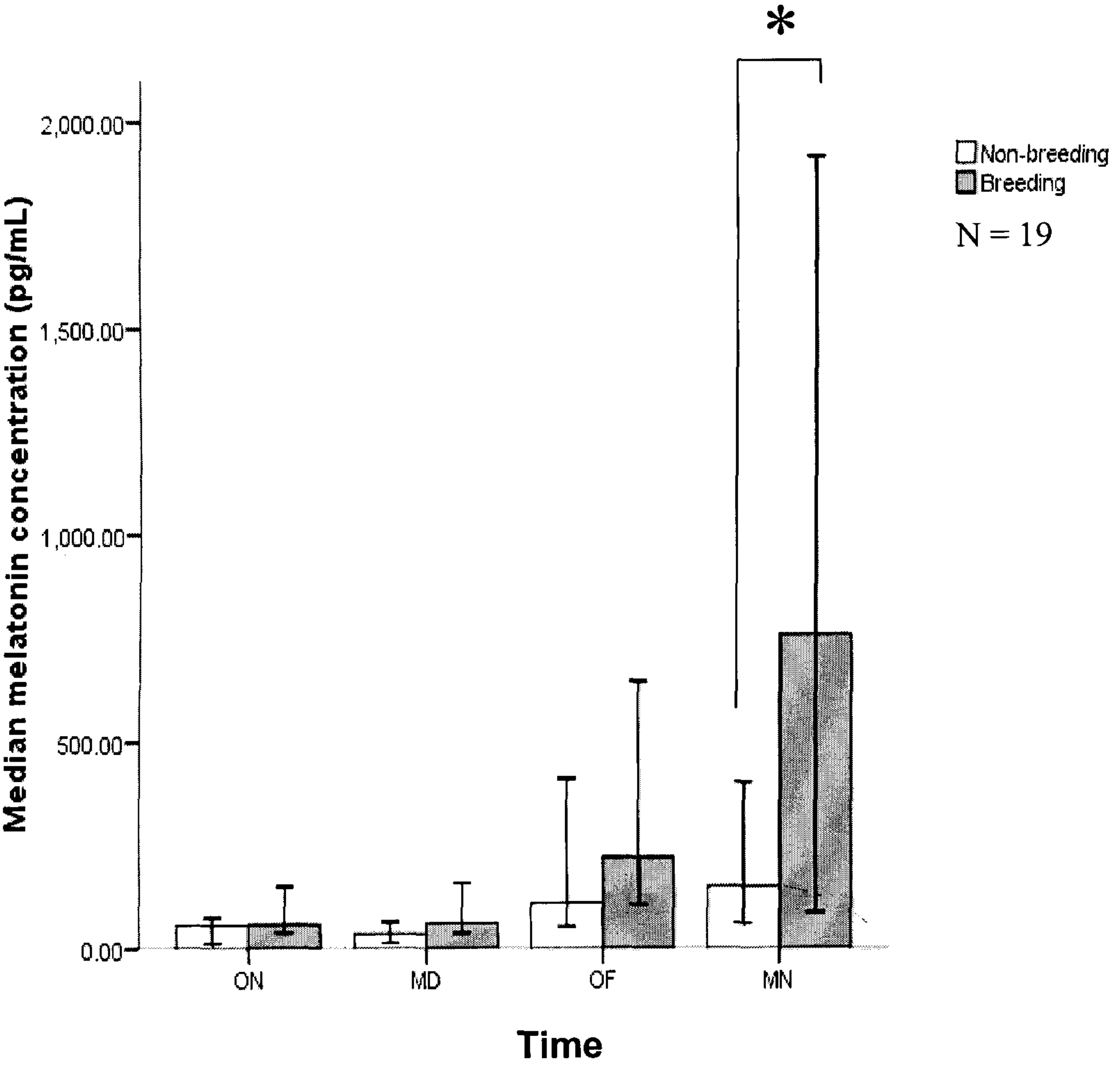

Fig. 2.6. Comparison of median melatonin concentrations of breeding and non-breeding chickadees blood sampled at various times during the day and night. Error bars represent 95% confidence intervals. Birds in breeding condition (*Median* = 757.25) had significantly more melatonin than birds in non-breeding condition (*Median* = 151.75), $Z = 2.27$, $p < .05$.

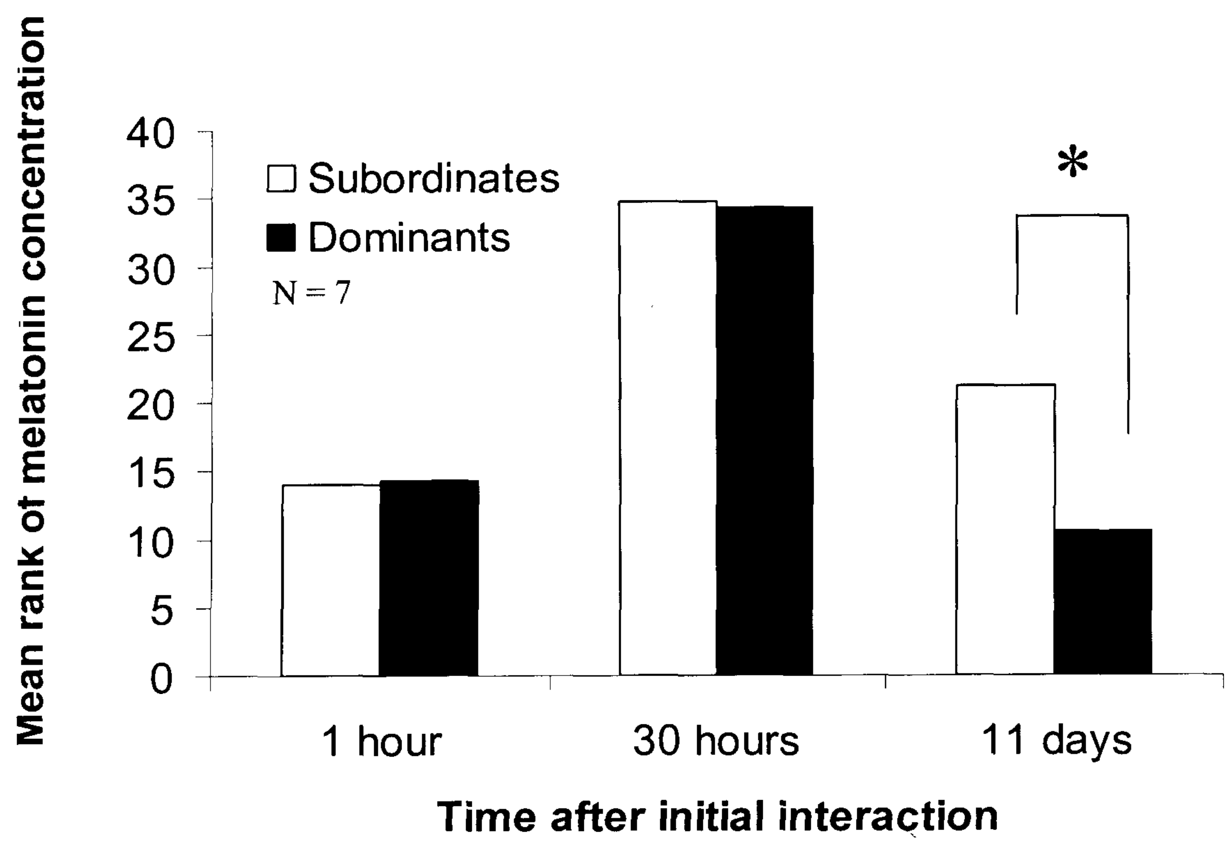

Fig. 2.7. Differences in mean rank of melatonin concentrations between dominants and subordinates at 1 hour, 30 hours, and 11 days after initial interaction for the first round of the paired interaction study. After the Bonferroni correction, the difference between melatonin levels of dominants and subordinates 11 days after initial interaction becomes insignificant.

paired interaction compared to dominants, $U = 10.50, p = .025, r = .60$ (Fig. 2.7). However, after application of the Bonferroni correction ($\alpha = .017$), this difference becomes non-significant. There were no other differences between melatonin levels of dominants and subordinates that were detectable.

Examination of melatonin at the three different times sampled indicates that melatonin levels were higher at night (30 hours after initial interaction; *Median* = 31.79) than during the day (1 hour and 11 days after pairing, respectively), $Z = 3.30, p <.005, Z = 2.98, p <.005$. No differences were observed between the samples taken during the day (Fig. 2.8).

