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No correlation between multi-locus heterozygosity and fitness in the common buzzard despite heterozygote advantage for plumage colour

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Abstract

Correlations between heterozygosity and fitness are frequently found but rarely well understood. Fitness can be affected by single loci of large effect which correlate with neutral markers via linkage disequilibrium, or as a result of variation in genome-wide heterozygosity following inbreeding. We explored these alternatives in the common buzzard, a raptor species in which three colour morphs differ in their lifetime reproductive success. Using 18 polymorphic microsatellite loci, we evaluated potential genetic differences among the morphs which may lead to subpopulation structuring and tested for correlations between three fitness-related traits and heterozygosity, both genome wide and at each locus separately. Despite their assortative mating pattern, the buzzard morphs were found to be genetically undifferentiated. Multilocus heterozygosity was only found to be correlated with a single fitness-related trait, infection with the blood parasite, *Leucocytozoon buteonis*, and this was via interactions with vole abundance and age. One locus also showed a significant relationship with blood parasite infection and ectoparasite infestation. The vicinity of this locus contains two genes, one of which is potentially implicated in the immune system of birds. We conclude that genome-wide heterozygosity is unlikely to be a major determinant of parasite burden and body condition in the polymorphic common buzzard.

Introduction

Understanding the genetic basis of fitness has long been an aim of evolutionary studies. One interesting and frequently reported observation is that many aspects of fitness correlate with genetic heterozygosity, a phenomenon often referred to as heterozygosity–fitness correlation (HFC). Across a wide range of species, many different components of fitness have been implicated, including birth weight, juvenile survival, parasite resistance, longevity, reproductive success and even behavioural traits such as territory holding and bird song complexity (Coltman *et al.*, 1999; Coulson *et al.*, 1999; Slate *et al.*, 2000; Amos *et al.*, 2001; Slate & Pemberton, 2002; Hoffman *et al.*, 2004).

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An interesting case of suspected HFCs is presented by the common buzzard, *Buteo buteo*, L. This species has three distinct colour morphs that have been known to mate assortatively, and the intermediate morph has the highest fitness (Krüger *et al.*, 2001; Chakarov *et al.*, 2008; Boerner & Krüger, 2009). Inheritance patterns suggest that these intermediate individuals are heterozygous for a single colour locus (Krüger *et al.*, 2001). Plumage colour in the common buzzard is largely determined by a mutation in Mc1R (M.A. Pointer, N.I. Mundy, O. Krüger, unpublished), a melanocortin receptor responsible for plumage and hair variation in many vertebrates (Mundy, 2005). Inheritance of morph is Mendelian, with dark and light individuals being homozygous for the respective alleles. Individuals with either of these extreme morphs are on average shorter-lived and produce fewer young in their lifetimes than the intermediate morph. This has led us to propose that the observed fitness differences are due to overdominance (heterozygote advantage originating

from a single overdominant locus) or heterosis (heterozygote advantage based on genome-wide effects) (Krüger *et al.*, 2001).

As the Mc1R gene has few known pleiotropic effects (Ducrest *et al.*, 2008), we have now collected genetic data from presumed neutral microsatellite markers to explicitly test whether individuals from the fitter intermediate morph are more heterozygous overall or whether any correlation exists between heterozygosity at marker loci and fitness-related traits. Such associations might plausibly occur as a result of population substructure linked to assortative mating, and the buzzard's mating pattern may create such population substructure. Buzzards generally choose partners of the same morph as their mother (Krüger *et al.*, 2001), presumably because they imprint on the mother who stays at the nest to brood young chicks and to distribute food delivered by the father.

Assortative mating has long been known to reduce heterozygosity at the locus or loci responsible for the trait that assortment is based on. However, such behaviour could lead to population stratification based on morph (Seiger, 1967). Since the extreme morphs are far rarer than intermediates, this could potentially also lead to inbreeding depression and enhanced levels of identity disequilibrium (ID: correlation in heterozygosity across loci resulting from consanguineous matings) and/or linkage disequilibrium (LD: association between alleles at different loci in gametes, for example due to selection or genetic drift; see Szulkin *et al.*, 2010) in dark and light individuals. Both LD and ID could result in correlations between functional and marker loci, making morph act as a surrogate marker, reflecting broader patterns of heterozygosity either within the same chromosome or even genome wide. On the other hand, this should be mitigated by the fact that many extreme morph individuals are born to intermediate mothers, a concept that seems supported by observations in two other bird species showing plumage polymorphism and assortative mating. Both these species exhibit high F_{ST} between morphs at Mc1R but little or no differentiation at neutral markers elsewhere in the genome (Mundy *et al.*, 2004). Here, we test whether assortative mating is strong enough to create sufficient population substructure to result in detectable inbreeding effects among the morphs of the common buzzard.

