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RESEARCH ARTICLE

Cryptochrome expression in the eye of migratory birds depends on their migratory status

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ABSTRACT

Most passerine birds are nocturnal migrants. When kept in captivity during the migratory periods, these species show a migratory restlessness, or *Zugunruhe*. Recent studies on *Sylvia* warblers have shown that *Zugunruhe* is an excellent proxy of migratory disposition. Passerine birds can use the Earth's geomagnetic field as a compass to keep their course during their migratory flight. Among the candidate magnetoreceptive mechanisms are the cryptochromes, flavoproteins located in the retina that are supposed to perceive the magnetic field through a light-mediated process. Previous work has suggested that expression of Cryptochrome 1 (Cry1) is increased in migratory birds compared with non-migratory species. Here we tested the hypothesis that *Cry1* expression depends on migratory status. Blackcaps *Sylvia atricapilla* were caught before fall migration and held in registration cages. When the birds were showing robust *Zugunruhe*, we applied a food deprivation protocol that simulates a long migratory flight. When the birds were refed after 2 days, their *Zugunruhe* decreased substantially, as is expected from birds that would interrupt migration for a refuelling stopover. We found that *Cry1* expression was higher at night than during daytime in birds showing *Zugunruhe*, whereas in birds that underwent the fasting-and-refeeding protocol and reduced their levels of *Zugunruhe*, night *Cry1* expression decreased to daytime levels. Our work shows that *Cry1* expression is dependent on the presence of *Zugunruhe* and not on species-specific or seasonal factors, or on the birds being active versus inactive. These results support the hypothesis that cryptochromes underlie magnetoreceptive mechanisms in birds.

KEY WORDS: Cryptochrome, *Zugunruhe*, Bird migration, Nocturnal migration, Magnetic orientation, Magnetoreception

INTRODUCTION

Several billions of birds migrate every year between continents. Small passerine birds are mostly nocturnal migrants, i.e. they have diurnal patterns of activity outside of migratory times but they migrate at night. Birds held in registration cages during the times of the year that correspond to their migratory phase show intense locomotor activity at night. This phenotype has been called migratory restlessness, or *Zugunruhe*, and has been shown to correlate to actual duration of migration in several warbler species of the genus *Sylvia* (Berthold, 1973). Recent work showed that in wild garden warblers, *Zugunruhe* is an excellent indicator of their tendency to resume migration after a stopover (Fusani et al., 2009; Goymann et al., 2010). *Zugunruhe* can be suppressed in captive *Sylvia* warblers by a 'fasting-and-refeeding' protocol that consists of depriving the birds of food for 2 days and reintroducing the food on the third day (the 'Biebach effect') (Biebach, 1985; Fusani and Gwinner, 2004). This experimental protocol presumably mimics the situation of a migrating bird that has been fasting during a long flight and subsequently interrupts migration temporarily upon reaching a suitable refuelling site (Biebach et al., 1986).

The capacity of animals to detect geomagnetic fields has significant biological importance as it is used by many invertebrates and vertebrates for compass orientation purposes, including the capability of maintaining a given course during long-distance migration (Wiltschko and Wiltschko, 2005). In birds, two models of magnetoreception have been proposed: a magnetite-based process (Kirschvink and Gould, 1981; Eder et al., 2012) and chemical-based reactions (Ritz et al., 2000; Rodgers and Hore, 2009). The chemical model of magnetoreception suggests that magnetic information is transmitted to the nervous system through the light-induced product of magnetically sensitive radical-pair reactions in specialized photoreceptors (Ritz et al., 2000; Ritz et al., 2004). Until now, the only molecules found to have such characteristics are the cryptochromes (CRY) (Schulten et al., 1978; Liedvogel et al., 2007; Biskup et al., 2009). The first genetic evidence for a cryptochrome-based magnetosensitive system in animals has been obtained in the fruit fly *Drosophila melanogaster*. Flies missing the gene *Cry* did not respond to a magnetic field under full-spectrum light in a binary-choice behavioural assay (Gegear et al., 2008). Furthermore, *Cry*-deficient flies did not respond to the magnetic field when the short wavelengths (<420 nm) were blocked (Gegear et al., 2008). The ability to sense a magnetic field in a light-dependent manner has also been demonstrated for the monarch butterfly (*Danaus plexippus*) and human cryptochromes (Gegear et al., 2010; Foley et al., 2011).