In the second round of paired interactions, melatonin concentrations of dominants and subordinates at the three different times sampled also differed significantly more than by chance, χ^2 (5, N=8) = 32.51, $p < .001$. However, no pairwise comparisons of melatonin levels between dominants and subordinates were significantly different. This suggests that the significant results obtained from the Kruskall-Wallis test may have been driven by differences within status groups (i.e. melatonin levels of dominants at night may have been significantly different from melatonin levels of dominants during the day). Results from the Wilcoxon signed-ranks test support this speculation, as nocturnal levels of melatonin (*Median* = 910.28) were significantly higher than daytime levels of melatonin (1 hour and 11 days after pairing, respectively), $Z = 3.52, p < .001$; $Z = 3.52, p < .001$, but there were no differences between daytime levels of melatonin (Fig. 2.9).

2.3.3 Melatonin was not correlated with aggressive behaviours

Frequencies of the different aggressive behaviours exhibited during paired interactions are shown in Table 2.2. The aggressive measures gargle, chase, and supplant

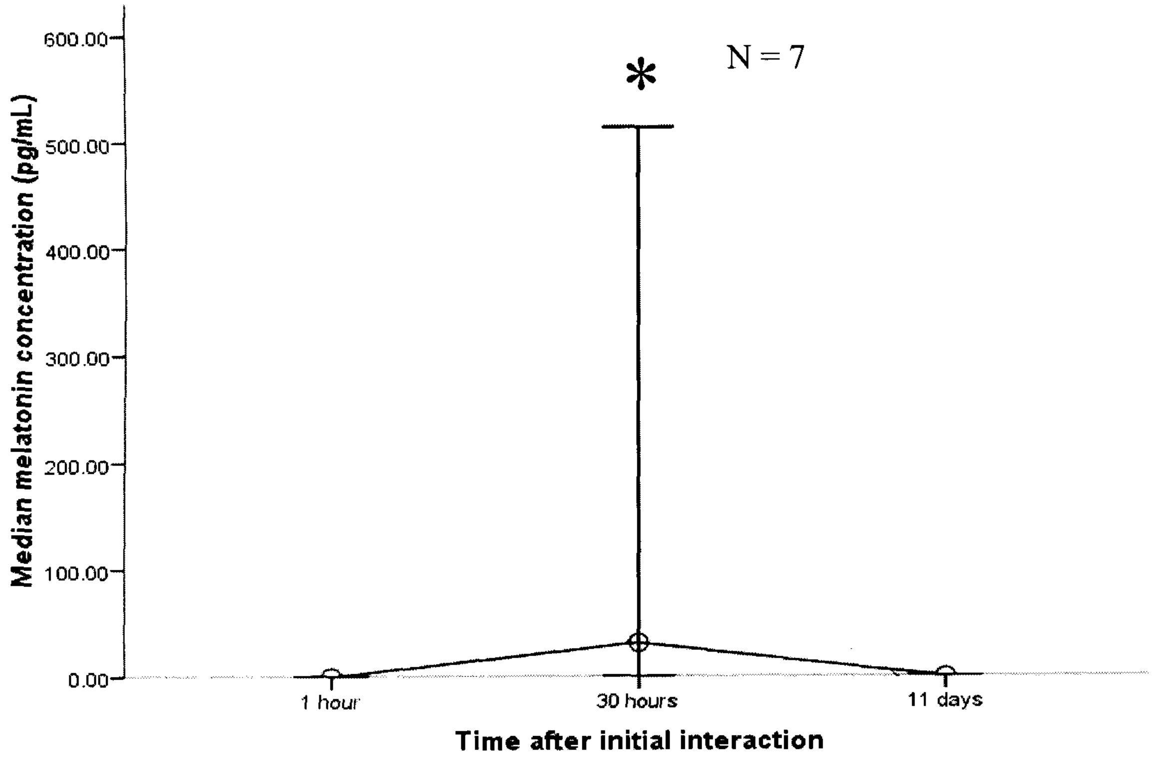

Fig. 2.8. Median melatonin levels at 1 hour, 30 hours, and 11 days after initial interaction of all subjects in the first round of the paired interaction study. Error bars represent 95% confidence intervals.

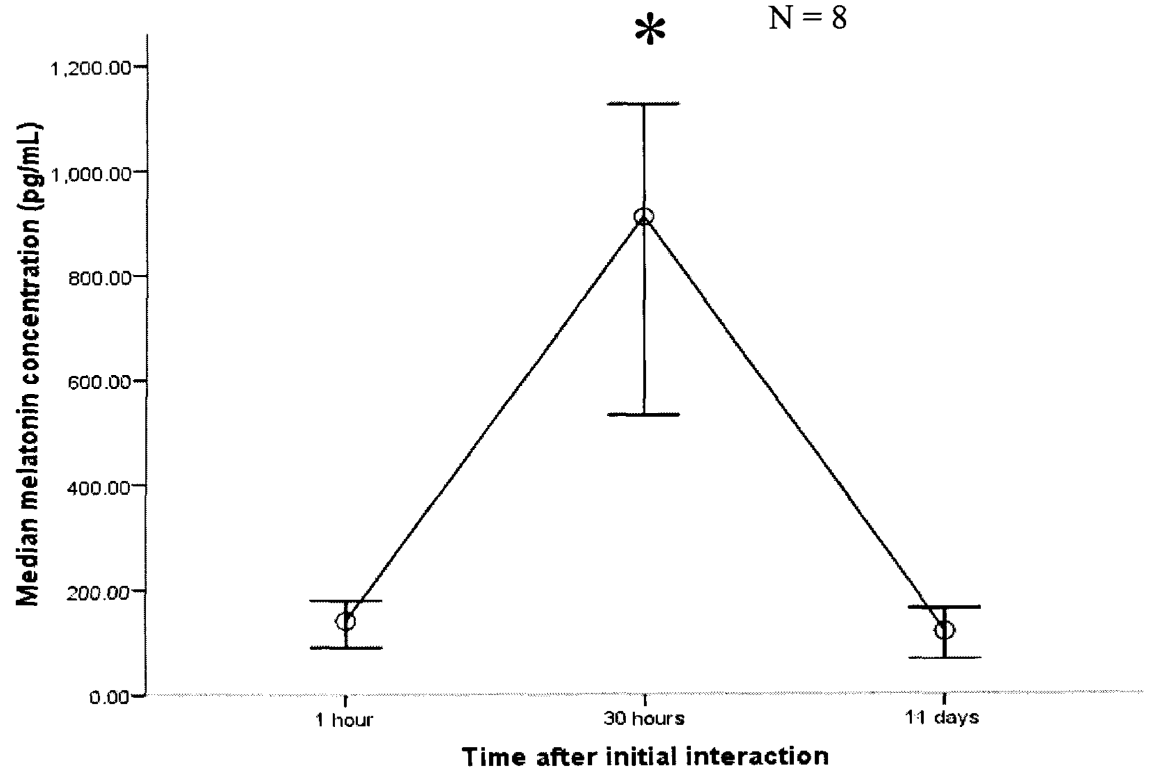

Fig. 2.9. Median melatonin levels at 1 hour, 30 hours, and 11 days after initial interaction of all subjects in the second round of the paired interaction study. Error bars represent 95% confidence intervals.

	Gargle	Gape	Supplant	Chase
Dominant	1050 1474	36 40	340 227	662 260
Subordinate	10 0	17 5	12 1	8 0

Table 2.2. Frequencies of different aggressive behaviours observed during paired interaction. The first line shows frequencies for the first round (N = 14) and the second line shows frequencies for the second round (N = 16) of interaction.

	Gargle	**Gape**	**Supplant**	**Chase**
Gargle	---	.266 .490	.605* .898**	.640* .957**
Gape	.266 .490	---	.397 .617*	.175 .535*
Supplant	.605* .898**	.397 .617*	---	.812** .912**
Chase	.640* .957**	.175 .535*	.812** .912**	---

* $p < .05$ ** $p < .01$

Table 2.3. Spearman's rho correlation coefficients between measures of aggression. The first line shows correlations for the first round of paired interaction (N=14) and the second line shows correlations for the second round of interaction (N=16).

were significantly correlated in the first and second round of interaction (see Table 2.3), so a principle components analysis was performed for each round of interaction. Two components were extracted from each PCA that explained 58.8 and 32.4% variance (91.2% cumulative) in the first round, and 70.5 and 25.1% variance (95.6% cumulative) in the second round. Chase and supplant loaded highly on PC1 in the first and second round of interaction – chase loadings were greater than .92 and supplant loading were greater than .87. Gargle loaded moderately on PC1 (.65) and loaded negatively on PC2 (-.72) in the first round but loaded highly on PC1 in the second round (.96). Gape loaded highly on PC2 in the first (.75) and second (.964) round. Therefore, I interpreted PC1 as representing behaviours used to threaten subordinates and PC2 as non-threatening displays of dominance status. However, no significant correlations were found between threatening behaviours or dominance status displays and melatonin levels in either the first or second round of interaction (see Table 2.4).