Beyond the population genetic effects able to cause HFCs, it has long been debated how many loci would be involved in generating them. Earlier studies of HFCs have stressed the distinction between two mechanisms potentially underlying the observed correlation of heterozygosity and fitness. One possibility is heterozygote advantage at particular genes ('direct effects hypothesis', or 'local effect hypothesis' if referring to neutral markers in the vicinity of the relevant gene), potentially allowing an individual to respond to a wider variety of environmental conditions (Brown, 1997; David, 1998; Hansson & Westerberg, 2002; Sellis *et al.*, 2011). The alternative is inbreeding, that is, higher genome-wide homozygosity increasing the expression of deleterious recessive alleles, causing inbreeding depression ('global effects hypothesis'; Ohta & Kimura, 1970). However, these two hypotheses are not mutually exclusive, and HFCs can lie on a continuum from those purely due to a single locus under balancing selection through to classical inbreeding depression (Balloux *et al.*, 2004).

Attempts to distinguish between genome wide and direct or local effects tend to be hampered by the relatively small number of genetic markers used in most studies, typically of the order of ten (e.g. Hansson & Westerberg, 2002; Hoffman *et al.*, 2010a,b). On the one hand, such small numbers of markers mean that only appreciably inbred individuals will contribute a detectable signal without very large sample sizes. Inbreeding at this level will be extremely rare in most real populations unless the effective population size is tiny, due either to a genuinely small census size or factors such as strong population substructure or polygyny (Balloux *et al.*, 2004; Pemberton, 2004; Slate *et al.*, 2004; Hansson & Westerberg, 2008; Grueber *et al.*, 2011). On the other hand, these are exactly the conditions that can make a species amenable to study, and many prominent HFC studies focus on small, isolated populations, often on islands where inbreeding becomes much more likely. However, in some circumstances, even small numbers of microsatellites may be informative, as recently illustrated by a study which found internal relatedness (IR) estimated from 11 microsatellites to be as powerful in detecting HFC as IR estimated from several hundred SNPs (Forstmeier *et al.*, 2012), and a number of studies have employed small numbers of microsatellite markers successfully (Merilä *et al.*, 2003; Hansson *et al.*, 2004; Da Silva *et al.*, 2009; Küpper *et al.*, 2010; Agudo *et al.*, 2012). Consequently, the debate continues (Shikano & Taniguchi, 2002; Hansson *et al.*, 2004; Chapman *et al.*, 2009; Mainguy *et al.*, 2009; Hoffman *et al.*, 2010a,b; Szulkin *et al.*, 2010), even though many would now agree that HFCs in larger populations where strong polygyny is absent are more likely to reflect local effects than inbreeding (Balloux *et al.*, 2004; Lieutenant-Gosselin & Bernatchez, 2006; Hansson & Westerberg, 2008; Da Silva *et al.*, 2009; Harrison *et al.*, 2011).

To explore the relationship between morph and genome-wide heterozygosity in the common buzzard, we analysed a panel of highly polymorphic microsatellite loci. In addition to population substructure, we sought to test (i) whether the greater fitness of intermediate morphs contributes to a more general HFC and (ii) whether the known buzzard HFC is due to genome wide or local effects.

Materials and methods

Study site and sampling

Buzzards were sampled during a long-term study in Eastern Westphalia, Germany. The population has grown from ca. 45 breeding pairs in 1989 to a current average of around 180 pairs per year and occupies a mosaic of fields, pastures and deciduous and mixed forest patches (see Krüger, 2004 for a detailed description). Breeding attempts were monitored from the ground (1989–2001) and by climbing nest trees (2002–2012), the latter allowing access to the chicks, sampling and measurement. Chicks were weighed to the nearest five grams, and tarsus length was measured to the nearest 0.1 mm. Parasite infestation was scored by counting the number of wing and leg pits showing signs of the blood-sucking fly *Carnus haemapterus* and using fixed, dried blood smears to count the blood parasite, *Leucocytozoon buteonis*, a pathogen causing an infection similar to avian malaria (formerly known as *L. toddi*; Valkiūnas *et al.*, 2010) (see Chakarov *et al.*, 2008 for a detailed description) (Table 1). When testing for HFCs, ectoparasite score, *L. buteonis* infestation (binary variable: infected or not infected) and body condition (residual body weight over tarsus length, calculated separately for each sex) were used as measures of fitness. Both parasitism load (Korpimäki *et al.*, 1995; Hakkarainen *et al.*, 1998; Whiteman & Parker, 2004; Parejo & Silva, 2009) and body condition (Kenward, 1978; Simmons, 1988; Whiteman & Parker, 2004; Dyrcz *et al.*, 2005; Sergio *et al.*, 2007) are known for their association with fitness in raptors and other birds, and all measures have been implicated in HFCs (Coltman *et al.*, 1999; Lieutenant-Gosselin & Bernatchez, 2006; Monceau *et al.*, 2013; Ruiz-López *et al.*, 2012; Voegeli *et al.*, 2012).

