nature ecology & evolution

ARTICLES

DOI: 10.1038/s41559-017-0236-1

A sex-chromosome inversion causes strong overdominance for sperm traits that affect siring success

Ulrich Knief[1,2], Wolfgang Forstmeier[1*], Yifan Pei[1], Malika Ihle[1], Daiping Wang[1], Katrin Martin[1], Pavlína Opatová[3,4], Jana Albrechtová[3,4], Michael Wittig[5], Andre Franke[5], Tomáš Albrecht[3,4] and Bart Kempenaers[1]

Male reproductive success depends on the competitive ability of sperm to fertilize the ova, which should lead to strong selection on sperm characteristics. This raises the question of how heritable variation in sperm traits is maintained. Here we show that in zebra finches (*Taeniopygia guttata*) nearly half of the variance in sperm morphology is explained by an inversion on the Z chromosome with a 40% allele frequency in the wild. The sperm of males that are heterozygous for the inversion had the longest midpieces and the highest velocity. Furthermore, such males achieved the highest fertility and the highest siring success, both within-pair and extra-pair. Males homozygous for the derived allele show detrimental sperm characteristics and the lowest siring success. Our results suggest heterozygote advantage as the mechanism that maintains the inversion polymorphism and hence variance in sperm design and in fitness.

Sperm competition and cryptic female choice are post-copulatory processes that determine male reproductive success and can lead to strong directional selection on sperm characteristics, including sperm morphology[1–4]. For example, in birds, species with more intense sperm competition, as reflected in higher levels of extra-pair paternity and larger testes relative to body size, show lower between-male variation in sperm length[5]. Sperm competition should hence deplete the additive genetic variance in those sperm traits that are the target of directional selection (those traits directly linked to reproductive success)[6]. However, sperm morphology is often heritable[7,8], leading to the question of how the genetic variation is maintained. To answer this, the genetic basis of variation in sperm characteristics needs to be identified, but this has only rarely been done[9].

A typical sperm cell consists of a head containing the genomic DNA and the acrosome, a midpiece that harbours the mitochondria for energy production and a tail that generates propulsion. Midpiece and tail together make up the flagellum[10]. In most bird species, midpiece and flagellum length show a linear relationship[11], but in the zebra finch (*Taeniopygia guttata*)—a small passerine bird that easily breeds in captivity and that has been a model species for investigations into sperm morphology and mechanisms of sperm competition[7,12]—this relationship is nonlinear with a distinctive L-shaped form[7,13,14]. Because variation in sperm morphology is highly heritable[7] (see also Supplementary Table 1), this is suggestive of a large genetic effect caused by a small number of loci.

Large genetic effects on phenotypes can be caused by chromosome inversions[15,16]. Inversions are structural mutations in which segments of DNA have been excised and reinserted in reverse sequence order without change in genic content[17]. Individuals that are heterozygous for the inversion pay a cost because an uneven number of crossovers within the inverted segment typically leads to unbalanced gametes (carrying deletions or duplications) and consequently embryonic death of the offspring[18–21]. In some taxa, the fertility costs for heterozygous individuals seem less severe, either because of a reduced recombination rate or because unbalanced chromosomes are removed prior to gamete maturation[17,22–24]. In general, inversion polymorphisms reduce the population recombination rate within the inverted region[25,26], which might link beneficial alleles together in a 'supergene'[27]. In such cases, the net fitness effect of an inversion can be positive. The inverted haplotype will then increase in frequency in the population and can eventually replace the ancestral state if the additive fitness benefits are independent of the environment. Alternatively, the inverted haplotype may be maintained in the population at intermediate frequency, resulting in a genetic polymorphism. This is likely when heterozygous individuals achieve higher fitness than either type of homozygotes (overdominance or heterozygote advantage)[28,29], when there is negative frequency-dependent selection[15,30,31] or when selection pressures change because of a variable environment (for example local adaptation with migration[32,33]).

Wild Australian zebra finches are polymorphic for four large inversions on chromosomes *Tgu5*, *Tgu11*, *Tgu13* and *TguZ*[24,34,35]. In each case, the inversion mutation that occurred in a single individual spread to an allele frequency between 40% and 53%, despite small but significant detrimental effects on the survival of embryos sired by heterozygous fathers[24]. It remains unresolved why the inverted alleles spread and how they are maintained in the population[24].

The Z chromosomes of wild zebra finches can be classified into three haplotypes: haplotype A (estimated allele frequency in the wild: 59.6%) represents the ancestral state, from which haplotype C (frequency: 7.4%) was derived by an inversion, which then

[1]Department of Behavioural Ecology and Evolutionary Genetics, Max Planck Institute for Ornithology, Seewiesen 82319, Germany. [2]Division of Evolutionary Biology, Faculty of Biology, Ludwig Maximilian University of Munich, Planegg-Martinsried 82152, Germany. [3]Institute of Vertebrate Biology, Czech Academy of Sciences, Brno 603 65, Czech Republic. [4]Faculty of Science, Charles University, Prague 128 44, Czech Republic. [5]Institute of Clinical Molecular Biology, Christian-Albrechts-University, Kiel 24105, Germany. *e-mail: forstmeier@orn.mpg.de

