TITLE: Experimental demonstration that offspring fathered by old males have shorter telomeres and reduced lifespans

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ABSTRACT

Offspring of older parents frequently show reduced longevity, but the mechanisms driving this so-called 'Lansing Effect' are unknown. While inheritance of short telomeres from older parents could underlie this effect, studies to date in different species have found mixed results, reporting positive, negative or no association between parental age and offspring telomere length. However, most of the existing evidence is from non-experimental studies in which it is difficult to exclude alternative explanations such as differential survival of parents with different telomere lengths. Here we provide evidence in the zebra finch that offspring from older parents have reduced lifespans. As a first step in disentangling possible causes, we used an experimental approach to examine whether or not we could detect pre-natal paternal effects on offspring telomere length. We found that zebra finch embryos fathered by old males have shorter telomeres than those produced by the same mothers but with younger fathers. Since variation in embryonic telomere length persists into post-natal life, and early life telomere length is predictive of longevity in this species, this experimental study demonstrates that a paternally-driven pre-natal telomere length reduction could at least in part underlie the reduced lifespan of offspring from older parents.

KEYWORDS: ageing, early development, Lansing effect, paternal inheritance, *Taeniopygia guttata*

INTRODUCTION

Parental effects, where the phenotype of one or both parent(s) affect offspring phenotype, represent a form of phenotypic plasticity spanning generations [1]. Parental effects can take many forms [reviewed in 2], but recent evidence indicates that parental age is a key aspect of the parental phenotype that can strongly influence offspring phenotype and have positive or negative fitness consequences [e.g. 3, 4, 5]. Numerous studies have shown a negative effect of advanced parental age at reproduction on offspring health and longevity in a wide variety of taxa, including humans [see e.g. 3, 4, 5, 6-9], a phenomenon often referred to as the 'Lansing effect' [8]. The 'Lansing effect' could, therefore, influence the population rate of genetic and phenotypic change, and ultimately affect population persistence and adaptation [2].

In species with parental care, there are several ways parental age could affect offspring phenotypes based on maternal and/or paternal effects operating pre- and/or post-natally [3, 6, 10-15]. One potentially important route that could explain a reduction in the lifespan of offspring from older fathers is via these offspring having shorter telomeres. Telomeres are repeated tandem sequences of nucleotides located at the ends of chromosomes that play a key role in preventing chromosome degradation and genome instability [16]. Average cellular telomere length (TL) decreases with age in many taxa, particularly during the growth period [e.g. 9, 17-19], and individuals with shorter TL have an increased risk of disease [20, 21] and reduced longevity [9, 22]. However, studies to date have provided mixed results; whereas in some mammal and bird species paternal age has been positively associated with offspring TL, in other vertebrates the pattern is reversed or absent [reviewed in 23].

57 Existing data on the effect of paternal age on offspring TL are based on cross-
58 sectional studies, mostly in humans, in which it has not been possible to examine offspring
59 produced by the same fathers reproducing at different ages [24-31]. This makes it difficult to
60 separate maternal and paternal pre- and post-natal effects. The epidemiological nature of
61 these studies also means that it is difficult to control for factors such as variation in the
62 survival of males with different TLs, the condition of males reaching different ages, their
63 previous reproductive investment, the lack of paternity confirmation, non-random mate
64 selection by females and a lack of control over the age at which offspring TLs are measured.
65 With respect to the latter, offspring TL has often been measured at very variable ages after
66 birth or hatching [19, 25-28, 32, 33]; this makes it difficult to separate the influence of
67 paternal age from environmental factors acting during early or late postnatal life that are
68 known to influence TL, and also allows potential bias from non-random mortality in the
69 offspring. Controlling the offspring postnatal environment is likely to be particularly
70 important since paternal age will potentially influence the quality of the postnatal
71 environment which can affect telomere loss [30].
72 Here we report a first experiment designed to tease apart some of these effects. This
73 work was carried out in the zebra finch *Taeniopygia guttata;* as in humans, zebra finch TL
74 declines with age, mostly during early life [9, 34], and postnatal TL dynamics are strongly
75 influenced by factors acting on prenatal (embryonic) TL [17]. We provide evidence
76 supporting the existence of a Lansing effect by examining offspring survival of zebra finches
77 produced by parents of different ages bred and maintained under standardised aviary
78 conditions. We then used a within-female experimental design to examine the effect of
79 paternal age on offspring telomere length: middle-aged zebra finch females reproduced
80 with a young (4 months of age) and an old male (4 years of age) within a short period of