Blood samples were collected for DNA analysis by puncturing the brachial vein. These were then stored in 95% ethanol at −20 °C. The morph of the chicks was scored either during sampling or, in case of young chicks still wearing their nestling down, during subsequent visits to the nest site around fledging date. Age at sampling was estimated from wing length, body weight and the eventual fledging date of the chicks. Molecular sexing was carried out according to the protocol of Griffith *et al.* (1998). To estimate food availability, vole abundance was estimated by counting entrances to vole burrows which were found re-opened after artificially closing them and categorizing the abundance as low, medium or high, following Heise & Wieland (1991). Overall territory quality was calculated as the proportion of years during which the territory was occupied since its first use in the course of the long-term study. This measure controls for the effect of increasing population density and the resulting splitting of territories over the course of the study and is a reliable predictor of reproductive success (Krüger *et al.*, 2012). Male lifetime reproductive success represents the number of fledglings the father of the chick has sired over his lifetime. We assume no extra-pair paternities, as these are probably extremely rare (Krüger *et al.*, 2001) and as less than 1% of chicks in our study show an 'impossible' morph considering the morphs of their mother and social father. Date of sampling was recorded as the number of days since an arbitrarily chosen start date (June 1st) to control for potential seasonal effects (Table 1).

Molecular analyses

For the analysis of heterozygosity, 177 individuals sampled over 7 years (2002 and 2004–2009) were used. To stratify the sample, individuals were selected in a way to balance the representation of all morphs, to maximize availability of fitness-related data and to minimize the number of close relatives (siblings and half-siblings). In the final data set, 47 chicks were dark, 59 light and 71 intermediate in colour. Individuals belonged to 117 different families, of which 30 were represented by two siblings, five by three siblings, two by four siblings and six of a mixture of full and half-siblings. 70 chicks had no close relatives (siblings, half-siblings, parents or offspring) in the data set. Family ID was initially included as a random factor in statistic modelling but dropped as it did not lead to significant improvements in the models. Genomic DNA was isolated from whole blood samples by Chelex extraction and PCR amplified using ^{33}Phosphorus radio-labelled nucleotides and microsatellite primers previously developed from a random genomic library in the common buzzard (Johnson *et al.*, 2005). To check their distribution and proximity to Mc1R, we mapped each marker against the genomes of the chicken *Gallus gallus* and the zebra finch *Taeniopygia guttata*. Results were very similar but closer matches were achieved in the comparisons with the zebra finch genome. However, even in the latter comparison, there was a wide range of reliability in the matches. Hence, we only present marker positions found in zebra finches (Table 2). As far as the mapping can be relied on, none of the markers were near Mc1R (chromosome 11) in either species. Of 24 polymorphic microsatellite loci tested, 18 amplified reliably (see Table 2 for a list of marker loci). PCR products were separated on standard 6% polyacrylamide sequencing gels and scored manually following autoradiography.

All microsatellite loci were tested for deviation from Hardy–Weinberg and linkage equilibrium using the software *Genepop on the web* (Rousset, 2008). Null allele frequencies were calculated following Brookfield (1996) using the Brookfield 1 estimator as implemented in the program *Microchecker* (Van Oosterhout *et al.*, 2004). Four of 18 loci (Bbu33, 42, 49 and 53) deviated

Table 1 Description of variables used in generalized linear mixed models to test for HFCs.

Variable	Description
Body condition	Residual body weight over tarsus length
Ectoparasite infestation	No. of wing and leg pits with ectoparasites or their faeces visible (0–4)
Blood parasite infection	0 = no; 1 = yes
Morph	1 = Dark; 2 = Intermediate; 3 = Light
Sex	0 = female; 1 = male
Age	Age in days since hatching
Date of sampling	Julian date of sampling
Territory quality	Proportion of years a territory was occupied since year of first time use
Male LRS	Lifetime reproductive success (no. of fledglings sired) of chick's father
Vole score	1 = low; 2 = intermediate; 3 = high
Year	Year of sampling

significantly from Hardy–Weinberg equilibrium in the direction of homozygosity excess, all of which showed evidence for the presence of null alleles at frequencies above 10% (Table 2). These were therefore excluded from the analyses of population structure. Heterozygosity was measured as internal relatedness (IR; Amos *et al.*, 2001), an improved measure of heterozygosity that weights allele sharing by allele frequency (Forstmeier *et al.*, 2012). IR was highly correlated ($r^2 > 0.9$; data not shown) with similar measures such as standardized heterozygosity (SH; Coltman *et al.*, 1999) and with heterozygosity weighed by locus (HL; Aparicio *et al.*, 2006). Consequently, we only present data for IR. To explore the possibility of linkage disequilibrium between morph and any of our markers, each marker was tested using a simple chi-squared test for a deviation from the null hypothesis of equal numbers of heterozygotes in each morph. None of the markers revealed an association. Furthermore, we calculated g_2, an estimator of heterozygosity disequilibrium, using RMES (David *et al.*, 2007). Again, there was no indication of significant levels of inbreeding ($g_2 = -0.008$; $P = 0.7$).

