It is hard to be small: Inbreeding depression depends on the body size in a threatened songbird

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Abstract

While inbreeding is known to affect individual fitness and thus population extinction risk, studies have under-represented non-model species of conservation concern, and rarely sought conditionality of inbreeding depression. Here, using SNPs identified with RAD-seq, we determined inbreeding depression in a threatened bird, the aquatic warbler *Acrocephalus paludicola*, and whether its magnitude depends on phenotypic and environmental factors. We found that the inbreeding coefficient (*F*) of adults with small tarsi was negatively associated with the seasonal breeding success (in males) and clutch size (in females), with the respective decrease in fitness in the most inbred relative to the least inbred individuals of ~89% and ~12%. In contrast, in adult males, for the average tarsus, wing and mass, support was low for *F* to be related to the long-term return rate to breeding grounds. For mean phenotypic covariates and male density, we also found low evidence that *F* is associated with the annual breeding success. Likewise, there was little support that mother *F* is related to egg hatch success and nestling survival, and – for average phenotypic traits, rainfall, temperature and nest density, and accounting for breeding peak – to clutch and fledged brood sizes. For nestlings, animal models showed that *F* is more negatively related to tarsus under higher temperatures and its effect varies by study year. However, for average brood size, temperature, rainfall and prey abundance, and when controlling for nestling sex, breeding peak and mother *F*, evidence for nestling *F* and tarsus association was weak. We conclude that (1) inbreeding depression on fitness components is stronger in smaller-bodied individuals, (2) considering interaction with phenotypic and environmental variables enables more accurate estimation of inbreeding depression, and (3) the inbreeding depression estimates will inform extinction risk analysis and conservation actions for the aquatic warbler.

Introduction

Inbreeding results from mating between kin, non-random mating or division of a population into isolated groups (Keller & Waller 2002). It leads to increased genomic homozygosity and accumulation of harmful mutations, which are typically recessive and thus more often expressed in highly homozygous, inbred individuals. Consequently, more inbred individuals have compromised fitness. Inbreeding depression is found across plant and animal taxa (Ralls *et al.* 1988, Keller & Waller 2002) and is especially strong in wild populations (Crnokrak & Roff 1999). Inbreeding negatively affects key fitness components, such as sperm quality (Gage *et al.* 2006, Hinkson & Poo 2020), embryo development (Noordwijk & Scharloo 1981, Kruuk *et al.* 2002, Hemmings *et al.* 2012a), juvenile survival (Kruuk *et al.* 2002, Kennedy *et al.* 2014), annual reproductive success (Huisman *et al.* 2016, Niskanen *et al.* 2020), and lifetime reproductive success (Grueber *et al.* 2010, Huisman *et al.* 2016, Harrisson *et al.* 2019). Therefore, inbreeding depression decreases population viability and contributes to extinction risk (Westemeier *et al.* 1998, Brook *et al.* 2002, O'Grady *et al.* 2006, Nonaka *et al.* 2019), and evaluating its impact is of particular importance in populations of conservation concern.

Inbreeding depression is expected to be more pronounced in harsher environments or individuals of lower condition (Armbruster & Reed 2005). For example, low food availability and high number of competitors enhance the inbreeding depression on survival in Darwin's finches (*Geospiza sp.*) (Keller *et al.* 2002). A long-term study on the great tit (*Parus major*) found that inbreeding by environment interactions, while generally weak, are stronger in adverse conditions more closely related to fitness (Szulkin & Sheldon 2007). Laboratory experiments on insects showed that inbreeding effects on offspring survival increase with mother age (Fox & Reed 2010) and environmental toxicity (Nowak *et al.* 2007). However, evaluation of the conditionality of inbreeding depression in wild populations has not been frequent, while determining its magnitude across individual traits and environmental conditions would yield more accurate inbreeding depression estimates, especially in threatened populations.

Here, we determine the strength of inbreeding depression on fitness-related traits in an IUCN-threatened songbird, the aquatic warbler *Acrocephalus paludicola*, a habitat specialist breeding in fen mires and a long-distance migrant (Le Nevé *et al.* 2018, Tanneberger *et al.* 2018b). Following the gradual disappearance of fens, which accelerated especially over the past 100-200 years due to peat extraction, changes in hydrological regime, drainage, eutrophication and succession, the extent of occurrence of the species shrank considerably (Tanneberger *et al.* 2018a). This led to a steep decline in the population size, amounting to 95% only between 1950-80, and its extinction from the western Europe by the end of the 20th century (Briedis & Keišs 2016, Flade *et al.*

2018). Today, the breeding range of the aquatic warbler is constrained to the east-central Europe, which holds c. 98% of the global population estimated at 12200 singing males (Flade *et al.* 2018). A recent study (Kubacka *et al.* 2024) demonstrated that genetic diversity in the species is low and comparable to that of other threatened bird species (Evans & Sheldon 2008, Vitorino *et al.* 2019). As it is habitat specialists that are more strongly affected by loss of genetic diversity (Matthews *et al.* 2014, Pflüger *et al.* 2019), it is crucial to determine the magnitude of inbreeding depression in the aquatic warbler. In addition, alongside the depleted genetic diversity, the high asymmetry of the male reproductive success (Dyrcz *et al.* 2002, 2005, Kubacka *et al.* 2025) and the promiscuous mating system (Leisler & Schulze-Hagen 2011) are expected to increase variation in the inbreeding rates, which facilitates detection of inbreeding depression (Balloux *et al.* 2004), thus making the species a good study model. Finally, the aquatic warbler faces a broad range of environmental conditions, as the breeding season strides the cool spring and the hot summer, water levels on the fen are changeable, and nests are built on the ground and thus exposed to environmental factors, all of which enable studying the conditionality of inbreeding depression.

Specifically, we aimed at 1) assessing the relationship of adult inbreeding rates with the reproductive success and survival, and of nestling inbreeding rates with the body size; 2) evaluating whether inbreeding depression is conditioned by phenotypic variables corresponding to individual quality and by environmental variables that are expected to affect adult survival and fecundity, and offspring body size; and 3) quantifying the inbreeding load and change in fitness associated with inbreeding.

Methods

Study area and sampling

The study was conducted between May-August of 2017-2023, in the Biebrza Valley (Poland), which holds about 25% of the global breeding population of the aquatic warbler. The breeding habitat is permanently water-logged open fen mire and to a less extent wet meadows, all dominated by sedges *Carex* spp. Three study areas were used, referred to as Ławki (N 53º17′11.4″, E 22º33′49.2″), Szorce (N 53º17′34.8″, E 22º37′15.9″) and Mścichy (53º25′41.7″ E 22º30′17.0″). Each study area consisted of two 10-20-hectare (ha) study plots, totalling 70 ha.

Between May-July of 2017-2019, adult individuals (past their 1st calendar year) were caught with mist-nets and marked with unique metal and colour rings. They were blood-sampled through puncture of the brachial vein with a sterile needle and collecting approx. 10-120 ul of blood with a capillary. Each blood sample was immediately placed in a vial with an o-ring seal, containing 2 ml of

96% EtOH, and shaken to avoid formation of large clots. We measured tarsus length (from the notch on the metatarsus to the top of the bone above the folded toes, with a calliper to the nearest 0.1 mm), wing length (with a ruler to the nearest mm) and body mass (with an electronic Kern CM 150-1N balance to the nearest 0.1 g).

Nests were located by search and observation of alarming females, every 2-7 days in each plot from late May to early June, and from late June until the end of July. We distinguished two breeding peaks, which correspond to the first and second breeding attempts that are typically separated by about 10 days when no nests are initiated (Kubacka *et al.* 2014). Nests were monitored every 1-5 days. In nests found at the egg stage or on day 1 post-hatch (day 0 being the last day when most offspring in the nest were still eggs), we determined the clutch size, hatched brood size and nestling survival (number of chicks surviving from hatching to fledging). After day 1 the risk is higher that a nestling dies and is removed by the female, which biases estimation of the above traits. In all the nests, we recorded the fledged brood size as the number of nestlings seen on the last check when the brood was still in the nest, as long as on the following check the nest did not bear any traces of predation and was recorded empty not earlier than on day 12 post-hatch (i.e. 3 days before the expected fledging).

