Variation in sleep behaviour and its underlying causes: a study in a free-living blue tit population

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Blue tit, *Cyanistes caeruleus*
Photo by Jan Wijmenga

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Summary

Most if not all animals spend a considerable amount of their time within a 24-hour day in the sleep state. Several benefits for the organisms have been ascribed to sleep and a reduction in daily sleep amount can have various deleterious effects. However, the daily sleep amount varies considerably between different species and varies also between individuals within the same species. This has been mainly described in several human populations and the underlying causes of the variation such as gender, age or genetic variability have been reported in the literature. The focus of this dissertation is to provide a detailed description of behavioural aspects of sleep in a wild bird population. Using little infrared-sensitive cameras I recorded blue tits while roosting in nestboxes at night.

In chapter 1, I showed that the amount of sleep per night in blue tits follows predominantly the length of the night, at least during the winter months from November to March and is interrupted by many (short) awake phases. The reduction in sleep duration between the longest and shortest night was about 4.8 hours and blue tits have to be able to tolerate such reduced sleep amounts in the course of the season. My results further demonstrate that in mid-winter during long nights the birds start to sleep relatively late in comparison to sunset suggesting that they need to expand their activities as much as possible to obtain enough food. Males and females clearly differ in their sleep behaviour. This suggests that the sexes either differ in their sleep requirements, in their sleep quality or in trade-offs with other activities. Repeated measurements of most of the individuals allowed me to determine the individual consistency in sleep behaviour.

In chapter 2, I could show that environmental factors such as night temperature and seasonal date not only influence temporal sleep parameters but also nocturnal sleep-wake cycles. Nighttime sleep could be categorized into being rhythmic or arrhythmic and differed in many aspects regarding this categorization. Birds showed significant

repeatability in rhythmicity, suggesting a genetic basis of this trait, but the period length of the rhythms was not repeatable.

After finding strong individual consistency and inter-individual as well as sex-specific variation in temporal organization of sleep I investigated the influence of sleep parameters on reproductive success in chapter 3. My results did not show any association between variation in sleep behaviour and reproductive success in females, but I found associations in males that contradicted my expectations. The probability of gaining extra-pair paternity was higher in males that began to sleep earlier and slept longer. Further, I showed that partners of a breeding pair were mated assortatively in regard to their awakening times in the morning, but that broods of pairs with more dissimilar awakening times had higher fledging success.

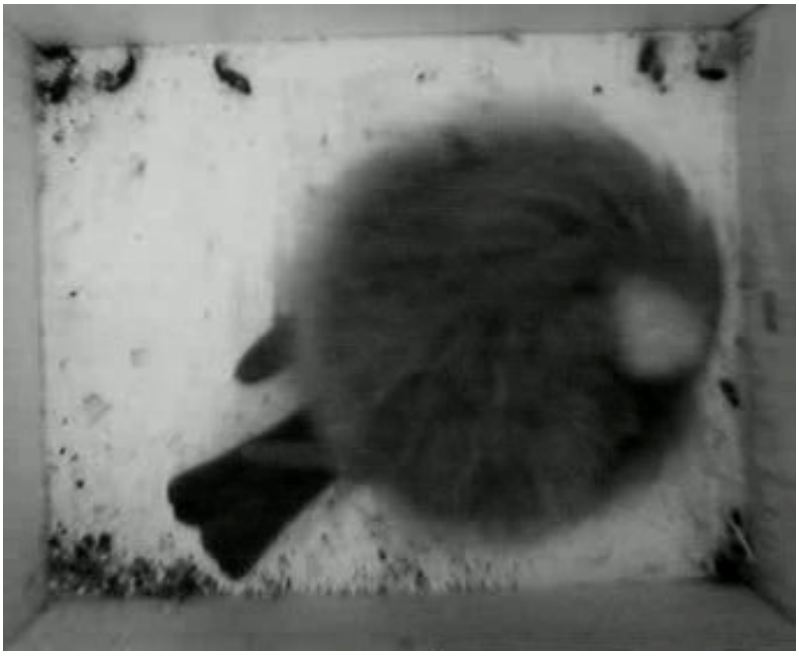
In the last chapter I aimed to examine the underlying genetic causes of the phenotypic variability. Therefore, I needed to identify informative polymorphisms in candidate genes of blue tits in a first step. In chapter 4, I describe the strategy that led to the identification of eleven polymorphisms with a high potential of being functional in genes which are involved in the regulation of circadian rhythms.

Finally, in chapter 5 I tested for associations between the described genetic polymorphisms and sleep phenotypes. My results revealed four significant associations between single nucleotide polymorphisms of three different genes and three sleep phenotypes. However, the number of significant results and the most significant result in my analyses could just as well have occurred by chance, given the large number of tests. I can therefore neither give evidence for an association between the genetic and phenotypic variations nor can I rule out such an association.

To conclude, in this dissertation I present several novel findings on the sleep behaviour of wild birds, with a special focus on temporal sleep parameters. My findings provide new insights on effects of environmental factors, sex, age and individuality on sleep behaviour and on the relationship between sleep and reproductive success. On the other hand, the

question about the genetic causes of inter-individual variation remains unanswered but gives possibilities for future research.

General Introduction



"If sleep does not serve an absolutely vital function, then it is the biggest mistake the evolutionary process has ever made." (Rechtschaffen 1971)

Sleep is a ubiquitous behaviour in the animal kingdom. It takes a considerable proportion of our lives every day. Not surprisingly, sleep has attracted the attention of many researchers over the past decades and which has led to numerous publications on this topic. Despite much attention, the reasons why we sleep are still not completely understood and are the focus of continuous debate among sleep scientists. The vast majority of studies on sleep have focused on mammals, particularly humans. It is rather straightforward to collect large sample sizes on human sleep habits by using questionnaires or observing subjects in sleep laboratories. Further, medical science has contributed knowledge about sleep in humans by examining sleep disorders and their underlying causes. However, studying sleep in animals and especially in wild animals

under natural conditions is far more challenging and therefore much less studies have been published. This dissertation has as its main aim to describe different aspects of sleep behaviour in a free-living population of a passerine bird – the blue tit *Cyanistes caeruleus*.

In the following sections, I will first give some general information about sleep in animals and briefly describe the organization, functions and regulation of sleep. Then, I will summarize the literature on sleep in birds. Finally, I will introduce the study organism, the blue tit, describe some general methods of the study and provide an outline of this thesis.

Sleep in the animal kingdom

All organisms on earth have to deal with daily changing environmental conditions that are caused by the earth's rotation. The most conspicuous change that occurs every day is the change between light and darkness, i.e. day and night. Living organisms have to cope with these extremely unequal states throughout their lives. Plants for example, can be observed to open and close their flowers according to the presence or absence of light. Most animals concentrate their main activities such as foraging/feeding, reproductive behaviour or territory defense on either day or night and are consequently well adapted in their physiology to the prevailing conditions during this portion of the 24-h cycle. The remaining time that is not filled with active behaviour, animals usually spend resting or sleeping.

Sleep is clearly among the most prominent of animal behaviours. Sleep has been found in most (if not all) species that have been studied so far (Cirelli and Tononi 2008; Siegel 2008), including the fruit fly *Drosophila melanogaster* (Hendricks et al. 2000), the zebrafish *Danio rerio* (Zhdanova et al. 2001; Yokogawa et al. 2007), the tree frog *Hyla septentrionalis* (Hobson et al. 1968), lizards (Ayala-Guerrero and Huitron-Resendiz 1991; Ayala-Guerrero and Mexicano 2008), birds (Amlaner and Ball 1983; Roth et al. 2006) and mammals (Zepelin et al. 1994). The definition of sleep is not straightforward

but it must be distinguished from rest and other distinct physiological states such as hibernation or torpor. A clear definition is necessary to determine whether a studied organism sleeps or not (Siegel 2008). Criteria that define sleep (at least in mammals) are usually immobility, an elevated arousal threshold, a stereotypic or species-specific posture, rapid reversibility through stimulation (Campbell and Tobler 1984; Cirelli and Tononi 2008) and closure of the eyes (Kavanau 1997). These are behavioural criteria and were described first. Later, electrophysiological criteria measured through electrical activity in the brain as indicators of sleep and wakefulness were also considered for a definition of sleep, predominantly in mammals and birds (Campbell and Tobler 1984).

The daily sleep amount of an animal can either be concentrated into a single sleep bout per 24 h, which is referred to as monophasic sleep (e.g. humans) or it can be partitioned into many sleep bouts alternated with waking phases, called polyphasic sleep (e.g. cats). Polyphasic sleep seems to be the ancestral situation in mammals and is associated with smaller body size and longer sleep durations (Capellini et al. 2008). Capellini et al. also hypothesized that polyphasic sleep is an adaptation to high predation risk, but they failed to find support for this idea. In contrast, they found polyphasic species to sleep in more protected sites. Most bird taxa are monophasic sleepers, but waterfowl and shorebirds are mostly polyphasic sleepers (Amlaner and Ball 1983). Not only does the phasing of sleep within a 24h-day vary between species, but also the daily amount of sleep. Some mammalian species such as horses and elephants only sleep 3-4 hours per day (Zepelin et al. 1994; Siegel 2005) while others, like the armadillo (*Chaetophractus villous*) spend up to 20 hours asleep (Affanni et al. 2001). At least two explanations are possible for this great inter-specific variation: the need for specific functional benefits of sleep varies between species (Zepelin et al. 1994; Lesku et al. 2006), or there are constraints on sleep time that differ between species (Allison and Cicchetti 1976). Species under higher risk of predation, for example, spend less time in a potentially "vulnerable" sleep state (Allison and Cicchetti 1976). Further, depending on foraging constraints and food availability, species vary in the amount of time that is available for sleep. Generally it has been observed that carnivores have the highest and herbivores the lowest daily sleep amount among mammals (Siegel 2005). In herbivores there is a negative correlation

between daily sleep duration and body mass, and there is an overall negative relationship between body mass and sleep amount in terrestrial mammals (Siegel 2005). Carnivores tend to sleep longer than predicted by their body mass alone. Because of their generally safe sleep places and the consumption of highly energetic food there is no necessity for continuous activity, which in turn allows them additional sleep time.

Organization of sleep

Mammals and birds are the only taxonomic groups that are known to exhibit two distinct brain states during sleep: rapid eye movement (REM) sleep - also called paradoxical sleep - and slow-wave sleep (SWS) - also called non-REM sleep (Campbell and Tobler 1984; Rattenborg 2006; Mignot 2008). During SWS the electroencephalogram (EEG) measurement of brain activity shows high-amplitude, synchronized, slow waves. The amount of slow-wave activity reflects sleep intensity. When slow-wave activity is high it is more difficult to awaken an animal from sleep. In contrast, during REM sleep the EEG is similar to the characteristics of wakefulness, with low-amplitude and high-frequency patterns. Loss of muscle tone (except in the ocular muscles) distinguishes REM sleep from wakefulness. During REM sleep, when most of our dreams occur, behavioural quiescence is maintained aside from frequent small twitches.

The two states, SWS and REM sleep, alternate in a “sleep cycle” that is repeated one or more times during a sleep bout (Szymczak et al. 1993; Zepelin et al. 1994) and is an example of an “ultradian” rhythm. Both total sleep duration and the duration of the individual sleep cycles (time taken to cycle from SWS through REM sleep to waking) varies between species. The length of the sleep cycles seems to be determined by body mass and brain size, at least in mammals, with small animals showing shorter sleep cycles (Siegel 2005) and spending more time in SWS (Allison and Cicchetti 1976). In addition to different physiological characteristics the two sleep states also seem to serve different functions.

Sleep functions and sleep deprivation

Why do animals sleep when they sacrifice opportunities to find mates, forage or invest in offspring during that time? Sleep is a largely exclusive state that precludes an animal from performing other tasks. Furthermore, sleep might not only be in conflict with other behaviours, it may even have substantial costs for animals. Compared to awake and alert animals, sleeping animals are relatively unresponsive and unaware of their environment and consequently experience an increased risk of predation and are less able to respond to changing environmental conditions.

To outweigh these costs, it seems likely that sleep must provide some vital functions to an organism. However, the functional benefits of sleep are still less well understood than those of other activities such as mating or foraging. On the other hand it has been proposed that sleeping may be less dangerous for an animal than roaming around, wasting energy and exposing itself to predators (Cirelli and Tononi 2008). In concordance with this the "null hypothesis" of the function of sleep states that it is not required, but just a behaviour that is adopted when animals cannot be usefully engaged in any other activity (Meddis 1975; Cirelli and Tononi 2008). The observed large variation in sleep duration across species could then easily be explained.

A variety of other, more complex functions of sleep have been described in the literature. SWS might be important for energy conservation and help to balance the costs of endothermy (Berger 1975; Berger and Phillips 1995), because it is associated with a reduction in body temperature and it is the state through which torpor and hibernation are entered. However, the *energy conservation hypothesis* remains controversial (Mignot 2008). Energy savings during sleep are small and dolphins for example show continuous movement during sleep (Zepelin et al. 1994). Sleep, and here especially REM sleep, seems to be beneficial to the brain as suggested by the fact that species with larger relative brain mass show a greater proportion of REM sleep (Lesku et al. 2006).

Sleep might play a role in *memory consolidation and learning* (Walker 2005; Walker and Stickgold 2006; Gais et al. 2007), as shown for humans (Plihal and Born 1997; Marshall et al. 2006) and birds (Jackson et al. 2008). However, this hypothesis also remains controversial (Vertes and Eastman 2000; Siegel 2001; Vertes 2004) and it is not totally clear what the molecular mechanisms are that make sleep beneficial for memory consolidation. Further, it has been suggested that sleep, and again especially REM sleep, is involved in *brain maturation*, supported by the finding that REM sleep decreases from birth to adulthood (Roffwarg et al. 1966; Jouvet-Mounier et al. 1969; Marks et al. 1995). The immune system may also benefit from sleep (Preston et al. 2009; Lange and Born 2011) and components of the immune system may even influence regulation of sleep (Opp 2005; Krueger 2008; Imeri and Opp 2009). There is no reason to believe that sleep only serves a single function, given these different possible benefits of sleep, but the functions are still a topic of much debate.

The importance and necessity of sufficient sleep for animals becomes most evident by the many studies that have reported negative consequences of long and short-term sleep deprivation. Among those are negative effects on vigilance, mood, cognitive performance and an increased risk of diabetes in humans, impaired recognition memory in mice or damage in brain and blood cells in rats (Ferrara and De Gennaro 2001; Van Dongen et al. 2003; Spiegel et al. 2005; Palchykova et al. 2006; Andersen et al. 2009). However, pigeons that were experimentally deprived of sleep did not show the “sleep deprivation syndrome” with hyperphagia, weight loss and debilitated appearance as rats did (Rechtschaffen and Bergmann 2002; Newman et al. 2009). Further, during migration birds are able to tolerate a natural sleep reduction for long periods of time without obvious deficits in cognitive function (Rattenborg et al. 2004). Nevertheless, birds and mammals show similarities in recovery sleep after sleep deprivation with elevated REM sleep and increased slow-wave activity during SWS (Tobler and Borbely 1986; Jones et al. 2008; Martinez-Gonzalez et al. 2008; Newman et al. 2009) suggesting a similar regulation of at least some aspects of sleep.

Regulation of sleep

Sleep is regulated by two principle mechanisms: a homeostatically controlled sleep drive and a circadian oscillator (Borbély 1982; Daan et al. 1984; Borbely and Achermann 1999; Landgraf et al. 2012). Sleep propensity builds up during waking and declines during sleep whereas the circadian oscillator is sleep-independent and determines the appropriate timing of sleep. The two processes are assumed to interact. Borbely and Achermann (1999) suggested a third process underlying sleep regulation, an ultradian process occurring within the sleep episode, which is represented by alternating non-REM and REM sleep states.

The anatomical substrate underlying the homeostatic process still remains elusive whereas the components and underlying mechanisms of the circadian oscillator have been described in detail. The first studies that identified genes as constituents of circadian clocks were carried out in fruit flies *Drosophila melanogaster* (Konopka and Benzer 1971), algae *Chlamydomonas reinhardi* (Bruce 1972), fungi *Neurospora crassa* (Feldman and Hoyle 1973) and eventually in mice (Vitaterna et al. 1994). Later, a variety of these so-called "clock genes", making up the intracellular machinery of the circadian oscillator, were discovered in other mammals and birds. They were found to be highly conserved between taxa (Dunlap 1999; Young and Kay 2001; Bell-Pedersen et al. 2005). Due to transcriptional-translational feedback loops the clock genes are transcribed rhythmically (Panda et al. 2002; Reppert and Weaver 2002).

In birds, the positive limb of the feedback loop is represented by the transcription factors CLOCK and BMAL1 which heterodimerize and activate the transcription of two *PERIOD* genes (*PER2, PER3*), two *CRYPTOCHROME* genes (*CRY1, CRY2*) and other clock controlled genes (Bell-Pedersen et al. 2005). PER and CRY proteins translocate back into the nucleus where they act as the negative limb of the loop by down-regulating transcription. Post-transcriptional control is mediated through phosphorylation of the proteins by casein kinases (CKIε and CKIδ) (Reppert and Weaver 2002). In addition to these core clock genes, several other genes are important for the regulation and

generation of circadian rhythms in behaviour. Circadian clocks operate in nearly all cells and tissues and are organized hierarchically. The main circadian pacemakers in birds are the pineal gland, the retina and the suprachiasmatic nuclei (SCN), regulating secondary circadian clocks in peripheral tissues (Gwinner and Brandstaetter 2001; Bell-Pedersen et al. 2005) and they are reset by external light stimuli.

Sleep in birds

Although many studies focused on the molecular basis and functions of sleep, sleep has received little attention from behavioural ecologists. In particular, sleep in birds has not been studied to a great extent yet and most studies on avian sleep are limited to captive animals with the focus on physiological characteristics (e.g. Ookawa and Gotoh 1965; Berger and Walker 1972; Walker and Berger 1972; Szymczak et al. 1996). However, studies on sleep in wild animals are important to understand ecological and evolutionary mechanisms. It has been shown that sleep behaviour in captive animals can vary tremendously from the behaviour of wild individuals (Rattenborg et al. 2008), therefore it is of great importance to study sleep in animals under natural conditions without direct human influences. The under-representation of literature covering sleep behaviour in wild birds may be related to the fact that sleeping birds are difficult to observe, especially at night, and that birds are easily disturbed. Some birds roost in cavities, some in trees or dense shrubs, and others on the water or out at sea.

Behavioural aspects of avian sleep have been investigated in a few studies. For example there are studies on vigilance in sleeping birds (Lendrem 1983; 1984; Dominguez 2003; Beauchamp 2011), on the influence of temperature on sleep behaviour in captive birds (Wellmann and Downs 2009), on effects of ectoparasite presence on sleep amount in incubating females (Christe et al. 1996; Tripet et al. 2002), on sleep depth in relation to the time of day (Dewasmes and Loos 2002), on tactile arousal thresholds in sleeping penguins (Dewasmes and Telliez 2000), and on responses to predator odour in sleeping great tits (Amo et al. 2011).

When asleep, birds adopt different postures (summarized in Amlaner and Ball (1983)): they either put their bill under the scapular feathers (e.g. commonly seen in small passerines), under the wings (e.g. penguins), or on the back (e.g. birds with long necks such as flamingos). Birds might sleep with the bill pointing forward, or put their head on the ground (e.g. large flightless species like ostrich and emu).

The total sleep duration per day varies between different bird species and so does the length of individual sleep bouts. Sleep bouts can be as short as a few seconds, but can also last several minutes or even hours (Klima 1966; Hamerstrom and Janick 1973; Szymczak et al. 1993; Gauthier-Clerc et al. 2002). Based on a literature analysis covering 32 bird species (16 families) Amlaner and Ball (1983) report an average total sleep time of 6.9 ± 0.7 (mean ± s.e.) hours, with a great variability of several hours between species. The greatest variability could be observed in passerines and other small birds and the best predictor of sleep duration was night length and latitude (Amlaner and Ball 1983).

Study species: the blue tit

I chose the blue tit (*Cyanistes caeruleus*) as study organism. Blue tits are small passerine birds (10.5-12 cm, 10-12 g) in the family Paridae, that are common throughout temperate and subarctic Europe and western Asia. In most of their range, blue tits are resident and non-migratory. Especially during winter blue tits can often be seen at bird feeders in urban areas as part of mixed-species flocks. They breed in deciduous or mixed woodlands (preferably oak) and use cavities such as tree holes as nesting sites, but also readily accept artificial nestboxes. This makes the blue tit an ideal study species in the field of ecology because breeding parameters can be easily measured. Nestboxes are also used for roosting at night during the winter months. The two sexes are similar in size and plumage characteristics, although females are slightly paler than males (less UV blue (Andersson et al. 1998; Hunt et al. 1998)). Most males and females form socially monogamous pairs in the breeding season with biparental brood care, but social polygyny is not uncommon (Kempenaers 1994). About 30-50% of females also engage in copulations with males other than their social mate (extra-pair males) leading to about 11-14% of all young being

extra-pair offspring (Kempenaers et al. 1997). Clutches contain in general 6-12 eggs that are incubated by the female only.

General methods

I carried out my study in a blue tit population near Landsberg am Lech in Southern Germany (Westerholz, 48°08' N, 10°53' E). The population was established in 2007 for a long-term study on the breeding biology of blue tits. The study area is an unmanaged part of mixed deciduous woodland, which is dominated by oak trees (*Quercus* sp.). It contains 277 nestboxes with an entrance hole of 26 mm. There are around 60-80 breeding attempts of blue tit pairs each breeding season and nestboxes are used for roosting at night between November and March by 14-77 (mean = 40) individuals. During regular night catches in winter, all new individuals roosting in nestboxes are banded, measured (tarsus and wing length, weight) and a blood sample is taken for genetic analyses. During the breeding season, a variety of breeding parameters of the birds are collected every year, such as date of the first egg of each breeding female, clutch size, number of hatchlings and number of fledglings. All adults are caught at least once to measure them, to equip them with unique rings and an RFID transponder and to take a small blood sample for genetic analyses. All nestlings are banded with a unique ring, measured and bled once at an age of 14 days. Therefore a comprehensive dataset on breeding success and fitness traits of individuals in this population is available for analyses.

I video-recorded the birds while they were present in the nestbox at night during the winter months. For this purpose I put together small camera systems, consisting of a black-and-white infrared-sensitive camera (Fig. 1a), attached to the nestbox lid and connected via a long cable to a digital recorder which was stored in a water-proof plastic box placed on the ground under the nestbox (Fig. 1b). Six LEDs emitting infrared light were placed around each camera lens. The plastic box also contained two rechargeable lead-acid batteries that supplied enough power for the whole system to record on two consecutive nights. Above each nestbox I fixed a small data logger to record temperature and light intensities. Blue tits are very territorial and use the same roost site over days or

even months, if they do not get disturbed. This allowed me to record most of the individuals repeated times (up to 22 times in the case of one individual).

Fig. 1a Mini-camera attached to the inside of a nestbox lid, surrounded by six infrared light LEDs

Fig. 1b Camera system installed at a nestbox in the forest

Fig. 2 Picture produced by the camera system

Outline of the thesis

The general aim of the work reported on in this thesis was to describe in detail the sleep behaviour of a free-living bird. More specifically, I investigated (1) variation in temporal parameters of sleep and the factors associated with this variation, (2) whether there is individual variation in sleep-wake cycles, i.e. in the level of rhythmicity and period length, (3) whether variation in sleep behaviour is associated with fitness-related traits, (4) whether there are naturally occurring polymorphisms in candidate genes with a high likelihood of being functional and (5) whether associations can be detected between these genetic polymorphisms and inter-individual variation in sleep phenotypes.

In chapter 1, I report on an investigation of temporal aspects of sleep behaviour in the field. I quantified and described the sleep behaviour of blue tits under free-living conditions. I focused on variation in sleep behaviour between individuals and investigated which of the environmental (season, local light conditions) and individual (age and sex) factors influence sleep parameters. Further, I investigated whether individuals show consistency in their behaviour and compared within- and between-individual variation.

Chapter 2 focuses on ultradian rhythms. Here, I looked more closely at the nocturnal sleep-wake cycles of blue tits and analysed whether there is rhythmicity and variation in period length. I tested whether factors such as sex, age, environmental parameters and individuality influence periodicity.

In chapter 3, I report on the relationships between inter-individual variation in temporal aspects of sleep and fitness-related traits. I used detailed information on breeding parameters of the individuals that were available from a long-term population study, and tested whether differences in sleep behaviour are related to variation in reproductive success.

In chapter 4, I describe two different search strategies to find likely functional polymorphisms in avian candidate genes that are involved in the regulation of circadian rhythms. This approach can be applied when searching for polymorphisms with a functional or adaptive relevance in non-model organisms for which whole genome sequences are not available.

In chapter 5, I report on the testing of associations between the polymorphisms in candidate genes for circadian rhythms described in chapter 4 and inter-individual variability in various sleep phenotypes. Because of the multiple-tests performed, the occurrence of false-positive results is enhanced. I applied a permutation procedure to evaluate my results study-wide.

Following the main chapters, I summarize and discuss the results and give an outlook for future research in a general discussion.

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Chapter One

Variation in sleep behaviour in free-living blue tits, *Cyanistes caeruleus*: effects of sex, age and environment

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ABSTRACT

Although sleep is fundamental for survival, not much is known about sleep behaviour in free-living animals and between-individual variation in sleep patterns has hardly been studied, except in humans. We analyzed sleep behaviour in a free-living population of blue tits in southern Germany. We recorded individuals roosting in nestboxes between November and April using infrared-sensitive cameras. We investigated the following sleep parameters: time of entering and leaving the nestbox, sleep onset, awakening time, sleep duration, midpoint of sleep, latency to sleep and frequency and duration of nocturnal awakenings. Sleep onset, awakening time and sleep duration followed seasonal changes in day length. Blue tits slept ca. 4.8 h longer in winter than in spring. During the night, birds woke up between 23 and 230 times, but this did not change seasonally. Local light conditions influenced awakening time: birds at brighter locations woke up earlier. Females slept on average 15 minutes longer per night than males and this sex difference became more pronounced in early spring. Although females spent a greater proportion of the night awake than males, they still slept more overall. First-year birds spent more time in the nestbox after waking up and left the nestbox later in the morning than older individuals. Repeatability estimates showed that individuals were consistent in their sleep behaviour over the two-year study period. Our results indicate that sleep patterns are individual-specific traits in blue tits. We suggest that the observed sex difference in sleep duration is caused by sexual selection.

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INTRODUCTION

Sleep is a widespread behaviour in the animal kingdom being found in most if not all animals (Cirelli and Tononi 2008; Siegel 2008). It is generally defined as a rapidly reversible state of immobility and greatly reduced responsiveness (Siegel 2008). Animals spend a considerable proportion of each day and of their entire lifetime asleep (Zepelin et al. 1994). Daily sleep duration can vary tremendously among species. For example, elephants and horses apparently require only a few hours of sleep, whereas the armadillo (*Chaetophractus villosus*), opossum (*Didelphis* sp.) and some species of bats spend about three quarters of a 24-hour day sleeping (Van Twyver and Allison 1970; Meddis 1975; Zepelin et al. 1994; Affanni et al. 2001). In addition to inter-specific variation in sleep requirements, there is also substantial variation in sleep duration among individuals of a single species. Individual variation in duration and other characteristics of sleep behaviour has only been studied in detail in humans (Webb and Agnew 1970; Webb and Friel 1971; Roenneberg et al. 2003; Dijk and von Schantz 2005; Buckelmüller et al. 2006) and is partly explained by effects of gender and age (Lindberg et al. 1997; Hume et al. 1998; Dijk et al. 2000; Jean-Louis et al. 2000; Van Cauter et al. 2000).

Because sleep is such a ubiquitous phenomenon, it most likely serves important adaptive functions. However, there is still considerable debate about what these functions are. The early hypothesis that sleep is simply a state animals adopt when they do not have anything else to do (Meddis 1975) is clearly untenable. Many studies have shown negative consequences for animals that were deprived of sleep. For example, sleep deprivation induced DNA damage in brain and blood cells in rats (Andersen et al. 2009) and negatively affected vigilance, mood and motor or cognitive performance in humans (Ferrara and De Gennaro 2001; Van Dongen et al. 2003). Sleep may have a restorative function for the brain (Tononi and Cirelli 2006; Mignot 2008) and it may be essential for memory consolidation (Solodkin et al. 1985; Walker and Stickgold 2006; Jackson et al. 2008; Mignot 2008). Sleep may also serve to conserve energy or to reduce exposure to predators (Meddis 1975; Zepelin et al. 1994; Lima and Rattenborg 2007), although this would also be accomplished through simple resting.

Despite the fact that sleep is ubiquitous and despite strong empirical evidence that sleep is important for an individual's health, sleep behaviour has received little attention in studies of animal behaviour and behavioural ecology. Most studies on sleep and its functions have been conducted in captivity on mammals, and there is an extensive literature covering sleep related issues in humans. Studies on sleep in birds are mostly limited to captive birds, especially pigeons and chickens, and focus on physiological characteristics of sleep (Ookawa and Gotoh 1965; Berger and Walker 1972; Van Twyver and Allison 1972; Walker and Berger 1972; Ayala-Guerrero 1989; Ayala-Guerrero et al. 2003). These studies have found that birds show two distinct sleep states (slow-wave sleep, SWS and rapid-eye-movement sleep, REM) characterized by changes in brain activity remarkably similar to those in mammals (Rattenborg 2006; Rattenborg et al. 2009). Furthermore, previous studies have shown that at least some bird species are able to engage in unihemispheric sleep (Szymczak et al. 1996; Rattenborg et al. 1999; Bobbo et al. 2002; Bobbo et al. 2006a; b; Fuchs et al. 2006).

Only a handful of studies considered behavioural aspects of sleep in birds. A few investigated sleep and vigilance in relation to season or to the position of individuals within a flock (Lendrem 1983; Gauthier-Clerc et al. 2000; Dominguez 2003). One study investigated the influence of temperature on sleep behaviour in three passerine birds in captivity (Wellmann and Downs 2009). Another study experimentally investigated the effect of the presence of ectoparasitic hen fleas in nests of great tits (*Parus major*) on the total amount of sleep (Christe et al. 1996).

To our knowledge, individual variation in sleep behaviour has not yet been systematically studied in any free-living bird. Studies in the wild are important because sleep behaviour of animals observed in captivity may differ substantially from behaviour shown in the natural environment (Dunnett and Hinde 1953; Rattenborg et al. 2008). The general aim of our study is to investigate sleep behaviour in a natural population of blue tits (*Cyanistes caeruleus*). The blue tit is a widespread and common passerine bird in Europe and parts of northern Africa. In Germany, blue tits are year-round resident and inhabit

deciduous and mixed woodlands, as well as parks and gardens. They breed in natural cavities, but readily accept artificial nestboxes. We made use of the fact that in winter blue tits regularly roost in the nestboxes, which allowed us to monitor their night-time behaviour without disturbance.

The specific aims of this study were threefold: (1) to describe seasonal variation in several variables reflecting the timing and duration of sleep; (2) to test whether factors such as local light conditions, temperature, individual age and sex explain some of the within-season variation; and (3) to estimate the proportion of variation in sleep behaviour that is individual-specific (individuality). Because most knowledge about inter-individual variation in sleep behaviour comes from studies in humans, we discuss our findings in comparison to human sleep patterns.

METHODS

Study Site and General Field Procedures

The study was carried out during the winter seasons 2007-2008 and 2008-2009 in a population of blue tits in southern Germany (Westerholz, 48°08'N 10°53'E). The study site (39 ha) is a natural reserve ("Naturwaldreservat") dominated by oak trees (*Quercus* sp.) and forms the north-eastern tip of a larger mixed deciduous forest. In November-December 2006, we put up 277 nestboxes (12 × 15 cm and 25 cm high) with an entrance hole of 26 mm, which excludes the larger great tit. Blue tits readily accepted nestboxes for roosting during winter and for breeding.

