Microsatellite heterozygosity and phenotypic quality in bluethroats (*Luscinia svecica*)

Master of Science thesis

by

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Forord

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Abstract

Heterozygosity has been considered as an important fitness-related trait among individuals in populations with reduced genetic diversity (i.e. inbred or bottle-necked populations), but it is not obvious why there should be a link between heterozygosity and fitness in outbred populations. Here I analyse heterozygosity of 43 microsatellite markers in breeding bluethroats (*Luscinia svecica*) and their offspring. I found no correlations in heterozygosity among subsets of these markers, suggesting that overall heterozygosity does not reflect the level of inbreeding in this population. However, heterozygosity was related to tarsus length in males and body condition (i.e. body mass controlled for body size) in both sexes. The heterozygosity seems to be due to positive effects in several markers, some of which were significant alone, suggesting that the fitness-heterozygosity correlations are driven by multiple local effects in the genome. Previous studies have found an effect of increased heterozygosity on extra-pair offspring (EPO) compared to within-pair offspring (WPO) in the same nest predicting that females seek extra-pair copulations to increase the heterozygosity of their offspring. This study found a trend in the same direction, though not statistically significant. The paternity analyses with such high number of markers revealed that some EPO can go undetected if just a few markers are used, even when such markers apparently have high exclusion probabilities. However, this potential bias did not seem to inflate the heterozygosity scores of EPO as opposed to WPO, as hypothesized by a recent study.

Introduction

Molecular markers have the last decades become the standard tool for studies of population genetics, paternity and genetic diversity. In intraspecific studies it is important to use genetic markers with a high degree of polymorphisms and microsatellites have proved very useful for this reason. Microsatellites are noncoding simple sequence length polymorphisms comprising tandem copies of usually , di-, tri- or tetranucleotid repeat units (Brown 2002). They mutate through replication slippage and cells do not appear to have any repair mechanism for reversing these mutations and hence new microsatellite alleles are generated relatively frequently (Ellegren 2004). In addition microsatellites are normally spread evenly in the genome and are easily amplified in PCR since the sequence seldom exceeds 300bp in length. Microsatellite markers have been used extensively for detecting paternity and calculating heterozygosity in natural populations (Blouin 2003; Kempenaers 2007).

Several studies of passerine birds, have found positive correlations between heterozygosity and various fitness-related traits such as survival, territory size, song diversity, male plumage characters, clutch size, fertilization-, hatching- and fledging success (see Kempenaers 2007 for a review). Hansson and Westerberg (2002)described three main hypotheses for the explanation of heterozygosity-fitness correlations. First, the 'general effect' hypothesis proposes that heterozygosity-fitness correlations reflects the level of inbreeding and are due to negative effects of homozygosity at genome-wide distributed loci (inbreeding depression) or with higher fitness for the heterozygous state than the two homozygote states (functional overdominace/heterosis). In this case the microsatellite markers are assumed neutral, but that there is a associative overdominace with functional loci. Second, the 'local effect' hypothesis states that microsatellites are in linkage disequilibrium with functional loci. The local effect hypothesis predicts that heterozygosity at particular microsatellite loci will correlate with fitness-related traits. The third hypothesis is called the "direct effect" hypothesis and assume that the correlation between heterozygosity and fitness is a result of the functionality of the scored microsatellite loci themselves. The direct effect hypothesis has received little support in studies using microsatellites, since these are assumed selectively neutral. I therefore choose to concentrate on the two other hypotheses in this study.

Several studies have questioned the general effect hypothesis as a general explanation for the observed heterozygosity-fitness correlations, especially the idea that heterozygosity across a relatively small number of microsatellite markers simply reflects inbreeding (Balloux et al. 2004; Pemberton 2004; Slate et al. 2004)**.** First, individual heterozygosity estimates

correlate only weakly with the individual inbreeding coefficient (Balloux et al. 2004; Slate et al. 2004) and second, random subsets of markers should correlate if a general effect is present (Kempenaers 2007) and this is often not the case (Hansson et al. 2004; Lieutenant-Gosselin and Bernatchez 2006; Tiira et al. 2006). Several studies have found relationships between single markers and fitness, suggesting that single markers are able to drive the overall correlation (Hansson et al. 2001; Coltman and Slate 2003; Tiira et al. 2006; Fossøy et al. 2009).

Irrespective of the exact mechanism, the existence of heterozygosity-fitness correlations suggests that heterozygosity could be used as a quality trait in mate choice (Brown 1997) and that individuals should be selected to optimize the heterozygosity of their offspring. Population studies during the last two decades have revealed that among investigated passerine birds, 86% produce extra-pair offspring (EPO) (Griffith et al. 2002). Females have normally been considered as the choosy sex in extra-pair mating due to their higher investment in each offspring (egg production). Several hypotheses have been proposed to explain why females engage in extra-pair copulations (EPC) leading to extra-pair paternity and both direct and indirect benefits have been suggested (Birkhead and Møller 1992; Kempenaers 2007).

Potential direct benefits include being allowed to forage inside the extra-pair males' territory, help with predator defence, brood care or simply ensure fertilization of the eggs (Sheldon 1994; Gray 1997). Indirect benefits are genetic effects that can be of an additive or non-additive nature. The first, often termed "the good genes effect" is the idea that females seek extra-pair copulation with males that have preferable traits or "good genes" that are inherited additively by the EPO (Mays and Hill 2004)**.** The females will then increase the fitness of their offspring. This means that the optimal male would be the same for every female. The second, often termed "the compatible genes effect" is the concept that it is the combination of the father and mother genotype that adds quality to the offspring (Zeh and Zeh 1996; Zeh and Zeh 1997). This means that what would be the optimal male to choose for one female might not be the optimal for another. One version of the compatible genes hypothesis postulates that females will seek EPC with males that have a genotype different from their own to increase the heterozygosity of their offspring (Kempenaers 2007)

Several studies have found that females are less genetically similar to EPC mates than their social males (Fossøy et al. 2008) and that EPO have higher heterozygosity than their within-pair siblings (Foerster et al. 2003; Stapleton et al. 2007; Fossøy et al. 2008). Wetzel

and Westneat (2009) have recently criticized studies that have used the same, small set of markers to determine both paternity and heterozygosity. They claim that including the paternity markers in the heterozygosity estimates will lead to a bias towards higher values of heterozygosity in EPO and extra-pair males (compared to within-pair males). This is because EPO would only be detected if the extra-pair male differs partially or wholly from the within-pair male. If the extra-pair male is very similar, or even identical on the limited set of markers, to the social male he cuckolds there is a higher probability that his EPO will not be detected. Wetzel and Westneat (2009) assumed that not all EPO are detected and predicted that including the paternity markers in the heterozygosity estimate will lead to EPO always appearing more heterozygous than their within-pair siblings. Wetzel and Westneat (2009) suggested that to expand the marker-set with higher level of polymorphisms and heterozygosity will reduce this bias, but to use two different sub sets of markers for paternity and heterozygosity will eliminate the bias entirely.

The bluethroat (*Luscinia svecica*) is a migratory passerine with a high frequency of extra-pair paternity (Johnsen et al. 1998a; Johnsen et al. 1998b). Previous studies lend support to female extra-pair mate choice based on compatible genes in this species. First, in both maternal and paternal half sibling comparisons, EPO had a higher swelling response to phytohaemagglutinin (PHA) than their withinpair siblings (Johnsen et al. 2000; Fossøy et al. 2008). Second, females had EPCs with males that were genetically dissimilar from themselves, which increased the heterozygosity of their EPO (Fossøy et al. 2008). Overall heterozygosity and PHA response were not correlated and hence there were multiple independent benefits for female promiscuity (Fossøy et al. 2008). Recently Fossøy et al. (2009) presented evidence for local heterozygosity effects on PHA response in bluethroat.