Mate choice and its potential to cause inbreeding

Mate choice in the common buzzard is assortative (Krüger *et al.*, 2001; see also Table S1 in the Supporting information), with a strong preference in each individual for the morph of its own mother (Table 3), presumably achieved through sexual imprinting during the nestling stage (Krüger *et al.*, 2001). When choosing a partner, buzzards thus have a reduced pool of preferred partners in the population. We tested whether this pool differed in size between the morphs, increasing the risk of inbreeding more in some morphs than in others. To calculate the preference of a given morph for each of the morphs, we assumed perfect preference for mother's morph. Therefore, relative preference by morph A for morph B was equal to the proportion of individuals from morph A which had B-coloured mothers. These proportions were empirically calculated from an extended data set of 1673 chicks and their mothers recorded in the population over 14 years (Table 3).

In order to estimate the number of available partners for individuals of a given morph, relative preference for each morph was multiplied by the average number of

Table 2 Position and structure of marker loci used. Positions are given as the chromosome number on which the best hit was found when BLASTing the flanking sequence of the marker against the reference genome *Taeniopygia guttata*. Due to the presence of null alleles, loci Bbu33, 42, 49 and 53 were excluded from further analyses.

Locus	Position in *T. guttata*	Identity, %	Total score	E-value	No. of alleles	Het_{exp}	Het_{obs}	*P* (HWE)	Null allele frequency
Bbu3	15	90	418	4e−115	5	129	127	0.316	0.004
Bbu6	5	75	277	2e−72	4	88	84	0.558	0.015
Bbu11	5	74	114	6e−24	4	90	90	0.254	<0.001
Bbu14	7	90	43	0.021	3	105	96	0.110	0.029
Bbu16	Z	83	46	0.004	3	48	50	0.061	−0.021
Bbu17	1	81	228	8e−58	4	99	94	0.681	0.016
Bbu22	1	100	34	1.3	3	21	20	0.186	0.005
Bbu26	24	78	208	4e−52	4	49	45	0.436	0.017
Bbu30	2	92	223	3e−32	4	33	29	0.014	0.019
Bbu33	3	78	199	3e−49	8	140	105	<0.001	0.109
Bbu34	1	83	171	1e−40	10	150	143	0.018	0.020
Bbu35	2	70	100	2e−19	4	24	23	0.594	0.003
Bbu36	1	91	105	1e−07	3	35	33	0.465	0.008
Bbu38	2	89	270	8e−71	2	15	14	0.294	0.007
Bbu42	4	78	170	2e−40	18	163	133	<0.001	0.086
Bbu46	2	84	322	6e−86	19	158	151	0.272	0.020
Bbu49	Z	72	179	3e−43	2	57	23	<0.001	0.143
Bbu53	19	77	82	6e−14	3	46	37	0.003	0.038

Table 3 Percentage of chicks having a mother of a given morph. Data derived from chicks recorded over a 14-year study period.

	Mother morph			
Chick morph	Dark	Intermediate	Light	N
Dark	26.6	72.4	1.0	214
Intermediate	8.4	75.3	16.3	931
Light	0.0	44.7	55.3	528

opposite-sex adults of that morph currently present in the study population. Again, average numbers of each morph were calculated from the long-term data set. The population size of preferred potential partners (P) was thus calculated as:

$$P_x = \sum(p_{\mathrm{ml}} * L + p_{\mathrm{mi}} * I + p_{\mathrm{md}} * D)$$

with p_{mx} as the probability of having had a mother of the light (l), intermediate (i) or dark (d) morph. L, I and D are the number of light (L), intermediate (I) and dark (D) individuals of the opposite sex present in a model population of 100 males and 100 females.

We tested whether this assortative mating among the morphs impacts overall levels of inbreeding and creates population substructure by comparing inbreeding coefficients F_{IS} between the morphs in an ANOVA, excluding siblings and half-siblings from the data set. F_{IS} and F_{ST} (Wright 1951) were calculated using the AMOVA function in GENALEX (Peakall & Smouse, 2006) with 9999 randomized permutations of the data set for estimating the statistical significance of F_{ST}. IR was compared between different morphs in two-sided t-tests, using both the full and the reduced data set which excluded known relatives.

