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Inbreeding effects on telomeres in hatchling sand lizards (*Lacerta agilis*): An optimal family affair?

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Abstract

Telomeres are nucleotide-protein caps, predominantly at the ends of Metazoan linear chromosomes, showing complex dynamics with regard to their lengthening and shortening through life. Their complexity has entertained the idea that net telomere length and attrition could be valuable biomarkers of phenotypic and genetic quality of their bearer. Intuitively, those individuals could be more heterozygous and, hence, less inbred. However, some inbred taxa have longer, not shorter, telomeres. To understand the role of inbreeding in this complex scenario we need large samples across a range of genotypes with known maternity and paternity in telomere-screened organisms under natural conditions. We assessed the effects of parental and hatchling inbreeding on telomere length in >1300 offspring from >500 sires and dams in a population of sand lizards (*Lacerta agilis*). Maternal and paternal ID and their interactions predict hatchling telomere length at substantial effect sizes ($R^2 > .50$). Deviation from mean maternal heterozygosity statistically predicts shorter offspring telomeres but this only when sibship is controlled for by paternal ID, and then is still limited ($R^2 = .06$). Raw maternal heterozygosity scores, ignoring absolute deviation from the mean, explained 0.07% of the variance in hatchling telomere length. In conclusion, inbreeding is not a driver of telomere dynamics in the sand lizard (*Lacerta agilis*) study system.

KEYWORDS
hatchling telomere length (T/S ratio), *Lacerta agilis*, parental effects, sand lizard, standardized heterozygosity (SH)

1 | INTRODUCTION

Telomeres are the nucleotide-protein caps that constitute the ends of linear chromosomes, protects the chromosomes from end-to-end fusion, and facilitate DNA integrity and stability (Blackburn, 1991; Monaghan & Haussmann, 2006). In recent years, telomeres have become increasingly appreciated biomarkers and indicators of proximate and ultimate health and fitness, reflected in, for example, better parasite resistance (Asghar et al., 2015), lower levels of free radicals (von Zglinicki, 2002), better social health (Epel et al., 2004), and a longer life span (Bauch et al., 2014; Heidinger et al., 2012). One explanation for this could be that individuals with relatively longer telomeres, or those that show slower attrition, are of better pheno-typic- or genetic quality, or have better antioxidation against reactive

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oxygen species (ROS, von Zglinicki, 2002). Also, when antioxidation fails, they may have better DNA repair systems, such as through the BER pathway, especially of the telomere sequence, or higher telomerase production (Henriques et al., 2013; Jia et al., 2015; Olsson et al., 2018a, 2018b; Ujvari et al., 2017). This suggests a polygenic, "multigene-target" for selection to act on and it would agree with logic that these organisms are potentially less inbred on average, with a lower risk of inbreeding depression and with more traits significant for maintaining high relative fitness.

Inbreeding can result from population bottlenecks and consanguineous matings, and we have shown in previous studies that sand lizards from a population with low genetic variation who mate with relatives show detrimental effects on offspring viability (Madsen et al., 2000; Olsson et al., 1996). Such processes often result in a genome-wide increase in homozygosity and can negatively affect fitness through two proposed mechanisms, over-dominance or partial dominance. At over-dominance, inbreeding depression is an effect of superiority of heterozygous individuals relative to homozygous ones at fitness-related loci. Conversely, partial dominance can result in inbreeding depression when individuals become homozygous for recessive or partially recessive deleterious alleles at fitness-related loci (Charlesworth & Willis, 2009).

Inbred individuals may suffer from poor ability to maintain sufficient energy and nutrient levels, in particular when foraging success is limited (Bebbington et al., 2016) or when suffering from strong intra- and interspecific competition (Sharp, 1984). If so, inbreeding effects on telomere length and attrition would be predicted to be particularly strong under more averse conditions (Bebbington et al., 2016). To the best of our knowledge, the only field study that has tested the effects of inbreeding on telomere traits under such conditions is the study by Bebbington et al. (2016) of Seychelles warblers (*Acrocephalis schellencis*). They found a significant negative effect of increased homozygosity on telomere length in juveniles in years of low food availability, but no effect in years of high food availability. The explanatory power was, however, low ($R^2 = .11$). In adults, the corresponding effect was statistically significant ($p = .04$), but with only one percent of the variance in telomere length explained by level of homozygosity ($R^2 = .01$).

In contrast to the intuition that inbred organisms ought to have shorter telomeres, there is currently an accumulation of literature that links longer telomeres, with higher variance in telomere length, to inbreeding in several taxa. In *Pennisetum* plants, there were significant differences in average telomere length among different inbred lines, ranging between c. 20 and 40 kb, whereas the wild type *Pennisetum* had an average telomere length of only half that, c. 10 kb (Sridevi et al., 2002). Similarly, in the fissiparous starfish (*Coscinasterias tenuispina*), clonal individuals with lower genetic diversity had significantly longer telomeres than sexually reproducing ones with higher genetic diversity (Garcia-Cisneros et al., 2015). In amphibians, inbreeding does not seem to be linked to telomere length (Sànchez-Montes et al., 2020). For birds, the information is less clear – three classes of telomeres based on length have been described and the characteristic 'mega telomeres' (up to several MB) seem to occur in all inbred lines of chicken (*Gallus gallus domesticus*), with differences in variation and diversity between lines (e.g., Delany et al., 2000). However, there seems to be no published information yet on "noninbred" lines – or any other bird species but the Seychelles warbler (Bebbington et al., 2016) with information on both level of inbreeding/genetic variation and telomere length. In mammals, and across all taxa, the most complete information is available for mice. Established inbred mice (*Mus spretus*) have much longer telomeres (30–150 kb) compared to wild-derived mice (8–10 kb; Hemann & Greider, 2000). Interestingly, inbred mice strains also show a high level of telomere hypervariability, suggesting an unusually high mutation rate in telomere-regulating genes (Kipling & Cooke, 1990), and "fairly" active telomerase in most somatic tissues (reviewed in Buckingham & Klingelhutz, 2011).

