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The Effects of Dietary Mercury Exposure on Male Fertility in the Zebra Finch

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The Effect of Mercury on Male Fertility in Domestic Zebra Finches

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COMPLIANCE PAGE

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ABSTRACT

Sperm traits, including morphology, number and function, have been linked to fertilization ability and offspring quality in several species. However, many of these traits are artificially influenced by anthropogenic pollutants. Mercury, a globally distributed heavy metal pollutant, has been linked to altered testicular morphology and reduced fertility in many vertebrates. In this study, we exposed domestic male zebra finches (*Taenopygia guttata*) to dietary mercury at concentrations found in prey species in a highly polluted watershed region. We then compared sperm traits from these males to those of controls, and found a significant effect of mercury on sperm length and variability. Mercury-dosed birds produced shorter, more variable sperm than control males. When paired to female birds with no history of mercury exposure, fewer sperm from mercury-dosed males were found on the egg perivitelline membrane, suggesting that fewer sperm managed to reach the egg within the female reproductive system. Finally, we also found that mercury-exposed males had lower sperm counts compared to control males. To examine if this effect persisted under more natural copulation, we mated male zebra finches to dummy females. We found a similar trend, suggesting that mercury exposure could result in males transferring fewer sperm to females. Taken together, these results suggest that mercury exposure could result in males that produce fewer, smaller, more variable sperm with a reduced ability to reach the egg in the female reproductive tract. This could have strong implications for post-copulatory sexual selection and gene transmission patterns in mercury-affected populations, and may have further consequences in terms of epigenetic effects or developmental disruptions that affect offspring quality.

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Dietary mercury reduces sperm function in male domesticated zebra finches

Introduction

In sexually reproducing species, successful fertilization is an essential prerequisite to reproductive success. Several decades of research in a variety of model systems have shown that several sperm traits, including concentration, morphology and speed, play a large role in successful fertilization (Simmons and Fitzpatrick 2012). Some of these sperm-traits are influenced by anthropogenic pollutants (Vecoli et al. 2016). This is cause for concern and could explain why much of the developed world is experiencing a sharp decline in male human fertility. Since the 1970s, human sperm counts in developed nations have dropped by over 50% (Levine et al. 2017). Reduced reproductive function in males has also been found in a wide range of free-living vertebrates, ranging from fish to mammals (Edwards et al. 2006), strongly suggesting that this trend is at least in-part due to environmental factors, particularly exposure to pollutants. Thus, there is an urgent need to explore the causal relationship between exposure to pollutants and sperm's morphology and function.

Due to their ubiquity and persistence in nature, endocrine-disrupting compounds and heavy metals have received particular attention for their detrimental effects on both humans and wildlife (Harrison et al. 1997; Wirth and Mijal 2010). Mercury, a persistent, globally distributed heavy metal toxicant, is also weakly estrogenic (Zhu et al. 2000) and has been found to negatively affect male reproductive processes at several stages (Tan et al. 2009). Mercury accumulates preferentially in the testes of a range of vertebrates, including birds (McNeil and Bhatnagar 1985), rodents (Moussa et al. 2011) and primates (Mohamed et al.

1987). Concomitantly, exposure to mercury has been linked to a reduced gonadosomatic index (ratio of testis mass to overall body mass) in fish (Webb et al. 2006) and rodents (Homma-Takeda et al. 2001). Within the testes across taxa, mercury causes a disruption in the function of Leydig and Sertoli cells essential to spermatogenesis (Ram and Joy 1988; Homma-Takeda et al. 2001). Functionally, exposure to mercury reduces sperm motility and proportion of normal sperm in primates (Mohamed et al. 1987), and is associated with infertility in humans (Dickman et al. 1998; Choy et al. 2002). Therefore, there is evidence that mercury can reduce male fertility in vertebrates.

However, there are limitations in how we can interpret previous studies. Many previous experiments have used artificial means of exposing subjects to mercury (such as high dose- injection) (Homma-Takeda et al. 2001; Yu et al. 2016), used levels of exposure much higher than those found in nature(Moussa et al. 2011), and/or did not assess the ability of the sperm to fertilize eggs. Here, we will address each of these limitations. Avian systems are particularly interesting in studies of sperm function, as sperm that successfully travel to the ovum are trapped in the outer perivitelline membrane (PVL) of the egg. The number of sperm trapped on this membrane offer a biologically meaningful proxy of sperm function in a non-terminal fashion (Birkhead and Fletcher 1998). Our lab has developed techniques which allow us to dose zebra finches (*Taenopygia guttata*) with environmentally available concentrations of methylmercury in their diets (Varian-Ramos et al. 2014; Buck et al. 2016; Chin et al. 2017; Whitney and Cristol 2017). Thus, we can dose animals in a realistic method and compare metrics of sperm production and sperm performance.