2.4 DISCUSSION

2.4.1 Daily and seasonal variations of plasma melatonin

Melatonin concentrations in black-capped chickadees were similar to concentrations found in other birds (e.g. Bentley, 2003; Brandstatter, 2003; Singh & Haldar, 2007; Zawilska et al., 2006). Importantly, this is the first experiment to quantify and compare daily fluctuations of melatonin in breeding versus non-breeding black-capped chickadees. Contrary to predictions, breeding chickadees had higher concentrations of melatonin than non-breeding chickadees, but only for samples taken at midnight. This result likely reflects my method of sampling. As I was interested in peak

	Threatening behaviours	**Non-threatening displays**
Melatonin concentration 60 min after initial interaction	.154	-.228
	.193	.092
Melatonin concentration 30 hr after initial interaction	.231	-.064
	-.451	-.181
Melatonin concentration 11 days after initial interaction	-.251	.223
	.081	-.212

Table 2.4. Spearman's rho correlation coefficients between PC1 (behaviours used to threaten subordinates) and PC2 (non-threatening displays dominance status displays). The first line shows coefficients for the first round of interaction and the second line shows coefficients for the second round of interaction. None of the melatonin concentrations taken after the initial interaction are significantly correlated with either of the two behaviours.

amplitude of nocturnal melatonin concentration, I only measured melatonin at a specific time point (i.e. midnight). However, duration of nocturnal melatonin rise is another valid method of examining changes in melatonin levels. This is because nocturnal melatonin increases gradually throughout the night until it reaches peak amplitude, after which levels begins to decline. Many animals exhibit seasonal variations in duration of nocturnal melatonin, whereby the duration of nocturnal melatonin peak appears to be inversely related to daylength. Longer days are associated with shorter nocturnal melatonin signal duration while shorter days are linked to longer nocturnal melatonin signal duration (see Nelson et al., 1990).

On the other hand, peak amplitude of nocturnal melatonin does not always follow the same seasonal pattern as duration of nocturnal melatonin increase. In sea bass, nocturnal plasma concentrations were always lower in autumn and winter, in comparison to spring and summer, regardless of nocturnal melatonin duration (Garcia-Allegue et al., 2001). Nocturnal plasma melatonin peaks were also more pronounced during spring and summer. Altogether, results from the current experiment replicate these findings by Garcia-Allegue et al. (2001), although future studies should aim to characterize both nocturnal melatonin rise and peak amplitude if possible. Doing so will provide a more complete picture of melatonin profiles and also help to distinguish between the potential effects of peak nocturnal melatonin amplitude and duration of nocturnal melatonin rise on dominant-subordinate aggression in black-capped chickadees.

2.4.2 Effect of dominance status on melatonin levels

This experiment is also the first to compare melatonin concentrations between birds of higher and lower dominance status. The results showed no differences in melatonin levels between dominant and subordinate chickadees. This was incongruous to my expectations that melatonin concentrations would be elevated in dominants during the day but elevated in subordinates at night. It also contradicts previous findings that melatonin differs in dominant and subordinate rainbow trout (Larson et al., 2004).

Two possible explanations for these inconsistent findings are (1) high variation in the EIA masked the effects of dominance status on plasma melatonin concentrations, or (2) melatonin is not involved in or affected by establishment and maintenance of dominance status in black-capped chickadees.

Melatonin concentrations were unaffected following dominant-subordinate interactions in this experiment, even though other studies have shown that animals subjected to stressful social encounters (which involved aggression) had altered melatonin concentrations (Heinzeller et al., 1988; Fuchs & Schumacher, 1990; Larson et al., 2004). One reason for this discrepancy could be that studies reporting these positive findings used the resident-intruder paradigm as the stressful stimulus. This suggests that perhaps the effect of social stress on melatonin observed in those studies is limited to situations involving territorial disputes. If this is the case, melatonin is likely not affected by chickadee dominant-subordinate relationships because dominant-subordinate interactions are more often used to resolve disputes over which individuals gain first access to resources rather than disputes that focus on exclusive access to certain areas or territories. Moreover, I introduced both members of a pair to a novel environment, so neither of the individuals was a prior resident in the test cage.

Finally, the current experimental results may contradict previous research due to a species-specific effect. Exogenous melatonin has different behavioural effects in different animals, decreasing aggression in a cichlid fish (Munro, 1986) but increasing aggression in song sparrows, for instance (B. Kriengwatana, unpublished data). In light of these conflicting findings, no firm conclusions can be made about the extent to which melatonin plays a role in dominance status formation in black-capped chickadees. Errors committed during EIA procedures also contribute to the uncertainty of the relationship between melatonin and dominance in this species.

2.4.3 Relationship between melatonin and aggressive behaviour

Results from this study indicate that melatonin levels are not significantly correlated to a variety of measures of aggression. I expected that a correlation between melatonin and dominant-subordinate aggression would exist because artificially increasing melatonin also leads to an increase in territorial aggression in song sparrows (B. Kriengwatana, unpublished data). However, melatonin has been reported to have either aggression-inducing or sedative effects depending on the animal studied. For example, melatonin increases aggression in some rodents (Paterson & Vickers, 1981; Jasnow et al., 2002), but decreases aggression in some types of fish (Munro, 1986; Satake, 1979). As melatonin also did not differ between dominants and subordinates, the cumulative evidence suggests that melatonin also does not regulate dominant-subordinate aggression in black-capped chickadees. That being said, it may still be possible that melatonin plays a role in regulating chickadee aggression, but the fluctuations in melatonin during the day may have been too small to detect.

In conclusion, findings from the current experiments suggest that black-capped chickadees do not exhibit seasonal differences in daily rhythms of melatonin secretions, with the exception of nocturnal melatonin concentrations. Melatonin also does not appear to be involved in regulating aggression in dominant or subordinate birds. Future studies should address whether aggressive dominant-subordinate relationships in black-capped chickadees are mediated by serotonin. Serotonin is a hormonal precursor to melatonin and numerous studies have linked higher levels of serotonin with decreased aggression in a wide variety of species (see Olivier et al., 1995; Sperry et al., 2005). Moreover, work by Lepage et al. (2005) showed that artificially increasing melatonin had no effect on dominance status in rainbow trout but administration of serotonin influenced dominance status by inhibiting aggressive behaviour in dominants, even though this treatment also resulted in elevated melatonin levels. Serotonin also fluctuates seasonally in humans (e.g. Serrias et al., 1989; Lambert et al., 2002), however, whether this is the case with birds has yet to be determined. Thus, more work is needed to investigate whether serotonin is a candidate in influencing aggression in the context of dominant-subordinate relationships in birds.

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CHAPTER 3

EFFECT OF EXOGENOUS MELATONIN ON BLACK-CAPPED CHICKADEE VOCALIZATIONS

3.1. INTRODUCTION

Seasonal differences in songbird vocalizations tend to correspond with seasonal changes in a network of brain nuclei that control singing (known collectively as the song control system) (e.g. Tramontin et al, 2000; Moore et al., 2003; Tramontin & Brenowitz, 2000). In most songbirds, HVC, the robust nucleus of the arcopallium (RA), and Area X are three song control regions that are typically larger during spring (Tramontin & Brenowitz, 2000). Additionally, volumetric increases in HVC during the breeding season are due to recruitment of new neurons (Tramontin & Brenowitz, 1999), which occurs at a time when songs are more stereotyped (Smith et al., 1997). The remarkable seasonal plasticity in structure and physiology of the avian song control system (see Brenowitz, 2004; Thompson & Brenowitz, 2005: Meitzen et al., 2007) is mediated primarily by the actions of gonadal sex steroids (Tramontin & Brenowitz, 2000; Ball et al., 2004; Soma et al., 2004), and to a lesser degree by other photoperiodically regulated hormones (Bentley, 1999). Growth of song control nuclei corresponds to times when circulating levels of gonadal sex steroids are high (Tramontin & Brenowitz, 2000. Photoperiod appears to regulate levels of these gonadal steroids by stimulating gonadal regression or recrudescence, as well as its actions on target tissues (see Bentley & Ball, 2000a). Testicular growth, elevated plasma testosterone, and development of song nuclei are seen in captive birds that are housed on long, spring-like daylengths (e.g. Brenowitz et al., 1991). Therefore, seasonal plasticity the song control system likely mediates seasonal variation of vocalizations in songbirds.