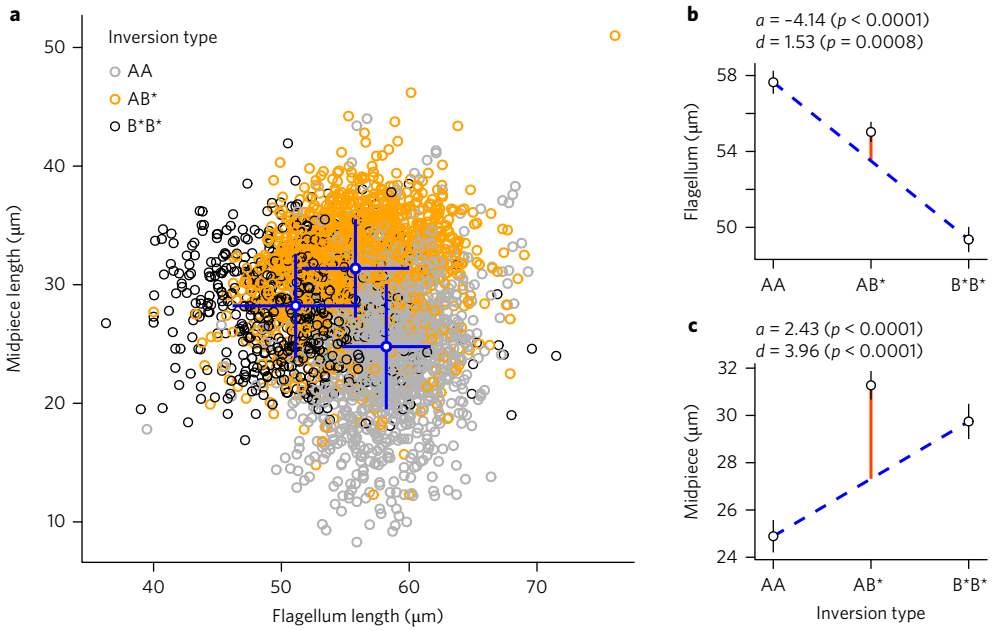

Figure 1 | Sperm morphology as a function of male Z-chromosome inversion type. a, Midpiece length plotted against flagellum length for 3,670 sperm (raw data; from 367 ejaculates from 158 males). Sperm of males that are homozygous for the ancestral allele (AA; $N=54$ males) are shown in grey, those of heterozygous males (AB*; $N=70$ males) in orange and those of males with two copies of the derived allele (B*B*; $N=34$ males) in black. Blue points and crosses mark the group means ± their standard deviations, illustrating that effect sizes are remarkably large (Cohen's $d>1$; see also results of discriminant analysis in the main text). **b**,**c**, Flagellum and midpiece length for each inversion type (estimate ± SE from mixed-effects models, Supplementary Table 1). Red lines indicate the dominance and blue dashed lines the additive effect. Above each graph, we indicate the additive (a) and dominance (d) estimates and their associated p-values from mixed-effects models (see Supplementary Table 1). In combination, the additive and dominance fixed effects explain 45.4% and 41.9% of the phenotypic variance in flagellum and midpiece length, respectively.

further evolved to haplotype B (frequency: 33%), presumably by a second inversion[24]. Putative double crossovers created some rare genotypes that are intermediates between A and C or between B and C, which we refer to as haplotype D (Supplementary Fig. 1). Haplotypes A and B apparently rarely or never recombine[24]. Here, we describe how the inversion on chromosome *TguZ* affects sperm morphology, velocity and fertilization ability, and we provide an explanation for why the inverted allele spread and how it is maintained in the population.

Results

In our two captive populations, one domesticated and one recently wild-derived, haplotypes A, B and C and some intermediates between B and C (which we refer to as D) segregate (Supplementary Fig. 1). Haplotypes B, C and D have similar effects on sperm morphology in comparison to the ancestral haplotype A (Supplementary Fig. 2 and Supplementary Table 2). We therefore focus here on the contrast between the ancestral haplotype A and all derived inversion haplotypes combined. For this purpose, we define haplotype category B* as the combination of all derived haplotypes (B, C and D). Thus, haplotype B* comprises all genotypes that go back to a single common ancestor in which the first inversion event occurred.

Males that are homozygous for the ancestral allele (genotype AA) have sperm with a relatively long flagellum (mean ± standard deviation = 58.21 ± 3.47 µm) and a relatively short midpiece (24.78 ± 5.28 µm; Fig. 1, Supplementary Table 1). Heterozygous males (AB*) have a shorter flagellum (55.79 ± 4.20 µm) and a considerably longer midpiece (31.37 ± 4.15 µm) than AA males, and males that are homozygous for the derived allele (B*B*) have an even shorter flagellum (51.11 ± 4.94 µm) combined with a midpiece of intermediate length (28.22 ± 4.39 µm; Fig. 1). Hence, the effects of the derived allele B* on flagellum length are largely additive (Fig. 1b), whereas the effects on midpiece length are strongly non-additive, with heterozygous males showing the longest midpiece (overdominance; Fig. 1c, Supplementary Table 1). The size of the observed effects is remarkable: the inversion genotypes (additive and overdominant effect combined) explain 45.4% and 41.9% of the phenotypic variance in flagellum and midpiece length, respectively. A simple discriminant analysis correctly classifies 70% of the males into one of the three genotypes based only on measurements of midpiece and flagellum length from a single sperm (versus 43% random probability of correct classification; Fig. 1a). This classification success further increases to 78% if averages from 10 sperm per male are used for analysis.

The observed differences in sperm morphology are biologically relevant, because they relate to sperm swimming speed (see also previous work[36]). In a sample of 355 ejaculates from 154 males, midpiece length related positively to sperm velocity (linear mixed-effects model (LMM): slope estimate ± SE = 0.58 ± 0.19, $z=3.07$, $p=0.0022$, Supplementary Fig. 3a), and there is a strong quadratic effect of flagellum length on sperm velocity, with intermediate morphotypes being the fastest (LMM: slope estimate of squared and mean-centred predictor ± SE = −0.12 ± 0.030, $z=-4.00$, $p=8.4\times10^{-5}$; Supplementary Fig. 3b and Supplementary Table 3). Together, midpiece and flagellum length strongly predict sperm velocity (Fig. 2a). The quadratic effect of flagellum length on sperm velocity is in line with previous findings[13], but differs from the positive linear relationship that had been reported earlier by the same group[36]. However, the limited number of long-flagellum males in the earlier study[36] may explain why the quadratic relationship was not detected.