To better understand what may genetically drive elongation of telomeres at inbreeding, Manning et al. (2002) conducted an inbreeding experiment with three outbred populations of *Peromyscus leucopus* mice (GS109, GS16A1, and GS16B). All mice derived from outbred *P. leucopus* stock, and all three lines, developed significantly longer telomeres in response to active inbreeding, but to differing degrees (Manning et al., 2002). The telomeres of the inbred GS16B line were three times as long on average as the outbred stock. Through hybridization of several parental strains with shorter and longer telomere phenotypes, F1 hybrids were obtained with intermediate telomere length to their parents. It was further deduced from telomere length distributions in F_2 offspring from a cross between outbred *P. leucopus* and the GS16B strain that more than five loci were involved in the regulation of telomere length. While it was repeatedly confirmed that inbreeding results in telomere elongation, the exact mechanism(s) underpinning this scenario is unknown.

Relative telomere length and attrition, and telomerase levels (Ujvari et al., 2017), are expected to be under stabilizing selection; in humans, broadly, arteriosclerosis is associated with "too short" telomeres, whereas "too long" telomeres are associated with elevated cancer risk (Olsson et al., 2018a, 2018b; Stone et al., 2016, for many examples of deviations from these patterns). Thus, regardless of whether telomeres have a causal role on fitness or are just biomarkers of "health and well-being", we may expect individuals with telomeres longer than "the shortest" to have higher viability and fitness – until they reach cell immortality and cancer (e.g., most often associated with telomerase upregulation; Stone et al., 2016). In contrast, compromised telomere regulation in inbred organisms, without opposing selection pressures in captive animals, suggest a similar outcome – that is, poor control of dynamics (length regulation) would predict deviations from optimal telomere length – potentially resulting in both "too long" and "too short" telomeres as an outcome of inbreeding. Thus, perhaps while maintenance of longer telomeres carries costs in the wild, longer telomeres can be maintained in captivity where benign conditions may release such selection pressures.

To understand the role of inbreeding in this complex scenario, it would be helpful to have a study system that under natural conditions can be used to assess the effects of inbreeding across a

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range of genotypes with known maternity and paternity in large samples of telomere-screened organisms, through long periods of time and multiple episodes of selection. We have access to such data and here assess inbreeding effects on telomere length in hatchling sand lizards (*Lacerta agilis*) in a sample of more than 1300 offspring from up to 520 known parents, collected over a decade in the wild.

2 | MATERIALS AND METHODS

2.1 | Model system

Details of field work, husbandry, and laboratory techniques are described in previous studies (Olsson, 1994; Olsson, Pauliny, Wapstra, Uller, Ujvari, et al., 2011a; Olsson, Pauliny, Wapstra, Uller, Schwartz et al., 2011b; Olsson & Shine, 1997). Therefore, such procedures are only summarized here.

Sand lizards are small ground dwellers (up to 20g) and have one of the largest distributions of any reptile species, c. 8000×5000km (Bischoff, 1984). Their main distribution is in central Europe. In Sweden, the distribution is fragmented, the genetic variation low, compared to continental Europe, and the species is considered vulnerable (Gullberg et al., 1999). In our study population (Asketunnan; N570 22′ E110 58′) matings between close kin are detrimental as revealed by malformations in inbred offspring (Bererhi et al., 2019; Olsson et al., 1996). Females lay a single annual clutch of five to 15 eggs, depending on female body size (Olsson, 1993). Approximately 1week before egg laying, when females show egg contours along their abdomens, gravid females were captured by noose or hand and brought to facilities at the University of Gothenburg, Sweden. They were marked by toe-clipping for identification (Olsson, 1994), and kept individually in cages (40×50×60 cm) containing a flat rock, placed on wet sand for egg laying, and a 40-W spotlight at one end to allow thermoregulation. Eggs were collected within hours of laying and incubated at 25°C, which optimizes hatching success and minimizes developmental asymmetries in this species (Zakharov, 1989). All clutches were incubated individually in separate boxes, all in the same incubator. Eggs hatched after approximately 40days. The juveniles were measured snout to vent to the nearest mm, weighed to the nearest 0.001g, marked by claw/toe-clipping and released at random locations at the Asketunnan study site.

2.2 | Genotyping, parentage assignment, and heterozygosity measurements

DNA was extracted from blood and tissue samples of a total of 4534 individual adults, juveniles, and embryos that died during incubation. Parentage was assigned using 17–21 microsatellite loci (see Olsson, Pauliny, Wapstra, Uller, Ujvari, et al., 2011a; Olsson, Pauliny, Wapstra, Uller, Schwartz et al., 2011b for a detailed description of methods). 452 eggs failed to hatch, of these we were able to extract DNA for genotyping from 204. From the 3627 eggs that hatched, a total of 2601 juveniles were successfully assigned a father (Olsson, Pauliny, Wapstra, Uller, Ujvari, et al., 2011a; Olsson, Pauliny, Wapstra, Uller, Schwartz et al., 2011b). All blood samples were stored at negative 80°C from sampling to laboratory procedures, and all DNA was stored at negative 80°C subsequent to extraction. The genotyped microsatellites were used to calculate individual heterozygosity as a proxy for inbreeding in the R package Rhh (Alho et al., 2010). Individual heterozygosity was estimated for 2983 observations, including juveniles and unhatched eggs. This was done using standardized heterozygosity (SH; Coltman et al., 1999), with an average of 19.6 loci used per observation, and taking on values between zero and two. Thus, SH represents the proportion of heterozygous typed loci divided by the mean heterozygosity of the typed loci. Elsewhere, heterozygosity–heterozygosity correlations (HHC) were calculated in Rhh to estimate the inbreeding signal as a proxy for genome-wide inbreeding (Bererhi et al., 2019) following (Balloux et al., 2004). In Bererhi et al. (2019), we also report the parameter g2 (David et al., 2007), which estimates identity disequilibrium (ID) in the form of correlations in heterozygosity or homozygosity across the genome (Weir & Cockerham, 1973). This is fundamental information for heterozygosity fitness correlations (HFC; Szulkin et al., 2010). The value of g2 will be positive if there is variance in inbreeding, and thus potential for HFCs to arise in a population (Szulkin et al., 2010). Departure from 0 for g2 was assessed in robust multilocus estimation of selfing (RMES) based on 1000 iterations (David et al., 2007). In our previous publication (Bererhi et al., 2019) we report that the HHC was positive and significant (mean = 0.1414; 95% confidence interval: 0.05559–0.19875), indicating the loci had sufficient information to provide estimates of inbreeding. The signature of inbreeding in the population was further confirmed by a positive, and statistically significant g2 (n = 3786; s [g2] = 0.0025; p =.005).