Interestingly, there are also seasonal changes in song nuclei that are not mediated by gonadal testosterone or other gonadal hormones (e.g. Bernard et al., 1997; Smith et al., 1997). Dawson and colleagues (2001) suggest that these changes may instead be regulated by other photoperiodically controlled hormones such as melatonin. Melatonin is a pineal hormone secreted primarily during dark phases of the photoperiod (Yamada et al., 1988), so fluctuating patterns of circulating melatonin can be used as a reliable signal of seasons. Melatonin treatment during long, spring-like days attenuates growth of HVC and decreases volume of Area X in European starlings (Bentley, 1999). This suggests that melatonin may modulate the effects of gonadal sex steroids by inhibiting growth of the song control nuclei. Melatonin receptors in multiple song control regions have also been identified in European starlings, house sparrows, canaries, and zebra finches (*Taeniopygia guttata*) (Gahr & Kosar, 1996; Bentley, 2003; Whitfield-Rucker & Cassone, 1996; Bentley et al., 1999). Although melatonin receptors are widely distributed in the avian brain (see Aste et al., 2001), only receptors in song control regions, such as Area X, exhibit seasonal regulation that occurs as a result of changes in photoperiod, reproductive state, and gonadal steroid levels (Bentley & Ball, 2000a). Together, the stimulatory effects of testosterone and the inhibitory effects of melatonin may interact to refine seasonal song nuclei plasticity (Dawson et al., 2001).

If melatonin is involved in regulating seasonal plasticity of song control nuclei, then it may also influence seasonal differences in song and singing behaviour. Preliminary evidence that melatonin affects song behavior comes from two studies on song sparrows and European starlings. Western song sparrows given melatonin implants were faster to sing in response to a simulated territorial intruder and had higher levels of the androgen dehydroepiandrosterone (DHEA) than those given empty implants (B. Kriengwatana, unpublished data). DHEA implants increase territorial singing rates in song sparrows (Soma et al., 2002), which suggests that melatonin may affect song by acting directly on its receptors in the brain or by moderating the actions of steroid hormones such as DHEA. The study on European starlings directly examined the effects of melatonin on song and found that photosensitive, male European starlings treated with testosterone and melatonin sang significantly less and had smaller HVC volumes than those treated with testosterone only (Bentley & Ball, 2000b). However, because melatonin treatment was coupled with testosterone, these findings could be due to the effects of melatonin alone or to the effects of melatonin in combination with testosterone. In addition to rate or frequency of song output, melatonin also has the potential to affect song quality and structure because songs tend to be more stereotyped during spring (e.g. Smith et al., 1997) when duration of nighttime melatonin release is shorter. I therefore decided to investigate the effects of melatonin on song behaviour in black-capped chickadees. Although the extent of seasonal plasticity of their song control nuclei differ between laboratory and field studies (see MacDougall-Shackleton et al., 2003; Phillmore et al., 2006; Smulders et al., 2006), chickadees exhibits pronounced seasonal variation in song production and stereotypy that is linked to annual changes in photoperiod (Phillmore et al., 2006; Avey et al., 2008).

Black-capped chickadees have a repertoire of learned vocalizations. Their characteristic *chick-a-dee* call is given by both sexes and is mainly used to coordinate winter flock activity in various situations (Ficken et al. 1978). *Chick-a-dee* calls are made most frequently in autumn and winter (Avey et al., 2008). Another vocalization, the *gargle,* is heard more often during the winter (Avey et al., 2008). It is given mostly by males during territorial and agonistic encounters but can also be given in sexual contexts (Ficken, 1978). *Gargles* are complex calls that consist of two to nine notes separated by short intervals. Gargle types are made from various combinations of these notes, and individual birds have gargle repertoires that include unique and shared gargle types (Ficken, 1978; Ficken et al., 1987). Gargling is also associated with dominance rank in winter hierarchies, as dominants were likely to gargle more than subordinates, and individuals that gave gargles always won aggressive interactions (Ficken et al., 1987). Interestingly, there appears to be no correlation between gargle repertoire size and dominance status (Ficken et al., 1987) even though chickadees perceive a rival with multiple gargle types as more threatening than a rival giving a single gargle type (Baker et al., 1996).

Male chickadees also sing a whistled two-note *fee-bee* song. The first note (*fee*) is always followed by a second note (*bee*) sung at a lower frequency. Chickadees can shift the absolute frequency of their *fee-bee* songs (Horn et al., 1992); however, other aspects of the song structure are highly uniform and vary very little between populations that are distributed across North America (Weisman et al., 1990; Kroodsma et al., 1999). The *fee-bee* is a long distance vocalization that can be used to locate winter flock mates in the non-breeding season and potential rivals in the breeding season, but is also sung in bouts during the dawn chorus in both seasons (Ficken et al., 1978, 1981). *Fee-bee* song production in the dawn chorus peaks once in winter and once in spring (Avey et al.,

2008) and is more stereotyped in the breeding season than the non-breeding season (Smulders et al., 2006). In the breeding season, total song length and *fee* note during dawn chorus were more consistent and stereotyped, whereas the same variables were shorter in the non-breeding season (Smulders et al., 2006).

Structure and production of dawn chorus songs can convey important information about the singer. Otter and colleagues (1997) found that a male's performance during the dawn chorus is correlated with his winter dominance rank. They suggest that females use their mate's performance during the dawn chorus as a signal of his relative quality by comparing his performance with those of neighboring males. Higher-ranking males began singing earlier, sang for longer, and sang at higher average and maximum rates than low-ranking males (Otter et al., 1997). When shifting pitches, higher-ranking males also maintained more consistent internote frequency intervals (ratio of frequency at the end of the *fee* note to the frequency at the start of the *bee* note) than lower-ranking males (Christie et al., 2004). This difference in dawn chorus song production ability and song structure between higher and lower-ranking males suggests that the ability to produce highly stereotyped dawn chorus *fee-bee* songs can function as an advertisement of male quality. Therefore, if melatonin contributes to *fee-bee* song stereotypy or production, then it could indirectly contribute to black-capped chickadee fitness.

This study investigates whether treatment with exogenous melatonin affects song stereotypy and production of vocalizations in non-breeding black-capped chickadees. Captive chickadee vocalizations were recorded at baseline, treatment, and post-treatment. I predict that melatonin administration will decrease *fee-bee* song stereotypy because song stereotypy is typically reduced in non-breeding conditions, when the duration of melatonin signal is prolonged. I also expect melatonin-treated birds to make more aggressive *gargle* vocalizations. This prediction is based on findings that melatonin may modulate actions of DHEA, an androgen that increases territorial singing in Western song sparrows.

3.2 METHODS

3.2.1 Subjects

Twenty black-capped chickadees were captured between February and March 2009 from six different sites in London, Ontario. Subjects were individually housed in 24 x 30 x 37 cm cages, given *ad libitum* food (mixture of sunflower seeds and Mazuri small bird maintenance diet) and water, and maintained on a 10L:14D light cycle. Subjects were given at least two weeks to acclimate to laboratory conditions before any procedures were performed.

3.2.2 Melatonin preparation

Melatonin was prepared and administered based on methods used by Goymann et al. (2008). I perceived this method as suitable for this experiment because I could control the timing and dose of application in an attempt to mimic prolonged plasma melatonin increases that coincide with shorter daylengths. Briefly, 12 mg of powdered melatonin (Sigma M 5250) was dissolved in 200 uL of ethanol and then mixed with 30 g of Eucerin cream (*Eucerinum anhydricum*; Beiersdorf AG, Hamburg, Germany), resulting in 0.4mg melatonin per gram cream. Melatonin-treated birds received approximately 50 mg of melatonin per day. The melatonin cream mixture was covered with foil to prevent exposure to light and stored at 4°C.

3.2.3 Procedure

Vocalizations were recorded at baseline, after 7 days of treatment (day 7), and at least 14 days after terminating treatment (post-treatment). The first two sets of recordings (baseline and at day 7) began within 30min after the start of the light cycle and continued for 6 hours. Post-treatment recordings were only 2 hours in duration but everything else was conducted similarly as baseline and day 7 recordings. At each recording session, the subject was isolated in a room adjacent to the colony room, and a small mirror (30cm x 20cm) was positioned next to their cage, within the subject's view. The recording device used was a solid state recorder (Marantz PMD671) attached to a directional microphone.

After baseline vocalizations had been taken, subjects were randomly assigned to either melatonin treatment or control treatment condition. Birds in the melatonin condition were treated with the melatonin cream and birds in the control condition were treated with Eucerin cream alone. Application of treatment was via a pipette tip that had been dipped into the melatonin or Eucerin cream. The cream was applied directly onto the skin on the backs of the birds (between the wings). On average, birds received 128 g cream (SD = 15 g) daily, 4 hours prior to the start of the dark cycle. The duration of treatment was 8 days. Blood samples were taken on day 8, two hours after application of treatment in order to check whether treatment had been effective.