Sperm of males that are heterozygous for the inversion (AB*, $N=68$) are somewhat faster (although not significantly; LMM: estimate ± SE = 3.66 ± 1.95 µm/s, $z=1.87$, $p=0.059$, $N_{total}=153$ males)

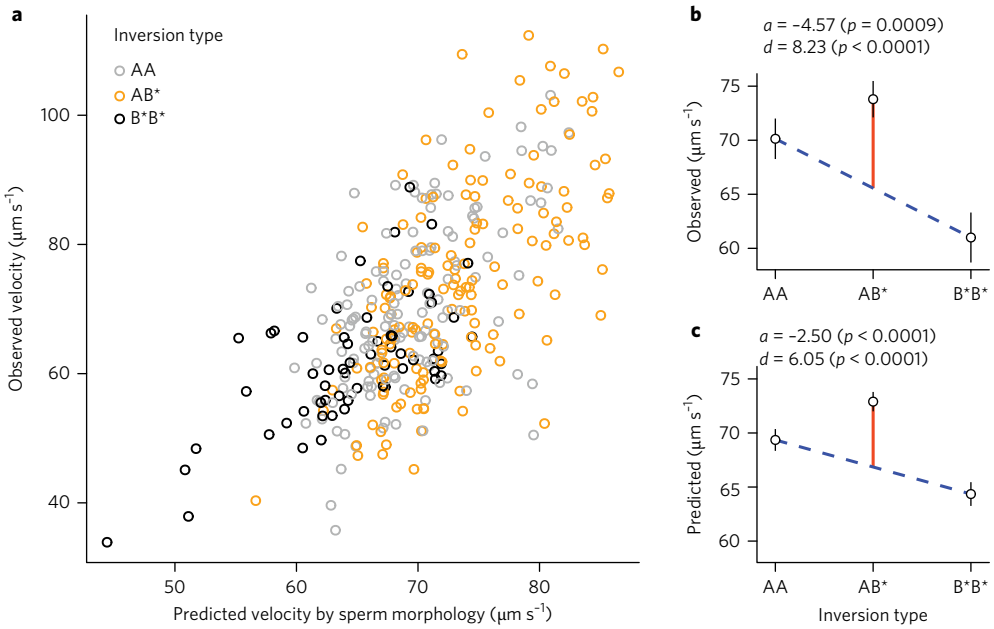

Figure 2 | Sperm swimming speed as a function of male Z-chromosome inversion type. a, Observed curvilinear velocity of sperm from 354 samples (average values based on a mean of 223 sperm per sample; samples from 153 males: 53 AA, 68 AB*, 32 B*B*) plotted against the predicted velocity from a mixed-effects model based on midpiece length and the square of scaled flagellum length (Supplementary Table 3). The colour of the dots indicates the Z-chromosome genotype. **b**,**c**, Observed and predicted sperm velocity (VCL) for each inversion type (estimate ± SE from mixed-effects models, Supplementary Table 4). Red lines indicate the dominance and blue dashed lines the additive effect. Above each graph, we indicate the additive (*a*) and dominance (*d*) estimates and their associated *p*-values from mixed-effects models (see Supplementary Table 4). In combination, the additive and dominance fixed effects explain 10.0% and 27.1% of the phenotypic variance in observed and predicted velocity, respectively.

than those of homozygotes for the ancestral type (AA, $N=53$), and clearly faster than those of homozygotes for the derived allele (B*B*, $N=32$; LMM: estimate ± SE = 12.80 ± 2.48 μm/s, $z=5.16$, $p=4.1\times10^{-7}$, $N_{total}=153$ males). This is true both for the observed velocity (Fig. 2b) and for velocity as predicted by the two morphological traits (Fig. 2c). The observation that the genetic effects on sperm morphology are largely able to explain the genetic effects on sperm velocity (compare the estimates of *a* and *d* between Fig. 2b,c) is in line with the idea that the inversion genotype affects sperm velocity through morphology. However, establishing that this is indeed a causal relationship would require experimentally changing sperm morphology within males of the same inversion genotype, which is hard to achieve. Allele B* has both a significant detrimental additive effect on observed velocity (LMM: estimate ± SE = −4.57 ± 1.37 μm s^{-1}, $z=-3.34$, $p=0.0009$, $N_{total}=153$ males) and a significant overdominant effect, lying above the estimates for the two groups of homozygous males (LMM: estimate ± SE = 8.23 ± 1.76 μm s^{-1}, $z=4.67$, $p=4.2\times10^{-6}$, $N_{total}=153$ males; Supplementary Table 4).

Faster sperm may reduce fertility problems and may be more successful in sperm competition[37,38]. If so, heterozygous males (AB*) should have lower infertility rates and should be more successful in siring offspring, either because they lose less paternity in their own brood, or because they sire more extra-pair offspring, or both. Analysing 11,616 eggs from 2,782 clutches laid by 440 females paired individually in cages to 435 males (in the absence of sperm competition) shows that heterozygous males did indeed fertilize a higher proportion of eggs than males from both homozygote groups (Fig. 3a, Supplementary Table 5). An analysis of total siring success of 482 males breeding in communal aviaries (based on 7,353 eggs from 1,996 clutches) revealed that heterozygous males also sired a higher number of eggs in an environment with opportunities for sperm competition (Fig. 3b, Supplementary Table 6). Here, effects were somewhat larger than for males breeding in single pairs in cages (fitness of heterozygous AB* males set to 1, fitness in cages: AA = 0.88, B*B* = 0.94; fitness in aviaries: AA = 0.87, B*B* = 0.83). When breeding in groups, heterozygous males sired both a higher proportion of the fertile eggs laid by their own female (within-pair success, Fig. 3c, Supplementary Table 7) and a higher number of extra-pair offspring (Fig. 3d, Supplementary Table 8), although the effects of the inversion genotype were smaller than those on sperm characteristics. As with patterns of sperm velocity (Fig. 2b), additive effects were generally negative: that is, males homozygous for the derived allele (B*B*) had the lowest within-pair and extra-pair siring success (Fig. 3).