In vertebrates, the night-migrating Passeriformes [the European robin (*Erithacus rubecula*) and the garden warbler (*Sylvia borin*)], the homing pigeon (*Columbia livia*) and the domestic fowl (*Gallus gallus*) are among the most extensively studied model species because they have been shown to be able to extract compass information from the Earth's magnetic field (Wiltschko and Wiltschko, 1972; Wiltschko and Wiltschko, 2002; Mouritsen et al., 2004; Wilzeck et al., 2010; Niessner et al., 2011). In these species, experimental evidence points to CRYs as the key molecules for light-mediated, radical-pair-based magnetic compass orientation (Solov'yov et al., 2010; Mouritsen et al., 2004; Niessner et al., 2011). In both migratory species, the European robin and the garden warbler, CRYs are expressed in the retina of the eye and their magnetic orientation performances under low-intensity monochromatic light are wavelength-dependent, i.e. birds are well oriented only when exposed to short wavelengths (<565 nm) (Rappl et al., 2000; Wiltschko and Wiltschko, 2001; Muheim et al., 2002).

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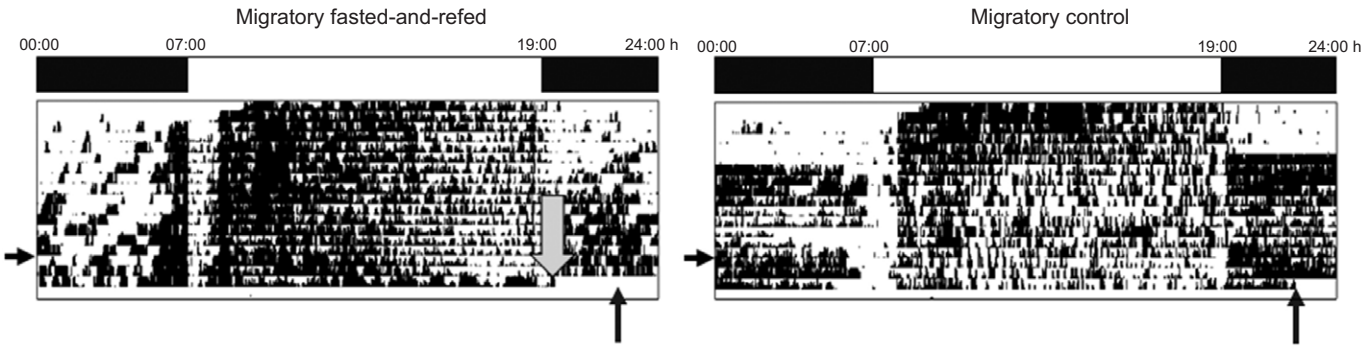

Fig. 1. Actograms showing the effects of the fasting-and-refeeding protocol on *Zugunruhe* in one treated (left) and one control (right) blackcap (*Sylvia atricapilla*). Each row represents the locomotor activity recorded during a single day, from top to bottom. The black and white bars at the top indicate dark and light phases, respectively. Both animals showed robust *Zugunruhe* in the 10 days preceding the beginning of experiments, which is indicated by the two small horizontal arrows. For the fasted-and-refed bird, the food was removed for 2 days and re-introduced in the morning of the last experimental day. The large grey arrow shows the time of interruption of *Zugunruhe* in the fasted-and-refed bird following food re-introduction (left). The recordings were terminated at 22:00 h (indicated by the two small black vertical arrows) to sample the tissue for gene expression studies.

In accordance, biochemical investigation showed that garden warbler CRY1a is excited by short-wavelength light, leading to the formation of radicals with millisecond lifetimes (Liedvogel et al., 2007). A comparison of the retinal expression levels of CRY between migratory garden warblers and non-migratory birds (zebra finch *Taeniopygia guttata*) showed higher levels of CRY at night compared with during the day in garden warblers, whereas in zebra finches the expression at night was lower than during the day (Mouritsen et al., 2004). These data suggested that higher expression of CRY is functional to an increased sensitivity to the magnetic field during oriented migration and indicated CRY1 as the main candidate for the light-mediated magnetoreception.