These studies were all based on a small (6-12) set of markers. My study has tested the relationship between heterozygosity and fitness in the bluethroat using a set of 43 microsatellite markers, which should make a more precise heterozygosity estimate and a more reliable paternity assessment. This study had three main aims. First, to test whether heterozygosity is correlated with fitness-related traits such as body mass, wing length, tarsus length, various measurements of colour, female fecundity and paternity of males with this larger marker set. Second, to test whether any such correlations would be attributed to local or general effects. Third, to test whether there are heterozygosity differences between EPO and within-pair offspring (WPO) based on a more precise heterozygosity estimate and more reliable paternity assessment, in other words can the results from Fossøy et al. (2008) study be

corroborated? In relation to this, I also performed an empirical test of Wetzel and Westneat's (2009) suggestion of a systematic bias in studies using small marker sets for both paternity assignment and calculating heterozygosity.

Materials and methods

Study area and species

Field work was carried out in the area around NHMs field station in Øvre Heimdalen valley (61°25'N, 8°52'E) in Øystre Slidre municipality, Oppland county during mid-May to mid-July 2008. The study area is in the sub-alpine vegetation zone situated about 1100 m above sea level (Vik 1978).

The bluethroat is a small (18 g) and highly dichromatic species where males have a complex throat ornament with high individual variation (Cramp 1988; Johnsen et al. 2001). It is migratory, socially monogamous and territorial and males arrive early in the breeding season (mid- May) to establish a territory. Females arrive somewhat later and starts building a nest on ground soon after pair-formation. The female lays 5-7 eggs and incubates them alone for 13-15 days (Cramp 1988). Both parents feed the nestlings which leave the nest after (10-14) days (Anthonisen et al. 1997)

Field methods

Most males were caught in their territories in the beginning of the breeding season using mists nets and playback of male and female song to attract them, although some males were caught later in the chick-feeding period. Most females were caught in the incubation period, or chick-feeding period when their nest had been located. Most males were controlled for social paternity through observations of chick feeding, nest building or other activities that connected them to the particular nest, although in some cases males were assumed social fathers because of territorial behaviour. All adults were measured for tarsus length (to nearest 0.1mm) using an electronic slide calliper, wing length (to nearest 0.5 mm) using a wing ruler, body mass (to nearest 0.1 g) using a pesola 50g spring balance. They were aged as yearlings or older on the basis of presence/absence of light tips on the greater wing coverts (Svensson 1992). Among females (n=27), only 3 were scored as young and all others as older females. This proportion of young in the sample is suspiciously small and there are good reasons to believe that some might have been misidentified regarding age. The whitish tips on the great wing coverts have a great deal of variability and young individuals can easily be identified as older. The covert tips can also be worn off during the season. I thus excluded age as a factor

in the female analyses. All adults were marked with a metal band and three additional colour bands all with a unique combination to distinguish them at distance in the field. All birds were bled (5-25µl) through puncturing of the brachial vein and the blood was stored in Queens lysis buffer (Seutin et al. 1991) for later genetic analyses.

Nests discovered in the incubation period were visited frequently around expected time of hatching, and nestlings were measured for body mass and blood sampled, through puncturing of the femoral vein, on day two after hatching. Broods were visited every second day afterwards until fledging, nestlings that survived to day eight were marked with a metal ring and measured for tarsus length. Nests discovered after hatching were weighed and blood sampled immediately.

Plumage measurements

Thirtyseven males were measured for reflectance from UV/blue feathers in the throat patch with an Ocean Optics USB2000 spectroradiometer, with a PX-2 pulsed xenon light source connected by a bifurcated fiber optics cable, (Ocean Optics BV, Duiven, The Netherlands). I calculated three colour parameters; hue, brightness and chroma (Andersson and Prager 2006). Hue is estimated as λ_{max}, the wavelength of peak of reflectance. Brightness (R_{av}) is estimated by average reflectance in the interval between 320-700 nm, which is the spectral range of most birds studied to date (Hart et al. 2000). Chroma (a measure of spectral purity) is the difference between R_{max} and R_{min} divided by average reflectance $[(R_{max}-R_{min})/R_{av}]$. Males were also measured with an electronic slide caliper for width (mm) of the chestnut band below the throat ornament. More details for all male plumage measurements can be found in Johnsen et al.(2001). Females were given a 1-10 colour score ranging from grey/dull to strongly chestnut/blue in throat colour, as described in Amundsen et al.(1997).

Genetic analyses

DNA was extracted from blood samples using QIAamp® DNA Blood kit (QIAGEN, Venlo, The Netherlands). Microsatellites were amplified by polymerase chain reaction (PCR), system 9700 (Applied Biosystems, Foster City, U.S.A), and ran on an ABI Prism® 3100XL Genetic analyzer (Applied Biosystems) using fluorescently labelled primers. Allele sizes were determined using ABI Prism® Genemapper™ Software version 4.0 (Applied Biosystems).

Eight markers; Ase 19, Cuμ4, Mcyμ4, PAT-MP 45, Ppi2, Fhu2, Lm6, PmaC25 used in previous studies (e.g. Fossøy et al. 2008, details in Table 1) were ran in separate PCRs. In addition, 37 new pied flycatcher (*Ficedula hypoleuca*) markers (Leder et al. 2008), also optimized for bluethroat, were sorted in five panels and run with multiplex PCRs (see Table 1 for details). Each DNA extract was diluted 1:3. To each PCR, I added 3 μl Qiagen Buffer (QIAGEN), 0,5 μl primer-mix, 1.5 μl water and 1 μl DNA extract. The primer-mix consisted of various volumes of forward and reverse primers from all markers in each panel (see details in Table 2). For all panels, the following PCR programme was used: 95°C for 15 min, 35 cycles of 94°C for 30 sec, annealing temperature for 1:30 sec, 72°C for 1 min, 60°C for 15 min. After PCR the samples were diluted 1:99 before preparation to the ABI. For panel 1-4 2 μl PCR product was added to ABI plate, for panel 5 1μl PCR product was added.

Parentage

I initially planned to divide the marker set in 6 highly polymorphic paternity markers used for paternity only and use the remaining markers for heterozygosity calculation, as recommended by Wetzel and Westneat (2009). However, since I discovered that 6 paternity markers were not able to detect all EPO (based on mismatches in the remaining markers), I decided to use all markers for assigning true parentage (but see below).

Mismatches between putative parents and offspring can emerge for at least four different reasons. The first, and obvious one, is a mismatch due to EPP. If individuals had mismatches on several loci (>6) from their putative father these individuals were defined as EPO. Mismatches can also emerge as a result of mutation, null-alleles and allelic drop-outs. These possibilities were considered in individuals that had 1-5 mismatches from their mother or social father. In microsatellite markers the alleles normally differ in +/- one or more units from each other. One unit will normally be one, two, three or four basepairs (occasionally more). Mutations are normally detectable as a mutation normally will be +/- one unit from the mismatching parents' allele. However mutations are rare and I chose a threshold at two mutations from father and/or mother in each individual. Some markers had a null-allele problem (see Table 1)which is when a mutation at the primer-seat leads to no amplification of the given allele in the PCR. This is also relatively easy to detect because the parent with the null-allele will appear homozygous and offspring which have inherited this allele appear homozygous for one of the other parent's alleles. Allelic drop-outs are more problematic. This

is when an allele appear in some individuals and "drops-out" in others. This may be due to primer competition which is a particular problem when running multiplex PCR because primers have to be adjusted to each other, not only the single marker. However, if some rules are followed there is a reasonable chance to identify allelic drop-outs. First, there can not be more than four alleles involved in parents and offspring. Second, the individuals with the drop-out have to be homozygous (offspring homozygous for one of the parent's alleles) and all individuals where the particular allele is visible have to have the same allele. All individuals had to be inspected for these problems due to the fact that individuals with null-alleles or allelic drop-outs should be scored as heterozygous since their apparent homozygosity is due to a methodological error. After corrections for mutations, null alleles and allelic drop-out, all offspring that were determined EPO had a minimum of seven mismatches with their putative father. To assign genetic extra-pair fathers I used CERVUS 3.0 (Marshall et al. 1998) to screen for potential candidates and used the same rules for assigning paternity as for excluding it.

