MOLECULAR ECOLOGY

Molecular Ecology (2013) **22**, 5430–5440 doi: 10.1111/mec.12493

Variation at phenological candidate genes correlates with timing of dispersal and plumage morph in a sedentary bird of prey

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Abstract

Polymorphic genes involved in the conserved molecular signalling of circadian and circannual clocks may play important roles in governing the timing of breeding and dispersal and thereby affect fitness in vertebrates. However, relatively few studies have explored associations between phenological candidate genes and behaviour, and these are somewhat biased towards particular taxonomic groups such as passerine birds and salmonid fish. Consequently, we assayed microsatellite polymorphisms within the exonic and 3′ untranslated regions of the regulatory genes CLOCK, NPAS2, ADCYAP1 and CREB1 in the common buzzard (*Buteo buteo*), a polymorphic raptor species with three plumage morphs that differ in key life history traits including lifetime reproductive success. In contrast to studies of passerines, CLOCK poly-glutamine (poly-Q) was found to be monomorphic in 976 common buzzard nestlings as well as in three other *Buteo* species. Moreover, none of the candidate genes were significantly associated with fledging dates, although intermediately melanized females were found to lay earlier on average than light or dark morph individuals, and their offspring carried longer ADCYAP1 alleles. In contrast, all three candidate genes explained significant variation in one or more measures of juvenile buzzard dispersal (resighting probability, timing of dispersal and distance dispersed). Our findings contribute towards a broader body of work on the adaptive significance of CLOCK polymorphism, while also building upon previous studies that have documented links between ADCYAP1 variability and the timing of migration.

Keywords: breeding phenology, *Buteo buteo*, dispersal, fitness, fledging, laying date, plumage polymorphism

Received 27 May 2013; revision received 19 July 2013; accepted 8 August 2013

Introduction

Understanding the genetic basis of behavioural variation in natural populations is one of the greatest challenges of contemporary biology. Among the most tractable behavioural traits are the timing of breeding and dispersal, which can be subject to strong selection in organisms inhabiting highly seasonal environments such as temperate birds (Visser *et al.* 2010; Liedvogel *et al.* 2011). The timing of long-distance migration is often consistent between years but other life history events like egg laying can vary greatly from year to year as a result of environmental conditions (Gwinner 1996, 2003; Helm *et al.* 2005). A mismatch between timing of reproduction, based on stable cues like photoperiod change on the one hand and shifting biotic and abiotic conditions on the other hand, can therefore have serious fitness consequences (Post & Forchhammer 2008). Moreover, after the breeding season, juveniles need sufficient time to accumulate resources and to achieve independence prior to the onset of winter (Visser *et al.* 2010).

Both circadian and circannual behavioural cycles are tuned to external timing cues like photoperiod and temperature (Gwinner & Scheuerlein 1998; Lehmann

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et al. 2012). Therefore, both rhythms are likely to rely at least partly on shared signalling cascades. This suggests that consistent among-individual differences may arise due to polymorphic genes regulating perception and reactions to photoperiod changes (Visser *et al.* 2010). Recently, a few candidate gene markers have been identified by mining circadian molecular pathways in the chicken genome for tandem repeats (Steinmeyer *et al.* 2009). This provides an excellent opportunity to explore the roles that these genes play in natural populations.

A number of genes involved in signalling cascades related to timing have been shown to carry length polymorphisms (Fidler & Gwinner 2003; Steinmeyer *et al.* 2009), allowing genetic variation to be conveniently assayed using PCR and capillary electrophoresis. For example, the genes CLOCK and its paralogue NPAS2 contain length polymorphisms, specifically at exonic poly-glutamine (poly-Q) repeat motifs. Latitudinal clines have been found in CLOCK poly-Q allele frequencies among populations of Chinook salmon *Oncorhynchus tshawytscha* and blue tits *Cyanistes caeruleus* (Johnsen *et al.* 2007; O'Malley & Banks 2008), providing a mechanism by which the timing of reproduction could be matched to the timing and duration of spring. Some evidence also suggests that individual CLOCK poly-Q length may influence the timing of reproduction in blue tits and barn swallows *Hirundo rustica* (Caprioli *et al.* 2012; Liedvogel *et al.* 2009; but see Liedvogel *et al.* 2012). However, although CLOCK poly-Q has been shown to be polymorphic in 65 of 69 populations across 12 passerine species studied to date (Johnsen *et al.* 2007; Liedvogel & Sheldon 2010; Dor *et al.* 2011, 2012; Mueller *et al.* 2011; Caprioli *et al.* 2012; Liedvogel *et al.* 2012; Peterson *et al.* 2013), its variation often fails to explain differences in timing among individuals and populations. Moreover, despite the potential of NPAS2 to overtake function from CLOCK and maintain alternative adaptive poly-Q variation (Debruyne 2008; Steinmeyer *et al.* 2009), only two studies on passerine bird species have so far assayed both CLOCK and NPAS2 (Mueller *et al.* 2011; Steinmeyer *et al.* 2012). Additionally, the tendency of avian studies to focus on small passerines introduces a strong phylogenetic bias that precludes a broader understanding of the role these and other genes might play in avian breeding phenology.