2.3 | Telomere length measurements: qPCR

DNA was extracted from blood and tissue samples using the Qiagen Puregene blood kit (Cat no. 158467). Telomere length was measured using quantitative polymerase chain reaction (qPCR) (Nussey et al., 2014). All qPCR work followed Cawthon (2002) and Criscuolo et al. (2009) with slight modifications (Axelsson et al., 2020). To evaluate DNA concentration and purity, we used a PHERAstar F5 spectrophotometer (BMG Labtech). The total yield averaged 527ng/µl per sample, with high molecular purity (mean A260/280 = 1.76; n = 2476). Samples with low yield and/or low quality were excluded, other samples were diluted to a working concentration of 20ng/µl. The limit for exclusion was set to 5 ng/µl and 1.4 for all our analyses but these applied only to juvenile samples. DNA concentrations ranged from 5.96ng/µl to 1090.61ng/µl. All samples were diluted to 20ng/µl, and nine samples that were <20ng/µl, were not diluted and the number of microliters of DNA added was increased to a total approximately 20ng/µl. The A260/280 range was 1.4–1.95.

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Previously published primers (Criscuolo et al., 2009) were used to amplify the control single copy gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and telomere primers (Tel1b and Tel2b). A standard curve was generated for GAPDH and telomere primers using six five-fold serial dilutions of DNA from an arbitrary *L. agilis* sample to generate a reference curve. Each run included a positive control, no template control and the same reference standard to control for the qPCR's amplification efficiency and set up to the threshold Ct value; every sample was run in triplicate. For maximized precision, if a triplicate had a standard deviation >0.3 it was rerun. The DNA concentrations at each point of the standard curve were: (1) 500 ng (log template DNA = 2.7), (2) 100 ng (log template DNA = 2.0), (3) 20 ng (log template DNA = 1.3), (4) 4 ng (log template DNA = 0.6), (5) 0.8 ng (log template DNA = −0.1), (6) 0.16 ng (log template DNA = −0.8).

All PCR analyses were performed on a RotorGene6000 (Qiagen). Using the product recommended PCR conditions, 11.25 µl SensiMix SYBR no-ROX Master Mix (Bioline) was included in the 20 µl final volume of both 200 nM concentration primer sets. Then, 1 µl DNA per well was added at a concentration of 20 ng/µl, except for nine samples that yielded less than 20 ng/µl, 2–4 µl was added for each of those samples.

Nonspecific products were not amplified, indicated by a single peak in the melt curve analysis for each reaction. The cycle at which the fluorescence level crosses the threshold, which is proportional to the quantity of DNA in a sample, is represented by the threshold cycle values (C_t). For each sample, for both genes, C_t values were obtained. Each sample's telomere length (T) was expressed relative to the single gene control (S; GAPDH). The Pfaffl method (Pfaffle, 2001) was used to calculate relative telomere length (T/S ratio) as the deviation in qPCR reading from a reference DNA sample (GAPDH), that is, relative telomere to single copy (T/S) ratio (adapted from Cawthon, 2002). Interassay coefficient of variation (mean ± STD) for qPCR runs for telomere (n = 123) and GAPDH (n = 176) amplification were 3.85 ± 0.59% and 7.73 ± 1.87%, respectively. Mean amplification efficiency across all qPCR runs (n = 299) was 1.999 ± 0.013 (STD). Raw data were also double-checked for baseline fluorescence, individual efficiencies, and window of linearity per amplicon using LinRegPCR 12.18 (Ruijter et al., 2009; Tuomi et al., 2010).

2.4 | Climatological data

Axelsson et al. (2020) reported effects of seasonal temperatures on telomere lengths in this population and we therefore included climatological data as covariates in our analyses. Climatological data was obtained from the Swedish Bureau of Meteorology and Hydrology (SMHI) for the relevant time period April to June in 1998–2006. This data was retrieved from the nearest climate data logger, located in Varberg, c. 50 km south of our lizard field site at Asketunnan. Both these localities are situated on the coast and at the same elevation and can be considered equivalent in terms of cloud cover, rainfall and sunshine (i.e., basking opportunities in spring/summer). Thus, data collected should reflect corresponding annual weather variation at the study site (Ljungstrom et al., 2015; Olsson, Pauliny, Wapstra, Uller, Ujvari, et al., 2011a; Olsson, Pauliny, Wapstra, Uller, Schwartz et al., 2011b) and captures year-to-year variation of a significiant driver of telomeres - temperature (Axelsson et al., 2020).

2.5 | Statistical analysis

In a previous publication, we demonstrated climatic effects on adult telomere length in the season following climatic monitoring (Axelsson et al., 2020). Although all the eggs in the current study were incubated at the same temperature (25°C), there could still potentially be effects on hatchling telomere length of the mean temperature prior to female capture, influenced by maternal basking behaviour leading up to oviposition. Ambient, hourly temperatures were averaged to daily means. All climatological data were then averaged to grand means per season to match the single observation of telomere length per individual and year. Elsewhere we have also demonstrated slight effects of adult sex in a much smaller sample on telomere length (Olsson, Schwartz, Wapstra, Uller, Ujvari, Madsen, & Shine, 2011, slightly longer in females and with sex-specific heritability; Olsson, Wapstra, Schwartz, Madsen, Ujvari, & Uller, 2011). We therefore also looked for such effects in the hatchlings and then controlled for sex in our analyses of inbreeding effects on hatchling telomere length.

