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Research article

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Individual heterozygosity may influence the expression of fitness-related traits, via genome-wide or local genetic effects. Earlier studies have shown negative relationships between heterozygosity and sperm variation, predominantly in captive, highly inbred populations. Little is known about the possible influence of variation in heterozygosity on sperm traits in wild, outbred populations. We studied two populations of the bluethroat, one from the widely distributed northern subspecies *Luscinia. s. svecica* and the other from the smaller, more patchily distributed subspecies breeding along the French coast of Brittany *L. s. namnetum*. The two subspecies differed significantly in body size, plumage colour, sperm traits and the degree of genetic diversity. However, there was no evidence that sperm traits (total length and motility) were influenced by the degree of heterozygosity at the individual level. In contrast, we found that male body size was positively related to heterozygosity across both populations, indicating a possible relationship between overall genetic diversity and general vigour or ability to obtain food. We conclude that sperm traits are unrelated to levels of heterozygosity in the studied outbred and weakly genetically depauperate bluethroat populations.

Keywords: heterozygosity-fitness relationships, microsatellites, sperm morphology, sperm motility, subspecies divergence

Introduction

Individual genetic diversity often shows positive relationships with fitness-related characters (Kempenaers 2007). Such heterozygosity-fitness correlations (HFC) may be mediated by genome-wide effects, affecting the individual's overall physical health (condition/state/viability), or local genetic effects, influencing traits more directly (Hansson and Westerberg 2002, Kempenaers 2007). HFCs are most likely to be found

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in small, genetically depauperate populations and populations with high levels of inbreeding, where reduced levels of heterozygosity can lead to increased expression of deleterious alleles and reduced fitness of individuals (Charlesworth and Willis 2009).

Most studies of HFCs have investigated external traits related to fitness, like measures of body size or secondary sexual traits (Kempenaers 2007). However, primary sexual traits like spermatozoa and pollen may also be influenced by individual genetic diversity. Indeed, a recent review found evidence for overall negative effects of inbreeding on gametic performance across a large number of animal and plant studies, the vast majority of which were on captive populations (Losdat et al. 2014). For example, a comparative study of several endangered, mostly captive mammal species showed that species with higher level of homozygosity had reduced sperm quality, both in terms of sperm abnormality and motility (Fitzpatrick and Evans 2009). Similarly, at the within-species level, experimentally induced inbreeding in zebra finches *Taeniopygia guttata* resulted in lower sperm motility and increased frequency of sperm damage, while there was no effect on the length of normal sperm cells (Opatová et al. 2016). Studies of wild populations are rare and have given mixed results. In a study of European rabbits *Oryctolagus cuniculus*, microsatellite heterozygosity was negatively correlated with the degree of sperm abnormality within and across populations (Gage et al. 2006), while a study of a moderately inbred wild population of song sparrows *Melospiza melodia* found no evidence of inbreeding depression on male sperm characteristics, using pedigree data (Losdat et al. 2018).

The bluethroat is a socially monogamous passerine, with a relatively high rate of extra-pair paternity (Questiau et al. 1999, Johnsen and Lifjeld 2003). The species consists of about 10 subspecies (Cramp 1988), which differ in coloration, size and sperm characteristics (Johnsen et al. 2006, Hogner et al. 2013). We investigate relationships between individual heterozygosity and sperm characteristics in two highly differentiated bluethroat subspecies, *L. s. svecica* and *L. s. namnetum*. The two subspecies differ in the color of the throat spot (chestnut vs white), the chroma of the blue feathers and in body size (Johnsen et al. 2006), as well as in sperm characteristics (Hogner et al. 2013, this study). The two subspecies also differ greatly in population size and hence degree of genetic diversity: *svecica* is widely distributed over most of the northern Palearctic and can be considered panmictic, while *namnetum* occurs in small isolated populations, scattered along the French Atlantic coast (Eybert et al. 2004, Johnsen et al. 2006, Marquet et al. 2014). Accordingly, *namnetum* shows lower microsatellite allelic richness and a higher estimated inbreeding coefficient than *svecica* (Johnsen et al. 2006), potentially increasing the likelihood of detecting relationships between variation in heterozygosity and sperm characters.