The genes ADCYAP1 (adenylate cyclase-activating polypeptide 1) and CREB1 (cAMP response element-binding protein 1) both contain polymorphic dinucleotide repeats in their 3′ untranslated regions (UTRs) (Steinmeyer *et al.* 2009). ADCYAP1 codes for a neuropeptide with a broad spectrum of functions (PACAP; Vaudry *et al.* 2009), and microsatellite length variation at this gene has been associated with the migratory status of blackcaps *Sylvia atricapilla* (Mueller *et al.* 2011) and with age- and sex-dependent survival in blue tits (Olano-Marin *et al.* 2011). CREB1 is a member of a gene family essential for both the maintenance of endogenous timing and light entrainment of the circadian clock (Lee *et al.* 2010; Asher & Schibler 2011) but, to our knowledge, no links between this gene and interindividual variability have yet been described. In addition to the timing of breeding, the tendency and timing of juveniles to disperse are also important aspects of animal life, although little is known about their driving factors. Such complex behaviours are most probably polygenic and under strong environmental influence (Liedvogel *et al.* 2012). Nevertheless, polymorphisms of candidate genes such as ADCYAP1 and CREB1 could play a role in dispersal decisions, along with epigenetic effects and the limited experience that juvenile individuals gather from interactions with parents and siblings (Sutherland 1988).

Birds of prey (Falconiformes) are an interesting case because this group comprises both migratory and non-migratory top predators that have to flexibly adjust their timing of breeding and dispersal to density fluctuations as well as the phenology of their prey species (e.g. Van Buskirk 2012; Lehikoinen *et al.* 2013). Additionally, many raptor species exhibit plumage polymorphisms (Fowlie & Krüger 2003; Krüger 2008). Melanin morphs are inherited as if encoded by a single autosomal locus (Krüger *et al.* 2001) and have in several instances been pinpointed to polymorphisms within the melanocortin-1 receptor MC1R (Mundy 2005; Gangoso *et al.* 2011; Johnson *et al.* 2012; Amar *et al.* 2013). Melanin polymorphisms can strongly influence many aspects of behaviour and may even elevate speciation rates (Ducrest *et al.* 2008; Krüger 2008). Species with polymorphisms in plumage melanization are interesting models to study variation in circadian genes as there is the potential for crosstalk between the two signalling cascades (Vaudry *et al.* 2009).

In common buzzards *Buteo buteo*, many aspects of behaviour, physiology and life history are morph dependent, such as habitat choice (Krüger 2002), mate-choice and aggression (Krüger *et al.* 2001; Boerner & Krüger 2009), blood parasite prevalence and infection intensity and ectoparasite infestation (Chakarov *et al.* 2008). Intermediately, melanized morphs are most frequent in all described common buzzard populations (e.g. central Germany dark ~10%, intermediate ~ 55%, light ~ 35%) (Krüger *et al.* 2001). In the common buzzard, plumage morph is fixed during an individual's lifetime and segregates in a simple Mendelian fashion (Krüger *et al.* 2001; Briggs 2010). However, plumage morph is not fully explained by MC1R polymorphism (M. A. Pointer, M. Boerner, N. Chakarov, O. Krüger & N. I. Mundy, unpublished). Overall, plumage morphs of common buzzards strongly differ in lifetime reproductive success (LRS), a key component of fitness (Krüger *et al.* 2001), with intermediately melanized morphs having the highest LRS.

Differences in fitness among the morphs could potentially be explained, at least in part, by differences in the timing of reproduction and/or dispersal, which in turn could be influenced by candidate genes for phenological traits. Consequently, we used the common buzzard system to test the following hypotheses: (i) plumage morphs differ in their timing of breeding; (ii) Candidate gene variation explains variation in the timing of breeding, after controlling for any variation attributable to morph; (iii) Candidate gene variation is associated with three dispersal-related traits: recapture probability, timing of dispersal and dispersal distance. To explore patterns of interspecific variability in a nonpasserine group, we also assayed these candidate genes in a variety of raptor species.

Methods

Common buzzard study population and field procedures

Common buzzards breed across Eurasia and comprise three melanin morphs termed as: 'dark', 'intermediate' and 'light' based on the proportion of dark feathers. Dark birds have a dark head, heavily speckled or dark breast and underwing coverts. Intermediate individuals have a dark head, intermediately speckled breast and underwing coverts. Birds with little to no breast and underwing coverts melanization, in extreme cases with light head and upperwing coverts belong to the light buzzard morph. Since 1989, we have intensively studied a population of common buzzards in an area of 300 km^2 in Eastern Westphalia, Germany (8°25′E, 52°06′ N). Each year, all woodlands in the area were searched for new buzzard nests and old nests were revisited. All occupied nests were regularly visited to determine the identity and morph of the breeding individuals and the approximate age of the nestlings. Conspicuous variation of plumage colour and individual maculation patterns combined with drawings, photographs and more recently wing-tagging of nestlings allowed individual recognition. Brood timing data were gathered between 1990 and 2012. This data set was used to analyse the effects of parental identity and melanin morph on breeding phenology.