3.2.4 Scoring vocalizations and song stereotypy

Songs were digitalized at 44.1 kHz sampling rate (16 bits per sample) and the following vocalizations were scored using Syrinx-PC 2.6 (John Burton, Seattle, WA): *chickadee, fee bee,* and *gargle,* and (see Fig. 3.1). Stereotypy of the *fee bee* song was also analyzed using Syrinx-PC. To improve image clarity and reduce background noise for

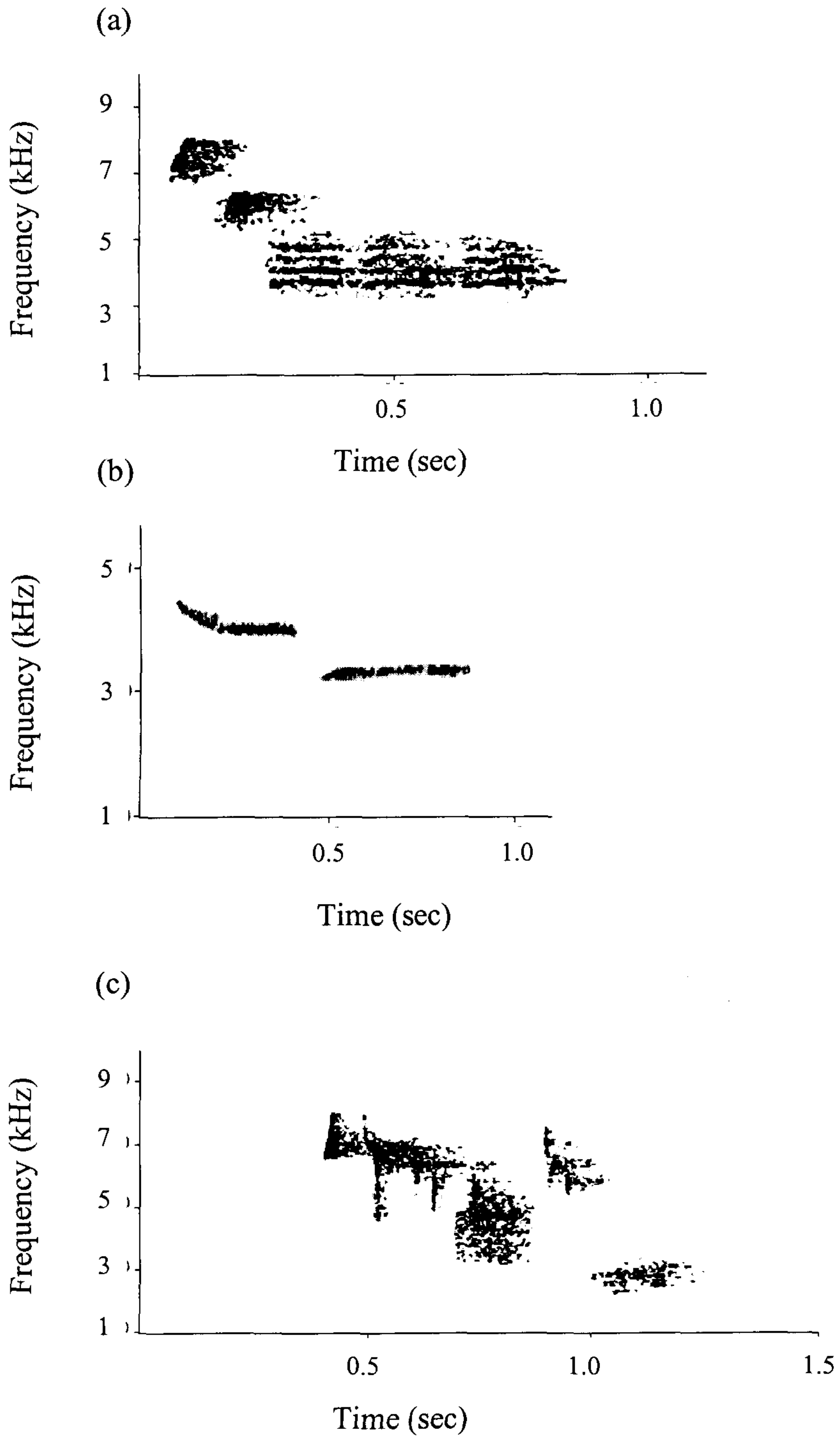

Fig. 3.1. Spectrograms of black-capped chickadee vocalizations: (a) chickadee call complex, (b) fee bee song, (c) gargle call. Spectrograms were not as clean as those found in other studies (e.g. Ficken et al., 1978) due to reverberations during recording.

stereotypy analysis, I adjusted the spectrogram FFT to 1024 points (Blackman), limited the frequency range to 1 kHz – 8 kHz, decreased spectral gain to -4 and kept temporal resolution fixed at 2.56 ms.

Ten songs were randomly selected from each subject at baseline and post-treatment recordings. If the subject did not produce at least 10 songs then all songs were analyzed. I examined six song features (see Fig. 3.2). These features were the frequency at the start of the fee note (fee_{start}), the frequency at the end of the fee note (fee_{end}), the frequency at the start of the bee note (bee_{start}), the frequency at the end of the bee note (bee_{end}), the glissando ratio (frequency at fee_{start} /frequency at fee_{end}), and internote interval (frequency at fee_{end} /frequency at bee_{start}). Chickadees can vary the relative and absolute pitch of their songs, so the glissando ratio and internote interval are used as reliable measures of absolute and relative pitch, respectively (see Weisman et al., 1900; Christie et al., 2004). Song length and amplitude are also important components of song stereotypy. However, I did not analyze these features because of the interference caused by reverberation in the testing room.

3.2.5 Blood sampling and melatonin assay

Blood samples were taken from the brachial vein, within 10 minutes of entering the room. Approximately 50 uL of blood was collected from each bird using a 26 ½ G needle and heparinized capillary tubes. Immediately after collection, blood was centrifuged and plasma was stored at -20°C.

Melatonin was quantified using a commercially available ELISA kit (Genway Biotech, San Diego CA). This assay employs competitive binding between biotinylated and non-biotinylated antigens for a fixed number of antibody binding sites.