The aims of this paper are threefold. First, we test whether previously found differences between the two subspecies in genetic variation and sperm characters are upheld in a larger sample and with a higher number of microsatellite markers, adding two aspects of sperm behavior (sperm velocity and proportion of motile sperm cells). Second, we test the hypothesis that individual heterozygosity influences sperm traits, predicting that the level of heterozygosity will be positively correlated with sperm length and sperm motility (assuming that length and motility are positively related to fitness; Simmons and Fitzpatrick 2012) and negatively correlated with sperm length variation (assuming that variation is negatively related to fitness; Immler et al. 2008). We also predict that the effects will be more pronounced in *namnetum* than in *svecica*, due to its lower genetic variation. Finally, we investigate relationships between heterozygosity and other fitness-associated characters (morphology and colouration) and age (two age-classes). The rationale behind these tests is that individual genetic diversity could be related to the development or maintenance of body size and ornamentation (Kempenaers 2007) and/or influence survival prospects (Cohas et al. 2009), leading us to predict positive relationships between heterozygosity and body size estimates and that older individuals will be more heterozygous than younger individuals.

Material and methods

Field procedures

We studied two geographically separate bluethroat populations, *L. s. svecica* in May–June 2007–2010 and 2012–2015 in Norway (Øvre Heimdalen; 61°25′07″N, 8°53′40″E) and *L. s. namnetum* in April 2011–2015 in France (Guèrande; 47°17′17″N, 2°28′13″W, Brière; 47°21′38″N, 2°12′05″W, Marais du Mès; 47°24′44″N, 2°24′45″W, Mont Saint-Michel; 48°40′43″N 1°28′10″W). The Norwegian locality is a sub-alpine mountain valley located about 1100 m above sea level. The French localities are salt marsh/reed bed habitats located at or close to the sea level on the coast of Brittany.

Birds were captured in their territories, using mist nets or song post traps combined with playback of male or female song and clap nets baited with mealworm. Importantly, males were captured at the same stage in the respective breeding period of each population, which is about two months earlier in France than in Norway. We measured tarsus length (to the nearest 0.1 mm) using a slide calliper, wing length (to the nearest 0.5 mm) using a wing ruler, body mass (to the nearest 0.1 g) using a Pesola 50 g spring balance, and the width of the red border (a chestnut-coloured band on the lower part of the breast of males; to the nearest 1 mm) using a slide calliper. The birds were aged (second year or older) by inspecting the median and greater coverts (Svensson 1992). Blood samples were collected by brachial venipuncture and stored in 2 ml Sarstedt tubes with 1 ml 96% ethanol for later genetic analysis. Sperm samples were collected using the cloacal massage technique (Wolfson 1952), collected in a capillary tube and instantly diluted in an Eppendorf tube containing preheated Dulbecco's Eagle Medium (DMEM) in 2012 and phosphate-buffered saline (PBS) in 2013–2015, for motility measures (details below). The remaining ejaculate was stored

in a 5% formaldehyde solution for later sperm morphology measurements. All of the captured birds were marked with a numbered aluminium ring and three colour bands for individual identification. After processing, the birds were released back in their territories. We sampled a total of 290 individual bluethroats (males: 273, females: 17) for this study, but the sample sizes vary in the different analyses for reasons explained below. Females were only included in the analyses of genetic differences between the populations. A number of males (n = 49) were caught and sampled on several occasions. We only used the measurements from one of these sampling events, to avoid pseudoreplication. As a general rule, we used the measurements from the first sampling event, unless there was another sampling event with more sperm cells measured.