We caught blue tits inside the nestboxes during monthly checks at night in winter (November-February in both seasons) and once during nestling feeding (May-June in the breeding seasons 2007 and 2008). We determined age by comparing the coloration of primary and secondary coverts and classified individuals as either yearling or adult (Svensson 1992). We marked each bird with a numbered metal band of the German ringing scheme and with a unique combination of three plastic colour bands. We also took a 10-50 µl blood sample from the brachial vein for genetic analyses and implanted a

RFID transponder (Biomark Inc., Idaho, U.S.A.) subcutaneously between the shoulder blades. This allowed us to determine the identity of individuals occupying a nestbox at night without disturbing the sleeping bird (see *Recording Sleep Behaviour*). Transponders were 8.5 mm long, 2.1 mm in diameter and weighed 0.067 g (0.6% of the average body mass of an adult blue tit). We did not encounter any problems during implantation and all birds behaved normally after release.

Recording Sleep Behaviour

Every four weeks we checked all nestboxes at night for sleeping birds using a handheld transponder reader (EUR 1000 Multi-Chip from EURO I.D., Weilerswist, Germany), which we moved around the outside of the nestbox. Our previous observations suggested that only rarely a bird carrying a transponder is missed using this technique. The following day, we installed infrared sensitive cameras in all boxes where a roosting blue tit had been detected. We also cleaned nestboxes of faeces to attain higher movie quality. Cameras were programmed to record on two consecutive nights between an hour before sunset and an hour after sunrise. We refer to these two consecutive nights as a recording session. We recorded 13-37 individuals (median 27) per night. We never observed two birds in a nestbox at the same time.

The infrared sensitive black-and-white cameras (S/W-Kamera modul 1 from Conrad Electronic, www.conrad.de) were attached to the nestbox lid. As a light source, we placed six LEDs emitting infrared light (peak wavelength 940 nm) invisible to the blue tit eye (Hart et al. 2000; Hart 2001) around each camera objective. We connected each camera to a digital recorder (Abus, TV8450), that saved the recording on a 2GB SD card. Power for all electrical devices was supplied by lead-acid batteries (Panasonic, LC-R067R2P). To obtain information about the microclimate at a nestbox, we fixed a Hobo Data Logger (Onset Computer Corporation, Bourne, MS, U.S.A.) just above each nestbox. The sensitive electrodes of the loggers pointed in the direction of the entrance hole and recorded light intensity (in lx) and temperature (in degrees C) at 1 min intervals. Based on the logged data we calculated mean light intensities at each nestbox in the evening (30

min before – 30 min after sunset) and in the morning (30 min before – 30 min after sunrise), and the mean night temperature (between 2300 and 0000 hours).

In the first season (early January to late March 2008) we recorded 69 individuals (45 males, 24 females) during four recording sessions (total of eight nights). Of these 69 individuals, 21 (30.4%) were yearlings. In the second season (mid-November 2008 to early April 2009), we recorded 60 individuals (37 males, 23 females) during six recording sessions (total of 12 nights). Seven of the 60 individuals (11.7%) were yearlings. Thirty-three individuals were recorded in both winters. Thus, in total we obtained 532 recordings of 96 different blue tit individuals (60 males, 36 females), with an average ± SD of 5.5 ± 3.7 recordings per individual (range 1-18). In 94% of cases individuals were recorded on both nights of one session.

Sleep Parameters

We used PowerDirector NE Express from CyberLink (www.cyberlink.com) to analyse the video recordings. We quantified 10 parameters of blue tit sleeping behaviour (for definitions, see below): (1) sleep onset; (2) awakening time; (3) entry time; (4) leaving time; (5) evening latency; (6) morning latency; (7) sleep duration; (8) midpoint of sleep; (9) frequency of awakenings per hour; and (10) the proportion of time spent awake per night (excluding evening and morning latency). We chose to record these 10 sleep parameters because they reflect parameters that are commonly used in human sleep studies, in particular those that assess individual 'chronotypes' (Hume et al. 1998; Park et al. 2002; Roenneberg et al. 2003; Roenneberg et al. 2004; Kronholm et al. 2006).

A bird was considered asleep when it showed the classical sleep position with the beak pointing backwards and tucked under the scapulars and feathers fluffed (Amlaner and Ball 1983). It was usually easy to distinguish sleep phases from awake phases, because awake birds were mostly active preening or moving around in the nestbox. Only a few cases were more ambiguous, when a bird sat quietly for some time with its head pointing forward. This typically happened in the morning before the bird left the nestbox and we considered these periods as awake phases.

We defined sleep onset as the time when the first sleep bout of at least 30 seconds started. In birds, sleep bouts can be as short as 10 seconds (as measured via brain activity patterns; Rattenborg, personal communication, (Szymczak et al. 1993) and we occasionally observed birds in the sleeping position for less than 30 s. However, we did not include these shorter bouts to define the onset of sleep, because usually birds still moved intensely during these first short sleep bouts. We defined awakening time as the time when the last sleep bout of minimum 10 s ended. We defined sleep duration as the difference between awakening time and sleep onset and calculated the midpoint of sleep by adding half of the sleep duration to the time of sleep onset. Awakenings during the night were counted when they lasted more than 2 s. If birds only turned their head from one side to the other within 1-2 s they were not considered to be awake. Sleep bouts between awakening phases had to last a minimum of 10 s, otherwise two consecutive awake phases were pooled (including the short sleep bout). We defined entry time as the time of entering the nestbox in the evening and leaving time as the time of leaving the nestbox in the morning. Generally birds entered the nestbox once in the evening and stayed inside until they left again the next morning. In four cases, we observed that a bird left again after entering in the evening to return 7-13 min later. In one case, a blue tit left the nestbox in the morning, but returned to it within 17 min. In these exceptional cases we used times of the second entry or first exit, respectively, as entry and leaving times. We defined evening latency as the difference (in min) between entry time and sleep onset and morning latency as the difference (in min) between awakening time and leaving time.

Data Analysis

We converted entry times, leaving times, times of sleep onset and awakening times to times relative to sunset or sunrise (reference data from the town of Kaufering which is ca. 7 km from our study site). We log-transformed evening and morning latencies, which reduced the right skew of these two parameters.

In a first step, we decomposed the phenotypic variance in relative time of entry, sleep onset, awakening and leaving, and in sleep duration into four main components: long-

term environmental (seasonal) effects; short-term environmental variation shared among nestboxes; consistency within individual birds; and residual variation (not shared among nestboxes). We did so by fitting univariate linear mixed-effect models that included sex and age (yearling versus adult) as fixed effects and with recording session, recording date and bird identity as random effects. We extracted the variance explained by the three random effects and the residual variance and calculated the proportion of the variance explained by these factors.

In a second step, we tested the effects of sex, age and light intensity at the nestbox on different sleep parameters, and we determined the variance components due to individual (individual consistency) and nestbox (spatial heterogeneity) after controlling for seasonal effects. In this analysis, temperature measurements were not included because differences between nestboxes are negligible. Some sleep parameters showed seasonal changes not only in their mean values, but also in their variances around the mean. This produces patterns of heteroscedasticity, which violates the assumption of identically distributed residuals and will affect the significance tests of fixed effects. Transformation did not solve this issue. Therefore, we standardized entry time, leaving time, sleep onset, awakening time, sleep duration, midpoint of sleep and morning and evening latency for the analyses by subtracting the date-specific mean from the raw values and dividing by the date-specific standard deviation. This standardization removed seasonal and date-specific changes in means and variances and both factors (recording session and date) were therefore excluded from models with standardized values. We fitted univariate linear mixed models with sex, age and mean light intensity at the nestbox as fixed effects and individual and nestbox identity as crossed random effects (in 40 nestboxes more than one and up to four individuals were recorded in different nights and 58 individuals were recorded in two to five different nestboxes). Variance components were converted to proportion of variance explained and their significance was tested by likelihood ratio tests.

The time when individuals entered the nestbox in the evening (entry time, standardized) and the time when they began to sleep (sleep onset, standardized) were highly correlated

(r_{528} = 0.98). Similarly, we found a strong correlation between the time when birds woke up (awakening time, standardized) and when they left the nestbox in the morning (leaving time, standardized: r_{519} = 0.82). Therefore, we further focus on sleep onset and awakening time.

We did not apply standardizations in the analysis of nocturnal awakenings, because seasonal effects for these traits were very weak. Therefore, we fitted separate linear mixed effect models for frequency of awakenings and proportion of time spent awake as dependent variable, including sex and age as fixed effects and bird identity, nestbox, recording date and recording session as random effects. As for the other parameters we then estimated variance components of the random factors.

We tested temperature effects on evening latency, morning latency, frequency of awakenings and proportion of time spent awake (the other parameters were strongly influenced by light). We calculated the average difference in mean night temperature and the average difference in sleep parameters between each of two consecutive recording nights and fitted linear models with difference in mean temperature as the independent variable and each sleep parameter as response variable.

Finally, we analyzed the relationship between sleep onset and awakening time and between evening and morning latency, by fitting models with awakening time (or morning latency) as the response variable and sleep onset (or evening latency), sex, age and mean light intensity as fixed effects, and bird and nestbox identity as random effects. However, before fitting the models, we decomposed the explanatory variable sleep onset (or evening latency) into its between-individual and its within-individual component (Van de Pol and Wright 2009) and fitted these two as separate predictors. This allowed us to evaluate within- and between-individual correlations separately.

Owing to occasional technical failure (i.e. discharged batteries, poor quality of the recording) and to the fact that information about age was not available for all individuals, we have varying numbers of missing values in the analyses. In the final models we

included 498-509 observations of 94 individuals in 127-128 different nestboxes for parameters 1-8. For parameters describing nocturnal awakenings (9, 10) we included 442 observations of 89 individuals in 119 nestboxes. We defined the beginning of the breeding season as the date when the first nest material was found in a nestbox in the study area. Following this definition, six recordings (in March and April) belonged to the breeding season, while all others (November - February) were considered the nonbreeding (winter) season.

All analyses were conducted in R 2.9.0 (http://cran.r-project.org) using the lmer function from the lme4 package for mixed models (Bates et al. 2008) and the cor.test function from the stats package for Pearson correlations.

Ethical Note

A recent study showed that subcutaneous implantation of passive integrated transponder (PIT) tags did not negatively influence fledging success, survival and recruitment of great tits (Nicolaus et al. 2008). These PIT tags are essentially the same as the RFID transponders we used. In our study population, RFID transponders have been applied since the beginning of 2007. We did not observe long or short-term effects of implantation on condition of adults and chicks. Recaptured individuals carrying a transponder did not significantly differ in their body mass from new captures without transponder (after correcting for age and tarsus length, $N = 225$, $P = 0.49$) and in all cases the transponder was well-placed and the lesion well-healed. Adults did not modify their feeding behaviour after implantation during the breeding season. There was no difference in mean brood mass between nests with newly implanted adults and recaptures ($N = 204$, $P = 0.76$; measured four days after capture). Permits were obtained from the Bavarian government and the Bavarian regional office for forestry LWF.

RESULTS

Seasonal Patterns in Timing and Duration of Sleep

The average time of sleep onset showed a strong seasonal pattern: individuals began to sleep earliest in December and latest in April (Table 1). Sleep onset relative to sunset also changed seasonally (Table 1, Fig. 1a). During the shortest days in November-January, most birds began to sleep after sunset when average light intensities were low (approaching 0 lx), whereas birds started to sleep before sunset in February-March at higher light intensities (50-400 lx). In April, the average onset of sleep coincided with sunset. In all cases, birds started to sleep before the end of civil twilight (when the centre of the sun is 6° below the horizon). Recording session explained 33% of the variance in relative sleep onset times, while recording date within sessions explained 14%. Individual identity explained an additional 23% of the variation.

The average awakening times showed a similar seasonal pattern (Table 1, Fig. 1b). Of the total variance in relative awakening time, 17% was explained by recording session, 15% by recording night (within session) and 39% by individual identity. Independent of the season, most blue tits woke up before sunrise but after the start of civil twilight (when the sun is 6° below the horizon), usually at low light intensities (0-20 lx).

Sleep duration increased from November to December and decreased from January to April (Table 2, Fig. 2). Relative sleep duration also followed the patterns of relative sleep onset and awakening time, with recording session explaining 26% of the variance, recording night within session 17% and individual identity 29%.

Variation in Times Awake in the Nestbox

Evening and morning latency did not show obvious seasonal changes (Table 2), and were usually short, with evening latency being shorter and less variable (mean ± SD = 3.1 ± 0.8 min,) than morning latency (5.6 ± 1.6 min). In the evening, after entering the box, birds moved or looked around in the nestbox, preened their plumage and fluffed up their feathers. After the birds woke up in the morning, we observed them stretching, preening,

moving inside the box and sitting at the entrance hole looking outside. Between two consecutive recording nights, birds on average had longer evening and morning latencies when mean night temperatures were higher (linear models; $N = 202$; evening latency: $d = 13.04 \pm 4.02$ SE, $Z = 3.25$, $P = 0.01$; morning latency: $d = 24.85 \pm 10.06$ SE, $Z = 2.47$, $P = 0.04$).

Table 1
Mean, SD (in min) and range (earliest-latest) of time of sleep onset and awakening for each recording date

Date	Session	Sunset	Sunrise	Sleep onset				Awakening time			
				N	Mean	SD	Range	*N*	Mean	SD	Range
12 Nov 2008	1	16:42	7:21	29	16:44	5.35	16:32-16:53	29	7:03	5.66	6:52-7:13
13 Nov 2008	1	16:41	7:22	29	16:42	6.38	16:30-16:53	28	7:06	5.32	6:56-7:18
10 Dec 2008	2	16:23	7:57	21	16:30	6.00	16:20-16:41	22	7:42	6.28	7:24-7:50
11 Dec 2008	2	16:23	7:58	26	16:31	7.06	16:16-16:41	26	7:40	7.03	7:23-7:50
2 Jan 2008	3	16:34	8:06	34	16:50	7.09	16:39-17:02	34	7:49	7.34	7:36-8:05
3 Jan 2008	3	16:35	8:06	33	16:48	9.42	16:20-16:58	31	7:48	7.13	7:22-7:59
14 Jan 2009	4	16:49	8:02	22	16:54	13.86	16:23-17:09	22	7:46	6.84	7:36-8:02
15 Jan 2009	4	16:51	8:01	22	16:59	12.55	16:29-17:14	22	7:49	7.51	7:35-8:05
30 Jan 2008	5	17:12	7:47	37	17:08	10.44	16:41-17:26	37	7:34	5.35	7:25-7:42
31 Jan 2008	5	17:13	7:46	34	17:15	13.10	16:47-17:35	28	7:37	7.50	7:25-7:49
11 Feb 2009	6	17:33	7:28	25	17:23	13.63	16:54-17:44	25	7:17	6.84	7:06-7:37
12 Feb 2009	6	17:34	7:27	23	17:25	15.62	16:48-17:50	24	7:14	6.05	7:04-7:30
27 Feb 2008	7	17:57	7:01	32	17:48	8.21	17:30-18:09	32	6:39	5.80	6:24-6:49
28 Feb 2008	7	17:58	6:59	33	17:48	8.91	17:26-18:06	33	6:48	6.02	6:35-7:09
18 Mar 2009	8	18:26	6:21	19	18:22	9.34	18:01-18:41	19	6:02	7.32	5:50-6:15
19 Mar 2009	8	18:28	6:19	22	18:05	10.87	17:44-18:22	22	6:04	6.23	5:55-6:19
27 Mar 2008	9	18:40	6:03	33	18:30	10.92	18:00-18:54	33	5:47	7.91	5:36-6:06
28 Mar 2008	9	18:41	6:01	28	18:39	9.93	18:19-18:56	28	5:43	9.23	5:32-6:02
7 Apr 2009	10	18:56	5:41	15	18:55	6.45	18:44-19:06	15	5:19	12.22	4:53-5:34
8 Apr 2009	10	18:57	5:39	13	18:56	7.65	18:43-19:07	11	5:19	13.55	4:47-5:36

Local times of sunset and sunrise (the following morning) are also given. All times refer to Central European Time.

On average ± SD, birds woke up between 38 ± 10 and 99 ± 50 times (range 23-230) on different recording nights with an average frequency of 2.8 ± 0.6 to 9.5 ± 4.8 awakenings per hour (Table 3). During these awakening phases birds usually preened, scratched, stretched legs and wings or moved inside the box. The number of awakenings per night did not change seasonally, but increased highly in April. Given the changes in sleep duration, the frequency of awakenings decreased from a mean ± SD of 3.8 ± 0.6 awakening phases per hour in November to 3.0 ± 0.6 in December and then increased to

9.5 ± 4.8 in April (Table 3). Recording session explained 32% of the variance in frequency of awakenings whereas recording night within session explained only 2%. The duration of the longest continuous sleep bout was highest in the first half of January and shortest in April (Table 3). Recording session explained 30% of the variance in the maximum duration of sleep bouts, whereas recording night explained 3%. On average, birds spent 92-99% of their sleep duration actually asleep, which corresponds to a total time spent awake at night of 12 ± 6 min SD (range 5-27 min) in February and 49 ± 25 min (range 17-93 min) in April (Table 3). Recording session explained 33% of the variance in the proportion of time spent awake, whereas recording night explained 7%. Birds woke up more frequently at night and spent a greater proportion of the night awake when the night was warmer (linear models; $N = 202$; frequency awake: $d = 0.18 \pm 0.05$ SE, $Z = 3.59$, $P = 0.007$; proportion awake: $d = 0.0033 \pm 0.00069$ SE, $Z = 4.80$, $P = 0.001$).

Table 2
Mean, SD and range (shortest-longest) of sleep duration (in h), evening latency (time difference between entry of nestbox and sleep onset, in min) and morning latency (time difference between awakening and leaving the nestbox, in min) for each recording date

Date	Sleep duration (h)				Evening latency (min)				Morning latency (min)			
	N	Mean	SD	Range	*N*	Mean	SD	Range	*N*	Mean	SD	Range
12 Nov 2008	29	14.3	0.2	14.1-14.6	29	2.9	1.1	1.5-6.8	29	5.2	3.5	0.4-12.7
13 Nov 2008	28	14.4	0.2	14.1-14.7	29	3.5	1.4	1.2-7.7	29	5.7	4.8	0.2-18.2
10 Dec 2008	21	15.2	0.2	14.8-15.5	21	2.2	1.2	0.9-4.7	22	6.6	6.3	0.2-23.7
11 Dec 2008	26	15.2	0.2	14.7-15.5	26	2.2	1.0	0.7-5.5	26	5.5	6.6	0.3-28.1
2 Jan 2008	34	15.0	0.2	14.6-15.4	34	2.8	1.8	1.3-11.7	34	7.2	6.1	0.8-27.7
3 Jan 2008	31	15.0	0.2	14.7-15.4	33	2.6	1.4	1.0-5.7	31	5.9	5.6	0.6-26.6
14 Jan 2009	22	14.9	0.3	14.5-15.4	22	2.8	0.8	1.0-4.4	22	6.4	6.3	0.3-28.1
15 Jan 2009	22	14.8	0.3	14.3-15.4	22	2.3	0.7	1.0-3.8	22	6.3	6.8	0.1-31.3
30 Jan 2008	37	14.4	0.2	14.0-15.0	37	2.5	1.0	0.9-5.5	37	4.8	3.4	0.4-12.5
31 Jan 2008	28	14.4	0.3	13.9-15.0	34	2.5	0.8	0.9-4.2	28	6.9	4.8	1.1-16.5
11 Feb 2009	25	13.9	0.3	13.5-14.4	25	2.6	1.7	0.8-8.6	25	7.8	8.3	0.5-36.1
12 Feb 2009	23	13.8	0.3	13.3-14.7	23	2.4	0.8	1.1-4.1	24	6.7	9.2	0.3-43.7
27 Feb 2008	32	12.9	0.2	12.5-13.2	32	3.0	1.7	1.0-9.2	32	3.1	2.6	0.1-9.3
28 Feb 2008	33	13.0	0.2	12.7-13.7	33	4.0	2.0	1.0-9.6	33	6.3	5.8	0.5-23.3
18 Mar 2009	19	11.7	0.3	11.4-12.2	19	3.9	2.1	1.1-9.1	19	2.9	2.4	0.3-7.5
19 Mar 2009	22	12.0	0.3	11.6-12.6	22	2.7	1.6	0.6-6.7	22	1.7	1.2	0.2-4.5
27 Mar 2008	33	11.3	0.3	10.8-11.8	33	3.5	1.9	0.9-7.5	33	3.1	2.9	0.5-14.4
28 Mar 2008	28	11.1	0.3	10.6-11.7	28	4.0	2.0	1.0-9.7	28	3.9	4.3	0.5-21.7
7 Apr 2009	15	10.4	0.2	9.8-10.7	15	4.6	2.2	1.3-8.6	15	5.4	5.1	0.3-17.2
8 Apr 2009	11	10.4	0.3	9.7-10.9	13	4.3	2.2	1.4-9.7	11	5.6	6.0	0.8-16.4

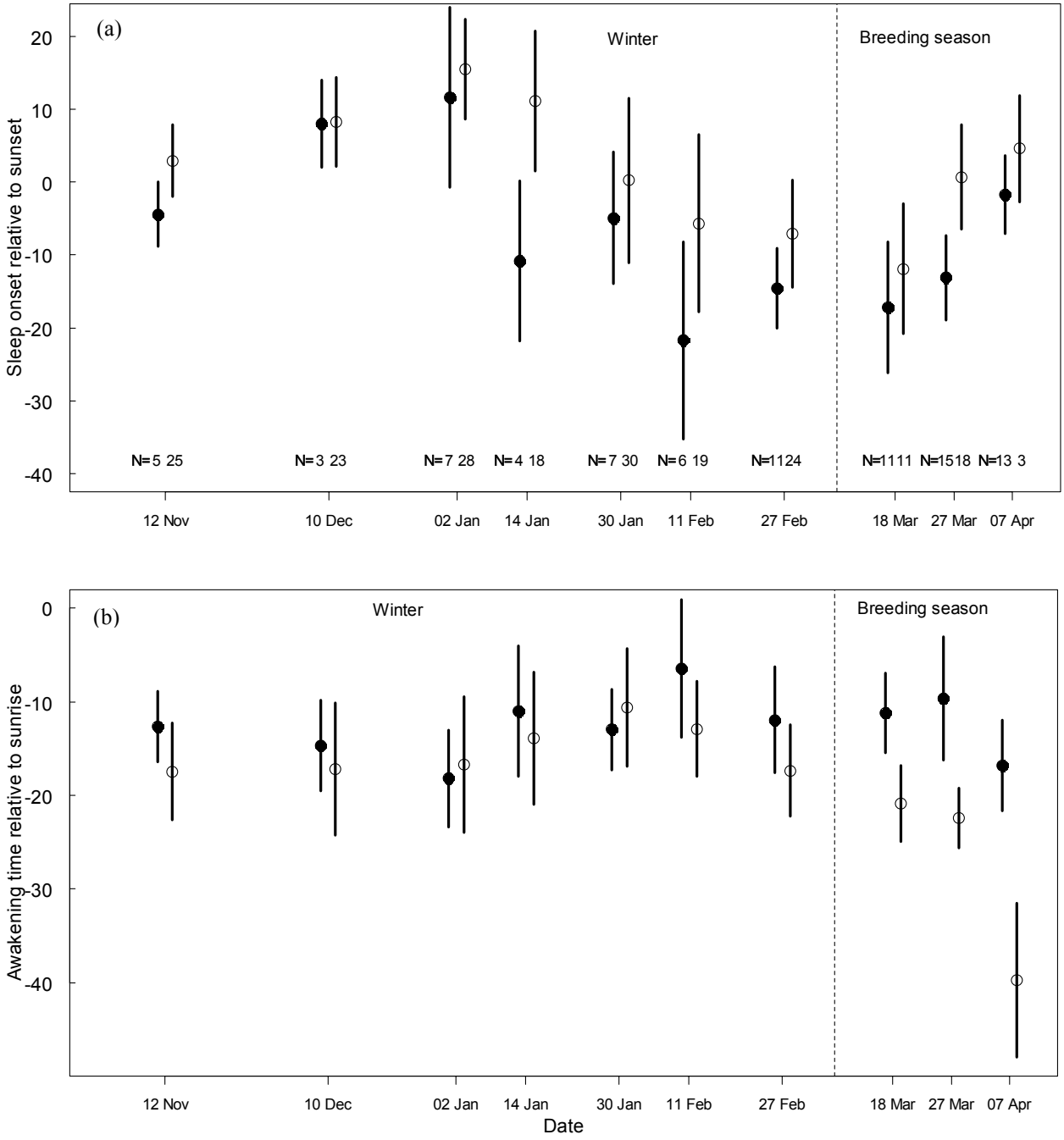

Figure 1. Seasonal changes in (a) sleep onset and (b) awakening time for female (filled circles) and male (open circles) blue tits. Times are given in minutes relative to sunset or sunrise (negative values indicate time before, positive values time after sunset/sunrise). Values of two consecutive nights within one recording session were averaged. Means and SD are shown. Sample sizes (number of individuals) are indicated above the *X*-axis.

Effects of Local Light Conditions

Each nestbox in the study area was exposed to slightly different light conditions due to variation in the direction of the entrance hole and variation in the surrounding vegetation. Light intensity measured at the nestbox in the evening had no effect on entry time, sleep onset, sleep duration and midpoint of sleep, but it significantly influenced evening latency (Table 4). However, in contrast to our expectations, birds started sleeping later in darker locations, i.e. they spent more time in the box before sleep onset and they slept less long in total. Light intensity at the nestboxes in the morning had no effect on morning latency, leaving time, sleep duration and midpoint of sleep, but significantly predicted awakening time (Table 4). As expected, birds that slept in brighter locations woke up earlier.

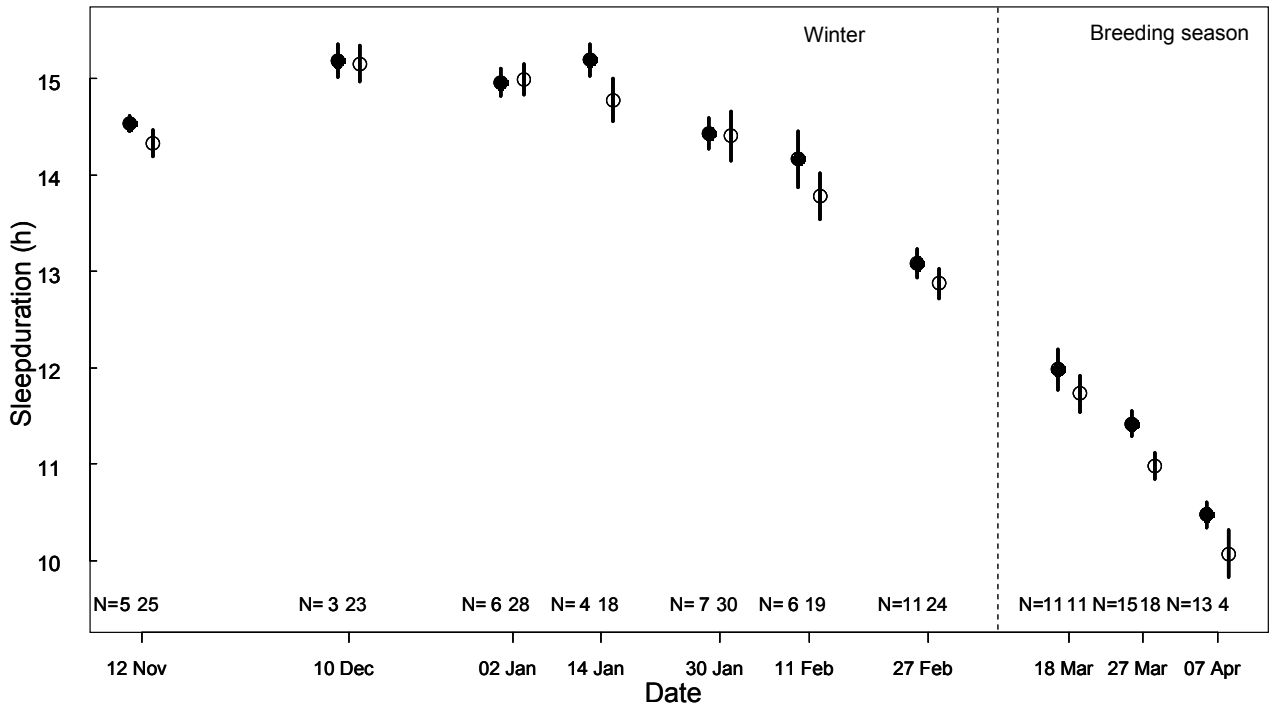

Figure 2. Seasonal changes in sleep duration (h) for female (filled circles) and male (open circles) blue tits. Values of two consecutive nights within one recording session were averaged. Means and SD are shown. Sample sizes (number of individuals) are indicated above the *X*-axis.

Table 3
Mean, SD and range of frequency of nocturnal awakenings (awakenings per hour), proportion of the night birds spent awake and duration of the longest continuous sleep bout (in min) for each recording date

Date	*N*	Frequency of awakenings			Proportion time spent awake			Maximum sleep bout (min)		
		Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
12 Nov 2008	24	3.8	0.6	2.5-5.6	0.03	0.01	0.01-0.05	93.5	27.5	45.8-147.8
13 Nov 2008	23	3.9	0.6	2.8-5.1	0.04	0.02	0.02-0.08	96.2	27.4	55.9-157.6
10 Dec 2008	20	3.3	0.6	2.6-4.5	0.02	0.01	0.01-0.04	112.1	39.6	60.0-211.4
11 Dec 2008	25	3.0	0.6	2.3-4.2	0.02	0.01	0.01-0.03	116.3	34.3	62.0-197.9
2 Jan 2008	28	3.3	0.8	1.9-5.1	0.02	0.01	<0.01-0.03	121.2	38.9	64.6-237.5
3 Jan 2008	26	3.5	0.8	2.0-5.1	0.02	0.01	0.01-0.03	123.1	38.0	68.9-246.6
14 Jan 2009	14	3.5	0.9	2.7-6.1	0.03	0.01	0.01-0.04	114.1	33.7	53.9-190.9
15 Jan 2009	14	3.1	0.7	2.1-4.6	0.02	0.01	0.01-0.04	129.8	61.7	51.7-245.5
30 Jan 2008	33	3.7	0.8	2.2-6.0	0.03	0.02	0.01-0.07	109.8	37.9	50.4-219.8
31 Jan 2008	26	3.8	0.8	2.3-5.7	0.03	0.01	0.01-0.07	101.4	25.5	54.4-155.5
11 Feb 2009	23	3.0	0.9	1.8-5.3	0.02	0.01	0.01-0.04	118.2	40.4	59.3-202.2
12 Feb 2009	21	2.8	0.6	1.9-4.2	0.01	0.01	0.01-0.03	120.2	45.5	45.4-195.2
27 Feb 2008	30	4.0	1.6	2.4-10.2	0.02	0.02	0.01-0.09	89.0	28.2	40.6-157.0
28 Feb 2008	32	5.2	1.6	3.2-9.6	0.04	0.02	0.02-0.11	71.6	20.6	34.7-131.8
18 Mar 2009	13	3.8	1.5	1.9-7.8	0.03	0.01	0.01-0.04	85.5	22.3	57.0-123.7
19 Mar 2009	16	3.2	0.9	2.0-5.1	0.02	0.01	0.01-0.03	115.3	34.0	69.5-191.9
27 Mar 2008	31	4.8	2.5	2.2-14.0	0.03	0.02	0.01-0.09	80.9	34.4	34.3-197.2
28 Mar 2008	26	4.5	1.3	2.8-7.6	0.04	0.02	0.01-0.07	71.6	21.9	34.4-122.9
7 Apr 2009	14	7.8	5.1	3.1-22.3	0.06	0.04	0.03-0.14	50.3	24.4	17.6-106.1
8 Apr 2009	11	9.5	4.8	4.3-20.5	0.08	0.04	0.03-0.15	39.6	17.6	20.8-74.1

Effects of Sex and Age

Sleep behaviour of blue tits was both age and sex dependent (Table 4). In the evening, males on average entered the nestbox and started to sleep 9 min later than females. In the morning, males woke up on average 6 min earlier than females and they left the nestbox on average 9 min earlier. This sex difference was most pronounced at the end of the winter. During the start of the breeding season (March-April), males on average woke up 15 min earlier than females (versus 3 min during the winter, November-February). Overall, there was a strong sex effect on sleep duration (Fig. 2), whereby males slept on average 15 min less than females. This effect was again most pronounced at the beginning of the breeding season; during March-April males slept 24 min less than females (versus 11 min during the winter, November-February).