Genetic sampling of buzzard chicks began in 2002, with all nestlings being sampled at around 3 weeks of age. Nests were climbed and nestlings brought to the ground and ringed. From 2007 onwards, nestlings were fitted with wing-tags, greatly improving the resighting probability. More than 90% of all successful buzzard nests in the area were sampled each year. Nestling tarsus was measured with a calliper to the nearest 0.1 mm, wing length with a ruler to the nearest 1 mm and weight with a Pesola spring balance to the nearest 5 g. We subsequently estimated age based on wing length, body weight and feather growth (Bijlsma 1997). Incubation time and growth of hatchlings are highly consistent (incubation varying 33–35 days, SD = 0.6 days and measurements of wing length at fixed days having SD < 7%) (Mebs 1964; Bijlsma 1997). Fledging is assumed to occur when nestlings are of mean age of 46 days (Mebs 1964). We further use the term 'timing of broods/breeding' to denote within-year time of fledging (mean of all nestmates) compared with other broods in the same year. A blood sample was taken from the brachial vein of each nestling and stored in ethanol or PBS–EDTA buffer. All field procedures were performed with permission from the local authority Kreis Gütersloh, permit nr: 4.5.2-723-Bussard.

Whenever fledged buzzards were resighted, the distance to the natal site and date of resighting were recorded. Buzzards that had dispersed further than 20 km by November 1 in their year of birth were classified as early dispersers, whereas animals that were recorded within 10 km of their natal territory between November 1 and the next breeding season were recorded as late dispersers (Walls *et al.* 1999).

Additional raptor samples

Samples from several other raptor species were collected as part of the standard treatment in the rescue and breeding facilities of Adlerwarte Berlebeck, Germany (20 red kites *Milvus milvus*, two black kites *Milvus migrans*, 15 northern goshawks *Accipiter gentilis*, one honey buzzard *Pernis apivous*, one long-legged buzzard *Buteo rufinus*, two upland buzzards *B. hemilasius*, one ferruginous hawk *B. regalis* and seven common kestrels *Falco tinnunculus*). Similarly, the Wildlife Rehabilitation and Breeding Centre-Green Balkans in Stara Zagora, Bulgaria, provided samples from 23 common buzzards, one long-legged buzzard, three honey buzzards, one peregrine falcon *F. peregrinus* and one saker falcon *F. cherrug*. All birds were of local origin, with the exception of the three *Buteo* species sampled at the Adlerwarte Berlebeck.

Molecular analyses

Genomic DNA was extracted from blood samples using a standard phenol–chloroform protocol. The sex of all sampled individuals was determined using a molecular protocol for raptors (Fridolfsson & Ellegren 1999). We used primers published by Steinmeyer *et al.* (2009) to amplify microsatellite-containing parts of ADCYAP1, CREB1, CLOCK and NPAS2 in a single multiplex, labelled with the dyes PET, FAM, FAM and VIC, respectively. A 10 μL polymerase chain reaction (PCR) volume with 20–200 ng DNA was amplified for 35 cycles using a Type-it microsatellite PCR kit (QIAGEN), following the manufacturer's standard protocols and using an annealing temperature of 56 °C. Diluted amplification products (1 μL; 1:20 dilution) were then resolved on an ABI 3730 Automated DNA Analyser (Applied Biosystems), and fragment lengths were scored for all individuals using GENEMARKER 1.95 (SoftGenetics LCC). To verify that our PCR products correspond to alleles of the genes in question, we PCR-amplified and sequenced up to four individuals that were homozygous for the various alleles identified at each of the genes. Sequencing reactions were performed with 2 mM Mg^{2+}, 40 μM dNTPs, 40 μM unlabelled forward and reverse primers and 20–200 ng DNA using a standard PCR cycle profile and an annealing temperature of 60 °C. The resulting products were purified using exonuclease and shrimp alkaline phosphatase and then cycle-sequenced in both directions using amplification primers and ABI BigDye Terminator. Sequencing products were cleaned using Sephadex columns and processed with an ABI 3730 Analyzer.

Statistical analyses

Tests for deviations from Hardy–Weinberg equilibrium (HWE) were performed separately for the four candidate loci based on the genotypes of 976 buzzards within GENEPOP 4.0 (Raymond & Rousset 1995; Rousset 2008). Distributions of alleles for all genes were compared between broods belonging to parents of different morphs using analysis of molecular variance (AMOVA) with 10 000 permutations within ARLEQUIN (Excoffier & Lischer 2010).

Because we were primarily interested in between-individual and not between-year differences in phenology, year-specific effects common to all breeding pairs were excluded by standardizing the timing of all broods in 1 year to a mean of zero and standard deviation of one. Morph effects on timing of breeding were analysed using a linear mixed effects model (GLMM) with maternal morph as fixed effect and paternal and maternal identity as random effects. Individual repeatability was estimated by dividing the variance explained by the respective random factor by the total variance, comprising the variance explained by random factors and the residual variance (including fixed effects). Because samples from breeding adults were not obtainable, parental genotypes could not be established. We therefore related nestling genotypes to timing of the brood, morphs of nestlings and parents. The effect of the mean allele length on timing of breeding was analysed using linear mixed effect models with the respective mean allele length (ADCYAP1, CREB1 and NPAS2 separately) of individual offspring and maternal morph as fixed effects and maternal identity as a random effect.

