

## Effects of chronic, parental pharmaceutical exposure on zebrafish (*Danio rerio*) offspring



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### ABSTRACT

In this study we explored how parental exposure to pharmaceuticals influences reproduction in offspring. Adult zebrafish (*Danio rerio*) were exposed for 6 weeks to 10  $\mu\text{g L}^{-1}$  of carbamazepine (CBZ) and gemfibrozil (GEM), two commonly prescribed drugs. Embryos were collected, reared in clean water until sexual maturity and then assessed for reproductive output, courtship, sperm function and organ histology. While 34% of the control pairs produced clutches, only 11% of the fish with CBZ exposed parents or 17% of the fish with GEM exposed parents produced clutches. Reciprocal crosses indicated that exposure in males had more profound reproductive effects. When a control F<sub>1</sub> male was crossed with either a F<sub>1</sub> female whose parents were CBZ or GEM exposed; no differences were observed in embryo production compared to controls. However, when a control F<sub>1</sub> female was crossed with either a CBZ or GEM F<sub>1</sub> male, 50% less embryos were produced. Male courtship was reduced in both CBZ and GEM F<sub>1</sub> fish but the deficits in courtship displays were drug specific. Compared to control males, the sperm from GEM F<sub>1</sub> males had shorter head lengths and midpieces whereas sperm from CBZ F<sub>1</sub> males had longer midpieces. Although it remains unclear how specifically these morphological differences influenced sperm velocity, the sperm from GEM F<sub>1</sub> males and from CBZ F<sub>1</sub> males swam faster than the sperm of control F<sub>1</sub> at 20 s post activation. No significant differences were observed in the histology of the liver, kidney and gonads across treatment groups. These data are important as they show that chronic, low dose pharmaceutical exposure of parental fish is sufficient to cause significant reproductive effects in offspring.

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### 1. Introduction

The effects of parental contaminant exposure on offspring are a sorely understudied area of toxicology. Multi- or trans-generational effects are typically easier to study in laboratory using species with short life spans (Dietrich et al., 2010; Lüriling et al., 2006; Péry et al., 2008) but can also be investigated with organisms that have lived for generations in polluted environments (Hontela et al., 1992; Nacci et al., 2002; Nye et al., 2007). In both cases, exposures are typically for the whole life cycle. Traditionally, life cycle assessments (LCA) utilize either a cradle-to-grave or cradle-to-gate approach (EPA, 1993). In both schemes, exposure is typically from the onset

of fertilization until terminal sampling (full life cycle) or until 24 h past sexual differentiation (partial life cycle, EPA, 1993). Rarely do studies extend beyond one generation. Yet, the effects documented in LCA may be caused during gamete production, embryogenesis, maternal deposition or uptake of contaminants into the egg, secondary sexual maturation or some combination of these important biological processes. The major challenge is that life cycle or multi-generational studies are difficult and time consuming to conduct and thus the number of studies that use vertebrates are limited. In a fast reproducing species such as zebrafish, these types of experiments are clearly possible. Given that many fish have migratory behaviours or distinct breeding grounds, quantifying the risks of parental versus embryonic contaminant exposures may be quite important and provide distinct data from traditional LCA approaches.

Studies that have looked at trans-generational impacts in aquatic species have generally focused on effects caused by estrogens (Greytak et al., 2010), estrogen like compounds (Brennan et al., 2006) or polychlorinated biphenyls (PCBs) (Nacci et al., 2002).

Abbreviations: CBZ, carbamazepine; GEM, gemfibrozil; CTL, control.

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The number of studies that have explored the trans-generational impacts with pharmaceuticals is extremely limited. To date, only one multi-generational assessment involving fish (Parrott and Bennie, 2009) has been published and the majority of studies that have been conducted use invertebrates (Dzialowski et al., 2006; Kim et al., 2012; Ortiz-Rodríguez et al., 2012). In fish, maternal deposition of contaminants is one primary route of exposure for the developing embryo (Abrams and Mullins, 2009), however there are other mechanisms by which the progeny may be impacted by parental exposure. For example, fluctuations in circulating estrogen concentrations within the mother, during gametogenesis, have been linked to impaired growth rate and survival amongst offspring (Migliaccio et al., 1996). Furthermore, elevated cortisol levels, induced from behavioral stress during courtship in females, impacts the morphology and yolk-sac size of F<sub>1</sub> offspring in coral fishes (McCormick, 1998) and cichlids (Mileva et al., 2011). Male mediated effects are often associated to impairments with spermatozoa and DNA damage which has been linked to the incidence of skeletal abnormalities amongst F<sub>1</sub> (Devaux et al., 2011). Such studies provide evidence suggesting that parental exposure to endocrine disrupting compounds may impair fish populations over several generations.