We also found a difference in morning latencies between females and males (Table 4). After waking up, females stayed on average 134 s longer in the nestbox than males. Midpoint of sleep and evening latency were not significantly affected by sex (Table 4). There was no significant difference in the frequency of awakenings per night between males and females (linear mixed model: d = 0.52 ± 0.39 SE, Z = 1.34, P = 0.18). However, we found that females spent a greater proportion of the time between sleep onset and awakening time actually awake (mean ± SD = 0.035 ± 0.02) than males (0.026 ± 0.01; linear mixed model: d = 0.01 ± 0.003 SE, Z = 3.26, P = 0.001).

An individual's age did not influence entry time, sleep onset, evening latency, awakening time, sleep duration or midpoint of sleep (Table 4). However, we found a significant effect on leaving time and morning latency (Table 4). Yearlings spent on average 2 min longer in the nestbox after waking up, and left the nestbox on average 4 min later than adult birds. This age effect was only pronounced during the winter (leaving time: d = -0.60 ± 0.16 SE, Z = -3.79, P < 0.001; morning latency: d = -0.49 ± 0.15 SE, Z = -3.18, P < 0.002) and disappeared during the breeding season (leaving time: d = 0.07 ± 0.17 SE, Z = 0.40, P > 0.6; morning latency: d = -0.37 ± 0.28 SE, Z = -1.35, P = 0.18).

Longitudinal analysis of seven individuals (six males and one female) that were recorded as yearlings in the first winter and as adults in the second winter confirmed the cross-sectional age effect on leaving time. Leaving times were significantly earlier when individuals were adult (paired t test: t_6 = -2.47, P = 0.05). Although not significant, the longitudinal comparison of morning latencies showed a similar trend. Individuals spent less time in the nestbox after waking up when they were adults (paired t test: t_6 = -1.22, P = 0.27).

Nocturnal awakenings were also age-dependent. Yearlings spent a slightly greater proportion of the night awake (mean ± SD = 0.032 ± 0.02) than adults (0.029 ± 0.02; linear mixed model: d = -0.008 ± 0.003 SE, Z = -3.09, P = 0.002) and adults had longer continuous sleep bouts than yearlings (d = 0.22 ± 0.09, Z = 2.47, P = 0.01).

Table 4
Effects of sex, age and light intensity on eight sleep parameters

Sleep parameter	*N*			Sex (females-males)			Age (adults-yearlings)			Light intensity around sunset (lx)			Light intensity around sunrise (lx)		
	Obs	Ind	Box	*d*	SE	*P*	*d*	SE	*P*	*b*	SE	*P*	*b*	SE	*P*
Entry time	509	94	128	-0.85	0.15	**<0.0001**	-0.01	0.13	0.92	-0.06	0.04	0.18			
Sleep onset	509	94	128	-0.83	0.15	**<0.0001**	-0.02	0.13	0.88	-0.07	0.04	0.09			
Awakening	500	94	127	0.74	0.17	**<0.0001**	-0.22	0.13	0.09				-0.14	0.04	**0.0006**
Leaving time	502	94	127	0.85	0.17	**<0.0001**	-0.38	0.13	**0.005**				-0.07	0.04	0.08
Evening latency	509	94	128	0.1	0.15	0.49	-0.04	0.13	0.77	-0.13	0.05	**0.01**			
Morning latency	500	94	127	0.32	0.16	**0.05**	-0.42	0.14	**0.002**				0.04	0.04	0.32
Sleep duration	498	94	127	0.9	0.15	**<0.0001**	-0.15	0.13	0.25	0.08	0.05	0.09	-0.08	0.04	0.07
Midpoint	498	94	127	-0.19	0.16	0.23	-0.11	0.13	0.46	-0.05	0.05	0.41	-0.1	0.05	0.06

Effect sizes were estimated in linear mixed effect models that included individual and nestbox identity as random effects. Effects of sex and age were estimated as differences in means *d* and effects of light intensity as standardized slopes *b*. Sample sizes *N* are given for the total number of observations (Obs), the number of individuals (Ind) and the number of different nestboxes (Box). Significant *P* values are shown in bold.

Individuality in Sleep Behaviour

After controlling for season, sex, age and light conditions there was still considerable variation in all sleep parameters and a substantial part of this variation was between-individual variation. Individuals were consistent in their sleeping behaviour, such that most investigated sleep parameters showed highly significant repeatabilities of about 0.4 (Table 5). Repeatabilities for the two sexes were generally similar, with the exception of awakening time and leaving time where females showed a higher and lower repeatability than males, respectively (details not shown). Note that repeatability estimates are less accurate for females, because we recorded considerably less females than males.

Table 5
Repeatabilities of sleep parameters for individual blue tits or for particular nestboxes

Sleep parameter	*N*			Individual			Nestbox		
	Obs	Ind	Box	*R*	χ^2	*P*	*R*	χ^2	*P*
Entry time*	509	94	128	0.38	55.92	<0.0001	0.08	5.48	0.02
Sleep onset*	509	94	128	0.41	65.21	<0.0001	0.06	3.77	0.05
Awakening time*	500	94	127	0.46	99.15	<0.0001	0.18	32.80	<0.0001
Leaving time*	502	94	127	0.41	84.63	<0.0001	0.23	41.29	<0.0001
Evening latency*	509	94	128	0.26	29.26	<0.0001	0.12	9.01	0.003
Morning latency*	500	94	127	0.38	76.43	<0.0001	0.20	25.82	<0.0001
Sleep duration*	498	94	127	0.38	69.74	<0.0001	0.19	32.71	<0.0001
Midpoint of sleep*	498	94	127	0.42	74.00	<0.0001	0.00	<0.0001	0.99
Frequency of awakenings (N/h)	442	89	119	0.44	65.01	<0.0001	0.09	9.08	0.003
Proportion time spent awake	442	89	119	0.25	65.30	<0.0001	0.18	45.09	<0.0001

Estimates are variance components from a linear mixed model for each sleep parameter with individual and nestbox identity fitted as random factors. Significance of the effects was calculated by likelihood ratio tests separately for the two components with one degree of freedom. Sample sizes *N* are given for the total number of observations (Obs), the number of individuals (Ind) and the number of different nestboxes (Box).
*Controlled for seasonal effects by standardization.

Sleep onset and awakening time were negatively correlated, both between individuals (linear mixed model: b = -0.26 ± 0.11 SE, Z = -2.35, P = 0.02) and within individuals (b = -0.13 ± 0.04 SE, Z = -2.96, P = 0.003). Thus, individuals that started sleeping earlier in the evening woke up later the next morning and vice versa (Fig. 3a). A negative correlation suggests that there are long-sleeping and short-sleeping individuals, whereas a positive correlation would have indicated that there are evening and morning types (birds sleep the same amount but shift the timing of sleep).

The time birds spent in the nestbox before sleep onset (evening latency) and the time they spent in the box after waking up (morning latency) were strongly positively correlated between individuals (linear mixed model: b = 0.49 ± 0.10 SE, Z = 4.67, P < 0.0001; Fig. 3b), but not within individuals (b = 0.05 ± 0.04 SE, Z = 1.07, P = 0.28). This means that some individuals showed longer latencies than others, while a day with a short latency in the evening did not predict a short latency in the morning (after accounting for between-individual differences).

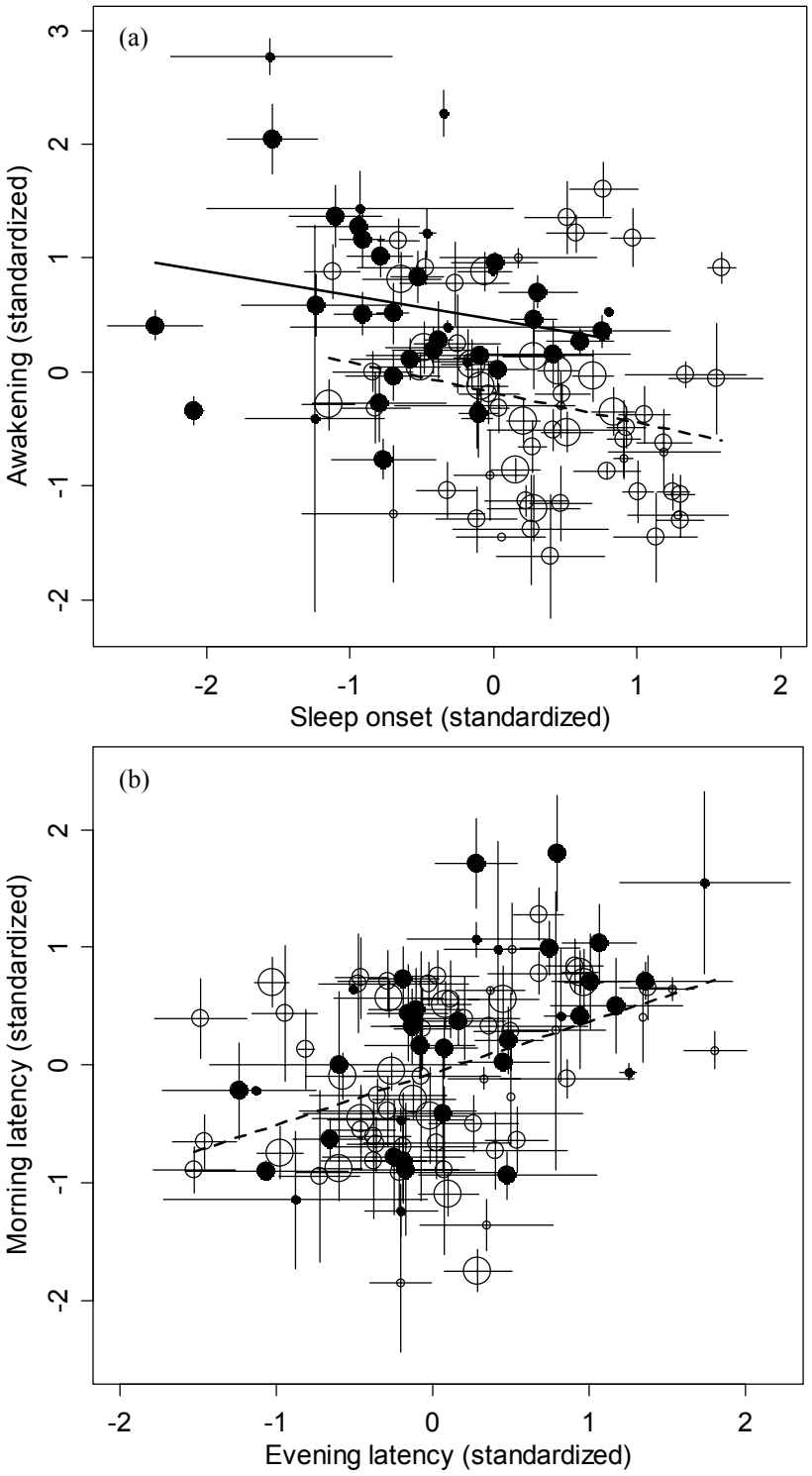

Figure 3. Between-individual correlations (a) between sleep onset and awakening time and (b) between evening and morning latency. Values were standardized within recording dates and averaged within individuals. Males are shown with open and females with closed circles. Points indicate means and lines standard errors. The size of the dots reflects the number of observations: smallest = 1-2, intermediate = 3-9, largest = 10-18 observations per individual. Total N=36 for females, 60 for males. (a) Regression lines for females (solid) and males (dashed). The slopes for males and females were not significantly different (t_{92} = 0.24, P = 0.81). (b) The dashed line shows the regression line for males and females combined.

DISCUSSION

Our study comprehensively describes the sleep behaviour of a population of free-living blue tits roosting in nestboxes. Most sleep variables changed seasonally, largely following changes in day length. We found additional effects of the light intensity near the roosting site (nestbox) on awakening time and evening latency. Males generally slept less than females by starting to sleep later and waking up earlier. This pattern was most pronounced in the early breeding season. First-year birds left the nestbox on average later than adults and they spent a greater proportion of the night awake, but overall they did not differ from adults in their sleep duration. Repeated measurements of the same individuals revealed a significant repeatability of the majority of sleep parameters, suggesting that sleep behaviour is an individual-specific trait.

Our results are restricted to individuals that used artificial nestboxes for roosting. Although there were ample free boxes available for roosting, a substantial proportion of the population did not roost in a nestbox. We do not know where these birds roosted, and we cannot exclude that their sleeping behaviour differed from birds that roosted in boxes. In this population, the risk of predation inside the nestbox is essentially zero, there is negligible intra- and inter-specific competition for nestboxes (an excess of boxes with small entrance hole excluding most other species is available in the study site), and birds are relatively well-protected from weather conditions. Hence, it can be expected that sleep disturbance is minimal in nestboxes, and this could affect the quality and quantity of sleep. Information on sleep behaviour of blue tits outside nestboxes is not available, and is difficult to obtain.

Seasonal Changes and Light Effects

Sleep onset and awakening time of blue tits were strongly related to sunset and sunrise. It is likely that light intensity itself functions as the main Zeitgeber. This is supported by our observation that light intensity in the morning predicted awakening time. Blue tits slept longest in December and January when the days were shortest, and progressively

slept less with increasing day length. Over the course of the study, sleep duration decreased by about five hours. This suggests that blue tits can tolerate a large range of sleep durations. We did not find evidence that blue tits compensated for the varying length of the night, for example by adjusting the amount of time they spent awake while in the nestbox. On the contrary, we observed a higher frequency of night-time awakenings in the early breeding season when nights became shorter compared to mid-winter. Thus, blue tits seemed to have less continuous sleep at the beginning of the breeding season.

As shown for migrating passerines, birds are indeed capable of dramatically reducing their daily amount of sleep, at least during migration, without showing typical consequences of sleep deprivation (Rattenborg et al. 2004). To cope with changing sleep duration blue tits could possibly adjust the depth of their sleep. Differences in sleep depth have already been observed in other species: blackbirds showed deeper sleep towards the end of the night, as measured by arousal thresholds (Szymczak et al. 1996) and king penguins, *Aptenodytes patagonicus*, increased sleep depth in the afternoon compared to the morning (Dewasmes and Loos 2002). However, white-crowned sparrows, *Zonotrichia leucophrys gambelii*, did not compensate for the reduction of sleep during migratory nights by increased sleep depth (Rattenborg et al. 2004).

Similar to mammals, birds show SWS and REM sleep that are unequally distributed across the night (Szymczak et al. 1993; Rattenborg et al. 2000). Each sleep state might serve a different function (Siegel 2005). When reducing the total amount of sleep, birds could change the proportion of these two states if one is more important for health or survival than the other. After short-term sleep deprivation pigeons, *Columbia livia*, showed a slight increase in REM sleep and a more frequent switching between SWS and REM sleep during the recovery night (Tobler and Borbely 1988; Martinez-Gonzalez et al. 2008). This shows that birds are able to alter sleep composition, at least in response to sleep deprivation.

In theory, birds could also compensate for varying sleep duration at night by periods of sleep during daytime. This is perhaps unlikely, because birds in general are monophasic sleepers (except for some shorebirds and waterfowl), who spend a distinct proportion of the 24h day asleep (Amlaner and Ball 1983). In blackbirds, diurnal sleep was extremely rarely seen (Szymczak et al. 1996). However, during periods of nocturnal migration Swainson's thrushes were shown to increase their daytime sleep as a response to reduced night-time sleep (Fuchs et al. 2006). We cannot exclude that blue tits slept outside the nestbox or in other nestboxes during daytime, but we never observed a blue tit sleeping in a nestbox during day (based on full daytime recordings on four recording sessions).

Meddis (1975) proposed that sleep is little more than a spare time activity which enforces inactivity during times of the day when it would be dangerous and/or energetically unproductive to be active. If indeed blue tits seek the secure shelter of a nestbox as soon as it gets too dark to orientate or engage in other activities such as foraging, one would expect that birds always enter the nestboxes at approximately the same light intensity. However, this was not the case. In mid-winter (November-January) birds entered and started to sleep when there was almost no detectable light, whereas later in the year birds entered the nestboxes before sunset when light intensity was still relatively high. This suggests that birds might be forced to expand their activities beyond the short winter days, most likely to obtain enough food to survive the night.

Most blue tits woke up before sunrise, independently of the season, which is in agreement with observations on other birds including the closely related great tit (Hinde 1952; Dunnett and Hinde 1953; Fisler 1962). Like our blue tits, great tits started to sleep earlier relative to sunset in spring than in mid-winter. Weather conditions also seemed to influence the decision of a bird to start roosting or to wake up, but this may be because of the association with variation in light intensity (Dunnett and Hinde 1953; Davis 1958). We observed the largest differences in relative sleep onset or relative time of waking up between two consecutive nights when weather conditions changed. Rain or snowfall resulted in earlier roost times and later wakeup times in connection with low light intensities.

In contrast to Wellmann and Downs (2009), who found no significant effect of temperature on the amount of sleep in three songbird species held in captivity, we found that changes in sleep behaviour of blue tits between two consecutive nights were related to changes in mean night temperature. We observed more nocturnal awakenings in warmer nights, as well as longer evening and morning latencies. However, the latter could also be influenced by weather or light conditions, because overcast and rainy nights were warmer than clear nights within the same recording session.

Unexpectedly, birds that roosted in nestboxes that received comparatively little light in the evening had longer sleep latencies. We had expected a positive correlation, under the hypothesis that the absence of light triggers the onset of sleep and the appearance of light triggers awakening. Birds that entered darker nestboxes should therefore spend less time awake in the box before sleep onset. This was obviously not the case. Why latency to sleep correlated negatively with light intensity remains puzzling.

Sex and Age

Our results clearly show that sleep behaviour is sex dependent. Males started to sleep later and woke up earlier than females and consequently males slept less per night. There are at least three explanations for a sex effect on sleep behaviour. (1) Male and female blue tits might have different sleep requirements, because of sex differences in physiology or in daytime activities. During the early breeding season, for example, females might have higher sleep requirements, because their bodies start to prepare physiologically for the energy demanding egg production and incubation. If females spend more time foraging and can obtain sufficient food faster, they could spend more time sleeping. (2) Males and females may differ in the quality of their sleep. For example, males might wake up less often during the night, or they might spend different proportions of their sleep time in the two sleep states. Indeed, we found that females were awake during the night slightly longer than males. However, after subtracting the amount of time spent awake per night from total sleep duration, males still slept significantly less than females. (3) Males and females may face different trade-offs with other activities.

The sex difference in total sleep duration was most obvious at the beginning of the breeding season, when male-male competition is most intense. Males need to establish and defend territories and they sing in the early morning (dawn chorus), particularly when their mate is fertile. Activities such as dawn song can already be performed early in the morning, at times when it may not be bright enough to forage efficiently (Verner 1965). Females spent more time in the nestbox after waking up ($P = 0.05$), which also supports the idea that males need more time to engage in other activities, such as singing and territory defence.

Yearlings woke up only slightly later than adults, but spent considerably more time in the nestbox and hence flew out later compared to adult birds. This pattern was also seen in a longitudinal analysis of the same individuals in different years. Although one would expect that first-year birds need more time to forage than adult birds (e.g. because of differences in experience and dominance), they may also face different trade-offs (e.g. higher risk of predation). We often observed individuals spending a considerable amount of time sitting at the entrance hole looking out and jumping back and forth between the entrance hole and the bottom of the box before eventually flying out. The effect of age on awakening and leaving time was only observed during winter and decreased towards the breeding season. These effects were also present when only males were considered, which is surprising, given that Poesel et al. (2006) showed that, during the breeding season, adult male blue tits started their dawn song earlier than yearling males. Males that began to sing earlier were more likely to gain extra-pair paternity and had more mating partners, increasing their reproductive success (Poesel et al. 2006). This suggests that awakening time or at least the time of leaving the nestbox may be influenced by sexual selection.

Sleep duration was unaffected by age, at least when comparing yearlings and adults. Apparently, first-year blue tits do not have different sleep requirements than adult birds. However, there might be an age effect on sleep behaviour earlier in life (before the first winter), or later in life (as observed in humans; e.g. Hume et al. 1998; Kronholm et al. 2006). In our study population yearlings seemed to have a more interrupted sleep than

older birds, since they spent a significantly larger proportion of the night awake and had shorter continuous sleep bouts.

Individual Consistency in Sleeping Behaviour

Despite strong effects of environmental factors on sleep behaviour in blue tits, we also found consistent variation between individuals in all investigated sleep parameters. Time of sleep onset differed as much as 56 min between individual males and 45 min between individual females. Similarly, individual variation in awakening time amounted to about 37 min in males and 41 min in females on a given day. Previous work also described substantial inter-individual differences in nightly resting times in blue and great tits. For example, Dunnett and Hinde (1953) studied four blue tits in aviaries and observed a range in emergence time from the nestbox of about half an hour on a given morning. Field observations by Hinde (1952) revealed differences of 35 min in roosting times (entering the nestbox) for both blue tits (N = 11) and great tits (N = 11-23) during a particular evening, and differences in emergence time (leaving the nestbox) of 14 min for blue tits (N = 3-6 individuals) and 18 min for great tits (N = 3-15 individuals) on a given morning.

In humans, it is well-known that individuals vary dramatically in their daily sleep duration (Aeschbach et al. 2001; Aeschbach et al. 2003; Van Dongen et al. 2005), with short sleepers sleeping less than six hours and long sleepers more than nine hours. There is also variation in the preferred sleep timing, usually referred to as ‘morningness’ and ‘eveningness’ (Kerkhof 1985; Van Dongen et al. 2005), although this may partly be due to social and cultural factors. We did not find evidence for the existence of morning or evening types in our population of blue tits. Instead, we found that relatively late sleep onset was linked with early awakening times and vice versa, both between and within individuals. This suggests that variation in the duration of sleep is more important, with populations consisting of long and short sleepers.

Most phenotypic sleep traits we investigated were highly repeatable within individuals and might therefore be regarded as ‘personality’ traits (Réale et al. 2007). The regulation

of sleep and sleep timing might be genetically determined or related to individual quality or long-term condition. There is general agreement that sleep regulation has a homeostatic and a genetic basis (Dijk and Czeisler 1995; Andretic et al. 2008; Vassalli and Dijk 2009). Sleep patterns in humans are not only repeatable but also heritable, as indicated by twin and family-based studies. For example, de Castro (2002) found that genetic influences on sleep patterns accounted for 21-41% of the variance. Klei et al. (2005) reported heritabilities of 12-29% for different sleep measures and Gottlieb et al. (2007) provided heritability estimates of 17-22% for sleep duration and bed time. Obviously, whether sleep behaviour is also heritable in birds remains to be shown.

Causes and Consequences of Variation in Sleep Behaviour

Further study is required to understand the causes and consequences of variation in sleep behaviour in natural populations of animals. For example, sleep demand could be condition-dependent, or dependent on daytime activity. Beginning to sleep early and waking up late may be costly in terms of lost opportunities, during winter when foraging time is limited, or during the breeding season when competition for mates and territories takes place.

Some of the variation in sleep duration may be explained by variation in infestation with parasites. As shown in great tits, females in nests that were experimentally infested with ectoparasitic hen fleas increased their investment in nest sanitation at a cost of sleep duration compared to females in non-infested nests (Christe et al. 1996). On the other hand, total sleep duration increased during infection in animals, including humans (Bryant et al. 2004).

In our study, most blue tits slept in nestboxes without any nest material, which may have reduced the number of ectoparasites. During handling in winter or during the nestling phase, we did not observe any parasites except feather mites, but a more detailed study on the effect of ectoparasites on sleep in blue tits would be useful. We would expect that ectoparasite presence or prevalence particularly influences the frequency or duration of the awake phases that interrupted sleep. These phases were usually rather short, only a

few seconds, but they could last up to about 20 min. The number of times an individual woke up within one night varied considerably (see Results). Birds often scratched or preened during these awake phases, suggesting that the total amount of time spent awake at night may be associated with parasite infestation. We note that the time an individual spent awake at night was the least repeatable of all tested sleep parameters, suggesting it may be more influenced by external factors such as parasite load or daytime activity levels.

Our results show that sleep duration of the same individuals also varied from one night to the next. It would be interesting to test whether this was related to daytime activity levels or to the amount of sleep acquired in previous nights (Horne and Minard 1985; Tobler and Borbely 1988; Driver and Taylor 2000).

Conclusion

This study shows that environmental factors (season, local light conditions and temperature) predict sleep parameters in free-living blue tits. Several aspects of sleep behaviour, in particular overall sleep duration, differed between males and females, or were dependent on the age of the individual. This might reflect sex- or age-dependent trade-offs between sleep and other activities. We found consistent variation between individuals in their sleep behaviour, suggesting that sleep behaviour is an individual-specific trait. It remains to be investigated to what extent these sleep patterns are heritable and whether and how sleep behaviour is related to other individual life-history traits and to fitness. Different sleep traits could be advantageous under different environmental conditions, or they could reflect individual health or quality.

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Chapter Two

Individual variation in sleep-wake rhythms in free-living birds

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ABSTRACT

Ultradian rhythms such as sleep-wake periodicities during the night might represent basic rest-activity cycles of organisms. However, in contrast to circadian rhythms, little is known about the underlying oscillators and molecular mechanisms of such rhythms. A fundamental step for the understanding of the mechanisms is the analysis of variation in sleep-wake cycles in free-living animals, which can help in estimating the relative importance of genetic and environmental influence on the rhythmicity. We analysed variation in the level of rhythmicity and period length in a natural population of blue tits (*Cyanistes caeruleus*). Our results indicate that the expression of periodicity in sleep-wake patterns, but not the period length, has a strong individual-specific basis. Within-individual repeatability estimates of periodicity ranged between 25 and 45% when data from males and females were combined. In addition, periodicity was influenced by specific environmental factors such as night temperature, season, and age of the individual. Most strikingly, decreasing night temperature negatively affected periodicity of sleep-wake patterns, potentially via a hypothermic response of the birds. Our results further suggest that period length is influenced by photoperiod. Blue tits showed longer sleep-wake rhythms when the nights were longer. These observations are consistent with a genetic basis for the incidence of rhythmic sleep-wake behaviour in addition to environmental modifications of their specific expression.

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INTRODUCTION

Much of the daily behaviour of animals as well as the underlying physiological processes show a rhythmic pattern. This rhythmicity can be categorized into circadian with a period length of around 24 hours and ultradian with a period length of less than 24 hours. Whereas circadian rhythms have been extensively studied, less attention has been devoted to ultradian rhythms. Most short-term rhythms differ from circadian rhythms in that they do not correspond to any known environmental periodicity (D'Olimpio and Renzi 1998). Hence, a pure endogenous control of ultradian rhythms has been proposed (Daan and Aschoff 1981; Wollnik 1989) and a genetic basis of short-term rhythms has been detected in mice (Dowse et al. 2010; Pasquali et al. 2010) and rats (Buettner and Wollnik 1984; Wollnik et al. 1987). Ultradian rhythms sometimes display remarkably precise oscillatory features and are important components in the temporal organization of behaviour (Daan and Aschoff 1981). Typical examples are the repetition of rapid eye movements (REM) approximately every 90 minutes during sleep in adult humans (Pace-Schott and Hobson 2002) and locomotor activity patterns in voles with a period length of 2-3 hours (Gerkema et al. 1990) and in mice with periods of 3-5 hours (Dowse et al. 2010). It has been suggested that ultradian periodicity is controlled independently of circadian rhythms (Wollnik 1989; Dowse 2008), but it has also been argued that these short-period oscillators could represent the actual frequency standard of the circadian clock system, in particular because clocks with shorter oscillator periods perform more precisely (Paetkau et al. 2006; Dowse et al. 2010). The neural pacemaker system and the molecular mechanism of ultradian clocks, however, are largely unknown (Gerkema and Daan 1985; Pace-Schott and Hobson 2002).

Although prominent in many biological systems, there is little known about the possible function of ultradian rhythmicity. In voles, the ultradian feeding and activity rhythms are adjusted to provide optimal timing of food intake and digestive pauses (Daan and Slopsema 1978). Also in voles, ultradian activity patterns are synchronized among conspecifics in order to reduce individual predation risk (Daan and Aschoff 1981). Moreover, the level of social synchronization and spatial cohesion was linked to the

presence of distinct ultradian rhythmicity in group activity of Japanese quails (*Coturnix japonica*) (Lumineau et al. 2001; Formanek et al. 2009). In the context of sleep it has been argued that frequent shifts between sleep phases with low and high arousal thresholds allow a periodic screening of the sleep environment for danger signals (Voss 2004). Indeed, behavioural reactivity, arousability and attention changes over the NREM/REM cycle with deep SWS and phasic REM sleep being the most vulnerable periods of sleep (Voss 2004). Activity rhythms during the day may also be related to sleep rhythms at night. These basic rest-activity cycles are considered relics from early ontogenetic feeding and rest cycles (Kleitman 1982; D'Olimpio and Renzi 1998). If true, the functional significance of nighttime rhythms needs to be considered in the context of daytime periodicities, such as variation in the timing of feeding or vigilance (Romeijn and Van Someren 2011).

Most studies on sleep and its function have been conducted on humans or on captive mammals, but little is known about the pattern and function of sleep cycles in free-living animals. Studying sleep in free-living animals under natural conditions may be important, because it has been shown that sleep behaviour of animals observed in captivity can differ substantially from behaviour shown in the natural environment (Rattenborg et al. 2008). Under natural conditions, intrinsic ultradian behavioural cycles of a day-active animal might best be studied during the night when there is little locomotor activity and little interaction with conspecifics. Also the night environment appears to be relatively homogeneous in temperature and light conditions. In a previous study we described that sleep in a free-living population of the blue tit *Cyanistes caeruleus*, a small passerine bird, is organized as short sleep bouts with regular interspersed awakenings (Steinmeyer et al. 2010). Here, we examine 1) whether these awakenings appear in a rhythmic fashion with an ultradian period length, 2) whether rhythmicity and period length are individually repeatable, 3) whether there are sex- or age-specific differences in rhythm parameters, and 4) whether and how environmental parameters influence ultradian periodicity. We calculate repeatability, a measure of the relative amount of between-individual variation, and use it as an indication of the relative significance of genetic versus environmental influence on rhythmicity.

METHODS

Study site and general field procedures

The study was carried out between January 2008 and April 2009 in a wild population of blue tits in a natural reserve in southern Germany (Westerholz, 48°08'N, 10°53'E). The study site with 277 wooden nestboxes is situated in a mixed deciduous forest with many oak trees (*Quercus* sp.). Adult birds were caught inside the nestboxes in winter (November – February) during monthly checks at night and once during nestling feeding (May – June) when chicks were nine to eleven days old. We banded each bird with a numbered metal band of the German ringing scheme and a unique combination of three plastic bands. We determined the age of the birds by comparing the coloration of primary and secondary coverts and classified individuals as yearlings or older (Svensson 1992). Upon first catch, we implanted each bird with a RFID transponder and took a 10-50 µl blood sample from the brachial vein for genetic sexing according to Griffiths et al. (1998).

Recording sleep behaviour and night temperature

To monitor sleep behaviour we recorded blue tits roosting in nestboxes with infrared-sensitive cameras monthly for two consecutive nights in the winters 2008 (January – March) and 2008/2009 (November – April). Cameras were attached to the nestbox lid, illuminated by six infrared light LEDs with a peak wavelength of 940 nm and connected to a small digital recorder that was programmed to record between an hour before sunset and an hour after sunrise. Dataloggers on top of each nestbox recorded temperature at 1 min intervals. We used mean temperature between sleep start and sleep end of the corresponding bird as night temperature. Each night the identity of the sleeping bird was determined with a hand-held RFID transponder reader from outside the nestbox. Wakeups occurred in 37 % of these identity checks and were discarded from the data.