Songbirds are vulnerable to the effects of mercury pollution due to their

relatively high trophic position. Some forest songbirds, such as the tree swallow (*Tachycineta bicolor*), have higher blood methylmercury concentrations than the fish-eating belted kingfisher (*Megaceryle alcyon*), despite not feeding directly on aquatic organisms that live in a contaminated river (Cristol et al. 2008a). Other than increasing mortality (Jackson et al. 2011), exposure to mercury causes a broad range of sub-lethal effects in songbirds. These include apparent effects on singing behavior (Hallinger et al. 2010), immune competence (Lewis et al. 2013), endocrine response to stress (Wada et al. 2009), cognitive abilities (Swaddle et al. 2017) and reproduction (Varian-Ramos et al. 2014).

Exposure to sub-lethal levels of mercury results in a 50% decline in the reproductive success of captive, domesticated zebra finches (*Taenopygia guttata*) (Varian-Ramos et al. 2014), though there appear to be no discernable effects on incubation or provisioning behavior (Chin et al. 2017). Additionally, while clutch size was not affected by mercury exposure, the proportion of chicks hatched was reduced when birds were fed diets containing 0.6 ppm ww methylmercury or higher (Varian-Ramos et al. 2014a; Chin et al. 2017). This reduction in hatching and fledging success, but not clutch size or parental investment, suggests a role for male-mediated reduction in reproductive success, perhaps through reduced fertility or offspring viability via epigenetic effects (Evans et al. 2017). In addition to its use in studies of mercury toxicity, the zebra finch has a well-established history as a model system for studies of sperm competition and sperm function (Birkhead and Fletcher 1995, 1998; Bennison et al. 2014; Hurley et al. 2018). We designed and conducted an experiment to examine the effects of sublethal dietary methylmercury exposure, at doses similar to those found in prey species at polluted watershed sites

(Cristol et al. 2008), on sperm morphology and function in male zebra finches.

We compared sperm morphology from male zebra finches exposed to dietary mercury for their entire lifetimes to birds that had never been exposed to mercury, to assess whether this dose of dietary mercury had any effects on sperm and fertility parameters. We predicted that mercury would have a detrimental impact on spermatogenesis, resulting in altered morphology. We then paired mercury-exposed and control males to females with no history of mercury exposure to assess the ability of sperm to reach the PVL of the egg, as a functional index of fertility. We predicted that mercury would interfere with spermatogenic processes resulting in altered sperm morphology and concentration, resulting in poorer sperm performance, i.e. reduced numbers of sperm reaching the PVL of eggs.

Methods

Experimental Design

We conducted all research at the College of William & Mary's aviary in Williamsburg, Virginia, USA, between December 2016 and July 2018. This study was carried out in accordance with the recommendations in the Guide of the Care and Use of Laboratory Animals of the National Institutes of Health. All procedures and protocols were approved and overseen by William & Mary's Institutional Animal Care and Use Committee (IACUC-2016-10-24-11515). We maintained the birds under controlled, monitored environmental conditions that facilitated breeding (14:10

light:dark photoperiod, approximately 21°C - 23°C, approximately 50% humidity). When not breeding, we housed birds in single-sex cages (76 cm × 45 cm × 45 cm, l × b × h), in groups of four to six, with *ad libitum* access to food, vitamin-enriched water, cuttlebone and grit.

We fed the birds a commercially available pelletized diet (Zupreem FruitBlend), dosing the feed for mercury-exposed animals with 1.2 ppm wet weight methylmercury cysteine, and that for control animals with a carrier solution of cysteine, but no mercury. We chose this concentration as it is similar in mercury content to some prey items consumed by songbirds living downstream of heavily contaminated areas in Virginia (Cristol et al. 2008), and has previously been found to reduce reproductive success in captive zebra finches (Varian-Ramos et al. 2014). The protocols for food preparation are described in detail elsewhere (Varian-Ramos et al. 2014). Briefly, methylmercury chloride and cysteine were mixed in degassed, deionized water to convert the methylmercury chloride to methylmercury cysteine, and thoroughly mixed in to the food. Control food was prepared similarly, using aqueous cysteine solution, but no mercury. Each batch of food was tested to ensure that it fell within 10% of the target concentration.

The dosed zebra finches used in this study were the offspring of age-matched birds (F_0) that had been kept on a mercury-dosed diet or a control diet for the entirety of their lives, including *in ovo* exposure. The F_0 parents themselves were bred from randomly chosen birds from a large, outbred zebra finch colony maintained at William & Mary, checking parental lineages to avoid inbreeding. The birds used in this experiment were bred in free-flight indoor rooms measuring 3 m x 3.6 m x 3 m (l x b x h). Each room contained between 31 and 38 pairs of birds.

Conditions within these rooms remained otherwise identical to those in the caged bird facility. F_0 birds could choose their mates and nesting sites. The lineage of individuals used in this study is thus unknown, but will not represent more than a single generation of possible inbreeding. Zebra finches also appear to have relatively low rates of extra pair-paternity, at least in wild populations (Birkhead et al. 1990).Two bouts of breeding were conducted for this study, each lasting approximately 3.5 months. Adolescents were removed and housed in single-sex cages to prevent them from breeding with their parents. The control and mercury breeding rooms were switched between breeding bouts, after thorough cleaning of both rooms, to minimize any overall room effects. After removal from the free-flight room, fledglings were housed in single-sex cages for the remainder of the experiment. Male cages were kept adjacent to female cages so that males were in constant visual and acoustic contact with females.