In 2017-2018, the tarsus and body mass of chicks were measured, as above, on days 2, 5 and 9 post-hatch, which fall at the beginning, in one third and in two thirds of the nest stage, respectively. Only nestlings from nests found at the egg stage or day 1-2 post-hatch were included in the analysis. We used the nestling tarsus length as it is one of the proxies for structural body size in birds (Rising & Somers 1989, Senar & Pascual 1997) and predicts survival to reproductive maturity (Gebhardt-Henrich & Richner 1998). Chicks were blood-sampled (approx. 10-80 µl) on day 9 post-hatch, as above.

Between May-July of 2018-2023, we searched the study plots and their surroundings up to 150 m off plot border for colour-ringed adults with the search effort of 0.2-3.9 person-hours per ha. The colour-ring code was read with an 80-mm-lens and 20-60 magnification scope. The position of each resighted individual was stored in a GPS receiver. We measured the long-term return rate by the number of years during which a given adult individual was resighted or recaught in the study plots, or in other breeding areas – as determined from the Polish Bird Ringing Database, over 4 years following the first observation as adult.

We obtained the daily sum of rainfall in mm and average daily temperature from the Polish Institute of Meteorology and Water Management (IMGW). To assess food abundance, in 2017-2018 we sampled arthropod prey within c. 30 m from the nest by sweep-netting (1 sweep per 1 second)

on 3 radially oriented transects between 3 m of the nest and 50 steps away from the nest. Collected arthropods were placed in a plastic bag, frozen upon arrival from the field, determined to the order and counted. We then quantified the amount of preferred prey as the total number of insects from the taxonomic groups known to be selectively brought by females to nestlings: *Odonata*, *Aranea*, *Lepidoptera* and *Orthoptera* (Schulze-Hagen *et al.* 1989). We estimated the male density with QGIS 3.10.7 (QGIS Development Team 2020) as the average number of ringed males observed within 100 m of each ringed male's position, and the nest density as the number of nests found per 10 ha of study plot. Following our previous study (Kubacka *et al.* 2025), based on all the captures and resights of the males, we determined the number of encounters of a male per plot in each breeding season, to account for excess zeros in the male breeding success (see *Statistical analysis*). We suspected that the low father-to-offspring assignment rate (see *Results*) could be caused mainly by late arrivals of some males to the study areas. Individuals with a low number of encounters were first captured during the second breeding attempt, or were rarely seen in the study plots.

DNA extraction and molecular sex identification

DNA was isolated with the Xpure™ Blood Mini kit (A&A Biotechnology, Poland). 200-300 µl of blood suspended in 96% EtOH were used per individual and the proteinase K digestion time was 2-3 hours. The remaining steps were performed according to the manufacturer's protocol. We analysed DNA of 175 adult males (1 was ringed as a chick), 320 chicks (2 were resighted as adult males) from 79 nests and 105 adult females. DNA concentration was measured with a Picogreen (Invitrogen) or Qubit (Thermo Fisher Scientific) instrument.

Sex of nestlings was determined by PCR amplification of sex-specific sequences in the CHD-W (c. 370 bp, females only) and CHD-Z genes (c. 330 bp, both sexes) (Griffiths et al. 1998) with a PCR kit (Eurx, Poland). Electrophoresis of PCR products ran for 90 mins at 100 V in a 3% agarose gel stained with Simply Safe (Eurx, Poland) and the gel was photographed under UV light.

Molecular marker identification and genotyping

To determine inbreeding rates and assign paternity, we used single nucleotide polymorphisms (SNPs), determined with 3-enzyme restriction site-associated sequencing (3RAD) (Bayona-Vásquez *et al.* 2019, Glenn *et al.* 2019). Inbreeding estimated with genomic markers better predicts the identity by descent and decrease in fitness, compared to inbreeding rates inferred from the pedigree (Hemmings *et al.* 2012b, Kardos *et al.* 2015, Huisman *et al.* 2016). First, we constructed a reference

176 genomic library, i.e. a catalogue of reliable loci excluding potential paralogs and artefactual alleles,
177 according to the Reduced-Representation Single-Copy Orthologs (R2SCOs) method (Driller *et al.*
178 2021), with modifications. We sequenced 2x300 bp reads for three females to reconstruct the entire
179 locus by merging overlapping reads, using samples with high DNA concentration (75.9, 53.7 and 76.6
180 ng/µl), each representing a different study location. After normalising the samples to 20 ng/µl, we
181 split each into 5 digestion reactions (200 ng each), totalling 1 µg digested DNA. The enzymes XbaI,
182 EcoRI-HF and NheI were used for the digestion, which ran at 37°C for 2 hours. Each digestion
183 reaction was ligated with a unique XbaI and EcoRI adapter pair (i.e. double-indexed) and the 5
184 reactions were pooled and purified (0.8x PCRclean magnetic beads - GCbiotech). We performed a
185 PCR with P5 and P7 customised ligation check primers, to ensure that the digestion was successful.
186 Each pooled sample was size-selected (350-580 bp) on a BluePippin machine (Sage Science). Each
187 size selection product was then split into 4 reactions and in a single-cycle PCR we added an iTru5-8N
188 index (with 8 random nucleotides, to mark unique DNA template molecules and remove PCR
189 duplicates during the bioinformatic analysis) (Hoffberg *et al.* 2016). After purification (0.8x beads) an
190 additional PCR step (8 cycles) completed the Illumina adapters with a unique iTru7 P7 primer per
191 reaction and a P5 primer (which has a flow cell binding site sequence) common to all the reactions.
192 The 4 PCR replicates of each sample were then pooled, purified (0.8x beads) and quantified (Qubit –
193 Thermo Fisher Scientific). Finally, the pools were sequenced with the Illumina MiSeq V3 at 600
194 cycles, yielding a total of 3.6 M reads per sample.

195 Next, we constructed population libraries (Supplementary Online Resource 1), consisting of
196 the remaining 597 samples. DNA concentration was normalised to 20 ng/ul or less (at least 8.14
197 ng/µl). We followed the protocol described above (Bayona-Vásquez *et al.* 2019), with modifications,
198 and produced 9 population libraries, which were processed in 3 batches. The libraries were
199 sequenced with 2x150 bp on Illumina instruments; 15% of PhiX was used to increase sequence
200 diversity. The number of sequenced reads obtained from each library is presented in Table S1a.

201

202 ***Bioinformatic analysis***

203 We first constructed a reference locus catalogue using the R2SCOs method (Driller *et al.* 2021),
204 which allowed us to select a set of high-quality single-copy orthologous loci to be used as reference
205 for the population analysis. The R2SCOs pipeline included de-replication of the pre-processed and
206 size-selected sequences (range 250-370 bp, for which we obtained comparable and high coverage),
207 setting the minimum coverage per unique sequence to define a putative allele equal to 3; and
208 clustering and definition of putative loci using the intra-specific identity threshold of 90%. As a next

209 step, the pipeline applies a set of filters to remove loci that are prone to generate spurious results
210 due to issues of either biological or technical background. Aiming to avoid sex-biased genotypes that
211 might interfere with the population analyses (Faux *et al.* 2020), we removed all the R2SCOs loci that
212 mapped to the sex chromosomes of the Eurasian blackcap (*Sylvia atricapilla*) genome (GenBank
213 accession number GCA_009819655.1). The final reference contained 10997 loci.

214 The raw data from the population libraries was mapped against the PhiX genome to filter
215 any remaining read from the Illumina library control. Next, we performed adapter trimming using
216 the Cutadapt software (Martin 2011). Then, reads were attributed to individuals based on the in-line
217 barcodes using FLEXBAR (Dodt *et al.* 2012). In part of the libraries (Supplementary Online Resource
218 1), PCR duplicates were filtered out using the Python script Filter_PCR_duplicates.py
219 (https://github.com/BeGenDiv/Arantes_et_al_2020). The 4 replicates were then concatenated. The
220 forward and reverse reads were merged with overlapping regions of at least 30 bp and maximum
221 length of 249 using the software PEAR (Zhang *et al.* 2014). All the merged reads (<249 bp) were
222 considered short fragments that were out of the target range, and we continued analysing the
223 unassembled read pairs, which were filtered for minimum quality (Q>30) and trimmed to 142 bp
224 with a minimum length of 130 bp using Trimmomatic (Bolger *et al.* 2014). We checked the presence
225 of the XbaI and EcoRI restriction sites in the paired-read ends using the script
226 checkRestrictionSites.py (https://github.com/BeGenDiv/Arantes_et_al_2020) and filtering out any
227 fragment resulting from star activity or NheI digestion. Finally, reads containing the internal
228 restriction site of XbaI, EcoRI or NheI were removed using the Filter_Reads.py script
229 (https://github.com/BeGenDiv/Arantes_et_al_2020). The filtered reads were mapped against the
230 reference locus catalog constructed with the R2SCOs method, using Bowtie2 (Langmead *et al.* 2009)
231 with default parameters and the flags '-no-mixed' and '-no-discordant'. In order to select a common
232 range of high-coverage loci for all individuals belonging to different libraries, we checked the length
233 distribution of the loci based on the .sam files, and extracted the range 250-310 bp with *samtools*
234 (Danecek *et al.* 2021) and a bash *awk* command.