When a bird showed the classical sleep position with the beak pointing backwards and tucked under the scapulars and the feathers fluffed we considered it asleep (Amlaner and

Ball 1983). Awake birds were usually moving the head, moving around in the nestbox or actively sorting feathers or preening and were therefore easy to distinguish from sleeping birds. We defined sleep onset as the time when the first sleep bout of at least 30 sec started and awakening time as the time when the last sleep bout of minimum 10 sec ended. Nocturnal awakenings had to last a minimum of 2 sec to be counted and be separated by at least 10 sec of sleep to be considered as individual wake phases. For a more detailed description of the recording and analysis of the sleep behaviour see Steinmeyer et al. (2010).

Spectral and statistical analyses

We transformed the behavioural sleep data of each recorded night into a time series of "proportion awake per minute interval". Hence a single night consists of between 582 (9.7 h) and 930 (15.5 h) time sampling intervals. Before analysis we discarded 15 datapoints each at the beginning and at the end of the night, in order to exclude behavioural patterns associated with falling asleep and waking up. We used the R package (R Development Core Team 2011) to plot the data, to calculate autocorrelations and to estimate spectral densities of the time series by periodograms. The periodograms are calculated using a fast Fourier transformation and smoothed by moving averages of 10 adjacent spikes. Period lengths in rhythmic nights (see definition below) were estimated using maximum entropy spectral analysis (MESA) (Burg 1967) implemented in a Fortran program (Dowse 2007). Prediction error filter coefficient was set to 90. MESA is described as a high resolution technique that is well-suited to short noisy time series (Dowse 2009; Dowse et al. 2010).

For each night and each individual, we classified the sleep behaviour as rhythmic or arrhythmic based on the following 2 criteria. Criterion 1 considers a night significantly rhythmic if the maximal observed spectral density outranges the maximal spectral densities from 1000 data permutations of the same night. Criterion 2 is more stringent and uses as a significance threshold for highly rhythmic nights two times the maximal spectral density of the permutations. This latter criterion highly correlates with a visual categorization of the periodograms into those with a single major periodicity (single

strong peak) and into those with noisy rhythms. We employed the maximal spectral density of the periodogram as a measure of the clarity in periodicity, because it relates to the variance explained by a single frequency.

Repeatabilities (general between years, months and days) of periodicity parameters for individual blue tits were calculated with the R package rptR (Nakagawa and Schielzeth 2010) and lme4 (Bates et al. 2011). For Gaussian data (e.g. spectral density and period length) we estimated repeatability by a LMM-based procedure using REML, its 95% confidence intervals by parametric bootstrapping (1000 runs) and significance by a likelihood ratio test. For binary data (e.g. categorization into rhythmic and arrhythmic nights according to criteria 1 and 2), with dispersion parameter fixed to 1, we calculated the residual variance as $\pi^2/3$ (Nakagawa and Schielzeth 2010). We calculated approximate confidence intervals of repeatability estimates in binary models according to formulae (6) and (7) in Nakagawa and Schielzeth (2010). All estimates were adjusted for potential confounding factors by including sex, age, temperature and seasonal date (linear and quadratic term) as fixed variables and date and nestbox as random factors. Seasonal date is defined as increasing day numbers starting from November 4th in each of the two study periods.

We fitted generalized linear mixed models to test the effects of sex, age (yearlings, adults), night temperature and seasonal date plus all interactions with sex using the R packages lme4 (Bates et al. 2011) and languageR (Baayen 2011). We included seasonal date (see definition above) with linear and quadratic term to consider potential effects of non-linear photoperiod changes. Seasonal date and temperature were centered prior to analysis. All models included date, nestbox and individual ID as random factors to account for random temporal and spatial effects and to avoid pseudoreplication. We report odds ratios with 95 % confidence intervals for the logistic regressions and unstandardized regression coefficients with 95% highest probability density intervals for the linear regressions. The latter were estimated by MCMC sampling of 10000 runs. An extended model for testing the specific effect of perceived risk on periodicity also

included the yes-no categorization into nights where the bird did or did not wake up due to the identity check.

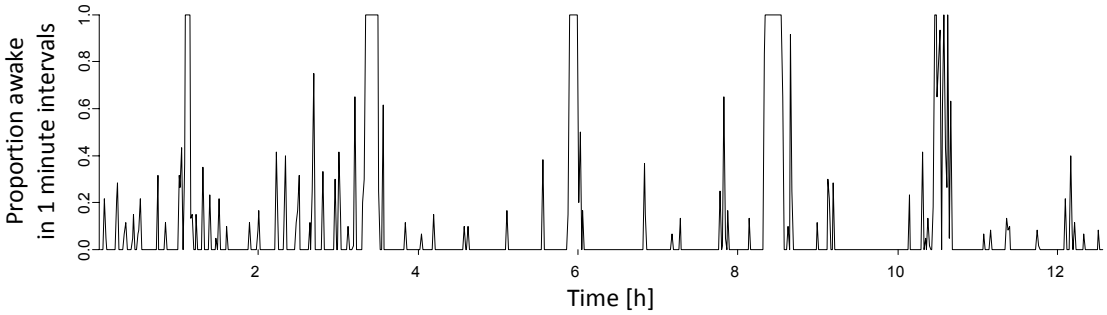
RESULTS

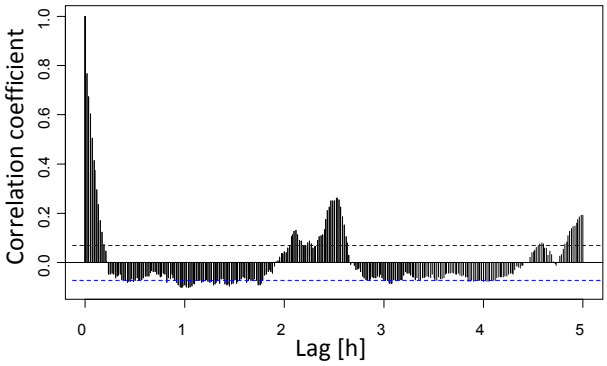
Rhythmicity

We recorded 129 nights for a total of 36 females and found significant rhythmicity of sleep in 108 nights (84 %) in comparison to permutated data (crit. 1). Following the more stringent criteria (crit. 2), 57 nights (44 %) appeared as highly rhythmic. All females except one showed at least one rhythmic night (crit. 1) and 25 females had at least one highly rhythmic night (crit. 2). We recorded 335 nights for 58 males in total, of which 271 nights (81 %) were significantly rhythmic (crit. 1) and 133 nights (40 %) were highly rhythmic (crit. 2). All males but two showed at least one rhythmic night (crit. 1) and 44 males had at least one highly rhythmic night (crit. 2). Rhythmic and arrhythmic nights differ in many aspects (compare figures 1 A and C): rhythmic nights show longer awake bouts, regularly alternating positive and negative correlation coefficients in the autocorrelogram and high spectral densities at specific frequencies/periods, whereas arrhythmic nights have in general fewer and shorter awake bouts. Average frequency of awakenings equals 3.1 and 4.2 per hour and duration of awakenings equals 15 and 27 sec in arrhythmic and rhythmic nights according to crit. 1, respectively. These differences are significant: Welch two sample t-tests, $P<0.0001$.

All repeatability estimates of rhythmicity for individual birds were significant (table 1). This holds true both for the categorizations into (highly) rhythmic and arrhythmic nights (according to crit. 1 and 2) and for the quantitative measure of spectral density. All three measures are highly inter-correlated and are suggestive of the same interpretation, i.e. a strong individually-based component in periodicity. Repeatability estimates for females were slightly higher than those for males, but this was not significant (all $P > 0.05$; t-test for R value difference after Fisher's z transformation).

A)


Proportion awake
in 1 minute intervals
0.0
0.2
0.4
0.6
0.8
1.0
2
4
6
8
10
12
Time [h]


Correlation coefficient
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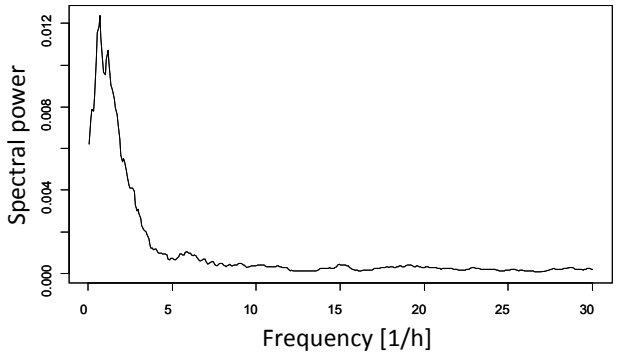
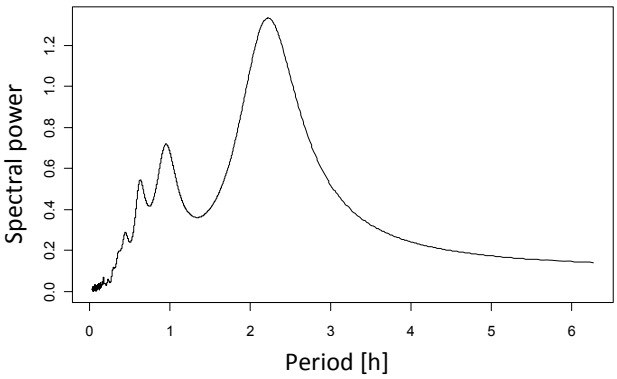
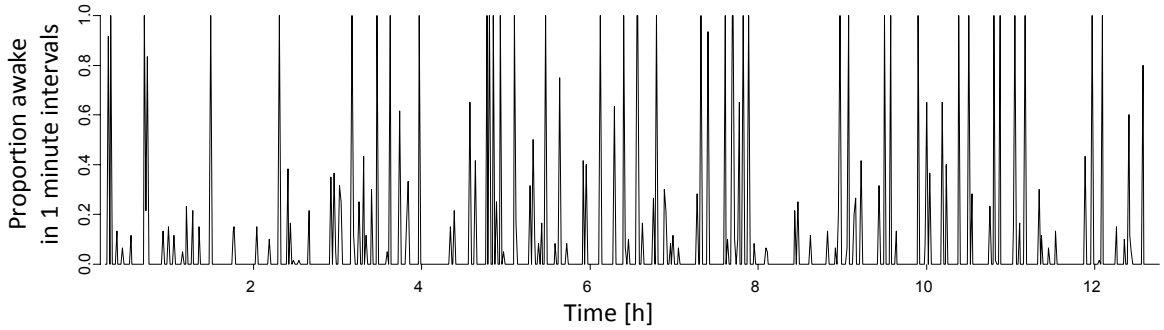
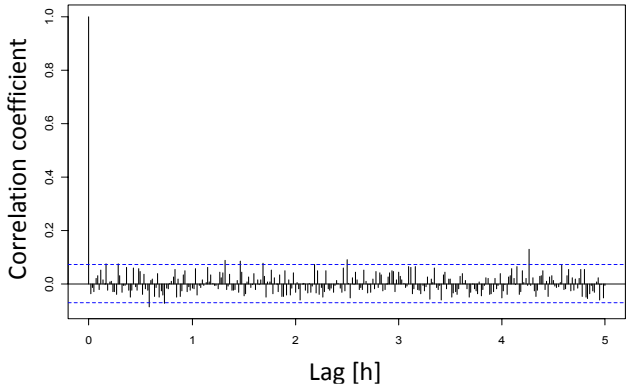
0.2
0.4
0.6
0.8
1.0
0
1
2
3
4
5
Lag [h]

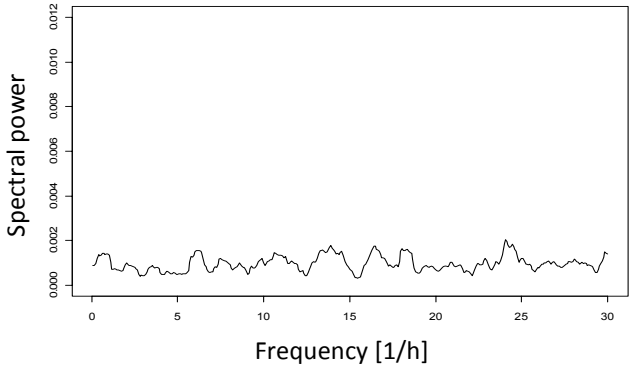

Spectral power
0.000
0.004
0.008
0.012
0
5
10
15
20
25
30
Frequency [1/h]


Spectral power
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
1
2
3
4
5
6
Period [h]

B)


Proportion awake
in 1 minute intervals
0.0
0.2
0.4
0.6
0.8
1.0
2
4
6
8
10
12
Time [h]


Correlation coefficient
0.0
0.2
0.4
0.6
0.8
1.0
0
1
2
3
4
5
Lag [h]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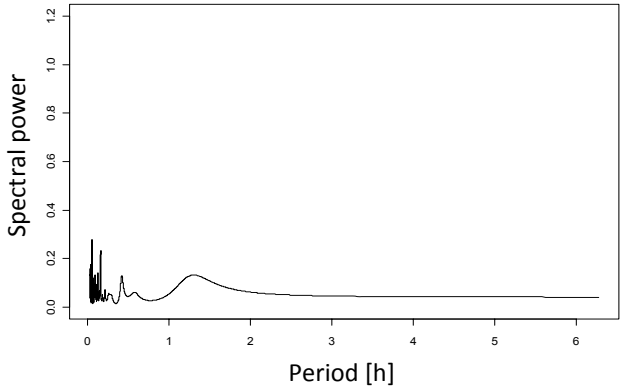
0.008
0.010
0.012
0
5
10
15
20
25
30
Frequency [1/h]


Spectral power
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
1
2
3
4
5
6
Period [h]

C)

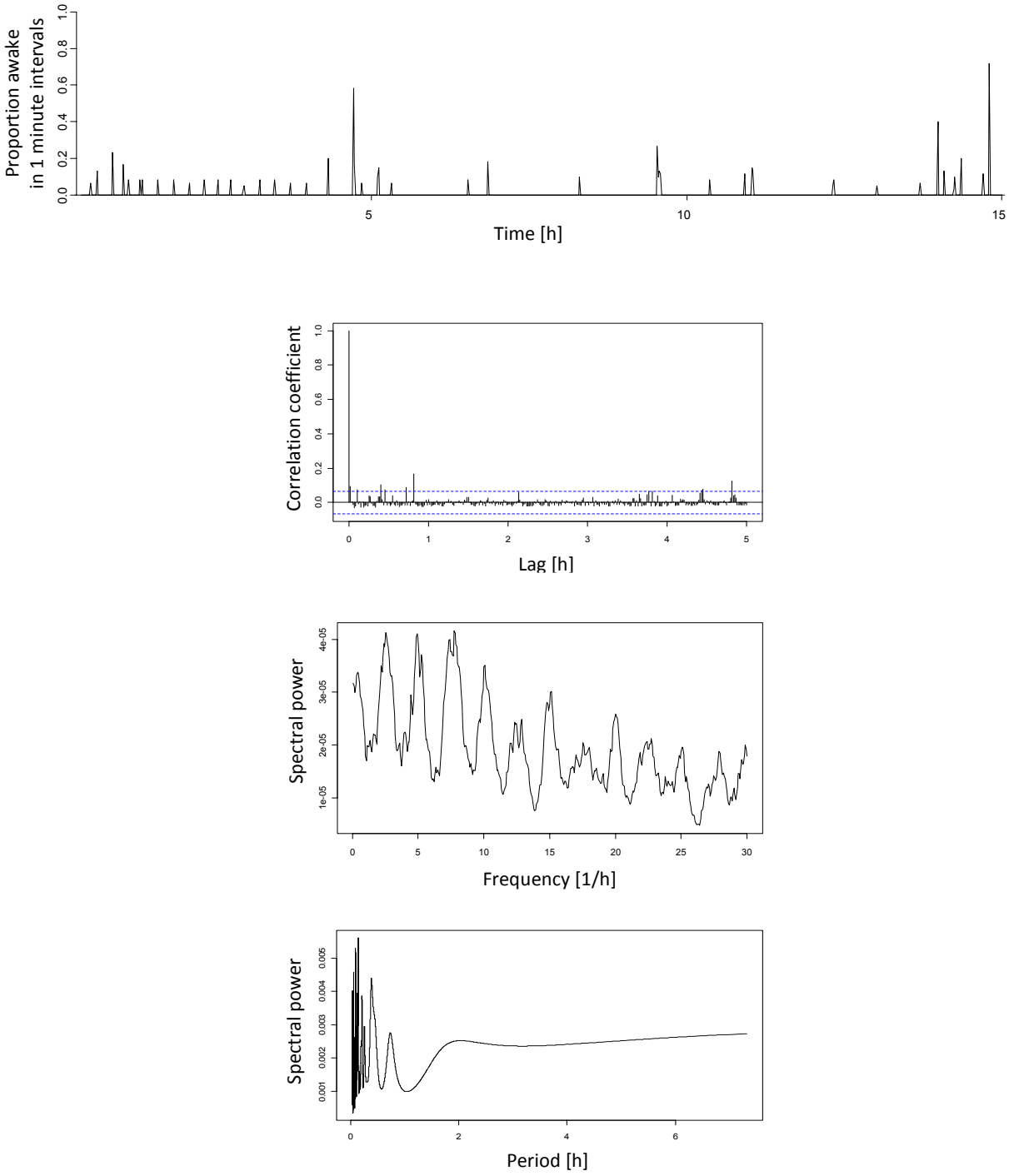


Figure 1. Sleep-awake patterns and corresponding autocorrelograms, periodograms and MESA spectrograms (in this order from top to bottom) of A) a rhythmic night (February 28th 2008 of female B2V8439), B) the permuted data of the same rhythmic night, and C) an arrhythmic night (December 11th 2008 of male B1P5982). For the latter, periodogram and MESA is shown with enlarged ordinate scales. The simple confidence intervals of the autocorrelograms are based on the assumption of uncorrelated series with no correction for multiple testing.

We found a strong temperature effect on rhythmicity (table 2). A one-degree increase in midnight temperature increased the odds (i.e. the relative chance) of having rhythmic nights by 48 or 23 % for the two definitions of periodicity (crit. 1 or 2), respectively. Similarly, spectral density, a basic quantitative component of the categorization into rhythmic sleep behaviour, also showed a positive correlation with temperature. In addition there was a linear seasonal effect on periodicity with an estimated 3 % decrease per day of the odds of showing rhythmic nights (table 2). For spectral density we also found that adults showed fewer rhythmic nights than yearlings (table 2). Periodicity was independent of sex (table 2). None of the interactions between sex and age, temperature or seasonal date was significant (not shown; all p-values > 0.05). We did not find an effect of wakeups during the nestbox checks on periodicity in the extended model (see methods).

Table 1
Repeatability (R) and 95% CI of periodicity parameters for individual blue tits

	All		Males		Females	
Periodicity parameter	Nights (Individuals)	R (CI)	Nights (Individuals)	R (CI)	Nights (Individuals)	R (CI)
Rhythmicity (crit.1)[a d]	442 (92)	**0.45 **** (0.35 - 0.55)**	317 (56)	**0.39 ** (0.26 - 0.52)**	125 (36)	**0.54 ** (0.36 - 0.72)**
Rhythmicity (crit.2)[a d]	442 (92)	**0.31 **** (0.21 - 0.42)**	317 (56)	**0.29 ** (0.17 - 0.42)**	125 (36)	**0.40 * (0.20 - 0.60)**
Spectral density [b]	442 (92)	**0.25 **** (0.14 - 0.35)**	317 (56)	**0.19 *** (0.04 - 0.33)**	125(36)	**0.35 **** (0.21 - 0.50)**
Period length (crit.1)[c]	347 (89)	0.09 (0 - 0.15)	247 (54)	0 (0 - 0.08)	100 (35)	0.14 (0 - 0.36)
Period length (crit.2)[c]	171 (67)	0.07 (0 - 0.23)	119 (43)	0 (0 - 0.18)	52 (24)	0 (0 - 0.47)

* $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$; estimates are adjusted for potential confounding variables (see methods); sample sizes here can differ from total nights recorded due to few missing values in the confounding variables; [a] categorization into rhythmic and arrhythmic nights according to criteria 1 or 2 (see methods); [b] log-transformed; [c] only rhythmic nights according to the indicated criteria; [d] logit scale repeatability estimates; significant results in bold.

Table 2
Effects of sex, age, temperature and seasonal date on sleep periodicity parameters. Odds ratios with 95% confidence intervals (in brackets) are given for the categorical response variables (rhythmicity according to criteria 1 and 2). Note that an odds ratio of 1 would indicate no effect. MCMC regression coefficients with 95% highest probability density intervals are given for the continuous response variables (spectral density, period lengths)

Periodicity parameter	Sex_{female}	Age_{adults}	Temperature	Seasonal date	$(Seasonal\ date)^2$
Rhythmicity (crit.1)	0.69 (0.02 - 23.45)	0.57 (0.14 - 2.23)	**1.48 *** (1.21 - 1.81)**	**0.97 ** (0.96 - 0.99)**	0.99 (0.99 - 1.00)
Rhythmicity (crit.2)	1.46 (0.24 - 8.91)	0.73 (0.31 - 1.71)	**1.23 **** (1.11 - 1.36)**	0.99 (0.98 - 1.00)	0.99 (0.99 -1.00)
Spectral density[a]	0.15 (-0.65 - 0.91)	**-0.46 * (-0.81 - -0.08)**	**0.16 **** (0.09 - 0.23)**	**-0.01 * (-0.01 - -0.0004)**	-0.0001 (-0.0002 - 0.0001)
Period length (crit.1)	-0.05 (-0.30 - 0.19)	0.05 (-0.06 - 0.16)	0.01 (-0.01 - 0.03)	**-0.002 ** (-0.004 - -0.001)**	**-0.00004 * (-0.0001 - -0.00001)**
Period length (crit.2)	-0.14 (-0.52 - 0.19)	-0.02 (-0.19 - 0.14)	0.002 (-0.028 - 0.031)	**-0.003 * (-0.005 - -0.0003)**	-0.00004 (-0.0001 - 0.0000)

* $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$; for sample sizes see table 1; all models included date, nestbox and ID as random factors; [a] log-transformed; significant results in bold.

Period length

Among the subset of significantly rhythmic nights, we found major period length estimates ranging between 0.9 and 6.2 hours with an accumulation around the median at 2.2 hours (see figure 2). After visual inspection of the data, we discarded the 16 nights (from 14 individuals) with outlying period lengths of more than 4 hours for further analyses, because of their complex patterns with mostly multiple periods. The remaining nights had a median period length of 2.14 hours with 5 % and 95 % quantiles of 1.49 and 2.83 hours, respectively. The subset of highly rhythmic nights (crit.2) showed a median period length of 2.21 hours with 5% and 95% quantiles of 1.54 and 2.94 hours, respectively. Repeatability of period length in rhythmic nights for individual birds was not significant, irrespective of the selection threshold used to define rhythmic nights (table 1).

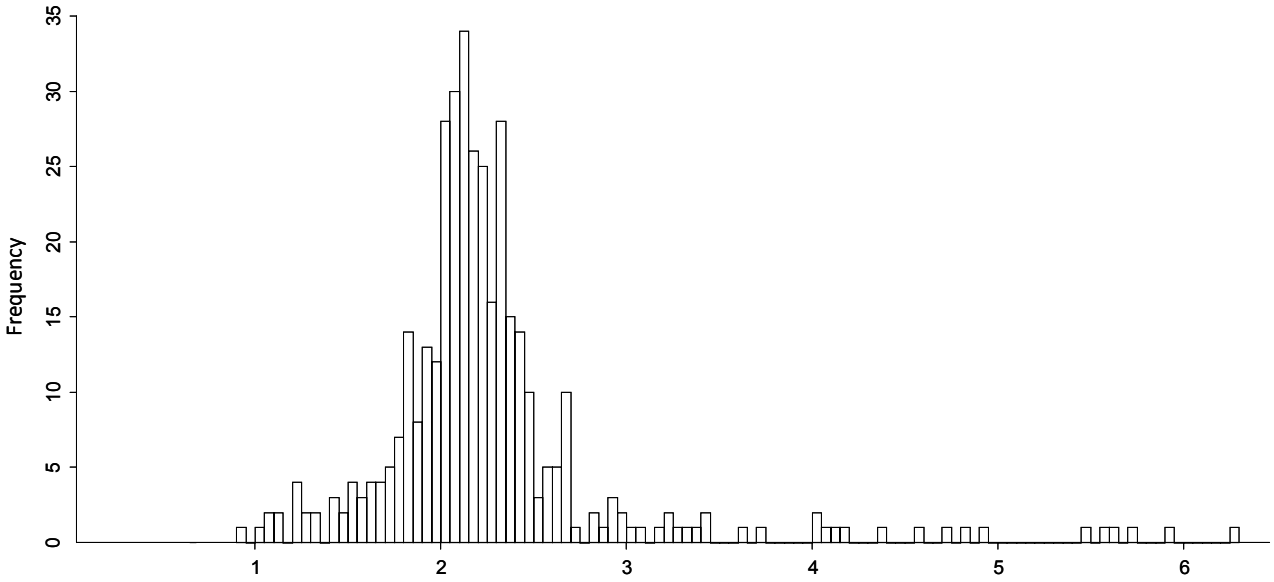


Figure 2. Histogram of estimated period lengths in the rhythmic nights selected by criterion 1.

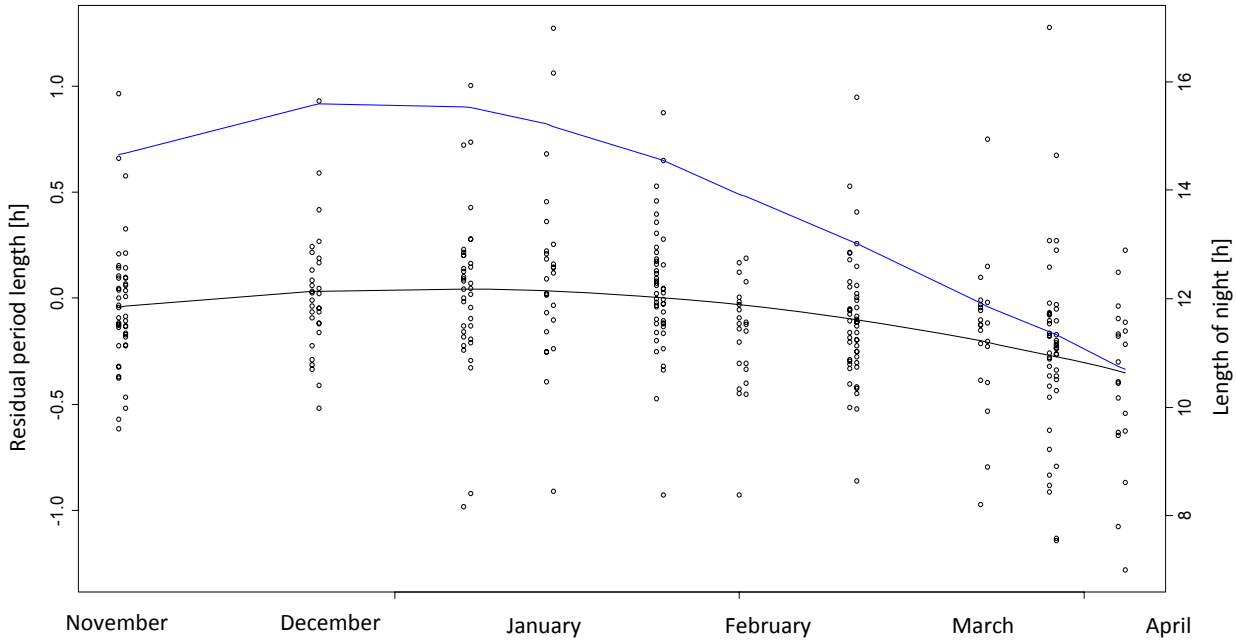


Figure 3. Partial residual plot showing the adjusted relationship between seasonal date (linear and quadratic term) and period length in rhythmic nights (crit.1). Model includes sex, age, temperature and all interactions with sex as fixed effects and date, nestbox and ID as random effects. The black line represents the adjusted prediction from the full model with the linear and quadratic term of seasonal date included. The plotted data include multiple measurements per ID. Night length is plotted in blue.

Season significantly affected period length (table 2): for the subset of rhythmic nights according to criterion 1 both a linear and a quadratic relationship with seasonal date was detected. Period length decreased by an estimated 7 to 11 seconds per day while approaching the breeding season. Again, males and females did not significantly differ in period length itself, or in interaction with age, temperature or season. The relationship between seasonal date and period length followed a curvilinear function, whose trajectory resembled the variation in night length, i.e. the time period between sunset and sunrise (figure 3). The arousal during the nestbox checks caused a reduction in mean period length of 0.16 hours (subset of highly rhythmic nights; extended model; P=0.03).

DISCUSSION

In females and males 82.5% of the recorded nights showed a signature of periodicity in awake-sleep patterns and about 42% of the nights had a single clear major rhythm of relatively long awakening bouts (several minutes). In addition to these regular long awakenings, blue tits often woke up for short periods (less than one minute) mainly in the first third of the night. These short awakenings were also seen in the arrhythmic nights, where most or all of the regular long awake phases were missing. These sleep-awake patterns suggest that arrhythmicity arises through an increase in sleep depth or an increase in arousal thresholds, thus interfering with the basic and regular sleep-awake rhythm. In line with this we found a strong correlation between the level of rhythmicity (spectral density) and the previously described sleep parameter 'proportion of time spent awake' (Steinmeyer et al. 2010) (r=0.79, P<0.0001, N=464). Phases of nocturnal hypothermia could alter the awakening threshold, although little is known about this relationship in birds (McKechnie and Lovegrove 2002). It is however known that in mammals circadian and ultradian rhythms in body temperature are coupled to rhythms in sleep and vigilance (Van Someren 2006; Romeijn and Van Someren 2011). The quite variable avian hypothermic responses are determined by a suite of ecological and physiological factors including food availability, ambient temperature, hormone levels, and breeding cycle (McKechnie and Lovegrove 2002). For blue tits it has been shown

that they can down-regulate their nocturnal body temperature by a few degrees Celsius during cold winter nights (Nord et al. 2009; 2011). In support of this explanation we found a strong relationship between night temperature and rhythmicity. On colder nights blue tits showed less rhythmicity coupled with fewer and shorter awake phases. In conclusion, rhythmicity is not a pure endogenous and fixed phenomenon, but its expression can be strongly modified by environmental factors such as temperature.

We found an age effect on spectral density, indicating that yearlings were more rhythmic than adults. Birds are known to be able to modify their nocturnal hypothermic response (and potentially concomitantly rhythmic sleep-awake patterns) in relation to the perceived risk of predation. For example, when pigeons were exposed during daytime to a model of a flying hawk, they did not decrease their body temperature as much as control pigeons during the following night (Laurila and Hohtola 2005). Work on zebra finches (*Taeniopygia guttata*) suggests an effect of stress hormones on energy metabolism and possibly on nocturnal arousal and sleep fragmentation (Spencer and Verhulst 2008). Assuming that older birds are more dominant (Korsten et al. 2007) and more experienced, they should be able to select apparently safer roosting places than yearlings. Great tits, for example try to avoid roosting cavities containing traces of predator presence (Ekner and Tryjanowski 2008). If the perceived risk of predation is higher in juveniles, because suboptimal nestboxes were chosen for roosting, regular and frequent awakening bouts would allow a periodic screening of the sleep environment for danger signals. Similar concepts of the maintenance of a protective field in novel or unpredictable sleep environments have been described in humans (Voss 2004). Regular visual inspection of the environment becomes more important, if other cues (e.g. olfactory cues) to detect predators are less effective (Amo et al. 2011). Our extended model results, however, do not corroborate our hypothesis of the effect of perceived risk on the strength of periodicity. Arousals during identity checks did not influence rhythmicity, but they did affect period length. Hence, perceived risk of predation seems to strengthen the protective field via faster sleep-wake cycles.