time. Freshly laid eggs were then artificially incubated for a fixed period under standardised conditions and TL in embryos measured. Therefore, this design allowed us to examine the influence of paternal age while avoiding potential confounding effects on offspring TL such as the variation in the age at which offspring TL was measured, and also effects of parent mate choice, maternal age, inter- and intra-individual variation in reproductive effort (e.g. pre- and post-natal parental care), variation in the offspring post-natal environment and differential postnatal survival of parents and offspring.

RESULTS

Effect of parental age on offspring longevity

We found clear evidence for a Lansing effect in this species (Table S1); offspring longevity was negatively related to maternal and paternal age but the later had a quadratic effect on offspring survival (Cox mixed-effect model; female age; Wald=4.958, DF=1, p=0.026; male age^2: Wald=6.519, DF=1, p=0.011; figure 1a,b and Table S1). Longevity did not differ between sons and daughters, nor did the influence of parental age on offspring longevity depend on offspring sex (Table S1). Interestingly, when we re-analysed the data but removing the offspring that died before reaching adulthood, only paternal age had a significant effect on offspring longevity (Table S2).

Effect of paternal age on embryonic telomere length and embryo size

Our experimental approach showed that paternal age had a significant negative effect on embryo TL ($F_{1,18.80}$=5.341, p=0.032; table 1); on average, the TL in embryos sired by old males was *ca*. 10 % shorter than in those with the same mother but sired by young males

(figure 2). The reduction in TL was independent of the positive effect of egg size on embryo TL (table 1), and was not influenced by the order in which females mated with the old and young fathers, whether the embryo came from the first or second breeding event (i.e. clutch number), the embryo's position within the clutch or its sex (see table 1). Paternal age had no effect on clutch size, egg mass or embryonic survival (P>0.27 in all models; see SM, and table S2), but embryos sired by old males developed faster than those sired by young males, as indicated by their larger body size at 4 days of age after controlling for egg mass (table 1; figure 3). This difference in embryonic growth rate did not create the effect of paternal age on embryo TL; the effect of paternal age on embryo TL was independent of embryo size when added into the model ($F_{1,19.77}$=5.514, p=0.029; see table S3) but embryo size was positively correlated to embryonic TL, regardless of paternal age (embryo size: estimate=0.138, $F_{1,112.89}$=7.736, p=0.006; embryo size x paternal age: $F_{1,111.44}$=1.562, p=0.214; see table S3).

DISCUSSION

We found evidence of a Lansing effect in our zebra finch population. This reduction in offspring longevity as the parental age at the time of conception increases is consistent with previous studies across a range of vertebrates including humans [see e.g. 3, 4, 5, 6-9]. By using a carefully controlled experimental approach, we show that offspring fathered by old males have significantly shorter telomeres early in prenatal life than embryos from the same mother but with a younger father. Since we know that in this species differences in embryo TL persist into adult life [17], and that TL at the end of the main growth period is predictive of longevity [9], this paternal effect is likely to affect offspring lifespan.

127 The negative effect of paternal age on offspring TL could come about by a number of
128 different routes, several of which we have excluded via our experimental design. Offspring
129 TL was measured at the embryo stage, thus excluding any effect of the post-natal
130 environment. The eggs were incubated in standardised conditions in incubators; thus
131 differences in incubation behaviour of males of different ages do not underlie this effect. TL
132 was measured at a fixed time period after incubation started, excluding another potential
133 factor, age at which offspring measurements are taken. Interestingly, the embryos of older
134 males developed faster than those from younger males, but a strong paternal age effect
135 remained when we included embryo size in our statistical models. Two potential
136 mechanisms could underlie this paternal age effect that we have found. It is possible that
137 females adjust egg composition in some way in response to male age, and that this gives
138 rise to shorter telomeres in the embryos. Birds have been shown to adjust egg size and egg
139 hormones in response to male attributes [35, 36]. Zebra finches have for example been
140 shown to increase testosterone levels in their eggs when mated with a perceived higher
141 quality male [35]. This hormone appears to have pro-oxidant effects in mammals and
142 evidence suggest that it may also contribute to dysregulate the antioxidant system of young
143 birds [37]. Consequently, it might be possible that the embryos fathered by old males were
144 exposed to higher levels of oxidative stress during development, which in turn, could
145 accelerate the post-fertilization loss of TL [38]. Alternatively, if egg antioxidants are reduced
146 when females are breeding with old males, this could also give rise to increased telomere
147 loss [39]. This egg composition effect is worthy of further investigation. To fully exclude the
148 possibility of female egg adjustments in response to male age, in vitro fertilisation would be
149 required.