235 We analysed the mapped reads with Stacks (Catchen *et al.* 2011, 2013), using the reference-
236 based pipeline. Genotypes were called and filtered with ref_*map.pl*, which uses the *gstacks* and
237 *populations* modules. In *gstacks*, the *var_alpha* (alpha threshold for discovering SNPs) was set to
238 0.01, and all the samples were assumed to belong to one population, because of the extensive gene
239 flow known in the aquatic warbler (Kubacka *et al.* 2024). Otherwise, default parameters were used.
240 In *populations*, we used: the minimum percentage of individuals in a population required to process
241 a locus r= 0.8, to avoid high missingness; the minimum number of populations a locus must be
242 present in to be processed p= 1; and the minimum minor allele frequency required to process a

nucleotide site at a locus min-maf= 0.005, which ensured that an allele is present in at least three samples to be processed (Rochette & Catchen 2017). To obtain independent SNPs, we wrote only the first SNP in a locus. The remaining parameters were retained at their defaults. After calling genotypes, we generated mean depth per individual with Vcftools (Danecek *et al.* 2011), to inspect coverage distribution. We removed 1 individual with mean depth <10 and repeated the SNP-calling step. We obtained 6267 loci, of which 4179 variant sites (SNPs) were retained after the *populations* filter, with mean ±SE observed heterozygosity in variable positions of 0.141 ±0.002. We checked whether individuals with the lowest coverage (mean depth of ≥10x and <30x, N= 46) affect loci-calling, by running *ref_map.pl* without them. The number of SNPs called without these individuals was 4171 and the mean ±SE observed heterozygosity was 0.143 ±0.002; we therefore retained them for further analysis.

We then used Vcftools for further SNP filtering (Table S1b). We removed genotypes with mean depth per SNP <10x and loci with >20% missing data. Next, we filtered out sites (N= 6) of mean depth >115, which formed the tail of the depth distribution, to remove potential collapsed paralogs. In the R environment (R Core Team 2024), with the *dartR* package (Gruber *et al.* 2018) we checked whether the loci are in the Hardy-Weinberg equilibrium (HWE), applying the false discovery rate correction and p-value of 0.05. Most loci not in HWE were heterozygote-deficient and we found a positive correlation between the locus Fis and missingness (r= 0.38), which decreased to r= 0.13 after the HWE-violating loci were removed. As this is indicative of null alleles, which artefactually lower heterozygosity (Waples 2015, De Meeûs 2018), we proceeded without the HWE-violating SNPs. Finally, using VCFtools, we calculated loci-pairwise r^2 to inspect distribution of linkage disequilibrium. The mean ±SD of r^2 was 0.003±0.012. We excluded one randomly selected SNP from each pair for which r^2 was >0.3, to remove the tail of r^2 distribution (Fig. S1). The final dataset consisted of 2948 SNPs (Table S1b). The mean ±SD read depth per locus and individual are provided in Fig. S2.

Paternity assignment

We selected a set of informative SNPs to maximise the statistical power of parentage assignment (Andrews *et al.* 2018, Thrasher *et al.* 2018). With Vcftools (Danecek *et al.* 2011), we filtered out SNPs with minor allele frequency <0.3, as rare SNP variants are not informative in parentage assignment (Huisman 2017), and missingness >0.1. The number of SNPs after filtering was 333 (Table S1b). Typically, 100-200 SNPs suffice for parentage assignment (Huisman 2017, Flanagan & Jones 2019). For 1 individual, which was RAD-sequenced twice, we removed the genotype with fewer loci typed.

276 The resulting genotypes dataset consisted of 320 offspring (316 with known mother) and 173
277 candidate fathers, with 1 mother typed in 88 SNPs and all the other individuals typed in >99 SNPs.
278 Paternity was then assigned with Cervus (Kalinowski *et al.* 2007), running the allele frequency
279 analysis, parentage analysis simulation and paternity analysis given known mother. The allele
280 frequency analysis showed the mean proportion of SNPs typed of 0.954. In the simulation, we
281 assumed: the allele frequencies generated in the previous step; 100000 offspring; 440 as the
282 average number of candidate fathers per offspring (based on the number of males assessed within
283 the study plots and 150 m around them, and summed over all the three study locations); 0.4 as the
284 proportion of candidate fathers sampled; 0.954 and 0.01 as the proportion of loci typed and
285 mistyped, respectively; 0.01 as the error rate in likelihood calculations; 88 as the minimum number
286 of typed loci; Delta as the statistic to determine confidence; and 99% and 95% as the strict and
287 relaxed confidence level, respectively. In the paternity analysis, we used the allele frequency and
288 simulation results generated in the previous steps. The offspring file contained candidate fathers,
289 and males known to not be sexually mature in the year when a given chick was born were excluded.
290 We ran the simulation and paternity analyses also for 95% and 80% strict and relaxed confidence
291 levels, respectively. In addition, we established paternity with Colony v. 2.0.7.1 (Jones & Wang
292 2010), providing information on known maternal sibships and excluded paternity, assuming
293 polygamy of both sexes, no inbreeding and default parameters otherwise. We quantified the male
294 seasonal breeding success as the number of offspring produced in the given year, as detected by
295 paternity assignment to the blood-sampled chicks.

296

297 ***Inbreeding rate and inbreeding load***

298 To assess inbreeding, we quantified the inbreeding coefficient *F* with a method of moments (Wang
299 2014) using the *–het* option in VCFtools (Danecek *et al.* 2011). To explore how well *F* corresponded
300 to inbreeding, with the R package *inbreedR* (Stoffel *et al.* 2016), we calculated g_2, an estimate of
301 identity disequilibrium which quantifies the covariance of heterozygosity at the studied loci
302 standardised by their average heterozygosity; hence, it corresponds to inbreeding variance in the
303 population (David *et al.* 2007). A correlation between a trait and heterozygosity will not arise when
304 $g_2 = 0$ (Szulkin *et al.* 2010). To avoid pseudoreplication, we used a dataset filtered to include all the
305 adult individuals and 1 nestling per nest. We used 1000 permutations over SNPs to evaluate whether
306 $g_2 > 0$ and 1000 bootstraps over individuals to determine its 95% confidence interval (CI).

307 To quantify the strength of inbreeding depression, we used the inbreeding load measured
308 with lethal equivalents (*B*) and percent change in fitness due to inbreeding (δ) (Morton & Crow

309 1956, Keller & Waller 2002). Assuming that mutations at different loci have independent effects on
310 fitness, the logarithm of a fitness-related trait is predicted to show a linear negative relationship with
311 the inbreeding coefficient. The inbreeding load is quantified as the negative of the slope describing
312 this relationship. To compare inbreeding load between populations and studies, the concept of
313 lethal equivalents was coined. The effect of a mutation on fitness can be lethal or have partial
314 detrimental effects. One lethal equivalent is the average number of alleles in a population which
315 cause death of the homozygous individual. The inbreeding load equals the average number of lethal
316 equivalents per haploid gamete found in the studied population. We calculated *B* using Equation II in
317 Box 3 from Keller & Waller (2002), predictions weighted-averaged across all the models and
318 categorical levels (see *Statistical analysis*), and fitness values for the maximum and minimum
319 inbreeding rates in our dataset. For the survival traits, we used Poisson models with the logarithm
320 link function, which yield unbiased estimates of inbreeding load for binomial traits (Nietlisbach *et al.*
321 2019). The δ refers to the change in a fitness-related trait in inbred individuals compared to outbred
322 individuals (Keller & Waller 2002). We calculated δ for the most inbred compared to the least inbred
323 individuals in the dataset (Harrisson *et al.* 2019, Sin *et al.* 2021).