On top of the described environmental and age effects we found a significant individual-specific component on the level of periodicity. Repeatability estimates range between 19 and 54% in males and females, respectively. This could indicate a genetic basis for the incidence of rhythmic sleep-awake behaviour. In contrast, repeatability of period length was estimated to be close to zero, leaving little margin for a genetic basis of period length in sleep-wake patterns of blue tits. These results are different from the ones observed among inbred mice strains kept under controlled conditions: the period length of ultradian rhythms, but not the power of these rhythms differed among mice strains (Dowse et al. 2010; Pasquali et al. 2010). In rats, however, strains also differed in the incidence of ultradian rhythms, which was shown to be heritable by cross breeding experiments (Wollnik et al. 1987). There is also variation in ultradian feeding activity rhythmicity among Japanese quail individuals, which appears to be positively correlated with the circadian rhythmicity (Lumineau et al. 2001; Formanek et al. 2009). One can speculate that if the level of rhythmicity reflects the level of hypothermic response in winter nights, then individual differences in basic metabolism could underlie the significant repeatabilities observed in blue tits. As expected, body temperature and metabolic rate covary, as shown in sleeping great tits (Amo et al. 2011). In this framework the pattern of sleep-awake cycles could be seen as an emergent trait based on the metabolic control to balance energy reserves of an individual and its expenditure during the night. Measures of resting metabolic rates are highly heritable in blue tits (Nilsson et al. 2009) and are expected to be related to other physiological, behavioural or life history traits as proposed by the pace-of-life syndrome concept (Reale et al. 2010). Further studies are needed to analyze the relationship between sleep-awake patterns and metabolic rate.

Very little is known about seasonal effects on ultradian rhythmicity in animals. For instance in free-living common voles (*Microtus arvalis*) a 2h-rhythm in day-time activity was evident in winter, but not during the breeding season in summer (Hoogenboom et al. 1984). Similarly, we found slightly more blue tits rhythmically sleeping in winter than in spring after adjustment for temperature. This pattern mainly arose by a decrease of rhythmic nights in March and April. Further analysis is needed to clarify whether this

pattern represents compensation for shorter nights or to physiological changes when approaching the breeding season.

Interestingly, period length in rhythmic birds covaried with photoperiod. Birds sleeping in shorter nights had shorter sleep-awake rhythms than birds sleeping in longer nights. A similar seasonal trajectory was observed for sleep duration (Steinmeyer et al. 2010). The change in predicted period length, however, did not completely compensate night length in a way that all nights would show the same number of sleep-awake cycles. Our results indicate that photoperiod length or light intensity may play a role in the control of ultradian periodicity, potentially via an interaction between light and neuronal components of the circadian system. A recent study in the social vole (*Microtus socialis*) revealed significant ultradian rhythms in metabolic rate with period length being inversely related to irradiance level during the photophase (Zubidat et al. 2010). A similar influence of light intensity on ultradian patterns of activity was found in the leaf-eared mice (*Phyllotis xanthopygus*) (Kramer and Birney 2001). Also in red deer (*Cervus elaphus*) higher frequencies in activity and feeding rhythms were found in spring, summer and autumn compared to winter (Berger et al. 2002).

Estimated period lengths of the sleep-wake rhythms were variable with a median of 2.14 hours for the rhythmic nights and 2.21 hours for the highly rhythmic nights. Ultradian periodicities of different behavioural traits have been reported with periods between about 1 hour and up to about 18 hours (Kleitman 1982; Dowse et al. 2010). Sleep-wake cycles in *Drosophila* with periodicities of 1.4 and 2.2 hours were regulated in a sex-dependent manner by diet (Catterson et al. 2010). In birds only little information about ultradian periodicity is available. Japanese quails showed a feeding activity rhythm with a period of approximately 40 min (Lumineau et al. 2001; Formanek et al. 2009). Barn owl (*Tyto alba*) nestlings changed period length of their rest/activity rhythms with age in the range between 2 and 12 hours (Wuntke 2003). We can only speculate about the significance of finding period lengths of about 2 hours, as there is nothing known about the molecular basis of the ultradian clock. The observed sleep-wake cycles could reflect basic rest-activity cycles, which are considered as a relic from early ontogenetic feeding

and rest cycles or NREM/REM sleep cycles (Kleitman 1982; D'Olimpio and Renzi 1998). Zebra finches showed frequent shifts between REM (or SWS) and intermediate stage (IR) sleep phases, but this was in the range of seconds (Low et al. 2008). Low et al. (2008) also showed a rhythm between predominantly SWS and REM sleep with a period of about 10 – 20 mins. Thus, the detected ultradian sleep-wake cycle does not directly correspond with REM/SWS cycles in birds. Further studies in a single species are warranted to investigate whether the sleep-awake periodicity represents a multiple of the REM/SWS sleep periodicity.

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Chapter Three

Individual variation in sleep behaviour in blue tits: assortative mating and associations with fitness-related traits

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ABSTRACT

Sleep is ubiquitous in animals, but there is great inter- and intraspecific variation in the daily amount of sleep that is needed. Considerable sleep loss is known to impair health and performance of individuals, but not much is known about the fitness consequences of naturally occurring variation in sleep behaviour. Here we test for assortative mating in sleep behaviour and for correlations between sleep phenotypes and reproductive success and survival in a free-living blue tit population. Partners of a breeding pair were mated assortatively in regard to their awakening times, but not for other sleep parameters. In female blue tits sleep parameters were not significantly correlated with laying date or clutch size. Females that had extra-pair young in their brood did not differ in awakening time, or in any other sleep parameter, compared to females without extra-pair young. In males, the probability of siring extra-pair young was related to sleep onset and sleep duration, but not as predicted. Males that began to sleep earlier and slept longer were more likely to sire extra-pair offspring. None of the sleep parameters were significantly correlated with survival of first-year birds. Our results suggest that there is no strong effect of variation in sleep behaviour on fitness in blue tits, at least under natural conditions. Such a relationship might only become evident when natural sleep patterns are disturbed, for example due to anthropogenic noise or light pollution.

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INTRODUCTION

Although the functions of sleep are not well understood, most researchers agree that sufficient daily sleep seems fundamental for well-being and health. This view is based on numerous studies that report on the negative consequences of sleep loss or deprivation (Ayas et al. 2003; Van Dongen et al. 2003; Patel et al. 2004; Vgontzas et al. 2004; Gottlieb et al. 2005; Spiegel et al. 2005; Gangwisch et al. 2006; Irwin et al. 2006; Lim and Dinges 2008; Van Cauter et al. 2008; Andersen et al. 2009; Cohen et al. 2009; Okun 2011). It has further been shown that daily sleep duration varies tremendously between species. Some animals need only a few hours of sleep whereas others spend a great proportion of their day in the sleep state (Van Twyver and Allison 1970; 1972; Meddis 1975; Zepelin et al. 1994; Affanni et al. 2001).

Studies in humans have also shown substantial between-individual variation in sleep duration and other aspects of sleep behaviour such as morningness-eveningness, nocturnal awakenings and sleep latency (Webb and Agnew 1970; Webb and Friel 1971; Burazeri et al. 2003; Roenneberg et al. 2003; Kronholm et al. 2006; Cavallera and Giudici 2008; Ohayon 2010; Kripke et al. 2011). However, studies on natural variation in sleep behaviour in other species are scarce. Anderson and McGrew (1984) report on asynchronous awakening times in groups of free-living Guinea Baboons (*Papio papio*) with juveniles becoming active earlier than adult males and Lodwick et al. (2004) found variation in the duration of active periods in chimpanzees in relation to sex, rank and reproductive status. In birds, two studies showed that manipulation of the flea density in the nest led to a decrease in sleep duration in incubating female blue tits *Cyanistes caeruleus* (Tripet et al. 2002) and great tits *Parus major* (Christe et al. 1996). In a large-scale study on free-living birds, we previously showed that individual blue tits vary consistently in several sleep parameters such as sleep onset, awakening time, sleep duration, evening and morning latencies and nocturnal awakenings (Steinmeyer et al. 2010), suggesting that sleep behaviour is an individual specific trait.

A question that remains unaddressed – except in humans – is whether and to what extent individual variation in sleep behaviour has fitness consequences. There are several reasons why one would expect such a relationship, in general, and in blue tits in particular. First, studies in humans have shown that extremely short as well as very long sleep durations are associated with reduced longevity (Kripke et al. 2002; Burazeri et al. 2003; Kripke et al. 2011; Kronholm et al. 2011), although this remains controversial (Horne 2011). Other studies showed that pregnant women suffering sleep deprivation had higher risks of preterm delivery and were more likely to have caesarean sections (Chang et al. 2010; Naghi et al. 2011). Second, in many bird species, being active early in the morning seems advantageous for males at least during the breeding season when territorial and reproductive behaviours are most prominent. For example, in blue tits and eastern kingbirds *Tyrannus tyrannus*, male success in gaining extra-pair paternity was associated with early dawn singing (Poesel et al. 2006; Dolan et al. 2007), and in eastern kingbirds early singing males were also paired to females breeding earlier in the season (Murphy et al. 2008) which is generally an indicator for higher breeding success. In accordance with the idea that sexual selection could act on being active earlier during the day or longer overall, we showed previously that sleep duration is on average shorter in male than in female blue tits, in particular closer to the breeding season (Feb-March) (Steinmeyer et al. 2010). If extra-pair copulations occur early in the morning we would expect a correlation between awakening time or sleep duration and male success in siring extra-pair young.

Our aims in this exploratory study were twofold. First, we tested for assortative mating in sleep behaviour in a wild population of blue tits to investigate whether individuals with similar or dissimilar phenotypes mate. In human couples, partners were assortatively mated in regard to their morningness-eveningness preference and this was due to initial assortment rather than convergence after pair formation (Randler and Kretz 2011). Second, we investigated correlations between individual sleep phenotypes and reproductive success and survival of blue tits. Here we focus on sleep duration, sleep onset, awakening time, frequency of awakenings and proportion of time spent awake as

measurements of sleep behaviour, because those parameters are the ones least influenced by environmental conditions and directly linked to sleep.

METHODS

Study site and field procedures

The study was carried out between January 2008 and June 2010 in a wild population of blue tits in a natural reserve in southern Germany (Westerholz, 48°08'N, 10°53'E). The study site (39 ha) is situated in a mixed deciduous forest and dominated by oak trees (*Quercus* sp.). In the winter of 2006, we put up 277 wooden nestboxes (dimensions: 12 x 15 cm x 25 cm) with an entrance hole of 26 mm diameter that were readily accepted by blue tits for roosting during winter and for breeding. Adult birds were caught inside the nestboxes in winter (November – February) during monthly checks at night and once during nestling feeding (May – June) when chicks were nine or ten days old. We banded each bird with a numbered metal band of the German ringing scheme and a unique combination of three plastic colour bands. We determined the age of the birds by comparing the coloration of primary and secondary coverts and classified individuals as yearlings or older (Svensson 1992), measured their tarsus, wing length (3rd primary) and body mass and - from all new captures - we took a 10-50 µl blood sample from the brachial vein for genetic analyses. Each individual also received a RFID transponder (Biomark Inc., Boise, Idaho, U.S.A.), implanted subcutaneously between the shoulder blades. In the breeding season we checked all nestboxes weekly during nest building, daily just before and during laying and at least weekly during the nestling stage. We recorded laying date (date of first egg), clutch size and the number of hatchlings and fledglings. We banded nestlings when they were 14 days old with a metal ring, measured them (tarsus length and body mass) and took a 10-50 µl blood sample from the brachial vein for molecular sexing and paternity analysis. In addition, we collected all unhatched eggs and dead nestlings and extracted DNA for sexing and paternity analyses.

Recording and analysis of sleep behaviour

A detailed description of how we recorded and analyzed sleep phenotypes can be found in Steinmeyer et al. (2010). In brief, we recorded blue tits roosting in nestboxes with infrared-sensitive cameras for two consecutive nights each month in the winters 2007-2008 (January – March), 2008-2009 (November – April) and 2009-2010 (November – March). Cameras were attached to the nestbox lid, and the box illuminated by six infrared light LEDs with a peak wavelength of 940 nm. Each camera was connected to a digital recorder (Abus, TV8450) that was programmed to record between an hour before sunset and an hour after sunrise. We used lead-acid rechargeable batteries (Panasonic, LC-R067R2P) as power sources for all electrical devices.

From the nighttime video recordings we quantified different parameters of blue tit sleep behaviour and here we focus on the following: (1) sleep onset, (2) awakening time, (3) sleep duration, (4) frequency of awakenings per hour throughout the night and (5) the proportion of time spent awake per night. We considered a bird asleep when it showed the classical sleep position with the beak pointing backwards and tucked under the scapulars and the feathers fluffed (Amlaner and Ball 1983). It was easy to distinguish sleep from wakefulness, because awake birds were usually actively preening and moving around in the nestbox. We defined sleep onset as the time when the first sleep bout of at least 30 s started and awakening time as the time when the last sleep bout of minimum 10 s ended. Sleep onset was highly correlated with the time of entering the nestbox in the evening (Pearson's R=0.96) and awakening time was highly correlated with emergence time in the morning (Pearson's R=0.80). We defined sleep duration as the period between awakening time and sleep onset. Nocturnal awakenings had to last a minimum of 2 s to be counted and be separated by at least 10 s of sleep to be considered as individual wake phases. We calculated the frequency of awakenings per hour by dividing the total number of wake phases (between sleep onset and awakening time) by the sleep duration. To calculate the percentage of time a bird spent awake per night we divided the sum of the durations of all wake phases between sleep onset and awakening time by the sleep duration and multiplied by 100. The frequency of awakenings and percentage of time spent awake was only analyzed for the winters 2007/08 and 2008/09.

Fitness measurements

We measured reproductive success of all recorded individuals that bred during the 2008-2010 breeding season. We used laying date, clutch size and the presence of extra-pair young in the brood as measures of female reproductive behaviour, and total number of sired eggs, i.e. the number of offspring sired with the social female (within-pair) plus the total number of extra-pair offspring sired, and extra-pair paternity gain (yes/no) as estimates of male reproductive success. Mean fledging mass (mass of the chicks on day 14 averaged per brood) and fledging success of a nest (measured as the proportion of fledglings over the number of hatchlings) were used to estimate the reproductive performance of a breeding pair. Nestboxes with complete brood failure where none of the eggs hatched or none of the chicks fledged and second nesting attempts were excluded from the analyses. We also determined the survival probability of first-year birds (yearlings) from winter to the subsequent breeding season as a proxy for fitness. We only considered yearlings because the exact age of many of the adult birds was unknown and mortality rate changes with age (Møller and De Lope 1999; Orell and Belda 2002).

Statistical analysis

We analyzed relationships between the sleep parameters and fitness measurements from the subsequent breeding season. For the analyses of assortative mating and individual reproductive success in males and females we centered sleep parameters within recording date and sex by subtracting the date-specific mean from the raw values in order to eliminate seasonal changes and day-specific differences due to e.g. weather. For analyses on reproductive performance of the pair we centered sleep parameters within recording date for both sexes together, which allowed for obtaining differences between males and females in raw units of minutes. Most individuals were recorded more than once (mean=3.8, N=206) per winter season, so we averaged the centered values within winter to obtain one measurement for each bird and parameter per year. Laying date was centered within year for the whole breeding population (including also females that we did not video-record during winter).

All statistical tests were conducted using the free software R 2.10.0 (http://cran.r-project.org). We tested for assortative mating using Pearson's correlation coefficients, based on all breeding pairs where the sleep of both partners had been recorded. Here, we used centered sleep parameters because using the raw values of the measurements is rather difficult. First, the sleep parameters vary greatly between recording dates and second the differences between males and females (especially in awakening times) increase close to the breeding season (Steinmeyer et al. 2010). We also looked at the synchrony of actual awakening times between both partners of a pair for two recording dates separately. This was only possible for the two dates where both partners of seven and six pairs respectively had been recorded. On all other recording dates both partners of less than five breeding pairs had been recorded and the two partners of six pairs had never been recorded in the same night. We analyzed the effects of individual sleep parameters on estimates of fitness using mixed effects models (Pinheiro and Bates 2000) with the add-on R package lme4 (Bates et al. 2008). Males and females were analyzed separately, except for survival, because here the female dataset was too small for meaningful analysis (N=10 females). All models included the fitness measure as the response variable, the sleep parameters as predictors, *age* (yearling or adult) and *year* (3 levels) as cofactors and individual identity as a random effect.

We used glmm's with binomial error structure to analyze the effect of sleep behaviour on 1) the probability of gaining extra-pair parenthood (i.e. the presence of at least one offspring in a nest other than the social nest for males and the presence of at least one extra-pair young in the social nest for females) and 2) on survival of first-year birds. For fledging mass and fledging success we fitted two models: (1) using the breeding pair's average sleep parameters (centered within date for both sexes) as predictors, (2) using the absolute difference in sleep parameters (centered within date for both sexes) of both partners within a pair as the predictor to estimate the effect of the pair similarity/dissimilarity on the success of the brood.

We analyzed the frequency of awakenings and proportion of time spent awake together in one model and also combined sleep onset and awakening time in one model. We could

not analyze nocturnal awakening parameters and the other sleep parameters together in the same model since we had a smaller dataset for the awakening parameters (two winter seasons instead of three). Sleep duration was always analyzed separately since it was highly correlated with sleep onset (Pearson's R=-0.86) and with awakening time (Pearson's R=0.65) and therefore would lead to high collinearity in the models. We thus ran three separate models to test five different sleep parameters. We are aware of the problem of multiple testing that arises from this approach, but this is an exploratory study and there are no previous studies available from which we could have deduced a priori hypotheses.

RESULTS

Assortative mating for sleep behaviour

Of all sleep parameters, only awakening time was significantly positively correlated between pair members (Table 1, Figure 1). Hence, early (late) awakening females were mated to males that also woke up relatively early (late). However, we did not find significant correlations between male and female actual awakening times (Pearson's correlations: R_{date1}=0.23, P_{date1}=0.62, N_{date1}=7; R_{date2}=-0.58, P_{date2}=0.22, N_{date2}=6).

Table 1
Test for assortative mating for sleep behaviour: Pearson's correlations between sleep parameters of pair members

Sleep parameter	R	*P*	*N*
Sleep duration	0.27	0.15	29
Sleep onset	0.20	0.30	29
Awakening time	0.40	0.03	29
Frequency awakenings	0.15	0.57	15
Percentage awake	0.02	0.95	15

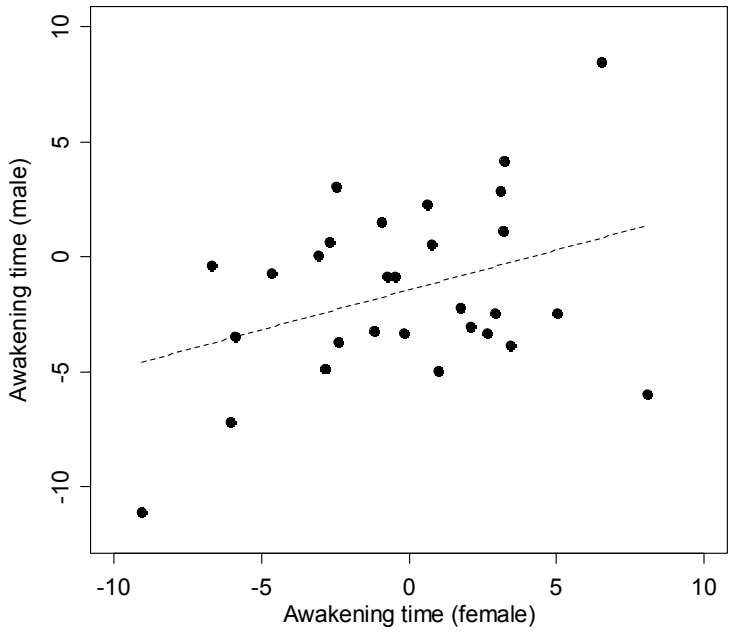

Figure 1. Awakening times of males in relation to awakening times of their female partner. Awakening times were centered within day and are plotted in minutes difference from the mean.

Sleep behaviour and female reproductive success

Laying date and clutch size varied significantly among years, but there was no significant effect of female age, nor of any of the sleep parameters (Table 2). If females that generally wake up earlier are more likely to engage in extra-pair copulations, we would expect awakening time or sleep duration to be related to extra-pair paternity. However, females that had extra-pair young in their nest did not differ significantly in any of the sleep parameters from females that did not have extra-pair young (Table 3). Neither year, nor female age was a significant cofactor in these models.

Table 2
Influence of sleep parameters on laying date and clutch size in female blue tits

	Laying date					Clutch size				
	Estimate	SE	Z	*P*	*N*	Estimate	SE	Z	*P*	*N*
Intercept	-2.53	1.42	-1.79	0.08	59	9.52	0.55	17.32	<0.0001	61
Sleep duration	<0.01	0.04	0.08	0.94	59	-0.01	0.02	-0.82	0.41	61
Season 2009[1)]	2.20	0.83	2.64	0.01	59	1.16	0.39	2.95	0.005	61
Season 2010[1)]	0.15	0.97	0.15	0.88	59	0.48	0.42	1.12	0.27	61
Age[2)]	-0.24	1.36	-0.18	0.86	59	0.82	0.50	1.65	0.10	61
Intercept	-2.34	1.43	-1.63	0.11	59	9.40	0.55	17.04	<0.0001	61
Sleep onset	0.03	0.06	0.53	0.60	59	-0.01	0.02	-0.32	0.75	61
Awakening time	0.10	0.11	0.96	0.34	59	-0.05	0.04	-1.36	0.18	61
Season 2009[1)]	2.10	0.84	2.50	0.02	59	1.21	0.40	3.02	0.004	61
Season 2010[1)]	0.05	0.98	0.05	0.96	59	0.51	0.43	1.20	0.24	61
Age[2)]	-0.36	1.37	-0.26	0.80	59	0.91	0.49	1.84	0.07	61
Intercept	-2.80	1.67	-1.67	0.11	36	10.31	0.84	12.35	<0.0001	36
Frequency awakenings	-0.18	0.24	-0.74	0.47	36	0.01	0.12	0.05	0.96	36
Percentage awake	-0.22	0.30	-0.73	0.47	36	-0.03	0.15	-0.20	0.84	36
Season 2009[1)]	2.71	0.60	4.54	<0.01	36	0.95	0.43	2.21	0.04	36
Age[2)]	-0.33	1.77	-0.19	0.85	36	-0.01	0.84	-0.01	0.99	36

[1)] compared to the reference season 2008; [2)] adults compared to yearlings

Sleep behaviour and male reproductive success

Sleep onset and sleep duration significantly affected male extra-pair success (Table 3). Males that sired at least one extra-pair young started to sleep earlier and slept longer than males without extra-pair young. In both cases the effect sizes were very small (1-2% increase in probability of obtaining extra-pair young, see Table 3) and the mean difference in sleep duration between males with and without extra-pair young were only about five minutes (Figure 2). Male extra-pair success did not differ between years and was not influenced by age.

Neither year, nor any of the tested sleep parameters correlated significantly with the total number of eggs a male sired but adults sired significantly more eggs than yearlings (Table 4).

Table 3
Effect of different sleep parameters on the probability of gaining extra-pair young in male and female blue tits. Estimated parameters are back-transformed from linear mixed models with binomial error structure with their 95% confidence intervals (CI, corrected for multiple comparisons, see Hothorn et al. (2008)) and represent a deviation from the random probability of gaining extra-pair young of 0.5

	Females					Males				
	Estimate	CI	Z	*P*	*N*	Estimate	CI	Z	*P*	*N*
Intercept	0.32	0.05-0.81	-0.84	0.40	61	0.43	0.15-0.77	-0.47	0.64	82
Sleep duration	0.50	0.48-0.51	-0.27	0.79	61	0.51	0.50-0.53	2.40	0.02	82
Season 2009[1)]	0.59	0.22-0.88	0.54	0.59	61	0.30	0.08-0.69	-1.30	0.19	82
Season 2010[1)]	0.46	0.14-0.82	-0.22	0.83	61	0.31	0.10-0.65	-1.43	0.15	82
Age[2)]	0.71	0.25-0.95	1.14	0.26	61	0.68	0.31-0.91	1.23	0.22	82
Intercept	0.30	0.04-0.81	-0.98	0.33	61	0.43	0.14-0.78	-0.49	0.63	82
Sleep onset	0.49	0.47-0.52	-0.62	0.54	61	0.48	0.47-0.50	-2.08	0.04	82
Awakening time	0.48	0.44-0.52	-1.35	0.18	61	0.51	0.48-0.55	1.13	0.26	82
Season 2009[1)]	0.60	0.21-0.89	0.60	0.55	61	0.30	0.07-0.70	-1.30	0.19	82
Season 2010[1)]	0.47	0.13-0.83	-0.19	0.85	61	0.31	0.10-0.67	-1.38	0.17	82
Age[2)]	0.73	0.26-0.96	1.27	0.20	61	0.68	0.30-0.91	1.24	0.21	82
Intercept	0.09	0.002-0.82	-1.49	0.14	36	0.31	0.07-0.72	-1.15	0.25	47
Frequency awakenings	0.34	0.16-0.59	-1.62	0.11	36	0.51	0.28-0.73	0.06	0.95	47
Percentage awake	0.58	0.41-0.72	1.21	0.23	36	0.45	0.23-0.70	-0.48	0.63	47
Season 2009[1)]	0.55	0.14-0.90	0.23	0.82	36	0.31	0.08-0.71	-1.19	0.24	47
Age[2)]	0.93	0.25-0.99	1.77	0.08	36	0.76	0.29-0.96	1.42	0.16	47

[1)] compared to the reference season 2008; [2)] adults compared to yearlings

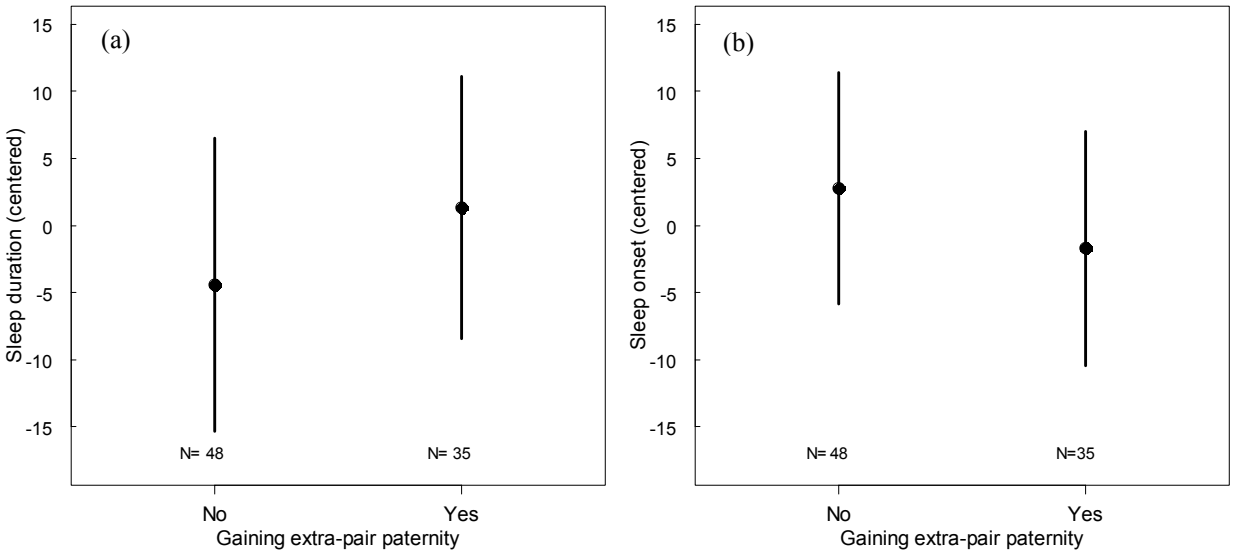

Figure 2. Sleep duration (a) and sleep onset (b) in minutes difference from the mean (± SD) for males that did not (=No) and did (=Yes) sire extra-pair young.

Table 4
Influence of sleep parameters on the number of sired eggs in male blue tits

	Estimate	SE	Z	*P*	*N*
Intercept	8.82	0.62	14.21	<0.0001	73
Sleep duration	0.03	0.02	1.47	0.15	73
Season 2009[1)]	0.22	0.74	0.30	0.77	73
Season 2010[1)]	-0.78	0.61	-1.29	0.20	73
Age[2)]	1.64	0.62	2.64	0.01	73
Intercept	8.78	0.63	13.85	<0.0001	73
Sleep onset	-0.04	0.03	-1.42	0.16	73
Awakening time	0.02	0.06	0.26	0.80	73
Season 2009[1)]	0.25	0.75	0.33	0.74	73
Season 2010[1)]	-0.72	0.63	-1.15	0.25	73
Age[2)]	1.63	0.63	2.60	0.01	73
Intercept	8.74	0.75	11.62	<0.0001	39
Frequency awakenings	0.43	0.47	0.92	0.36	39
Percentage awake	-0.11	0.52	-0.20	0.84	39
Season 2009[1)]	0.15	0.79	0.19	0.85	39
Age[2)]	1.74	0.90	1.93	0.06	39

[1)] compared to the reference season 2008; [2)] adults compared to yearlings

Reproductive performance of the pair

Because we found some evidence for assortative mating in regard to sleep behaviour, we tested the effect of similarity between partners and the average of the sleep parameters of both partners on the performance of the brood. We did not find any correlation between the average sleep parameters of a pair and mean nestling mass or fledging success (Table 5). However, we found a significant positive effect of the difference in awakening times on fledging success of the brood with broods from more dissimilar pairs in regard to the partners' awakening times having a higher fledging success (Table 5). Mean nestling mass and fledging success varied significantly among years, and were affected by age of the male.

Table 5
Influence of the difference (Δ) in sleep parameters between the male and female of a breeding pair, as well as the pair's average of the male and female sleep parameter (mean) on success of the brood, measured as average nestling body mass (at day 14) and fledging success (number of fledglings over number of hatchlings)

		Nestling body mass				Fledging success			
	N	Estimate	SE	Z	*P*	Estimate	SE	Z	*P*
Intercept	27	8.38	0.80	10.49	<0.0001	1.65	0.88	1.88	0.06
Sleep duration									
Δ	27	0.01	0.02	0.44	0.67	0.03	0.02	1.08	0.28
mean	27	0.001	0.03	0.04	0.97	0.02	0.03	0.61	0.54
Season 2009[1)]	27	1.12	0.49	2.27	0.03	1.59	0.71	2.25	0.02
Season 2010[1)]	27	0.14	0.46	0.30	0.77	-0.33	0.46	-0.73	0.47
Age male[2)]	27	1.11	0.61	1.81	0.09	1.01	0.53	1.89	0.06
Age female[2)]	27	-0.07	0.45	-0.15	0.88	-0.84	0.65	-1.30	0.20
Intercept	27	8.51	0.81	10.49	<0.0001	1.53	0.89	1.72	0.09
Sleep onset									
Δ	27	-0.01	0.02	-0.30	0.77	-0.004	0.03	-0.14	0.89
mean	27	-0.02	0.03	-0.61	0.55	0.003	0.04	0.09	0.93
Awakening time									
Δ	27	0.05	0.04	1.44	0.17	0.12	0.05	2.29	0.02
mean	27	-0.06	0.06	-0.93	0.36	0.07	0.07	0.90	0.37
Season 2009[1)]	27	1.16	0.49	2.35	0.03	1.66	0.72	2.31	0.02
Season 2010[1)]	27	0.19	0.46	0.41	0.69	-0.34	0.46	-0.75	0.45
Age male[2)]	27	0.94	0.64	1.48	0.16	1.19	0.59	2.02	0.04
Age female[2)]	27	-0.17	0.46	-0.37	0.72	-0.80	0.65	-1.22	0.22
Intercept	15	6.34	0.81	7.88	<0.0001	-0.94	1.37	-0.68	0.50
Frequency awakenings									
Δ	15	-0.10	0.34	-0.28	0.79	-0.62	0.60	-1.03	0.30
mean	15	-0.23	0.31	-0.74	0.48	-1.02	0.60	-1.68	0.09
Percentage awake									
Δ	15	-0.01	0.20	-0.07	0.94	0.66	0.42	1.59	0.11
mean	15	-0.24	0.23	-1.07	0.31	-0.45	0.50	-0.90	0.37
Season 2009[1)]	15	1.19	0.33	3.56	0.01	1.62	0.83	1.95	0.05
Age male[2)]	15	2.37	0.37	6.36	0.0001	2.28	1.07	2.12	0.03
Age female[2)]	15	0.97	0.53	1.83	0.10	1.05	1.01	1.03	0.30

[1)] compared to the reference season 2008; [2)] adults compared to yearlings

Yearling survival

We did not find significant effects of any of the sleep parameters on survival of first-year birds in our study population. Survival did not significantly differ between years or between sexes. However, birds with earlier awakening times seemed to be somewhat more likely to survive from winter to breeding season (Table 6). The effect of sleep

duration was in the same direction, with birds that slept shorter being more likely to survive (Table 6).