150 An alternative route is, however, via changes in sperm TL with male age. The
151 reduction in TL in the embryos fathered by old males that we have found supports recent
152 experimental evidence in mice [40] and correlative studies in other bird and lizard species
153 [28-30]. These results, however, contrast with previous epidemiological studies in other
154 vertebrate species such as humans or apes [24-27, 33] where the opposite relationship
155 between paternal age and offspring TL was found. It is possible that, in contrast to long-lived
156 mammals such as humans or apes, sperm TL in short-lived vertebrates such as the zebra
157 finch decreases with male age so that embryos fathered by older males inherit shorter
158 telomeres. Indeed, it has recently shown that old male mice *Mus musculus* have shorter
159 sperm TL compared to young males [40]. Although suggestive, whether or not this can also
160 occur in other taxonomic groups such as birds is still unknown. For such studies, it is
161 important that within-male measurements are made to exclude the possibility that males
162 with longer telomeres in all tissues, including sperm, are more likely to survive and remain
163 reproductively active into old age. No such studies have yet been carried out.
164 We also found that embryos sired by old males developed faster than those sired by
165 young males as indicated by their larger body size at 4 days of age after controlling for egg
166 mass. This, while not responsible for the effect on TL, is in itself very interesting. It is likely to
167 reflect a faster cell division rate as a result of a maternal influence on egg composition since
168 this can influence development rate of the embryos [41]. While it might be assumed that
169 the faster growth of embryos fathered by old males would contribute to the loss of TL [34,
170 42, 43], embryo size was in fact positively rather than negatively related to embryo TL
171 irrespective of male age. This positive covariation has been reported in other vertebrate
172 species [44] and contrasts with the prevailing view of accelerated growth as costly in terms
173 of telomere shortening [42, 43]. Instead, it suggests that individuals that best tolerate

telomere shortening may perform best [see e.g. 39, 45], thus being able to invest more resources in costly physiological functions such as cell growth and division rate.

METHODS

Effect of parental age on offspring longevity

In order to investigate the existence of a Lansing effect in the zebra finch, we analysed the longevity data of a cohort of birds produced in our animal facilities and for which the age of both parents at the time of reproduction was known. In brief, 44 adult male and female zebra finches from our stock population were paired as a part of our long-term breeding program between 2012 and 2014. These birds were not involved in any experimental treatment and pairs were formed from genetically unrelated birds. On the day of pairing male and female age were highly correlated (Pearsons´ correlation coefficient: $r=0.97$, $N = 44$, $p<0.001$), with the average age (days) being 242 days ± 76SD (range 170-496 days) for males and 258 days ± 124 SD (range 183-660 days) for females. Pairs were housed in individual breeding cages (60-50 cm and 50 cm high) with nesting material and an external, cage-mounted nest box, in a room maintained at 20.5 ± 2C under full spectrum, artificial light (16:8 h light:dark cycle). The birds' diet comprised an ad libitum supply of commercial mixed seeds (Johnson & Jeff, U.K.), oyster shell grit, cuttlefish and water. Nests were monitored daily and the exact date of hatching of each offspring recorded. At 20 days of age, all chicks were individually marked with a metal ring and around 30 days of age, they were separated from their parents.

Due to space limitations, it was impossible to keep all the offspring from all broods for the remainder of their lives and therefore only one randomly selected bird of each

original brood (N = 44) was retained. These birds were then moved to single-sex holding cages and maintained under the same aviary conditions as described above. The survival of these birds was monitored daily until July 2017 and the exact date of death of all birds recorded. The cause of death was generally unknown but was considered to be due to natural causes if carcases did not have obvious signs of infection or injury [9].