324

325 ***Statistical analysis***

326 We ran the analysis in the R environment 4.4.2 (R Core Team 2024) and applied the information-
327 theoretic approach, in which an a priori set of biologically plausible candidate models is built and
328 each model in the set obtains a relative rank indicating how well it explains the data (Burnham &
329 Anderson 2002). To rank models within a candidate set, we used the Akaike information criterion
330 corrected for small sample size (AICc) and – in animal models (see below) – the deviance information
331 criterion (DIC). In addition, we generated the following quantitative measures of relative support for
332 each model: model likelihood (relative likelihood of the model in the candidate set, given the data),
333 Akaike/DIC weight (ωAICc/ωDIC; probability that the given model is the best approximating model in
334 the candidate set), evidence ratio (showing the probability of the best model relative to other
335 models in the set) and cumulative AICc/DIC (sum of AICc/DIC weights of a given model and all the
336 higher-ranking models) (Symonds & Moussalli 2011).

337 Each candidate set included a null model assuming that the response variable is constant, or
338 that it varies with a variable strongly affecting the response that was common to all the models. All
339 the models in the set except the null model included *F*. For selected candidate sets, we restricted the
340 number of variables in a model to allow 10-15 data points per each non-intercept estimate and
341 reduce the risk of over-parametrisation; for models using the binomial distribution, we ensured that

342 there are 10-15 data points of the less probable event per estimate (Harrell 2015). To account for
343 model uncertainty, the estimates of regression coefficients and their confidence intervals were
344 weighted-averaged (by the ωAICc/ωDIC) across all the models in a set that contained the given term
345 (i.e. natural model-averaging), as we expected small effects (Grueber *et al.* 2011, Symonds &
346 Moussalli 2011). Model selection and averaging were carried out with package *AICmodavg*
347 (Mazerolle 2023). All the input numerical explanatory variables were standardised to the mean of
348 zero and SD of 1 (i.e. z-score standardisation). The weather variables, nest density and prey
349 abundance were z-score standardised within the year and breeding peak; in the chick tarsus analysis,
350 the weather variables were also standardised within the day. Sum contrasts were used (i.e. with the
351 intercept being the corrected mean), to enable comparison of effects of categorical and numerical
352 variables. For visualising interactions, predictions were calculated using packages *AICmodavg*
353 (Mazerolle 2023) and *MuMIn* (Bartoń 2019) for the model containing the term, on the link scale and
354 back-transformed. We visualised results with package *ggplot2* (Wickham 2016) and custom code.

355 We evaluated the relationship between *F* as an explanatory variable and the fitness-related
356 (male long-term return rate, male breeding success, clutch size, hatch success, nestling survival and
357 fledged brood size) and condition-related traits (chick tarsus on days 2, 5 and 9 post-hatch) as
358 response. We also determined whether the *F* effect is conditioned by phenotypic (tarsus, wing and
359 body mass in adults; sex in offspring) or environmental variables (male density, nest density, rainfall,
360 temperature, breeding peak, prey abundance and brood size). Candidate sets were built as follows:

361 (1) *Male inbreeding and long-term return rate*. We did not consider the female return rate due to
362 only 8 recoveries, which did not ensure a reliable regression analysis (Harrell 2015). The male 4-
363 year-return rate showed a right-skewed distribution. In order to select an appropriate count
364 model, following the recommendations by Zuur & Ieno (2021), we ran an intercept-only GLM
365 Poisson (*P*) model with log-link with the return rate as response, and calculated its
366 overdispersion and zero-inflation using package *DHARMa* (Hartig 2022). The *P* model showed
367 overdispersion (dispersion statistic 1.44, $P<0.001$) but no zero-inflation (ratio of observed to
368 simulated zeros 1.09, $P= 0.194$). We then ran an intercept-only negative-binomial (NB) model
369 using package *MASS* (Venables & Ripley 2002), which showed no overdispersion (1.00, $P=0.997$)
370 and no zero-inflation (1.00, $P= 1.000$). Therefore, we continued with the NB distribution to
371 construct a candidate model set. We included the following terms: body mass, tarsus and wing
372 length, ringing area and year, their interaction with *F*, *F* squared, body mass squared, tarsus
373 squared, and wing squared (Table S2a).
374 (2) *Male inbreeding and seasonal breeding success*. The male breeding success had a large number
375 of zeros and, corresponding to previous observations (Dyrcz *et al.* 2002, 2005, Kubacka *et al.*

2025), was right-skewed. We proceeded as in point (1) and constructed a null GLM *P* log-link model with the number of encounters and ringing year as explanatory variables. The *P* model was overdispersed (dispersion statistic 4.31, $P<0.001$) and zero-inflated (ratio of observed to simulated zeros 1.70, $P<0.001$). We then fitted a *NB* model with the same terms, which was not overdispersed (0.99, $P= 0.965$) and not zero-inflated (1.02, $P= 0.786$). Hence, we proceeded with the *NB* distribution. While 24/145 males bred both in 2017 and 2018, we treated these breeding events as independent after ascertaining that the residuals within these males were uncorrelated in a null GLM *NB* model (Zuur *et al.* 2009, 2013). The covariates included were body mass, tarsus and wing length, local male density, study area and ringing year, their interactions with *F*, and *F*, mass, wing and tarsus square effects. All the models included the number of encounters and ringing year (Table S2b).

(3) *Female inbreeding and clutch size*. This candidate set was constructed using linear models. As covariates, we included the mother body mass, tarsus and wing length, and their quadratic effects, breeding peak (present in each model as it strongly predicts the clutch size), sum of rainfall during and mean daily temperature averaged for 15 days prior to start of incubation, study area and year, and the interactions of all the covariates with mother *F*. We restricted the number of non-intercept estimates in a model to 6 (Table S4a).

(4) *Female inbreeding and egg hatching success*. The hatching success was coded as 1 for eggs that hatched and 0 otherwise, with the egg being the subject. The candidate set was built using generalized linear mixed models (GLMMs) with package *lme4* (Bates *et al.* 2015) and the binomial distribution with logit link. Nest ID was entered as a random factor. As covariates, we included the breeding peak, sum of rainfall and mean average daily temperature during incubation, clutch size, study area and year. Due to the low number of unhatched eggs ($N= 23$), we limited the number of non-intercept estimates in a model to 2 and did not consider interactions (Table S4b).

(5) *Female inbreeding and nestling survival*. The nestling survival was coded as 1 for nestlings that survived until fledging and 0 otherwise, with the nestling being the subject. The candidate set was constructed with GLMMs, as above. As covariates, we included the mother body mass, wing and tarsus length, breeding peak, sum of rainfall and mean average daily temperature from hatching to fledging, hatched brood size, study area and year. Due to the low number of nestling deaths ($N= 22$), we limited the number of non-intercept estimates in a model to 2 and did not consider interactions (Table S4c).

(6) *Female inbreeding and fledged brood size*. We considered only successful nests (i.e. those that fledged at least 1 chick), as nests fail completely for reasons that are mostly independent of the

410 female (e.g. predation). The candidate set was built using linear models. As covariates, we
411 included the mother body mass, wing and tarsus length and their quadratic effects, breeding
412 peak, sum of rainfall and mean average daily temperature from hatching to fledging, study area
413 and year. We also considered interactions of the covariates with *F*. The number of non-intercept
414 estimates in a model was <8 (Table S4d).

415 (7) *Nestling inbreeding and tarsus length on days 2, 5 and 9 post-hatch*. In natural populations,
416 phenotype-associated inbreeding can occur, e.g. if the wing length of a parent is correlated with
417 its relatedness to the mate. This creates a bias on the inbreeding depression especially in
418 morphological traits, as part of variation in the trait results from genetic covariance with the
419 relatedness between parents. Therefore, as recommended by Becker *et al.* (2016), we applied
420 the animal model. We used package *MCMCglmm* (Hadfield 2010), with the random effects being
421 the breeding value ('animal'), chick ID, nest ID and father ID (from the Colony results). We
422 calculated the genomic relatedness matrix using package *AGHmatrix* (Amadeu *et al.* 2023) and
423 the Van Raden matrix. We applied an uninformative prior and set the number of MCMC
424 iterations to 1.2 million, burnin to 10000 and thinning to 100. The candidate set considered the
425 following covariates: measurement day (present in all models), chick sex, brood size, breeding
426 peak, sum of rainfall during the previous 14 days, mean average daily temperature over the
427 previous 4 days, prey abundance, area and year, and their interaction with chick *F*; and mother
428 *F*, to account for an effect of maternal inbreeding on brood provisioning and parent-offspring
429 correlation in *F* (Nietlisbach *et al.* 2016) (Table S6).