Discussion

In birds, males are the homogametic sex (ZZ), and, during avian evolution, genes with a male-biased expression adaptively translocated onto the Z chromosome[39]. Thus, genes that are linked to male function—the most prominent of which is spermatogenesis[40]—are expected to reside on the Z chromosome. This may explain why the effect sizes of the Z-chromosome inversion on sperm morphology and velocity shown here (Figs. 1,2, Supplementary Table 2) are much larger than any of the previously reported effects of the four inversions on other morphological traits in the zebra finch (5.9-fold and 11-fold larger additive and dominant effects, respectively, compared with the largest of 2 × 40 effect-size estimates[24]; see also ref. [41]).

Genetic variation in sperm design is extraordinarily large in zebra finches[7], which is puzzling if selection depletes the additive genetic variance[6]. One answer is that post-copulatory sexual selection on sperm design is actually weak in zebra finches[7], because they are socially monogamous[42–44] with low levels of sperm competition[43,45–47]. Here we provide an alternative although not exclusive explanation, namely that a heterozygote advantage preserves variation in sperm design. Concordantly, an independent genome-wide analysis found that the inversion on chromosome Z is indeed the main locus contributing to variation in sperm design[41]. Our results suggest a causal scenario from inversion genotype via

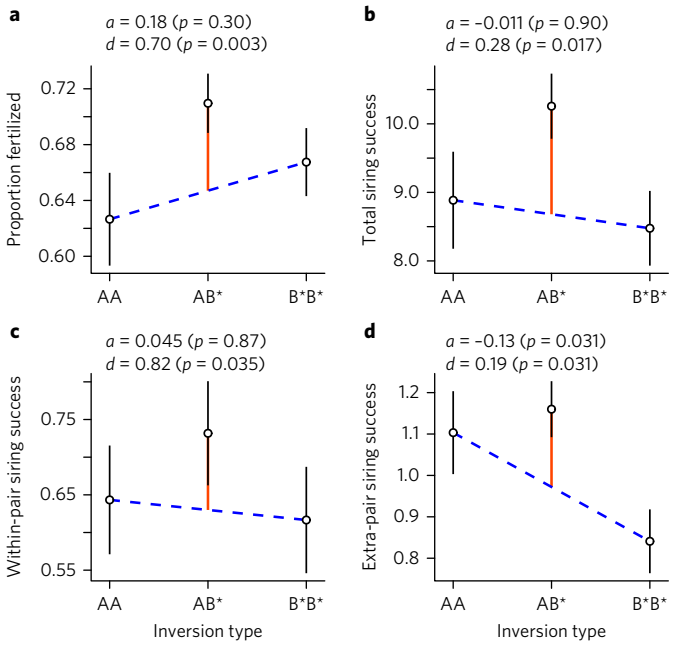

Figure 3 | Male siring success as a function of male Z-chromosome inversion type. a, Male fertility (proportion of eggs laid by the female that was fertilized by the male: that is, showed a visible embryo) measured in the absence of sperm competition (breeding pairs kept separately in cages) of 435 males as a function of their Z-chromosome inversion type (N = 68 AA, 216 AB*, 151 B*B*). For the purpose of illustration only, we analysed the data as the proportion of fertilized eggs in a clutch in a Gaussian mixed-effects model (weighted by the number of eggs in a clutch; N = 2,782 clutches). **b**, Total siring success of 482 males in the presence of potential sperm competition (communal breeding aviaries) as a function of inversion type (N = 110 AA, 219 AB*, 153 B*B*) for three datasets combined (see Methods). **c**, Within-pair siring success (proportion of eggs laid by the social partner sired) of 428 males in the presence of potential sperm competition (communal breeding aviaries) according to their inversion type (N = 95 AA, 193 AB*, 140 B*B*) in a combined analysis of three datasets (see Methods). For the purpose of illustration only, we analysed the data as the proportion of a clutch sired by a male in a Gaussian mixed-effects model (weighted by the number of eggs in a clutch; N = 1,758 clutches). **d**, Extra-pair siring success (square-root-transformed number of extra-pair young sired per male and per breeding season) of 482 males in the presence of potential sperm competition (communal breeding aviaries) as a function of inversion type (N = 110 AA, 219 AB*, 153 B*B*) for three datasets combined (see Methods). Shown are estimates ± SE from mixed-effects models. Red lines indicate the dominance and blue dashed lines the additive effect. Above each graph, the additive (a) and dominance (d) estimates and their associated p-values from the appropriate binomial and Gaussian mixed-effects models are indicated (see Supplementary Tables 5–8).

sperm morphology and function to overdominant fitness effects. The observed pattern of overdominance explains the evolutionary maintenance of the Z-chromosome polymorphism and of Z-linked variance in sperm design, because heterozygous males by necessity produce 50% homozygous sons. However, considerable phenotypic variance in sperm morphology still exists within inversion genotypes (Fig. 1a). This might be because selection is indeed too weak in this system[7] as argued above. Alternatively, the large effect variants on the Z chromosome may interact with other loci (epistasis), thereby potentially maintaining polymorphisms elsewhere in the genome (for example one allele being favoured in AA males and the other in B*B* males). Gene expression analyses and eQTL studies of males from lines selected for short and long sperm show that most but not all expression differences map to the Z-chromosome inversion, but that causal variants seem to be restricted to the Z chromosome[41], which at least does not contradict such epistatic interactions.