Effect of paternal age on embryonic development

To investigate the influence of paternal age on embryonic development and telomere length (TL), in 2016 we performed an experimental study in a separate group of adult zebra finches from those mentioned in the previous section. We randomly paired thirty-two middle-age zebra finch females (age range 1.73-1.76 years) with either a young (N=16; age range 0.37-0.40 years) or an old (N=16; age range 4.04-4.19 years) male. Zebra finches can live up to 5 years in the wild [46] and up to 8 years in captivity [9], although post-fledging mortality starts to sharply increase from 13 months of age [47] and by 3-4 years of age they show clear signs of physiological ageing [48, 49]. All birds used in the experiment came from our stock population and had previously been kept in single-sex groups. All pairs were formed on the same day and comprised unfamiliar and genetically unrelated birds that were not involved in any experimental treatment. Breeding pairs were kept under the same breeding conditions (e.g. cage size, diet, temperature and light conditions) as those described above (see the previous section).

Each breeding pair was provided with a nest-box that was inspected once daily between 7:00-10:00 hr, and any new egg was marked and weighed using an electronic balance (±0.01 g). To avoid any effect of differences in incubation effort (e.g. incubation

220 temperature, onset or consistency of incubation) which could influence embryonic
221 development or embryo TL [17], every newly laid egg was immediately replaced by a
222 dummy clay egg (which maintained the laying pattern of the females), and the fresh egg was
223 placed in an external incubator at 37.5 °C and 80% relative humidity (Octagon 20 ECO
224 Incubator; Brinsea Products Ltd, Standford). Eggs were candled every morning (between 9-
225 11 am) with a cold LED light (Mini Bonfire 200 lumens; Hong Kong) in order to determine
226 whether the embryo was alive. If there were no signs of embryo development by day 5 of
227 incubation, the eggs were considered not fertile [50]. After 5 days of artificial incubation, all
228 eggs were removed from the incubator and immediately stored at -80^{o}C for later molecular
229 determination of embryo sex and TL (see below). Clutches were considered complete if no
230 new eggs were laid for 4 days [17]. All breeding pairs were separated once the first clutch
231 was complete and the birds transferred to single-sex groups.

232 One month after this first reproductive event, the females were paired again and
233 allowed to produce a clutch for second time, but this time with a male of the opposite age
234 group to the one they were paired with previously. We used the same set of males (and
235 females) during the two consecutive reproductive events. As before, mates were both
236 unrelated and unfamiliar. Both male and female body mass was measured (± 0.01 g) on the
237 day they were paired in both reproductive events. Male and female body mass had no
238 effect on embryo size (male mass: $F_{1,31.81}$=0.088, p=0.774; female mass: $F_{1,36.31}$=0.707,
239 p=0.406) or embryonic TL (male mass: $F_{1,27.15}$=0.032, p=0.859; female mass: $F_{1,36.58}$=0.478,
240 p=0.494) when tested into in the models (see statistical section). Egg fertility was lower in
241 the second clutches but did not differ with respect to paternal age (male age: $F_{1,28}$=1.37,
242 p=0.251; clutch number: $F_{1,68}$=16.69, p<0.001; clutch number x male age: $F_{1,60}$=0.46,
243 p=0.499).

244

245 **Embryo size measurements**

246 We measured embryo size via digital image analysis. This allowed a non-destructive reliable
247 measurement of embryo body size (see validation in the Supporting Material, SM) without
248 the risk of damaging telomeric DNA regions e.g. through exposure to the oxygen-rich air of
249 the atmosphere [51]. To maximize our sample size, we measured the size of the embryos at
250 4 days (i.e. one day before the telomere measurements) since at earlier/later stages most of
251 the embryos are too small/big to be accurately measured in the digital images *(pers. obs.*).
252 In total, we successfully measured 136 embryos (1^{st} reproduction: N=76; 2^{nd} reproduction,
253 N=60). Embryo size measurements were repeatable and highly correlated with the real mass
254 of the embryo (see SM Material and Methods for further details). We, therefore, used the
255 embryo size (in pixels) as a proxy of embryo mass in our experiment.