430

431 ## Results

432 *Parentage assignment and inbreeding statistics*

433 Of the 320 assignments, in further analysis we used 223 that were of ≥99% confidence in both the
434 father-offspring pair and trio (i.e. father-mother-offspring), and 2 that were of ≥99% confidence in
435 father-offspring pair, but their mother was not sampled and trio confidence could not be
436 determined. We excluded 1 assignment for which the trio confidence was 99%, but the pair
437 confidence was <95%. Of the 173 candidate fathers, 58 were assigned paternity with confidence as
438 above. The range and median number of mismatches were: 0-3 and 0 for mother-chick; 0-5 and 1 for
439 the 225 trios with the most likely father; and 0-49 and 39 for the 225 trios with the second most
440 likely father. We obtained same assignments with the strict and relaxed confidence of 80% and 95%,
441 respectively. All the Colony paternity assignments matched the 225 Cervus assignments and had the
442 probability of 1.

The mean ±SD *F* was 0.005 ±0.043, median 0.002 and range –0.077 to 0.138 for adult females; and mean ±SD 0.028 ±0.048, median 0.030 and range –0.101 to 0.168 for adult males (Fig. S3). Two nestlings with unusually low inbreeding coefficients were removed from the tarsus analysis as outliers. The mean ±SD offspring *F*, excluding these outliers, was 0.007 ±0.046, median 0.006 and range –0.096 to 0.175. The *g2* was estimated at 0.0009 (95% confidence interval 0.0002 to 0.0015) (Fig. S4). There was a moderate positive correlation between the mother *F* and the mean within-mother chick *F* (Pearson *r*= 0.30, 95% CI 0.05 to 0.52).

Inbreeding depression on fitness components in adults

In adult males, the return rate was predicted neither by the male *F* nor by the *F* by phenotype interactions, as the interaction models received weak support, and the CIs of these terms overlapped zero (Fig. 2, Tables 2a & 3a). The *F* and tarsus squared model obtained top support (ωAICc=0.28), was 8.8 times more likely than the *F* and tarsus model, and 3.5 times more likely than the null model. The *F* squared models were also well supported (sum of ωAICc= 0.47) but only weakly more probable compared to the null model (Table S2a). The inverse quadratic relationships with the tarsus and *F* indicated that return rates are the highest for males with average tarsi and *F* values, respectively.

While the male *F* was not a good predictor of the breeding success for average phenotypes and male densities (Fig. 2, Table S3b), the *F* by tarsus interaction model scored the highest (ωAICc=0.47), was 4.9 times more probable than the null model and 16.7 times more probable than the *F* and tarsus model (Table S2b). The remaining interaction effects obtained low support. In males with small tarsi, the *F* estimate was negative, and in males with exceptionally large tarsi it was positive, however, the latter result was based on very few observations and thus uncertain (Fig. 3).

For average covariates, the female *F* relationship with the clutch size was unsupported (Fig. 4, Table S5a). However, the *F* by tarsus interaction model obtained top support (ωAICc=0.52), was 4.3 times more probable than the *F* and tarsus model, and clearly more parsimonious than the null model (Table S4a). The remaining interaction effects were unsupported. In females with small tarsi, the *F* effect on the clutch size was negative, while in those with the largest tarsi it appeared positive; however, the latter result was based on very few observations and thus uncertain (Fig. 5). The tarsus models ranked the highest (sum of ωAICc 0.99), pointing to the tarsus as the strongest and positive predictor of the clutch size (Fig. 4, Tables S4a&S5a).

474 Mother *F* showed no clear relationship with the hatching success and nestling survival (Fig.
475 4, Tables S5b-c). For the hatching success, the null model scored the highest (ωAICc=0.21) and
476 support was spread between several models (Table S4b). For the nestling survival, the *F* and body
477 mass model obtained clear support from the data (ωAICc=0.70) (Table S4c), showing that mother
478 body mass was a strong negative correlate of nestling survival (Fig. 4, Table S5c).

479 Similarly, the relationship between the mother *F* and fledged brood size was not supported
480 for average phenotypic and environmental covariates (Fig. 4, Table S5d). The null model ranked the
481 second and the *F* by body mass interaction model was 4.4 times more probable than the *F* and body
482 mass model, however, it was nearly as parsimonious as the null model, indicating very low support
483 (Table S4d). The remaining interaction effects were not supported. The breeding peak was a
484 relatively strong predictor of the fledged broood size (Fig. 4, Table S5d).

485

486 *Inbreeding depression on nestling tarsus*

487 The 95% credible interval of the model-averaged chick *F* effect spanned zero, indicating no support
488 for an association between *F* and tarsus for average covariates (Table S7). Evidence was spread
489 between several animal models (Table S6). The *F* by rainfall and *F* by temperature interaction models
490 scored best (ωDIC=0.12 and 0.11, respectively). The *F* by temperature interaction estimate was
491 negative, suggesting that the *F* effect becomes more negative with increasing temperatures and
492 varies by year. We obtained low evidence for the remaining interactions (Tables S6 & S7).

493

494 *Inbreeding load and change in fitness*

495 For average phenotypic traits, the inbreeding load on male return rates was moderate, with nearly
496 half the number of years returned to the breeding grounds in the most inbred males, compared to
497 the least inbred ones, and the inbreeding load on the male breeding success was high (Table 1).
498 However, as the 95% CIs of the male *F* effects spanned zero, these inbreeding load estimates bear
499 high uncertainty. The males with average small tarsi incurred strong inbreeding load, with almost
500 90% reduction in the number of young for the most inbred males, compared to the least inbred
501 ones. In contrast, in males with average large tarsi the inbreeding load was negligible (Table 1).

502 The female inbreeding load on the clutch size was low for the average covariates. However,
503 mothers with average small tarsi bore higher, although moderate inbreeding load, with about 10%
504 reduction in the clutch size for the most inbred relative to the least inbred females, while in mothers

with average large tarsi the inbreeding load was very low (Table 1). The mother inbreeding load on the hatching success, nestling survival and fledged brood size was also low. The overall decrease in fitness between laying a clutch and brood fledging totalled 0.6 haploid lethal equivalents (Table 1).

Discussion

Weak inbreeding depression for average phenotypic and environmental variables

For the mean value of the phenotypic traits and male density in the case of the breeding success, inbreeding depression on the long-term return rates and seasonal breeding success of adult males obtained low support from the data. Male aquatic warblers show high post-breeding annual return rates (Dyrcz & Zdunek 1993, Bellebaum 2018), which are comparable to survival rates of other migratory passerines in the central European latitude (Scholer *et al.* 2020). Hence, the 4-year return rate is a strong predictor for the lifespan survival. Given the median return rate of 1 year among the surviving males, most males reproduce over 1 breeding season, indicating that the seasonal breeding success is a proxy for the lifetime reproductive success. Therefore, our results do not suggest that the inbreeding rate in adult males is associated with their adult lifespan nor the lifetime reproductive success.

Avian studies on the inbreeding depression on adult survival have been scarce and showed weak, e.g. B= 1.7 (Keller 1998), moderate, e.g. δ= 49% (Harrisson *et al.* 2019) or strong effects of inbreeding, e.g. up to B= 7 in the house sparrow (*Passer domesticus*) (Niskanen *et al.* 2020). Evidence for male inbreeding effects on the annual reproductive success has also been variable, ranging from weak support (Sin *et al.* 2021), through moderate effects, e.g. δ= 42% (Harrisson *et al.* 2019) to clearly negative inbreeding depression in the house sparrow, B= 6 (Niskanen *et al.* 2020). The B= 4.9 and δ= 69% that we obtained for aquatic warbler males appear to be high inbreeding load on the annual reproductive success, however, these estimates bear high uncertainty.