Heterozygosity

Since all markers were used to determine paternity, I also used all markers in heterozygosity calculations. Since not all markers were typed for each individual, I calculated standardized heterozygosity (SH) by dividing the proportion of heterozygous loci for an individual by the mean observed heterozygosity for all loci typed for that individual (Coltman et al. 1999). SH correlated positively with the plain heterozygosity values (H) (r=0.992, P=<0.001, n=84). I was interested in finding out whether heterozygosity-fitness correlations were due to single loci and hence caused by a local effect or a general genome-wide effect. To test this, I correlated various subsets of markers to detect markers with high influence on the correlations. I divided the marker-set in two random subsets consisting of 21 and 22 markers, respectively subset 1 and 2. I sorted the markers from highest to lowest observed heterozygosity (Hobs – see Table 1) and then assigned every second marker to each subset, to avoid bias in overall heterozygosity. I was also interested to see if the markers that had been used in the previous study (Fossøy et al. 2008) was correlated to the new expanded marker-set, hence I made a subset consisting of the 8 old markers (subset 3) and all the new 35 markers (subset 4). I also analysed the effect of individual markers on the fitness traits that correlated significantly with standardized heterozygosity using Pearson correlation.

Heterozygosity and extra-pair paternity

Wetzel and Westneat (2009) predicted that only the most heterozygous EPO from the most heterozygous extra-pair fathers would be detected in small marker sets. To test this, I based paternity and heterozygosity estimates on six paternity markers (Ase19, Cuμ2, Mcyμ4, PAT-MP 2-43, Ppi2 and Fh448) with a combined inclusion probability of 0.9999. I used the conventional criterion of two or more mismatches with the putative father to define EPO in these analyses. I used the same statistical analysis for comparison of EPO and WPO as described below.

Statistical analyses

Statistical analyses were run in SPSS 16.0., STATISTICA 7 and GLMStat 5.5.1. I used general linear models (GLM) to test heterozygosity-fitness correlations. I controlled for age (males only) and catching day and removed factors by backward stepwise exclusion when $p > 0.1$. For body mass I also controlled for tarsus length. Response variables were checked for normality using Kolmogorov-Smirnov and Shapino-Wilk tests in SPSS 16.0. Chroma showed a skewed distribution and was hence tested with a non-parametric Spearmans rank correlation. I also tested whether heterozygous males had higher probability of losing/gaining paternity with a generalized linear model with binominal distribution and logit link. To test for relationship between heterozygosity and amount of within-pair paternity, I used a generalized linear model (GLZ) with the number of offspring sired by the attending male as dependent variable, brood size as binomial denominator and binomial error distribution (GLMStat 5.5.1). I did comparisons of heterozygosity in maternal half siblings, using linear mixed model analyses (restricted maximum likelihood (REML)), brood identity was included as a random factor. Figures were made using SPSS 16.0. and Origin® version 7 (OriginLab Corporation). I used CERVUS 3.0 to calculate locus characteristics (Table 1).

Results

Parentage

Of the 29 nests, 15 (52%) contained one or more EPO, and 54 of 152 (36%) chicks were sired by an extra-pair male. I managed to detect the genetic father of 19 (35%) of the 54 EPO. In broods containing EPO, eight were mixed including both WPO and EPO, seven nests consisted only of EPO. Among WPO, some did have mismatches with their parents : 31 had one, 21 had two, 12 had three, nine had four and eight had five mismatches with father and/or mother. Of these 192 mismatches, 92 were null alleles, 46 mutations and 53 allelic dropouts**.** With a score for all 152 offspring at 43 loci there is potential for errors at 13 072 alleles and the error rate is hence 0.015.

The six paternity markers detected only 44 of the 54 EPO. However, of the ten remaining offspring, two had one mismatch in the six paternity markers, three were only scored of five markers, and for four offspring the mother and/or the social father was not scored for all markers, reducing the exclusion probability. Nevertheless, one individual did not mismatch at any of the six paternity markers while clearly being an EPO based on the additional markers (7 mismatches in total).

Heterozygosity

The microsatellite markers ranged from 3-35 alleles per marker (mean±SD: 15.35±8.40). Observed heterozygosity (Hobs) ranged from 0.159 – 0.964 (mean±SD (0.676±0.219). Especially the markers EST9, Fh356, Fh408 and Fh224 had high frequencies of null alleles (F(null)>0.3). Details can be found in Table 1.

If heterozygosity at a number of markers is representative for the entire genome, I would expect a positive correlation between heterozygosity scores for subsets of markers. However, this was not the case (Table 4). Some of the sub set shared markers and hence did correlate, but the two most random subsets 1 and 2, which were the halves of the overall marker set, and was sorted based on observed heterozygosity, did not correlate (Figure 1)

Heterozygosity and fitness-related traits

Heterozygosity correlated positively with tarsus length (Figure 2) and body mass (Figure 3), and negatively with chestnut band width for males (Figure 4). For tarsus length, 27 of the 43 markers were positively related to heterozygosity and two markers, Fh431 (n=42, r=0.353, p=0.022) and EST46 (n=39, r=0.538, p=<0.001) were significant. On body mass there was no significant effect on any single marker, but 25 of the 43 markers had a positive effect. The markers Fh350, Fh310, Fh405, Fh221, Fh224 and ZF-S8 all had p>0.1. The correlation found for the chestnut band was highly influenced by one outlier, with the lowest heterozygosity and the highest chestnut band score. When this individual was removed from the analysis, the result was no longer significant (n=40, F=0.881, p=0.354). When I investigated single markers effect for the chestnut band, 25 of the 43 markers had a negative r-value, but no single marker seemed to influence strongly. There were no significant correlations between heterozygosity and colour measurements (chroma, hue, brightness), or wing length (Table 3A). I found no correlations between heterozygosity and paternity; paternity (number of WPO controlled for brood size and age) ($F_{1,26}$= 1,661, p=0.201) and probability of losing paternity (χ^2=0.005, df=1, p=0.944).

For females there was a significant correlation between body mass and heterozygosity (Figure 5). There were no other significant correlations between heterozygosity and morphology or colour (Table 3B). I found no correlations between female heterozygosity and clutch size (F=3.94, p=0.374)

For all adults combined, controlled for sex, there was an overall significance for both body mass (estimate±SE: 4.36±1.17, F= 13.86, p=<0.001) and tarsus length (estimate±SE: 2.49±0.99, F= 7.15, p=0.009).