256

257 **Telomere length analyses and molecular sexing**

258 The DNA of the whole defrosted embryos was later extracted with DNeasy Blood and Tissue
259 kit (Qiagen, Crawley, UK), following the manufacturer's protocol. The TL of these embryos
260 (76 from the 1st and 60 from the 2nd breeding event; all sampled at the same age) was
261 measured by real-time quantitative PCR as described previously for zebra finch embryos
262 [17] (a detailed description of the methodology is provided in SM). The sex of the embryos
263 was determined by PCR amplification of the CHD1-W and CHD1-Z genes using the primers
264 P2 and P8 as described in [52].

265

266 **Statistical analyses**

267 *Longevity study: Effect of parental age on offspring longevity*

We used Cox mixed-effects regression models to test the influence of parental age on offspring longevity (i.e. survival). The model included sex as a fixed factor and maternal age, paternal age and their quadratic terms as covariates. The interactions between maternal and paternal age with the sex of the offspring were also tested. Because three of the females reproduced twice (but with different partners), we also initially included the identity of the female as a random factor. However, the random factor was never significant ($P<0.05$) and hence, removed to avoid model over-parametrization. Because few birds died before reaching adulthood (N=4), we re-ran the survival models after removing these individuals to avoid the risk that these early mortalities may confound any effect of parental age on offspring longevity (i.e. natural mortality at adulthood). These complementary analyses provided similar results and are provided in the SM (see Table S1).

Paternal age experiment: Effect of paternal age on embryo size and embryonic TL

We used linear mixed effect models (LMM) to test the effect of paternal age (young vs old) on embryo TL and embryo size. In the models, we controlled for several potential confounding variables such as the females' clutch number (1^{st} or 2^{nd}), the sex of the embryo, egg mass and position of the egg in the clutch (i.e. whether the first-, second-laid egg etc). Any effect of paternal age on embryo TL may be related to an effect of paternal age on embryo growth rate. Thus, we re-ran the model including embryo size and its interaction with paternal age as covariates.

Complementary analyses: Effect of paternal age on clutch size, egg mass, embryo sex and survival

We also assessed the effect of paternal age on clutch and egg traits (i.e. clutch size, egg mass and embryo sex) and embryo survival using linear mixed effect models (LMM) and generalized mixed effect models (GLMM) as appropriate. Paternal age had no effect on any of the above-mentioned variables. Additional details on statistical analyses are provided in SM (Material and Methods).

In all the above models the identity of the parents was included as a random factor to account for non-independence of eggs and/or clutches from the same mother and/or father. The interaction between male age and clutch number was also initially included in models but was never found to be significant and so was removed from the analyses. When needed, variables were log-transformed (embryo size and TL) to improve data distribution. Residuals obtained from the models were always normally distributed. Analyses used Satterthwaite's approximation for degrees of freedom. To avoid inflating the type I error we did not apply model selection in any analyses, and so report results for full models after removing non-significant interactions as recommended by [53]. Data are presented as means ± standard error (se), and the significant level was set at P=0.05.

ETHICS

All animal experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals and granted by U.K. Home Office (Project Licence No. 60/4109).

DATA ACCESSIBILITY

Data are deposited in the Figshare digital repository: DOI10.6084/m9.figshare.5271712.

COMPETING INTEREST

We have no competing interests.

AUTHORS' CONTRIBUTION

The study was conceived by J.C.N., P.M. and N.B.M. Data were collected and analysed by J.C.N and the manuscript writing was by J.C.N., P.M. and N.B.M.