The effect of the mean allele length of polymorphic genes on the propensity of buzzards to be early or late dispersers was analysed using binomial GLMMs with year as random factor. The effect of mean allele length on the probability of being resighted was analysed using binomial GLMMs with year, brood and maternal identity as random factors. In addition, brood rank estimated from differences in tarsus length, a measure for brood hierarchy, was used as a fixed effect. The effect of mean allele length on the dispersal distance (log-transformed) was analysed using a linear mixed effect model with the respective genotype as fixed effect and brood identity as random effect. Sample sizes for different genes differed because CREB1 amplification was generally weaker, and this locus could not be scored in 103 nestlings. GLMMs were implemented using the packages lme4 in R 2.15.1 (R Development Core Team 2012). All figures show raw relationships between variables without controlling for the covariates included in statistical models.

Results

Candidate gene polymorphism

We gathered data on the timing of fledging of 1430 common buzzard broods belonging to 594 individually identified females and 584 males over 23 consecutive years. We analysed genetic material from 976 nestlings belonging to 479 broods from 275 mothers sampled between 2008 and 2012 and from 25 additional buzzards that were ringed before 2008 and subsequently resighted or otherwise recovered. Within this large sample of individuals, no length polymorphisms were detected within CLOCK in common buzzards, with only a single allele observed comprising eight glutamine-coding codons (Q_8, Table 1). NPAS2 carried two alleles (Q_8 and Q_9), but had very low heterozygosity while ADCYAP1 and CREB1 had three alleles each (Table 1). CREB1 was polymorphic not for the CA-repeat motif found in blue tits (Steinmeyer *et al.* 2009) but for a TA-repeat residing 104 bp downstream (Supplementary Material on Dryad, doi:10.5061/dryad.r058p). None of the three polymorphic candidate genes deviated significantly from HWE (NPAS2 $P = 1.000$, ADCYAP1 $P = 0.090$, CREB1 $P = 0.232$).

To determine whether the variability described above is representative of the species as a whole and to explore the extent to which this pattern can be generalized to other raptor species, we assayed microsatellite length variation at the four candidate genes in common buzzards from Bulgaria and in 11 different bird of prey species, including four representatives of the genus *Buteo*. Although sample sizes are variable and in some cases small, all four buzzard species were monomorphic for CLOCK and only northern goshawks and red kites showed any evidence of variability (Table 1). ADCYAP1 was polymorphic in six of the raptor species while CREB1 and NPAS2 were only polymorphic in two and three species, respectively.

Individual repeatability of brood timing

Timing of breeding varied substantially in our study population during the period 1990–2012, with the earliest brood fledging on June 1 and the latest on July 28, and

Table 1 Microsatellite length variation within four candidate genes in 11 raptor species. Allele sizes are given together with their frequencies and number of repeats in parentheses. Q stands for poly-glutamine, CAG repeat number. For the common buzzard, the German and Bulgarian populations were not significantly differentiated at NPAS2, ADCYAP1 and CREB1 (exact G tests, $P = 0.294$, 0.056 and 0.778, respectively). H indicates heterozygosity of respective genes in buzzard populations

	Gene/fragment size (allele frequency, number of repeats)			
	ADCYAP1	CREB1	CLOCK	NPAS2
Common buzzard *Buteo buteo* *Germany; n = 976*	146 (0.02, (TC)7) 152 (0.81, (TC)10) 156 (0.17, (TC)12) H 0.312	531 (0.01, (TA)5) 533 (0.95, (TA)6) 535 (0.04, (TA)7) H 0.093	185 (1.00, Q_8)	167 (0.99, Q_8) 170 (0.01, Q_9) H 0.014
Common buzzard *B. buteo* Bulgaria*; n* = 23	146 (0.04) 152 (0.68) 156 (0.28) H 0.475	533 (0.93, (TA)6) 535 (0.07, (TA)7) H 0.125	185 (1.00, Q_8)	167 (0.98, Q_8) 170 (0.02, Q_9) H 0.044
Long-legged buzzard *B. rufinus* *n* = 2	152 (1.00)	533 (1.00)	185 (1.00, Q_8)	167 (1.00, Q_8)
Upland buzzard *B. hemilasius* *n* = 2	152 (1.00)	533 (1.00)	185 (1.00, Q_8)	167 (1.00, Q_8)
Ferrunginous hawk *B. regalis* *n* = 1	152 (1.00)	533 (1.00)	185 (1.00, Q_8)	167 (1.00, Q_8)
Honey buzzard *Pernis apivorus* *n* = 4	142 (0.12) 148 (0.12) 150 (0.26) 152 (0.12) 154 (0.26) 156 (0.12)	535 (1.00)	188 (1.00, Q_9)	179 (1.00, Q_{12})
Northern goshawk *Accipiter gentilis* *n* = 15	—	534 (1.00)	188 (0.10, Q_9) 194 (0.90, Q_{11})	158 (0.03, Q_5) 173 (0.97, Q_{10})
Red kite *Milvus milvus* *n* = 20	135 (0.08, (TC)6) 139 (0.92, (TC)8)	532 (0.15, (TA)5) 534 (0.85, (TA)6)	179 (0.18, Q_6) 185 (0.82, Q_8)	164 (0.97, Q_7) 167 (0.03, Q_8)
Black kite *M. migrans* *n* = 2	139 (1.00, (TC)8)	534 (1.00, (TA)6)	185 (1.00, Q_8)	164 (1.00, Q_7)
Common kestrel *Falco tinnunculus* *n* = 7	161 (0.08) 163 (0.75) 165 (0.17)	542 (1.00)	182 (1.00, Q_7)	169 (1.00, Q_9)
Peregrine falcon *F. peregrinus* *n* = 1	163 (0.50) 169 (0.50)	545 (1.00)	182 (1.00, Q_7)	169 (1.00, Q_9)
Saker falcon *F. cherrug* *n* = 1	163 (0.50) 169 (0.50)	545 (1.00)	182 (1.00, Q_7)	169 (1.00, Q_9)