Heterozygosity and extra-pair paternity

There were eight nests with mixed paternity that could be used in half-sibling comparisons. There was no significant difference in heterozygosity between EPO and WPO in mixed broods ($F_{1, 40.1}$=1.338, p=0.254), but I found the same positive trend as Fossøy et al. (2008) where EPO had higher heterozygosity than WPO (Figure 6).The paternity markers were not able to detect all EPO. In one brood, the single EPO was not detected,, and three full loss (only EPO) broods became mixed broods since the paternity markers did not manage to detect all EPO in the brood. The new analysis thus included 10 mixed broods. The results went in the opposite direction than expected based on Wetzel and Westneat's prediction, that is, the difference between EPO and WPO became smaller ($F_{1, 56.0}$=0.789 p=0.378). Wetzel and Westneats prediction would imply more heterozygous EPO than WPO in this test as compared to the test with all 43 markers, but the EPO were actually less heterozygous with paternity assessment based on six markers.

Discussion

The main difference between this and previous studies lies in the number of microsatellite markers. First, with this large marker set heterozygosity estimates will be more precise, which should increase the power to detect heterozygosity-fitness correlations in the population. Indeed, I found several significant correlations between overall heterozygosity and phenotypic characters. Second, an expanded marker set made it possible to test whether any heterozygosity-fitness correlations were due to local or general heterozygosity effects. My results were most consistent with local effects, since there was no correlation between heterozygosity estimates based on two random subsets of markers and single-marker analyses highlighted certain markers with strong associations. Third, the expanded marker set made it possible to test if the previous finding that EPO have higher heterozygosity than their half siblings (Fossøy et al 2008) could be corroborated. This was of particular interest since the former bluethroat study and other such studies have recently been criticized for having a bias towards increased heterozygosity values in EPO, and an expanded marker set was suggested as one possibility to reduce this bias (Wetzel and Westneat 2009). My study supports Wetzel and Westneat's claim that small marker sets will leave some EPO undetected, but this did not seem to introduce the predicted bias in heterozygosity.

I found significant positive correlations of heterozygosity on tarsus length in males and body mass in both sexes. The tarsus is a trait that is more or less fully grown at the end of the chick period. A long tarsus may thus relate to good conditions while growing up. This suggests that more heterozygous chicks compete better for food in the nest and/or are more resistant to diseases, thereby increasing the growth rate which leads to longer tarsus at fledging. Body mass was controlled for tarsus length and hence a measure of overall condition (García-Berthou 2001). Individuals in better condition have possibilities to invest more in each offspring and possibly have increased resistance to diseases and enhanced survival (Perrins 1965; Nur 1984)**,** all traits that are directly linked to fitness. There are not many studies that have revealed a relationship between heterozygosity and condition, but a previous study in threespined sticklebacks (*Gasterosteus aculeatus*) (Lieutenant-Gosselin and Bernatchez 2006) found this effect. The reason why this effect was not found in females is unknown, but it could be due to a lack of statistical power. Note that the effect was significant in a combined analysis with both sexes. The negative correlation between chestnut-band width and heterozygosity is surprising since this ornament character has previously been found related to age and male paternity success (Johnsen et al. 2001). However, one data

point adds very significantly to the overall result, and the significance disappeared when this individual was removed from the analysis. Further studies are needed to determine whether the negative relationship between chestnut-band width and heterozygosisty is biologically significant or spurious.

Heterozygosity-fitness correlations have previously been assumed to reflect the level of inbreeding in the population (David 1998; Hansson and Westerberg 2002). The population in this study should be highly outbred due to the continuous distribution of bluethroats in Scandinavia and Russia, and the low level of philopatry in the local population (A. Johnsen and J. T. Lifjeld, unpublished data). Nevertheless, overall heterozygosity-fitness correlations have been found (Fossøy et al. 2008). There are mainly two hypotheses that have been proposed to explain this. First, Markert et al. (2004) suggested that individual heterozygosity might capture some of the inbreeding depression that was not accounted for by the inbreeding coefficient, or second, the observed effects could reflect local effects between individual markers in linkage disequilibrium with functional loci with a fitness effect (Hanson and Westerberg 2004). The latter hypothesis has been invoked to explain why several single locus effects have been found in heterozygosity-fitness correlations (Merilä et al. 2003; Hansson et al. 2004; Van Oosterhout et al. 2004; Lieutenant-Gosselin and Bernatchez 2006). When looking for each marker's influence on the fitness-related traits showing correlations with overall heterozygosity in this study, it looks like several markers add up the overall significance for body mass, but that tarsus length is more strongly associated with two loci that were significant alone. Hence, heterozygosity-fitness correlation in this species seem to be due to the impact of several local effects rather than a general heterozygosity effect. This is corroborated by the fact that heterozygosity estimated by two random subsets of the markers was not significantly correlated, which would be predicted if heterozygosity reflected inbreeding (Kempenaers 2007).

I found no significant correlation between heterozygosity and male UV/blue throat colour measurements. Previous studies have found strong relationships between male colour, female mate choice and paternity in bluethroats (Johnsen et al. 1998a; Johnsen et al. 2001) and colour is hence strongly related to fitness. However, these effects seem to be unrelated to heterozygosity.

I found no correlations between heterozygosity and paternity or female clutch size, unlike other studies of passerines (Foerster et al. 2003; Tomiuk et al. 2006). Fossøy et al. (2008) found a significant effect in one of the four years for total fertilization success in the

same bluethroat population. However, total fertilization success was not investigated in the present study since a precise estimate of this would require far more sampling of the potential extra-pair males in the area.

There were no significant differences in heterozygosity between WPO and EPO in mixed broods, but there was a positive trend towards higher heterozygosity in EPO as found in Fossøy et al. (2008). It is necessary to point out that these two studies, although representing the same population, are not directly comparable. First, the higher heterozygosity of EPO found in Fossøy et al. (2008) was based on a large, four-year dataset combined. For each year the magnitude of the effect fluctuated, but showed the same trend in all years. Sample size was also larger every single year in the Fossøy et al. (2008) study, ranging from 13 to 33 mixed broods each year. Hence, the lack of significant effect in the present study may be due to a lack of statistical power. Another question is the reliability of detecting social fathers. My sample had a very high proportion of nests with full loss of paternity for the social fathers. The proportion was 7/29=0.24, compared with 16/191=0.08 in the same area over four years in Fossøy et al. (2008). This difference in proportion of full loss nests might be due to random effects of the small sample size, or it might be due to misidentification of some social males. Most social males were observed feeding chicks or helping with nest building and hence obviously connected to the particular nest. However, some males were caught earlier in the season by the nest and thereby assumed as the social father. This group included five of the seven social fathers in full loss broods. There are possibilities for misidentification in this group.

An important finding in this study was that a few paternity markers do not detect all EPO. The main argument for paternity assessment based on a limited number of markers in previous studies has been a high overall exclusion probability (Jamieson 1994). However, it is important to note that the exclusion probability gives the likelihood of detecting EPO based on one mismatch. Normally, two mismatches have been the criterion to assess paternity and hence the effective exclusion probability is lower. In addition, a correct exclusion probability requires a genotype score at every locus. A missing genotype in the offspring will make it more difficult to detect mismatches. Missing genotypes at parents is even more problematic, because you can not be sure from whom a particular allele is inherited and hence may not be able to detect a mismatch where present. Whether these assumptions have been taken into account when using high exclusion probability as an argument for complete paternity assessment in previous studies is rarely mentioned. When I based paternity assessment on six

highly polymorphic markers, which is about the same as that used in several previous studies (Foerster et al. 2003), I was only able to detect 44 of the 54 EPO. Even if only one of these were genotyped at all paternity markers and had no mismatching locus, the exclusion probability was 0.9999, implying that these six markers should only miss out one of 9999 offspring (not one of 152). It seems that exclusion probability as calculated e.g. in Cervus is too optimistic. This study thus supports the claim by Wetzel and Westneat (2009) that paternity studies based on small marker sets will underestimate true paternity. However, Wetzel and Westneat (2009) also predicted that the lack of total detection of EPO would lead to a bias towards higher heterozygosity in EPO, in studies of heterozygosity-differences between EPO and WPO. By running the same linear mixed model analyses as used in the original EPO/WPO comparison, I thus expected to find a higher heterozygosity difference in these comparisons than the one based on all 43 markers. The result went in the opposite direction, as thetrend towards more heterozygous EPO was weakened when the test was based on six paternity markers compared to the test based on 43 markers. This conclusion was the same whether I based the heterozygosity estimate on the six markers used for paternity or all 43 markers used in the original test. It should be noted, however, that the individual that had full score at all six loci and no mismatch was the individual with the lowest heterozygosity among the 10 EPO not detected by the six selected paternity markers, adding some support for Wetzel and Westneat's (2009) prediction that less heterozygous EPO are less likely to be detected.