Gemfibrozil (GEM) is a fibric acid derivative which is clinically prescribed to lower plasma triglycerides and total cholesterol (Spencer and Barradell, 1996). Although its exact mechanism of action is unclear, GEM is a peroxisome proliferator believed to elicit its effect by binding to the nuclear transcription factor peroxisome proliferator-activated receptor- $\alpha$  (PPAR $\alpha$ ) and promoting hepatic uptake and metabolism of free fatty acids (Martin et al., 1997). Peroxisome proliferators have been heavily studied in mammalian systems and chronic exposure to them has been linked to the onset of hepatic carcinoma (Gonzalez, 1997) and efficiency reduction in the anti-oxidant defense system in rodents (O'Brien et al., 2001). Little work has been done to characterize the impact of GEM on fish, however goldfish (*Carassius auratus*) exposed to 1.5  $\mu\text{g L}^{-1}$  of GEM had decreased plasma testosterone (Mimeault et al., 2005) and adult zebrafish (*Danio rerio*) chronically exposed to 0.5 and 10  $\mu\text{g L}^{-1}$  of GEM show decreased reproductive output, atretic oocytes, and altered kidney histology (Galus et al., 2013b).

Carbamazepine (CBZ) is primarily prescribed as an anticonvulsant and mood stabilizer and is frequently used in the treatment of epilepsy and bipolar disorder (Brodie et al., 1995). CBZ is believed to have two target sites within vertebrates, triggering unique cellular responses. Its anticonvulsive properties are believed to occur through modulation of voltage gated sodium channels, maintaining the channels in an inactive state and reducing neuronal excitability (Brodie et al., 1995). However, its anti-depressive properties are believed to occur by lowering the turnover rate of arachidonic acid while keeping docosahexaenoic acid unaltered in brain (Rao et al., 2008). Japanese medaka (*Oryzias latipes*) acutely exposed to 6150  $\mu\text{g L}^{-1}$  of CBZ showed reduced feeding behaviour and swimming speed (Nassef et al., 2010). Sperm of the common carp (*Cyprinus carpio L.*), incubated for 2 h with 2000  $\mu\text{g L}^{-1}$  of CBZ had reduced velocity (Li et al., 2010). Lastly, adult zebrafish chronically exposed to 0.5 and 10  $\mu\text{g L}^{-1}$  of CBZ showed decreased reproductive output, decreased plasma ketotestosterone in males, and altered kidney histology (Galus et al., 2013b).

Of the small number of studies that examined the effects of GEM and CBZ in fishes, most involved concentrations well above what would be considered environmentally relevant and most exposure were acute. Both GEM and CBZ have been detected in waste water effluent and surface waters with average effluent concentrations ranging from 0.84 to 4.76  $\mu\text{g L}^{-1}$  and 0.87 to 1.2  $\mu\text{g L}^{-1}$ , respectively (Andreozzi et al., 2003; Kolpin et al., 2002; Metcalfe et al., 2003; Paxéus, 2004; Petrović et al., 2003; Zhang et al., 2008). Like

other pharmaceuticals in the aquatic environment, CBZ and GEM enter receiving waters via waste water effluent discharge. Their release into surface waters is due to the low removal efficiency of these compounds in waste water treatment, which is usually between 40% and 50% for GEM and <20% for CBZ (Galus et al., 2013a; Metcalfe et al., 2003). Both GEM and CBZ have been detected in fish tissues such as muscle (0.4, 0.52 bio-concentration units) and adipose tissue (20.8, 4.16 bio-concentration units) (Zhang et al., 2010).