The aim of this work was to test the hypothesis that the expression of *Cry1* at night in migratory birds depends specifically on migratory status. Therefore, we predict that birds of a same species, at the same time of the year, will show differences in the pattern of expression of *Cry1* according to the presence or absence of *Zugunruhe*. Specifically, we studied whether *Cry*1 expression could be inhibited by inducing a temporary interruption of *Zugunruhe* by applying the fasting-and-refeeding protocol described above. The study was carried out with a long-distance migratory population of the blackcap *Sylvia atricapilla* (Linnaeus 1758), a congener of the garden warbler that responds very well to the fasting-and-refeeding protocol (Fusani and Gwinner, 2004). First, we identified *S. atricapilla* orthologues of *Cry1a* and *Cry1b*. Then, we compared the expression pattern of these genes in the eye of blackcaps showing *Zugunruhe* with that of birds in which *Zugunruhe* had been reduced through the fasting-and-refeeding protocol. Our results show that interruption of the migratory behaviour significantly alters the expression of *Cry* in the eye of blackcaps, and provide new insights on the role of cryptochromes in the magnetic compass orientation of night-migrating birds.

RESULTS

Effects of the fasting-and-refeeding treatment on body mass

Birds that were assigned to the migratory groups had a larger body mass compared with diurnal birds that showed no nocturnal migratory restlessness (20.6±0.6 g versus 16.3±0.8 g, respectively; t_{22}=2.933, P=0.008), as expected because of the fat deposition that typically accompanies the migratory status. Before the beginning of the treatment, there was no significant difference between the body mass of migratory birds in the fasted-and-refed and control groups (means ± s.e.m. averaged over the 4 days preceding food deprivation: migratory controls, 19.6±0.67 g, N=6; migratory fasted-and-refed, 21.6±1.0 g, N=7; t_{14}=1.713, P=0.109). The fasting-and-refeeding protocol affected body mass substantially after 2 days: in migratory controls, body mass was reduced to 0.1±0.5 g compared with the previous 4 days, whereas migratory fasted-and-refed birds lost 2.3±0.2 g (t_{14}=3.975, P<0.001).

Effects of the fasting-and-refeeding treatment on locomotor activity

The fasting-and-refeeding protocol had clear-cut effects on *Zugunruhe* and diurnal locomotor activity (Fig. 1, Fig. 2B). The ANOVA showed highly significant effects of the experimental phase ($F_{2,32}$=17.791, P<0.001) and of the interaction between phase and treatment ($F_{2,32}$=9.254, P<0.001), but not of the treatment alone ($F_{1,16}$=0.009, P=0.927). This was due to the fact that the treatment affected locomotor activity only during and after the food deprivation. In the 2 days of food deprivation, migratory fasted-and-refed birds showed more intense diurnal activity compared with migratory controls (23.6±3.3 versus 9.9±1.6; t_{16}=3.471, P=0.003), but no significant differences in *Zugunruhe* (19.1±4.3 versus 23.7±4.8, respectively; t_{16}=0.711, P=0.487). On the day of refeeding, on the contrary, *Zugunruhe* was significantly reduced in the migratory fasted-and-refed group compared with migratory controls (t_{16}=3.290, P=0.005; Fig. 2B), whereas diurnal activity showed the opposite pattern (t_{16}=2.946, P=0.009; Fig. 1, Fig. 2B).

Identification of *S. atricapilla* cryptochromes

We cloned *S. atricapilla Cry1a* and *Cry1b* orthologues. The blackcap *Cry1a* full-length cDNA includes a 1863 bp open reading frame (ORF) encoding a 620 amino acid protein, whereas the full-length *Cry1b* cDNA contains a 1764 bp ORF encoding a 587 amino acid protein. Both deduced amino acid sequences show a high degree of similarity (>92%) with homologous sequences of other avian species in which these genes have been cloned (Haque et al., 2002; Möller et al., 2004; Mouritsen et al., 2004; Liedvogel and Mouritsen, 2010). Between the blackcap CRY1a and CRY1b, the similarity is 95%; they differ in the C-termini region that in CRY1a is longer than 33 amino acids. The presence of two *Cry1* isoforms (alternative splicing products) was also shown in the migratory songbird *E. rubecula* (Möller et al., 2004). Inspection of the protein primary structure reveals that domains indicative of functional