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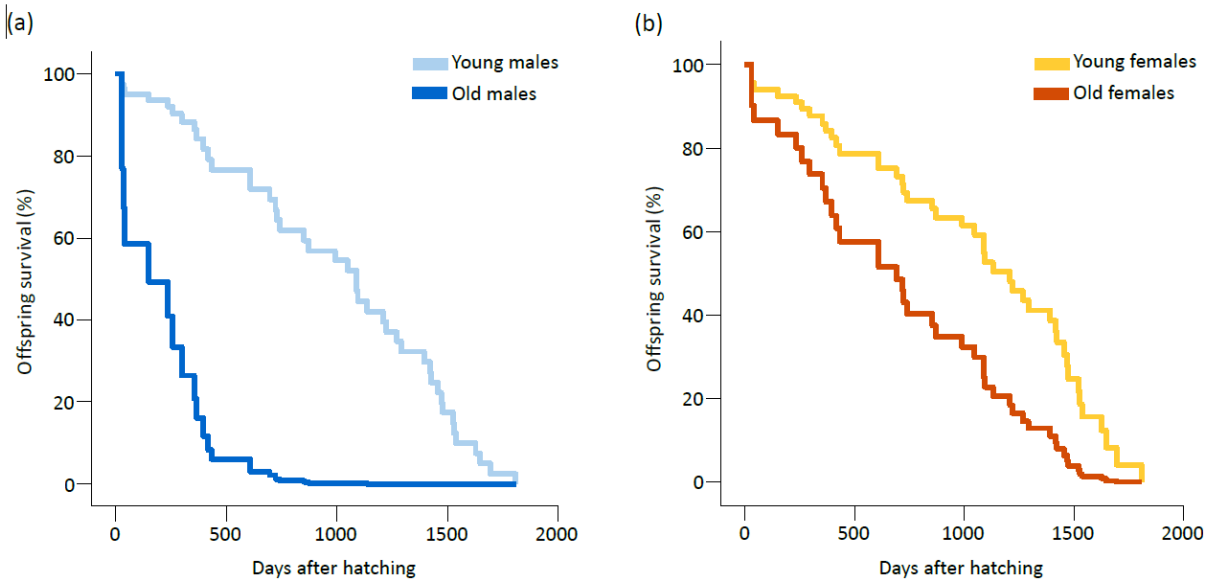
461
462
463 **Table 1.** Summary of linear mixed models (LMM) for the effects of paternal age at
464 conception and covariates on embryo telomere length (TL) and size (estimated body mass)
465 of zebra finch embryos. Measurements were made after 120h (TL) and 96 h (embryo size) of
466 artificial incubation respectively. Significant t-test are denoted by an asterisk '*'.
467
468 **Figure 1**. Offspring longevity in relation to a) paternal and b) maternal age at conception.
469 Paternal and maternal age was included as a covariate in a Mixed Effect Cox Regression
470 Model – see main text for details. For illustrative purpose, the graphs show survival curves
471 for offspring from two different (arbitrary) age classes of father or mother.
472
473 **Figure 2**. Effect of paternal age on (log) embryo telomere length (TL; mean T/S ± se) after
474 five days (120 h) of artificial incubation (N=139).
475
476 **Figure 3**. Effect of paternal age on (log) embryo size (mean ± se) after four days (96 h) of
477 artificial incubation (N=136).

478 Table 1

		Embryo telomere length					Embryo size				
Factor	Description	estimate	df	t	F or Z	P	estimate	df	t	F or Z	P
Intercept		0.147	109.36	2.120*			4.148	79.41	39.34*		
Clutch number			124.56		1.742	0.189		109.84		0.006	0.940
	First	0.014	124.56	1.320			0.001	109.84	0.075		
	Second	0					0				
Male age					5.341	**0.032**		19.15		5.061	**0.036**
	Old	-0.042	18.80	2.311*			0.056	19.15	-2.250*		
	Young	0					0				
Sex			104.52		0.405	0.526		119.16		2.235	0.138
	Female	-0.005	104.52	-0.636			0.024	119.16	1.495		
	Male	0					0				
Egg order			94.82		0.075	0.996		106.84		3.953	**0.003**
	First	-0.008	98.86	-0.428			-0.042	112.79	-1.125		
	Second	-0.006	96.20	-0.299			-0.070	112.04	-1.867		
	Third	-0.008	92.33	-0.441			-0.118	108.05	-3.205*		
	Fourth	-0.003	91.97	-0.187			-0.104	107.36	-2.775*		
	Fifth	-0.004	91.19	-0.203			-0.058	105.21	-1.145		
	Sixth	0					0				
Egg mass		0.119	106.74	2.194*	4.815	**0.030**	0.297	64.75	3.685*	13.577	**<0.001**
Random factors											
Male ID		0.001			2.06	**0.039**	0.002			1.556	0.120
Female ID		0.002			2.58	**0.010**	0.001			0.968	0.333