Although morph is central to the main hypotheses being tested, it is possible that other factors such as geography or habitat have caused some level of genetic substructuring. To test for substructure without making *a priori* assumptions about possible causal factors, we used the program STRUCTURE version 2.3.3 (Pritchard *et al.*, 2000) to find the most likely number of clusters up to a maximum of nine. This allowed for testing whether each of the three habitat types which are geographically separated in the study area (deciduous forest north-east of the mountain ridge, deciduous forest on the ridge and coniferous forest south-west of the ridge) contains three subpopulations separated by morph. STRUCTURE uses a Bayesian approach to determine both the most likely number of distinct genetic clusters in the sample (K) and which individuals are most likely to belong to each cluster (the membership of each individual is estimated as q, which varies between 0 and 1 with the latter indicating full membership). We ran five independent runs for K = 1–10 using 10^6 MCMC iterations after a burn-in of 10^5, the correlated allele frequencies model and assuming admixture. The most likely number of genetic clusters was evaluated using the maximal average value of Ln $P(D)$, a model-choice criterion that estimates the posterior probability of the data.

Statistical analyses

The relationship between health and environmental parameters was explored using generalized linear models (GLMs). Three response variables were considered: body condition (continuous), ectoparasite infestation (continuous) and blood parasite infection (binary). Predictor variables were vole abundance, age, male lifetime reproductive success, territory quality and date of sampling (continuous), morph (factor) and year of sampling (factor) (Table 1). For each response variable, a separate data set was created, excluding only those cases containing missing data for the variable in question. Since sample sizes are modest, it is important not to over-parameterize the model. Consequently, we used the function 'step' with an initial starting model including all predictors but no interactions and an upper limit of all two-way interactions during model search, implemented with both backward and forward simplification aimed at finding the lowest possible Akaike's information criterion (AIC). For comparison, we also used the package 'bestglm' (McLeod & Xu, 2011), choosing the 'leaps' algorithm to explore parameter space and BIC as the goodness of fit criterion. 'Bestglm' generally yielded very similar results. However, since 'bestglm' does not fit interaction terms, we decided to present only the results obtained using bidirectional simplification in 'step'.

To determine whether there was any appreciable genetic influence on health, the above process was repeated with an extra genetic variable, either IR or heterozygosity at one particular locus. We felt that inclusion of multiple genetic terms would likely lead to over-parameterization. To maximize sample size, for each analysis (n = 19 genetic factors: IR + 18 microsatellites), the full data set was subsetted to exclude missing data and both a nongenetic model and a model including one genetic term were fitted. Best-fit models achieved using 'step' as above were then compared using the 'ANOVA' function in R, with the resulting P-value indicating whether inclusion of the genetic terms resulted in a significant improvement in model fit.

Although already close to the maximum number of predictor variables that can reasonably be fitted, we were also concerned by the possibility of year or family effects. As an additional test, we therefore fitted models with two random factors, year and family ID. Neither of these had significant influence. Consequently, for simplicity, we present only the results for GLMs without random factors.

Results

Mate choice

The model for the number of potential partners available suggested that each morph has a similarly sized pool of preferred mating partners to choose from. The average estimated population size of preferred breeding partners was very similar for all morphs: 45.9 for dark individuals, 51.1 for intermediate and 44.5 for light individuals in a model population of 100 females and 100 males. The even distribution was mainly due to the high numbers and breeding success of intermediate pairs. Intermediate individuals were the most common morph (60% of individuals), followed by light (32%) and dark (8%). Since intermediate pairs are the most successful and produce chicks of all morphs, large numbers of dark and light chicks had intermediate mothers (72.4% of all dark chicks and 44.7% of all light chicks). The probability of chicks of each morph to have a mother of a given morph is shown in Table 3.

Differentiation between morphs

To find evidence of population subdivision along the lines of the colour morphs, we used the AMOVA function in GENALEX to test the significance of Wright's *F*-statistics. Overall F_{ST} was 0.001 ($P = 0.319$) with a nonsignificant proportion of the variation explained by morph, 5% among individuals and 95% of variation within individuals. There were no significant differences in F_{IS} between the morphs ($F_{IS\text{-}Dark} = 0.018$; $F_{IS\text{-}Intermediate} = 0.054$; $F_{IS\text{-}Light} = -0.014$; $F_{2,39} = 0.689$, $P = 0.508$) and pairwise comparisons of F_{ST} between morphs were also nonsignificant (Dark vs. Intermediate: $F_{ST} = 0.002$, $P = 0.283$; Dark vs. Light: $F_{ST} = 0.006$, $P = 0.075$; Intermediate vs. Light: $F_{ST} < 0.001$, $P = 0.435$). Similarly, Bayesian cluster analyses yielded the greatest support for a single population ($K = 1$). Models assuming division into several clusters ($K = 2$ to $K = 9$) received steadily decreasing support, offering no support for geographical population structure or differentiation between the morphs (Fig. S1). At the individual level, heterozygosity did not differ among the morphs ($ANOVA_{IR}$: $F_{2,114} = 0.511$, $P = 0.602$; Fig. 1).