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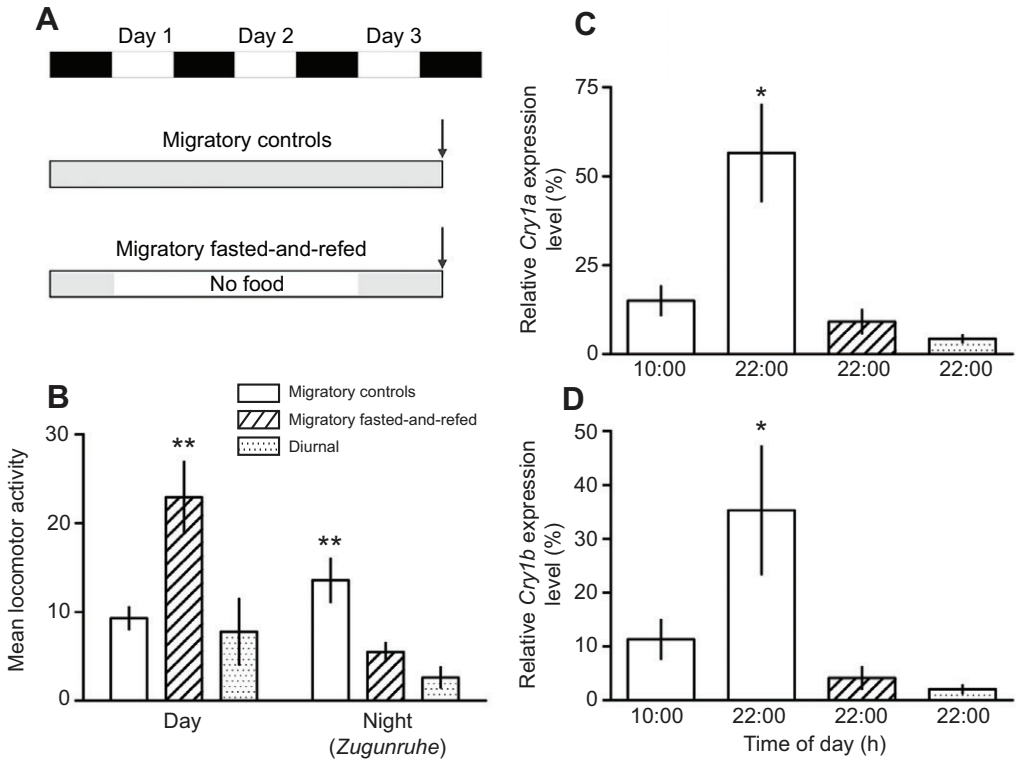

Fig. 2. Effects of the fasting-and-refeeding protocol on locomotory activity and on *Cry1a* and *Cry1b* expression levels in blackcaps (*Sylvia atricapilla*). (A) Schematic representation of the fasting-and-refeeding protocol. The black and white bars at the top represent dark and light phases, respectively. The fasted-and-refed birds were deprived of food from lights on of Day 1 to lights on of Day 3. The arrows indicate the termination of the experiments at 22:00 h of Day 3. (B) Locomotor activity (means ± s.e.m.) of migratory (open bars) and migratory fasted-and-refed birds (hatched bars) on the day of refeeding, and of diurnal birds (dotted bar) in the 5 days preceding the sampling. Migratory fasted-and-refed birds showed more intense day activity and less intense *Zugunruhe* compared with migratory controls. (C) *Cry1a* and (D) *Cry1b* expression levels (normalized for the maximum level detected for each gene) in the eye of migratory control (open bars), migratory fasted-and-refed (hatched bars) and diurnal blackcaps (dotted bars). Migratory fasted-and-refed birds that interrupted *Zugunruhe* in response to the food protocol showed significantly lower expression of both genes at night compared with migratory control birds showing *Zugunruhe*. The night expression of *Cry1a* and *Cry1b* in birds that had interrupted migration was in fact similar to that of diurnal birds, and did not differ from that found in migratory control birds during daytime. Asterisks indicate significant differences between treatments (*P<0.05; **P<0.01).

proteins, such as DNA photolyase and FAD (flavin-adenine dinucleotide) binding, are all present in both CRYs.

Cry1 mRNA levels in the eye

Our results revealed a significant difference for both *Cry1a* and *Cry1b* expression levels (Kruskal–Wallis test, *Cry1a*: K_4=13.7, P=0.003; *Cry1b*: K_4=13.3, P=0.004) in the eye of blackcaps with different migratory behaviour (Fig. 2B,C). Specifically, at 22:00 h, i.e. 3 h after lights off, higher levels of both *Cry1a* and *Cry1b* mRNA were found in the eye of birds showing *Zugunruhe* compared with blackcaps in which the *Zugunruhe* was experimentally turned off. The expression level of *Cry1a* and *Cry1b* at 22:00 h in blackcaps with diurnal activity was similar to that in birds showing no *Zugunruhe*, and significantly lower than that in those showing *Zugunruhe* (Dunn's multiple comparison test, P<0.05). Furthermore, *Cry* expression levels during the day (10:00 h, i.e. 3 h after lights on) were not different from levels found during the night in birds in which *Zugunruhe* was absent (Fig. 2B,C).