Transgenerational maternal effect from mere female size on hatchling telomere length was dismissed (p = .11) and will therefore not be further reported on here. Linear mixed modelling was performed in Proc Mixed, Statistical Analysis Systems (SAS) 9.4 to assess covariation of telomere length (T/S ratio) with temperature and sex as fixed effects and inbreeding traits as the target predictor of analysis. All mixed models were run with Kenward-Roger's correction to calculate degrees of freedom and control for type I error (for description of random effects see below).

The total maternal effect is summed up in the maternal identification number ("Maternal ID") and can be partitioned into the heterozygosity score ("MomSH") and any residual effects captured in the female ID variance component; this will also contain environmental- and genetic "noninbreeding" effects and their interactions. In lizards, the partitioning of yolk precursors (vitellogenin) is simultaneous among the secondary ovarian follicles (Monaghan et al., 2020). Eggs from the same clutch will therefore be more similar in terms of yolk provisioning of nutrients, steroids etc within than among clutches. Furthermore, unlike in birds and mammals (Copenhaver et al., 2019), squamate females produce a new germline bed each year (Monaghan et al., 2020). Thus, there should be limited attrition effects on hatchling telomere length from having oocytes stored and suffering from exposure to reactive molecules for long periods of time (unlike in vertebrate endotherms; Olsson et al., 2018a). There is no behavioural maternal care past hatching.

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Our work on sand lizard mating systems demonstrates that c. 80% of sand lizard clutches are sired by more than one male (Gullberg et al., 1997; Olsson et al., 2019) and that the polygynous males do not defend a geographically defined territory that potentially could vary in quality among territorial males and their females. Instead, males defend a female of immediate interest within an "umbrella territory" that travels with the male between females during the mating season (Gullberg et al., 1997). Thus, males contribute nothing but semen towards the offspring. There is no behavioural paternal care. Consequently, male contributions towards hatchling telomere length are most probably only genetic. Paternal, solely genetic effects can be partitioned into the paternal heterozygosity estimate ("DadSH") and any residual effects left in the male ID variance component ("paternal ID"). Significant interactions between male and female IDs would indicate that a male or female produce offspring with different length telomeres at hatching, depending on how their genotypes and maternal environment(s) interact. Thus, estimating paternal genetic effects on offspring telomere length variance has the added complication that a male's variance component will be affected by the multiple environments of the females with which he has sired offspring. We therefore used two approaches in our analyses: (i) We entered female and male IDs and their interaction into the analyses as random factors. (ii) In a second set of analyses, we contrasted the sex-specific statistical effects of males and females by only using the partners' ID as a random effect (i.e., capturing a single maternal environment for maternal full- and half sibs, but with multiple female environments for halfsibs sired by polygynous males). This approach allows us to expose the relative difference of controlling for sibship in terms of genetic effects (paternal ID) within a single female's environment, versus controlling for multiple maternal environments ("maternal ID's"), when males are polygynous.

We first ran models assessing directional effects of inbreeding on hatchling telomere length and then analysed corresponding effects of absolute deviations from mean heterozygosity on hatchling telomere length (essentially analysing quadratic selection but not assuming the observations follow an inverted U shape). Two observations were removed from the analyses, since the hatchling telomere outliers deviated by more than 14 standard deviations from the mean. Directional- and stabilizing effects were analysed independently of each other (following the logic of selection analysis in quantitative genetics; Lande & Arnold, 1983), since a parameter squared to analyse quadratic effects introduces collinearity and inflated risk of type I error.

Parameter and model selection considered a combination of controlling for previously published and established effects, maximizing the number of observations from our genotyped and qPCR assessed telomere lengths, and observing shifts in Akaike indices, the latter while acknowledging that shifts in sample sizes between analyses due to lack of complete data for all predictors influences Akaike indices (Wang et al., 2011).

In a last section of analyses, we calculated R^2s and intraclass correlation coefficients (ICC) following Nakagawa et al. (2017) for results indicating ongoing quadratic (stabilizing) selection on telomere length deviation from the mean level of inbreeding. These analyses were based on a data set restricted to 1434 offspring from 191 females and 180 males with no missing values on any trait so that comparisons of shifts in magnitude of variance components between models, with and without selected predictors and random effects, could be made based on an identical data set. It seems warranted to explain these methods in more details to the reader and, citing Nakagawa et al. (2017), their approach starts with a case of Gaussian error distributions based on a linear mixed effects model (LMM) analysing a two-level data set where the first level corresponds to observations (telomere length at hatching in our case) and some grouping factor at the second level (individual ID in our case) with k fixed effect covariates:

$$y_{ij} = \beta_0 + \sum_{h=1}^{k} \beta_h x_{hij} + \alpha_i + \varepsilon_{ij}$$

$$\alpha_i \sim \text{Gaussian}\left(0, \sigma^2\alpha\right)$$

$$\varepsilon_{ij} \sim \text{Gaussian}\left(0, \sigma^2\varepsilon\right)$$

where y_{ij} is the jth observation of the ith individual, x_{hij} is the jth value of the ith individual for the hth of k fixed effects predictors, β_0 is the (grand) intercept, β_h is the regression coefficient for the hth predictor, α_i is an individual-specific effect, ε_{ij} is an observation-specific residual.

We fit models with the random factors maternal and paternal ID, and their interaction, where the covariance parameter estimates were obtained from the covariance parameter estimates' table in SAS 9.4, Proc MIXED (Wang et al., 2011), and VarF.

$$\sigma^2 f = \text{var}\left(\sum_{h}^{k} \beta_h x_{hij}\right)$$

calculated as mariginal predictions from the same model.