yearly standard deviations in timing of breeding varying from 3.3 to 7.9 days. Individual female buzzards showed consistency in the timing of their broods between years, with female identity ($n = 597$) explaining 34.7% of the variation in standardized timing of 1430 broods ($\chi^2_{1,1430} = 148.49$, $P < 0.001$). When both male and female identities were included as random factors, female identity explained 23.5% and male identity a further 15.2% of the total variation in timing of breeding ($\chi^2_{1,1430}$=20.047, $P < 0.001$).

Effects of melanin morph and candidate genes on timing of breeding

Melanin morph of female buzzards had a significant effect on the standardized timing of broods in models accounting for identities of both breeding partners ($\chi^2_{2,1430} = 7.335$, $P = 0.026$, Fig. 1). Broods of intermediate females were timed earlier than broods of both dark and light females. Morphs differed in standardized timing of breeding by 0.2–0.3 standard deviations, which is equivalent to 0.7–2.4 days depending on the year.

No allele frequency differences were found between nestlings of different morphs (AMOVA of nestlings belonging to one morph joined in dummy broods of four nestlings: ADCYAP1, $F_{CT} < 0.001$, $P = 0.376$; NPAS2, $F_{CT} < 0.001$, $P = 0.997$; CREB1, $F_{CT} < 0.001$, $P = 0.672$). However, a significant difference in ADCYAP1 allele distributions was found between nestlings with different maternal melanin morphs (AMOVA: $F_{CT} = 0.028$, $P < 0.001$, permuting actual broods between maternal morphs). Intermediate female buzzards had nestlings with significantly greater mean ADCYAP1 allele length than either dark or light females (Fig. 2), but no such pattern was evident for paternal morph (AMOVA: $F_{CT} = 0.002$, $P = 0.714$). No differences were found among the morphs at the remaining candidate genes. Mean allele length of NPAS2, ADCYAP1 and CREB1 in nestlings did not correlate with timing of the brood they belonged to (Fig. 3: NPAS2 $\chi^2_{1,972} = 0.400$, $P = 0.527$; ADCYAP1 $\chi^2_{1,972} = 0.002$, $P = 0.966$; CREB1 $\chi^2_{1,873} = 1.555$, $P = 0.212$).

Effects of melanin morph and candidate genes on timing of dispersal

All three polymorphic candidate genes were significantly associated with some aspect of early dispersal (Table 2). ADCYAP1 and CREB1 had no effect on resighting probability or distance at resighting of juvenile buzzards. However, buzzards that were resighted after ringing and buzzards resighted close to their natal sites carried longer NPAS2 alleles (mean distance ± SE per genotype: $\text{NPAS2}_{167/167} = 57.8 \pm 6.2$ km, $n = 153$; $\text{NPAS2}_{167/170} = 13.8 \pm 9.7$, $n = 8$). Resighted buzzards were also more likely to be dark or intermediate than light (Table 2).

The tendency of juvenile buzzards to disperse early in the season as opposed to remaining around the natal territory was associated with the mean length of ADC-

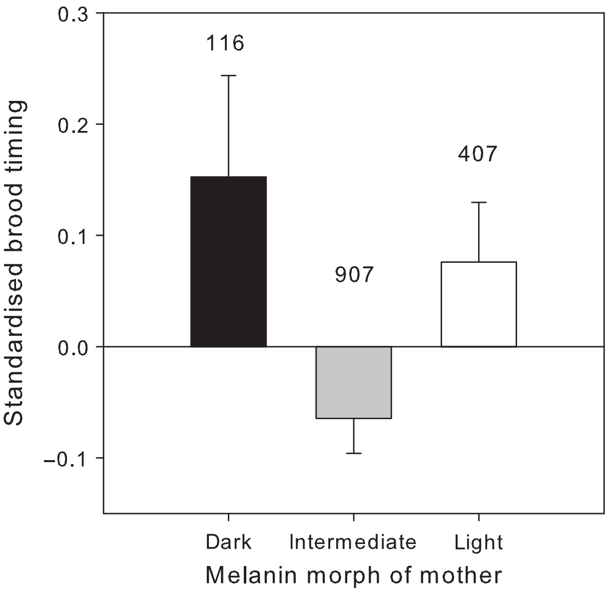

Fig. 1 Mean (± SE) standardized timing of broods of female buzzards of different melanin morphs from the period 1990–2012.