Because the inversion does not seem to have any fitness consequences in females[24], its overdominant effects in males[24,41] might explain the spread of the inversion allele and its current frequency in the wild zebra finch population. The inverted allele would only have started spreading if its benefits in terms of reduced infertility and higher siring success were larger than its cost of slightly elevated embryo mortality in heterozygous males[24]. However, in the wild (and also in wild-derived populations; see Supplementary Fig. 4f,i), reported levels of extra-pair paternity are low[43,45–47]. The frequency of extra-pair paternity may have been higher at the time when the inversion first occurred and started to spread in the population, or the benefits of reduced infertility and higher success in sperm competition might have been enough to outweigh the small cost in terms of embryo mortality.

The zebra finch belongs to the grassfinch family (*Estrildidae*), in which inversions with presumably beneficial additive effects arise and spread to fixation on average once per 2.26 million years[48]. Several grassfinch species have a segregating inversion polymorphism on the Z chromosome[34,49]. Our results demonstrate how such an inversion with detrimental additive but strong overdominant effects is kept polymorphic. In this case, the inversion polymorphism maintains genetic variation in sperm design, despite presumably constant directional selection on aspects of sperm morphology[1–4]. The polymorphism described here and in ref. [41] has a large effect on a relatively simple phenotypic trait and thus forms a suitable system to study the molecular basis of overdominance in detail[50].

Methods

Study species. Zebra finches are socially monogamous, forming strong pair bonds that last for a lifetime[42–44], but they sometimes reproduce outside the pair bond ('extra-pair paternity'). In the wild, only 2–3% of all offspring are sired by an extra-pair male[45,46]. However, when wild-caught birds were allowed to reproduce in large outdoor aviaries, 12% of the offspring were extra-pair[47], which corresponds well with the 9% of extra-pair offspring observed in our recently wild-derived population[43]. Moreover, birds from populations that have been domesticated for 50–100 generations show much higher levels of extra-pair paternity in communal breeding aviaries (25–30% of extra-pair young[51–53]). Hence, using such populations has the advantage that it increases power for detecting effects on two components of fitness: siring extra-pair young and avoiding loss of within-pair paternity.

Study populations. We studied two genetically independent captive populations of Australian zebra finches (*Taeniopygia guttata castanotis*), one domesticated and one recently wild-derived, held at the Max Planck Institute for Ornithology in Seewiesen, Germany (birds originated from populations no. 4 and no. 18 described previously in ref. [34]). Housing, breeding, banding, blood sampling for parentage assignment, measuring and observing captive zebra finches do not qualify as animal experimentation according to the relevant national and regional laws and are fully covered by our housing and breeding permit (no. 311.4-si, Landratsamt Starnberg, Germany). The domesticated population (birds from generations P and F1) was brought from Sheffield University to Seewiesen in 2004 and then bred to generation F4 in 2009. We then used birds from all previous generations (P, F1–F3) to breed lines selected divergently for breeding values of male courtship rate (two high lines, two unselected control lines and two low lines[55]). We measured levels of extra-pair paternity (see below) in most of the founders (F1–F3) and in the third generation of the selection lines (S3). The wild-derived population consisted of birds from generations F1 and F2 that were bred at the University of Bielefeld and brought to Seewiesen in 2009. We bred an F3 generation in 2011 and studied sperm traits and extra-pair paternity in these birds in 2012–2013[43,56].

Inversion genotyping. We successfully genotyped 948 wild Australian zebra finches, all 74 founders that produced offspring in the wild-derived population and 88 out of 105 founders that produced offspring in the domesticated population, for 442 SNPs located on chromosome *TguZ* with an Illumina Infinium iSelect HD Custom BeadChip[57]. Using only genotypic data on the wild birds, we identified the three inversion haplotypes by principal component analysis[24] and found that 104 single-nucleotide polymorphisms (SNPs) uniquely identified the inversion types, meaning that composite linkage disequilibrium of individual SNPs with one of the inversion haplotypes was $r^2 > 0.98$ (ref. [24]). From these tag SNPs, we selected

six SNPs to subsequently genotype all birds in the two study populations for their inversion type using the Sequenom MassARRAY iPLEX platform[58] at the Institute of Clinical Molecular Biology at Kiel University. We showed previously that the six tag SNPs extend over the largest physical distance of the inverted region and are fully informative about an individual's inversion genotype when using an unanimity decision rule (that is, all tag SNPs must specify the same type; missing data are not allowed)[24]. Genotypes were called using the MassARRAY Typer (v4.0) software with default settings. Further details on the genotyping methods and quality checks are described elsewhere[24,59].

In the wild-caught Australian zebra finches, ancestral haplotype A had an allele frequency of 0.578, derived haplotype B of 0.319 and derived haplotype C of 0.080. Additionally, rare intermediate haplotypes that originated from putative double crossovers between haplotypes A and C and between haplotypes B and C segregated at a joint allele frequency of 0.023 (ref. [24]).

In the pedigree founders of the wild-derived captive population, ancestral haplotype A and derived haplotype B had an allele frequency of 0.632 and 0.124, respectively. Derived haplotype C was absent, but instead two intermediate haplotypes (jointly referred to as type D) were present with an allele frequency of 0.246. We clarified the formation of the two intermediate haplotypes by scrutinizing the alleles of all 104 SNPs in a linear sequence in relation to the WUSTL 3.2.4 assembly[60]. For most of the inverted region, the haplotypes were derived from haplotype C, with a relatively small region stemming from haplotype B (Supplementary Fig. 1). This region reached maximally from 37–50 megabases, suggesting that the D haplotypes were formed through double crossovers between inversion types B and C. The six tag SNPs used for genotyping (see above) were selected such that in the case of an individual carrying an intermediate haplotype D, not all SNPs unambiguously indicate one of the three main inversion haplotypes. Thus, we could track the intermediate haplotypes through our pedigree. Because similar intermediate haplotypes occur also in the wild, the D haplotypes probably originated in the wild and increased in frequencies in captivity due to founder effects and genetic drift.