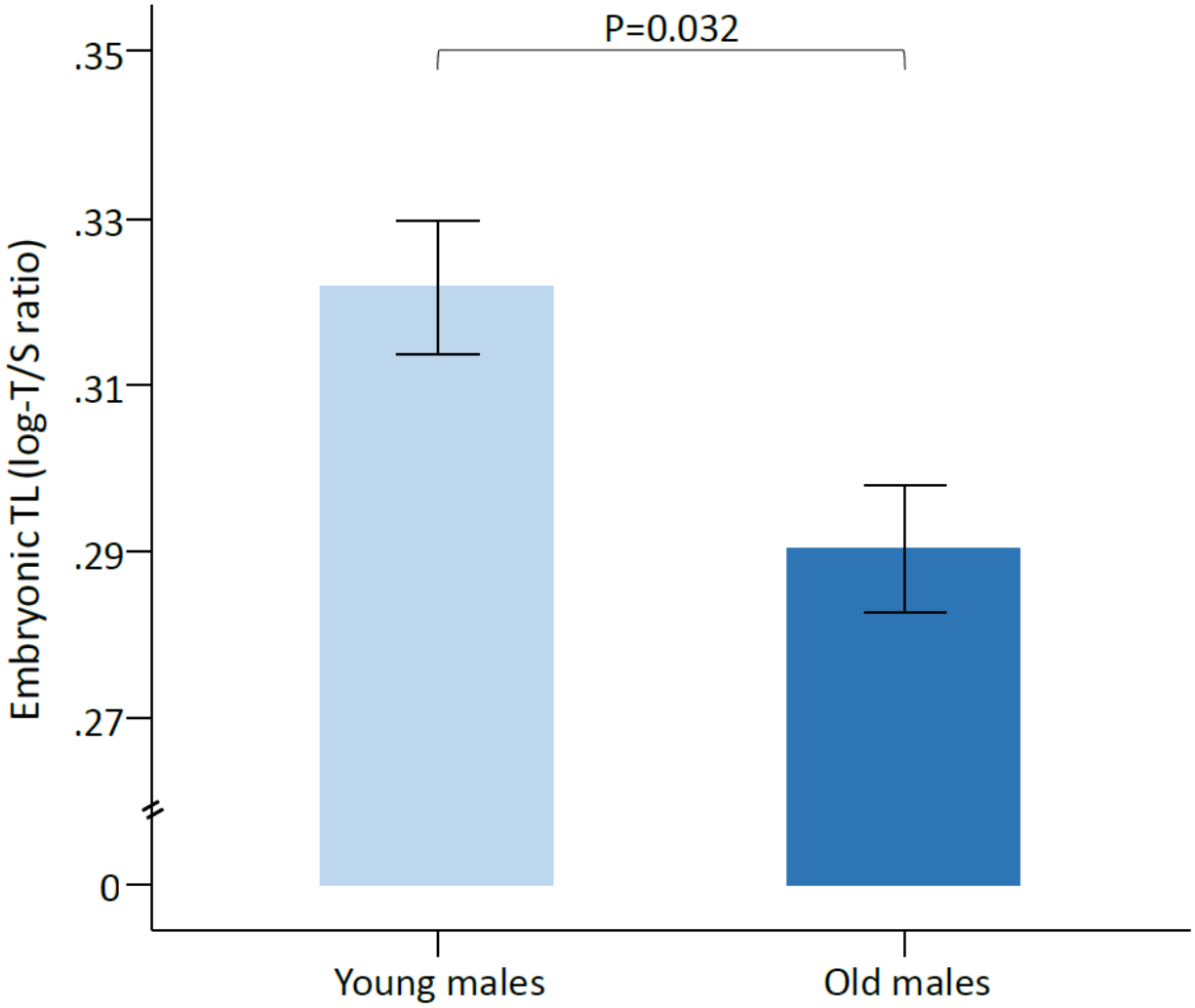
479

480


(a)
Young males
Old males
Offspring survival (%)
100
80
60
40
20
0
0
500
1000
1500
2000
Days after hatching
(b)
Young females
Old females
Offspring survival (%)
100
80
60
40
20
0
0
500
1000
1500
2000
Days after hatching