DNA extraction and microsatellite typing

DNA was extracted using the Omega Bio-Tek E-Z 96 Blood DNA Kit (D1199-01), following the protocol of the manufacturer. Twenty-one microsatellites were amplified by polymerase chain reaction (GeneAmp® PCR System 9700 (Applied Biosystems)). The markers were originally isolated from pied flycatcher *Ficedula hypoleuca* and zebra finch *T. guttata* (Karaiskou et al. 2008, Leder et al. 2008), and optimized for the bluethroat. They were sorted in five panels (1–5) and run using multiplex PCR (Supporting information). Each 10 µl PCR reaction consisted of 5 µl 2 × Qiagen Multiplex Master Mix (Qiagen), 1 µl primer-mix, 3 µl RNase-free water and 1 µl DNA extract. For all panels, the following PCR program was used: 95°C for 15 min, then 34 cycles of 94°C for 30 s, 59°C (panel 1) or 56°C (panels 2–5) for 1:30 s, 72°C for 1 min, before a final elongation step of 60°C for 15 min. To confirm amplification success, we tested 3 µl of the PCR product on an 1% agarose electrophoresis gel. After PCR, the products were diluted 1:99, after which 2 µl (1 µl for panel 5) of product was combined with 9.5 µl HiDi and 0.5 µl Liz 600 and ran on an ABI Prism® 3130XL Genetic analyzer (Applied Biosystems) using fluorescently labeled primers. Allele sizes were determined using GeneMapper™ Software ver. 4.0 (Applied Biosystems).

We performed Hardy–Weinberg and null-allele tests in Cervus ver. 3.0.7 (Supporting information for marker characteristics). Two markers with low variability (three alleles in each) showed relatively high estimated frequencies of null alleles (FH356: 0.118, FH413: 0.095). Assuming that null alleles were randomly distributed in the two populations, we have chosen to include these markers in our analyses. Overall genetic differentiation was estimated by the F_{ST} index (Weir and Cockerham 1984) using FSTAT, with the significance level estimated from 10 000 randomisations. Allelic richness, adjusted to the smallest number of individuals typed for a given marker and subspecies (n = 31), was calculated in FSTAT. In total, we obtained multilocus genotypes for 158 individuals (*svecica*: 120 (107 male, 13 female), *namnetum*: 38 (34 male, 4 female)), with an average ± SD of 20.75 ± 0.67 (range 16–21) markers per individual. There was an incidental significant difference in the number of typed loci for the two subspecies (*svecica*: 20.86 ± 0.04 (SE), *namnetum*: 20.34 ± 0.18, Welch two-sample t-test, t = 2.82, df = 41.1, p = 0.007).

Heterozygosity

We calculated individual heterozygosity as the number of heterozygous loci divided by the number of loci typed for that individual. Not all markers are represented for all the individuals, therefore we also calculated standardized heterozygosity by dividing the proportion of heterozygous loci for an individual by the population-specific mean observed heterozygosity for all loci typed for that individual (Coltman et al. 1999). Unmodified heterozygosity was used in the analysis of subspecies differences (since the standardization removed the subspecies differences), while standardised heterozygosity was used in all other analyses.

Sperm analyses

Sperm morphology

Approximately 10–15 µl of diluted, fixed sperm was spread out on a microscope slide with a pipette and dried overnight. The slide was then washed with distilled water, to remove salt crystals, and left to dry for at least one hour. From each male, 10 or 30 (below) normal sperm cells were photographed, using a Leica DFC420 camera mounted on a Leica DM6000 B digital light microscope to obtain digital images at magnification of 160×. Sperm morphometry was performed using the image analysis software Leica Application suite ver. 4.1. Sperm cells consist of three components; head, midpiece and tail, which were measured separately. The total length of the sperm cell was calculated by adding the length of these three components. Since four different persons were involved in the measurement of sperm morphology and preliminary analyses indicated large inter-measurer differences in the estimation of head length ($F = 371.1$, $p < 0.0001$, effect size (95% CI): 0.59 (0.53–1.00)), we only used total sperm length in further analyses. There was, however, a significant albeit weaker inter-measurer difference also in total sperm length ($F = 6.63$, $p = 0.011$, effect size (95% CI): 0.02 (0.00–1.00), hence we included measurer ID in all initial multivariable models involving total sperm length. We calculated the within-male coefficient of variation of total sperm length, CVwm = SD/mean × 100. For about half of the 262 males (n = 134), 30 sperm cells were measured, while for the rest (n = 128) 10 sperm cells were measured. The percentage of males with only 10 cells measured was the same in both subspecies (49%). We used average total sperm length in the analyses. We obtained sperm length estimates from 262 males. Thirty-eight of these were sampled in the years 2007–2011 and their sperm morphological measurements were included in a previous paper (Hogner et al. 2013). These measurements were not included in analyses of population differences in the present paper, which thus comprise data from 224 males. Data from *svecica* from 2013–2015 were taken from Sætre et al. (2018), where they were used in a different context.