In conclusion, this study suggests that heterozygosity-fitness correlations in the bluethroat are due to local effects, ranging from a few to several loci that together drives the overall correlation. Findings of various correlations between heterozygosity and fitness components will then depend on the marker set used, since various fitness components will be in linkage disequilibrium with different loci. This might lead to a selection for females to chose extra-pair males that increase the heterozygosity of their offspring. This has previously been found in this population, and this study found a trend in the same direction. This study lends support to Wetzel and Westneat's (2009) critique that many EPO might go undetected if paternity is based on a limited set of markers, however the suggestion that the higher heterozygosity in EPO compared to WPO is biased due to shared markers was not supported in this study.

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Tables and figures

Table 1 Characteristics of all microsatellite used. *n* is the number of individuals, *k* is the number of alleles, H(Obs.) observed heterozygosity, H(Exp.) expected heterozygosity, PIC is the polymorphic information content, HW test of Hardy-Weinberg equilibrium and F an estimate of proportion null alleles.

Microsatellite	*n*	*k*	H(Obs.)	H(Exp.)	PIC	HW	F(null)
Ase19	78	17	0.744	0.848	0.828	NS	0.0649
Cuμ4	81	15	0.84	0.86	0.839	ND	0.01
Mcyμ4	84	20	0.964	0.907	0.894	ND	-0.0338
PAT-MP 2-43	81	19	0.914	0.865	0.846	NS	-0.0342
Ppi2	82	30	0.951	0.954	0.946	ND	0.0087
FhU2	83	10	0.446	0.515	0.496	ND	-0.0018
Lm6	79	9	0.62	0.52	0.48	NS	0.0888
PmaC25	86	8	0.547	0.517	0.436	NS	-0.1077
ZF-S9	83	7	0.434	0.446	0.416	NS	-0.0411
ZF-S8	77	15	0.805	0.783	0.757	NS	0.0187
EST10	75	14	0.787	0.835	0.812	NS	-0.0174
EST16	74	8	0.473	0.551	0.495	NS	0.0201
EST31	80	6	0.238	0.477	0.439	***	0.0652
EST9	72	35	0.917	0.963	0.954	ND	0.3561
GG-C25	79	6	0.734	0.776	0.736	NS	0.0206
ZF-C59	76	8	0.461	0.605	0.557	NS	0.0235
EST46	79	13	0.785	0.805	0.774	NS	0.1162
EST62	80	11	0.663	0.686	0.639	NS	0.009
FH350	82	5	0.159	0.204	0.195	ND	0.0156
FH407	77	31	0.896	0.939	0.929	ND	0.1347
Fh310	79	25	0.81	0.87	0.852	ND	0.0179
Fh326	73	12	0.548	0.743	0.709	NS	0.0357
Fh336	78	24	0.808	0.918	0.906	ND	0.1416
Fh361	82	5	0.439	0.657	0.604	NS	0.0599
FH304	70	23	0.457	0.903	0.889	ND	0.1942
FH356	84	3	0.167	0.167	0.158	ND	0.3258
FH403	81	19	0.728	0.813	0.786	NS	0.0189
FH405	64	26	0.453	0.909	0.894	ND	0.0537
FH408	79	25	0.924	0.947	0.938	ND	0.3342
FH413	81	3	0.568	0.532	0.444	NS	0.0093
Fh344	78	7	0.731	0.764	0.723	NS	-0.0416
EST17	81	13	0.716	0.879	0.86	ND	0.0159
Fh431	84	10	0.738	0.703	0.663	NS	0.1007
Fh448	80	21	0.9	0.922	0.91	NS	-0.0366
Fh452	79	7	0.582	0.502	0.418	ND	-0.0756

Fh465	78	31	0.936	0.916	0.904	NS	-0.0135
Fh466	73	15	0.795	0.781	0.744	NS	-0.022
Fh221	77	15	0.701	0.838	0.814	ND	0.083
Fh224	56	23	0.357	0.93	0.916	ND	0.4424
Fh225	70	24	0.914	0.897	0.884	NS	-0.0173
Fh227	81	11	0.667	0.661	0.607	ND	-0.0106
Fh230	76	16	0.868	0.846	0.823	ND	-0.0203
Fh359	73	15	0.904	0.895	0.879	NS	-0.0094

Table 1 continued

Table 2 Characteristics of panels, with information about annealing temperature, volume of forward and reverse primer (100 µ/mol), dye type and size range of alleles.

Microsatellite	Panel	PCR annealing temperature	Volume of each primer to primer mix (110 samples)	Dye	Range
ZF-S9	1	59	1.5	NED	135-185
ZF-S8	1	59	6	NED	225-265
EST10	1	59	1.5	PET	130-175
EST16	1	59	4	NED	280-320
EST31	1	59	5	FAM	325-360
EST9	1	59	1.5	VIC	375-455
GG-C25	1	59	1.5	FAM	225-265
ZF-C59	1	59	8	PET	280-320
EST46	2	56	2	NED	200-250
EST62	2	56	3	VIC	385-430
FH350	2	56	3	NED	100-140
FH407	2	56	3	FAM	170-250
Fh310	2	56	2	PET	280-340
Fh326	2	56	3	NED	320-350
Fh336	2	56	3	PET	125-205
Fh361	2	56	3	NED	370-400
FH304	3	56	4	VIC	200-280
FH356	3	56	3	PET	350-390
FH403	3	56	3	FAM	100-190
FH405	3	56	8	PET	95-200
FH408	3	56	4	NED	115-290
FH413	3	56	3	VIC	370-570
Fh344	3	56	4	VIC	300-330
EST17	4	56	3	NED	280-430
Fh431	4	56	2	NED	165-220
Fh448	4	56	2	PET	110-200
Fh452	4	56	2	VIC	265-320
Fh465	4	56	10	FAM	165-280
Fh466	4	56	4	VIC	125-175
Fh221	5	56	3	VIC	140-190
Fh224	5	56	4	NED	320-405
Fh225	5	56	3	PET	340-390
Fh227	5	56	3	PET	215-250
Fh230	5	56	5	VIC	330-370
Fh359	5	56	3	FAM	190-240

Table 3. General linear models testing the relationship between heterozygosity and phenotypic characters. Each variable is controlled for age (males) and capture date, body mass also for tarsus length. Factors were removed in a backwards stepwise fashion $p>0.1$. (A) male heterozygosity and (B) female heterozygosity.