Heterozygosity–fitness correlations

Overall, we found little evidence for associations between the three fitness measures and single-locus heterozygosity. One locus, Bbu30, was retained in models for both types of infection, though significance estimated by deletion of the genetic term was at best marginal (blood parasites, $P = 0.04$; ectoparasites, $P = 0.02$) and could therefore both be due to type I error. Nonetheless, since the two potentially significant genetic terms are at the same locus, we BLASTed

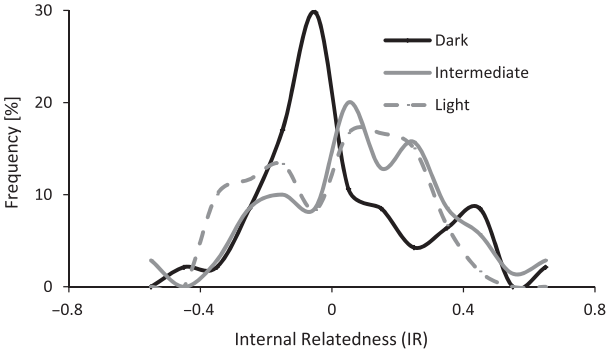

Fig. 1 Frequency distribution of heterozygosity (measured as internal relatedness, IR) of the three colour morphs. There was no significant difference between morphs ($P = 0.602$).

(Altschul *et al.*, 1990) the sequence of Bbu30 to the chicken genome and found CUB and multiple Sushi Domains 3 (CMSD3) and Tricho-rhino-phalangeal syndrome 1 (TRPS1) to be the closest genes. These are located on chromosome 2 of the chicken, whereas Mc1R is found on chromosome 11.

IR calculated over all of the loci was retained as a significant term only in the model explaining blood parasite infection status (Tables 4 and 5; parameter coefficients for all genetic models (best model plus IR) are shown in Table 6). This model suggests that in years of low food abundance, individuals with higher IR are less likely to be infected with *L. buteonis*.

Apart from the genetic terms, the three sets of models yield contrasting results. Our measure of body condition yields a best-fit model where, although the AIC is lower than the null model, the amount of deviance explained is not significant (Table 4). The two parasite models both explain a significant proportion of the deviance. For endoparasite infection, the best model contained the predictors age, sex, vole abundance, morph and male lifetime reproductive success besides IR (Table 5). In contrast, the model for ectoparasite infestation did not contain a genetic term but was overall strongly significant at $P = 1 \times 10^{-11}$ (Table 4).

Discussion

Morph differentiation and inbreeding

We assessed the potential for inbreeding to create HFCs and differences in heterozygosity among the plumage morphs in the common buzzard. We found no evidence of differentiation between the morphs suggesting the existence of population substructure or inbreeding, and little evidence to suggest that HFCs are prevalent. Significant differences in F_{ST} among the buzzard morphs have been found at Mc1R, the locus responsible for the plumage polymorphism (M.A. Pointer, N.I. Mundy, O. Krüger, unpublished), but this did not hold up for

Table 4 Comparisons between best models for all three response variables and their respective null models.

Response Variable	Model	Residual DF	Residual deviance	DF	Deviance	*P*
Blood Parasite Infection	Null	100	24.16	17	9.27	<0.001
	Best	83	14.89			
Ectoparasite Level	Null	168	157.14	10	49.77	<0.001
	Best	158	107.37			
Body condition	Null	176	847582	1	10438	0.14
	Best	175	837144			

Table 5 Best models explaining variation in blood parasite infection and ectoparasite burden, respectively. The best model for condition was no significant improvement over the null model. Coefficients for the best model with the term IR added can be found in Table 6.

Model	Variable	Df	Deviance	Residual Df	Residual Dev.	*P*
Blood infection	NULL			100	24.16	
	Age	1	0.87	99	23.29	0.038
	Voles	1	0.07	98	23.22	0.559
	Sex	1	0.91	97	22.31	0.033
	Morph	2	0.42	95	21.89	0.351
	Paternal LRS	1	0.05	94	21.84	0.618
	IR	1	0.13	93	21.7	0.386
	Voles × IR	1	1.82	92	19.89	0.001
	Sex × Paternal LRS	1	0.84	91	19.05	0.031
	Voles × Morph	2	1.11	89	17.94	0.045
	Morph × Paternal LRs	2	1.29	87	16.65	0.028
	Age × Sex	1	0.68	86	15.97	0.051
	Age × IR	1	0.45	85	15.52	0.114
	Sex × Morph	2	0.62	83	14.89	0.176
Ectoparasites	NULL			168	157.14	
	Territory quality	1	0.82	167	156.31	0.27
	Age	1	10.22	166	146.1	<0.001
	Sex	1	0.09	165	146.01	0.72
	Morph	2	14.64	163	131.37	<0.001
	Paternal LRS	1	5.74	162	125.63	0.004
	Territory quality × Sex	1	4.63	161	121	0.01
	Sex × Morph	2	6.9	159	114.11	0.01
	Sex × Paternal LRS	1	6.74	158	107.37	0.002

the presumed neutral microsatellite markers used in our study. Furthermore, assortative mating following filial imprinting does not lead to a reduced gene pool in the rarer morphs. This is probably because most of the rarer morph individuals are born to intermediate mothers and thus prefer intermediate partners as adults. Over the past 14 years, only 27% of dark chicks had dark mothers and thus preferred dark partners, with the vast majority of dark individuals (72%) preferring intermediate partners. For light chicks, the proportion born to an extreme morph mother is higher than in dark birds, 45%. The remaining 55% of light chicks had intermediate mothers, leading to a strong preference for this morph as mating partner and thereby contributing to the panmictic population structure. Thus, gene flow between morphs should be high and not conducive to inbreeding and population substructuring.