Testing of pharmaceuticals, such as CBZ and GEM, for effects on different life stages and across generations is necessary to ascertain if impacts from these pseudo-persistent contaminants are likely to have population level impacts. This will require chronic, low level exposures of adults and embryos as well as multi- or trans-generational and/or life cycle assessments. To date, most of the studies of CBZ and GEM have been conducted on adult fish; embryonic exposures and multi-generational impacts are almost non-existent. Previously we showed that parental exposure did not increase embryo mortality, abnormalities or altered hatching rates in zebrafish (Galus et al., 2013b). Yet, the direct exposure of zebrafish embryos to 0.5 and 10  $\mu\text{g L}^{-1}$  GEM and 0.5  $\mu\text{g L}^{-1}$  CBZ increased mortality (Galus et al., 2013b). To our knowledge, only one study has conducted a full life cycle exposure with GEM and no such work has been done with CBZ. Fathead minnow (*Pimephales promelas*) exposed to 0.01–1  $\mu\text{g L}^{-1}$  GEM had normal embryogenesis, growth, survival, development, and egg production (Parrott and Bennie, 2009). In exposed fish the only endpoint impacted was greater deformities in clutches laid by the F<sub>1</sub> (Parrott and Bennie, 2009).

Given the environmental presence and reproductive effects of CBZ and GEM in zebrafish, the purpose of this study was to investigate whether parental exposure to CBZ and GEM was sufficient to cause reproductive consequences to the first filial generation (F<sub>1</sub>). We chronically exposed adult zebrafish to aqueous CBZ and GEM, at concentrations known to cause decreased reproduction in the parental generation, and then reared the offspring to sexual maturity in clean water. Our experimental procedure differed from conventional life cycle assessment methods as our exposure was primarily limited to the parents and the resultant embryos were exposed to aqueous GEM or CBZ for <1.5 h. At sexual maturity, F<sub>1</sub> zebrafish were examined for effects on reproduction (breeding success, clutch size), sperm function (morphology and velocity), male courtship behaviours (frequency and duration), and organ histology. These data are important to help understand if chronic, low dose pharmaceutical exposure to parental fish is sufficient to cause significant effects in offspring that have not been directly exposed to these compounds.

## 2. Methods

### 2.1. Compounds tested

All pharmaceuticals were purchased from Sigma–Aldrich (Sigma–Aldrich, Canada). Gemfibrozil (GEM) and carbamazepine (CBZ) were dissolved in dimethyl sulfoxide (DMSO). The final nominal concentration of each pharmaceutical in exposure tanks was 0 control (CTL) and 10  $\mu\text{g L}^{-1}$ . Nominal concentrations of pharmaceuticals were not verified in this study as compound preparation was identical to a prior study and the concentrations and stability of the compounds with this experimental design has been published elsewhere (Galus et al., 2013b). This concentration represents the highest expected in most environments; prior work has shown similar reproductive and histological effects of these drugs at this and a lower, more typical environmental concentration

(Galus et al., 2013b). All tanks had a final concentration of DMSO at 0.004%.

## 2.2. Adult exposures

Fish care is described in detail elsewhere (Galus et al., 2013b) and was in accordance to McMaster University's animal care policies and under approved animal use protocols. Briefly, fish were kept in our facility for one month to acclimatize and monitor reproductive output. Fish were sorted into a 1:1 sex ratio, based on a visual examination, and reproductive output was assessed 3–5 times per week for a minimum of 2 weeks prior to the onset of the experiment. Only fish from tanks with comparable reproductive output were included in the experiment. All fish were between 8 and 9 months of age. Pairs in tanks were randomly assigned to a treatment group and moved to exposure tanks 3 days prior to the start of exposure in order to acclimate.

Triplicate, 10 L exposure tanks housed 30 zebrafish at a density of 4 fish L<sup>-1</sup>. Water chemistry in exposure tanks did not differ from that of the holding tanks and was comprised of distilled water with 12 mg L<sup>-1</sup> sodium bicarbonate and 60 mg L<sup>-1</sup> sea salts (Instant Ocean, Spectrum Brands, USA). Tanks were recirculating, maintained at 28–29 °C and pH of 7.0–7.5 with a 90% renewal every 3 days, for six weeks. Weekly measurements of pH, dissolved oxygen, conductivity, nitrate, nitrite, ammonia, alkalinity, and general hardness were conducted. Zebrafish were kept on a white, fluorescent, artificial light cycle of 14-h:10-h light:dark and fed twice a day with a commercial flake food (Tetramin Tropical Flakes, Tetra, USA) and once per day with live, adult *Artemia* (GSL Brine Shrimp, USA).