For this procedure, Nakagawa et al. (2017) define two types of R^2:

Marginal $R^2_{\text{LMM(m)}}$

$$R^2_{\text{LMM(m)}} = \sigma_f^2 / \left(\sigma_f^2 + \sigma_\alpha^2 + \sigma_\varepsilon^2\right)$$

and

Conditional $R^2_{\text{LMM(c)}}$

$$R^2_{\text{LMM(c)}} = \left(\sigma_f^2 + \sigma_\alpha^2\right) / \left(\sigma_f^2 + \sigma_\alpha^2 + \sigma_\varepsilon^2\right)$$

Thus, the marginal $R^2_{\text{LMM(m)}}$ estimates the proportion of total quantified variance explained by the fixed factors only, whereas the conditional $R^2_{\text{LMM(c)}}$ estimates the proportion of variance explained by the summed fixed and random factors. In some cases further explained in the results section, we selectively dropped random or fixed factors to quantify their relative importance for explaining

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variance in hatchling telomere length. In all such cases we recalculated all variance components (since e.g., σ^2_α will change depending on the presence of fixed factors and how well these fit the data).

We then also calculated two corresponding types of ICC, defined with respect to whether fixed effect variance has been fit or not:

$$\mathrm{ICC}_{\mathrm{LMM(adj)}} = \sigma^2_\alpha / (\sigma^2_\alpha + \sigma^2_\varepsilon).$$

$$\mathrm{ICC}_{\mathrm{LMM}} = \sigma^2_\alpha / \left(\sigma^2_f + \sigma^2_\alpha + \sigma^2_\varepsilon\right).$$

Thus, these ICCs quantify the proportion variance explained by the random factors, with the adjusted $\mathrm{ICC}_{\mathrm{LMM(adj)}}$ based on the empty model (i.e., no fixed effects). The *R*-square and ICC measures can then be calculated for GLMM situations where distribution-specific effects are incorporated in the calculations (as per Nakagawa et al., 2017). Here, however, we are dealing with very large sample sizes and approximately normally distributed data so the LMM approaches suffice.

3 | RESULTS

3.1 | Empty model

The empty model was run with maternal (N = 240) and paternal ID (N = 270) and their interaction as random factors and hatchling telomere length as response variable (Intercept estimate = 0.90 ± 0.005, SE, t = 182.1, p < .0001, based on 1687 offspring). The model was then rerun with the parental ID interaction backwards-eliminated (likelihood ratio test [LRT], X^2 = 46.0, df = 1, p < .00005), paternal ID eliminated (increases sample size of offspring to 1795 [306 females], LRT X^2 = 63.1, df = 1, p < .00005 – that is, better fit without Paternal ID in the model when sample sizes also increases by 66 females and 108 offspring). With Maternal ID eliminated, sample sizes increased to 1804 offspring (LRT, X^2 = 396.1, df = 1, p < .00005). Thus, maternal and paternal ID, and their interaction, all influenced telomere length at hatching. Also, offspring telomere length at hatching varies with the partner(s) ID for both the male and female.

3.2 | Parental and offspring heterozygosity

We first modelled the effect of maternal and paternal level of heterozygosity (MomSH, N = 233, and DadSH, N = 266, respectively) on the heterozygosity level of their offspring (JuvSH, N = 2329), with maternal and paternal IDs and their interaction as random effects in the model. These effects were significant (MomSH, β = 0.12 ± 0.036, t_{182} = 3.40, p = .0008; DadSH, β = 0.18 ± 0.035, t_{196} = 3.40, p < .0001) with a significant interaction between the maternal and paternal IDs (LRT, X^2 = 52.6, df = 1; p = .0005; MomID, LRT X^2 = 266.5, p = .0005; DadID, LRT X^2 = 114.2, p = .0005; Supporting Information). The best Akaike fit is the full model, with a significant interaction, which demonstrates that (unsurprisingly) offspring heterozygosity is determined by the level of heterozygosity of each parent but, in addition, also what combination of partners have produced a given offspring (a significant maternal - paternal ID interaction; Supporting Information).

3.3 | Telomere length at hatching - effects of juvenile inbreeding, juvenile sex and climate

To make our results comparable to previously published information (e.g., Bebbington et al., 2016), we first analysed the effect of a hatchling's level of heterozygosity on its telomere length (Figure 1). This result was positive and significant (parameter estimate, β = 0.036 ± 0.01, SE, t_{1656} = 3.52, N = 1658, p < .0002). Thus, nothing else considered, less inbreeding (more heterozygosity) yields longer telomeres at hatching. In a subsequent analysis, both male ID (LRT, X^2 = 8.0, df = 1, p < .005) and female ID (LRT, X^2 = 507.8, df = 1, p < .0005, N_{females} = 268) significantly affected the hatchling heterozygosity-telomere length relationship. However, again, the interpretation of the independent effects of maternal and paternal IDs were made complex by their significant interaction (N_{males} = 266, N_{females} = 236, $N_{\mathrm{offspring}}$ = 1567; LRT, X^2 = 62.4, p < .0005; Supporting Information); thus, male and female ID effects on hatchling telomere length are different depending on which partner they pair with to sire offspring (details in Supporting Information).

The analyses of hatchling sex and climate (mean spring temperature in the current year of analysis) both showed effects on hatchling telomere length while controlling for parental IDs and their interaction (N_{females} = 229, N_{males} = 256, $N_{\mathrm{offspring}}$ = 1348; Significant parental ID effects presented in Supporting Information). The analysis revealed significant positive effects of average spring temperature on telomere length (parameter estimate, β = 0.03 ± 0.004, SE, t_{1084} = 7.20, p < .0001), and that daughters hatch out with significantly shorter telomeres than sons in this population (parameter estimate contrasting daughters against sons set to zero, parameter estimate for daughters, = −0.028, ±0.003, SE, t_{1119} = −8.55, p < .0001). Hatchling heterozygosity (JuvSH) was unrelated to hatchling telomere length (parameter estimate = 0.006 ± 0.009, t_{1282} = 0.61, p = .543) when parental identities were controlled for.