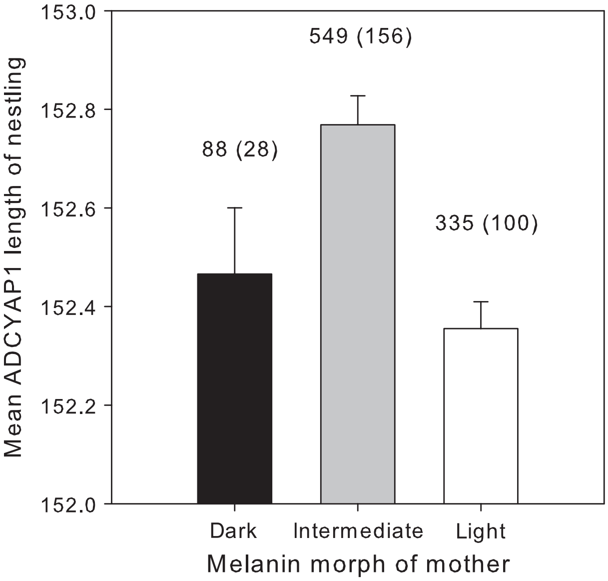

Fig. 2 Mean (± SE) allele length of ADCYAP1 of nestlings belonging to mothers of different melanin morphs. Nestling sample sizes are given above the bars with corresponding numbers of mothers shown in parentheses.

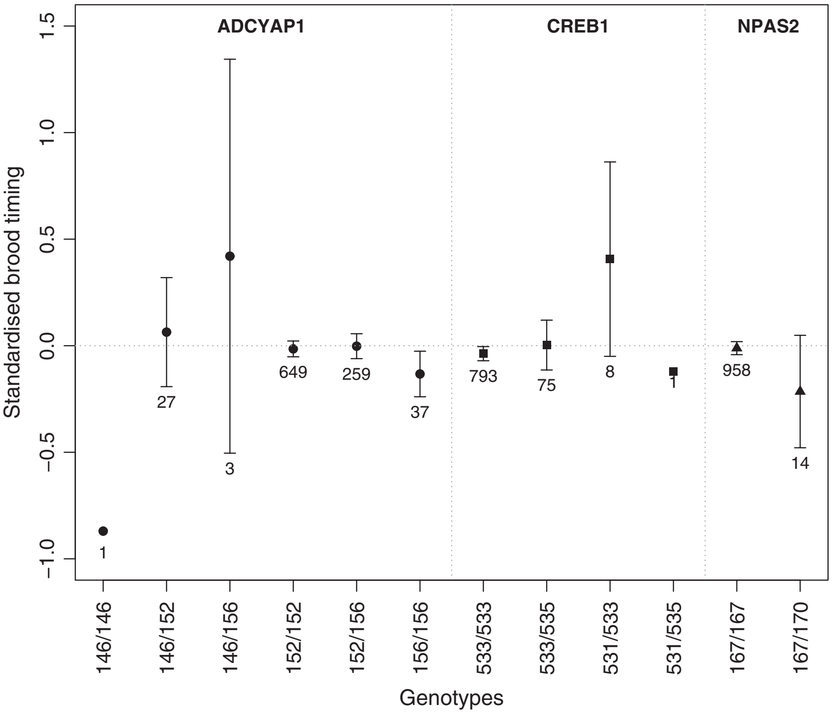

Fig. 3 Mean (± SE) standardized fledging date of nestlings per genotype for ADCYAP1, CREB1 and NPAS2. On the *x*-axis, the allele composition of each genotype is shown. Numbers in the graph indicate the sample size per genotype.

Table 2 Model results explaining resighting probability, distance at first resighting and timing of dispersal (resighting more than 20 km away from the natal territory before November 1 or <10 km after November 1) with microsatellite length at three candidate genes and plumage morph in juvenile common buzzards (see Materials and Methods for details). Models controlled for year of fledging and in the case of resighting probability also for brood identity. Maximal available sample sizes for each explanatory variable were used causing deviations in degrees of freedom

Variable explained	d.f.	χ^2	*P*	d.f.	χ^2	*P*
	ADCYAP			CREB1		
Resighting probability	1,974	1.259	0.262	1,875	0.893	0.345
Distance of first resighting	1,160	0.07	0.792	1,146	0.106	0.745
Early vs. late dispersal	1,39	4.162	0.041	1,35	4.872	0.027
	NPAS2			Morph		
Resighting probability	1,955	13.441	<0.001	2,956	11.745	0.003
Distance of first resighting	1,160	4.25	0.039	2,160	0.607	0.738
Early vs. late dispersal	1,39	0.228	0.633	2,42	6.353	0.042

YAP1 and CREB1 and the melanin morph of the individual (Table 2). Early dispersers had longer ADCYAP1 alleles and shorter CREB1 alleles (Fig. 4). Only around two-thirds of all intermediate and light buzzards were early dispersers, whereas all five resighted dark buzzards were far from their natal sites early after fledging (Fig. 5). To correct for multiple testing, we also calculated Fisher's combined probability (across the three response variables) for each of the different predictor variables. The resulting values (ADCYAP1: 0.048, CREB1: 0.043, NPAS2: 0.0002, morph: 0.0075) indicate that all four variables affect some aspect of dispersal.