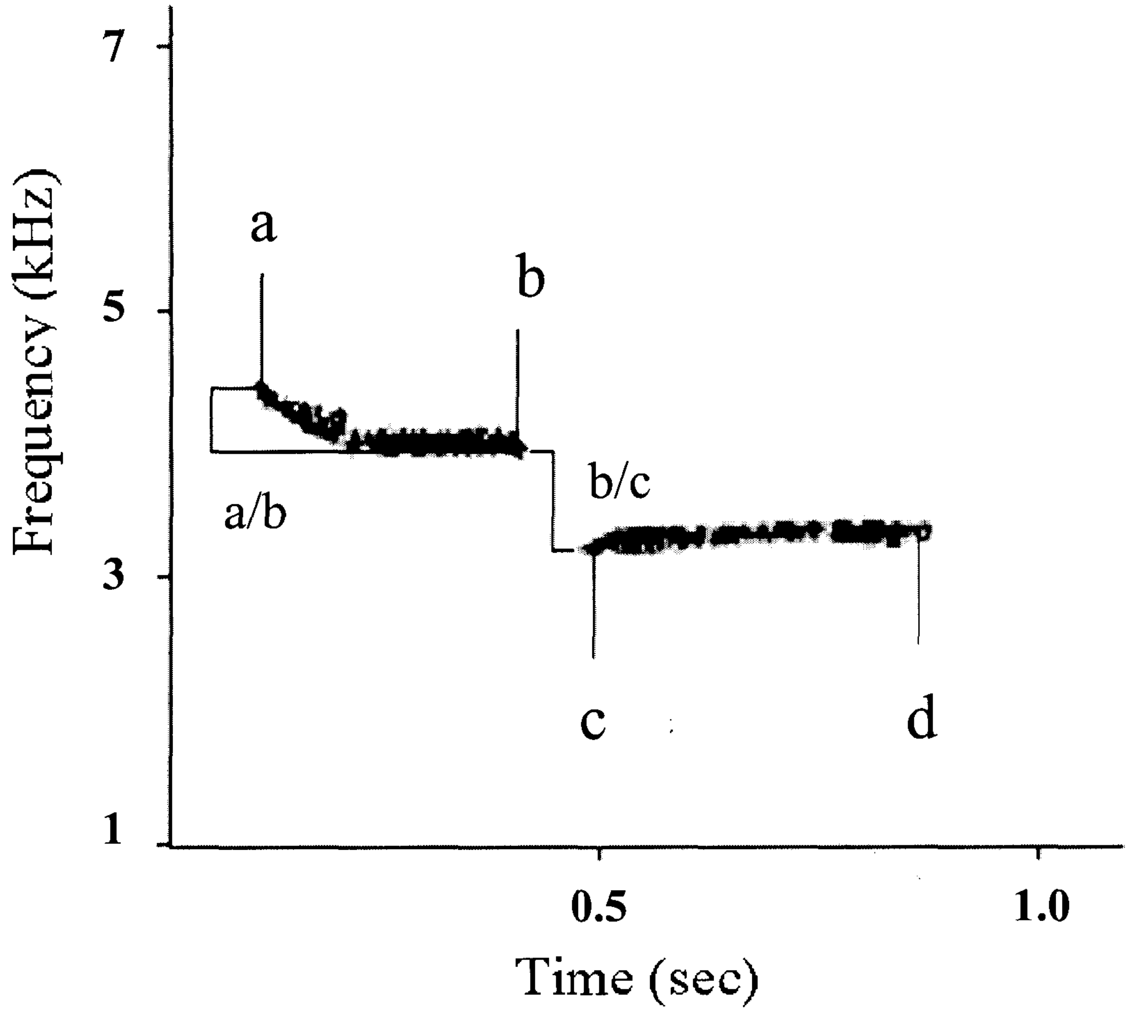

Fig. 3.2. Spectrograph representing a black-capped chickadee song. Song features analyzed included the frequency at start of fee (a), frequency at end of fee (b), frequency at start of bee (c), frequency at end of bee (d), the glissando ratio (a/b), and the internote interval (b/c).

Quantification of melatonin in unknown samples is interpolated from a response curve generated using standards of known concentration. Assay procedures were performed according to the protocol provided with the kit. The intra-assay coefficient of variation was 3.24%.

3.2.6 Statistics

The first aim of this study was to see whether birds treated with melatonin would produce significantly different numbers of vocalizations from birds not treated with melatonin. To test this, I used an ANOVA to analyze mean differences between the number of vocalizations produced by birds in melatonin and control groups, at baseline, treatment day 7, and post-treatment. The number of *chickadee*, *fee bee*, and *gargle* vocalizations, as well as the total number of vocalizations was examined. Group and recording trial (e.g. baseline) were considered fixed factors, and $p < .05$ was regarded as significant. I also reported partial eta squared as a measure of effect size. One bird appeared sick during the post-treatment recording so its post-treatment data were excluded from the analysis.

The second aim of this study was to determine whether birds treated with melatonin would produce songs that were significantly more or less stereotyped than birds not treated with melatonin. For each song feature, I calculated the mean and standard deviation for each bird and then used those measures to determine the coefficient of variation (CV = SD * 100 / mean). I compared mean CV values of each song feature of birds in the melatonin versus control group at baseline and post-treatment using an ANOVA, in order to determine whether birds in the melatonin group sang songs that significantly differed in structure from the control group after melatonin treatment.

The recording trial (i.e. baseline and post-treatment) and group were defined as fixed factors, $p < .05$ was regarded as significant, and partial eta square was reported as a measure of effect size. Additionally, a repeated measures ANOVA was performed on the all six measures of song stereotypy for individuals that sang during baseline and post-treatment recordings.

I also took blood samples at treatment day 7 to check whether melatonin delivery was effective. A one-sample t-test was used to examine whether birds treated with melatonin had higher concentrations of circulating melatonin than birds not treated with melatonin. Again, $p < .05$ was regarded as significant. All statistical analyses and graphs were generated using the statistical software SPSS version 16.0.

3.3 RESULTS

3.3.1 Melatonin did not affect number of vocalizations produced

Chickadees treated with melatonin ($n = 8$ at baseline; $n = 6$ at post-treatment) did not differ significantly in the number of *chickadee, fee bee, gargle,* or total vocalizations produced after treatment, compared to chickadees not treated with melatonin ($n = 5$ at baseline; $n = 6$ at post-treatment). However number of vocalizations produced by subjects in both groups varied significantly depending on the recording trial (i.e. baseline, day 7, post-treatment). Specifically, there was a main effect of recording trial on *chickadee* [$F(2, 44) = 7.13$, $p < .01$, $\eta_p^2 = 0.245$], *fee bee* [$F(2, 44) = 5.37$, $p < .01$, $\eta_p^2 = 0.196$], *gargle* [$F(2, 44) = 5.11$, $p < .05$, $\eta_p^2 = 0.189$], and total vocalizations [$F(2, 44) = 12.08$, $p < .01$, $\eta_p^2 = 0.355$] (see Fig. 3.3).

Post hoc analysis on the vocalization measures for the three recording trials was

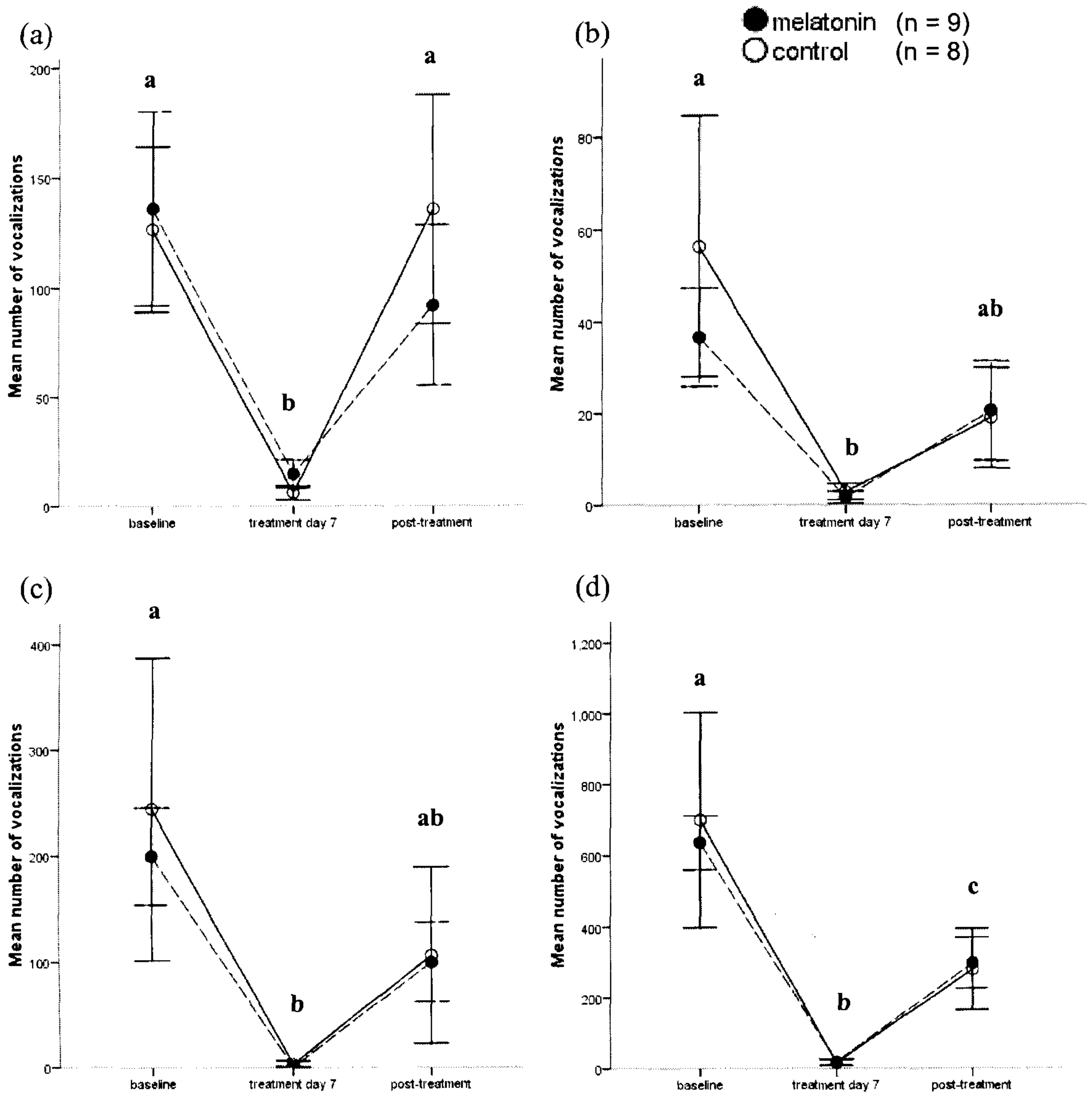

Fig. 3.3. Mean number of (a) *chickadee*, (b) *fee bee*, (c) *gargle*, and (d) total vocalizations at baseline, treatment day 7, and post-treatment. Closed circles with dashed lines represent melatonin group and open circles with solid lines represent control group. Error bars represent ±1 SEM. There was a main effect of recording trial (i.e. baseline, day 7, post-treatment) on mean number of all the measured vocalizations produced by both groups.

performed using Tukey HSD. The number of *chickadee* vocalizations produced decreased at treatment day 7 compared to baseline ($p = .04$) and post-treatment ($p = .011$). The number of *fee bee* vocalizations produced decreased at treatment day 7 compared to baseline ($p = .007$), but there were no differences between the number of *fee bee* vocalizations produced at treatment day 7 and post-treatment. Similarly, the number of *gargle* vocalizations produced decreased at treatment day 7 compared to baseline ($p = .007$), but there were no differences between the number of *gargle* vocalizations produced at treatment day 7 and post-treatment. Total number of vocalizations also decreased at treatment day 7 and post-treatment compared to baseline ($p < .001$ and $p = .028$, respectively). Moreover, birds produced less total number of vocalizations at treatment day 7 compared to post-treatment ($p = .091$).