After 4 weeks of exposure an egg trap was placed in each tank before the onset of light, the mechanical filters shut off and embryos were collected for 1.5 h. Embryos were placed in petri dishes containing E3 embryo rearing media (NaCl 5 µg L<sup>-1</sup>, KCl 0.17 µg L<sup>-1</sup>, CaCl<sub>2</sub> 0.33 µg L<sup>-1</sup> and MgSO<sub>4</sub> 0.33 µg L<sup>-1</sup>) at a density of 50 embryos per plate and incubated at 28.5 °C. Embryos were examined under a microscope at 6, 24, 48, 72 and 96 hours post fertilization (hpf). Embryos that were dead or abnormal and any post-hatch chorions were removed. Media was replaced as needed to maintain water quality. Embryos were reared to sexual maturity using a modified version of the rearing protocol outlined by Lawrence (2007). Specifically, larvae were fed 4× per day (2 h between feedings), starting at 5 days post fertilization (dpf), using a 50:50 mixture of spirulina (Aquatic eco-systems, USA) and grade 0 hatchfry encapsulon (Argent labs, USA) dissolved in 50 mL of distilled water and vortexed thoroughly. A pasteur pipette was used to provide 1 drop of food per plate and the mixture was evenly distributed throughout the E<sub>3</sub> media. Starting at 8 dpf, fry were fed a combination of the spirulina and encapsulon mixture as well as live first instar *Artemia* larvae (GSL Brine Shrimp, USA). At 8 dpf, fry were transferred to 500 mL tanks containing 200 mL of the E<sub>3</sub> media at a density of 50 fish tank<sup>-1</sup>. Between 11 and 14 dpf, fry were slowly transitioned from E<sub>3</sub> media to tank water, in order to minimize osmotic shock, by incrementally adding system water to the existing 200 mL of E<sub>3</sub> media. System water was added such that the final composition of tank contained 10, 25, 50, 80 and 100% system water each subsequent day. Special attention was provided to the thoroughness of water change out in the dishes and tanks, especially during feedings with the spirulina mixture, to prevent the formation of an algal bloom. From 15 dpf onwards, fry were fed 3× per day, twice with a combination of grade 0 and grade 3 hatchfry encapsulon and once with live 1st instar and adult *Artemia*. Fry remained in 500 mL

tanks until their head width was ≥500 µm (~4–5 weeks post hatch), they were then transferred to holding tanks in a semi-recirculating system (28 °C, pH 7–8, dissolved oxygen ≥87% and conductivity 470–455 µS) with ≥10% daily renewal (Aquatic

habitats, FL, USA). Once fry reached 8 weeks post hatch they were transitioned to the adult diet as described above and raised until they were 6–7 months post hatch.

## 2.3. Pairwise and reciprocal breeding

The reproductive capabilities of the first filial (F<sub>1</sub>) generation were assessed via mating crosses between pairs from within treatment and between pairs from reciprocal crosses across treatment groups. Reproductively mature males and females, between 8 and 9 months of age, were separated into holding tanks, transferred into pairwise breeding tanks (500 mL) overnight to assess reproduction, and then returned to their respective separate-sex holding tanks and allowed 3 days recovery. Each pairwise tank held 1 male and 1 female either from within a treatment group (exposed male and exposed female) or across treatment groups (reciprocal cross: an exposed male and a control female or a control male and an exposed female). Fish were placed in a tank with an insert and divider to separate the sexes and kept overnight (12 h prior to breeding) at 28.5 °C. Before first light, dividers were removed and the pair was allowed to interact and breed for 1.5 h after onset of light; additional inserts at the bottom of the tank acted as a barrier between the zebrafish and the fertilized eggs to prevent clutch cannibalism. After 1.5 h of breeding, fish were returned to their holding tank and the clutch, if present, was collected and counted. Embryos were transferred into petri dishes containing E<sub>3</sub> media and assessed for viability at 6 hpf. In total, we made 75 pairwise within treatment crosses (CTL, CBZ and GEM) and reciprocal crosses between a F<sub>1</sub> CTL male + F<sub>1</sub> CBZ female; and a F<sub>1</sub> CTL male + F<sub>1</sub> GEM female. There was elevated aggression among the F<sub>1</sub> CBZ and GEM males that induced F<sub>1</sub> CTL female mortality thus, only 55 reciprocal crosses were completed for F<sub>1</sub> CBZ males with F<sub>1</sub> CTL females and 28 reciprocal crosses for F<sub>1</sub> GEM males with F<sub>1</sub> CTL females.