Discussion

CLOCK

CLOCK, the usual suspect for genetic associations with timing of breeding or dispersal in birds, was found to be monomorphic in a large sample of common buzzards. There was also no evidence of compensatory diversification in its paralogue NPAS2, despite the potential of this gene to function as a replacement or addition to CLOCK for adaptive variation in regulation of timing (Debruyne 2008). Moreover, our limited interspecific sampling suggests that CLOCK polymorphism may also be absent in other *Buteo* species, as well as potentially from several other raptors. With such limited levels of intra- and interspecific variability, it thus appears that CLOCK poly-Q polymorphisms are unlikely to be of widespread importance for the timing of reproduction or dispersal in birds of prey. While CLOCK is crucial for the maintenance of circadian

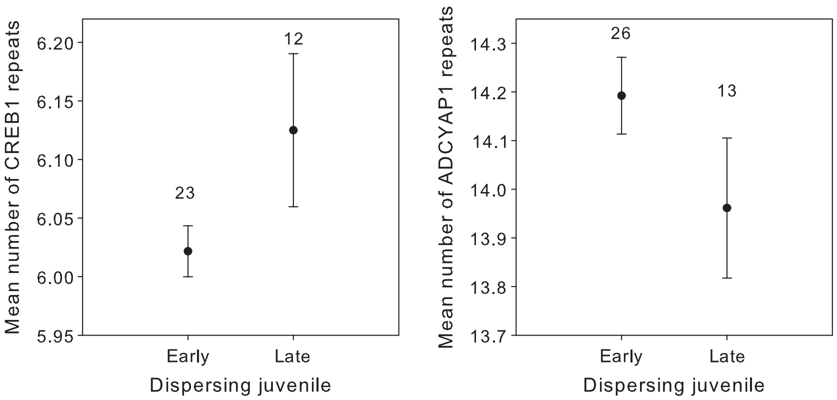

Fig. 4 Mean (± SE) number of dinucleotide repeats in the variable 3′ untranslated region of CREB1 and ADCYAP1 in buzzards dispersing early (more than 20 km before November 1) or late (detected within 10 km of natal nest after November 1).

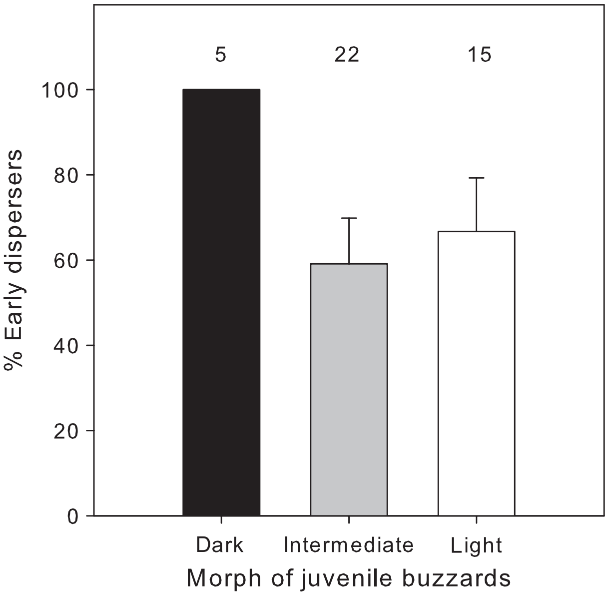

Fig. 5 Frequency of early (more than 20 km before November 1) vs. late dispersers (detected within 10 km of natal nest after November 1) belonging to different melanin morphs among resighted juvenile buzzards.

rhythms, the adaptive value of its poly-Q polymorphism may therefore be a special case (Liedvogel *et al.* 2012). Alternatively, prey-specialized raptors have to adapt their breeding to the prey population fluctuations. Therefore, such groups may be least dependent on potentially adaptive variation in the molecular mechanics of the circannual rhythm. However, an important caveat is that we only genotyped the known CLOCK poly-Q polymorphism, whereas it is possible that other polymorphisms reside within other parts of the gene that we did not study.

Timing of breeding

Within our intensively studied common buzzard population, individual effects on brood timing were strong, with up to 34% of the variation in timing being explained by the identity of the mother. In birds, breeding early within seasons is often related to better body condition, including lower stress levels of females (e.g. Tobler *et al.* 2007; Gladbach *et al.* 2010; Goutte *et al.* 2010, 2011). Differences among the three maternal morphs support an association between morph and control of timing. Because the start of egg laying is determined by mothers, maternal genetic make-up could influence an individual's decision to start laying. Improved body condition of intermediately melanized female buzzards due to heterozygote advantage might also allow them to lay earlier than the other morphs. Unfortunately, these effects are currently difficult to disentangle in our system owing to a paucity of data on female body condition.