A)			All markers		
Response variable	Factor	*n*	Estimate±SE	Test statistic	*p*
Tarsus length	Heterozygosity	43	3.33±1.22	$F= 7.40$	0.010
Body mass	Heterozygosity	43	3.01±1.01	$F= 8.83$	0.005
	Age			$F= 6.96$	0.012
Wing length	Heterozygosity	43	1.30±2.68	$F= 2.35$	0.630
Chestnut-band width	Heterozygosity	43	-5.09±2.48	$F= 4.20$	0.047
Colour: Hue	Heterozygosity	37	3.72±8.14	$F= 0.18$	0.651
Colour: Brightness	Heterozygosity	37	-2.67±6.39	$F= 0.004$	0.951
	Age			$F=3.66$	0.064
Colour: Chroma	Heterozygosity	37		$r= 0.16$	0.349
B)					
Response variable	Factor				
Tarsus length	Heterozygosity	27	1.26±1.42	$F= 0.80$	0.380
Body mass	Heterozygosity	27	6.30±2.44	$F= 6.68$	0.016
Wing length	Heterozygosity	27	4.42±3.45	$F= 1.57$	0.222
Colour score	Heterozygosity	20	-2.10±5.19	$F= 0.16$	0.690

Table 4 Correlations between subsets of markers, all adults *n*=84 included. Subset 1 and 2 constitute two halves of the marker set (see methods). Subset 3 consist of the six paternity markers, and subset 4 the remaining 37 markers.

		All markers	Subset 1	Subset 2	Subset 3
Subset 1	*r*	0.76			
	p	<0.001			
Subset 2	*r*	0.73	0.14		
	p	<0.001	0.21		
Subset 3	*r*	0.26	0.09	0.29	
	p	0.018	0.40	0.008	
Subset 4	*r*	0.92	0.75	0.63	-0.10
	p	<0.001	<0.001	<0.001	0.37

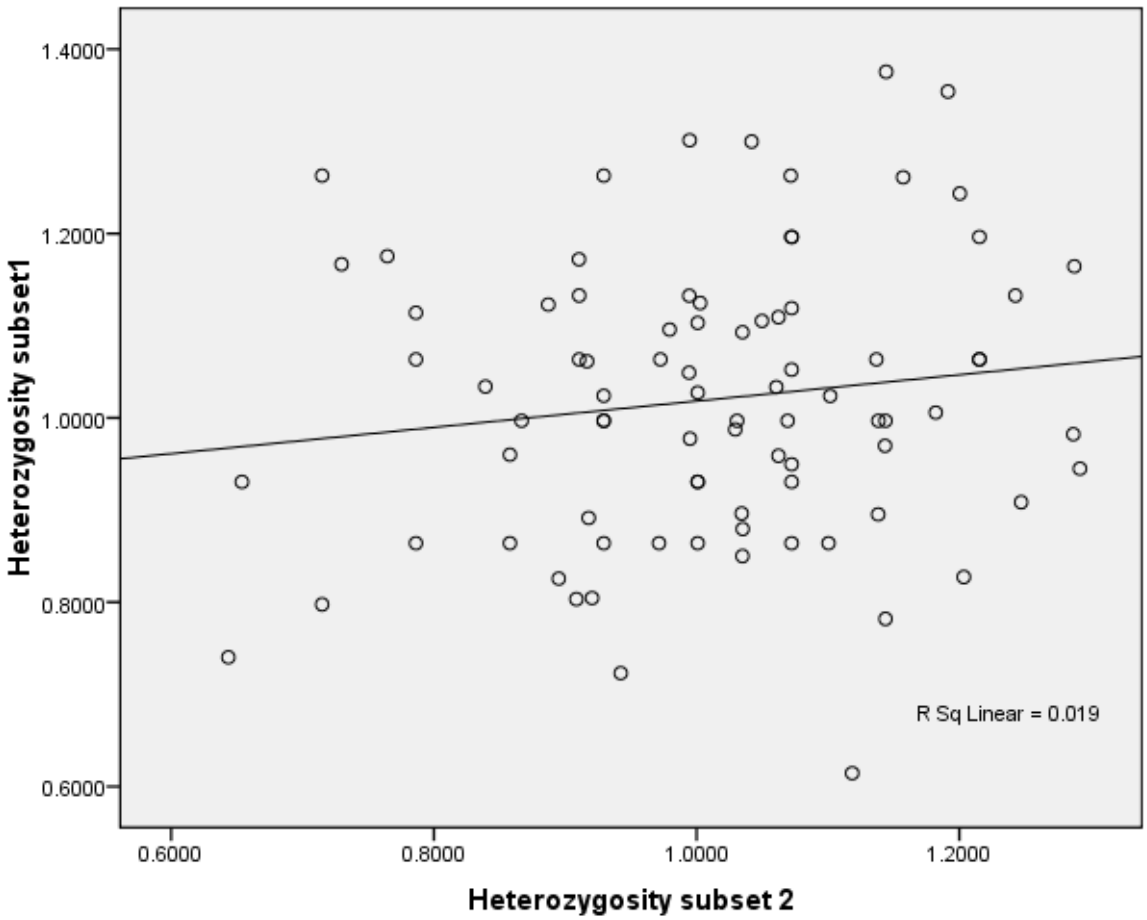

Fig. 1 Correlation between two random subsets of microsatellite markers (n=84, p=0.214, r=0.137). The regression line is shown for visual purposes.

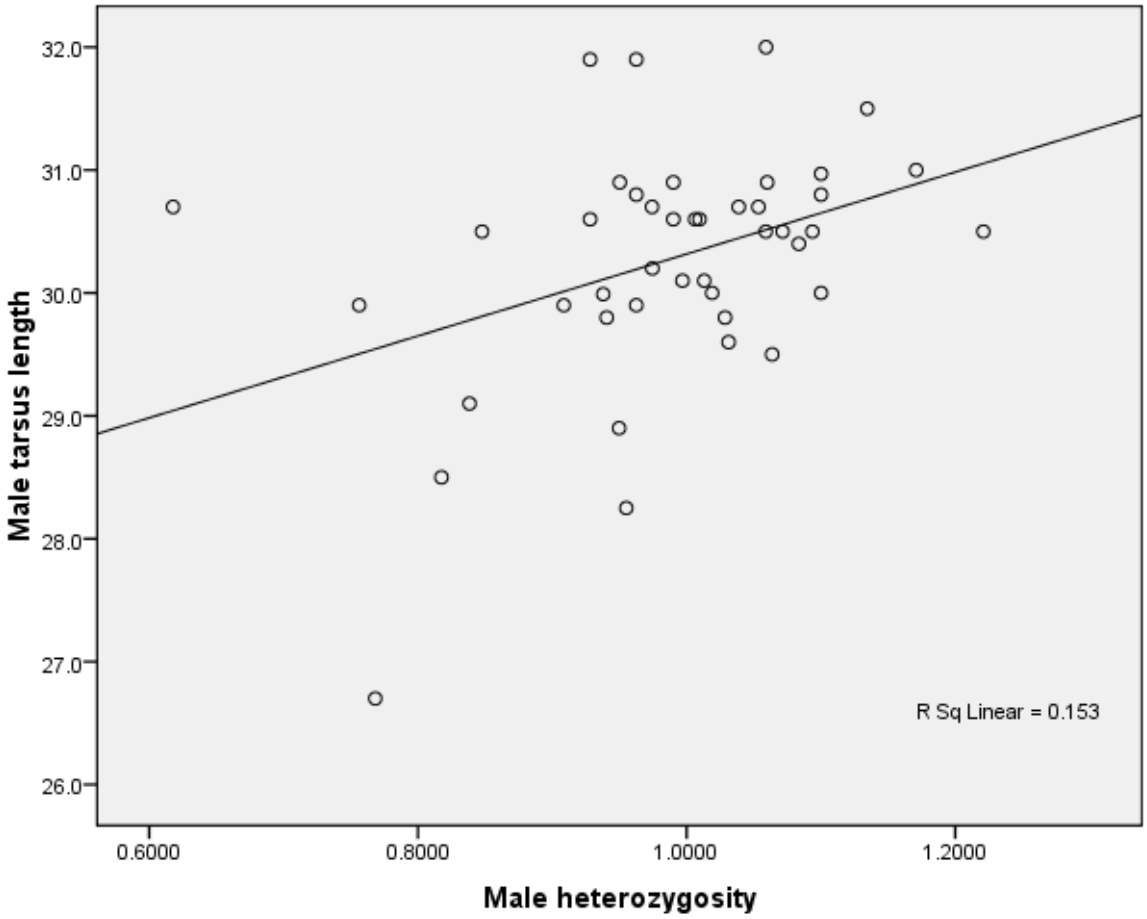

Fig. 2 Correlation between male heterozygosity (SH) and tarsus length (n=43, r=0.31, p=0.010). The regression line is shown for visual purposes.