DISCUSSION

The aim of our work was to verify whether the expression of cryptochromes, the best candidate magnetic-sensory molecules required for light-mediated magnetoreception, depends specifically on the migratory status of birds. For this purpose we compared mRNA *Cry1* levels in the eye of blackcaps showing migratory restlessness – *Zugunruhe* – with those of birds in which *Zugunruhe* had been temporarily switched off by exposing them to a protocol that simulates a long migratory flight and the subsequent stopover. Our data show that *Cry1a* and *Cry1b* expression in the blackcap eye is higher at night than during the day in birds expressing *Zugurnuhe*, and that nocturnal *Cry1a* and *Cry1b* expression is significantly reduced (to day levels) when *Zugunruhe* is suppressed through the fasting-and-refeeding protocol, and is low in birds that show spontaneously a diurnal pattern of activity, i.e. had spontaneously terminated *Zugunruhe*.

A previous study had shown higher cryptochrome levels at night compared with during the day in the retina of birds in migratory status (Mouritsen et al., 2004). In addition, CRY1 expression levels in the retina at night were significantly higher in migratory garden warblers than in non-migratory zebra finches (Mouritsen et al., 2004). However, because the comparison was limited to two species, we do not know whether this difference reflects species-specific patterns. Similarly, the finding of a different pattern of *Cry1* expression between birds sampled in migratory versus sedentary periods could be due to unknown seasonal factors unrelated to the migratory status. The present investigation fills the gap by showing differences in *Cry1* expression at night between migratory and non-migratory birds of the same species and population and at the same time of year.

The behavioural paradigm used in this study consists of the induction of an interruption of *Zugunruhe* by means of a fasting-

and-refeeding protocol. This is a robust and reliable protocol (Biebach, 1985; Gwinner et al., 1985; Gwinner et al., 1988; Fusani and Gwinner, 2004) that provides an alternative and complementary approach to the study of migratory restlessness behaviour in orientation cages such as Emlen funnels (Emlen and Emlen, 1966; Mouritsen et al., 2004). *Zugunruhe* has been recognized as a strong proxy for migratory status for more than 200 years [Naumann, 1795-1817, cited in Berthold (Berthold, 1988)], and our recent studies in garden warblers have shown that *Zugunruhe* is an excellent indicator of the actual migratory disposition in *Sylvia* (Goymann et al., 2010). We did not attempt here to test whether the birds were showing oriented movements in their cages, because our main interest was to understand the pattern of expression of the putative magnetoreceptive mechanism rather than the response of birds to migratory cues. However, there is no reason to think that birds in migratory status showing robust *Zugunruhe* would have functional magnetic sensing only when using these cues. On the contrary, our paradigm is very robust because we compare birds in identical experimental conditions, i.e. controlling for location, day, species and population effects.

Our results provide novel, additional evidence for a role of cryptochromes in magnetoreception in birds because they allow us to rule out other factors that could have been responsible for the reported differences in the pattern of expression following the study of Mouritsen and colleagues (Mouritsen et al., 2004). Nocturnal migrants are diurnal during non-migratory periods, thus differences in expression could have resulted from a constitutive pattern associated with the activity status – active versus inactive. On the contrary, our results show that *Cry1* expression is significantly higher at night than during the day in birds showing *Zugunruhe*, which are therefore active across the 24 h. These results appear to contrast with unpublished data mentioned by Mouritsen and colleagues (Mouritsen et al., 2004), according to which garden warblers that do not show *Zugunruhe* also show high levels of nocturnal CRY1 expression. We do not know the reasons for these discrepancies. They might depend on differences in the experimental design between the two studies, e.g. in the time of day and night sampling.

Magnetosensitivity is not a unique function of cryptochromes. These molecules have been shown to play a crucial role in the circadian clock, with differences between species (Oztürk et al., 2007). For instance, in some invertebrates, CRY acts as circadian photoreceptor (Busza et al., 2004), whereas in other invertebrates and vertebrates it plays a role as transcriptional repressor (Sancar, 2004; Zhu et al., 2005). Because CRYs are expressed in circadian manner in many bird organs including the eye (Haque et al., 2002; Tu et al., 2004), future investigations should verify a daily variation of light-mediated magnetoreception in both migratory and resident birds. Moreover, we cannot exclude a role of the other cryptochromes (Möller et al., 2004; Mouritsen et al., 2004; Liedvogel and Mouritsen, 2010). Recent investigations showed that CRY4 is expressed in the retina of chickens and garden warblers and changes its structure in a light-dependent manner (Liedvogel and Mouritsen, 2010; Watari et al., 2012).