Sperm morphology

To assess morphology of sperm cells, we collected ejaculates from sexually mature male birds (n = 24 each control and mercury-dosed birds) housed in single-sex cages via cloacal manipulation (Rowe et al. 2015). After collection, sperm were fixed in a 5% solution of formalin in phosphate buffered solution (PBS). Sperm morphology was analyzed by placing 10 µL of a fixed sperm sample and allowing it to air dry for at least 5 mins (Rowe et al. 2015). High-magnification images (600×) of the sperm were then captured using a digital camera (Fastec TS5, Fastec, USA) fitted to a light microscope (Olympus CX60, Olympus, USA). Ten morphologically normal sperm (i.e., without obvious deformities such as a missing tail or head) were used for analysis, giving us measures for the following traits: (a) sperm head length,

(b) midpiece length, (c) flagellum length, and (d) total length (i.e. the sum of the previous three measurements). Every measurement was obtained blind to treatment group.

Sperm function

In this study, “sperm function” is defined as the ability of the sperm to travel through the female reproductive tract and reach the egg. To assess sperm function in the absence of female-mediated effects of mercury exposure, none of the females used in this study were ever exposed to mercury. Female birds used in the sperm function study did not share any ancestors with the male birds (described above) for at least three generations. All birds, including mercury-exposed males, were fed a control Zupreem diet (0.0 ppm Hg) for the duration of our breeding experiments, as it was not possible to expose males to mercury without exposing females. Males were also fed control food 24 hours prior to introduction to avoid contaminating the breeding cages with their mercury-laden feces. Each male and female pair (control, n = 14; mercury, n = 8) were placed in a wire cage (approximately 0.4 x 0.6 x 0.4 m) with a plastic nesting box and ad libitum nesting material. Pairs that did not initiate egg-laying within a 6-week period of introduction were removed from the study. Previous research has indicated that the half-life of mercury in zebra finch blood is approximately 3.5 weeks post-exposure (Whitney and Cristol 2017), thus, we felt that it was possible that any effects may not have been apparent after a six week period. Eggs were collected and stored at -20°C on the day they were laid, but replaced with eggs that had been laid by other non-breeding females in the colony. This replacement procedure allowed us to dissect and examine experimental eggs while encouraging females to lay more eggs to complete a clutch. Only clutches

containing at least three eggs were used for analyses.

We followed established protocols to collect and process the PVL in each experimental egg(Birkhead et al. 1994; Johnsen et al. 2012; Hurley et al. 2017). Briefly, eggs were defrosted from -20°C in a 4°C refrigerator overnight, then opened with fine scissors and the yolk separated from the albumen. The perivitelline layer was cut open and lifted off the yolk using a pair of fine tweezers. Frozen yolks remain semi-solid, making this separation straightforward. The membrane was cut in two halves, ensuring the germinal disk remained unaffected, and was placed in a glass dish containing 1x PBS and we used a hair loop (described in Birkhead et al. 2008) to clean off excess yolk with gentle agitation.

We mounted the washed PVL on a microscope slide and incubated it in the dark with 15 µL of Hoechst 33342 fluorescent dye for 10 mins before viewing the membrane under a fluorescent microscope (Olympus BX51, Olympus, USA) and examining it at 200×. We counted the number of sperm on the PVL, blind to treatment group.

Statistical Analysis

We tested whether sperm size varied between control (n = 24) and mercury-exposed (n = 24) males using an independent-samples t-test, assuming unequal variances. As we noted that variances were not equal between these two treatments, we also compared the coefficient of variation for sperm tail length within the ejaculates (among 10 sperm cells) of control and mercury males, also using an independent-samples t-test.

To examine whether the number of sperm found on the PVL was influenced by whether a (control) female mated with a control or mercury-dosed male, we

performed a one-way ANOVA with average (per egg within a clutch) number of sperm on the PVL as the dependent variable and male treatment (control, n = 14; mercury, n = 8) as a fixed factor and clutch size as a covariate. We included clutch size as a covariate as it is possible that sperm numbers might be depleted as more eggs are formed and travel down the reproductive tract of females, potentially clearing sperm as the eggs are laid.

All statistical analyses were performed in IBM SPSS Statistics v23, employing two-tailed tests of probability.

Results

Males exposed to mercury in their diet produced sperm cells with marked morphological differences compared to controls (Table 1.1). On average, sperm lengths of mercury-exposed males were 17% shorter than those of control males. In addition, the relative amount of variation in length within the ejaculates of mercury-dosed males was larger than the variation in length of control males ($t_{39.1} = 3.78$, $p = 0.001$; average CV (± 95% confidence interval) of tail length for control males = 9.77 (± 0.681), average CV (± 95% confidence interval) of tail length for mercury-dosed males = 12.13 (± 1.01)). Hence, the ejaculates of mercury-dosed males, compared with controls, are typified as containing more variable sperm that have shorter tail lengths (Figure 1.1).

Table 1.1. Mercury dosed male zebra finches have significantly altered sperm morphology compared to controls. Mean traits are shown ± SD.