Motility

Immediately after the sperm were collected (in the field, see above), the samples were diluted in pre-heated DMEM (2012) or PBS (2013–2015) set to 40°C. DMEM was initially used because it is a standard dilution medium for assessing sperm motility, while PBS was used later in order to have a protein-free diluent for another experiment (Cramer et al. 2016). Then 3–5 µl of the diluted sperm was placed in a pre-heated microscopy counting chamber (depth 20 mm; Leja Products BV, Nieuw-Vennep, the Netherlands) and mounted on a MiniTherm stage warmer (Hamilton Thorne Biosciences, Beverly, MA; 2012–2013) or a Tokai Hit TP-S glass stage (2014–2015, Gendoji-cho, Fujinomiya-shi, Shizuoka-ken, Japan) maintained at a constant temperature of 40°C. Sperm movement was then recorded using a phase contrast microscope (model CX41, Olympus, Japan) with a connected digital video camera (model HDR-HC1C, Sony, Tokyo, Japan) (Laskemoen et al. 2013b). Each of the sperm samples were recorded for about 30 s and 6 frames were used to optimize the recording of the sperm cells. For males included in experiments described in Cramer et al. (2016), only control conditions were included here (see also data description in Sætre et al. 2018).

For measuring the motility of the sperm cells, a computer-assisted sperm analysis (HTM-CEROS II Sperm Analyzer; Hamilton Thorne Research, Beverly, MA) was used. The sperm analyser was set at a frame rate of 50 Hz and 25 frames (i.e. sperm cells were tracked for 0.5 s). Each analysis was visually examined, and cell detection parameters were adjusted using the two interactive quality control plots as well as directly from visual examination of each recording. Three estimates of sperm velocity were calculated: straight line velocity (VSL), average path velocity (VAP), and curvilinear velocity (VCL). To remove the potential effect of drift in the chamber, sperm cells with VAP less than 10 µm s^{-1} and VSL ≤ 5 µm s^{-1} were counted as static and excluded from the swimming speed analysis. We used VCL as our measure of sperm velocity, but all three measures where highly intercorrelated (all $r > 0.92$, all $p < 0.0001$) and analyses using the other two estimators gave qualitatively similar results (not shown). Only recordings with a minimum of 10 motile sperm cells surviving the quality filters were included in the analyses (average number of motile sperm cells: 88.5, range 10–509). Proportion of motile sperm was calculated as the number of motile sperm (here including non-static sperm cells with VAP ≤ 10 µm s^{-1} and VSL ≤ 5 µm s^{-1} as non-motile cells) divided by total number of sperm in the frames. We obtained sperm motility estimates from 134 males (*svecica*: 87, *namnetum*: 47), all sampled in 2012–2015.

Statistical analyses

All statistics were performed in R ver. 4.2.2 (www.r-project.org). In the analyses of subspecies differences, we used non-parametric Wilcoxon rank sum test with continuity correction for all sperm- and morphological variables except total sperm length, due to significant departure from normality in Shapiro–Wilk tests. For total sperm length, we used a Welch two-sample t-test. For the pairwise comparison of allelic richness in the 21 microsatellite markers, we used a Wilcoxon signed rank test with continuity correction. For individual heterozygosity, which did show a significant departure from normality ($p = 0.012$), we performed both a Wilcoxon rank sum test and a general linear model (GLM), the latter to be able to control for the difference between the subspecies in the number of typed loci.

