

Effects of ageing and inbreeding on the reproductive traits in a cichlid fish I: the male perspective

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Inbreeding and ageing can affect characteristics of reproductive physiology, influencing an individual's fecundity, fertility, and thus fitness. The effects of inbreeding and age are expected to depend on several factors such as inbreeding history of a population, lifespan, or environmental influences. Here, we investigated the impact of inbreeding and age on gonadal and sperm traits in males of *Pelvicachromis taeniatus*, a small West African cichlid featuring an inbreeding mating system. Lab-bred inbred and outbred, young and aged males were compared. Microsatellite heterozygosity was additionally taken into account. While age effects were generally present with aged males having larger testes and longer sperm but less sperm than young ones, inbreeding effects were less strongly pronounced and age specific. Negative inbreeding effects were generally absent in young males suggesting purging of deleterious alleles after continuous inbreeding in the natural *P. taeniatus* population. In aged males, inbreeding affected testes mass and sperm number. Age-dependent inbreeding effects are consistent with the mutation accumulation model of ageing. Generally, our results suggest that ageing can influence gamete performance and fitness and, thus, has evolutionary consequences.

ADDITIONAL KEYWORDS: inbreeding depression – microsatellite heterozygosity – purging – senescence – sperm competition – sperm length – sperm number – West African cichlid.

INTRODUCTION

Inbreeding and ageing can affect fitness-related traits. Inbreeding increases the frequency of homozygotes (Hartl & Clark, 1997) facilitating the expression of recessive deleterious alleles (partially recessive mutations), and decreases the frequency of heterozygotes. As a consequence, inbred organisms often show inbreeding depression that is defined as the reduction in fitness in inbred offspring compared with outbred offspring (Charlesworth & Charlesworth, 1987; Charlesworth & Willis, 2009). Inbreeding depression can result, for example, in higher juvenile mortality (Greenwood, Harvey & Perrins, 1978; Keller, 1998; Bean *et al.*, 2004). However, when inbreeding is persistent, inbreeding depression can become less severe

due to purging (Lande & Schemske, 1985; Pusey & Wolf, 1996), that is the selection against deleterious, recessive alleles in an inbred population (Kristensen & Sørensen, 2005). Several studies show that deleterious inbreeding effects might be reduced due to purging (reviewed in Crnokrak & Barrett, 2002). Thus, benefits of inbreeding (inclusive fitness benefits) might, in some cases, 'even-out' or even exceed its costs (e.g. Parker, 1979; Waser, Austad & Keane, 1986; Kokko & Ots, 2006; but see Ross-Gillespie, O'Riain & Keller, 2007), and inbreeding tolerance (Bilde *et al.*, 2005) or mating preferences for kin might evolve (Thünken *et al.*, 2007).

Inbreeding can affect reproductive traits. Across the animal kingdom, inbreeding most often has negative effects on ejaculate quantity and quality (mammals: Gomendio, Cassinello & Roldan, 2000; Fitzpatrick & Evans, 2009; crustaceans: Weeks *et al.*, 2009; insects: Nakahara & Tsubaki, 2008; Robinson, Kennington &

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Simmons, 2009; Pedersen *et al.*, 2011), while there have been fewer studies dealing with inbreeding effects on gonadal and sperm traits in fishes (e.g. Zajitschek *et al.*, 2009; Mehlis *et al.*, 2012; Gasparini *et al.*, 2013).

Ageing is defined as the progressive loss of function associated with decreasing fertility and increasing mortality with advancing age (Charlesworth & Hughes, 1996; Kirkwood & Austad, 2000). According to evolutionary theory, the ultimate cause of ageing is the weaker natural selection on old life stages than on early ones (Medawar, 1952; Williams, 1957; Hamilton, 1966). There are two main hypotheses explaining the underlying mechanisms of ageing: (1) mutation accumulation theory by Medawar (1952) states that accumulation of deleterious alleles reduces fitness late in life and (2) according to antagonistic pleiotropy theory by Williams (1957), however, pleiotropic genes cause directional selection for earlier maturity and increased performance early in life leading to a reduced performance late in life.