3.3.2 Melatonin did not affect song stereotypy

When song stereotypy of individuals across trials (i.e. baseline and post-treatment) was examined, song stereotypy of chickadees treated with melatonin (n = 8 at baseline; n = 5 at post-treatment) was not significantly different from song stereotypy of chickadees that were not treated with melatonin (n = 5 at baseline; n = 6 at post-treatment). However, mean glissando ratio CV of melatonin-treated birds were significantly lower at baseline and post-treatment trial when compared to control birds, $F(1, 20) = 8.38, p < .01, \eta_p^2 = .295$ (Fig. 3.4). There was also a main effect of trial on mean glissando ratio CV of birds in both groups, $F(1, 20) = 18.77, p < .001, \eta_p^2 = .484$. Specifically, mean glissando ratio CV was reduced at post-treatment compared to baseline in both groups (Fig. 3.4). Results of treatment on song stereotypy are summarized in Table 3.1.

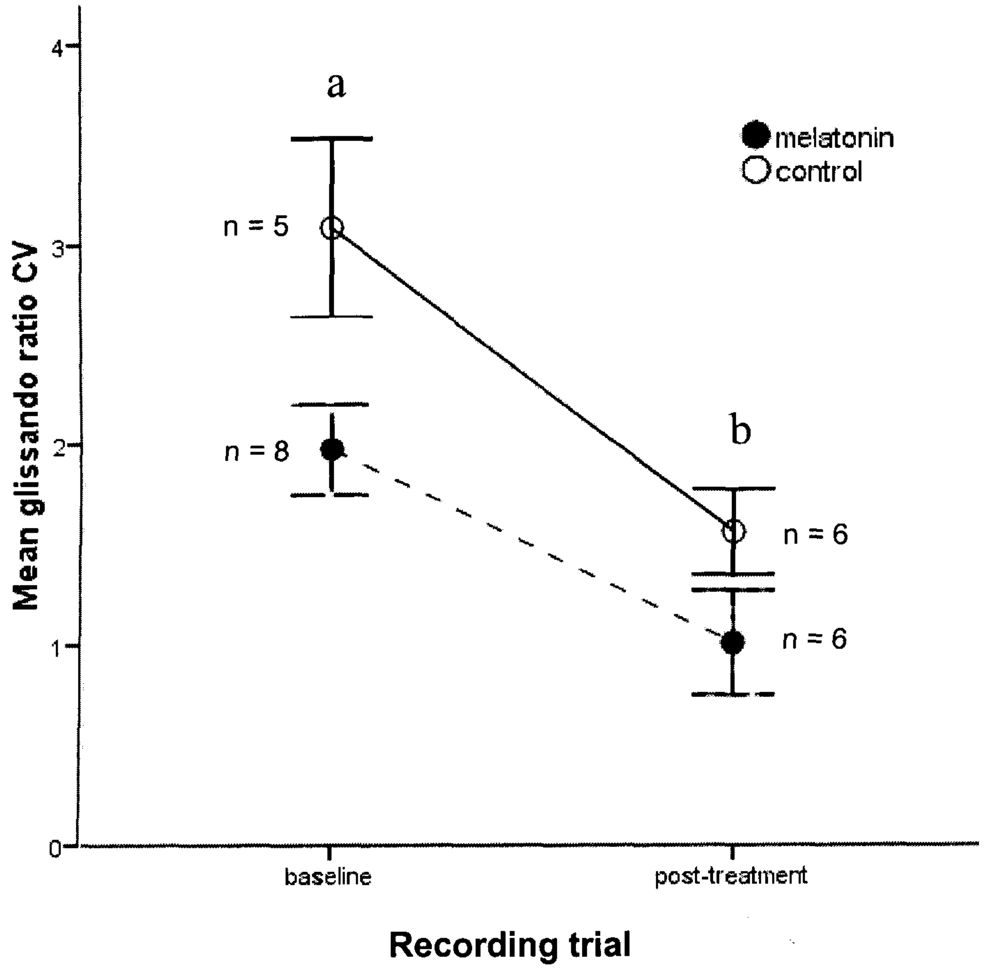

Fig. 3.4. Mean glissando ratio CV of birds in melatonin and control groups. Closed circles with dashed lines represent melatonin group and open circles with solid lines represent control group. Error bars represent ±1 SEM. Both groups showed significantly lower mean glissando ratio CVs at post-treatment compared to baseline, *F*(1, 20) = 18.78, *p* < .001. Birds in the control group had significantly higher mean glissando ratio CV at baseline and post-treatment when compared to birds in the melatonin group, F(1, 20) = 8.38, *p* < .01.

BASELINE (melatonin: n = 8, control: n = 5)

	melatonin			control		
	mean (kHz)	SD (kHz)	Means of CV	mean (kHz)	SD (kHz)	Means of CV
$\text{fee}_{\text{start}}$	4.21	0.17	6.77	4.24	0.12	4.28
fee_{end}	3.85	0.24	6.67	3.98	0.20	4.79
$\text{bee}_{\text{start}}$	3.46	0.17	5.43	3.44	0.12	4.24
bee_{end}	3.53	0.15	4.94	3.50	0.09	3.22
glissando ratio	0.25	0.05	1.98	1.07	0.09	3.09
internote interval	1.14	0.03	2.66	1.16	0.02	2.48

POST TREATMENT (melatonin: n = 6, control: n = 6)

	melatonin			Control		
	mean (kHz)	SD (kHz)	Means of CV	mean (kHz)	SD (kHz)	Means of CV
$\text{fee}_{\text{start}}$	4.34	0.19	5.46	4.27	0.27	4.21
fee_{end}	4.08	0.25	5.96	3.97	0.25	4.73
$\text{bee}_{\text{start}}$	3.47	0.22	5.50	3.43	0.15	4.37
bee_{end}	3.55	0.23	5.10	3.54	0.13	3.78
glissando ratio	0.29	0.50	1.01	0.30	0.05	1.56
internote interval	1.26	0.26	2.83	1.16	0.04	3.01

Table 3.1. Frequency of fee bee song among black-capped chickadees before and after treatment. CV = [SD/mean] *100 .

Repeated measures ANOVA of all six measures of song stereotypy for individuals that sang during baseline and post-treatment recordings (melatonin: n = 5; control: n = 5) revealed that these individuals did not differ in degree of song stereotypy before or after treatment.

3.3.3 Plasma melatonin levels were higher in melatonin-treated birds

Melatonin concentrations of birds treated with melatonin (n = 8) was significantly higher than melatonin concentrations of birds not treated with melatonin (n = 8), $F(1, 14) = 11.29, p < .01, \eta_p^2 = .446$ (Fig. 3.5)

3.4 DISCUSSION

Findings from this study suggest that exogenously increasing plasma melatonin has no effect on number of vocalizations or song stereotypy in black-capped chickadees. Both treatment groups did however, make fewer vocalizations during treatment and showed reduced glissando ratio CV after treatment. Even so, I suspect that these effects were produced as a result of the Eucerin cream administration, or from the stress of being handled daily in order to receive treatment. My reason for suspecting this is because birds in both melatonin and control groups did not seem to be in optimal health while receiving treatment (personal observation). They appeared to be losing a lot of feathers, and the area of skin where the cream was applied was often raw or flakey. Goymann et al. (2008) originally gave a one-time application of melatonin via Eucerin cream to garden warblers and European stonechats and they did not report the treatment to have any impact on the health of their subjects. Consequently, although this method was effective at raising plasma melatonin concentrations in melatonin-treated subjects in this study, I advise