In the analyses of relationships between heterozygosity on the one hand, and sperm and morphological variables on the other, we performed Spearman correlations and multivariable GLMs. For all GLMs, model assumptions were checked manually by inspecting qq-plots and plots of homoscedasticity. For variables showing significant subspecies differences (total sperm length, CVwm of total sperm length, tarsus length, wing length, body mass and red border width; Table 1), we centred the variables with respect to subspecies, using the package misty (Yanagida 2022). The full models included the following independent variables: standardised heterozygosity, subspecies and the interaction between standardised heterozygosity and subspecies (all models), measurer-ID and number of sperm cells measured (total sperm length and CVwm of total sperm length), number of motile cells in calculation (average VCL), and time of capture and measurer-ID (tarsus length, wing length, body mass and red border width). Variance inflation factors, calculated using the package car (Fox and Weisberg 2019), were all below 3.46, indicating low to moderate collinearity among the independent variables (Montgomery and Peck 1992). We present the full models, after removing non-significant interaction terms

Table 1. Tests of subspecies differences in sperm characters and male morphology.

	Luscinia svecica svecica			*Luscinia svecica namnetum*				
Variable	Mean	95% CI	n	Mean	95% CI	n	Statistic	p
Total sperm length (um)	210.63	209.62–211.60	129	204.20	202.99–205.44	95	t = 7.75	< 0.0001
CVwm[1] of total sperm length	1.67	1.58–1.76	129	1.88	1.76–2.01	95	W = 4847	0.008
Average VCL[2]	151.64	145.13–157.73	87	153.29	145.59–160.58	47	W = 1994	0.82
Prop. of motile sperm	0.45	0.41–0.49	87	0.45	0.39–0.52	47	W = 2073	0.90
Tarsus length (mm)	30.12	29.98–30.27	146	27.60	27.46–27.75	109	W = 15539	< 0.0001
Wing length (mm)	75.94	75.64–76.24	147	68.40	68.08–68.72	110	W = 16155	< 0.0001
Mass (g)	16.92	16.80–17.05	145	14.99	14.84–15.15	108	W = 14992	< 0.0001
Red border width (mm)	7.55	7.21–7.91	147	8.24	7.82–8.68	97	W = 5829	0.015

[1]CVwm = coefficient of variation within males, [2]VCL = curvilinear velocity

($p > 0.1$). Effect sizes are given as partial eta squared, calculated in the package effectsize (Ben-Shachar et al. 2020).

Results

Population differences in genetics and morphology

The two populations were significantly differentiated ($p = 0.002$), with an overall F_{ST} value of 0.068 (95% CI: 0.051–0.087). The *namnetum* population exhibited less variation in the microsatellites than the *svecica* population, as evidenced by a significantly lower allelic richness at the population level (Wilcoxon signed rank test, $V = 210$, $p < 0.0001$) and lower heterozygosity at the individual level (average proportion of heterozygous loci *svecica* ($n = 120$): 0.67 (95% CI: 0.66–0.69), *namnetum* ($n = 38$): 0.54 (95% CI: 0.53–0.58); Wilcoxon rank sum test, $W = 3832$, $p < 0.0001$). The latter test was also significant when controlling for number of typed loci in a GLM (subspecies: $F_{1, 155} = 55.03$, $p < 0.0001$, number of typed loci: $F_{1, 155} = 2.22$, $p = 0.14$).

Svecica males had significantly longer sperm and a lower CVwm of total sperm length than *namnetum* males (Table 1), but there were no differences in sperm velocity or the proportion of motile sperm (Table 1). The well-documented larger body size of *svecica* compared to *namnetum* was reflected in all three morphological traits. There was also a significant difference in red border width, with *namnetum* males having wider red borders than *svecica* males (Table 1).

Heterozygosity and sperm characteristics

There were no significant relationships between individual heterozygosity and total sperm length, variation in total sperm length, sperm velocity or proportion of motile sperm cells, neither in GLMs (Table 2) nor in Spearman correlation tests (all $|R_{sp}| < 0.07$, all $p > 0.70$). In the GLM analyses, the number of cells measured was positively related to CVwm of total sperm length, and subspecies identity was significantly related to the proportion of motile sperm cells (*namnetum* showing a higher proportion of motile sperm than *svecica*). Note that the latter relationship was not significant in the larger dataset testing for subspecies differences (Table 1).