Data on the effect of age on sperm quality and quantity are often contradictory across the animal kingdom and even within closely related taxonomic groups: some studies report a decline of sperm quality with age (Sloter *et al.*, 2006; Thongtip *et al.*, 2008), while others find no decline or even an increase of sperm quality with age (Casselman & Montgomerie, 2004; Gasparini *et al.*, 2010). And still other studies find indirect evidence that old males' sperm negatively affects female fecundity and offspring survival (Price & Hansen, 1998; Jones, Balmford & Quinnell, 2000). Although fishes show large variation concerning lifespan (reviewed in Reznick, Ghalambor & Nunney, 2002), only few studies deal with fertilization success and gonadal traits of ageing males, often reporting trade-offs between sperm traits (e.g. Vuthiphandchai & Zohar, 1999; Gasparini *et al.*, 2010; Mehlis & Bakker, 2013; Gasparini, Kelley & Evans, 2014).

Inbreeding and ageing effects may act together and thus have the potential to strongly affect individual fitness (Keller, Reid & Arcese, 2008; Decanini, Wong & Dowling *et al.*, 2013). However, little is known about interactive effects of inbreeding and ageing (Biemont, 1976; Laws *et al.*, 2010; Tan, Pizzari & Wigby, 2013). Theory predicts that inbreeding depression increases with age when ageing effects are based on mutation accumulation but not when based on antagonistic pleiotropy (Charlesworth & Hughes, 1996; Keller *et al.*, 2008). Therefore, the examination of interactive effects of ageing and inbreeding allows a conclusion about the underlying mechanisms of ageing (Charlesworth & Hughes, 1996; Wilson *et al.*, 2007; Goymer, 2008; Keller *et al.*, 2008).

Here, we investigate the effect of age and inbreeding on male reproductive traits in the West African cichlid fish *Pelvicachromis taeniatus*. This species shows active inbreeding preferences – in laboratory experiments, both sexes preferred kin over non-kin as mating

partners (Thünken *et al.*, 2007; see also Thünken *et al.*, 2012). Thus far, no evidence for inbreeding depression has been found in traits that are usually highly affected by inbreeding in fishes (Waldmann & McKinnon, 1993) such as juvenile survival or growth (Thünken *et al.*, 2007). As the natural population is highly inbred (Langen *et al.*, 2011), deleterious alleles might be purged from the population. We analyzed gonadal and sperm traits of young, that is 1 year old, and aged, that is 4 years old, F1-inbred and F1-outbred *P. taeniatus* males. Additionally, individual heterozygosity was calculated based on a microsatellite analysis to take potential inbreeding of the past into account. We expected no negative inbreeding effects in young males due to purging induced by long-lasting inbreeding in the natural population (Langen *et al.*, 2011). Generally, the mutation accumulation hypothesis of ageing predicts that ageing and inbreeding effects interact (Charlesworth & Hughes, 1996; Keller *et al.*, 2008). Thus, the comparison of ageing effects in inbred and outbred individuals is an indirect way to examine mutation accumulation in later life stages. We expect that ageing effects are more pronounced in inbred males compared with outbred males as purging acts less effective in old fish.

MATERIAL AND METHODS

STUDY SPECIES AND EXPERIMENTAL FISH

Pelvicachromis taeniatus is a monogamous, cave breeding cichlid with biparental brood care (Langen, Thünken & Bakker, 2013). These fish are sexually dimorphic for size and colour, which is most pronounced during the reproductive phase (Baldauf *et al.*, 2009, 2011). Clutch and larvae are mainly parented by the female in the cave, while the male defends the territory against intruders. Thereafter, both parents care for the free-swimming offspring for a couple of weeks.

In 2003 and 2006, adult breeding pairs of *P. taeniatus* were caught in the Moliwe River in Cameroon (West Africa, 04°04'N/09°16'E) and transported to the Institute for Evolutionary Biology and Ecology in Bonn, Germany. First, F1 offspring of the wild parents were raised. To create the inbred and outbred fish, brother–sister pairs and unrelated, randomly chosen pairs were bred in large tanks (50 × 30 × 30 cm³, 45 L; group size ranged from 20 to 30 individuals). Tanks were separated by opaque grey plastic partitions to avoid visual contact between families. The water temperature was kept at 25 ± 1 °C. Tanks were equipped with sand, Java moss, and an internal filter but without caves to avoid reproduction.