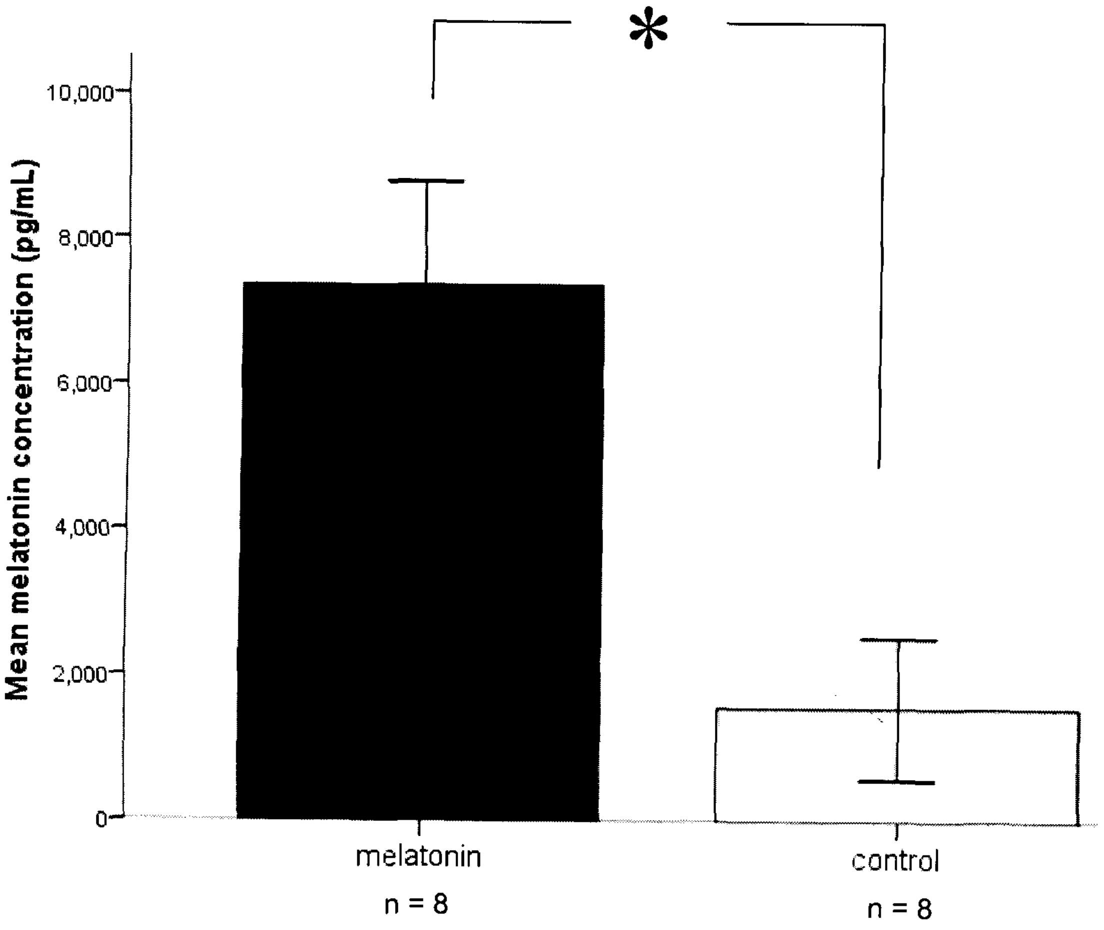

Fig. 3.5. Melatonin concentration differences between melatonin and control groups, *F*(1, 14) = 11.29, $p < .01$. Error bars represent ±1 SEM.

restricting this method of administration to experiments that require only single or short-term applications, and I would not recommend using this technique for an extended treatment regimen.

An explanation for why I found no differences in the number of vocalizations between the melatonin and control group is that any group differences present during treatment may have been too small to detect. In other words, group differences may have been masked by the depression of vocalizations likely caused by method of treatment and or handling stress.

Interestingly, both experimental groups exhibited greater song stereotypy (reflected in the lowered glissando ratio CV) after treatment when compared to baseline. The reason for this effect may be that at post-treatment, the birds are being recorded in the testing room for the third time and thus may have had sufficient time to habituate to the room.

Lastly, melatonin may indeed have no effect on vocalizations or song stereotypy in black-capped chickadees. This explanation falls in line with results obtained by Smulders et al. (2006), who reported finding no seasonal changes in song control nuclei plasticity in black-capped chickadees. Despite showing seasonal variations in vocalization production, vocalizations are still produced year-round (Phillmore et al, 2006; Odum, 1942), thus any plasticity of the song control regions is likely to be attenuated because the areas are continually active. Therefore, if chickadees show none or marginal changes in song control nuclei in response to seasons, then it follows that prolonging elevated levels of melatonin that mimic shorter daylengths in winter will also have negligible effects on these brain areas. This may explain why the expected changes in song production were not observed in this study. Moreover, in order to affect singing and vocalizations, changes in plasma melatonin produced by treatment of exogenous melatonin would have to affect song control regions. As I did not examine brain tissue in this experiment, I could not determine whether exogenous melatonin actually reached the brain to produce any significant changes. Subsequently, I cannot say with certainty that the melatonin treatment had any direct or indirect effects on song control regions.

An issue that affected the interpretation and generalization of results from this study is the fact that melatonin levels were elevated supraphysiologically in melatonin-treated birds. Future experiments should try to limit the dose of melatonin application to physiologically relevant concentrations in order to make the results applicable to natural populations. Future studies in this direction should also consider replicating this experiment, but compare the effects of melatonin administration on vocalizations in breeding and non-breeding birds. This is because increasing melatonin may not have affected song stereotypy in non-breeding black-capped chickadees because their songs may have already been less stereotyped than they were in breeding season (as a result of their transition into non-breeding condition). Analysis of volumes and neural characteristics of song control regions, as well as melatonin receptor functioning in these areas should also be investigated in order to get a more comprehensive understanding of the role of melatonin in black-capped chickadee songs and vocalizations.

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GENERAL DISCUSSION

The aim of the current studies were to (a) describe changes in diel changes in plasma melatonin concentration and (b) determine whether melatonin was involved in formation of dominance status by affecting aggression or vocalizations in black-capped chickadees. In these studies, black-capped chickadees had melatonin levels that were similar to other birds (see Bentley, 2003; Brandstatter, 2003; Singh & Haldar, 2007; Zawilska et al., 2006), and that they also had lower nocturnal concentrations in the non-breeding season compared to the breeding season. Overall, my results suggest that melatonin does not play a role in aggression, vocalizations, or establishment of dominance status in black-capped chickadees. I found no differences in melatonin levels between dominant and subordinate individuals and found no relationship between melatonin and aggressive behaviour. Previous work has reported that a positive association between melatonin and aggression exists (e.g. Paterson & Vickers, 1981; Demas et al., 2004, Heinzeller et al. 1988), however, the hypothesis that melatonin does not mediate black-capped chickadee aggression is further supported by my finding that exogenous melatonin does not affect number of aggressive vocalizations or song stereotypy. Moreover, dominant birds tend to produce aggressive vocalizations more often and also show greater song stereotypy, thus the absence of an effect of melatonin on vocalizations provides additional evidence that there is likely no relationship between melatonin and dominance status.

Nevertheless, these results should be interpreted with caution, as certain components of the experimental method and analysis were not ideal and could use improvement. Specifically, these findings may have been affected by the fact that I only measured melatonin at certain time points but did not investigate duration of nocturnal melatonin elevation, by high inter- and intra-assay variation in the EIAs, and by administration of supraphysiological doses of melatonin. The use of a topical cream as a method of exogenous melatonin delivery was also inappropriate for this experiment as the health of the animals may have been compromised by possible ingestion of the cream or handling stress. Furthermore, I did not examine brain tissue and thus am unable to confirm whether any changes in behaviour were caused by the effect of melatonin on neural components.

Future studies can overcome the limitations of this study by ensuring that both nocturnal melatonin rise and peak nocturnal amplitude are measured, by restricting application of exogenous melatonin to physiologically relevant concentrations, and_also by verifying whether melatonin treatment has direct effects on the brain by conducting studies on neural tissue. In addition, researchers interested in pursuing this relationship should consider comparing the effects of melatonin on vocalizations in breeding and non-breeding birds, as well as distinguish between the effects of serotonin and melatonin on aggression and dominance status.

This research expands knowledge of seasonal endocrine profiles of black-capped chickadees and also contributes to the body of research regarding the behavioral effects of melatonin in different animals. Although I have found no evidence that melatonin regulates dominance status, aggression, or vocalizations in the black-capped chickadee, there are many other endocrine mechanisms that have yet to be investigated that have the potential to regulate chickadee winter behaviour. The effects of serotonin on aggression in dominance interactions and the effects of exogenous melatonin on vocalizations of birds in breeding conditions are just two examples of prospective studies that should be undertaken in order to gain a deeper understanding of mechanisms influencing winter behaviour of black-capped chickadees.