Trait	Control Males	Mercury Males	*T*	df	p
Total length (µm)	78.18 ± 2.86	64.78 ± 3.35	-14.906	44.933	2.2e-16
Head length (µm)	9.71 ± 0.50	9.02 ± 0.31	-5.7002	38.466	1.412e-06
Midpiece length (µm)	25.79 ± 0.83	23.38 ± 0.43	-12.62	34.613	1.657e-14
Flagellum length (µm)	42.67 ± 3.33	32.32 ± 2.87	-11.542	44.993	4.843e-15
Total length $CV_{within\ male}$	9.77 ± 2.73	12.13 ± 6.69	3.775528	46	0.000228

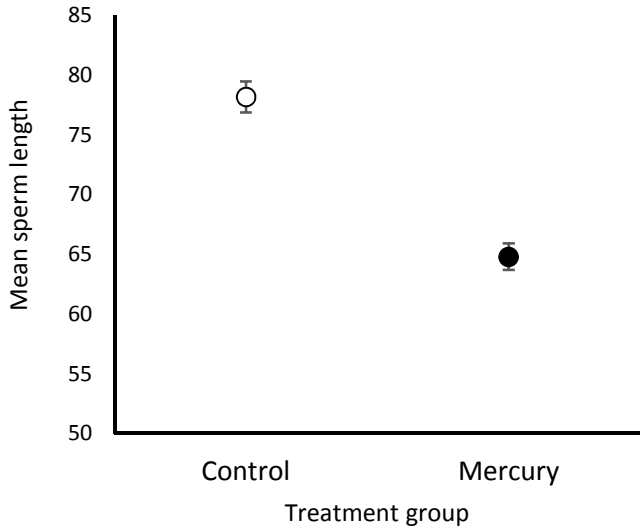

Figure 1.1. Mean (± 95% confidence interval) length of sperm of control (open circle) or mercury-dosed (filled circle) males. Note that the y-axis does not start at zero, for ease of seeing confidence intervals.

Approximately 33% more sperm were found on the PVL when females were mated to control males as opposed to when they were mated to males who were fed a mercury diet ($F_{1,19}$ = 7.71, p = 0.012; figure 1.2). There was little effect of clutch size on the number of sperm cells found on the PVL ($F_{1,19}$ = 2.06, p = 0.167).

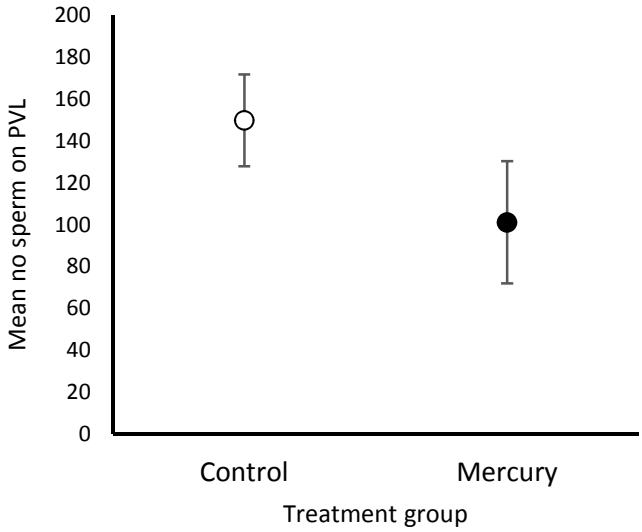

Figure 1.2. Mean (± 95% confidence interval) number of sperm, per egg within a clutch, that were found on the PVL of eggs when mated with either control (open circle) or mercury-dosed (filled circle) males.

Discussion

In this study, we show that exposure to dietary methylmercury significantly reduces sperm length in male zebra finches, and reduces their ability to reach eggs. Additionally, mercury-exposed birds had more variable sperm than control males, suggesting that mercury exposure interferes with quality-control mechanisms during spermatogenesis. We suggest that the reduction in sperm count at the PVL provides compelling evidence that exposure to environmentally available levels of methylmercury could have significant implications for male infertility.

Methylmercury is capable of crossing the blood-testis barrier, where it causes damage to testis tissue and affects spermatogenesis by increasing levels of oxidative stress (Martinez et al. 2014). Oxidative stress has been linked to poor sperm performance in barn swallows (*Hirundo rustica*) at a radiation-contaminated site (Bonisoli-Alquati et al. 2011). Mercury-induced oxidative stress could result in

sperm damage through a variety of mechanisms, such as lipid peroxidation in sperm membranes or premature acrosome reactions (Hermosell et al. 2013). It remains unclear if mercury also inhibits enzymes necessary to protect cells from oxidative stress in the testis, and how chronic exposure may affect antioxidant mechanisms (Bando et al. 2005). These issues remain important avenues of investigation into proximate causes of subfertility following pollutant exposure.