Experimental fish were bred in spring 2006 and in summer 2009. Between March and September 2010, gonadal traits at age of 1 and 4 years, respectively,

inbred and outbred males were examined. In total, thirty-three 1-year-old virgin males (referred to as young males) originating from 17 families (14 inbred fish from 7 families and 19 outbred individuals of 10 families) and thirty-three 4-year-old virgin males (referred to as aged males) from 13 families (16 inbred from 6 families and 17 outbred from 7 families) were analyzed in terms of age and inbreeding effects.

Reproductively active males ($N = 66$) were removed from group tanks and kept isolated in tanks ($30 \times 20 \times 20 \text{ cm}^3$, water level 12 cm, 7.2 L) filled with 1-day-old tap water and equipped with sand, an air-stone, and a breeding cave for a period of 2 weeks. Tanks were lit with a fluorescent lamp (Lumilux de Luxe daylight, Osram, 36 W, light–dark cycle 12L:12D), and the water temperature was maintained at $25 \pm 1 \text{ }^\circ\text{C}$. To avoid visual contact between individuals, tanks were separated by grey opaque plastic partitions. Fish were fed daily ad libitum on defrosted *Chironomus* larvae.

Isolated males were allowed visual contact with a brightly coloured, reproductively active female on a daily basis over a period of 14 days to ensure sperm production. Under a constant water temperature, production of new sperm takes ~10 days in cichlids, and sperm tubules are always filled with ripe sperm (Fishelson, 2003). Every day, females were chosen randomly from different families and presented to the males in a transparent plastic box ($18.5 \times 11.5 \times 13.5 \text{ cm}^3$) in front of their isolation tanks for 30 min. All males responded to the female's presence by showing courtship behaviour.

After 14 days, standard length (SL) and body mass of each fish was noted. Both inbred and outbred 4-year-old males showed physical signs of ageing, for example crooked backs.

PREPARATION

Males were killed by decapitation, and testes were extracted surgically. Each testis was weighed three times with a fine scale (XS205 Dual Range, Mettler Toledo, error $\pm 0.1 \text{ mg}$). A mean value per testis and the total gonad mass were calculated. The left testis was stored in a tube containing 440 μL of tap water and was used for the quantification of sperm traits, while the right testis was stored in a tube containing 440 μL of 4% formalin for further examinations. A fin clip of each male was taken and stored in 99.8% ethanol in a 1.5 mL tube for later DNA extraction.

SPERM TRAITS

The left testis of each male was mortared in the tube, and 12 μL of the mixed sperm suspension was placed on a Neubauer counting chamber (Labor Optik, chamber depth: 0.1 mm, smallest square: 0.0025 mm^2). Under a microscope (Universal Zeiss, $\times 40$ object lens,

$\times 10$ eyepiece lens, $\times 1.25$ Optovar) with $\times 500$ magnification, sperm were counted in 64 smallest squares that were evenly spread over the entire counting chamber, and the total sperm number per testis was calculated (see Mehlis *et al.*, 2012). Another 12 μL sperm suspension was spread over a microscope slide. After the slide was air-dried, 10–30 sperm per male (depending on how many sperm were found, mean = 26 ± 7) were digitally photographed at $\times 500$ magnification. Total sperm length (head + midpiece + flagellum) was measured three times on photos using Image J version 1.43. For each male, a mean value for sperm length was calculated, and the maximum sperm length (longest sperm length) was noted.

MICROSATELLITE ANALYSIS

Using nine microsatellites already established in *P. taeniatus* through cross-species amplification (for further details on GenBank accession number and primer sequences, see Langen *et al.*, 2011, 2013), a microsatellite analysis was conducted on the one hand to determine the effects of experimental inbreeding on heterozygosity and on the other hand to be able to take into account potential variation in individual heterozygosity.

DNA extraction was conducted using the QIAGEN DNeasy Blood and Tissue Kit (for further details, see Langen *et al.*, 2011). Polymerase chain reactions (PCR) were multiplexed with up to three microsatellite loci in one PCR (MIX A: GM006, UNH934, US758/773; MIX B: GM120; MIX C: UNH911, UNH855, GM553; MIX D: UNH871, MIX E: UNH971). PCR volumes, PCR amplifications, and PCR profile are used as described in Langen *et al.* (2013).