Table 6
Effect of sleep parameters on the probability of survival of yearling blue tits from winter to the following breeding season. Estimated parameters are back-transformed from linear mixed models with binomial error structure with their 95% confidence intervals (CI, corrected for multiple comparisons, see Hothorn et al. (2008)) and represent a deviation from the random probability of survival of 0.5

	Estimate	CI	Z	*P*	*N*
Intercept	0.40	0.16-0.70	-0.83	0.41	43
Sleep duration	0.49	0.47-0.5	-1.42	0.16	43
Season 2009[1)]	0.84	0.19-0.99	1.36	0.18	43
Season 2010[1)]	0.78	0.32-0.96	1.59	0.11	43
Sex[2)]	0.79	0.23-0.98	1.35	0.18	43
Intercept	0.41	0.15-0.73	-0.70	0.48	43
Sleep onset	0.50	0.48-0.53	0.43	0.67	43
Awakening time	0.47	0.43-0.51	-1.79	0.07	43
Season 2009[1)]	0.85	0.17-0.99	1.37	0.17	43
Season 2010[1)]	0.75	0.27-0.96	1.36	0.17	43
Sex[2)]	0.79	0.21-0.98	1.30	0.20	43
Intercept	0.48	0.22-0.75	-0.16	0.87	28
Frequency awakenings	0.43	0.18-0.73	-0.56	0.58	28
Percentage awake	0.54	0.31-0.76	0.42	0.67	28
Season 2009[1)]	0.85	0.19-0.99	1.38	0.17	28
Sex[2)]	0.47	0.05-0.94	-0.12	0.91	28

[1)] compared to the reference season 2008; [2)] females compared to males

DISCUSSION

To our knowledge this is the first study that looked at fitness consequences of variation in sleep behaviour in a wild bird population. Such a link can be assumed given that all animals sleep and that an experimental reduction in daily sleep time has several negative consequences. Further, in birds being active early in the morning during the female fertile period is advantageous, at least for males (Poesel et al. 2006; Dolan et al. 2007; Murphy et al. 2008). We found assortative mating of blue tit social partners for awakening time and against our expectation we found that males with earlier sleep onset and longer sleep duration were more likely to sire at least one extra-pair young. In addition we found that

broods of dissimilar pairs in regard to the female's and male's awakening time had a higher fledging success.

Assortative mating for individual sleep patterns

Our data suggest that blue tit breeding pairs were mated assortatively in regard to their relative awakening times in the morning. We found a similar trend for assortative mating for sleep onset and consequently also for sleep duration. So far, assortative mating for sleep related parameters has only been shown in humans. Partners of a married couple were similar in their morningness-eveningness disposition (husband-wife correlation: 0.23), even after correcting for age and length of marriage, suggesting that the correlation reflects assortative mate choice rather than effects of cohabitation during marriage (Hur et al. 1998).

Similarities in timing of daily sleep/activity patterns might increase the probability that pair members meet, e.g. early active females might be more likely to encounter early singing males. Unfortunately, we know too little about the process of mating and pair-bonding to assess the importance of timing of activity, except in the context of extra-pair paternity (discussed below). Nevertheless, in early spring, males woke up and became active significantly earlier than females (on average 10-20 min) (Steinmeyer et al. 2010). Hence, females that woke up relatively early would resemble males that woke up relatively late, arguing against the hypothesis that assortative mating for awakening time is linked to an increased probability of encountering potential mates. When looking at the absolute awakening times of partners of a pair on days on which both partners had been recorded we did not find a correlation between the males' and females' awakening times. Here, we could only analyse two recording dates because on all other dates partners of too few pairs had been recorded. Our data suggest that birds mated assortatively in regard to their phenotypic traits (waking up early or waking up late in comparison to the population) rather than true synchrony in awakening times.

From an evolutionary point of view, it also remains unclear why assortative mating for the timing of sleep/activity would be important. In fact, one could argue that it would be

advantageous for species with biparental care to mate disassortatively because this would maximize the daily period over which young would receive care. Similarity or dissimilarity between partners of a breeding pair in regard to the most relevant sleep parameters (sleep onset, awakening time and sleep duration) did not correlate with nestling mass but indeed we found that fledging success in broods where partners had more dissimilar awakening times was higher. Hence, there is some evidence that disassortative mating in blue tits is related to the success of the brood.

Sleep behaviour and female reproductive success

In females we could not find any significant correlation between sleep parameters and fitness measurements.

We might have expected a link between the frequency of nocturnal awakenings or proportion of nighttime spent awake with one of the fitness measurements, such as laying date. If fragmentation of sleep indicates sleep quality we would have expected birds with fewer awakenings and hence better sleep quality to have higher fitness. At least in mammals it could be shown that stress leads to more fragmented sleep with more frequent arousals (Tiba et al. 2003; Gronli et al. 2004). Similarly, infestation of the nest with parasites (fleas) led to reduced nightly sleep and more frequent nocturnal awakenings in great tits (Christe et al. 1996). In our study, no ectoparasites were found on the birds caught in winter, and the recordings were made during cold winter months when nestboxes did not contain nest material.

On the other hand, there are also reasons to assume a positive relationship between an individual's condition and the number of nightly awakenings. Several studies suggest that individuals in better body condition do not sleep as deep as those in poor condition and therefore wake up more often during the night. For example, blue tits that carried less fat reserves reduced their rest-phase body temperature more than birds with larger fat scores (Nord et al. 2011) and lower body temperatures result in higher arousal thresholds and consequently lead to less frequent awakenings (Geiser and Ruf 1995).

Sleep behaviour and male reproductive success

We found significant correlations between sleep parameters of male blue tits and fitness measurements: males with earlier sleep onset and longer sleep durations were more likely to gain extra-pair paternity. Assuming that sleep behaviour in the winter and early spring reflects sleep behaviour during the breeding season, this is the opposite pattern we expected. Males that started their dawn singing earlier (naturally, or under influence of artificial night lighting) were more likely to gain extra-pair young and had more mating partners (Poesel et al. 2006; Kempenaers et al. 2010). Similarly, male eastern kingbirds that began singing earlier relative to sunrise sired the most extra-pair young (Dolan et al. 2007). Hence, if anything, we expected that males with inherently earlier awakening times or shorter sleep duration would be more successful at gaining extra-pair paternity. Similarly, one could argue that early-rising females should be more likely to have their young sired by extra-pair males (Halfwerk et al. 2011), but this was not supported by our data. Females in dissimilar pairs (in regard to awakening time) or females with earlier awakening times were not more likely to obtain extra-pair young (LMM: Estimate=0.01, SE=0.02, Z=0.48, P=0.64). Possibly, awakening times of males and females might be regulated differently during the fertile period and not reflect those measured during winter and early spring. Unfortunately, male sleep behaviour cannot be investigated during the breeding season, because males do not sleep inside nestboxes.

Conclusion

Altogether we did not find strong evidence that individual sleep behaviour influences reproductive success and survival in blue tits, although we could previously show consistent inter-individual variation in sleep parameters. Possibly there is no (strong) relationship between sleep behaviour and reproductive success. If under strong selection individual variation in sleep phenotypes might be smaller than observed. Here, we investigated natural variation in sleep behaviour. Effects on fitness-related traits might only be visible if natural sleep patterns get disturbed, e.g. due to anthropogenic noise or light pollution. This could be the focus of further experimental work.

Alternatively, sleep behaviour of birds during winter and early spring – even though repeatable within and across years (Steinmeyer et al. 2010) – might not reflect sleep and

activity patterns during the breeding season, when other selection pressures act. Indeed, it is known that birds can seasonally modify their "normal" sleep patterns, for example during migration (Rattenborg et al. 2004).

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Chapter Four

Search for informative polymorphisms in candidate genes: clock genes and circadian behaviour in blue tits

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ABSTRACT

The identification of functional polymorphisms in genes that underlie behavioural trait variation is a challenging but intriguing task in evolutionary biology. Given the wealth of genomic data and the increasing number of genotype-phenotype association studies in model organisms, one can ask whether and how this information can be used for non-model organisms. Here we describe two strategies to search for likely functional polymorphisms in candidate genes in a bird species that has been intensively studied by behavioural and population ecologists, the blue tit *Cyanistes caeruleus*. In the first approach we searched for repeating elements in coding regions of the genome using information about repeats in *Gallus gallus* genes. The rationale is that tandem-repeat elements have a high potential to be polymorphic and functional. The second strategy aimed to replicate reported genotype-phenotype association studies by extrapolating results from model organisms to our study species. Both strategies showed high success rates with respect to finding homologous gene regions and potentially informative genetic variants in the genes *AANAT*, *ADCYAP1*, *CKIε*, *CLOCK*, *CREB1*, *NPAS2* and *PERIOD2*.

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INTRODUCTION

Genome-wide association studies are becoming an increasingly effective tool for identifying genetic factors contributing to complex traits (Amos 2007). This approach, however, is only applicable to genetic model species with available genome sequences and genome-wide polymorphism data. The majority of species including several ecological model species lack these data and it is unlikely that this situation will substantially change in the near future. Therefore the candidate gene approach is an appropriate choice when searching for functional or adaptively relevant polymorphisms in genetic non-model organisms (Tabor et al. 2002; Fitzpatrick et al. 2005). In this approach, functional genes identified from studies in genetic model organisms, such as *Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus musculus* or *Homo sapiens* serve as 'candidate genes' for similar phenotypic traits in other organisms. The structure of many genes and their cellular functions are highly conserved between evolutionary divergent animal taxa which makes this approach promising (Fitzpatrick et al. 2005). For example, information about the genetic basis of complex traits obtained from *Drosophila* has been used as a model for human traits and diseases (Mackay and Anholt 2006).

Once a gene is identified it is even more intriguing to find functional genetic polymorphisms in the non-model species that vary with the trait of interest. Various research strategies to detect functionally important genetic variation in natural populations have been proposed (Vasemagi and Primmer 2005), and several species-specific polymorphisms in candidate genes have been detected e.g. (Abzhanov et al. 2006; Fidler et al. 2007). The latter study replicated previously reported associations between a dopamine receptor D4 variant and human personality in a wild bird species. Interestingly, the avian functional polymorphism – although different in type - was located in the same genomic region (exon) as the mammalian polymorphism (Fidler et al. 2007).

In this paper we describe two different strategies to find naturally occurring polymorphisms in candidate genes, which are likely to have functional consequences for

circadian behavioural and physiological rhythms in birds. The circadian clock is perhaps the aspect of animal behaviour most fully characterised at the molecular level (Bell-Pedersen et al. 2005). This knowledge can be utilized in a candidate gene approach to analyse the influence of natural genetic variation on circadian behaviour. Our species of interest is the blue tit *Cyanistes caeruleus*, which is a common European passerine bird. Free-living populations of blue tits are studied by many research groups across Europe, mostly in the context of population or behavioural ecology. Johnsen et al. (2007) for example reported a polymorphic tandem repeat in the *CLOCK* gene, one of the core genes involved in generating endogenous rhythms. The authors reported a correlation between the *CLOCK* repeat length and latitude of different blue tit populations.

Strategy I: search for tandem repeats in exonic regions

Rationale and approach

The first strategy was to look for simple tandem repeat elements in exons of the candidate genes, either in protein coding regions or in untranslated regions (UTRs). It has been shown that the mutation rate of microsatellites is higher than that of non-repeat sequences (Jeffreys et al. 1988). These mutations are a consequence of different mechanisms such as unequal crossingover, gene conversion or replication slippage, that lead to a change in sequence length (Nikitina and Nazarenko 2004). Several studies have shown that the number of repeats at mini-/microsatellite loci can influence different aspects of gene function. In particular, increasing length of trinucleotide repeats are associated with various inherited neurodegenerative disorders in humans (e.g. fragile X syndrome (Verkerk et al. 1991), Huntington disease (Mirkin 2007), Spinocerebellar ataxias (Orr and Zoghbi 2007) and Friedreich ataxia (Campuzano et al. 1996)). Many of these disorders involve repeats which produce polyglutamine tracts in the amino acid sequence, i.e. they directly affect protein structure. Gene expression can also be altered by other mechanisms such as degradation of mRNA, decrease in protein production, an increase in DNA methylation resulting in the absence of gene expression, repressed transcription through increased nucleosome stability or gene silencing (Pieretti et al. 1991; Wang et al. 1994;

Imagawa et al. 1995; Choong et al. 1996). The relevance of tandem repeats in gene regulation was emphasized by a study showing that trinucleotide repeats in *Saccharomyces cerevisiae* are more frequent in open reading frames (ORFs) of genes that encode proteins involved in the regulation of transcription than in any other type of ORF (Young et al. 2000). Furthermore, dinucleotide repeats have the ability to form left-handed Z-DNA which plays a role in regulation of transcription due to altered DNA structure and protein binding affinity (Comings 1998).

In order to find repeating elements with a high probability of showing functional polymorphisms in clock genes, we first queried the National Center for Biotechnology Information (NCBI) database on the web (http://www.ncbi.nlm.nih.gov/) with the following key words: "biological rhythm* or biological timing or circadian rhythm* or central clock and Eukaryota". Secondly, we queried the University of California Santa Cruz (UCSC) Genome browser (http://www.genome.ucsc.edu/cgi-bin/hgGateway) searching for "Simple tandem repeats within RefSeq exons of chicken". The NCBI query resulted in a list of 206 genes that have been reported to have a function related to the endogenous clock. The UCSC query resulted in a list of 438 genes that are known to contain simple tandem repeats in exons of annotated genes of the chicken (*Gallus gallus*). The intersection between the two sets of genes contained the following five genes: *CLOCK* (*circadian locomotor output cycles kaput protein*), *NPAS2* (*neuronal PAS domain protein 2*), *ADCYAP1* (*=PACAP*, *adenylate cyclase activating polypeptide 1*), *CREB1* (*cAMP responsive element binding protein 1,* containing two distinct repeats), *CSNK1A1 (casein kinase 1 alpha 1*) (see Table 1). *CSNK1A1* contains a repeat sequence with a period length of about 1,700 base-pairs. Therefore it is unlikely to show a polymorphism and consequently was not considered further.

We then BLASTed the chicken mRNA sequence of the exon comprising the repeat against the NCBI databases "nucleotide collection" and "Non-human, non-mouse ESTs" limiting the search to "Aves" and against the zebra finch (*Taeniopygia guttata*) WGS database of the NCBI trace archives (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The chicken sequence and an aligned sequence of a second bird species (in these cases sequences of

zebra finch or wild turkey *Meleagris gallopavo*) were used to generate primer oligonucleotides flanking the requested exon. For designing forward and reverse primers for PCR amplification we used the program PrimaClade (http://www.umsl.edu/services/kellogg/primaclade.html), which is suited to design primers from multiple-species alignments. Primers were between 18 and 26 base-pairs in length and had one to three degenerated positions if necessary (Supplementary Table). After amplifying the targeted sequence of the blue tit genome in a thermocycler (Supplementary table) we ran the PCR products of each gene from 16 presumably unrelated adult individuals on a 10% polyacrylamid gel. If bands on the gel showed any inter-individual difference due to length variance of the amplified products we confirmed the presence of a polymorphism by running the fragments on an ABI 3130 sequencer. For this purpose we used fluorescently labeled forward primers in the PCR reactions.

RESULTS

We amplified each of the requested exons of the four candidate genes in the blue tit genome (Table 1). Four of the five simple tandem repeats of interest were also found in the blue tit (Table 2). Furthermore, these repeats were polymorphic: in a sample of 148 presumably unrelated blue tits sampled in 2007 in our study population (Westerholz, 48°08'N 10°53'E, Southern Germany) the number of alleles varied between 5 and 7 (Table 2). Sequencing one carrier of each occurring allele confirmed that the detected variation in length of the alleles is caused by different copy numbers of the repeating elements. Genotype proportions were in Hardy-Weinberg equilibrium (Table 2, all $P>0.1$). We also tested whether the developed primers for the tandem repeats could be used in another songbird, the blackcap (*Sylvia atricapilla*). In a sample of 70 individuals from Southern France (43°31'N 4°43'E), we could directly genotype all four microsatellites. All markers were in Hardy-Weinberg equilibrium except for *NPAS2* which turned out to be monomorphic in this sample. Heterozygosity values ranged between 0.30 and 0.69.

Table 1
Genes involved in the endogenous clock that contain a repeat element in RefSeq exons of chicken

Gene name	Gene function	Repeat element	Average copy number	Position of repeat in gene of chicken
ADCYAP1 (=PACAP)	Neurotransmitter	Dinucleotide	25	3' UTR
CLOCK	Transcription factor	Trinucleotide	22.3	Exon 20
CREB1	Transcription factor	Dinucleotide	24	3' UTR
		Dinucleotide	21.5	3' UTR
CSNK1A1[a]	Regulation of signal transduction	Repeat of 1,738 base-pairs	2.0	Exon 6 and adjacent intron
NPAS2	Transcription factor	Trinucleotide	8.3	Exon 20

[a] Not considered further in our search strategy since the contained repeat element is unlikely to be polymorphic due to its period length

Table 2
Detected repeats in clock genes of blue tits

Gene name	Sequence of repeat 5'->3'	No. of alleles	Allele size (bp)/frequency	H_{Obs}[b,c]	H_{Exp}[b,c]	χ^{2c}	P^c
ADCYAP1	$(CT)_5TT(CT)_{12-16}$	7	162/0.44, 164/0.42, 163[a]/0.08, 166/0.03, 165[a]/0.01, 158/0.01, 160/0.003	0.68	0.62	2.24	0.13
CLOCK	$(CAA)_2(CAG)_{5-9}(CAA)_{1-2}CAGCAA$	5	200/0.65, 203/0.18, 197/0.09, 206/0.07, 194/0.01	0.57	0.53	1.85	0.17
CREB1	$(CA)_{6-7}CC(CA)_3(CC)_{0-1}((CA)_3CC)_{0-1}(CA)_{2-9}TC/CC(CA)_3$	7	548/0.85, 546/0.05, 556/0.02, 540/0.02, 534/0.02, 550/0.01, 538/0.01	0.27	0.27	12.24	0.97
NPAS2	$CAG(CAA)_{0-1}(CAG)_{4-7}(CAA)_{0-1}CAGCAACAG(CAA)_2$	5	178/0.39, 181/0.21, 184/0.19, 175/0.19, 172/0.02	0.75	0.73	2.75	0.84

Sequences and the number of alleles are based on a sample of $N = 148$ presumably unrelated individuals
[a] Alleles with one base-pair difference in length are caused by at least one additional polymorphism next to the simple tandem repeat.
[b] Observed (Obs) and expected (Exp) heterozygosity
[c] Calculated with Cervus 3.0 (http://www.fieldgenetics.com/pages/home.jsp)

Strategy II: search based on reported genotype-phenotype associations

Rationale and approach

The second strategy to find functionally important polymorphisms in genes involved in generating endogenous rhythms was based on reported genotype-phenotype associations in model organisms. First, we searched Web of Science (http://apps.isiknowledge.com) for articles reporting an association between polymorphisms in clock genes and a relevant phenotype. We used key words such as: "polymorphism AND sleep", "circadian rhythm AND polymorphism", "clock gene AND sleep", "circadian rhythms AND gene", "polymorphism AND clock" or in general "polymorphism AND bird AND association".

In total, 24 studies tried to link a naturally occurring allelic variation in a clock gene with a phenotype that is influenced by the endogenous circadian clock (Table 3). Phenotypes investigated in humans were either extreme diurnal preferences classified as morningness or eveningness (Horne and Ostberg 1976), or a variety of sleeping patterns, including various sleep disorders (Table 3). In non-human animals, studies reported the period length of different behavioural rhythms. For further work, we selected those studies that reported a significant association between genotype and phenotype.

Second, we attempted to localize the position of the investigated polymorphism in the homologous bird gene. In the case of single nucleotide polymorphisms (SNPs) located in protein-coding regions, we proceeded as follows. First, we identified the position of the amino acid encoded by the polymorphic codon in the organism as reported in the original paper. We then aligned the amino acid sequence of the entire protein from the reported organism with the chicken genome in the UCSC browser. If we found a good alignment between the protein region of interest and a sequence in the chicken genome, we aligned the complete protein of chicken from the NCBI database entry to the chicken genome in the UCSC browser. In the resulting protein-DNA alignment the intron-exon structure of the chicken gene is shown, and we could determine the exon comprising the potentially polymorphic codon.

For SNPs located in UTRs of a gene and for the tandem repeat in exon 18 of *PERIOD 3* (see Table 3), the respective mRNA sequence of the model organism obtained from the NCBI database was directly aligned to the chicken genome. This was done by a NCBI "BLAST" (Altschul et al. 1997, Benson et al. 2008, http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and UCSC "BLAT" (Kent 2002, http://www.genome.ucsc.edu/cgi-bin/hgBlat?command=start&org=Chicken&db=galGal3&hgsid=104455251) homology search. The best alignment was then tested for consensus in the region of the studied polymorphism.

To amplify the sequences of interest in the blue tit we designed PCR primers as described above for the tandem repeat sequences. The goal was to amplify the entire exon containing the homologous position of the polymorphism of interest. Degenerated primers were developed from the alignment between chicken and zebra finch, or, if no zebra finch sequence was available, between chicken and human, Japanese quail (*Coturnix japonica*) or starling (*Sturnus vulgaris*) sequences (Supplementary Table). After PCR amplification of genomic DNA from blue tits (Supplementary Table), the PCR products of 10-14 presumably unrelated adult individuals were directly sequenced. Electropherograms were studied by manual inspection and sequences then aligned by using the programme Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and screened for sequence variations in either heterozygous or homozygous forms.

The ABI PRISM® SNaPshot™ Multiplex Kit (Pati et al. 2004) was then used to genotype 149 presumably unrelated adults sampled in our population in 2007 for each detected exonic SNP.

RESULTS

In total, we studied polymorphisms at 18 different sites (17 SNPs and one VNTR = variable number tandem repeats) in 10 genes for which a significant association to a behavioural trait was reported (Table 3). Fourteen of these published polymorphisms were discovered in human genes, the remaining four in *Drosophila*, mouse (*Mus*

musculus) and Syrian hamster (*Mesocricetus auratus*). For seven of the SNPs we could identify the exact position in the homologous exon of four different chicken genes. At six sites the amino acid and the nucleotides in the mRNA coding for this amino acid were identical between chicken and the studied organism. At one SNP site (exon 4 of *AANAT*; Table 3) the coded amino acid was not identical between chicken and the reported species, but we still found high similarity at the surrounding amino acids. We obtained a specific PCR product for the blue tit of these seven target regions. For the marker *CKIδ* no polymorphisms were detected in the sample of 10 blue tit sequences.

Overall, we discovered seven exonic SNPs in the amplified fragments of blue tit DNA in the genes *AANAT* (2 SNPs), *PERIOD 2* (2 SNPs) and *CKIε* (3 SNPs). SNP sites were located between 1 and 79 base-pairs away from the position of the reported SNP in the model-organism. All detected SNPs in blue tits were silent (synonymous).
For six of these seven SNPs we genotyped 149 individuals and detected two alleles at each locus (Table 4). Genotyping of one SNP in *CKIε* failed so far. Genotype frequencies for the six SNPs were not significantly different from those expected under Hardy-Weinberg equilibrium (Table 4, all $P>0.3$). We found significant linkage disequilibrium between the two SNPs in the gene *PERIOD2* and between the two SNPs in the gene *CKIε* ($D'_{(PERIOD2)}=0.995$, $P_{(PERIOD2)}=0.035$; $D'_{(CKI\varepsilon)}=0.998$, $P_{(CKI\varepsilon)}<0.0001$).

Table 3 Reported studies in different species that aimed to link a circadian phenotype with a certain polymorphism in one of the clock genes

Gene	Species	Region	Original name of polymorphism[a]	Amino acid change	Phenotype	Assocication	Publication
CLOCK	Human	Exon 17	A1982G	Yes	DSPS and N-24	No	(Iwase et al. 2002)
		Exon 17	G1955A	Yes	DSPS and N-24	No	(Iwase et al. 2002)
		3' UTR	T3111C	No	Morningness-eveningness	Yes	(Mishima et al. 2005)
					Morningness-eveningness	Yes	(Katzenberg et al. 1998)
					Morningness-eveningness, DSPS	No	(Robilliard et al. 2002)
					DSPS and N-24	No	(Iwase et al. 2002)
					Morningness-eveningness, DSPS	No	(Pedrazzoli et al. 2007)
		5' UTR	T257G	No	Morningness-eveningness, DSPS	No	(Pedrazzoli et al. 2007)
PERIOD1	Human	Exon 18	T2434C	No	Morningness-eveningness	Yes	(Carpen et al. 2006)
		Exon 18	A2548G	No	Morningness-eveningness	No	(Katzenberg et al. 1999)
PERIOD2	Human	Exon 17	A2106G	Yes	ASPS	Yes	(Toh et al. 2001)
		Promotor	C-1228T	No	Morningness-eveningness	No	(Carpen et al. 2005)
		5'UTR	C111G	No	Morningness-eveningness	Yes	(Carpen et al. 2005)
		Exon 23	G3853A	Yes	Morningness-eveningness	No	(Carpen et al. 2005)
PERIOD3	Human	Exon 15, 17, 18, 20	T1940G, C2590G, T3110C, A3473A, del(3031-3084nt)	Yes	DSPS (haplotype analyses across all five loci)	Yes	(Ebisawa et al. 2001)
		Exon 18	4-/5-repeat =		Morningness-eveningness	Yes	(Jones et al. 2007)
			del(3031-3084nt)		Morningness-eveningness, DSPS	Yes	(Archer et al. 2003)
		Exon 15	T1940G	Yes	Morningness-eveningness	Yes	(Johansson et al. 2003)
AANAT[c]	Human	Promotor	T-542G	No	Sleep pattern	No	(Wang et al. 2004)
		Promotor	G-263C	No	Sleep pattern	Yes	(Wang et al. 2004)
					DSPS	No	(Hohjoh et al. 2003)
		Exon 4	C702T	No	DSPS	No	(Hohjoh et al. 2003)
		Exon 4	C756T	No	DSPS	No	(Hohjoh et al. 2003)
		Exon 4	G619A	Yes	DSPS	Yes	(Hohjoh et al. 2003)
CKIδ[d]	Human	Exon 2	T44A[b]	Yes	FASPS	Yes	(Xu et al. 2005)
CKIε[e]	Human	Exon 9	G1223A	Yes	DSPS and N-24	Yes	(Takano et al. 2004)
MTNR1a[f]	Human	Exon 1	C160T	Yes	DSPS and N-24	No	(Ebisawa et al. 1999)
		Exon 2	C470T	Yes	DSPS and N-24	No	(Ebisawa et al. 1999)
MTNR1b[g]	Human	Exon 1	G71A	Yes	DSPS and N-24	No	(Ebisawa et al. 2000)
		Exon 1	C196T	Yes	DSPS and N-24	No	(Ebisawa et al. 2000)
PRNh[f]	Human	Exon 2	D178N[b]	Yes	Fatal familial insomnia	Yes	(Tafti et al. 2005)
TIMELESS	Human	Exon 20	A2634G	Yes	Morningness-eveningness	No	(Pedrazzoli et al. 2000)
HLA DR1	Human				DSPS	No	(Hohjoh et al. 1999)
doubletime	*Drosophila*	Exon 3	dbt[L]	Yes	Period of behavioural rhythm	Yes	(Kloss et al. 1998)
		Exon 3	dbt[S]	Yes	Period of behavioural rhythm	Yes	(Kloss et al. 1998)
Rab3a[i]	Mouse	Exon 2	A3144G	Yes	Shortened circadian period	Yes	(Kapfhamer et al. 2002)
CKIε[e]	Hamster	Pos. 532	R178C[b]	Yes	Free-running rhythm	Yes	(Lowrey et al. 2000)

DSPS, Delayed sleep phase syndrome; (F)ASPS, (Familial) advanced sleep phase syndrome; N-24, Non-24-hour sleep-wake syndrome; SNP, Single nucleotide polymorphism; VNTR, Variable number of tandem repeats
[a] Usually refers to nucleotide exchange and the nucleotide position
[b] Amino acid change and its position in the protein
[c] Arylalkylamine N-acetyltransferase
[d] Casein kinase 1 delta
[e] Casein kinase 1 epsilon
[f] Melatonin 1a receptor
[g] Melatonin 1b receptor
[h] Prion protein
[i] Ras-associated binding protein 3a

Table 4 Single nucleotide polymorphisms (SNP) in three clock genes of blue tits. Major nucleotides are underlined. Sample size $N = 149$

Gene name		Nucleotide variants	Major allele frequency	H_{Obs}[b,c]	H_{Exp}[b,c]	χ^{2}[c]	P[c]
AANAT	SNP1	C/T	0.91	0.18	0.17	1.48	0.36
	SNP2	G/A	0.93	0.13	0.13	0.77	0.62
PERIOD2	SNP1	G/A	0.87	0.22	0.23	0.10	0.74
	SNP2	C/T	0.91	0.17	0.17	0.05	1
CKIε - tau	SNP1	C/T	0.94	0.12	0.11	0.62	0.65
	SNP2	C/T	0.57	0.45	0.49	1.06	0.32
CKIε - dbt	SNP3	T/G	-[a]	-[a]	-[a]	-[a]	-[a]

[a] Information not yet available
[b] Observed (Obs) and Expected (Exp) heterozygosity
[c] Calculated with R (http://www.r-project.org) using the package "genetics"

DISCUSSION

We reported high success rates for two strategies to detect potentially functional polymorphisms in candidate genes of interest. The first approach was to search for tandem repeats in exonic regions of genes. All four target regions that contained tandem repeats in chicken genes could be amplified in the homologous blue tit genes. Backström et al. (2008) designed primers at conserved sites between chicken and zebra finch, and found cross-species primer amplification success for blue tits of 83% (N=122 markers tested). Difficulties referring to cross-species primer design turned out to be of minor importance in both strategies of our study. Only three out of eleven pairs of primers designed from chicken and another bird reference species did not amplify the homologous fragment in blue tits and this problem could be solved by designing new sets of primers. Furthermore, four of the five studied tandem repeats could be found in the

blue tit and they were polymorphic. Thus, gene-associated tandem repeats seem to be highly conserved among the class of Aves.

In contrast, it is estimated that only 13-25% of anonymous microsatellites developed for specific passerines co-amplify in other passerine species (Primmer et al. 1996; Hansson et al. 2005; Dawson et al. 2006). However, this relatively low cross-species amplification success may be due to variable primer binding sites, and not to the absence of the tandem repeat itself. In general, tandem repeats have a high potential to be functional and polymorphic (Contente et al. 2002; Iglesias et al. 2004; Nikitina and Nazarenko 2004). Therefore, this group of markers – if available - could be a first choice for testing genotype-phenotype associations.