In the domesticated population, all three main inversion haplotypes segregated (allele frequencies in pedigree founders: A = 0.333, B = 0.419, C = 0.248)[24].

Measurement of sperm traits. We obtained sperm samples from 159 males of the domesticated and the wild-derived population by cloacal massage (domesticated: $N = 56$, sampled in July 2011, wild-derived: $N = 103$, sampled up to four times in April 2012 ($N = 103$), August 2012 ($N = 92$), April 2013 ($N = 76$), August 2013 ($N = 41$)[56,61]). Overall, the sampling method was successful in 368 out of 383 attempts. Sperm samples (~0.5–3 µl) were immediately diluted in pre-heated (40 °C) Dulbecco's Modified Eagle's Medium solution (Advanced D-MEM, Invitrogen, USA). For analysis of velocity, we pipetted an aliquot onto a standard count slide (depth: 20 µm, two chambers, Leja, The Netherlands) that had been placed on a heating table kept at 40 °C. The rest of the sperm sample was fixed in 250 µl ~5% formalin solution for later analyses of morphology.

For 355 samples (out of 368) that contained sufficient sperm, we recorded sperm velocity for 45 s at eight different fields of the slide with a digital camera (UI-1540-C, Olympus) mounted on a microscope (CX41, Olympus) under 100× magnification. Each field of recording was later analysed by a CEROS computer-assisted sperm analysis (CASA) system (Hamilton Thorne, USA). All tracked objects were visually inspected by J.A., and non-sperm objects and static spermatozoa were excluded from the analysis (see also previous work[56,62,63] for a similar approach). As the medium did not contain any component to guide the spermatozoa towards one direction, we used curvilinear velocity (VCL) rather than straight-line velocity as our measurement of sperm swimming speed[63]. On average, velocity measurements were based on 223 sperm cells per sperm sample (median: 163, 95% CI: 15–700, range: 5–1,118). Log-transformed number of measured sperm cells per sample showed a moderate positive correlation with average velocity ($r = 0.29$, $N = 355$ samples), but was not related to inversion genotype (LMM: $p = 0.28$, d.f. = 2, $N = 354$ samples).

For each of 368 samples fixed in 5% formalin solution, we placed ~6 µl on a slide, air-dried it and inspected it under a light microscope (BX51, Olympus) under 400× magnification. For each sample, we photographed 10 intact spermatozoa with a digital camera system (DP71, Olympus) and analysed them with the software QuickPHOTO Industrial 2.3 (Olympus). For each sperm, we measured head (including the acrosome), midpiece and tail length to the nearest 0.1 µm. Flagellum length was calculated as the sum of midpiece and tail length[10]. For analyses (except in Fig. 1a), we used average values of the 10 sperm.

Measuring male infertility. We used a dataset[64] on 11,616 eggs laid by females kept in single pairs in isolated cages, in which we determined the fertility status of each egg (30.2% unfertilized eggs, $N = 874$ pairs, $N = 440$ females and $N = 435$ males from the domesticated population; Supplementary Table 9). Eggs were classified as unfertilized when no embryo was visible. This definition may include cases in which the zygote died before any visible embryonic development (but this is probably rare[64]). In aviaries, infertility rates are lower (around 10–20%). However, we did not use aviary data here, because egg dumping by females[65] and extra-pair mating[53] make the assignment of unfertilized eggs to females and their social partners uncertain, which might blur effects of the inversion genotype on male fertility.

Measuring male siring success. Data of male siring success stem from seven breeding experiments (involving mostly males different from those used for assessing male infertility; details in Supplementary Table 10) that group into three larger datasets. These are (1) published data from our domesticated population from 2005–2009 (generations F1–F3[53]), (2) unpublished data from selection lines that were bred from the same population from 2014–2015 (generation S3), and (3) partly published data from our wild-derived population from 2012–2013[43]. Briefly, in each of these experiments, usually groups of six males and six females were allowed to breed freely in large aviaries. In three out of the seven breeding experiments, males were allowed to breed a second time in an aviary with new females, such that we obtained repeated measures for $N = 322$ males from two breeding seasons. In total, birds were allowed to breed in 131 aviaries for a period of 49–113 days (length of the period for egg-laying; Supplementary Table 10). Each bird was observed several times per day to obtain information about its social pairing status on a daily basis. We sampled DNA from all eggs ($N = 7,406$ eggs from 2,004 clutches) that were neither infertile nor disappeared during breeding (for example broken or eaten by birds, or fallen out of nest) and used 10–20 microsatellite markers to assign them to their genetic mothers and fathers[43,53,66,67] ($N = 482$ potential fathers). Large-scale SNP genotyping of the surviving offspring[68] revealed that our parentage assignment was practically error free (error rate < 0.1%).

We quantified three components of male siring success. (1) Total siring success is the total number of eggs sired by a male in an aviary ($N = 7,353$ eggs laid by 454 females with 482 potential sires). All eggs were included independent of the males' or females' pairing status. Total siring success is the combination of the male's fertilization ability, his ability to avoid paternity loss in his own brood and his extra-pair siring success (see below). (2) Within-pair siring success is the proportion of the total number of eggs laid by the social female that is sired by the social male ($N = 6,620$ eggs laid by 425 females, of which 5,093 were sired by the social male and 1,527 were sired by 267 extra-pair males). Eggs were included only if they were laid by a female during the period for which she was monogamously paired with the focal male. In other words, eggs laid prior to social pairing were excluded from the analysis, because they cannot be classified as within-pair or extra-pair ($N = 733$ eggs). Note that the pairing status of a potential extra-pair male is irrelevant here. (3) Extra-pair siring success is the sum of all eggs sired by the focal male during the period he was paired but which were laid by females other than his social partner. Eggs of all females were included, independent of their pairing status ($N = 7,353$ eggs laid by 454 females, of which $N = 1,813$ were extra-pair eggs sired by 256 out of 482 potential extra-pair males).