Genotypes were scored on an ABI 3500 (Applied Biosystems); a blank sample and a positive control per PCR was run, and the error rate of a random subset of ~10% of all samples of each microsatellite was calculated (for further details, see Langen *et al.*, 2013). After comparing allele sizes, the percentage of mistypes was calculated revealing an error rate of 0%. Microsatellite statistics were conducted using the Excel Microsatellite Toolkit (Park, 2001) and Genepop v 4.1.4 (Raymond & Rousset, 1995). The observed and expected heterozygosity (H_{obs} and H_{exp}), deviation from Hardy–Weinberg equilibrium (p_{HWE}), mean number of alleles (A), and the inbreeding coefficient (F_{IS}) were calculated. Additionally, the heterozygosity of each individual over the nine loci was calculated.

COEFFICIENT OF VARIATION

As age and inbreeding can affect between- and within-male variation (Birkhead & Montgomerie, 2009), we also estimated variation in sperm length.

The coefficient of variation between males was calculated (CV_{bm}) according to the equation $CV_{bm} = SD / \text{mean} \times 100$ and, likewise, the coefficient of variation in sperm length within males (CV_{wm}) (Laskemoen et al., 2013). Additionally, all CV values were adjusted for sample size [$CV_{adj} = CV \times (1 + (1/(4n)))$] (Sokal & Rohlf, 1995).

STATISTICAL ANALYSES

Statistics were performed with the statistical software package R version 2.9.1 (R Development Core Team, 2009). When data were normally distributed according to the Shapiro–Wilk test, parametric tests were used. Otherwise, data were transformed to achieve normal distribution. Testis mass and total sperm number were logarithmically transformed to achieve normal distribution of data. *P*-values are two tailed throughout.

Linear mixed effect (lme) models were conducted in order to account for family identity of individual fish using the ‘nlme’ package. They were based on likelihood ratio tests (LRT). Non-significant interactions ($P > 0.05$) and factors ($P > 0.05$) were removed from the model. We examined the effects of age class (young vs. aged) and breeding line (inbred vs. outbred) on body size, testis, and sperm traits. Family identity was entered as random factor. To control for variation in body size, we added body size as covariate. Furthermore, lme models with heterozygosity (instead of breeding line), age, and body size as explanatory variables were calculated. The degrees of freedom always differed by one.

Data on sperm morphology are only present for 53 males because not enough sound structured sperm were found (less than five) in 13 males. For one young inbred male, testis mass could not be calculated because only one testis was found. Two DNA samples were missing, reducing the sample size for microsatellite analysis to 64.

RESULTS

EFFECTS OF AGEING AND INBREEDING

Aged males were significantly larger (LRT, $N = 66$, $\chi^2 = 32.182$, $P < 0.001$) and heavier (LRT, $N = 66$, $\chi^2 = 18.791$, $P < 0.001$) than young ones, while inbreeding had no significant effect on body size (LRT, $N = 66$, $\chi^2 = 1.542$, $P = 0.217$) and mass (LRT, $N = 66$, $\chi^2 = 1.425$, $P = 0.233$).

Age and inbreeding interactively affected variation in testis mass and sperm number (Table 1). While young males generally had more sperm than aged males (inbred_{young} vs. inbred_{aged}: LRT, $N = 36$, $\chi^2 = 2.394$, $P < 0.001$; outbred_{young} vs. outbred_{aged}: LRT,

$N = 30$, $\chi^2 = 14.677$, $P < 0.001$; Fig. 1A), but lighter testes (inbred_{young} vs. inbred_{aged}: LRT, $N = 29$, $\chi^2 = 6.709$, $P = 0.01$; outbred_{young} vs. outbred_{aged}: LRT, $N = 36$, $\chi^2 = 16.271$, $P < 0.001$), significant inbreeding effects were only present in aged males (for sperm number, see Table 1, Fig. 1A; testis mass: $N = 33$, $\chi^2 = 3.670$, $P = 0.055$) but not in young ones (LRT, $N = 33$, $\chi^2 = 0.525$, $P = 0.469$; Fig. 1A; testis mass: LRT, $N = 32$, $\chi^2 = 2.568$, $P = 0.109$).