Our second strategy was to focus on gene regions (particularly exons), for which positive associations with phenotypic traits had already been reported. Our analysis showed a high success rate in finding a polymorphism in the target region. In summary, we were able to detect 1-2 polymorphisms in four blue tit regions that showed high homology to the target regions of human, *Drosophila* and hamster. Although the detected SNPs in blue tit genes are silent, they might have functional consequences by mechanisms affecting mRNA structure (Shen et al. 1999; Duan et al. 2003; Kimchi-Sarfaty et al. 2007) or pre-mRNA splicing (Cartegni et al. 2002). In total we sequenced about 2,180 base-pairs of genomic blue tit DNA, of which 1,630 base-pairs were exonic and 550 base-pairs intronic. Thus SNPs in exons occurred on average every 233 base-pairs in the single blue tit population we studied. This is more frequent than the estimate reported for collared flycatchers *Ficedula albicollis* (on average one SNP in every 550 base-pairs of coding sequence; Backström et al. (2008)), but it has to be considered that our calculation is based on a rather short DNA sequence of about 2.2 kb.

Both search strategies revealed only a small number of polymorphisms with the likelihood of being functional. This can now be tested by association with behavioural traits of interest. Thus, the advantage of this approach – in contrast to large-scale genotyping – is that it reduces the multiple testing problem and the costs. A prerequisite

for these approaches is that the genes underlying phenotypic traits are already known. Many other strategies, for example QTL mapping (Flint and Mott 2001), candidate gene approach (Tabor et al. 2002) and targeted mutation (knock-out and knock-in technologies (Austin et al. 2004; Rago et al. 2007)) have recently been developed to find the genetic basis of phenotypic traits. The approach described here allows us to investigate in a following study whether particular genetic polymorphisms are associated with variability in phenotypes, under the assumption that the function of a gene is conserved between different animal taxa. The candidate-gene strategy is appropriate for the majority of organisms where no databases for genetic polymorphisms are available. A clear disadvantage of that approach in contrast to whole genome studies is that relevant polymorphisms might be missed.

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Supplementary Table: Sequences of primers used to amplify candidate regions in the blue tit

Gene name	Forward primer 5'-> 3'	Reverse primer 5'-> 3'	T_m* (°C) forward /reverse	Expected length (bp)	PCR conditions[d]	
					$MgCl_2$ (endconcentration)	$T_{annealing}$ (°C)
AANAT	CRGCRCTGACCCTRCACA	GTGCTGCATCTCSRYGAAG	59/58	260[b]	2.0 mM	57
CKIδ	ATTGCTGCWGGMGARGAGGTT	TCCWCCCTGCATCATYTTGT	59/56	100[b]	2.0 mM	56
CKIε	GCAAGARGTGTCACGGAT	CTAAGCAAACACTGGTCC	54/53	460[b]	2.0 mM	49
CKIε (tau)	GCTGGTGTGGAGGGTTAAAT	TCCCAGGTGGGTGTTGAT	57/56	435[b]	1.5 mM	55
CKIε (dbt)	ATGATCTTCTCAGCAGGGGA	GAGAGTAGGCACAAATGCTTCC	57/60	250[b]	1.0 mM	55
PERIOD2	CTCTACTGTGTTGAAGKCATCTG	CTAATTCAGGTTGTGGYTTTTTG	59/56	170[b]	2.0 mM	55
ADCYAP1	GATGTGAGTAACCAGCCACT	ATAACACAGGAGCGGTGA	57/53	166[c]	1.5 mM	51
CLOCK[a]	TTTTCTCAAGGTCAGCAGCTTGT	CTGTAGGAACTGTTGYGGKTGCTG	58/64	285[c]		
CREB1	GGTCAGGCAGTTAAGATATTG	GTCTTACCAGTGGTTCCTTTAR	55/57	556[c]	2.0 mM	53
NPAS2	CTGTGGTAAATTTGATGATTCTGA	ACACCAAGTTCTTTGCACAATG	55/56	184[c]	2.0 mM	55

* approximate melting temperature

[a] primers according to Johnson et al. 2007

[b] approximate length

[c] contains maximum number of repeats obtained in the sample of 148 blue tit individuals

[d] PCRs were conducted in a final volume of 20 µl containing 1 µl of genomic DNA and 0.5 U Taq DNA polymerase (Fermentas), and a final concentration of 200 µM dNTPs, 0.5 µM of each of the forward and reverse primers, varying $MgCl_2$ concentrations (see table) and 1X Taq buffer with $(NH_4)_2SO_4$ (Fermentas). PCR cycling profiles for all markers began with an initial denaturation at 95ºC for 3 min and then proceeded with 30-35 cycles of 95ºC for 30 sec, annealing temperatures according to the table for 30 sec, and 72ºC for 1 min, followed by a final extension of 72ºC for 7 min.

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Chapter Five

Testing for associations between candidate genes for circadian rhythms and individual variation in sleep behaviour in blue tits

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ABSTRACT

The regulation of sleep in animals is controlled by environmental factors, homeostatic mechanisms and endogenous circadian oscillators. The molecular mechanisms underlying such circadian oscillators have been described in detail and a variety of genes that are components of these molecular clocks have been reported. In addition to inter-specific variation in the temporal organization of sleep, there is significant intra-specific variation in different organisms. From numerous studies in humans it is known that polymorphisms in the regulatory clock genes are causing such variation but knowledge about associations between naturally occurring polymorphisms and sleep patterns in wild animals is scarce. In this study, we investigated the phenotypic sleep correlates of eleven previously described polymorphisms in seven candidate genes within a free-living blue tit *Cyanistes caeruleus* population. We detected associations between four single nucleotide polymorphisms and three of the nine tested sleep parameters representing temporal organization. Awakening time was associated with polymorphisms in *AANAT* and *PERIOD2*, morning latency with a polymorphism in *CKIε* and the duration of the longest sleep bout with a second polymorphism in *AANAT*. However, by a permutation procedure we showed that the number of significant results and the most significant association has a study-wide likelihood of 46.7% and 5.9% respectively. Further replication studies are needed to evaluate the potential associations.

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INTRODUCTION

Almost all organisms live in a constantly fluctuating environment. Many of these fluctuations occur predictably over the course of the day due to the earth's rotation. It is beneficial for an individual to anticipate the diurnal changes in order to adjust its physiology and behaviour accordingly (Hastings 1997; Emerson et al. 2008), for example searching an appropriate roosting place in the evening or awakening in the morning are key behaviours in the diurnal activity cycles. This anticipation is enabled by molecular clocks which show an endogenous rhythm of approximately 24 hours. To be effective, biological clocks need to be entrained by environmental signals such as light, so that the rhythms they drive run at exactly 24 hours.

In birds the main circadian pacemakers are the pineal gland, the retina and the suprachiasmatic nuclei (SCN) that regulate secondary circadian clocks in the peripheral tissues (Gwinner and Brandstaetter 2001; Bell-Pedersen et al. 2005). The importance in generating rhythms by the pineal gland and the retina varies between species, but both these organs secrete melatonin into the bloodstream with a circadian periodicity (Bell-Pedersen et al. 2005). Generally, the circadian oscillator is made up by an intracellular machinery with a number of so-called "clock genes" which are transcribed rhythmically due to transcriptional-translational feedback loops (Young and Kay 2001; Panda et al. 2002; Reppert and Weaver 2002; Hastings and Herzog 2004). The following genes have been discovered to be key elements of the circadian clock mechanism in birds: *CLOCK, BMAL1, NPAS2, PERIOD2, PERIOD3, CRY1, CRY2* and *CKIε* (Chong et al. 2003; Doi et al. 2004; Bell-Pedersen et al. 2005). Beside these core clock genes a variety of other genes is involved in controlling circadian rhythms in behaviour, e.g. genes involved in light entrainment of the molecular clocks or in the downstream control of circadian behaviour (diurnal hormone levels) (Fidler et al. 2004; Nagy and Csernus 2007; Shimizu and Fukada 2007). Temporal sleep patterns in animals are supposed to be directly influenced by the circadian clock in addition to a homeostatic regulation (Dijk and Czeisler 1995; Dijk and Archer 2009)

Reports of behavioural associations with naturally occurring polymorphisms or spontaneous mutations in animal clock genes are rare. For example, a study on Syrian hamsters *Mesocricetus auratus* identified a mutation in *CKIε* leading to aberrant circadian phenotypes (Lowrey et al. 2000). Microsatellite variation in the *CLOCK* gene was found to be associated with variation in timing of breeding in females of a free-living blue tit population (Liedvogel et al. 2009) and the *ADCYAP1* microsatellite marker was linked to variation in migratory behaviour in blackcaps *Sylvia atricapilla* (Mueller et al. 2011). In humans however, a number of naturally occurring polymorphisms in clock genes were shown to be associated with sleep behaviour or disorders. In the *CLOCK* gene two different SNPs could be linked to variation in morningness/eveningness or sleep duration (Katzenberg et al. 1998; Mishima et al. 2005; Allebrandt et al. 2010). Further, different SNPs and a variable number tandem repeat polymorphism in the three *PERIOD* genes and in the casein kinases I epsilon and delta (Takano et al. 2004; Xu et al. 2005) were associated with morningness/eveningness or advanced or delayed sleep phase syndrome (Ebisawa et al. 2001; Toh et al. 2001; Archer et al. 2003; Johansson et al. 2003; Carpen et al. 2005; Carpen et al. 2006; Jones et al. 2007).

Previously, we identified eleven possibly functional polymorphisms in seven different clock genes in a natural blue tit *Cyanistes caeruleus* population (Steinmeyer et al. 2009). We followed two search strategies to find informative genetic polymorphisms related to circadian behaviour. First, we looked for exonic simple tandem repeat elements in candidate genes, i.e. genes that are known to be involved in the regulation of circadian rhythms, in the chicken genome and tried to identify these repeat elements in the homologous blue tit genes. Second, we used reported genotype-phenotype associations in model organisms as a template and attempted to localize the position of the underlying polymorphisms in the homologous blue tit genes. These two strategies led to the detection of four polymorphic microsatellite markers in the genes *ADCYAP1, CLOCK, CREB1, NPAS2* and two single nucleotide polymorphisms (SNPs) in *AANAT,* two SNPs in *PERIOD2* and three SNPs in *CKIε* in a population of blue tits (Steinmeyer et al. 2009). Given that the investigated clock genes and their proteins are highly conserved between different taxa and that they play a crucial role in the generation of circadian rhythms in an

organism, it is likely that polymorphisms in these genes influence temporal sleep patterns. In a study on sleep behaviour in the same blue tit population we found consistent inter-individual variation with high repeatability values in different sleep parameters, especially with regard to temporal measurements of sleep (Steinmeyer et al. 2010). The high repeatability estimates suggest substantial heritability of sleep parameters.

Here, we test for associations between the previously described set of candidate polymorphisms in avian clock genes and the observed variation in sleep phenotypes in blue tits. We use sleep parameters as quantifiable phenotypes since sleep is directly controlled by the circadian clock and represents a distinct behavioural state, which is easy to measure. We test for (a) general associations between the different genotypes at the candidate loci and nine different sleep parameters and (b) specific associations between the average allele length in individuals for microsatellites or the allele copy number for SNPs and the nine sleep parameters. We then (c) evaluate the single-locus results at a study-wide level using a permutation procedure.

METHODS

Field procedures and recording of sleep phenotypes

Birds were recorded in a wild population of blue tits, using artificial nestboxes for breeding and roosting, in southern Germany (Westerholz, 48°08’N, 10°53’E). We caught adult birds inside the nestboxes during monthly checks at night in winter (November – February) and once during the period of nestling feeding (May – June). Birds were equipped with a numbered metal band of the German ringing scheme, a unique colour combination for identification and a RFID transponder. We determined age of the birds (Svensson 1992) and took a blood sample from the brachial vein for genetic analyses. We banded nestlings with a metal ring when they were 14 days old and took a blood sample for paternity analysis. Every 4 weeks during three winter seasons (January 2008 - March 2010), birds were monitored for two consecutive nights while roosting in nestboxes by using a little infrared camera attached to the nestbox lid and connected to a digital

recorder. From the video recordings we measured the following sleep parameters: (1) sleep onset (the time when the first sleep bout of at least 30 s started), (2) awakening time (the time when the last sleep bout of at least 10 s ended), (3) sleep duration (time difference between sleep onset and awakening time), (4) midpoint of sleep (adding half of the sleep duration to the time of sleep onset), (5) evening latency (time difference between entering the nestbox in the evening and sleep onset), (6) morning latency (time difference between awakening time and leaving the nestbox in the morning), (7) longest sleep bout (duration of the longest sleep phase that was not interrupted by an awake phase), (8) frequency of nocturnal awakenings (average number of awake phases per hour), and (9) proportion of time spent awake (total duration of all awake phases divided by the sleep duration). We further measured light intensity (in lux) with data loggers at the nestboxes and calculated mean light intensities at each nestbox in the evening (30 min before – 30 min after sunset) and in the morning (30 min before – 30 min after sunrise). For more details on recording procedure and quantification of sleep parameters see Steinmeyer et al. (2010).

Genotyping of informative polymorphisms

Gene regions containing the polymorphic microsatellite markers were amplified in multiplex polymerase chain reactions (PCRs) using a Qiagen Multiplex PCR Master Mix. We used the primers for *ADCYAP1*, *CLOCK*, *CREB1* and *NPAS2* published in Steinmeyer et al. (2009) with forward primers end-labelled with fluorescent dyes. We separated and detected PCR products on an ABI 3130 *xl* Genetic Analyzer and used the software GeneMapper 4.0 for analyses. We amplified four gene regions containing seven SNP markers in PCRs using the primers for *AANAT*, *CKIε*, *CKIε*-tau and *PERIOD2* published in Steinmeyer et al. (2009). We genotyped SNP markers using the SNaPshot Multiplex Kit (Applied Biosystems) containing fluorescently labeled ddNTPs. SNaPshot products were separated and detected on an ABI 3130 *xl* Genetic Analyzer and analysed with GeneMapper 4.0. Altogether we obtained the genotypes at all 11 loci (Table 1) for 149 individuals (99 males and 50 females) for which sleep parameters had been recorded (total of 803 night recordings).

Statistical analysis

We calculated Hardy-Weinberg Equilibrium for microsatellite markers using Cervus 3.0.3 (http://www.fieldgenetics.com/pages/home.jsp) and for SNPs using the genetics package (Warnes 2011) with the free software R 2.10.0 (http://cran.r-project.org). Linkage Disequilibrium between pairs of markers that are located within the same gene region was also calculated with the genetics package in R. For both analyses we excluded 31 individuals from the dataset because they were offspring or siblings of other individuals in the dataset.

We used two different encodings of the genotypes, to test for an association between genetic markers and different sleep parameters. First, we used the basic full genotype model, treating each genotype as a different level. This allows to distinguish between each individual genotype (e.g. 162/162 and 161/163). Second, we used more powerful encoding models by calculating the mean allele length per individual for microsatellites and the number of one of the two alleles (the most common) per individual for SNPs. Although more powerful (due to a reduction in degrees of freedom), these models assume a linear allele length effect (microsatellites) and an allele dosage effect (SNPs).

We tested associations between individual sleep parameters and genotypes using linear mixed-effects models with normal error distribution while correcting for sex, age (yearling or adult) and light intensity at the nestbox as fixed effects. These covariates, except for light intensity in the models with frequency of awakenings, proportion of time spent awake and longest sleep bout as response variable, were selected because of their shown influence on the sleep parameters (Steinmeyer et al. 2010). To account for the fact that some of the recorded birds were related (parent-offspring or sibling relationships) we defined a unique number for each family and included individual identity (to account for repeated measures of individuals) nested within family identity as random effects in the models. Sleep parameters were standardized for each recording date by subtracting the date-specific mean from the raw values and dividing by the date-specific standard deviation, which removes seasonal and date-specific changes in means and variances.

To evaluate the study-wide significance, we conducted 1000 permutations by assigning multi-locus genotypes randomly to the individual ID and creating hereby 1000 new datasets. Both, the covariance structure among the different sleep parameters and among the genetic markers remained unchanged. The repeated measures of the same individual were assigned to a single individual in the permuted dataset, to keep the variance explained by individuality (random factor) unchanged in the models. We compared the number of significantly associated loci per permutation to the number of significant associations in our original datasets and estimated the probability to obtain a p-value in the permutations that is smaller than the minimum p-value of the original data. We performed the permutation procedure separately for the full genotype models and the specific genotype models. All calculations were carried out in R 2.10.0 using the nlme package for linear mixed models (Pinheiro et al. 2-01).

RESULTS

Genetic polymorphisms

Microsatellite markers revealed between four and nine alleles and SNPs two different alleles (Table 1). All markers except for *CREB1* were in Hardy-Weinberg Equilibrium (Table 1). Given the number of tests (9 independent markers in the 7 gene regions, see below) this deviation from Hardy-Weinberg Equilibrium does not remain study-wide significant after Bonferroni correction. We found high correlations between the *CKIε* SNP and the *CKIε*-tau SNP 1 (r=0.67, P <0.0001) and between *CKIε*-tau SNP 1 and SNP 2 (r=0.29, P <0.0001). Although located in the same gene region, there was no significant correlation between *CKIε* SNP and *CKIε*-tau SNP 2 (r=0.12, P=0.06), between SNP 1 and SNP 2 in *AANAT* (r=-0.03, P =0.67) and between SNP 1 and SNP 2 in *PERIOD2* (r=-0.09, P =0.17).

Table 1
Allele number and names, allele frequency, observed (H_{Obs}) and expected (H_{Exp}) heterozygosity and Hardy-Weinberg test results (p-values) of all microsatellite and SNP markers. Sample size equals 118 presumably unrelated blue tit individuals

Marker	No of alleles[a]/alleles[b]	Major allele frequency	H_{Obs}	H_{Exp}	P
ADCYAP1	9	0.47	0.64	0.61	0.25
CLOCK	4	0.62	0.60	0.56	0.09
CREB1	6	0.81	0.3	0.33	0.01
NPAS2	6	0.37	0.75	0.74	0.32
AANAT SNP 1	C/T	0.89	0.20	0.20	1
AANAT SNP 2	A/G	0.91	0.17	0.16	0.59
CKIε SNP	A/G	0.84	0.28	0.27	0.73
CKIε-tau SNP 1	C/T	0.92	0.15	0.14	0.62
CKIε-tau SNP 2	C/T	0.51	0.50	0.50	1
PERIOD2 SNP 1	A/G	0.83	0.29	0.28	1
PERIOD2 SNP 2	C/T	0.96	0.08	0.07	1

[a] for microsatellites
[b] for SNPs (major nucleotides are underlined)

Associations in the original dataset

The full genotype models applied to all microsatellite and SNP markers revealed in total four significant associations with sleep parameters (Table 2). SNP 2 in *AANAT* and SNP 2 in the gene *PERIOD2* associated significantly with awakening time, after controlling for an effect of sex (standardized effect size=0.75, SE=0.13, $P<0.0001$; females waking up later than males) and local light conditions (standardized regression coefficient=-0.09, SE=0.03, $P<0.01$; birds in brighter locations waking up earlier). Individuals that are homozygous for the G-allele at SNP 2 in *AANAT* (85% of all typed individuals) woke up earlier (on average 2.5 minutes) than those with G/A genotypes (15% of all typed individuals). Homozygous individuals for the C-allele at SNP 2 in *PERIOD 2* (92% of all typed individuals) also woke up earlier (about 3 minutes) than heterozygotes with C/T (8% of all typed individuals). *CKIε*-tau SNP 2 associated significantly with the length of morning latency, after controlling for an effect of sex (standardized effect size=0.33, SE=0.12, $P<0.01$; with females having longer morning latencies). Individuals with C/T genotypes (49% of all typed individuals) showed the shortest morning latencies, those

with T/T genotypes (27% of all typed individuals) the longest. The average difference in morning latencies between these two genotypes was about 1.8 minutes. SNP 1 in the gene *AANAT* associated with the duration of the longest sleep bout, after controlling for an effect of age (standardized effect size=0.31, SE=0.13, *P*=0.02; longer sleep bouts in adult birds compared to yearlings). Carrying two copies of the T-allele (0.01% of all typed individuals, i.e. just one individual) was associated with the longest sleep bouts whereas individuals with two copies of the C-allele (77% of all typed individuals) showed the shortest sleep bouts (about 47 minutes difference). Longest sleep bouts of heterozygotes were on average 17 minutes longer than those of individuals with C/C genotypes.

The mean allele length models revealed no significant association between the four tested microsatellite markers and the nine sleep parameters (Table 3). In models testing allele dosage effects between the SNPs and sleep parameters we found three significant associations (Table 3). SNP 2 in *AANAT* and SNP 2 in *PERIOD2* associated with awakening time. These models captured the same information as the full genotype models described above, because only two different genotypes were detected in both markers (*AANAT*: G/G and G/A, *PERIOD2*: C/C and C/T) in our dataset. Further, we found a significant negative association between the number of C-alleles at locus *AANAT* SNP 1 and the duration of the longest sleep bout. This association was also significant when the full genotype model was applied.

Evaluation by permuted datasets

In the permuted datasets with the full genotype models we found between zero and 12 significant results, with a peak at 2 significant results per permutation (Figure 1a). The probability to get the same or a greater number of significant results as in our original dataset was 46.7 %. The smallest significant p-value which we obtained in the full genotype models for the association between *AANAT* SNP 1 and the duration of the longest sleep bout was *P*=0.005. The probability to obtain a p-value smaller than this at any locus in the permuted datasets was 17.6 %.

In the permuted datasets with mean allele length of the microsatellites and allele dosage of the SNP markers we obtained between zero and 17 significant results, with a peak at 4 significant results per permutation (Figure 1b). With a probability of 78 % we obtained the same or a greater number of significant results as in our original dataset by chance. The smallest p-value that we observed in the original dataset occurred in 5.9 % of the permutations.

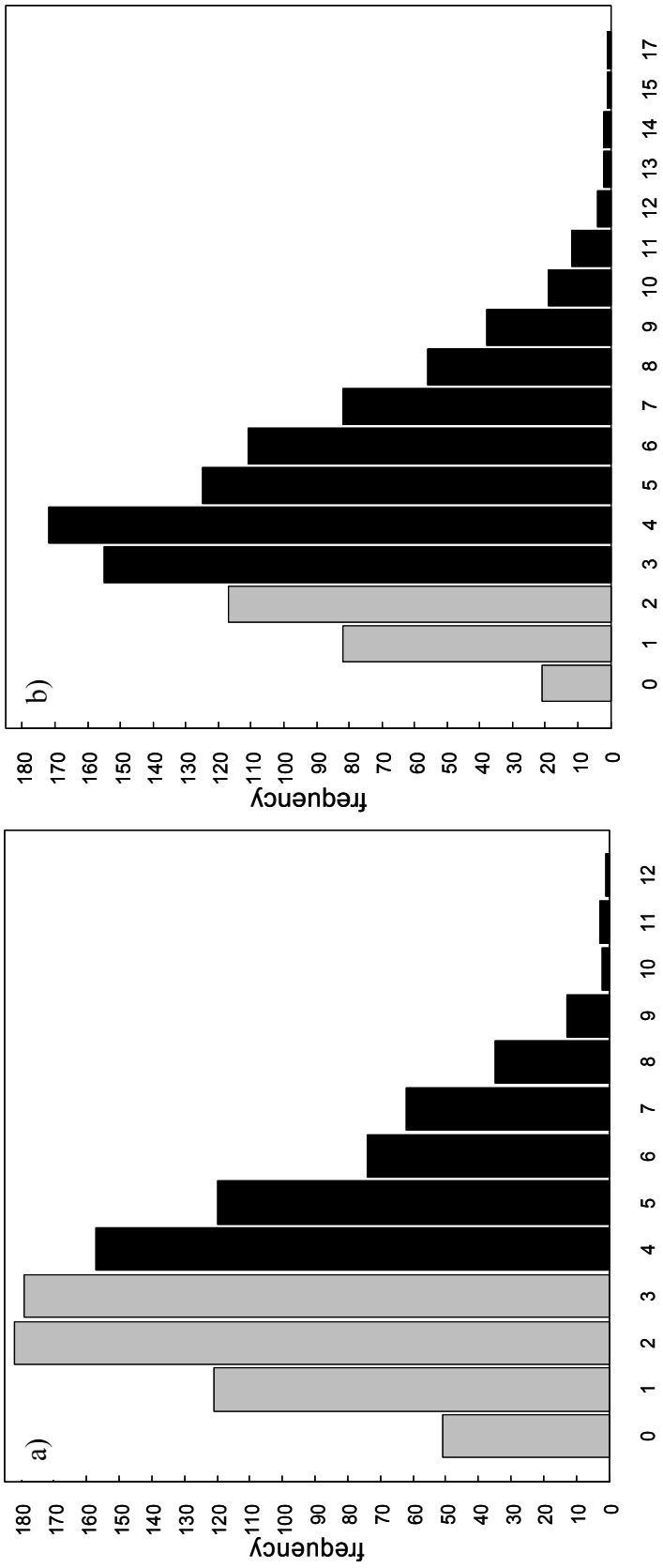

Figure 1. Frequency distribution of number of significant results obtained in permuted datasets (1000 permutations) testing (a) full genotype models (b) mean allele length for microsatellites and allele dosage models for SNPs. Black bars indicate permutations with the same or a greater number of significant results as observed in the original datasets

Table 2
Results (overall p-values) of linear mixed models testing for an association between various sleep parameters and polymorphic markers in blue tits in a full genotype model. Significant values are shown in bold.

Marker	Sleep onset	Awakening time	Sleep duration	Midpoint of sleep	Evening latency	Morning latency	Longest sleep bout	Frequency awakenings	Proportion awake
ADCYAP1	0.071	0.345	0.126	0.089	0.278	0.711	0.416	0.975	0.305
CLOCK	0.555	0.666	0.304	0.781	0.964	0.963	0.088	0.573	0.064
CREB1	0.564	0.767	0.421	0.680	0.787	0.775	0.339	0.380	0.501
NPAS2	0.237	0.500	0.298	0.222	0.859	0.793	0.209	0.314	0.396
AANAT SNP 1	0.669	0.093	0.763	0.110	0.770	0.454	**0.005**	0.238	0.422
AANAT SNP 2	0.415	**0.014**	0.111	0.682	0.921	0.886	0.616	0.905	0.208
CKIε SNP	0.924	0.507	0.904	0.620	0.947	0.495	0.261	0.372	0.233
CKIε-tau SNP 1	0.707	0.457	0.903	0.472	0.696	0.617	0.339	0.492	0.635
CKIε-tau SNP 2	0.931	0.310	0.754	0.594	0.815	**0.027**	0.585	0.872	0.946
PERIOD2 SNP 1	0.192	0.974	0.577	0.146	0.884	0.247	0.891	0.594	0.726
PERIOD2 SNP 2	0.434	**0.040**	0.087	0.586	0.442	0.356	0.278	0.276	0.207

Table 3
Results of linear mixed models testing for an association between various sleep parameters and mean allele length of microsatellite markers or presence of the most common allele in SNP markers, respectively, in blue tits. Standardized regression coefficients with 95% confidence intervals (in brackets) are given. Significant values are shown in bold.

Marker	Sleep onset	Awakening time	Sleep duration	Midpoint of sleep	Evening latency	Morning latency	Longest sleep bout	Frequency awakenings	Proportion awake
ADCYAP1	0.03 (-0.11-0.08)	0.04 (-0.12-0.20)	0.01 (-0.14-0.15)	0.04 (-0.10-0.19)	0.10 (-0.05-0.24)	0.03 (-0.13-0.18)	-0.15 (-0.33-0.02)	0.001 (-0.20-0.20)	0.06 (-0.13-0.25)
CLOCK	-0.03 (-0.25-0.18)	-0.05 (-0.29-0.18)	0.02 (-0.20-0.23)	-0.04 (-0.26-0.18)	-0.04 (-0.25-0.18)	0.07 (-0.15-0.29)	-0.23 (-0.53-0.07)	-0.03 (-0.37-0.31)	-0.06 (-0.38-0.26)
CREB1	0.05 (<0.001-0.10)	-0.01 (-0.06-0.05)	-0.05 (-0.10-0.002)	0.04 (-0.01-0.09)	0.02 (-0.03-0.07	0.002 (-0.06-0.05)	0.00 (-0.07-0.08)	0.03 (-0.06-0.11)	0.06 (-0.01-0.14)
NPAS2	0.02 (-0.03-0.07)	-0.01 (-0.06-0.05)	-0.01 (-0.06-0.04)	0.01 (-0.04-0.07)	0.02 (-0.03-0.07)	-0.01 (-0.07-0.04)	0.01 (-0.05-0.07)	0.05 (-0.03-0.12)	0.03 (-0.04-0.10)
AANAT SNP 1	-0.11 (-0.36-0.14)	-0.17 (-0.44-0.10)	0.02 (-0.23-0.27)	-0.22 (-0.47-0.03)	0.07 (-0.18-0.32)	-0.13 (-0.39-0.14)	**-0.53 ** (-0.79- -0.26)**	0.29 (-0.04-0.62)	0.16 (-0.15-0.48)
AANAT SNP 2	0.13 (-0.18-0.44)	**-0.43 * (-0.77- -0.10)**	-0.25 (-0.56-0.06)	-0.06 (-0.38-0.26)	0.02 (-0.30-0.33)	-0.03 (-0.37-0.31)	0.10 (-0.30-0.51)	-0.03 (-0.48-0.43)	-0.28 (-0.70-0.14)
CKIε SNP	-0.04 (-0.27-0.18)	-0.14 (-0.38-0.10)	-0.04 (-0.26-0.18)	-0.12 (-0.34-0.11)	-0.04 (-0.26-0.19)	-0.04 (-0.28-0.19)	0.22 (-0.04-0.49)	-0.22 (-0.52-0.08)	-0.22 (-0.51-0.06)
CKIε-tau SNP 1	-0.06 (-0.37-0.25)	-0.14 (-0.47-0.20)	0.02 (-0.29-0.33)	-0.12 (-0.43-0.20)	0.06 (-0.25-0.37)	-0.08 (-0.40-0.24)	0.19 (-0.18-0.56)	-0.15 (-0.58-0.27)	-0.10 (-0.49-0.30)
CKIε-tau SNP 2	-0.02 (-0.17-0.14)	-0.06 (-0.24-0.11)	0.03 (-0.12-0.19)	-0.06 (-0.22-0.10)	-0.03 (-0.19-0.13)	-0.11 (-0.28-0.06)	0.10 (-0.09-0.30)	-0.01 (-0.23-0.21)	0.01 (-0.20-0.21)
PERIOD2 SNP 1	-0.21 (-0.43-0.01)	0.01 (-0.23-0.26)	0.12 (-0.11-0.34)	-0.23 (-0.45-0.04)	-0.01 (-0.24-0.21)	0.17 (-0.07-0.41)	-0.04 (-0.31-0.22)	0.16 (-0.14-0.45)	0.07 (-0.21-0.35)
PERIOD2 SNP 2	0.16 (-0.23-0.55)	**-0.48 * (-0.91- -0.06)**	-0.34 (-0.73-0.04)	-0.12 (-0.52-0.29)	0.16 (-0.24-0.55)	0.20 (-0.22-0.61)	-0.32 (-0.88-0.24)	0.36 (-0.27-1.00)	0.40 (-0.20-0.99)

* $P < 0.05$; ** $P < 0.01$

DISCUSSION

We found four significant associations between our candidate loci and the sleep parameters awakening time, morning latency and duration of the longest sleep bout in full genotype models and three associations with awakening time and duration of the longest sleep bout with the specific models. The three association signals of the specific models were a subset of the four association signals of the genotype models. A priori we had good reasons to select the eleven polymorphisms which we tested in the association analyses because all selected microsatellites and SNPs except for one (*CKIε* SNP) are located in exonic regions of genes related to circadian rhythms and therefore likely candidates to be functional in the context of sleep variation (Steinmeyer et al. 2009). Previous studies in other organisms have shown significant associations between polymorphisms in a homologous or nearby codon and sleep disorders or abnormalities in sleep/wake rhythms. We selected sleep parameters that represent the temporal organization of sleep, which is supposed to be regulated by the circadian clock. Further, in a previous study we showed that most of these sleep parameters are highly repeatable within individuals (repeatability measures between 0.38 and 0.46) and hence can be regarded as individual-specific traits that might be genetically determined (Steinmeyer et al. 2010).