3.4 | Effects of parental- and hatchling (JuvSH) inbreeding on hatchling telomere length

We then added the heterozygosity indices for both parents to the analysis to test for parental heterozygosity effects, specifically, while removing any other parental identity effects by keeping maternal and paternal IDs, and their identity interaction, in the model. Neither paternal, maternal, nor juvenile heterozygosity, significantly predicted offspring telomere length at hatching with parental ID's as random effects in the model (p > .664, 0.174 and 0.854, respectively; $N_{\mathrm{offspring}}$ = 1340, N_{males} = 252, N_{females} = 223; Electronic supplement), while hatchling sex and spring temperature still did

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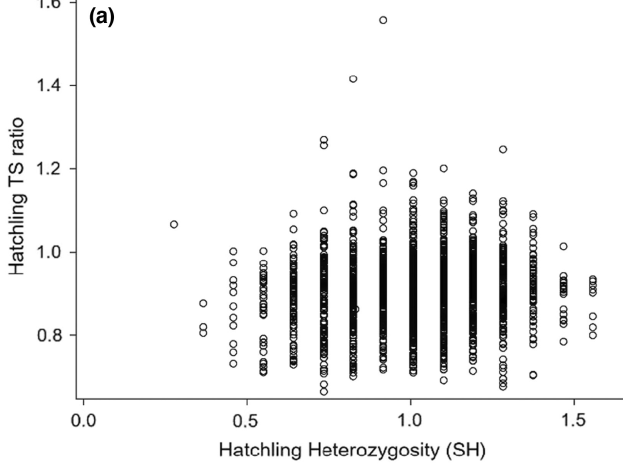

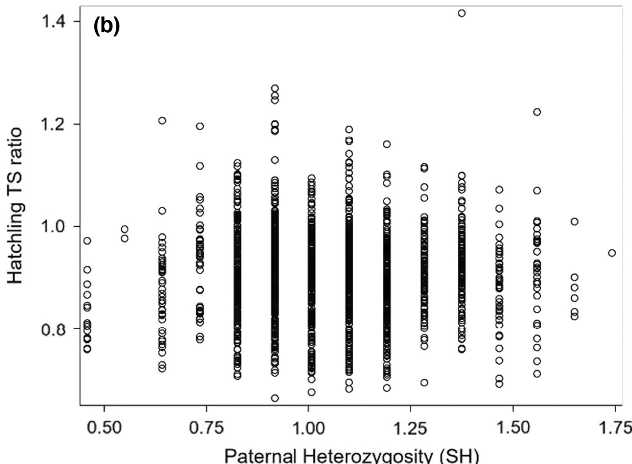

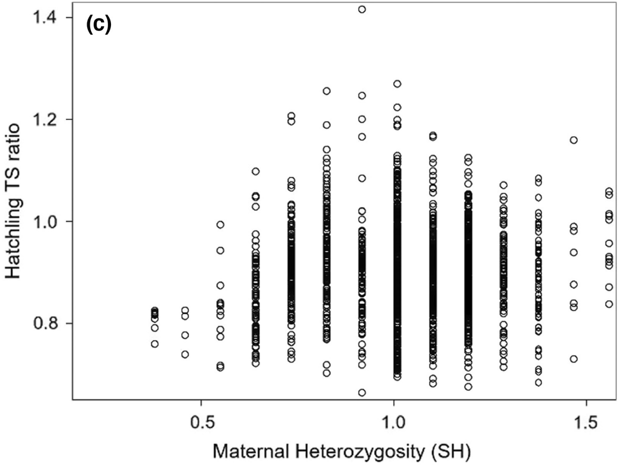

FIGURE 1 (a–c) From top to bottom, relationship between standardized heterozygosity (SH) and hatchling telomere lengths (as T/S ratios) for (a) hatchling SH, (b) paternal SH and hatchling telomere length, and (c) maternal SH and hatchling telomere length

(p <.0001; Electronic supplement). Likelihood ratio tests showed that the male*female interaction was significant (LRTs, X^2 =57.1, df = 1, p <.0005), and so were paternal ID (X^2 =144.0, df = 1, p <.0005), and maternal ID (X^2 =326.7, df = 1, p <.00005). When ID of the opposite sex parent only was entered, both parental ID effects were still nonsignificant (DadSH, p =.743, MomSH, p =.112; Supporting Information).

3.5 | Is hatchling telomere length under stabilizing selection?

The effect on hatchling telomere length of absolute juvenile deviation from mean heterozygosity was nonsignificant with maternal (N = 229) and paternal (N = 256) IDs, and their interaction, kept as random factors in the model ($P_{\text{juv deviation from mean}}$ =0.240, juvenile sex and thermal effects remained significant; Random effects are presented in Supporting Information).

We then tested for effects on hatchling telomere length of parental deviation from mean heterozygosity, first with parental IDs and their interaction in the model, then by controlling for sibship only using the opposite-sex parent's ID. No paternal- or hatchling absolute deviation from mean heterozygosity was significant, regardless of what random effects were kept in the model (p >.27 for all analyses; Figures 1a,b; Supporting Information). Maternal effects showed weak evidence of stabilizing selection in the full model (including maternal and paternal ID and their interaction), with deviation from mean inbreeding trending towards shorter hatchling telomeres (p =.107; Figure 1c). When paternal ID only was kept as a random factor, more mean heterozygosity-deviant females had young with shorter telomeres (p =.004; Figure 1c). The general agreement of distributions is obvious from visual inspection of Figures 1a–c (plots of telomere length against deviation from mean heterozygosity), but the peak is sharper in adult females.

3.6 | Effect sizes and explained variance

We pursued our analyses with an assessment of effect sizes of fixed and random effects, quantified as intraclass correlation coefficients (ICCs) and R^2s (following Nakagawa et al., 2017), specifically with respect to the effects on hatchling telomeres of maternal deviation from mean inbreeding. To estimate the proportion explained variance by each fixed effect we first calculated marginal R^2s separately for each fixed effect with the random effects maternal-, paternal IDs and their interaction in the model. Hatchling sex explained 3.1% of the variation in hatchling telomere length (i.e., marginal R^2 =0.031), whereas hatchling sex and random effects together explained 57.9% of the hatchling telomere length variation (i.e., conditional R^2 =0.0579). Mean annual temperature explained 4.9% of variation in hatchling telomere length, whereas the summed proportion of temperature and random effects explained 54.2% of hatchling telomere variation. Finally, these calculations showed that the marginal R^2 for female deviation from mean heterozygosity was 0.06 and the corresponding conditional R^2 equalled 0.55. Finally, we entered all three fixed factors (offspring sex, ambient temperature and deviation from mean maternal heterozygosity) into the model which gave a marginal R^2 of 0.087 (thus, the fixed effects are not

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strictly additive), and a conditional R^2 of 0.568. Raw maternal heterozygosity, ignoring deviation from the mean, had an R^2 of 0.0007.