Heterozygosity–fitness correlations

There was only very limited evidence for genome-wide HFCs. Our measure of heterozygosity, IR, was only retained in one of the models tested, suggesting that multilocus heterozygosity, and therefore, true heterosis may play little role in the buzzard chicks' ability to reduce ectoparasite burden or increase body condition. However, heterozygosity did affect blood infection status in conjunction with food abundance. In years of low food abundance, chicks had a higher chance of being *Leucocytozoon*-free if they had high heterozygosity. The trend was absent in years of higher food abundance. Resistance against this parasite therefore appears to be condition dependent, with chicks being more successful at avoiding infection under abundant food supply. IR was also retained in the model in an interaction with age, even though this interaction was not significant and may have been an artefact of differential survival. Using new sampling approaches, we hope to shed more light on the effects of individual and environmental factors on survival in the future.

Of course, IR might also correlate with other fitness-related traits we did not consider. For example, Hoffman *et al.* (2006) found no correlation between heterozygosity and pup survival in Antarctic fur seal

Table 6 Coefficients of the best models with IR for blood infection, ectoparasite load and condition.

Model	Variable	Estimate	Std. Error	*t* value	*P*
Blood Infection	Intercept	1.49	0.33	4.5	<0.001
	Age	0.03	0.01	3.36	0.001
	Voles	−0.24	0.12	−2.04	0.04
	Sex	−0.87	0.33	−2.63	0.1
	Intermediate	0.06	0.31	0.19	0.85
	Light	−0.19	0.34	−0.57	0.57
	Paternal LRS	0.0	0.02	−0.19	0.85
	IR	−0.41	0.78	−0.52	0.6
	Voles × IR	1.09	0.29	3.76	<0.001
	Sex × Paternal LRS	0.04	0.02	2.42	0.02
	Voles × Intermediate	−0.07	0.16	−0.42	0.68
	Voles × Light	0.39	0.17	2.32	0.02
	Intermediate × Paternal LRS	0.0	0.02	−0.07	0.94
	Light × Paternal LRS	−0.52	0.02	−2.25	0.03
	Age × Sex	−0.03	0.01	−2.11	0.04
	Age × IR	0.06	0.03	1.89	0.06
	Sex × Intermediate	−0.36	0.22	−1.64	0.1
	Sex × Light	−0.37	0.23	−1.58	0.12
Ectoparasites	Intercept	−0.25	0.42	−0.5	0.55
	Territory quality	−1.65	0.48	−3.42	<0.001
	Age	−0.03	0.01	−3.67	<0.001
	Sex	0.49	0.5	0.98	0.33
	Intermediate	−0.88	0.23	−3.82	<0.001
	Light	−1.15	0.22	−5.2	<0.001
	Paternal LRS	0.0	0.02	−0.02	0.99
	IR	−0.25	0.28	−0.9	0.37
	Territory quality × Sex	2.57	0.64	3.98	<0.001
	Sex × Intermediate	1.0	0.34	2.98	0.003
	Sex × Light	1.13	0.34	3.33	0.001
	Sex × Paternal LRS	0.08	0.03	3.05	0.003
Condition	Intercept	19.22	15.03	1.28	0.2
	Voles	−11.7	8.0	−1.46	0.15
	IR	27.67	21.8	1.27	0.21

but a strong relationship with adult male reproductive success (Hoffman *et al.*, 2004) and tooth size, a proxy for body size (Hoffman *et al.*, 2010b), suggesting that heterozygosity influences fitness post-weaning. Whether a similar pattern holds for buzzards remains to be determined. However, the fact that IR only plays a very limited role for our fitness measures and that we have failed to find heterozygosity differences between the morphs despite reduced fitness (LRS) in the rarer, homozygous morph emphasizes that HFCs might be common but not necessarily universal. Indeed, the growing body of literature reporting HFCs may create a somewhat distorted impression because many negative results go unreported (Chapman *et al.*, 2009; Hedrick, 2012).