In summary, this work adds to a series of studies that support the hypothesis that cryptochromes are responsible for magnetoreception in nocturnally migrating passerines (Rappl et al., 2000; Ritz et al., 2000; Wiltschko and Wiltschko, 2001; Muheim et al., 2002; Mouritsen et al., 2004; Liedvogel et al., 2007; Rodgers and Hore, 2009). A conclusive demonstration of the role of cryptochromes in magnetoreception in birds requires the ability of suppressing or altering the activity of these proteins *in vivo*. One could test, for instance, the capacity of orientation to magnetic cues of transgenic (*Cry*-null) migratory birds, or alternatively of birds with *Cry* silenced by RNA interference. However, to date, all attempts of creating a transgenic migratory bird have failed, and many gene manipulation techniques are difficult to apply to non-classical animal models such as migratory birds.

MATERIALS AND METHODS

Animals

Blackcaps, *Sylvia atricapilla*, from a long-distance migratory population (Berthold, 1973; Berthold, 2001) were trapped with mist-nets at the Pape Ornithological Station, Latvia (56°9′48″N, 21°1′35″E), between the end of August and the beginning of September 2011. Immediately after capture, the birds were held in individual cages under natural photoperiod and given water and a mixture of dry insect food, boiled egg and banana (Fusani and Gwinner, 2004) and mealworms. The birds were left undisturbed until the mid-afternoon, when they were checked for body mass changes and food intake. Birds that did not eat and whose body mass had decreased more than 1.5 g were immediately released. Five days after the last birds had been trapped, the birds were moved to transportation cages, with water and mealworms, and transported by air and car to the University of Ferrara, Ferrara, Italy. Here, they were held in an aviary (300×200×100 cm) with water and food provided *ad libitum* until the beginning of the experiments. Temperature was kept at 25±1°C and relative humidity at 70%. The photoperiod was reduced gradually to simulate southward migration until 21 September, when it reached 12 h:12 h light:dark, and was kept so subsequently. The capture and transportation of the birds, and all experimental procedures, were conducted under permission of The Nature Conservation Agency of Latvia (permit no. D3.6/37 of 26/08/2011), the IATA Guidelines, the University of Ferrara and the Italian Ministry of Health.

Experimental design

The experiments started in mid-October 2011, approximately 6 weeks after the birds were transported to Ferrara. The animals were moved into custom-built individual fabric cages where they were visually isolated from each other. Each cage had an infrared activity sensor set on the side opposite to the cage opening, which was connected with an electronic system (Ersacto, University of Groningen) that recorded the locomotor activity within the cage at 2-min intervals. The recording system has been described in detail elsewhere (Fusani et al., 2009; Fusani et al., 2011). The photoperiod was 12 h:12 h light:dark, with lights on at 07:00 h. Temperature was kept at 25±1°C and relative humidity at 70%.

Birds were assigned to two groups: migratory (N=20) or diurnal (N=4). We assigned to the migratory group birds in marked migratory conditions, i.e. those with a large body mass and which showed robust, consistent locomotor activity during the dark phase and reduced activity during the light phase. This pattern is typically expressed by caged migratory birds during autumn and spring and is traditionally called *Zugunruhe* (see Fig. 1). We assigned to the diurnal group those birds that showed activity mostly during the day and were not very active at night because of the spontaneous termination of *Zugunruhe* (Fig. 2A). A subset (N=13) of the migratory birds was further randomly divided in two groups: fasted-and-refed (N=7) and controls (N=6). We formed experimental blocks of three to four birds (one to two from each group) spaced apart by 1 week to test a few birds at a time and collect samples as close as possible to the defined time points. The body mass of the birds was recorded daily between 08:00 and 09:00 h. The animals of the migratory fasted-and-refed group received no food for 2 days, but fresh water was given as usual. Food deprivation is not stressful for birds with large fat reserves (Schwabl et al., 1991). On the third day, at lights on, fresh food was reintroduced into the cages (Fig. 2A). This protocol had been used in a series of previous studies in blackcaps and garden warblers (Biebach, 1985; Fusani and Gwinner, 2004) and we expected it to induce a suppression or strong reduction of *Zugunruhe* in the night following food refeeding. Migratory controls received food and water as usual along the experiments. In the night of the third day of experiments, at 22:00 h, the migratory fasted-and-refed (N=7) and migratory control birds (N=6) were