Similarly to males, for the mean value of the phenotypic and environmental covariates, we found weak support for the relationship between an adult female's inbreeding rate and her short-term fitness components. These results do not support mother inbreeding depression on offspring production from clutch laying until fledging in the aquatic warbler. However, it is the earliest developmental stages that could respond more negatively to inbreeding (Noordwijk & Scharloo 1981, Hemmings *et al.* 2012b), and it is inbreeding of the offspring that could affect its survival more strongly.

536 Previous studies on birds found the parental inbreeding to be little related to egg production
537 (Keller 1998, Szulkin *et al.* 2007, Harrisson *et al.* 2019). In the takahe (*Porphyrio hochstetteri*),
538 parental inbreeding was unassociated with hatching rates (B= –0.7) and fledging success (B= 3.3)
539 (Grueber *et al.* 2010), but in another study the latter decreased by 30% in mothers with the pedigree
540 $f>0$ (Jamieson *et al.* 2003). Negative effects of maternal inbreeding were demonstrated in two
541 studies on passerine birds for the hatching success (δ= 20-36%) and fledgling production (δ= 26%)
542 (Keller 1998, Harrisson *et al.* 2019). In our study, although the clutch size manifested the highest
543 inbreeding load of the female fitness components studied, it was relatively low, and so was the
544 inbreeding load from clutch laying to brood fledging.

545 Neither did we find unambiguous evidence that, for average values of the phenotypic and
546 environmental covariates, and while accounting for mother inbreeding, the offspring inbreeding rate
547 is associated with the tarsus length, and this association remained constant across the three nestling
548 ages. This does not support inbreeding depression on the skeletal body size in aquatic warbler
549 nestlings, for typical individuals and conditions. In contrast, in the collared flycatcher (*Ficedula*
550 *albicollis*), inbred fledglings had smaller tarsi compared to outbred ones (Kruuk *et al.* 2002), but no
551 such relationship was found in another species (Sin *et al.* 2021).

552

553 *Effects of inbreeding depend on body size in adults and on weather in offspring*

554 We demonstrated that inbreeding depression on the male seasonal breeding success and clutch size
555 is conditional on the tarsus length of males and females, respectively. In birds, tarsus length is a
556 proxy for the structural body size (Rising & Somers 1989, Senar & Pascual 1997), implying that the
557 inbreeding costs on these fitness-related traits increase in small-bodied individuals, relative to large-
558 bodied individuals. In some passerines, the tarsus or a body size index is positively associated with
559 the clutch size in females (Alatalo & Lundberg 1986, Sedinger *et al.* 1995, Garamszegi *et al.* 2004, this
560 study) and with sperm quality and functionality traits and testis size in males (Brown & Brown 2003,
561 Forstmeier *et al.* 2017), which translates into sperm production (Parker & Pizzari 2010, Hayward &
562 Gillooly 2011). Therefore, it could be more difficult for small inbred individuals to compensate for
563 their lower fecundity traits, than it is for large inbred ones.

564 The inbreeding depression on the breeding success in the small-bodied males appears
565 strong. For example, the B of 9 and ~89% decrease in the male annual reproductive success are
566 comparable to the high inbreeding depression in the house sparrow (Niskanen *et al.* 2020). In small-
567 bodied females, the inbreeding load on clutch sizes (B= 2.8) and the decrease in the clutch size
568 produced (~11%) were moderate and did not translate to decreased fledgling production. Our

finding of inbreeding depression on the clutch size in small females stands in contrast with previous studies (Keller 1998, Szulkin *et al.* 2007, Harrisson *et al.* 2019).

We did not obtain firm support for environmental dependence of the chick inbreeding effects on tarsus, except for a weak interaction with the average daily temperature. This interaction suggests that chick *F* was more negatively associated with the tarsus in higher temperatures in the preceding days. As temperature was standardised within year, breeding peak and day, its effect was independent of these factors. Elevated temperatures could correlate with lower abundance or availability of prey, and thus more inbred chicks could cope worse with being fed less during warmer days. As the effect of nestling *F* varied between the two study years, the magnitude and direction of inbreeding effects on nestling body size could differ between breeding seasons.

Conclusions and conservation implications

We sought to determine the magnitude of the inbreeding depression on fitness components and nestling growth in a threatened passerine, the aquatic warbler, and to inspect whether the inbreeding effects on these traits are modulated by the phenotype and environment. We demonstrated, to our knowledge for the first time, that in birds, inbreeding depression on adult fitness components could depend on the body size, with smaller individuals paying higher costs of inbreeding. This suggests that birds raised in poor environmental conditions, which negatively affect the adult body size (De Kogel 1997, Searcy *et al.* 2004, Cleasby *et al.* 2011) could have lower fitness if they are inbred. Our study also points out that accounting for the body size (and possibly other phenotypic and environmental variables) allows to determine inbreeding depression more precisely. This is of considerable importance especially in the case of species of conservation concern, where nuanced knowledge on inbreeding depression will be informative for adequate estimation of extinction risk. Finally, our observation that nestling inbreeding association with tarsus is conditioned by the temperature and year partly corroborates that unfavourable environmental conditions could exacerbate inbreeding depression. It also implies that survival to reproductive maturity, of which the chick tarsus length is a proxy, could be negatively affected for nestlings growing in excessively warm ambient temperatures.

For the aquatic warbler, our study shows that for average phenotypic and environmental covariates, inbreeding effects on the studied fitness traits are weakly supported. However, we were able to estimate inbreeding depression on fitness traits only in adults. Weakly negative inbreeding effects on short-term fitness components can accumulate to high inbreeding load over the complete

life history continuum (Szulkin *et al.* 2007, Grueber *et al.* 2010, Harrisson *et al.* 2019, Niskanen *et al.* 2020). Future studies on the inbreeding depression in the aquatic warbler should include the missing fitness components, e.g. survival from viable egg to sexual maturity, juvenile survival and adult female survival. Notwithstanding, our results will be informative for a population viability analysis accounting for inbreeding depression (O'Grady *et al.* 2006, Nonaka *et al.* 2019) and for conservation actions such as translocation.

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Figures and tables

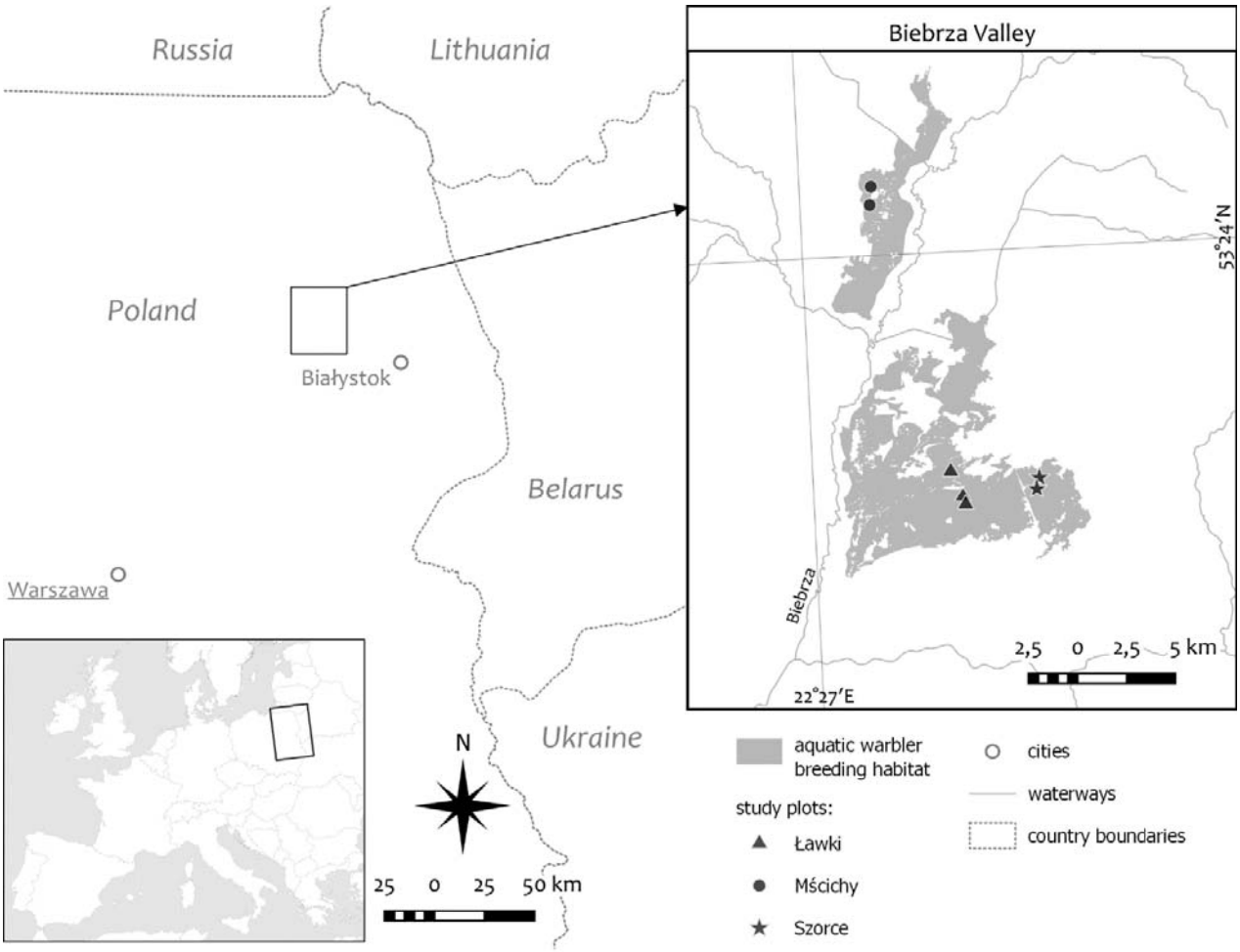
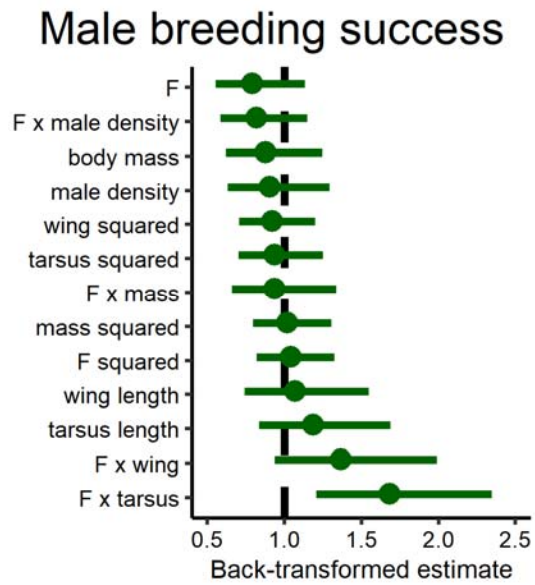