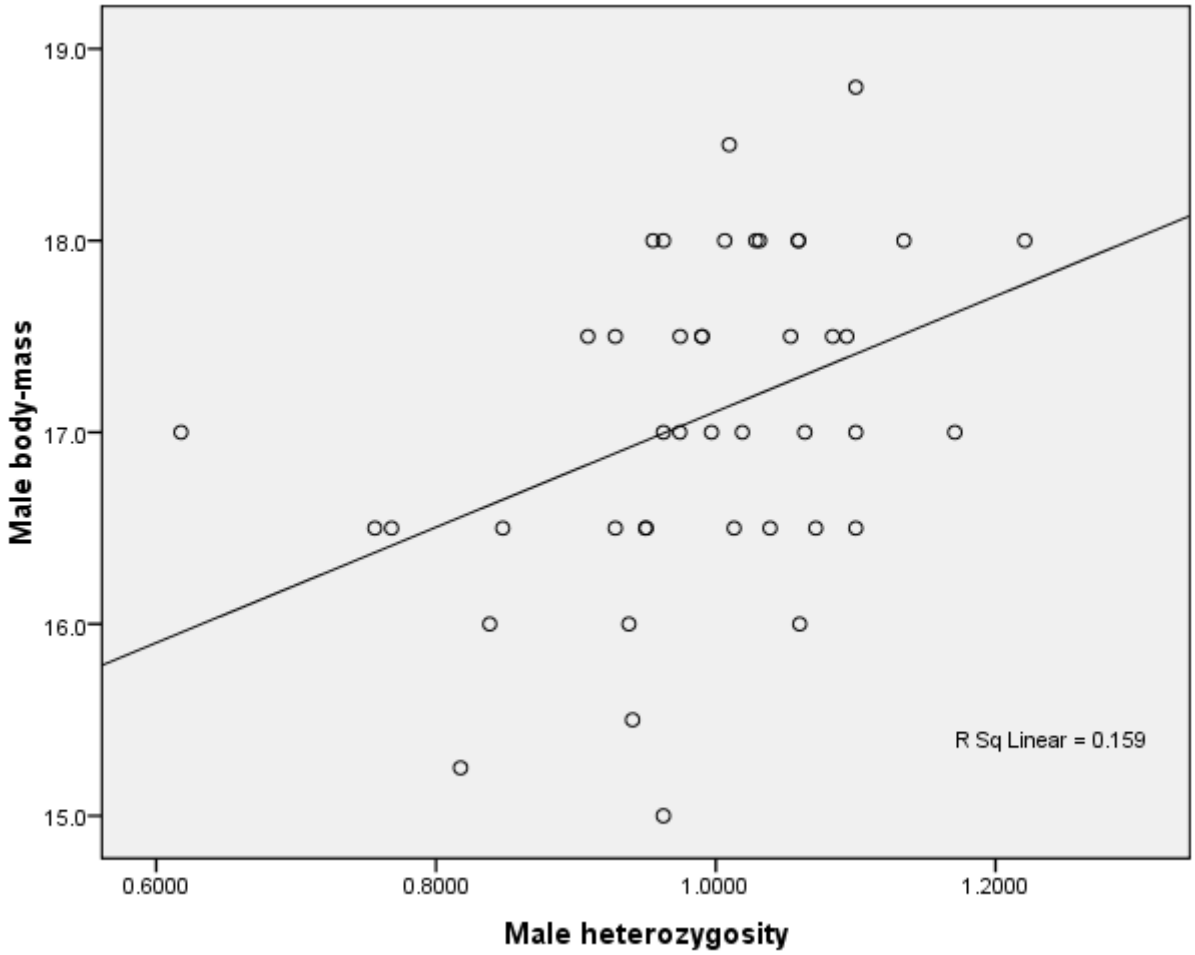

Fig. 3 Correlation between male heterozygosity (SH) and body mass (n=43, r=0.398, p=0.008). The regression line is shown for visual purposes.

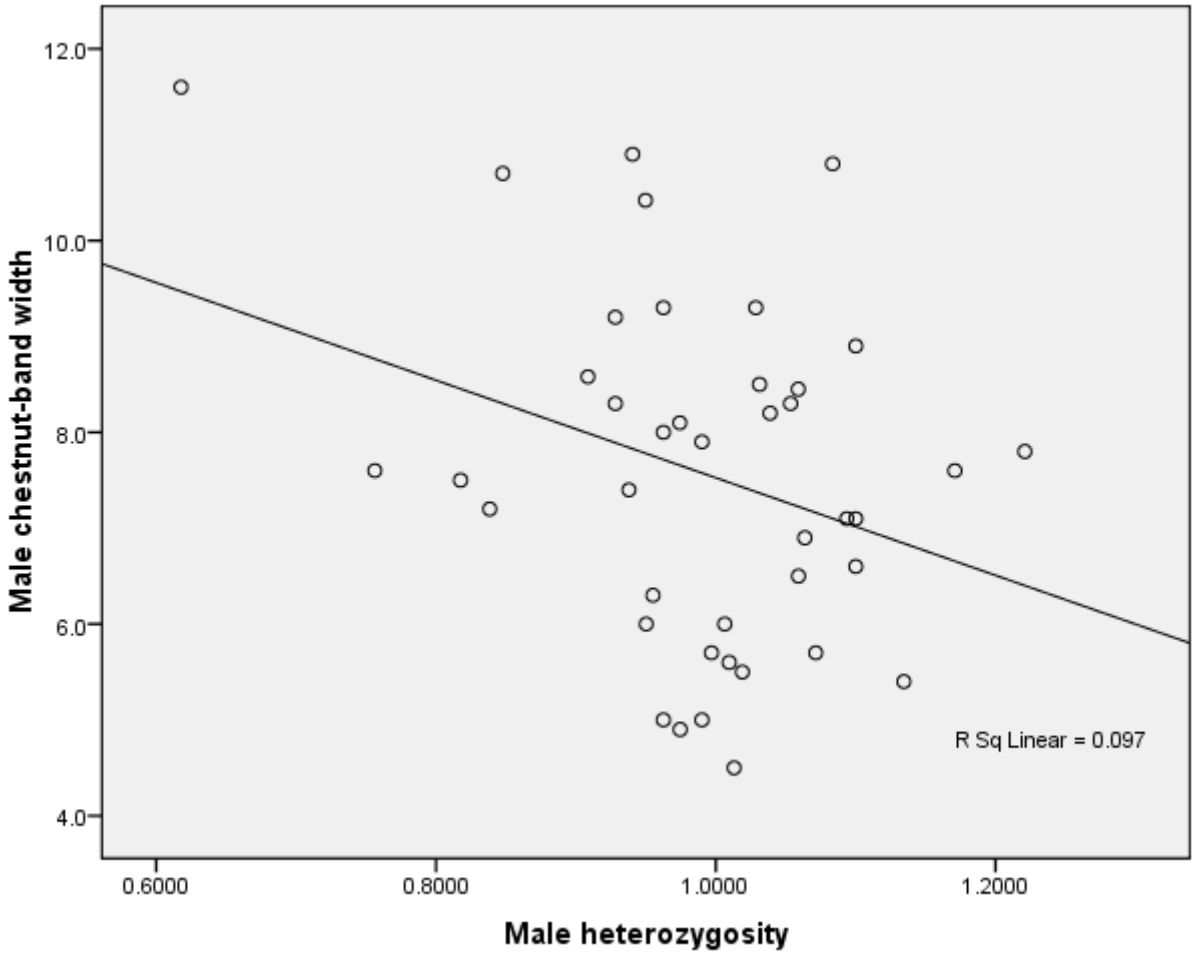

Fig. 4 Correlation between male heterozygosity (SH) and chestnut-band width (n=41, r=-0.312, p=0.047). The regression line is shown for visual purposes.