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Table 1. Nucleotide sequences of primers used in PCR

Primer	Direction	Sequence (5′→3′)	Use
Sa_CRY1a_R1822	R	5′-ACTTTTGGTCCAACGCTCTG-3′	RTPCR
Sa_CRY1b_R1736	R	5′-TGATGTTTTGTCTGGTTTTCCA-3′	RTPCR
Sa_Cry1a/b_F582	F	5′-CCTTGAAGAGCTGGGTTTTG-3′	RTPCR
Sa_Cry1b_R1704	R	5′-AGCCACTGCCACAATACCTT-3′	RTPCR
Sa_Cry1a/b_F243	F	5′-AGATGTTTTCCCCAGGCTTT-3′	RTPCR
Sa_Cry1a_R1197	R	5′-CCACATCCAACTTCCAGCAT-3′	RTPCR
Sa_Cry1b_F1706	F	5′-CAGCAAGGTATTGTGGCAGTGGCTGT-3′	RACE
Sa_Cry1a_F1784	F	5′-TTAGTGCAGGGAAACGCCCAAATCC-3′	RACE
Sa_Cry1a/b_R66	R	5′-CACTCCAGCTTCACTGGCCAGCTTC-3′	RACE
Sa_Cry1a/b_334	R	5′-CTGGCCATACTGCAGAAGGCAGACC-3′	RACE
SAcry1a/bFOR	F	5′-CGTACACAGGTTCCGCAAG-3′	Cloning
SAcry1aREV	R	5′-GGTCCAACGCTCTGAGTTTC-3′	Cloning
SAcry1bREV	R	5′-GTTGCAAGGATTTGGAGAGC-3′	Cloning
SAb-actin_F	F	5′-TGGATTTCGAGCAGGAGATGGC-3′	RTPCR
SAb-actin_R	R	5′-AATGCCAGGGTACATTGTGGTACC-3′	RTPCR
SAGAPDH_F	F	5′-ACTCTACTCATGGCCACTTCCG-3′	RTPCR
SAGAPDH_R	R	5′-ATGTTCTGAGCAGCACCTCTGC-3′	RTPCR
SAcry1a_qPCR_F	F	5′-GCTCATGGAGACAATCAGCA-3′	qPCR
SAcry1a_qPCR_R	R	5′-ACTTTTGGTCCAACGCTCTG-3′	qPCR
SAcry1b_qPCR_F	F	5′-GGAGCTGGTGATGGTCATTC-3′	qPCR
SAcry1b_qPCR_R	R	5′-TGGTTTTCCATAGTTGCAAGG-3′	qPCR
SAb-actin_qPCR_F	F	5′-TGGATTTCGAGCAGGAGATGGC-3′	qPCR
SAb-actin_qPCR_R	R	5′-AATGCCAGGGTACATTGTGGTACC-3′	qPCR
SAGAPDH_qPCR_F	F	5′-TGGTGATGCTCCCATGTTCGTGAT-3′	qPCR
SAGAPDH_qPCR_R	R	5′-CACGATGCCGAAGTTGTCATGGAT-3′	qPCR

weighed and then killed by cervical dislocation and decapitation. After the enucleation, using the standard procedure for preparing the eyecup, we cut the anterior part of the eye to remove the cornea, the lens and the vitreous body. Then we cut the ocular muscles and the optic nerve, if present. We did not dissect the retina from the eyecup to avoid any damage to the outer layers of the retina. Our enucleated eye samples basically included retina, pigment epithelium, choroid and sclera. After preparation, the eyecup was rapidly transferred into Trizol for RNA extraction (see below). During the same days, we sampled tissues from the remaining seven birds of the migratory group at 10:00 h, and from the diurnal group at 22:00 h.