Heterozygosity, morphology and age

Heavier males were more heterozygous than lighter ones across both subspecies (Table 3, Fig. 1a; Spearman correlation test: $R_{sp} = 0.21$, $p = 0.02$). Furthermore, there was a significant interaction effect between heterozygosity and subspecies on wing length (Table 3). Posthoc correlation tests within each subspecies showed a significantly positive correlation in *namnetum* ($R_{sp} = 0.35$, $n = 33$, $p = 0.044$; Fig. 1b) and no significant correlation in *svecica* ($R_{sp} = -0.07$, $n = 98$, $p = 0.47$; Fig. 1b). There were no significant relationships between heterozygosity, and tarsus length and red border width, respectively (Table 3; Spearman correlation tests: both $R_{sp} < 0.10$, both $p > 0.28$), and the two age-classes did not differ in heterozygosity (Welch two-sample t-test, $t = 1.01$, $df = 89.58$, $p = 0.32$). Among the other independent variables, body mass was also significantly related to the time of day the measurements were done, and body mass and red border width were significantly related to the identity of the measurer.

Discussion

We found significant genetic and morphological differentiation between the subspecies, and that *namnetum* is genetically depauperate compared to *svecica*, based on presumably neutral genetic markers. Individual heterozygosity was unrelated to sperm characters, but positively correlated with body mass across both subspecies and with wing length in *namnetum*.

Table 2. Multivariable GLM analyses of relationship between standardised heterozygosity and sperm characters.

Response	Independent	Effect size (95% CI)	df	F value	p
Total sperm length	Heterozygosity	3.2×10^{-4} (0.00–1.00)	1	0.039	0.84
	Subspecies	0.03 (0.00–1.00)	1	3.30	0.072
	Measurer-ID	0.04 (0.00–1.00)	3	1.78	0.16
	Number of cells	9.9×10^{-3} (0.00–1.00)	1	1.23	0.27
	Residuals		124		
CVwm[1] of total sperm length	Heterozygosity	2.3×10^{-3} (0.00–1.00)	1	0.29	0.59
	Subspecies	0.01 (0.00–1.00)	1	1.85	0.18
	Measurer-ID	0.05 (0.00–1.00)	3	2.37	0.07
	Number of cells	0.10 (0.03–1.00)	1	14.35	< 0.001
	Residuals		124		
Average VCL[2]	Heterozygosity	3.7×10^{-4} (0.00–1.00)	1	0.025	0.87
	Subspecies	6.5×10^{-4} (0.00–1.00)	1	0.044	0.83
	Number of motile[3]	0.04 (0.00–1.00)	1	3.19	0.079
	Residuals		68		
Prop. of motile sperm	Heterozygosity	4.1×10^{-3} (0.00–1.00)	1	0.29	0.59
	Subspecies	0.08 (0.01–1.00)	1	6.16	0.016
	Residuals		69		

[1]CVwm = coefficient of variation within males, [2]VCL = curvilinear velocity, [3]number of motile cells on which the velocity estimate was based

Table 3. Multivariable GLM analyses of relationship between standardised heterozygosity and male morphological characters.

Response	Independent	Effect size (95% CI)	df	F-value	p
Tarsus length	Heterozygosity	0.02 (0.00–1.00)	1	2.64	0.11
	Subspecies	3.0×10^{-3} (0.00–1.00)	1	0.38	0.54
	Measurer-ID	0.02 (0.00–1.00)	3	0.65	0.58
	Time of day	4.1×10^{-3} (0.00–1.00)	1	0.51	0.48
	Residuals		124		
Wing length	Heterozygosity	4.3×10^{-4} (0.00–1.00)	1	0.053	0.82
	Subspecies	1.7×10^{-4} (0.00–1.00)	1	0.021	0.88
	Measurer-ID	0.06 (0.00–1.00)	3	2.62	0.054
	Time of day	4.1×10^{-3} (0.00–1.00)	1	0.51	0.48
	Heterozygosity x Subspecies	0.03 (0.00–1.00)	1	4.40	0.038
	Residuals		123		
Body mass	Heterozygosity	0.06 (0.01–1.00)	1	8.35	0.0046
	Subspecies	$2.5 \times ^{-4}$ (0.00–1.00)	1	0.030	0.86
	Measurer-ID	0.14 (0.05–1.00)	3	6.77	<0.001
	Time of day	0.08 (0.02–1.00)	1	10.33	0.0017
	Residuals		122		
Red border width	Heterozygosity	2.8×10^{-4} (0.00–1.00)	1	0.032	0.86
	Subspecies	0.03 (0.00–1.00)	1	3.44	0.067
	Measurer-ID	0.10 (0.03–1.00)	2	6.58	0.0020
	Time of day	0.02 (0.00–1.00)	1	2.02	0.16
	Residuals		114		