Fig. 1 Map of the study area.



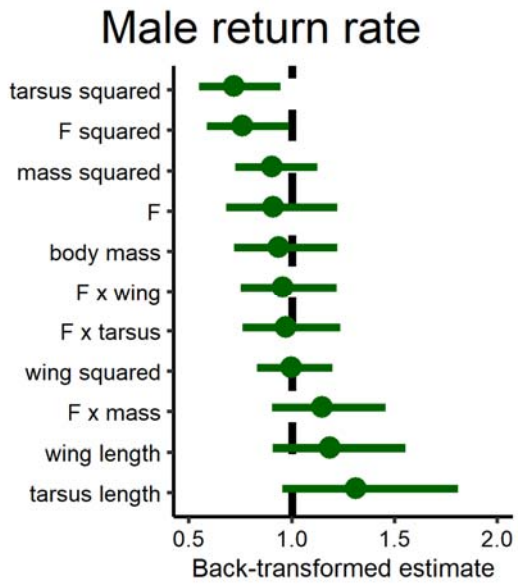


Fig. 2 The estimates (denoted by circles) of effects of the male inbreeding coefficient *F*, phenotypic variables and their interaction with *F* on male fitness components: 4-year return rate to breeding grounds (left) and seasonal breeding success (right). The whiskers represent 95% confidence intervals and the vertical dashed line refers to no effect. See Table S3 for the summary of the estimates.

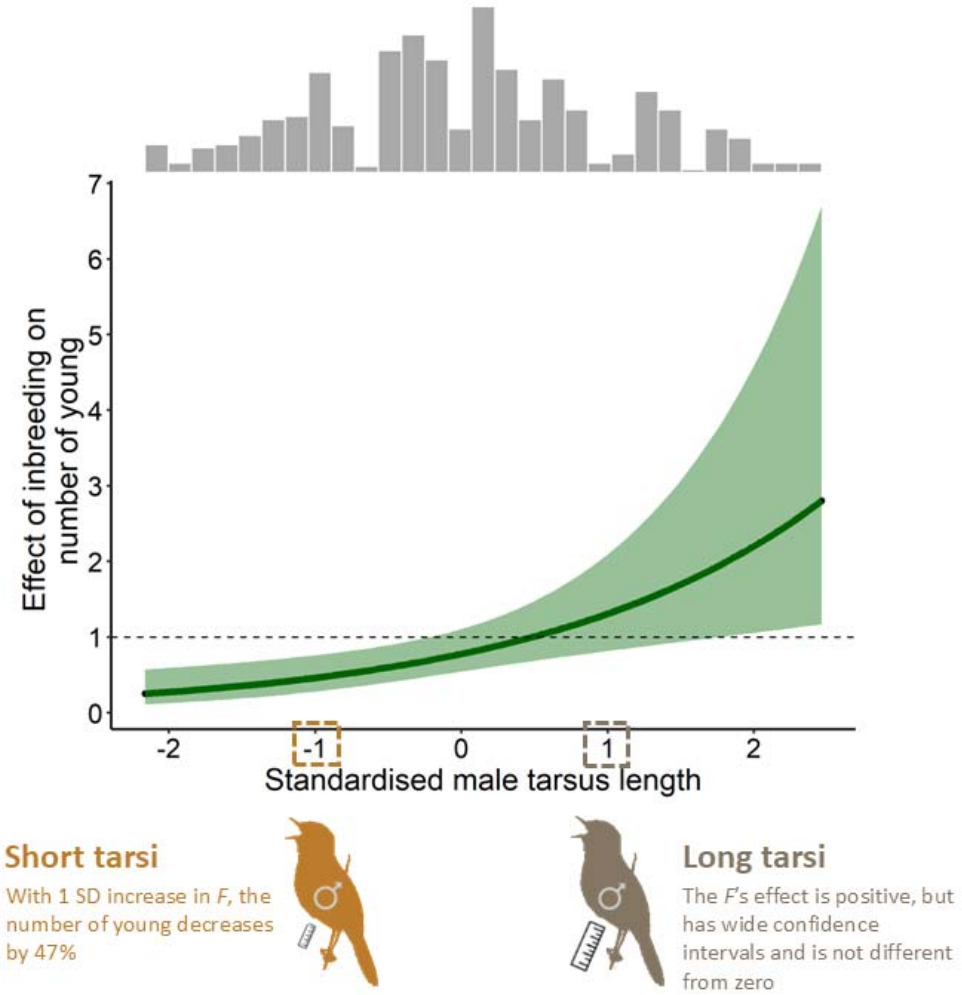


Fig. 3 The effect of interaction between the male tarsus length and inbreeding coefficient *F* on the seasonal breeding success. The graph shows how the back-transformed statistical effect of *F* changes against standardised tarsus lengths, and the bands show the 95% confidence interval of the effect. The aquatic warbler pictograms at −1SD and +1SD present examples of how to interpret the graph. The horizontal dashed line corresponds to no effect. The histogram shows frequency of tarsus lengths.