Surprisingly, variation in mean nestling ADCYAP1 allele length was also significantly associated with the melanin morph of their mothers. Although nothing is known about differences in translation of ADCYAP1 alleles, longer alleles correlate with potentially beneficial earlier migration in blackcaps (Mueller *et al.* 2011). In buzzards, longer ADCYAP1 alleles could potentially lead to better control of behaviour and allow females to lay early (Smith 2004; Hashimoto *et al.* 2011). We could not detect an effect of mean nestling allele length on brood timing. A correlation between the timing phenotype of a laying female and the genotype of her offspring might be obfuscated by paternal allele input. Similarly, the effect of the male on the timing of breeding as a result of provisioning rate or stimulation of the female may obfuscate the allele input from the matriline.

Dispersal

We found average ADCYAP1 allele length to be greater in early dispersing juvenile buzzards. This is consistent with recent findings that migratory blackcap populations carry longer alleles at this locus than nonmigratory populations and that individuals of this species carrying longer ADCYAP1 alleles show a tendency towards greater migratory restlessness (Mueller *et al.* 2011). Indeed, juvenile dispersal and migration behaviours are probably derived from a similar condition of restlessness that may generally be more strongly expressed in the presence of longer ADCYAP1 alleles (Ritchison *et al.* 1992).

Despite their low heterozygosity, CREB1 and NPAS2 were also found to be significantly associated with dispersal behaviour. Juvenile buzzards dispersing late after fledging or not at all had more CREB1 repeats than buzzards dispersing early. Individuals heterozygous for NPAS2 were more likely to be resighted and then to be close to their natal territory. Due to a higher density of informed observers, reported resightings are more probable within our study area than outside it. Therefore, individuals that do not disperse have a higher probability of being seen, and both resighting probability and distance to the natal territory might reveal similar information about NPAS2 genotypes.

Future directions

Allele frequencies of ADCYAP1 in nestlings covaried significantly with the plumage morph of their mothers. Promising candidate genes for morph determination are involved in the pro-opiomelanocortin (POMC) signalling pleiotropy (Ducrest *et al.* 2008). PACAP, the product of ADCYAP1, stimulates the expression of POMC (Vaudry *et al.* 2009). Additionally, in white-crowned sparrows *Zonotrichia albicollis*, plumage morph is reflected by a single-nucleotide polymorphism in vasoactive intestinal polypeptide (VIP) (Romanov *et al.* 2009). VIP is a close relative of ADCYAP1 and both share two of three receptors (Vaudry *et al.* 2009). Finally, ADCYAP1 resides on a chromosome 2, while POMC and VIP on chromosome 3 in chicken. In white-crowned sparrows, both chromosomes have inversion variants that co-occur in an interchromosomal or quasi-linkage and cause the plumage polymorphism (Thomas *et al.* 2008; Romanov *et al.* 2009; Ellegren 2010). It is unclear whether the same mechanism could play a role in the profound fitness differences among the buzzard morphs, given the variation we have described in ADCYAP1 allele lengths. Bringing advanced genomic methods to bear on this problem may therefore provide a fertile avenue for future research.

In conclusion, our study found that CLOCK, the best-studied gene in respect of time-dependent behavioural traits, appeared to be monomorphic in a large clade of birds of prey, showing that CLOCK poly-Q polymorphism is not necessary for adaptive variation in phenology. Nevertheless, we found significant associations between length polymorphism at other candidate genes and several different aspects of dispersal but not timing of breeding. Similar studies of other nonpasserine bird taxa are warranted to explore the extent to which these and other candidate genes impact behavioural traits more widely.

Acknowledgements

We are grateful to Miriam Liedvogel for initially simulating us to perform this study and to Staffan Bensch and three anonymous reviewers for comments on earlier versions of this manuscript. We thank Thomas Grünkorn for indispensable help in the sampling of German common buzzards. Jörg Westphal and Benjamin Aschmann from the Adlerwarte Berlebeck and the staff of Wildlife rehabilitation and breeding centre-Green Balkans, Stara Zagora, generously provided us with samples from other birds of prey. We are grateful to Katrin Lehmann, Elke Hippauf and Prisca Viehoever for help in the molecular analyses. NC is supported by the Volkswagen Foundation within its Evolutionary Biology initiative, Grant I/84 196. RMJ is supported by a grant from the German Science Foundation (DFG, KR 2089/5-1). MB holds a Marie-Curie Fellowship of the European Union (275049). JIH is funded by a Marie Curie Career Integration Grant (PCIG-GA-2011-303618). OK holds a Heisenberg Professorship of the German Science Foundation (DFG, KR 2089/2-1).

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This study was part of the PhD thesis of N.C. on fitness-related correlates of plumage polymorphisms and part of the long-term study on common buzzards coordinated by O.K. Conceived: N.C. & O.K. Data collected: N.C., M.B. & O.K. Data analysed: N.C., R.M.J. & J.I.H. Wrote the paper: N.C, J.I.H, R.M.J, M.B. & O.K.

Data accessibility

All used data, including sequenced alleles and analysis scripts are uploaded in Dryad repository, doi:10.5061/dryad.r058p. Sequenced CLOCK, ADCYAP1 and NPAS2 fragments were shorter than 200 bp precluding their entry in GeneBank.