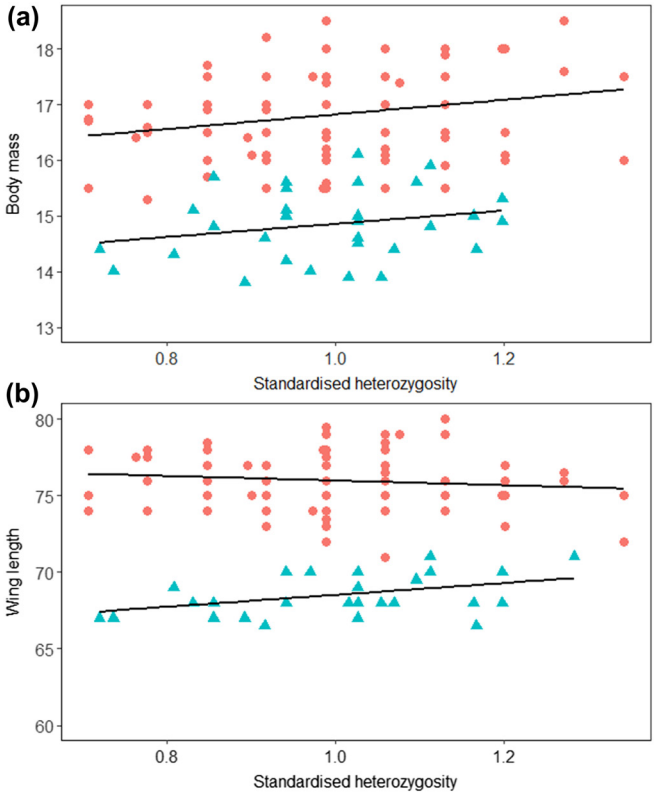

Figure 1. Relationship between standardised heterozygosity and male body mass (upper panel) and male wing length (lower panel) in two bluethroat subspecies, *Luscinia s. svecica* (red circles) and *L. s. namnetum* (green triangles). The lines represent linear regression lines.

Genetic differentiation between *namnetum* and *svecica* corroborates earlier studies, showing that these bluethroat subspecies are genetically distinct (Questiau et al. 1998, Johnsen et al. 2006, Hogner et al. 2013). This is not surprising given that the subspecies are geographically separated and highly unlikely to interbreed. They are also morphologically distinct, both in terms of plumage characteristics and size (Cramp 1988, Johnsen et al. 2006; this study). Here, we also show that *svecica* males have significantly longer sperm than *namnetum* males, a relationship that was not significant in a previous study with smaller sample sizes (Hogner et al. 2013). Furthermore, the variation in total sperm length was larger in *namnetum* than in *svecica*. These differences did not translate into differences in sperm behaviour, however, as there were no differences in velocity and proportion of motile sperm. The relationship between sperm length and sperm velocity is not straightforward, neither in birds or other animal taxa (Humphries et al. 2008, Rojas Mora et al. 2018, Cramer et al. 2021), and one might not expect differentiation in sperm length to translate into differentiation in velocity at the between-population level.