Our finding that mercury-exposed birds have altered sperm traits may have important consequences for fertilization success. Zebra finches appear to require that a minimum number of sperm reach the fertilization area before successful development of the zygote (Hemmings and Birkhead 2015), and longer sperm were more likely to reach the PVL of the egg in a competitive scenario (Bennison et al. 2014). These effects may be magnified in the field, where birds presumably do not have *ad lib* access to food and water resources, and energetic demands are much higher. While we did not quantify hatching success in this study, we have found that mercury-exposed pairs have lower hatching success in previous research (Varian-Ramos et al. 2014; Chin et al. 2017).

In the zebra finch, sperm length appears to be an important factor in reaching the egg. In birds that were artificially selected to have either long or short sperm, long sperm were more successful at reaching the PVL than short sperm, regardless of mating order (Bennison et al. 2014). Sperm size in captive, domesticated zebra finches is strongly heritable (Birkhead et al. 2005). Together, this suggests that sperm size is an important sexually selected trait that could be masked by methylmercury exposure. Zebra finches exhibit higher rates of intra-male ejaculate variation than similar songbirds (Hemmings et al. 2016), generally thought of as a

consequence of their relatively low rates of extra-pair paternity (Birkhead et al. 1990). Species with higher rates of postcopulatory sexual selection tend to have more uniform sperm (Immler et al. 2008). In species with higher rates of sperm competition, males that feed in mercury contaminated areas or on prey species such as spiders may suffer significantly reduced paternity as a consequence of altered sperm performance. This may be particularly significant in migratory species where there are numerous stopover sites, as birds feeding in certain areas have elevated mercury levels compared to those at less polluted sites (Kopec et al. 2018).

Sperm morphology is generally assumed to be under the control of the diploid male (Birkhead et al. 2005). However, there is increasing evidence that the haploid genes of sperm cells influence sperm phenotype (Immler 2008), and that variation within sperm phenotype could have consequences for offspring development (Immler et al. 2014). In this context, our finding of altered morphology combined with increased intra-male variation in sperm morphology could suggest several avenues for further investigation. Zebra finches appear to show a certain degree of intra-ejaculate sperm selection (Hemmings et al. 2016), favoring particular midpiece: flagellar ratios. Zebra finch females can store sperm from different males in different sperm storage tubules, and sperm phenotype has a role in mediating mating effects in fertilization (Hemmings and Birkhead 2017). Thus, altered sperm size may have strong consequences for post-copulatory sexual selection. Interestingly, the strongest effect of mercury on sperm morphology was a reduced flagellar length. In fruit flies (*Drosophila melanogaster*), the length of sperm flagella has been demonstrated to have evolved through post-copulatory sexual selection,

though this has yet to be investigated in a vertebrate model (Miller and Pitnick 2002). Overall, by altering spermatogenic processes, mercury exposure could potentially affect which sperm cells reach the eggs.

In this context, effects on overall reproductive success may be much stronger than effects on fertility per se. This could have far-reaching consequences in terms of haplotypes or epigenetic markers that are passed down between generations. In evidence of this idea, short-term food restriction in male guppies (*Poecillia reticulata*) was found to result in juveniles that were smaller than those fathered by non-restricted males (Evans et al. 2017). In zebrafish (*Danio rerio*), embryonic mercury exposure resulted in altered behavioral phenotypes and sperm epigenetic markers two generations after exposure (Carvan et al. 2017), further highlighting the importance of understanding how mercury pollution may impact gene transmission and the health of future populations.

Dietary mercury exposure reduces sperm number in the zebra finch in a context-dependent manner

Introduction

Sperm competition, often defined as the competition between sperm of two different males (Parker, 1970), is widely recognized as one of the most important forces of post-copulatory sexual selection in males (Simmons and Kotiaho 2002; Immler et al. 2011). Many models of mating behavior begin by assuming a strong asymmetry in investment, with males producing very large numbers of tiny sperm (Bateman 1948), to the extent that some modelling approaches suggest that extreme anisogamy and sperm competition are responsible for the evolution and maintenance of two sexes (Parker 1982). However, there is considerable variation across animal taxa, with males of some species, such as *Drosophila* producing few, very large sperm cells, and others, including humans and the majority of passerine birds, conforming to the numerous, tiny sperm phenotype (Pitnick et al. 1995). In trying to understand the relationship between sperm competition and sperm phenotype, comparative studies have reported both positive (Gage 1994) and negative relationships (Stockley et al. 1997) between sperm size and sperm competition, as well as the lack of a relationship between these factors (Hosken 1997). While research on sperm number across taxa is scarcer, theoretical approaches strongly suggest a trade-off between sperm size and number (Parker 1982; Immler et al. 2011).

In passerine birds, models of postcopulatory sexual selection have often assumed a passive 'raffle mechanism', with no adaptive form of sperm displacement

or cryptic choice (Colegrave et al. 1995). Modeling approaches using data on sperm size, count and mating system using several species of birds appear to support the "raffle" hypothesis (Immler et al. 2011), and higher rates of postcopulatory sexual selection are associated with a reduction in intra-male sperm variation in passerines (Immler et al. 2008). While direct experimental studies on sperm number and paternity are difficult to conduct, researchers have found positive relationships between fertilization success and cloacal protuberance volume in tree swallows, *Tachycineta bicolor* (Laskemoen et al. 2010), which has been found to be positively correlated with sperm number (Peer et al. 2000). Taken together, it appears that birds invest in higher sperm number rather than sperm size – opposite to the trend found in several species of insects such as Drosophila, where sperm numbers are very low, but each sperm cell is very large (Immler et al. 2011). While more recent research paints a somewhat more complex picture (Hemmings et al. 2016), the number of sperm still appears to be important in achieving successful fertilization (Bennison et al. 2014). Thus, higher-quality males would be at a selective advantage if they maximized their sperm number, as it would boost their chances of achieving successful fertilization.