Analysis plan. Sample sizes reflect the maximum available data. No data selection was done conditional on the outcome of statistical tests. We collected and analysed data on sperm traits and measures of reproductive success independently and blindly with respect to the males' inversion genotypes. Analysis strategies were chosen without regard to statistical significance. We report all results, all data exclusions, all manipulations and all measures in the study[69]. Part of our study was independently replicated and the results corroborated[41].

Statistical analyses. All analyses were conducted using R (v3.3.1)[70]. We used the lmer() and glmer() functions of the lme4 package (v1.1–12)[71] for fitting linear and generalized linear mixed-effects models, respectively, and the pedigreemm package (v0.3–3)[72] for fitting the pedigree structure as a random effect. We estimated the variance explained by the fixed effects of our mixed-effects models[73] as marginal R^2-values, using the r.squaredGLMM() function of the MuMIn package (v1.15.6)[74]. To obtain p-values, we performed likelihood ratio tests comparing models with and without a specific fixed effect. We used the lda() function of the MASS package (v7.3–45)[75] for discriminant function analyses. Model fit was visually validated (by qq-plots of residuals and plots of residuals against fitted values). To check consistency of model outputs, we ran all linear mixed-effects models that included the pedigree structure also in ASReml-R (v3)[76] and used the pin() function of the nadiv package (v2.13.2)[77] to calculate standard errors for heritability estimates. All estimates were highly consistent between ASReml and R; because all analyses can be repeated in R (freeware), we do not report the ASReml outputs here.

We fitted linear mixed-effects models with each sperm trait (head, midpiece and flagellum length, velocity) as dependent variable, with the inversion type as a fixed effect and with the pedigree structure as a random effect. Sample sizes were lowered by one male that had not been genotyped for the inversion. Because the sperm morphology of some males had been measured up to four times, we also fitted male identity as a permanent environment (pe) random effect[78]. This effect was always estimated as close to zero (ratio of the male identity variance to the total variance $pe^2 < 0.5\%$) and likelihood ratio tests comparing models with and without the male identity effect yielded p-values > 0.9. Fixed-effect z-values and p-values also did not differ between models with and without the male identity effect. However, model convergence was sometimes impaired when fitting the male identity random effect, and we thus dropped it from the final models. Thus, in the final models, the pedigree controls both for the pseudoreplication at the individual level and for the pseudoreplication due to relatedness (although the additive genetic variance might be slightly overestimated[79]). We also tested whether sperm traits differed between the domesticated and wild-derived

males by fitting population as a factor with two levels. This was only the case for flagellum length (somewhat larger in the wild-derived population, LMM: estimate ± SE = 2.00 ± 0.95 µm, $z = 2.11$, $p = 0.034$, $N = 158$ males), but for consistency we dropped population from all models because estimates were similar and led to the same biological conclusions (LMMs: all $p > 0.1$ for sperm head and midpiece length and sperm velocity; model output together with the raw data; see 'Data availability'). We decomposed the effect of the inversion genotype into an additive component (a, the number of derived allele copies, 0, 1 or 2, as a covariate) and a dominance component (d, homozygous = 0, heterozygous = 1), and contrasted the ancestral allele A with each of the derived types (B, C and D) in separate models, while Z-transforming the dependent variable to obtain effect size estimates (Supplementary Fig. 2 and Supplementary Table 2). Because effects were similar for each of the contrasts (A versus B, A versus C, and A versus D; see Supplementary Fig. 2), we ran the same mixed-effects model contrasting ancestral allele A against all derived alleles (B* = B, C and D combined; Supplementary Tables 1 and 4).

We included midpiece and flagellum length—the two traits significantly affected by the inversion type (Supplementary Table 1)—in two discriminant function analyses to predict an individual's inversion genotype: (1) based on a single sperm cell ($N = 3{,}670$ sperm), and (2) based on average values of 10 sperm per sample ($N = 367$ ejaculates). We evaluated whether the discriminant function analyses were robust to violations of the assumption of independence of data (multiple sperm per ejaculate and multiple ejaculates per male) using a subsampling procedure. We randomly sampled one sperm cell per male ($N = 158$ sperm), performed the discriminant function analysis and recorded the overall accuracy of the prediction of an individual's inversion genotype. We repeated this 10,000 times and calculated the mean accuracy of the prediction, yielding a value of 69.6% (95% quantile range: 63.9%–75.3%), only slightly smaller than the accuracy using all 3,670 sperm (70.5%). We calculated the expected random probability of classifying a male's inversion genotype correctly using a similar simulation procedure in which we first randomized the genotypes among all 3,670 sperm, performed the discriminant function analysis and then recorded the overall accuracy of the prediction of an individual's inversion genotype. We repeated this 10,000 times and calculated the mean accuracy of the prediction.

To investigate whether sperm morphology explained variation in sperm velocity, we first fitted a linear mixed-effects model with VCL as dependent variable, and head, midpiece and flagellum length, as well as their squared terms (after mean-centring) and all two-way interactions between the three linear terms, as explanatory variables while controlling for pedigree structure (fitted as a random effect). To evaluate multicollinearity between all main effect predictors, we estimated their variance inflation factors (VIFs) using the corvif() function[80] in R. VIFs were maximally 1.14, indicating no problems with collinearity[80]. Non-significant explanatory variables were then removed to create a minimal model. Finally, we used the predict() function in R to calculate the predicted VCL from the final model.