Aged males had longer sperm (Table 1; Fig. 1B). Inbreeding had no significant effect on total sperm length, but on within-male sperm length variation with inbred males having more variable sperm (Table 1; Fig. 1B, C). The adjusted coefficient of variation in sperm length between males, CV_{bm} , amounted to 39.831 and the average adjusted coefficient of variation in sperm length within males, CV_{wm} , to 36.150. In young males, CV_{bm} was 42.327 and in aged males 35.427. Descriptive statistics of measured traits are given in Table 2.

EFFECTS OF MICROSATELLITE HETEROZYGOSITY

Microsatellite analysis revealed that inbred males were less heterozygous than outbred males and deviated significantly from HWE ($A = 4.11$, $H_{exp} = 0.441$, $H_{obs} = 0.346$, $p_{HWE} < 0.001$, $F_{IS} = 0.215$; young inbred males: $A = 3.44$, $H_{exp} = 0.423$, $H_{obs} = 0.379$, $p_{HWE} = 0.167$, $F_{IS} = 0.109$; aged inbred males: $A = 3.33$, $H_{exp} = 0.440$, $H_{obs} = 0.319$, $p_{HWE} < 0.001$, $F_{IS} = 0.283$). Outbred males had a negative inbreeding coefficient and a higher heterozygosity than inbred males, but did not significantly deviate from HWE after Bonferroni correction ($A = 4.33$, $H_{exp} = 0.491$, $H_{obs} = 0.516$, $p_{HWE} = 0.035$, $F_{IS} = -0.050$; young outbred males: $A = 3.78$, $H_{exp} = 0.483$, $H_{obs} = 0.461$, $p_{HWE} = 0.339$, $F_{IS} = 0.048$; aged outbred males: $A = 3.67$, $H_{exp} = 0.494$, $H_{obs} = 0.583$, $p_{HWE} = 0.227$, $F_{IS} = -0.188$). While in most cases variation in testis and sperm traits was better explained by age than by heterozygosity (Table 1), there was a tendency to an interaction of heterozygosity and age on sperm number (LRT, $N = 64$, $\chi^2 = 3.366$, $P = 0.067$; Table 1). Therefore, sperm number was analyzed separately for the two age classes. While heterozygosity had no significant influence on sperm number in young males (LRT, $N = 33$, $\chi^2 = 0.306$, $P = 0.580$; Fig. 2), sperm number of aged males was positively correlated to heterozygosity (LRT, $N = 31$, $\chi^2 = 5.392$, $P = 0.020$; Fig. 2).

DISCUSSION

In the present study, we investigated the effects of age and inbreeding on male gonadal and sperm traits in the small cichlid fish *P. taeniatus*. Age had a strong

Table 1. Results of the linear mixed effect models examining the effect of age and inbreeding (or microsatellite heterozygosity, respectively) on different gonadal traits of male *Pelvicachromis taeniatus*

Dependent variable	Effects		<i>N</i>	Δ d.f.	χ^2	<i>P</i>	
Testis mass	Fixed effects	Breeding line × age	65	1	7.609	0.006	
		Breeding line	65	1	0.216	0.642	
		Age	65	1	1.886	0.170	
		SL	65	1	27.603	< 0.001	
	Random effect	Family	65	1	2.074	0.150	
	Fixed effects	Heterozygosity × age	63	1	0.566	0.452	
		Heterozygosity	63	1	0.791	0.374	
		Age	63	1	2.107	0.147	
		SL	63	1	27.212	< 0.001	
	Random effect	Family	63	1	1.826	0.177	
Sperm number	Fixed effects	Breeding line × age	66	1	7.107	0.008	
		SL	66	1	0.006	0.937	
		Breeding line	66	1	2.851	0.091	
		Age	66	1	13.770	< 0.001	
	Random effect	Family	66	1	2.417	0.120	
	Fixed effects	Heterozygosity × age	64	1	3.366	0.067	
		SL	64	1	0.099	0.753	
		Heterozygosity	64	1	3.770	0.052	
		Age	64	1	13.348	< 0.001	
	Random effect	Family	64	1	2.710	0.100	
	Sperm length	Fixed effects	Breeding line × age	53	1	1.027	0.311
			Breeding line	53	1	0.797	0.372
SL			53	1	3.712	< 0.054	
Age			53	1	17.982	< 0.001	
Random effect		Family	53	1	2.795	0.095	
Fixed effects		Heterozygosity × age	51	1	1.591	0.207	
		Heterozygosity	51	1	0.021	0.884	
		SL	51	1	3.546	< 0.060	
		Age	51	1	18.007	< 0.001	
Random effect		Family	51	1	2.483	0.115	
CV_{wm}		Fixed effects	Breeding line × age	51	1	0.159	0.691
			Age	51	1	0.204	0.651
	SL		51	1	3.609	0.058	
	Breeding line		51	1	5.061	0.025	
	Heterozygosity × age		51	1	0.494	0.482	
	Age		51	1	< 0.001	0.988	
	Heterozygosity		51	1	3.546	0.060	
	Random effect	SL	51	1	18.007	< 0.001	
	Random effect	Family	51	1	< 0.001	> 0.999	