The strongest a priori candidate for showing an association in our study was the microsatellite marker in the *CLOCK* gene that codes for a polyglutamine (poly-Q) repeat. This poly-Q region was found to serve as a transcriptional trans-activation domain (Gekakis et al. 1998) and appears to be highly evolutionary conserved (Saleem et al. 2001). The *CLOCK* protein is one of the core elements in the circadian oscillator hence a modified protein structure is most likely to influence circadian behaviour. In humans, the CAG-tandem repeats being responsible for the poly-Q sequence variation in birds were not polymorphic (Saleem et al. 2001), but other polymorphisms were found in the coding regions of the gene that have phenotypic effects, for example influence diurnal preference or sleep duration (Katzenberg et al. 1998; Mishima et al. 2005; Allebrandt et al. 2010). In blue tits and Chinook salmon *Oncorhynchus tshawytscha* however, a latitudinal cline in

allele frequencies at the *CLOCK* poly-Q locus has been identified in an inter-population comparison, suggesting that this gene shows local adaptation to latitudinal gradients in photoperiod (Johnsen et al. 2007; O'Malley and Banks 2008). Liedvogel et al. (2009) reported that timing of breeding and incubation duration correlated with the number of poly-Q repeats in a population of blue tits, with early breeding females having fewer poly-Q repeats. Despite these expectations of *CLOCK* having an influence on circadian or circannual rhythms, we did not find an association between the avian *CLOCK* polymorphism and sleep behaviour.

Another polymorphism in our list of tested candidates that is presumably coding is the trinucleotide repeat in the gene *NPAS2*. Again, this polymorphism leads to a variable number of poly-Q repeats in the protein. To our knowledge there is no study that describes an association between a naturally occurring polymorphism in the *NPAS2* gene and variation in circadian behaviour, although *NPAS2* is one of the core clock genes. We also did not find any evidence for an association with the sleep parameters we investigated.

All other polymorphisms that we tested, except for one intronic SNP in the *CKIε* (not considered further in Steinmeyer et al. (2009)) were exonic but non-coding because they are either located in UTRs (untranslated regions) of the gene or are silent nucleotide exchanges that do not lead to a modification of the amino acid sequence. All the originally associated SNP polymorphisms in the organisms that we used as a template were coding, i.e. resulted in an amino acid change. Nevertheless, non-coding polymorphisms might also have functional consequences. For example, microsatellites with varying length can alter gene expression through an influence on mRNA degradation, DNA methylation or nucleosome stability (Pieretti et al. 1991; Wang et al. 1994; Imagawa et al. 1995; Choong et al. 1996). The microsatellites in the genes *ADCYAP1* and *CREB1* are located in the 3'UTRs which are known to be regulatory elements also involved in post-transcriptional processes (Bartel 2009; Chatterjee and Pal 2009). Simple sequence repeats in 3'UTRs have been suggested to cause transcription slippage and therefore modify regulatory functions (Li et al. 2004). Silent SNPs can

affect mRNA structure or pre-mRNA splicing and therefore can have functional consequences (Shen et al. 1999; Cartegni et al. 2002; Duan et al. 2003; Kimchi-Sarfaty et al. 2007) as might be the case in an association between a SNP in the human *PERIOD2* gene and diurnal preference (Carpen et al. 2005).

Although the observed number of associations is not study-wide significant, we discuss why some of the singly significant genotype-phenotype associations are worth further investigation. First, single nucleotide polymorphisms in the *CKIε* gene were good candidates, because associations with circadian rhythm disorders have already been shown in a variety of other species. In humans a non-synonymous SNP was associated with delayed sleep phase syndrome and non-24h sleep-wake syndrome (Takano et al. 2004), a non-synonymous point mutation in Syrian hamsters shortens the period length of circadian rhythms (Lowrey et al. 2000) and point mutations in the homologous gene (*doubletime*) of *Drosophila* either shorten or lengthen the period of the behavioural rhythm (Kloss et al. 1998).We detected three different SNPs in the homologous blue tit *CKIε* and one of them (*CKIε*-tau SNP 2) showed a significant association with morning latency in the full genotype model. This SNP is located close (5 base-pairs) to the position of the mutation detected in the hamster *CKIε* gene which influences the length of the free-running circadian rhythm. This association was absent when tested in the allele dosage model. Indeed the strongest difference was observed between the heterozygotes (C/T) and homozygotes for the T-allele. Second, variants in *AANAT* which codes for the rate-limiting enzyme (arylalkylamine-*N*-acetyltransferase) in the synthesis of melatonin, and thus being involved in the regulation of daily oscillations of the melatonin level (Klein and Weller 1970; Binkley et al. 1973; Ganguly et al. 2002) are likely to affect phenotypes. It has been suggested that a point mutation in the promoter region of the human *AANAT* gene is a determinant of late/short sleep patterns (Wang et al. 2004) and that another single nucleotide polymorphism (causing an amino acid substitution) is associated with DSPS (Hohjoh et al. 2003). We observed a linear effect of the presence or absence of one of the two alleles of SNP 1 in the gene *AANAT* on the duration of the longest continuous sleep bout. Heterozygous individuals showed intermediate durations of the longest sleep bouts, whereas homozygotes for the C-allele had the shortest and

homozygotes for the T-allele the longest sleep bouts. However, there was only one individual carrying two T-alleles. Although this was the strongest observed association in our study, permutation analyses in the full genotype models and allele dosage models showed that such small p-values can be obtained with a chance of 17.6 and 5.9%, respectively. The second SNP in *AANAT* is located in close proximity to the first and showed an association with awakening time. Third, various studies have also reported associations between polymorphisms in all three human *PERIOD* genes and diurnal preferences or sleep disorders (Ebisawa et al. 2001; Toh et al. 2001; Carpen et al. 2005; Carpen et al. 2006; Jones et al. 2007). We found a possible association between SNP 2 in *PERIOD2* and awakening time. Interestingly, the SNP in the blue tit gene was near (~ 50bp) the missense mutation in the homologous human *PERIOD2* that is assumed to cause advanced sleep phase syndrome which is characterized by extremely early sleep onset and awakening time (Toh et al. 2001).

Although we carefully selected candidate polymorphisms, we still performed a large number of tests (eleven markers and nine phenotypes with partial interdependencies), which increases the risk of false positive results (type I error). This is a general known issue in genome-wide association studies, usually examining thousands of SNPs, but also in medium-sized candidate-gene approaches. With independent markers Bonferroni corrections can be applied, but this inflates type 2 errors (false negatives) (Johnson et al. 2010). Here, we evaluated the multiple test results by a permutation procedure that maintained the partial dependency structure among phenotypes and among genotypes of different loci (linkage disequilibrium structure). Our results suggest that the number of significant associations we found could have occurred by chance alone (47 or 78% likelihood for the full genotype or specific models, respectively). Even the smallest p-value for the association between *AANAT* and the longest sleep bout was observed in 5.9 % of the random datasets. On the other hand, effect sizes of genotype-phenotype associations are expected to be rather small. The sample size of 149 individuals in our study is relatively small for genotype-phenotype association studies and may not be sufficient to rule out a potential association. We therefore consider our candidate gene

approach on the temporal organization of sleep as a preliminary analysis to generate hypotheses that need further evaluation.

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General Discussion

Although sleep is a widespread and fundamental behaviour in the animal kingdom it has hardly been studied in animals living under natural conditions. The importance of studies carried out under natural conditions in order to understand ecological and evolutionary mechanisms is emphasized by findings of Rattenborg et al. (2008). The authors reported that sleep behaviour in captive animals (sloths, *Bradypus variegatus*) varied considerably from sleep of wild conspecifics. Thus, the major objective of this thesis was to investigate behavioural aspects of sleep in a common passerine bird, the blue tit, and investigate the underlying genetic basis of the sleep parameters. This is the first study in which sleep phenotypes have been recorded for a large sample of free-living birds (151 individuals). I applied behavioural criteria to define sleep in the blue tits: following Amlaner and Ball (1983), a bird was considered asleep when it showed the classical sleep position with the beak tucked under the scapulars and feathers fluffed. When birds were not in this sleep position, they were actively preening, scratching or moving around in the nestbox and therefore considered awake.

I examined individual variation in sleep parameters and the factors that influence these parameters and I was able to provide evidence that temporal organization of sleep in blue tits is strongly affected by specific external factors, differs between males and females and is highly repeatable. From this I concluded that sleep behaviour might represent an individual specific trait comparable to personality (chapter 1).

High repeatability is also present in periodicity of sleep-wake patterns but not in period length as reported in chapter 2. Sleep-wake rhythms were influenced by environmental conditions as well, such as night temperature and photoperiod. The results from the first two chapters show that sleep is not a static behaviour in blue tits but changes seasonally for several of the measured components and might show a trade-off with other physiological demands or activities.

In a next step (chapter 3) I investigated whether individual-specific variation is associated with fitness-related traits, such as timing of egg laying, success in gaining extra-pair paternity or survival and whether males and females mate assortatively or disassortatively in regard to their sleep phenotypes. Although I had good reasons to predict such a link given some existing knowledge from the literature, I did not find strong evidence that sleep behaviour is associated with fitness in blue tits. Either, the studied parameters did not influence reproductive measurements at all or such relationships were not evident under fluctuating natural conditions and would only be observable when sleep is disturbed experimentally.

Finally, I aimed to uncover the genetic causes underlying the phenotypic variation described in the first chapter with a candidate gene approach. This approach is an appropriate choice in studies of genetic non-model organisms for which whole genome sequences are not available. Although I successfully identified a set of genetic variants in different blue tit genes with a high likelihood of being functional (chapter 4), I did not find conclusive evidence that single polymorphisms were associated with phenotypic variation in sleep behaviour in my study population (chapter 5). All singly significant associations I observed could have occurred by chance and emphasize the importance of repeated testing. The results can be seen as generating hypothetical associations that need further testing.

Roosting in nestboxes – costs and benefits

Bird species that use nestboxes as breeding sites often use them for roosting as well. Among the species most commonly found to be roosting in nestboxes in Germany are great tits (*Parus major*), nuthatches (*Sitta europaea*), blue tits and tree sparrows (*Passer montanus*) whereas great spotted woodpeckers (*Dendrocopos major*), lesser spotted woodpeckers (*Dendrocopos minor*), starlings (*Sturnus vulgaris*), marsh tits (*Poecile palustris*) and short-toed treecreepers (*Certhia brachydactyla*) can occasionally be found

(Winkel and Hudde 1988). Interestingly, blue tits of two study populations in Corsica never use nestboxes for roosting at night whereas those of populations in mainland southern France regularly do (Dhondt et al. 2010). Roosting in nestboxes (or cavities in general) has both benefits and costs. One obvious advantage is energy saving due to thermal benefits, especially when temperatures are low in winter (Walsberg 1986; McCafferty et al. 2001; Paclik and Weidinger 2007; Velky et al. 2010). Consequently, overnight weight loss can directly be reduced (Dhondt and Eyckerman 1979). Second, predation risk (especially by owls) is reduced in nestboxes. For example, great tits that regularly slept in nestboxes were less often predated by owls than birds that did not roost in boxes (Drent 1987). A third advantage is that birds can occupy and defend potential breeding sites before the breeding season actually starts.

On the other hand, it can be disadvantageous to roost in nestboxes if they contain ectoparasites from the previous breeding season, especially when old nest-material is still present. Nocturnal predation risk by small mammals and snakes can also be increased in nestboxes (Dunn 1977; Orell 1989; Sorace et al. 2000), because there is no chance of escaping once a bird has been detected. In our study population, there is basically no risk of predation or inter-specific competition inside the boxes. The small-holed nestboxes can only be entered by very few species and snakes have never been encountered in the study area. Further, all nest-material is removed after each breeding season which minimizes ectoparasite abundance. Therefore the nestboxes I used for the nighttime recordings represent optimal roosting sites for blue tits. Nevertheless, intra-specific competition might occur, leading to higher occupancy rates by dominant males. Indeed, the usage of the nestboxes as roosting sites by the two sexes varied between months in our study population. At the beginning and in the middle of winter about 80% of the recorded birds were males whereas 50-70% of the recorded birds in spring were females. Adult birds were more abundant throughout the three study winters than yearlings. The proportion of recorded birds that were categorized as yearlings was between 12 and 31% each winter, whereas the proportion of yearlings during the following breeding seasons was between 37 and 53%. Although there were plenty of empty nestboxes available within the study site, it is clear that not all birds present in the population roosted in a

nestbox. Hence, I cannot rule out that birds roosting in the boxes represent a sub-sample of the whole population with specific characteristics, e.g. being high quality or dominant individuals. While sleeping in nestboxes, the blue tits might behave differently compared to birds roosting in other sites. On the other hand, nestboxes represent relatively standardized conditions for all individuals, allowing direct between-individual comparisons.

Factors explaining individual variation in sleep parameters

Environmental factors

In a comparative study on temporal sleep characteristics in birds Amlaner and Ball (1983) showed great differences in total sleep duration between bird species. They found a very strong effect of latitude and daylength on sleep duration. Species living at higher latitudes slept in total shorter ($r = -0.62$) and so did species that experienced shorter night length ($r = -0.7$). Overall, daylength accounted for about 50% of the variance in sleep duration. In my analyses on temporal aspects of sleep behaviour I found that sleep duration within the same species changes over the course of the season in accordance with changing daylength. Blue tits slept the longest on long winter nights in December and January and decreased their nighttime sleep by about 5 hours with decreasing length of the night towards spring (chapter 1). The same pattern has been observed in captive starlings (*Sturnus vulgaris*), rooks (*Corvus frugilegus*) and White-crowned sparrows (*Zonotrichia leucophrys gambelii*) (Szymczak 1986; 1987; Jones et al. 2010). Obviously, light conditions strongly affect the decision of the birds whether to sleep or not to sleep. Moreover, when weather conditions were bad (e.g. rain or snowfall), blue tits tended to start sleeping earlier and wake up later compared to nice days, but weather effects cannot be disentangled from light effects in my dataset. These findings are in accordance with the null hypothesis of sleep, which asserts that sleep is the safest and most efficient state when no other activities are possible (Meddis 1975).

Nevertheless, the time of entering the nestbox and sleep onset in our blue tit population was not triggered by the same light conditions throughout the season. In mid-winter, blue

tits began to sleep relatively late when there was almost no light detectable whereas they advanced the onset of sleep in relation to sunset in spring (chapter 1). To obtain enough food in order to survive the night, the birds might be forced to expand their active periods as long as possible during mid-winter even though light conditions might be suboptimal. Alternatively, blue tits might have a certain sleep demand per day and therefore start to sleep relatively early in spring when light conditions would still allow other activities such as foraging. This would coincide with other functions of sleep, e.g. restorative functions.

Other parameters of sleep also changed over the course of the season. Towards early spring, I observed an increased frequency of nocturnal awakenings and shorter continuous sleep bouts compared to mid-winter nights (chapter 1). This contradicts the assumption that birds compensate for shorter sleep duration by increasing sleep intensity (at least behaviourally measured). Further, period length of sleep-wake cycles was shorter when nights were shorter (chapter 2). Altogether, the results suggest that physiological conditions, influencing sleep behaviour vary between winter and spring. Further studies on sleep quality (EEG measurements) should shed more light on the regulation of seasonal sleep compensation.

One proposed function of sleep is the conservation of energy (Berger and Phillips 1993; Berger and Phillips 1995). Therefore one could assume that animals increase sleep duration or intensity when exposed to low ambient temperatures. King penguins (*Aptenodytes patagonicus*), for example, reduced their sleeping time when ambient temperature increased over 10°C (Dewasmes et al. 2001). However, other published studies show opposite results. In glaucous-winged gulls (*Larus glaucescens*), sleep decreased when birds were exposed to thermal loading but did not change under cooling manipulations (Opp et al. 1987). Total sleep time in little penguins (*Eudyptula minor*) was reduced during exposure to cold as a result of a reduction in length of sleep periods (Stahel et al. 1984) whereas sleep time in emperor penguins (*Aptenodytes forsteri*) did not differ under cold and thermo-neutral conditions (Buchet et al. 1986). Similar results were reported by Wellmann and Downs (2009). In a study on three African passerine birds the

authors did not find differences in sleep amount between two temperature conditions (5 and 25°C). In my observations on blue tits, I cannot easily uncouple ambient temperature effects on sleep parameters from light conditions, as mean night temperatures are the lowest in mid winter when darkness is longest. However, when comparing two consecutive nights, on which daylength is approximately the same I found differences in the frequency of nocturnal awakenings and the proportion of time spent awake according to variation in temperature. During warmer nights, blue tits were more awake (chapter 1) and they were more likely to show rhythmic sleep-wake cycles (chapter 2).

Birds, besides mammals, are the only taxonomic group that was found to engage in the two electrographically distinguished sleep states SWS and REM sleep (Rattenborg 2006; Rattenborg et al. 2009). These sleep states alternate throughout a sleep bout, presumably serve different functions (see General Introduction) and seem to be influenced by temperature. In response to higher ambient temperatures, rooks were observed to change the percentage of REM sleep from 1% to about 3% and increase the duration of sleep cycles (SWS+REM) by several minutes (Szymczak 1989). The reduction in awakenings and rhythmicity during nights with lower temperatures which I observed in the blue tits might be linked to an altered composition of SWS and REM phases. This again might be caused by a controlled down-regulation of body temperature during sleep (hypothermia). It is known from other studies that blue tits are able to reduce their body temperature by up to 5°C while sleeping (Nord et al. 2009; Nord et al. 2011).

Age effects

Sleep in humans is strongly age and gender dependent. Frequent nocturnal awakenings, difficulties to fall asleep and early awakening are the main issues found in older people (Bliwise 1993; Prinz 1995; Ancoli-Israel 1997; Van Someren 2000). From early childhood to adolescence total sleep duration decreases conspicuously (Iglowstein et al. 2003) and sleep duration is further reduced in older people (Hume et al. 1998; Dijk et al. 1999). In accordance with earlier awakening times in elderly, the mid-point of sleep is advanced compared to young people (Hume et al. 1998). In addition, age-dependent

differences in the amount of SWS and REM sleep were found (Hume et al. 1998) and sleep-wake patterns in elderly people were generally more regular (Kramer et al. 1999).

What causes these age-related changes, predominantly phase advances? Presumably, alterations of the circadian timing system with ageing are the reason for age-related changes in sleep structure and disruptions in sleep-wake cycles (Cajochen et al. 2006; Nakamura et al. 2011). The observed earlier awakening times in older people were found to be accompanied by advances of the core body temperature and melatonin rhythms (Dijk et al. 2000). Not only in humans, but also in hamsters (*Mesocricetus auratus*) the circadian clock is influenced by age (Turek et al. 1995).

My results showed that most temporal sleep parameters in blue tits were unaffected by age (chapter 1), at least in the two defined age classes. Sleep onset, awakening time, sleep duration and midpoint of sleep did not differ between yearlings and adults. However, age effects on sleep behaviour might only become observable when comparing very old and young (immature) individuals. We found differences in morning latency between yearling and adult birds, but we argued that these were more likely caused by experience or other trade-offs than by endogenous factors or sleep requirements (chapter 1). In addition, adult birds showed fewer rhythmic nights than yearlings (chapter 2). Differences in subcutaneous fat depots between juveniles and adults could be a possible explanation for this observation. In a study on body hypothermia in response to increased roost-site temperature, blue tits with greater fat reserves maintained their body temperature at higher levels compared to individuals with lower reserves (Nord et al. 2011). Generally, adult birds are more likely to be dominant, which positively affects individual food predictability, and it is hypothesized that with increasing resource predictability energy reserves should decrease (Ekman and Lilliendahl 1993; Gosler 1996). Subordinate juvenile blue tits might therefore carry higher fat reserves (Gosler 1996), allowing them to maintain higher body temperatures during sleep, which may lead to more regular and longer nocturnal awakenings.

Sex effects and reproductive success

Longer total sleep time and difficulties of maintaining sleep have been reported by human females (Lindberg et al. 1997). Chronotype distribution also seems to be related to gender in humans, with eveningness being present more often in men and morningness more often in women (Lehnkering and Siegmund 2007). In birds, only one other study reported variation in sleep behaviour between males and females. This study showed that in captive starling males slept less than females (Szymczak 1985). I also found significant differences in sleep parameters between male and female blue tits (chapter 1). First, females generally entered the nestbox and began to sleep earlier than males. Second, females woke up and left the nestbox later than males. Consequently, sleep duration in females was on average 15 min longer than in males. Third, females spent more time in the nestbox after waking up in the morning and fourth, females spent a greater proportion of the night awake. However, the observed differences were not consistent throughout the winter season. The differences between males and females in awakening time and sleep duration were largest in early spring, at the beginning of the breeding season. This suggests that sleep behaviour might also be shaped by sexual selection. Indeed, the breeding season poses different constraints, requirements and physiological demands on the two sexes. Early in the season, males may be more engaged in territory defense and male-male competition.

The results from chapter 1 led to the assumption that there is high pressure on waking up early in the morning in general, as awakening times were always before sunrise. Sexual selection might additionally act on starting activities early in the morning in males, leading to stronger sex differences. This is supported by other studies showing higher reproductive success for males that start dawn chorus early in the morning (Poesel et al. 2006; Dolan et al. 2007; Murphy et al. 2008). Interestingly, I did not find evidence that early awakening males had higher fitness than those with comparatively late awakening times (chapter 3). More specifically, my results did neither show an effect of awakening time on extra-pair success nor an effect of any sleep parameter on the number of sired eggs. Contrary to the predictions, I have found that males with early sleep onset and long sleep durations were more likely to sire at least one extra-pair young. It appears that long-

sleeping males in winter might be of high quality or dominant individuals that can 'afford' long sleep durations (due to high food availability) and are more likely to be chosen by females as extra-pair partners. However, I did not record sleep behaviour in the breeding season and therefore I do not have information about sleep durations of males during the time of the season when extra-pair copulations take place.

In females, variation in sleep behaviour may also influence fitness, given the importance of sleep for health and body maintenance (see General Introduction) and the overall costs of reproduction. Females that are less efficient in foraging during the day may need to expand their daytime activity as much as possible. Those females might in general show later sleep onset and earlier awakening times and have lower breeding success due to poor body condition. However, I did not find any correlations between sleep parameters and female fitness measurements such as laying date, clutch size and obtaining extra-pair young in females (chapter 3). It has been reported that females using nestboxes for roosting during winter have in general comparatively early laying dates (Dhondt and Eyckerman 1979). Indeed, females that I recorded during winter initiated clutches significantly earlier in the following breeding season than all other females after correcting for age (LMM for three study years combined: Estimate=-1.8, SE=0.7, Z=-2.5, P=0.01). Individual sleep behaviour of those females might therefore not further influence laying date.

Individuality

Besides the environmental effects (photoperiod, local light intensity), and the effects of sex and age I found considerable variation in sleep parameters between individuals (chapter 1). Sleep duration for example varied by as much as 68 minutes in adult males on one recording night and by 52 minutes in adult females. The maximum variation in sleep onset times of adult males on the same day was about 60 minutes and 44 minutes in adult females; differences in individual awakening times were up to 27 minutes for males and 41 minutes for females. In general, blue tits did not show continuous sleep throughout the night, but many sleep bouts interrupted by short awakenings. The number

of these awakenings varied between 35 and 154 times in males and between 32 and 230 times in females within the same night.

Different factors might influence the individual quantity and quality of sleep at night. As described in the introduction, sleeping animals are exposed to higher predation risks. Hence the perceived risk of predation may affect sleep. For example, rats have been shown to change their circadian sleep period from night to day following changes in predator activity (Fenn and Macdonald 1995). During the breeding season male mallards (*Anas platyrhynchos*) with conspicuous nuptial plumage increased their peeking rates (short eye-openings) compared to non-breeding periods when carrying more cryptic plumage (Lendrem 1983). Another example is a study, where barbary doves (*Streptopelia risoria*) were exposed to a potential predator (ferret, *Mustela furo*), which led to higher peeking rates than a group of birds without predator encounters (Lendrem 1984). Perceived predation risk can also influence the degree of nocturnal hypothermia (Laurila and Hohtola 2005) which again is associated with changes in sleep patterns. However, there is no evidence that birds can perceive and react to predator odour once they have fallen asleep (Amo et al. 2011).

In general, experiences made during the day might modify subsequent sleep. At least in humans it has been experimentally shown that sleep characteristics after a day with novel, stimulating environments differ from sleep following stereotypic days (Horne and Minard 1985). Inter-individual differences in sleep behaviour may also be related to aspects of body condition. More specifically, fat scores can differ between individuals and affect sleep parameters (as discussed above under age effects). Further, sleep can be affected by parasite infections. For example, experimentally increased ectoparasite load in the nest led to reduced sleeping time of brooding female great tits (Christe et al. 1996) and altered sleep bouts in female blue tits (Tripet et al. 2002). Although nestboxes were free of nest material when I recorded blue tits, and we never observed fleas and only rarely feather mites when handling the birds during night catches, the possibility remains that some individuals carried ectoparasites or were infected with endoparasites.

Repeated measurements of sleep parameters of the majority of individuals allowed us to estimate how consistent individual blue tits were in their sleep behaviour, i.e. how much of the total phenotypic variance is explained by between-individual variance, referred to as repeatability (Nakagawa and Schielzeth 2010). Repeatability estimates of most sleep parameters were relatively high (0.25-0.46) and also the expression of rhythmicity in nocturnal sleep-wake cycles was significantly repeatable (chapter 1 and 2). The human literature also suggests that there is great variability in individual timing and duration of sleep and in sleep architecture. Short sleepers characteristically need less than 6 hours of sleep per night, whereas long sleepers spend about 8 hours or more asleep (Webb and Agnew 1970; Webb and Friel 1971; Krueger and Friedman 2009; Kripke et al. 2011). Those groups show differences in EEG patterns, suggesting that sleep "efficiency" varies between individuals (Webb and Agnew 1970). I used the identity checks at night to test arousal thresholds of the birds, as the hand-held transponder reader device emits a standardized "beep" sound of about 2 s when it detects a transponder. Birds that did or did not wake up due to the check did not differ in any of the sleep parameters, giving no evidence for different sleep depth in long or short sleepers.

Two chronotypes have been characterized in humans: evening-types (staying up late at night, getting up with difficulty, being tired when waking up) and morning-types (waking up early, early sleep onset, being refreshed on waking up) (Roenneberg et al. 2003; Cavallera and Giudici 2008). Further, EEG characteristics during SWS were variable between individuals but stable within individuals when recorded on several nights, providing a unique "fingerprint" (Buckelmüller et al. 2006). Besides cultural and life-style factors influencing sleep length and timing in humans, there is evidence for a genetic component as well. Twin studies provide convincing evidence that sleep architecture, variance in slow-wave sleep, diurnal preference and sleep EEG profiles are highly heritable (Webb and Campbell 1983; Linkowski 1999; Koskenvuo et al. 2007; De Gennaro et al. 2008). Broad sense heritability for several self-reported sleep measures was estimated as 21-41% (de Castro 2002).

In order to understand the biological basis and manifestations of a trait or disorder it is important to understand the underpinning genetic factors. Much research has focused on the genetics of sleep during the past. Especially in the context of human sleep disorders a variety of genetic variants have been discovered that seem to be associated with sleep traits. In animals however, research has focused predominantly on the description of aberrant circadian rhythms under free-running conditions, due to artificially introduced mutations or gene knock-outs. For non-model organisms, where genomes are not sequenced and family pedigrees are not available, some commonly used strategies to find the genetic basis of phenotypic traits (e.g. linkage studies, genome-wide association studies) are not applicable. In chapter 4, I summarize studies that describe published associations between polymorphisms in clock genes and variation in sleep behaviour (mainly in humans) and describe how they can be used in a candidate gene approach. Ultimately, I was not able to pinpoint a single detected polymorphism in blue tits which causes the observed phenotypic variability in sleep behaviour (chapter 5), although several associations were suggestive. Further work is needed to resolve the genetic basis of sleep variation in birds.

Future directions

This study reports for the first time details about sleep behaviour of a large sample of free-living passerine birds and investigates some of the causes and consequences of the observed intraspecific variation. Our results can be used for further research to examine additional factors causing variation between sexes, age classes and individuals. For example, it would be worthwhile to measure individual body mass and score subcutaneous fat as indicators of general body condition close to the date of recording and search for correlations with sleep parameters. Also a more in-depth study of parasite abundance in relation to sleep might be worthwhile. Another aspect, which is linked to body mass and fat score, is the basal metabolic rate (BMR) of individuals. BMR can be influential on hypothermia and variation in BMR may therefore explain variation in sleep behaviour.

I described the successful identification of likely functional polymorphisms in candidate genes. However, all detected SNPs in the blue tit genes were synonymous, i.e. the amino acid sequence remains unchanged and protein function might not be influenced. A further search for non-synonymous polymorphisms in the exons of core clock genes might thus be warranted to further test for associations with variability in sleep behaviour. This needs large-scale sequencing, a project which is now underway. Our results do suggest that some polymorphisms may explain variation in sleep behaviour in blue tits. Hence, an alternative strategy could be the replication of this study in another population of blue tits or in a related species. If an association between one of the reported polymorphisms with the same phenotypic trait would be found, this would provide strong supporting evidence for a true association.

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Author Contributions

Chapter 1

C.S. contributed to the study concept and design, practical work, video and data analyses and writing; H.S. to the data analyses and writing; J.C.M. to the study concept and design, practical work, discussion and provided comments on the manuscript; B.K. to the study concept and design, discussion and writing.

Chapter 2

J.C.M. contributed to the study concept and design, data analyses and writing; C.S. to the study concept, data analyses and provided comments on the manuscript; B.K. provided comments on the manuscript.

Chapter 3

C.S. contributed to the study concept and design, practical work, data analyses and writing; J.C.M. to the study concept and design, practical work, discussion and provided comments on the manuscript; B.K. to the study concept, discussion and writing.

Chapter 4

C.S. contributed to the study concept and design, practical work, data analyses and writing; J.C.M. to the study concept and design, data analyses and writing; B.K. to the study concept and writing.

Chapter 5

C.S. contributed to the study concept and design, data analyses and writing; B.K. to discussion and provided comments on the manuscript; J.C.M. to the study concept and design, data analyses and writing.

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Working experience

Jul-Aug 2001	Practical assistant at the Maine Medical Center, USA
Aug-Oct 2004	Field assistant at the Institute for Zoo and Wildlife Research (IZW) Berlin, Germany; fieldwork in Namibia, Africa
Dec 2006-Feb 2007	Practical assistant at the University of Otago (Department of Zoology), Dunedin, New Zealand

Awards and Scholarships

2001	Winner of the "Karl-von-Frisch" student award
2004	Deutscher Akademischer Austauschdienst (DAAD)
2011-2012	Christiane Nüsslein-Volhard-Foundation

Publications

Steinmeyer C., Mueller J.C., Kempenaers B. 2009. Search for informative polymorphisms in candidate genes: clock genes and circadian behaviour in blue tits. Genetica 136 (1): 109-117

Steinmeyer C., Schielzeth H., Mueller J.C., Kempenaers B. 2010. Variation in sleep behaviour in free-living blue tits, *Cyanistes caeruleus*: effects of sex, age and environment. Animal Behaviour 80 (5): 853-864

Conference Presentations

Description and genetic basis of variation in sleep behaviour in a wild blue tit population
EGI Student Conference in Oxford, England: 7.-9.01.2009 (Poster)

Sleeping behaviour of wild blue tits *Cyanistes caeruleus*
Graduiertentreffen Verhaltensbiologie (DZG and Ethologische Gesellschaft): 11.-13.11.09 in Seewiesen, Germany (Talk)

Sleeping behaviour of wild blue tits *Cyanistes caeruleus*
EES (Munich Graduate Program for Evolution, Ecology and Systematics) Conference: 11.-12.10.2011 in Munich, Germany (Talk)

Declaration

Ehrenwörtliche Versicherung

Ich versichere hiermit ehrenwörtlich, dass die vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt ist.

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Erklärung

Hiermit erkläre ich, dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist, und dass ich mich anderweitig einer Doktorprüfung ohne Erfolg nicht unterzogen habe.

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