We tested the effect of the inversion types on both observed and predicted sperm velocity by fitting linear mixed-effects models with either observed or predicted VCL as dependent variable, inversion type as two fixed effects (the additive component and the dominance component, $a + d$), and the pedigree structure as a random effect (Supplementary Table 4). Sample sizes were lowered by one male that had not been genotyped for the inversion. Using the predicted VCL as the dependent variable may give an impression of circularity, because the same data were used to generate parameter estimates that were subsequently used for prediction. However, we use predicted VCL values to assess a plausible connection between inversion genotype and sperm velocity through effects on sperm morphology. An association between predicted velocity and inversion genotype is a necessary—though not sufficient—condition for a causal relationship.

We tested the effect of the inversion types on male fertility using a generalized linear mixed-effects model with the fertility status of each egg as a binary response variable (fertilized = 1, unfertilized = 0) and the inversion type of the father ($a + d$) as fixed effects. We controlled for the same fixed and random effects as in ref. [64], namely the inbreeding coefficient and the age of both parents, their pairing duration at egg laying and the laying sequence of eggs within a clutch (all covariates) and clutch, mother, father, pair and experiment identity as random effects (Supplementary Table 5). Often entire clutches appeared unfertilized, which may be due to pairs failing to copulate[64]. Thus, we repeated the analysis on a reduced dataset that excluded all completely infertile clutches (leaving $N = 2{,}190$ out of 2,782 clutches) and show that this led to the same conclusions (Supplementary Table 5).

To test the effect of the inversion types on male total siring success, we fitted linear mixed-effects models with the total number of eggs sired by a male (within a given breeding season) as the dependent variable. We fitted inversion type ($a + d$) as fixed effects and controlled for the following covariates (fitted as fixed effects): (1) the number of days the focal male was present in an aviary; (2) male inbreeding coefficient F_{Ped} calculated using Pedigree Viewer (v6.5)[81]; (3) adult sex ratio (proportion of males) in the aviary (0.5, except in the first experiment where it ranged from 0.4 to 0.6). As random effects we included male identity (because males bred in two breeding seasons in experiments 1, 5, and 6) and aviary identity.

We tested the effect of the inversion types on within-pair siring success using a generalized linear mixed-effects model with paternity of each egg laid by the social female as a binomial dependent variable (within-pair = 1, extra-pair = 0). We fitted inversion type ($a + d$) as fixed effects and controlled for the following factors or covariates (all fitted as fixed effects): (1) pair-bond duration in days, calculated as the date on which the egg was laid minus the date of pairing ($\log_{10}(n + 1)$-transformed); (2) male inbreeding coefficient; (3) adult sex ratio (proportion of males) in the aviary; (4) pair status: in both experiments 6 and 7, females were paired either to a preferred male or to a random male prior to their release into the aviary[43], which we accounted for as a fixed effect with three levels (chosen, non-preferred, divorced; force-pairing failed in 26% out of 140 cases and birds divorced). As random effects we included female identity, male identity, social pair identity and clutch identity. In experiments without repeated measures, female, male and pair identities were strongly aliased, so we dropped variance components that were estimated as zero from the model. Clutches were defined as all eggs laid by a female with laying gaps less than 6 days.

To test the effect of the inversion types on male extra-pair siring success, we fitted linear mixed-effects models with the number of extra-pair young that a male sired while being socially paired (within a given breeding season) as the dependent variable (square-root-transformed to approach normality). We fitted inversion type ($a + d$) as fixed effects and controlled for the number of days the focal male was paired to his social mate (including 0 days for unpaired males, which effectively forces the regression line through the origin). This also controls for variation in the duration of the breeding experiments. We also controlled for male inbreeding coefficient and adult sex ratio in the aviary by including these factors as covariates (as in the model for within-pair siring success described above). As random effects we included male identity (because males bred in two breeding seasons in experiments 1, 5 and 6) and aviary identity.

For total (within-pair and extra-pair) siring success, we fitted the models for each of the three datasets (domesticated F1–F3, domesticated S3 and wild-derived F3) separately and then combined them into a single model, in which we included population as a factor with two levels (domesticated versus wild-derived; see Supplementary Tables 6–8).

Code availability. All statistical models fitted in this study are accessible through the Open Science Framework (https://osf.io/d4m45/).

Data availability. Inversion genotype data, phenotype data, infertility and siring success data, and all ASReml-R model outputs are accessible through the Open Science Framework (https://osf.io/dkqth/ and https://osf.io/d4m45/). Sperm morphology and velocity data can also be accessed at Dryad (http://dx.doi.org/10.5061/dryad.4h245).

Received: 14 December 2016; Accepted: 6 June 2017;
Published online: 17 July 2017

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Acknowledgements

We thank T. Aronson, E. Bolund, S. Janker, H. Schielzeth, J. Schreiber and O. Tomášek for help with data collection, M. Schneider and G. Hemmrich-Stanisak for molecular and genomic work, and S. Bauer, E. Bodendorfer, J. Didsbury, A. Grötsch, A. Kortner, P. Neubauer, F. Weigel and B. Wörle for animal care and help with breeding. This work was supported by the Max Planck Society (B.K.) and by the Czech Science Foundation (project no. P506/12/2472 to T.A.).

Author contributions

U.K., M.W. and A.F. genotyped all birds. T.A., J.A. and K.M. collected sperm samples. P.O. measured sperm morphology. J.A. measured sperm velocity. W.F., M.I., D.W. and K.M. collected breeding data. U.K., W.F. and Y.P. analysed the data. U.K., W.F. and B.K. wrote the manuscript with help from T.A. All authors contributed to the final manuscript. W.F., T.A. and B.K. conceived of the study.

Competing interests

The authors declare no competing financial interests.

Additional information

Supplementary information is available for this paper at doi:10.1038/s41559-017-0236-1.

Correspondence and requests for materials should be addressed to W.F.