In discussions of sperm number, however, it is essential to recognize that ejaculate composition is strategic (Hunter et al. 2000; Wegener et al. 2013). Thus, the conditions under which an ejaculate is collected or studied may influence the outcome of the study. In this context, there is some speculation in the literature that the results of cloacal sperm manipulation techniques may not accurately represent male investment that would occur during a true copulation (Pellatt and Birkhead 1994; Girndt et al. 2017). This could occur for several reasons: for instance: (1)

excess pressure could result in fecal contamination of sperm samples, which could damage sperm cells by exposing them to altered pH or osmotic pressure compared the any fluids in the epididymis; (2) improper handling technique could also damage the cloaca; (3) inadequate pressure may not completely empty the glomeruli, where mature sperm are stored. This technique also would not allow us to detect any strategic allocation of ejaculates, as males may not transfer the entire contents of the glomeruli during any given copulation attempt. Mating is an important part of pair bonding in the zebra finch (Zann 1996), and males in our colony have been observed attempting to mate several times through the day – it is possible that only some a portion of sperm are transferred during each mating bout. Thus, manual extrusion may not be an accurate method of measuring sperm count in terms of the number of sperm transferred to the female.

In the previous chapter, we have shown that dietary exposure to methylmercury both reduces sperm size and increases intramale sperm variability. However, it is possible that birds that make shorter sperm may be able to make more sperm cells, i.e. purchase more tickets in the fertilization raffle, or could transfer a higher number of sperm in an individual mating to compensate for overall lower sperm production. In order to investigate these possibilities, we examined whether sperm count differs between mercury-exposed and control animals using both a dummy female and cloacal manipulation. We then compare these methods of sperm collection in terms of cell count and assess whether mercury affects sperm allocation under both artificial and ‘natural’ sperm collection techniques.

Methods

We used the same general experimental design as described in the previous chapter. We conducted two sets of sperm counts: one set using cloacal manipulation, and one set collected using dummy females.

Cloacal manipulation: As the ejaculates from a single male zebra finch can vary, three samples were collected from each male with a two-week resting period between each collection. The exact period required for spermatogenesis in the zebra finch remains unknown, but sperm numbers increase up to 7 days after ejaculation, and a two-week period roughly corresponds to a spermatogenic cycle in the Japanese quail (*Coturnix coturnix*) (Jones and Lin 1993; Birkhead and Fletcher 1995). Hence, we felt that two weeks was a conservative period for sperm to be replenished. Immediately following manipulation, sperm were fixed in a 5% solution of formalin in PBS and stored until analysis.

Dummy female: We followed closely the protocol described in Pellatt and Birkhead 1994. Briefly, a deceased female zebra finch without obvious physical damage or degradation was freeze-dried in a solicitation position (Morris 1954). After about 7 days, when the specimen was thoroughly freeze-dried, it was attached to a wooden perch using thin wire or epoxy, as needed. A hole was cut around the cloacal opening so that we could remove the cloaca with a blunt metal probe and create a small cavity within the body of the model. We then created a false cloaca using a 20 mm length of silicone tube with a 0.5 mm thick wall. We placed the tube over a metal probe and then folded one end back over itself twice to create an opening crudely resembling a true cloaca. We then folded the other end of the tube back on itself and used cotton thread to seal it shut firmly. We removed all feathers immediately adjacent to the cloaca to prevent any interference during copulation.

Immediately before inserting the false cloaca in the model cavity, we added 5 µL PBS into the tube's chamber, such that the meniscus protruded slightly outward. The same set of five female models were used throughout this experiment, though silicone cloacae were not reused to prevent any contamination.

On the day prior to use in the experiment, male birds were transferred from their single-sex group housing (described previously) to a smaller, individual cage measuring approximately 30 cm × 23 cm × 38 cm (l × b × h). The male remained in visual and auditory contact with other birds, and continued to receive *ad libitum* access to food and water. On the morning of the experiment, the perch with the freeze dried female was introduced to the male's cage within two h of the lights turning on and the male was observed for a period of 20 mins. Pilot trials indicated that most matings occurred in the first 10 mins, after which mating was unlikely. An observer was present in the room during all trials and observed the male mating with the dummy female. Immediately following the mating, the false cloaca was removed and held over an Eppendorf tube containing 295 µL 5% formaldehyde in PBS. We cut the thread that sealed the blind end of the tube and washed out its contents into the Eppendorf using a pipette containing some of the collecting solution, giving a total volume of approximately 300 µL, with some loss due to evaporation.