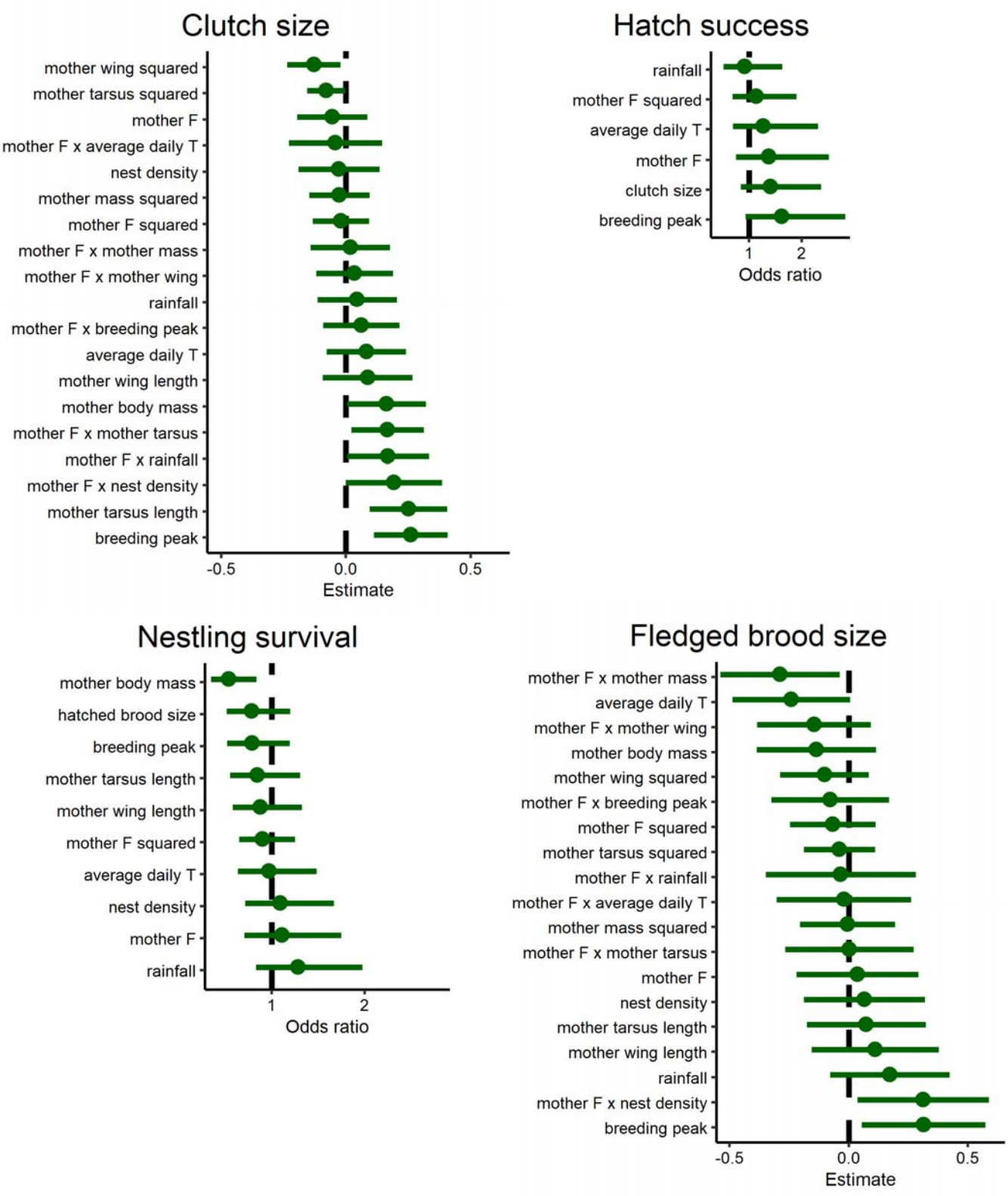


Fig. 4 The estimates (denoted by circles) of effects of the mother inbreeding coefficient *F*, phenotypic variables and environmental variables and their interaction with *F* on female fitness components. The whiskers represent 95% confidence intervals and the vertical dashed line refers to no effect. See Table S5 for the summary of the estimates.

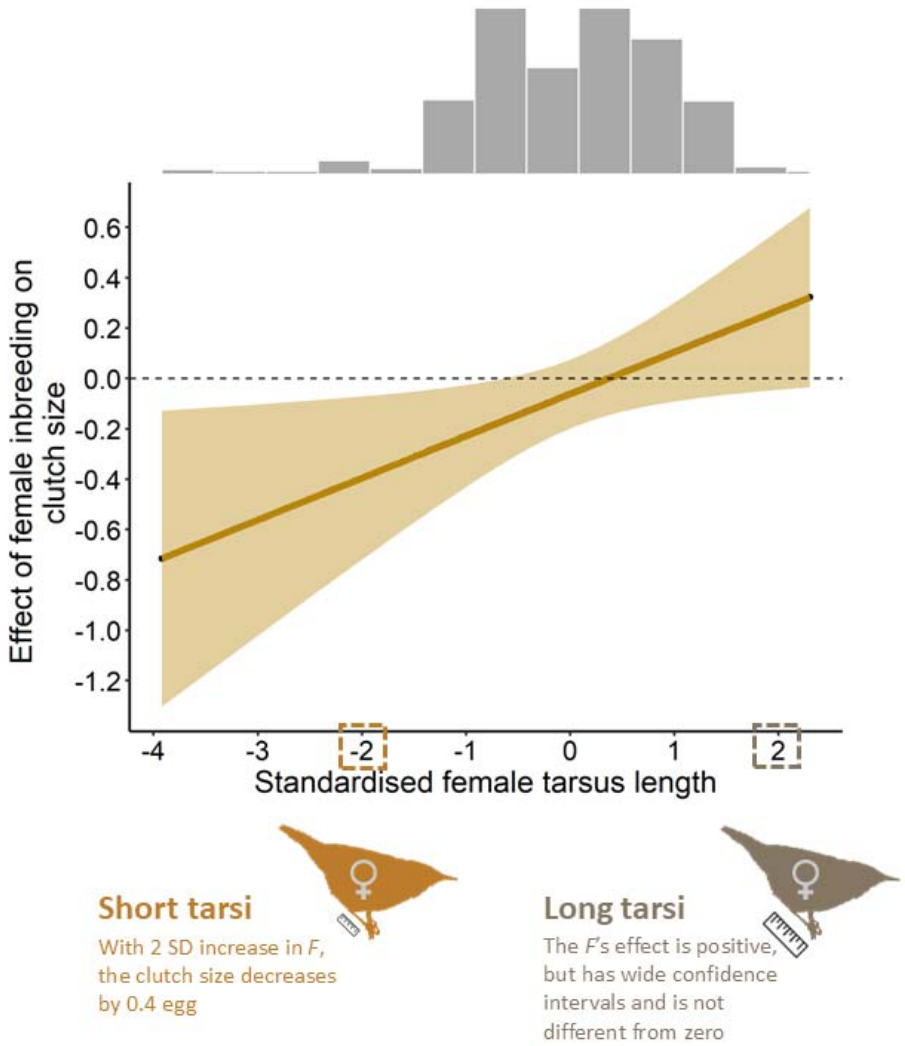


Fig. 5. The effect of interaction between the mother tarsus length and inbreeding coefficient *F* on the clutch size. The graph illustrates how the statistical effect of the mother *F* changes along the value of the tarsus. The aquatic warbler pictograms at –2 SD and +2 SD present examples of how to interpret the graph. The bands show the 95% confidence interval of the effect, the horizontal dashed line corresponds to no effect, and the histogram shows the frequency of tarsus lengths.

Table 1. Inbreeding load (*B*) and change in the fitness components due to inbreeding (δ).

Fitness component	Inbreeding determined in:		Standardised *B*	Native scale *B*	Change in trait $-\delta$
Male long-term survival	adult male		0.11[1]	2.45[1]	−48.3%[1]
Male seasonal breeding success	adult male		0.21[1]	4.87[1]	−69.3%[1]
Average small males	adult male		0.40[1]	9.27[1]	−89.4%[1]
Average large males	adult male		0.01[1]	0.21[1]	−5.0%[1]
Clutch size	adult female		0.06	1.26	−5.0%
Average small females	adult female		0.12	2.83	−11.5%
Average large females	adult female		−0.01	−0.28	+1.1%
Hatch failure	adult female		−0.01[2]	−0.17[2]	+3.5%[2]
Nestling mortality	adult female		0.01[2]	0.15[2]	−2.9%[2]
Fledged brood size in successful nests	adult female		−0.03	−0.66	+3.4%

The *B* is expressed in lethal haploid equivalents and was calculated from weighted-averaged predictions (Keller & Waller 2002, Box 3, Eqn II) of respective candidate model sets (Tables S2, S4 & S6) on the link scale, which were then weighted-averaged between the study areas, years and breeding peaks (if applicable). The native scale *B* was calculated by back-standardisation. The δ is reported as the change in fitness of the most inbred relative to the least inbred individuals in the dataset, and was calculated as above, with the final predictions back-transformed to the response scale. For clarity, $-\delta$ is given, i.e. the minus in front of the δ means a decrease in the trait value, while the plus indicates an increase.

[1] negative binomial distribution with log-link used
[2] poisson distribution with log-link used