481

482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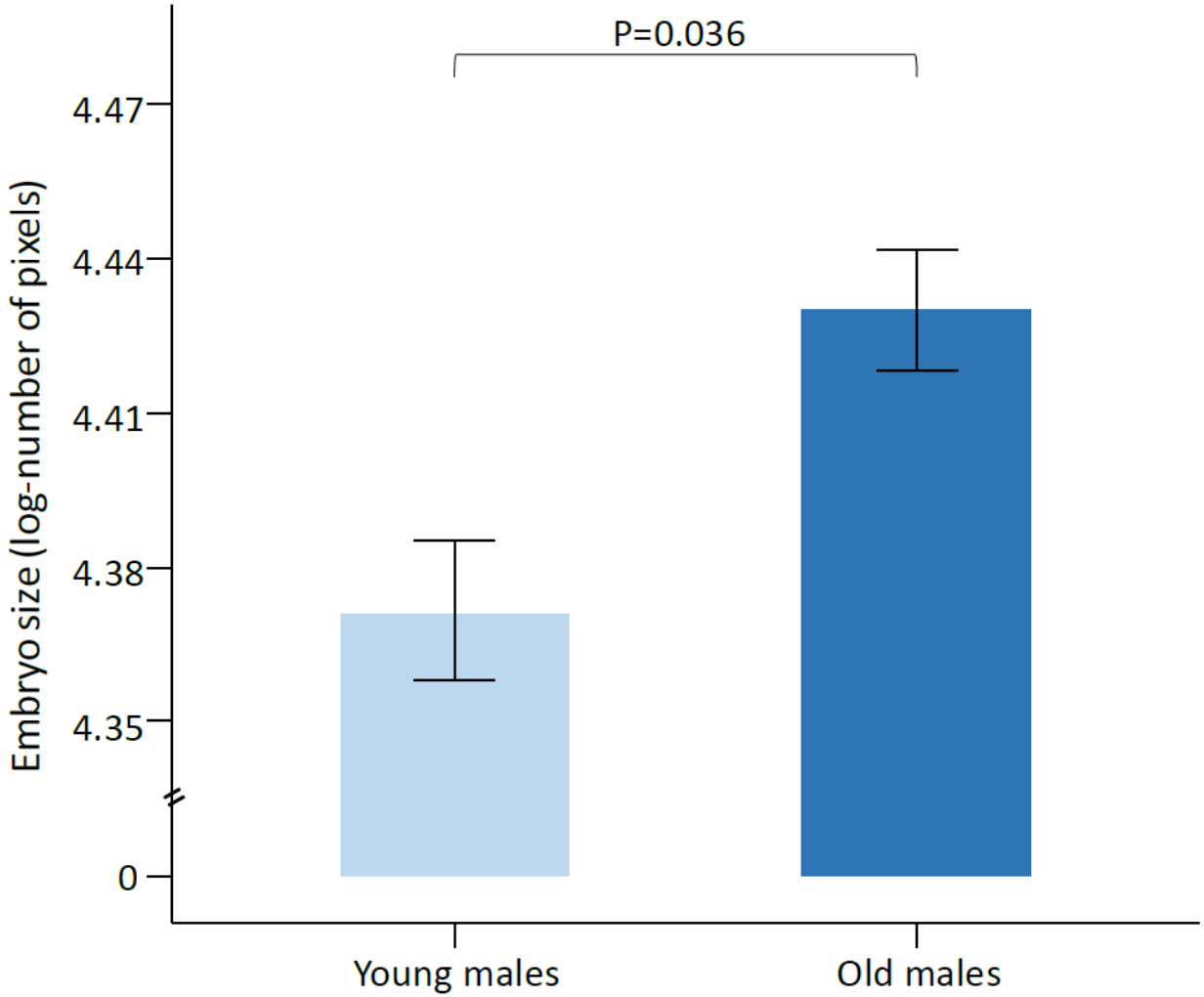
.33
.31
.29
.27
0
Embryonic TL (log-T/S ratio)
Young males
Old males

483

484


P=0.036
4.47
4.44
4.41
4.38
4.35
0
Embryo size (log-number of pixels)
Young males
Old males

485