The adjusted ICCs calculated with no fixed effects in the model showed a maternal identity $ICC_{LMM(adj)}$ of 0.20 (i.e., ICC based on the same mother and different fathers), whereas the corresponding values for paternal ID was 0.10 (same father, different mothers), and the ICC for the same father and mother was 0.48, and 0.53 with fixed effects included (i.e., ICC_{LMM}; Nakagawa et al., 2017).

4 | DISCUSSION

The most profound outcomes in our analyses are the significant effects of parental IDs and their interactions explaining about half of the variance in hatchling telomere length, along with a few percent of variation significantly and consistently explained by ambient temperature and offspring sex. The two parental ID traits are potentially affecting hatchling telomeres via different routes - genes and maternal environments (e.g., nutritional, hormonal and immunological yolk resources, female thermoregulatory behaviour prior to laying).

We maximized our sample sizes in order to increase the power to partition out the immediate effects of parental and hatchling inbreeding effects on hatchling telomere length, while controlling for two already established predictors of telomere length and attrition, sex and ambient temperature. The high level of multiple paternity offers both advantages and complications for analysing environmental- and genetic effects on offspring traits; polyandry offers a strong "pseudoexperimental" design when natural variation allows the comparison of male genetic effects on the same maternal environmental and genetic background. On the other hand, controlling for pseudoreplication becomes complex when there are significant maternal-by-paternal ID interactions at genetic-genetic and, potentially, environmental-genetic levels that differ between full sibs and half sibs. When there is mixed paternity, a female's maternal effects on half sibs can be controlled for by maternal ID, while separating out inbreeding effects using MomSH as a fixed effect. Half sibs within a female will thus have different paternal genetic effects, and inbreeding effects can simultaneously be partitioned out using DadSH as a fixed factor. However, paternal effects estimated with the paternal ID will, when a male has part of paternity in several clutches, include variance components from all his females' maternal effects in his explained hatchling telomere length variance. Thus, a male's variance in offspring telomere length will be inflated by variance stemming from offspring with a number of females providing different developmental, maternal environments. The corresponding effect in females will be a constant maternal effect across siblings, but with potentially different paternal genetic effects on halfsibs' telomeres, and with – potentially - different paternal genetic interactions within the same female environment.

To contrast the sex-specific effects, we also controlled for the opposite-sex partner IDs only in a separate set of analyses when analysing stabilizing selection. Our analyses showed no significant stabilizing selection arising from paternal- and hatchling deviation from mean heterozygosity. However, the corresponding analyses showed that females trended towards significant deviation from mean heterozygosity in the full model with significance maintained ($p < .004$) when only the paternal effect was kept as a random factor. Our focus here is not the maternal effect per se, which while significant is trivial, but the sex differences there seem to be in parental effects. This effect is not only apparent in the significant differences in stabilizing selection, but more importantly in the differences in R^2 values and ICCs. The adjusted ICCs were twice as high for maternal identity ($ICC_{LMM(adj)} = 0.20$; i.e., ICC based on the same mother and different fathers) as for paternal ID (0.10; same father, different mothers). The ICC for the same father and same mother was 0.48 (and 0.53 with fixed effects included, i.e., ICC_{LMM}; Nakagawa et al., 2017).

We also investigated hatchling sex effects since our previous work has established sex differences in telomere dynamics, with adult males having slightly shorter telomeres than females, at least under some conditions (Axelsson et al., 2020; Olsson, Pauliny, Wapstra, Uller, Ujvari, et al., 2011a; Olsson, Pauliny, Wapstra, Uller, Schwartz et al., 2011b). Somewhat to our surprise, males in this population with low-level genetic variation hatched out with c. 4% longer telomeres than females on average and we therefore controlled for hatchling sex in all inbreeding analyses. An interpretation of the sex difference in hatchling telomere length from an evolutionary standpoint is that, since telomere length early in life seems to predict life span in sand lizards and other taxa (Bauch et al., 2014; Heidinger et al., 2012; Olsson, Pauliny, Wapstra, Uller, Ujvari, et al., 2011a), there may be ongoing selection across life history stages for longer hatchling telomeres in males than females (e.g., Cheverud, 2000 and references therein). If so, males, with higher telomere attrition than females, may arrive at approximately the same length (or slightly shorter) telomeres late in life as do females. One would assume that this is close to an optimized telomere length in response to selection, after attrition, since both "too long" and "too short" telomeres are expected to be under selection (as outlined at length in the Introduction).

Parental and hatchling inbreeding estimates consistently had very minor effects on hatchling telomere length in our analyses. Assuming that telomeres are indeed biomarkers of overall health and vigour as outlined in the introduction, the lack of stronger inbreeding effects are puzzling and provokes discussion. Why are inbreeding effects not stronger? A full discussion of the validity of multilocus microsatellite markers from a perspective of inbreeding analysis and heterozygosity-fitness (HFC) analyses is outside the scope of this study, but suffice to say that results have varied across taxa, that heterozygosity-based measures outperform d^2-based measures, and that power to detect HFCs is low when 10 or fewer markers are typed (Slate & Pemberton, 2002). Other work has shown that sample sizes in excess of 600 individuals are necessary to detect reasonable effect sizes with maintained power (Coltman & Slate, 2003). We used 17–21 microsatellites and 1348–2329 hatchlings, depending on analysis; thus, 21 microsatellites is a substantial set of markers in a natural population and the sample sizes are in

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the upper tail end of published field studies with this level of resolution. Furthermore, we make the observation that our microsatellites were initially selected for paternity analysis and therefore chosen based on their above average variability (Olsson, Pauliny, Wapstra, Uller, Ujvari, et al., 2011a; Olsson, Pauliny, Wapstra, Uller, Schwartz et al., 2011b). Thus, from this perspective inbreeding could be underestimated in our work (with our markers being more variable than the true genome-wide average). However, given the consistently minute effects of inbreeding on hatchling telomere length, we doubt this explains our low level inbreeding effects on telomere traits.