Although overall heterozygosity, measured as IR, showed a link only with endoparasites, heterozygosity at one locus, Bbu30, revealed a significant link to both blood and ectoparasites, suggesting that it may be linked to a functional locus. This may be supported by the fact that ectoparasite infestation and blood infection intensity are negatively correlated with each other across morphs (Chakarov *et al.*, 2008). Although increasing melanization was accompanied by increasing levels of ectoparasite infestation, the opposite was true for infection levels with the blood parasite *L. buteonis*. However, the same was not true for blood infection status. The exact link between these three measures therefore remains unclear. Therefore, a more conservative interpretation of our results would be type I error, which would imply that among the very few individually significant associations between heterozygosity and fitness, it is merely chance that two involve the same locus. Taking the more optimistic view of a potential link between both effects and Bbu30, we BLASTed the Bbu30 sequence against the chicken genome. The nearest genes are CUB and multiple Sushi Domains 3 (CMSD3) and Tricho-rhino-phalangeal syndrome 1 (TRPS1). The closest, CMSD3, at 156,263 bases away has multiple domains with homology to complement

control proteins, so is plausibly part of the immune system. Of course, we cannot exclude the possibilities of linkage with loci farther away, or of Bbu30 being a functional locus itself. Both of these options remain tantalizing possibilities which could make the common buzzard one of the rare species with true heterozygote advantage (Hedrick, 2012).

Putting aside any genetic effects, the best model for blood parasite infection is in line with previous studies that stressed the importance of age, morph and food availability (Chakarov *et al.*, 2008). Again, ignoring the weak genetic effects, all other terms fitted in the model for ectoparasite burden explain appreciable variation in burden (range = 6% to 12.1%, though with some overlap between predictors). The largest effects are associated with sex and morph. However, territory quality, vole abundance and male lifetime reproductive success are also all retained strongly suggesting that, more generally, good levels of nutrition help chicks to keep any ectoparasite infestations under control.

The least successful model involves body condition. Here, the best model retains multiple predictor variables, but none of these should be taken seriously because overall the model does not explain significantly more of the null deviance than expected by chance. Quite why condition is so poorly correlated with other factors is unclear. Our measure, sex-specific residuals of mass and body size, is widely used and has been confirmed as a reliable indicator of condition in wild animals (Schulte-Hostedde *et al.*, 2005).

Heterosis and plumage polymorphism in the common buzzard

Our analysis offers little support for the idea that genome-wide heterozygosity explains the higher reproductive success of the intermediate buzzard morph or maintains polymorphism in the population. *Leucocytozoon* infection alone is unlikely to account for the variation in LRS (Chakarov *et al.*, 2008). However, buzzards are large, partly migratory birds which can disperse well over 100 km, and it has been suggested that they form a continuous, panmictic population across their entire European range (Olsson, 1958; Meunier, 1961; Mebs, 1964; Walls & Kenward, 1998). The low support for genome-wide HFCs is therefore consistent with a large population size (Balloux *et al.*, 2004; Slate *et al.*, 2004) and may suggest a low overall variance in inbreeding coefficient, *F*. Thus, the role of inbreeding depression for fitness differences appears unlikely but cannot be ruled out.

If genome-wide HFCs seem unlikely, any genetic benefits associated with morph are presumably due to one or a number of individual loci and their interactions. We found no evidence of such an effect, but the number of markers we were able to deploy covers only a small fraction of the genome, so other effects are likely to have been missed. In particular, a candidate for a large single-locus effect is the main locus responsible for the colour polymorphism, Mc1R, whose role remains a puzzle. Epistatic effects of Mc1R or regulatory elements associated with it are currently not known, and documented pleiotropic effects remain rare (Mundy, 2005; Ducrest *et al.*, 2008). For other large-effect loci to be closely correlated with colour morph, they would have to be in tight linkage disequilibrium with Mc1R (Balloux *et al.*, 2004; Slate *et al.*, 2004). A potential mechanism to create such linkage is a population bottleneck. Schreiber *et al.* (2001) postulated one or several bottlenecks in the recent history of the common buzzard to account for low allozyme heterozygosity in the species. As the distance across which linkage disequilibrium is maintained declines rapidly over generations of population expansions, this would suggest that Mc1R may lie in a gene-rich region. In this case, it seems possible that one of these other genes is under balancing selection yet close enough to Mc1R for its fitness consequences to be reflected in plumage. This idea is supported by the large number of phenotypic differences between the morphs (Krüger, 2002; Chakarov *et al.*, 2008; Boerner & Krüger, 2009) which make feather coloration itself unlikely to account for the large differences in survival and reproductive success between the morphs. Alternatively, a regulatory element associated with Mc1R may affect loci responsible for the manifold differences observed between morphs and the marker Bbu30. Given the important role of Mc1R in colour polymorphisms across vertebrates, the mechanism underpinning heterozygote advantage for colour morph clearly deserves further attention.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Morph combinations observed in breeding pairs over the entire study period.

Figure S1 Support by Bayesian cluster analyses (mean Ln P(D) ± standard error) for K genetic subpopulations in the buzzard population.

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