Cloning and sequencing

Total RNA was isolated from blackcap eyes using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The amount, quality and composition of isolated RNA were analysed by BioSpec-nano (Shimadzu, Kyoto, Japan). One microgram of total RNA was incubated with DNase I (Invitrogen) at room temperature for 30 min and then at 85°C for 15 min to inactivate the enzyme. DNase-treated RNA was used to perform cDNA synthesis in a final volume of 20 µl, using a mix of 0.4 µl random primers (3 µg µl^{-1}) and 0.6 µl of oligo dT 12-18 (0.5 µg µl^{-1}) and 200 units of SuperScript II reverse transcriptase (Invitrogen). The reaction was performed at 42°C for 50 min, followed by an inactivation step of 15 min at 70°C. Blackcap *Cry1a* and *Cry1b* were amplified by PCR with primers designed by Primer3 software (Rozen and Skaletsky, 1999) on the basis of the sequences of the *Sylvia borin* homologues. Specifically, because garden warbler *Cry1a* and *Cry1b* are alternative splicing products whose sequence differs only in the 3′ end, we designed forward primers common for the two isoforms and reverse primers specific for *Cry1a* and *Cry1b* (see Table 1). The PCR was conducted in a total volume of 50 µl, containing 2 µl of blackcap cDNA, 2 units of *Taq* DNA Polymerase (Invitrogen) with 0.4 µmol l^{-1} of both primers. Thermal cycling conditions were as follows: 2 min denaturation at 94°C; followed by 40 cycles of a 30 s denaturation step at 94°C, one annealing step for 30 s at a temperature specific to every pair of primers and one elongation step for 1 min at 72°C and a final 7 min elongation at 72°C. Negative control reactions containing RNA or water instead of cDNA were always included in the PCR reactions. Bands of the predicted sizes were cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). The blackcap *Crys* cDNA fragments were sequenced and compared with the GenBank database by using the BLAST algorithm. Full-length cDNA was completed with 5′-3′ RACE using a SMART RACE cDNA amplification kit (BD Bioscience-Clontech, Mountain View, CA, USA) according to the manufacturer's recommendations. To confirm the existence of the two *cry* isoforms, we cloned and sequenced the complete coding sequences of blackcap *Cry1a* and *Cry1b*. The sequences were deposited in GenBank (accession nos KC691287 and KC691288, respectively). Amplification of the partial nucleotide sequence of *S. atricapilla β-actin* and *GAPDH* was performed using primers (Table 1) that were designed on the basis of the sequences of garden warbler orthologues. The PCR product was then cloned as described above and the sequences were deposited in GenBank (accession nos KC691289 and KC691290, respectively).

Gene expression analysis

One microlitre of 1:4 diluted first-strand cDNA was PCR amplified with a Chromo4 Real-Time PCR Detection System (Bio-Rad, Milan, Italy) using iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Thermal cycling conditions were as follows: 3 min denaturation at 95°C, followed by 40 cycles of a 5 s denaturation step at 95°C and an annealing–elongation step for 20 s at 60°C. After amplification, melting curve analysis to confirm the specificity of the amplicon was performed from 60 to 95°C, with increments of 0.5°C 10 s^{-1}. All samples were run in triplicate. Gene-specific primers (Table 1) were used to amplify fragments of 120–150 bp in length.

We verified the efficiency of the primers by performing standard curves for all genes investigated. Moreover, the dissociation curve was used to confirm the specificity of the amplicon. The relative levels of each RNA were calculated by the $2^{-\Delta\Delta C_T}$ method (where C_T is the cycle number at which the signal reaches the threshold of detection) (Livak and Schmittgen, 2001). *β-actin* was used as housekeeping gene. Each C_T value used for these calculations is the mean of three replicates of the same reaction. To ascertain that the apparent changes in the expression of *Cry1a* and *Cry1b* mRNA were not artefacts of normalizing to the *β-actin* housekeeping gene, a control experiment was conducted normalizing to another reference gene, *GAPDH*. Nearly identical expression profiles were observed when *Cry1a* and *Cry1b* transcript levels were normalized to *β-actin* or *GAPDH* mRNA.

Statistical analysis

All results are reported as means ± s.e.m. Student's *t*-test, ANOVA, Kruskal–Wallis one-way ANOVA and Dunn's multiple comparison test were

used to determine significant differences ($P<0.05$) between groups using GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA, USA). To analyse the activity, we first ran a repeated-measures ANOVA with experimental phase as the within-subject factor and treatment (fasted-and-refed and control) as the between-subject factor. We divided the experiment into three phases: the 4 days before food deprivation; the 2 days of food deprivation; and the day of refeeding, i.e. the last experimental day. For the analyses, we used the average activity across each phase.

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Competing interests
The authors declare no competing financial interests.

Author contributions
L.F., C.B. and A.F. conceived and designed the study and wrote the paper. L.F. and C.B. conducted the fieldwork and the behavioural experiments. E.F. carried out the laboratory work and participated in the interpretation of the results.

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