SL was included as covariate and family as random factor. The *N*, Δ d.f., χ^2 , and *P*-values are given. Significant differences (*P* < 0.05) are marked in bold. Δ d.f., difference of degrees of freedom; CV_{wm} , coefficient of variation within males; *N*, sample size; SL, standard length.

effect on testis mass, sperm number, and sperm size, whereas inbreeding effects were only found in some traits of older males.

Older males had a smaller number of sperm than younger males. This finding was in accordance with expectations as mutations of germ line occur more

frequently with increasing age (Hansen & Price, 1999; Ellegren, 2007; Pitnick, Dobler & Hosken, 2009), potentially leading to a reduced sperm number. This result is consistent with findings in the striped bass (*Morone saxatilis*), where 12-year-old males had less sperm than 3-year-old males (Vuthiphandchai &

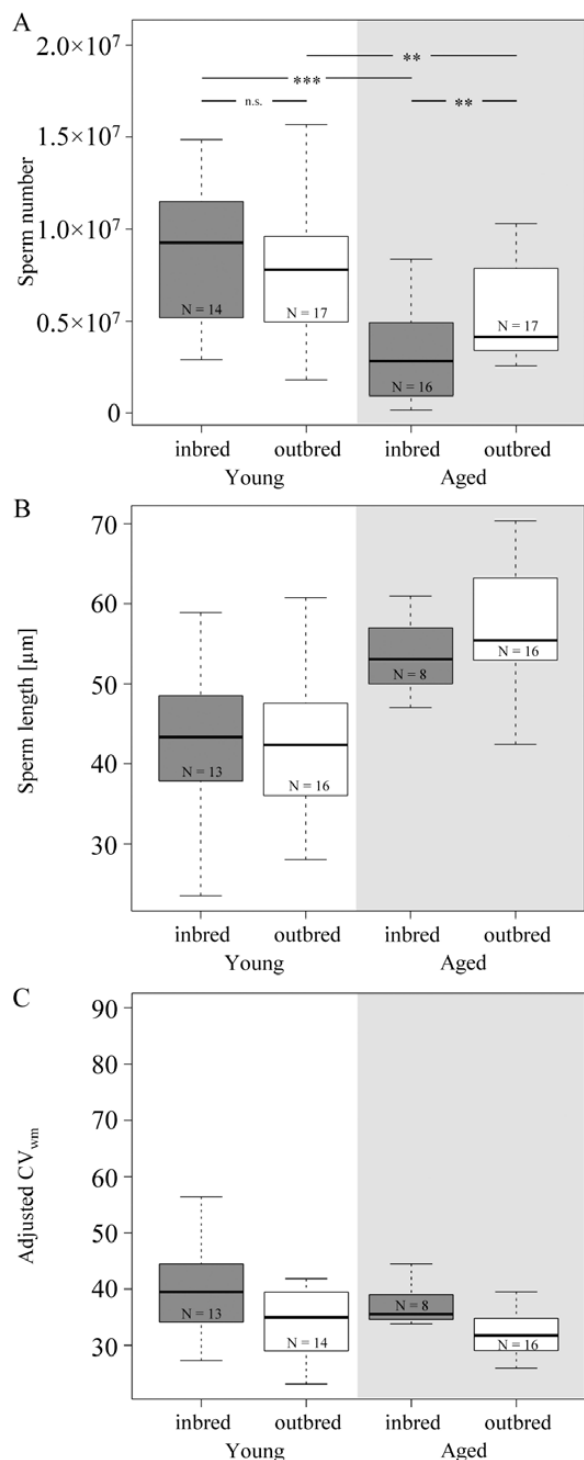


Figure 1. Boxplots of (A) sperm number, (B) sperm length, and (C) adjusted coefficient of variation in sperm length within males, CV_{wm} , of young (white area) and aged (light grey area) inbred (dark grey boxes) and outbred (white boxes) males. Shown are median, quartiles, and whiskers. $**P < 0.01$, $***P \leq 0.001$, $^{NS}P > 0.05$.