Individual heterozygosity did not correlate with any of our measures of sperm variation, and these results were similar in the two subspecies. There are several possible explanations for these negative results. First, previous demonstrations of negative impacts of low genetic variability on sperm characters have often been based on captive and highly inbred individuals/populations (Losdat et al. 2014) and it is plausible that the lack of effects is due to our study populations being predominantly outbred. Even though genetic variation was increased by including specimens from the relatively less genetically diverse *namnetum* population, sperm traits were unaffected. Second, there is a potential ambiguity in analyses of heterozygosity–fitness relationships in sperm traits, since

sperm cells are haploid while our heterozygosity estimates are based on blood samples reflecting the diploid genotype. It is unclear whether one should expect a relationship between inbreeding (and heterozygosity) and gametic traits, since inbreeding depression depends on genetic dominance effects that only pertain to diploid entities (Losdat et al. 2014). However, to the extent that spermatogenesis occurs in close contact to, and perhaps largely under the genetic control of, diploid Sertoli cells, it is unclear how substantial this disconnect is (Losdat et al. 2014). Third, the use of microsatellite markers to estimate levels of inbreeding has been questioned (Balloux et al. 2004, Pemberton 2004), although recent work suggests that studies using > 20 microsatellites can reveal meaningful patterns, albeit with lower statistical power (Kardos et al. 2016, Nietlisbach et al. 2017). We emphasize that in our study systems, inbreeding sensu stricto is uncommon at best. Hence, the variation in heterozygosity at our presumably neutral genetic markers is unlikely to stem from variation in current inbreeding levels but could reflect varying degrees of past inbreeding or bottleneck effects. Finally, several studies have found effects of heterozygosity on the degree of sperm damage/abnormality (Gage et al. 2006, Fitzpatrick and Evans 2009, Opatová et al. 2016), and in the absence of such data we cannot exclude the possibility of similar effects in bluethroats. Sperm head damage affected about 18% of *svecica* sperm cells in our recent comparative study (which did not include *namnetum* samples; Støstad et al. 2019), hence this possibility deserves further study.

We found that heavier males were more heterozygous than lighter males, independent of subspecies status. All males were captured in the pre-fertile or fertile period, and the analysis controlled for significant variation in the diurnal timing of capture, which was related to body mass as in many other avian study systems (Meijer et al. 1994, Cooper 2007). The correlative nature of our study does not permit inference about the causality of the positive relationship between heterozygosity and body mass, but it is conceivable that more heterozygous males are more vigorous and better able to obtain food and/or maintain metabolic balance than less heterozygous ones. There was a significant interaction effect of heterozygosity and subspecies status on wing length, with longer-winged males being more heterozygous in *namnetum*, while there was no such relationship in *svecica*. Collectively, our results support the idea that more homozygous individuals are at a selective disadvantage (Kempenaers 2007), assuming that a lower body mass and shorter wings translate to lower survival and/or reproductive success.

The *namnetum* population is less genetically variable than the *svecica* population, as expected from its lower population size and more patchy distribution. The population seems to be stable in size and even expanding its range (Marquet et al. 2014, Chiron 2017), and it remains to be seen whether the lower genetic variation will impact this marginal population negatively in the long run. At the very least, it does not seem to influence the size or variability of their spermatozoa. We conclude that sperm traits are unaffected by individual microsatellite heterozygosity in the studied outbred *svecica* and weakly genetically depauperate *namnetum* bluethroat populations, adding to the small body of literature suggesting that variation in individual genetic diversity has little effect on sperm characteristics in wild animal populations.

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Arild Johnsen: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Funding acquisition (equal); Project administration (equal); Supervision (equal); Writing – original draft (equal); Writing – review and editing (equal). **Kristine Wold-Dobbe**: Data curation (equal); Formal analysis (equal); Writing – original draft (equal). **Camilla Lo Cascio Sætre**: Data curation (equal); Formal analysis (equal); Writing – review and editing (equal). **Marie-Christine Eybert**: Data curation (equal); Resources (equal); Writing – review and editing (equal). **Matthieu Marquet**: Data curation (equal); Resources (equal); Writing – review and editing (equal). **Patrick Bonnet**: Data curation (equal); Resources (equal); Writing – review and editing (equal). **Emily R. A. Cramer**: Data curation (equal); Formal analysis (supporting); Writing – review and editing (equal).

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Data availability statement

Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.s7h44j1bz (Johnsen et al. 2023).

Supporting information

The Supporting information associated with this article is available with the online version.

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