Males were returned to their original cages immediately after mating with the dummy female. This method was largely successful; out of 20 trials with both control and mercury-exposed males, 11 out of 20 control males and 14 out of 20 mercury-exposed males successfully copulated with the female models. Two mercury-exposed birds and one control animal appeared to mate with the model, but no

sperm were found in the cloaca.

Quantifying sperm number. After collection of samples using either technique, cells were loaded on to a hemocytometer (improved Neuebauer chamber, Fisher Scientific, USA). All cells were counted across two grids of known volume (0.1 mm^3). This was corrected by a factor of 10,000 to obtain the cell concentration in cells per milliliter. The sperm concentration, corrected for dilution, was calculated using the formula:

$$Sperm\ count/mL = \frac{average\ \#\ cells\ counted}{Volume\ of\ solution\ (mL)} \times 10^4$$

Each ejaculate sample was measured three times to reduce measurement error.

Results

Mercury-dosed males did not differ from control males in terms of body mass, testis weight, testis size, or the gonadosomatic index measured as a ratio of tarsus length: testis volume. However, they did have significantly shorter tarsi than control males (Table 2.1).

Table 2.1 Mercury dosed males differed from control males in their mean tarsus length, but not body mass or any testis measures.

Trait	**Control Males**	**Mercury Males**	**t**	**df**	**p**
Tarsus length (mm)	15.63333	15.13913	-2.13	44.887	0.03869
Body mass (g)	15.63667	15.44609	-0.52222	43.247	0.6042
Testis volume (mm^3)	271.846	258.826	-0.44382	44.816	0.6593
Testis dry weight (g)	0.009774	0.009218	-0.73685	43	0.465215
GSI (tarsus length:testis volume)	0.3068937	0.3118683	-0.14441	44.996	0.8858

Mercury dosed males appear to produce less sperm on average than control males (t = -3.92423, df = 46, p = 0.000289, Figure 2.1). This effect was only found when sperm samples collected via cloacal manipulation were compared. In the samples collected via the dummy females, this effect was not found (t = -1.34121, df = 20, p = 0.097443, Figure 2.2) though the overall trend of mercury- dosed males producing fewer sperm persisted.

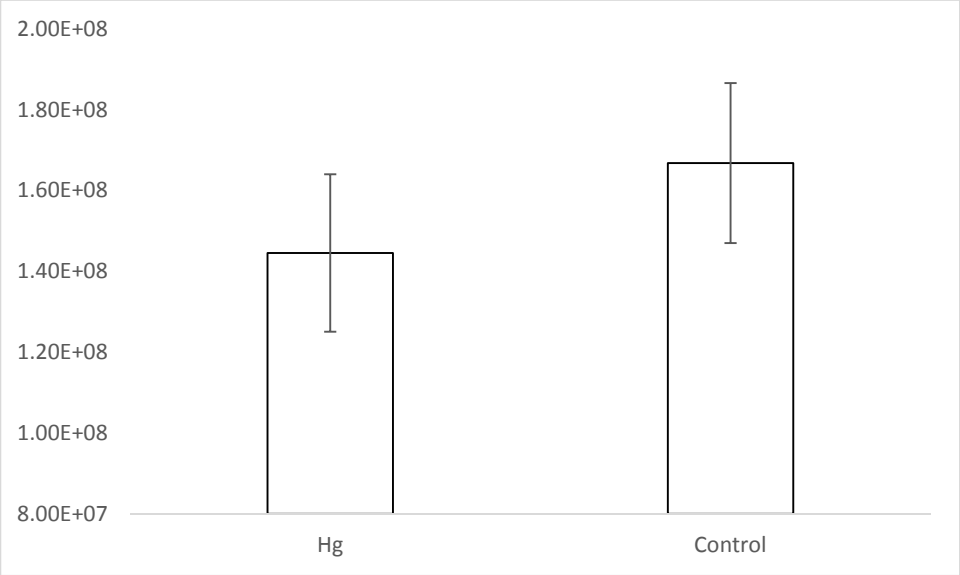

Figure 2.1. Mercury dosed males produced fewer sperm than control males, when sperm counts were compared using manual collection. Note that the y-axis does not begin at zero.