## 2.4. Courtship behaviour

To accurately assess courtship behaviours, pairwise breeding was set up as described above. Before the onset of light all pairwise tanks were covered to maintain darkness until they could be filmed. The covers and dividers separating the pair were removed and courtship behaviours recorded for 10 min on a Panasonic Lumix digital camera, mounted on a tripod. Once 10 min elapsed, the assessed animals were set aside until all pairs were observed.

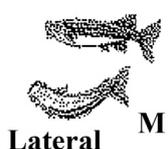
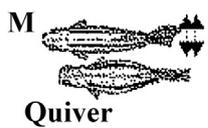
The videos were analyzed using VLC media player (v. 2.0.6, available at <http://www.videolan.org/vlc/index.html>) at 40% the original speed for total time (duration) and the number of occurrences (frequency) of characteristic male behaviours displays (Table 1). We focused on male behaviours because the male is more active during courtship and has a distinct set of behavioural displays that are used to entice the female to copulate. In contrast, the female does not display but simply accepts (follows) or declines (avoids) the male's displays (Cole and Smith, 1987; Darrow and Harris, 2004; Spence et al., 2008). The courtship behaviours were scored blind to the treatment group.

### 2.4.1. Sperm collection, velocity and morphology

Sperm samples were collected from CTL, CBZ and GEM F<sub>1</sub> males to assess sperm morphology and swimming velocity. To maximize the volume of sperm collected, all sperm extractions were carried out between 8:30 a.m. and 10:30 am, the peak zebrafish breeding times. To stimulate sperm production, males were held separately from females and then transferred into a tank with reproductively mature, ripe females for one day. The next morning the males were transferred individually into 0.6 mM tricaine solution buffered to pH 7.0 with 1 M Tris in clean fish water. Fish were anesthetized, removed and blotted with a kimwipe. The urogenital opening was

**Table 1**

Pictogram describing adult courtship behaviours amongst zebrafish. >The primary male courtship displays in zebrafish are depicted. The letter 'M' next to the fish denotes the male and the arrows show a typical path the male would take during a particular display. The images used were adapted from Darrow and Harris (2004).

Behavior	Description	Pictogram
Chasing	Rapid swimming movement directly toward the female terminating 2–3 cm before contact	 <b>Chase M</b>
Leading	Rapid straight line, zig-zag, or figure 8 pattern in front of or around the female attempting to lead her to a spawning site	 <b>Leading M</b>
Lateral display	Parallel positioning to the female and display of caudal fins	 <b>Lateral M</b>
Nudging	Contact with the snout to position the female in direction of the spawning site	 <b>Nudge M</b>
Quivering	Parallel positioning to the female delivering a series of small amplitude, but high frequency undulations against her body	 <b>Quiver M</b>

thoroughly dried to prevent premature activation of sperm. Males were placed belly up in a sponge holder and a 10  $\mu$ l microcapillary tube was placed on the urogenital opening. Using a pair of fine forceps, the abdomen was gently massaged, in the direction of the cloacae, while suction was applied to the microcapillary tube. On average, males yielded 1–3  $\mu$ l of good quality sperm, identified through its milky white appearance, where poor quality sperm was often translucent or transparent. Animals recovered from anesthesia in clean fish water, were returned to the holding tanks and given 10 days rest to recover before the second collection. Sperm was collected a second time so that both sperm velocity and morphology could be measured in each animal. Sperm collections were completed with 91% survival.