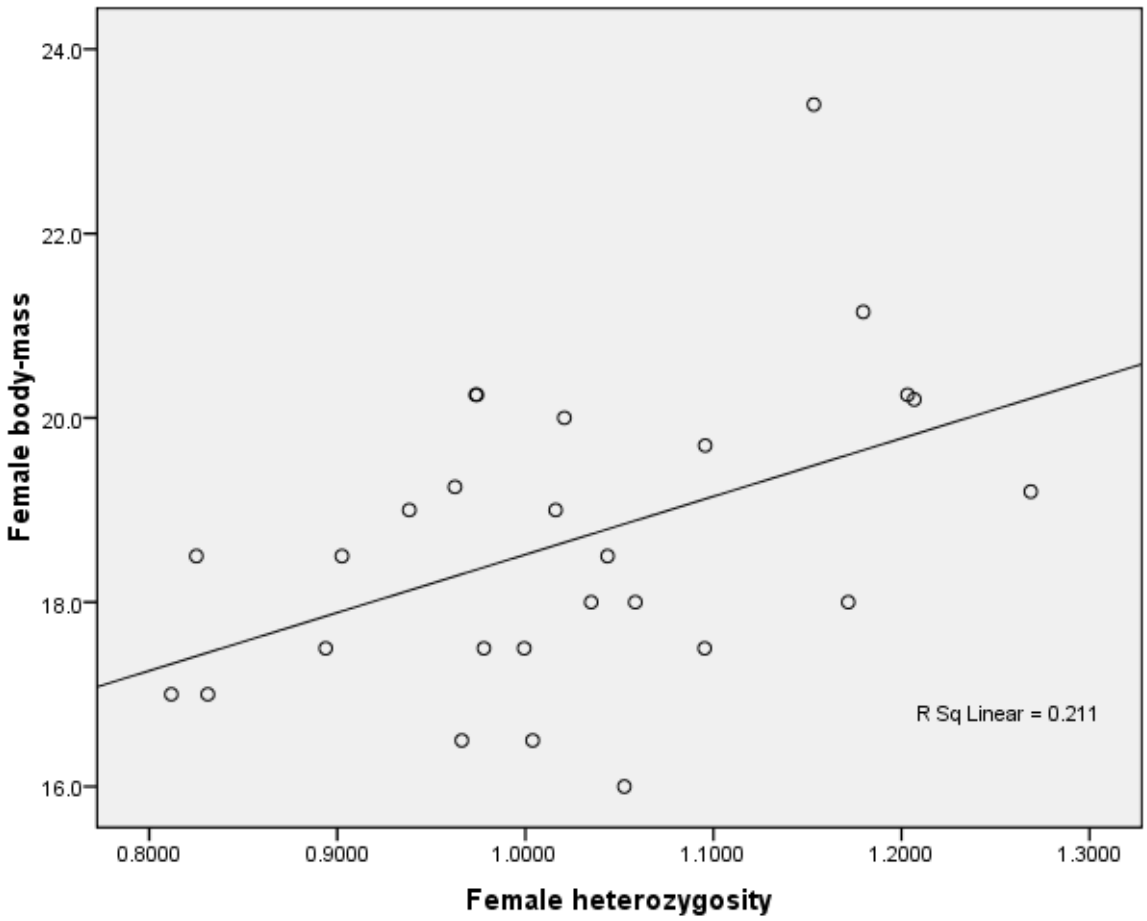

Fig. 5 Correlation between female heterozygosity (SH) and body mass (n=27, r=0.459, p=0.016). The regression line is shown for visual purposes.

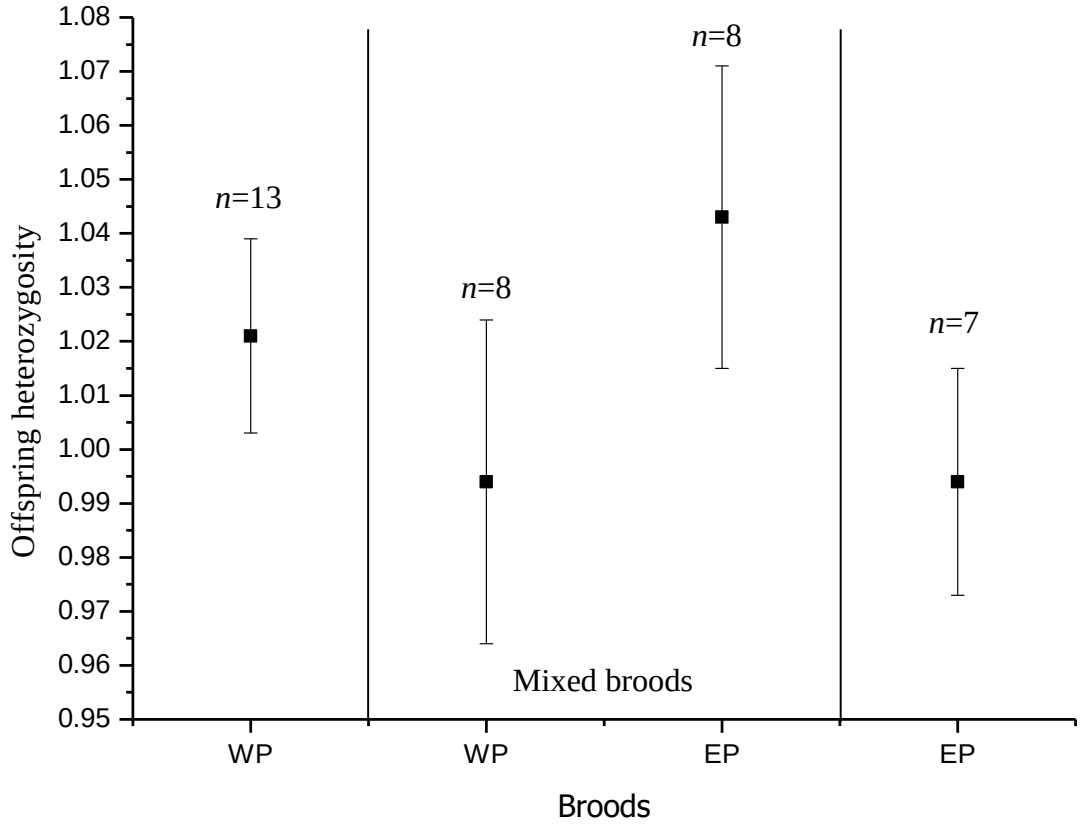

Fig. 6 Offspring heterozygosity in relation to brood paternity