Zohar, 1999). Sperm limitation may have serious consequences because of the fertilization ‘mode’ in *P. taeniatus*: as females sequentially spawn only a small fraction of eggs at once, the male must repeatedly release sufficient sperm to fertilize the eggs. Thus, it is advantageous for a male to have many sperm.

Testis mass was significantly higher in old males. As variation in testis mass was explained by male size and age class, the results suggest – in contrast to sperm number – a mere allometric relationship rather than an ageing effect. Testes of old males may consist of more non-sperm-producing tissue or the sperm-producing tissue could be inhibited in old males resulting in a production of less sperm. Structural changes of sperm-producing tissue as well as thickening of connective tissue have been reported in cichlids by Fishelson (2003).

In *P. taeniatus*, we found that older males had longer sperm but less sperm than younger males. Production of large sperm is often associated with higher costs for the male, leading to a reduction of sperm number (e.g. Parker, 1993). Such a trade-off between sperm length and sperm number has been reported in several animal species (Pitnick, 1996; Immler et al., 2011) but not in fishes (Stockley et al., 1997; Balshine et al., 2001; Fitzpatrick et al., 2009). At the proximate level, it may be possible that old males selectively resorb short sperm to produce longer sperm at the cost of sperm quantity. In old males, unemitted spermatozoa could be phagocytosed and resorbed, and new germ cells may proliferate of larger size. Resorption of sperm has been reported for several fish species (e.g. Billard and Takashima 1983; Besseau and Faliex 1994; Chaves-Pozo et al., 2005). In cichlids, age effects on testis and sperm have rarely been reported. In an analysis of testes of different cichlid species, Fishelson (2003) found major alterations in testis structure and development of gametes including cell apoptosis, cell degeneration, formation of large spermatogonia, and sperm head mutations in older males, pointing to an accumulation of deleterious mutations of the germ line with advancing age. In other fishes, however, contrasting results are reported. For example, in salmon, sperm competition success did not correlate with male age in vitro (Hoysak, Liley & Taylor, 2004), while older males of the guppy (*Poecilia reticulata*) had significantly longer but slower sperm than younger males, and a higher sperm number (Gasparini et al., 2010).

Young and aged males originated from two different cohorts, which theoretically could contribute to the observed ageing effects as old males could have gone through selection so that only males of lower sperm quality could have survived until the age of 4 years.

Table 2. Descriptive statistics of measured traits

Trait	Young		Aged	
	Inbred	Outbred	Inbred	Outbred
SL (cm)	5.56 ± 0.51 (N = 14)	5.37 ± 0.39 (N = 19)	6.55 ± 0.37 (N = 16)	6.31 ± 0.33 (N = 17)
Body mass (g)	3.94 ± 1.05 (N = 14)	3.62 ± 0.72 (N = 19)	5.40 ± 0.91 (N = 16)	4.85 ± 0.92 (N = 17)
Testis mass (mg)	2.61 ± 1.28 (N = 13)	2.47 ± 2.70 (N = 19)	3.95 ± 1.25 (N = 16)	6.45 ± 3.39 (N = 17)
Sperm number	8.77 × 10 ⁶ ± 3.76 × 10 ⁶ (N = 14)	8.06 × 10 ⁶ ± 4.64 × 10 ⁶ (N = 19)	3.19 × 10 ⁶ ± 2.57 × 10 ⁶ (N = 16)	5.91 × 10 ⁶ ± 3.95 × 10 ⁶ (N = 17)
Sperm length (µm)	42.40 ± 12.97 (N = 13)	43.02 ± 9.74 (N = 16)	53.53 ± 4.92 (N = 8)	56.93 ± 7.48 (N = 16)
Head length (µm)	4.73 ± 0.49 (N = 13)	4.62 ± 0.61 (N = 16)	5.20 ± 0.17 (N = 8)	4.82 ± 0.36 (N = 16)
Maximum sperm length (µm)	83.20 ± 24.05 (N = 13)	79.50 ± 17.44 (N = 16)	100.10 ± 12.92 (N = 8)	97.30 ± 14.59 (N = 16)

Means ± SD and sample sizes are given for 1- and 4-year-old inbred and outbred males. SL, standard length.