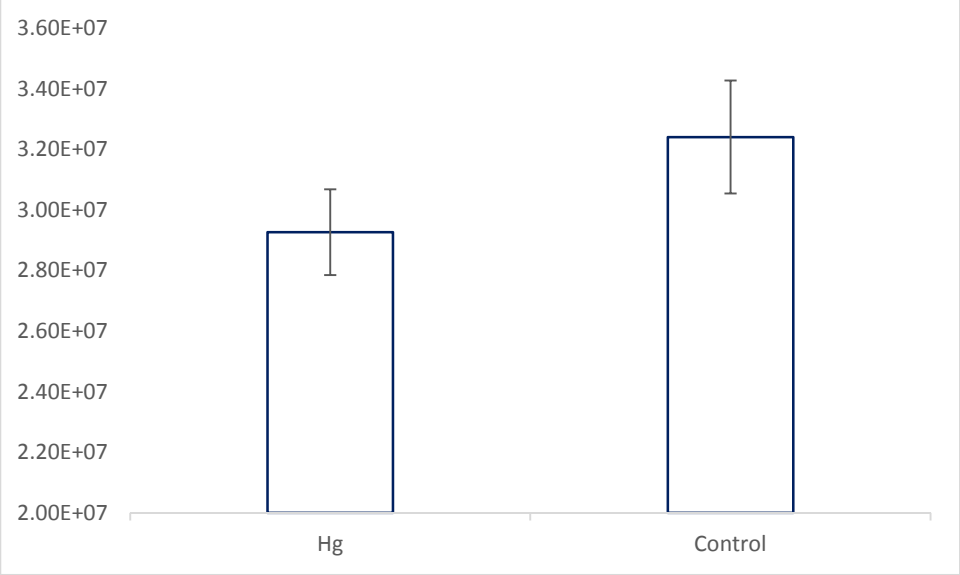

Figure 2.1. No difference between sperm counts among mercury dosed and control males was detected using the dummy females. Note that the y-axis does not begin at zero.

Discussion

In this study, we show that mercury-dosed males do not differ from control males in terms of body mass, testis size or testis weight. We demonstrate that mercury-dosed males have shorter tarsi and fewer sperm on average than control males, though this effect was only seen when comparing ejaculates collected via cloacal manipulation. We thus demonstrate that the reduction in sperm size due to mercury exposure is not compensated for by an increase in sperm numbers. A reduction in numbers in addition to altered morphology provides a good explanation for the reduced ability of these sperm to reach ova, as discussed in the previous chapter.

Though condition-dependent theories of resource allocation are useful heuristics, an operational definition of "condition" is much more elusive. Some researchers define condition as the residual reproductive value of an individual (Tomkins et al. 2004). In this context, mercury almost certainly reduces condition, as mercury dosed zebra finches fledge 50% fewer chicks than control pairs (Varian-Ramos et al. 2014c). Previous research indicates a weak link between phenotypic traits and ejaculate characters in domestic zebra finches (Birkhead and Fletcher 1995), suggesting that indicators of condition may be difficult to identify in this species.

Several researchers use body size as a proxy for condition, in taxa ranging from scathophagic flies (Blanckenhorn and Hosken 2003) to songbirds (Calhim and Birkhead 2009), but conclude that body size measures may not, in fact, be a good

proxy. This could be due to several factors, ranging from poor repeatability (Milenkaya et al. 2014), to incorrect assumptions regarding the independence of traits measured and their allometric relationships (Green 2001). Our study found no effects of mercury on overall body mass, nor on testis size. Testis size has been found to correlate positively with both plumage color and parasite load in wild greenfinches (*Carduelis chloris,* Merilä and Sheldon 1999), suggesting that higher-quality individuals may have larger testes, they also pay a higher cost in terms of reduced immune function. The lack of an effect on mass and testis traits in our study could potentially be attributed to the artificial conditions our animals experience in captivity, as they do not need to forage for food or compete for resources in the way a free-living bird might. In wild conditions, any effects may be significantly exaggerated. Importantly, previous research indicates that mercury dosed zebra finches do have weakened immune responses (Lewis et al. 2013) and may carry higher parasite loads (Ebers Smith et al. 2018), suggesting that any tradeoffs between reproductive status and physiological condition could be affected by exposure to mercury.

Historically, sperm competition outcomes in birds have been assumed to be a result of last-male precedence (Lessells and Birkhead 1990) with the last male to mate with a female being the one to fertilize the eggs. This was thought to be a consequence of sperm either displacing or stratifying over existing sperm in sperm storage tubules (SSTs) such that it would leave the tubule first. While this model has since been modified, passive sperm loss remains an important mediator of fertility in the zebra finch (Hemmings and Birkhead 2017), suggesting that a reduction in sperm numbers would reduce fertility in male finches and could ultimately result in

fewer fertilizations. Compounding this problem, mercury-dosed males appear to have shorter sperm, which are less likely to be released from SSTs after ovulation (Hemmings and Birkhead 2017). This suggests that mercury exposure could lead to significant selective disadvantages for fertilization success in songbirds.

For a thorough understanding of avian sperm biology from a resource-allocation perspective, it would be ideal if ejaculates could be collected under conditions that mimic natural ones as closely as possible. Previous research has indicated that sperm morphology does not differ between the dummy and cloacal manipulation techniques (Girndt et al. 2017), but sperm numbers were not compared. Our study found about one magnitude of difference in sperm count between cloacal manipulation and the stuffed dummy technique. This suggests that male birds may employ strategic allocation of sperm – though the effect may be masked through artificial collection techniques. Unfortunately, researchers have found very low rates of success in employing the female dummy model in species other than zebra finches, suggesting that zebra finch males may either be less selective about mating attempts, or that aspects of mate choice may have been bred out of captive colonies of birds which have gone through multiple generations of forced pairing. While we did not notice an effect of mercury on sperm counts using the model, we also used fewer samples. Future research involving multiple models to test the repeatability of sperm traits collected using this technique may help us better understand sperm allocation strategies in male birds.

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