In our previous work, we have also shown sex-differences in telomeric relationships with life history traits; female telomere length significantly predicts life span and lifetime reproductive success and differ from corresponding relationships in males (which were not significant per se). However, when heritabilities of telomere length, age-specific or not, were calculated on sex-specific data, son-sire heritability was about twice that of daughter-dams (Olsson, Pauliny, Wapstra, Uller, Ujvari, et al., 2011a; Olsson, Pauliny, Wapstra, Uller, Schwartz et al., 2011b). An obvious reflection on this is that if indeed telomere length is more closely linked to fitness in females, classic quantitative genetic theory predicts heritability to be lower in females as a consequence of stronger past and present natural selection (Falconer & Mackay, 1996; Lynch & Walsh, 1998).

Telomeres are primarily restored through the action of telomerase (TA; Quach et al., 2017). The enzyme includes the TA component *TERC*, which binds to the 3′ overhang of telomeric DNA and is a template for the addition of repeats, a protein component called TA transcriptase *TERT*, and accessory proteins. *TERT* variants associated with longer telomeres confer higher intrinsic epigenetic age acceleration (IEAA; Lu et al., 2018), with independent effects of telomere length and IEAA, suggesting a critical role for *TERT* in regulating the epigenetic clock. Thus, we would expect these relations to have direct or indirect effects on telomeres and life history parameters and be under selection in the wild. Given the variation in TA production in different tissue of ectothermic taxa (Henriques et al., 2013), ectotherms are likely to be valuable for studies of ongoing evolution of TA repression dynamics in the wild, since covariation between TA and lifetime fitness can be studied in real time.

Bebbington's model species, the Seychelles warbler, most often only produce a single offspring (so pseudoreplication is less of a problem; David Richardson, personal communication) but may live for up to 19 years. Sand lizards also live through multiple breeding events in the wild, which we control for in our mixed models (in Sweden, breeding annually for a maximum of c. 10 years, in continental Europe up to c. 16 years; Strijbosch, 1988). At large, our methods are very similar to those of Bebbington et al., building on qPCR with GAPDH as the reference gene and Rhh for analysing inbreeding coefficients, and so are our results; in our previous work we report an average SH of 0.99 (SD = 0.22, $n = 3043$) for the same population with a g2 parameter of 0.0025 ($p = .005$; Bererhi et al., 2019). Bebbington et al. (2016) recalculated their SH vales into homozygosities (i.e., 2–SH) so back-transforming them yields an average juvenile SH of 1.03 (SD = 0.21), and a g2 parameter of 0.007 ($p = .001$; Bebbington et al., 2016, p. 2954). Our magnitude and effect sizes are thus very similar to those of Bebbington et al. (2016) with the main difference being that Bebbington et al. (2016) demonstrated a statistically significant negative, directional effect size of increased homozygosity on telomeres of 1.1% (R^2), whereas in our study, the statistically significant effect was on the maternal deviation from mean heterozygosity with an effect size of 0.006 (R^2). This effect was only significantly detected in mothers and not fathers (and not the offspring themselves), which we suspect may be because maternal variance components are estimated on offspring with the same maternal environment, whereas those from fathers are detected on multiple female environments (probably reducing the detected male genetic effect relative to genetic and environmental effects in females).

In summary, our analyses show considerable effects on hatchling telomere length of parental IDs, their interaction, ambient spring temperature and juvenile sex. The significant paternal ID effects are more than likely to be genetic given that semen is the only transgenerational vector of paternal effects, although these are not only due to inbreeding. That said, we emphasize the complication of estimating paternal effects across multiple maternal environments. Furthermore, given that adult males and females are approximately equally inbred/heterozygous, there seems to have been no sex-specific mortality making one parent more likely to genetically compromise offspring viability or telomere length from a heterozygosity perspective.

While the current work was in review, Pepke et al. (2022) published an analysis of sex-specific inbreeding effects (shorter telomeres in response to inbreeding in male house sparrows, *Passer domesticus*), following an artificial selection experiment in the wild (Pepke et al., 2021). It is a little hard to interpret these observations in comparison with our own population, which exhibits a naturally, relatively low level of genetic variation, and the study by Pepke et al. (2022) openly questions whether some of the results can be explained by heterosis effects following artificial selection (if so, heterotic birds have longer telomeres – which would inflate the T/S ratio variance). That said, work of this kind offers an elegant way in which to analyse how strongly ongoing selection may change telomere dynamics.

To conclude, in our study maternal IDs explain approximately twice the variance in offspring telomere length that paternal IDs do. It seems appropriate to say that, with R^2s the magnitude of 0.0007–0.06, inbreeding is unlikely to be part of these sex-specific effects. We also draw the readers' attention to the differences in procedure for calculating maternal and paternal effects in polygynandrous species, with additional variance contributed by multiple maternal environments for polygynous males, but with no such contributions from multiple male environments in polyandrous females.

AUTHOR CONTRIBUTIONS

Mats Olsson planned the study. Mats Olsson and Erik Wapstra conducted fieldwork. Emily Miller, Nicky Rollings and Tonia Schwart conducted molecular analyses. Mats Olsson and Badreddine Bererhi conducted statistical analyses. Mats Olsson wrote the first

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draft of the manuscript and all authors contributed to writing and commenting on the manuscript thereafter.

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CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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DATA AVAILABILITY STATEMENT

All data supporting this work have been made available in the Dryad Digital Repository (https://doi.org/10.5061/dryad.v15dv41zh; Olsson, 2022).

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