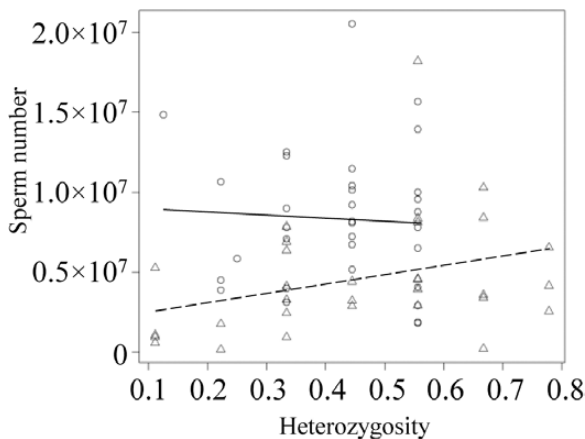


Figure 2. Relationship between heterozygosity at nine microsatellite loci and sperm number in young (○, solid line) and aged (△, dashed line) males. The lines are the least-squares linear regression lines.

However, we think that such strong selection is not very likely under laboratory conditions.

The absence of inbreeding depression in young males is in accordance with previous findings in juvenile *P. taeniatus* of the Moliwe population (Thünken *et al.*, 2007). The most probable explanation for the absence of inbreeding depression in this species is purging. In inbred populations, the frequency of deleterious mutations can be reduced by purging so that the mutational load is reduced (Charlesworth & Willis, 2009). Consequently, with increasing levels of inbreeding, the benefits of inbreeding should more easily override the costs of inbreeding (Lehtonen & Kokko, 2011). Another explanation for the absence of inbreeding depression in young males could be that selection for reproduction at younger ages could maximize fitness before deleterious alleles start to effect male fertility, which could explain why inbreeding

effects only occur in old males. However, as only old males showed inbreeding depression, purging is the most plausible explanation in this species. To confirm the purging hypothesis, populations with different inbreeding histories should be compared in future studies. We found significant inbreeding effects only in old males, suggesting that age effects are more strongly expressed in inbred males compared with outbred males. Old inbred males had less sperm than old outbred ones. Also, in old males, sperm number was positively correlated with heterozygosity, underlining the inbreeding effects on sperm in old males.

These results are in accordance with theory that predicts an increase of inbreeding depression with age under mutation accumulation (Charlesworth & Hughes, 1996; Keller *et al.*, 2008). Similar results were found in Soay sheep and red deer as well as in a freshwater snail (Wilson *et al.*, 2007; Escobar *et al.*, 2008). Purging effects decrease with age due to the fact that selection against deleterious mutations is less strong in older individuals compared with younger ones. This effect might be even stronger when the extrinsic mortality is high. Life expectancy of *P. taeniatus* under natural conditions is probably not as high as under laboratory conditions, for instance, due to an enhanced predation risk for small fish and thus a higher extrinsic mortality in the wild. In our study, old males were larger and heavier than young ones and already showed symptoms of ageing in body shape (with some males having crooked backs). As a consequence, natural selection probably cannot effectively act against age-specific deleterious mutations expressed in old individuals.

In conclusion, our study demonstrates that ageing and inbreeding can affect gonadal and sperm traits. While age effects were generally present with aged males having larger testis and longer but less sperm than young ones, inbreeding effects were less pronounced and age specific. Negative inbreeding

effects were generally absent in young males suggesting purging of deleterious alleles after continuous inbreeding in the natural *P. taeniatus* population. In aged males, inbreeding affected testis mass and sperm number. In accordance with other studies (reviewed in Kidd, Eskenazi & Wyrobek, 2001; Pizzari et al., 2008; Johnson & Gemmell, 2012), our results suggest that ageing can influence gamete performance and fitness, which should have an effect on sexual selection and, thus, has evolutionary consequences.

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