We measured the height of sperm collected within the capillary tube as an estimate of sperm volume and qualitatively assessed the sperm quality by the white colour and opaqueness of the sample; the whiter and more opaque a sample was, the better the yield. Following collection, sperm was diluted in 100–400  $\mu$ l of fish water. Dilution volume was determined based on both quality and volume of sperm collected. On average, a height up to 2 mm was diluted between 100 and 200  $\mu$ l and 2–5 mm was diluted in between 200 and 400  $\mu$ l. The entire sample was then expelled into a well slide (Fisher Scientific, Ottawa, Ontario), preplaced on a pre-focused Olympus CX41 light microscope (Olympus, America Inc., USA) and then covered with a coverslip. Video recording began a few seconds before the sperm was added to the well slide to ensure that the sperm was recorded at the earliest time possible. Video capture was done at 200 $\times$  magnification, under phase contrast, with a Prosilica EC-650 digital camera (Prosilica, Burnaby, British Columbia, Canada) mounted on the Olympus CX41 microscope (Olympus America Inc.). Videos were recorded using Astro IIDC (v. 4.04.00) software at 60 frames  $s^{-1}$ .

Sperm velocity was measured for 1 s at 20, 30, 40 and 50 s post activation (using a CASA (v.12) computer-assisted sperm

analysis system (Hamilton-Thorne Research, Beverly, Maine, USA) with parameters optimized for zebrafish sperm (Frames: 60 Hz, Contrast: 55, Cell size: 10, Optics magnification: 4.66). The researcher was blind to the treatment group when measuring sperm velocity. The median sperm velocity (VAP; median smooth path velocity) was calculated for all spermatozoa whose forward movement was traced for at least 0.33 s ( $\geq 20$  frames) at each time period after activation.

The second sperm sample collected was expelled into 1 mL of 10% neutral buffered formalin and thoroughly mixed to prevent clumping of spermatozoa. The samples were formalin fixed for 48 h. Ten microliters of fixed sperm was diluted 1:1 with nanopure water on a glass microscope slide, covered with a coverslip and examined under phase contrast at 400 $\times$  magnification using a Leica DMBL microscope (Leica Microsystems Inc., Buffalo, NY, USA). Using a mounted Prosilica EC-650 digital camera, pictures of 25 spermatozoa  $male^{-1}$  were taken and analysed to the nearest 0.1  $\mu$ m using National Institutes of Health's ImageJ software (v. 1.47j, available at <http://rsb.info.nih.gov/ij/>). Measurements of the sperm head length and width, mid-piece, mid-piece + flagellum were calculated by drawing a freehand line over each sperm section. Flagellum length was obtained by subtracting the length of midpiece from the combined mid-piece + flagellum measurement.

## 2.5. Histology

Methods for histological procedures were followed in accordance to (Galus et al., 2013b). Briefly, whole body (open body cavity) zebrafish were fixed in formalin for a minimum of 48 h and then rinsed and stored in ethanol until decalcified with Richard Allen De-cal (Fisher Scientific, Ottawa, ON) following the manufacturer's protocol. Decalcified samples were then embedded in paraffin, serially sectioned in the parasagittal plane at 5  $\mu$ m and stained with hematoxylin and eosin Y (Richard Allen). Histopathological scores

for each organ were assigned based on screening of at least 2 sections, in order to rule out staining artifacts and staining differences. The slides were read blind and the scores for histopathology in the organs were adapted from (Bernet et al., 1999), as outlined in Galus et al. (2013b).

## 2.6. Statistics

Normality of the data was determined using the Shapiro-Wilk's test. Differences in breeding success were analyzed using a series of Mann–Whitney rank sums tests, comparing each cross with the success of CTL pairs. Power analysis for clutch size was conducted using G\*Power 3 (Dusseldorf University). Data for the frequency and duration of courtship displays was not normal, thus data was log transformed and significant differences determined using one-way ANOVA. We compared the courtship frequency and duration in control pairs to the rates observed in the reciprocal and other within treatment pairs. Due to variations in samples size; embryo production, sperm morphology and velocity were analyzed using a Mann–Whitney rank sums test. Differences in embryo production were compared to the CTL pair and differences in sperm measurements were compared to CTLs at each time point; not across time points. Histological data was analyzed based on the percent incidence of histopathology and mean pathological score, within each exposure and analyzed using a student's *t* test. All statistical tests, except for the power analysis, were conducted using SigmaPlot 11.0 (Systat Software). Following statistical analysis, pairwise differences were determined using a Holm–Sidak post hoc test. Where applicable, all data was expressed as mean  $\pm$  SEM. The significance level was set at  $p \leq 0.05$ .

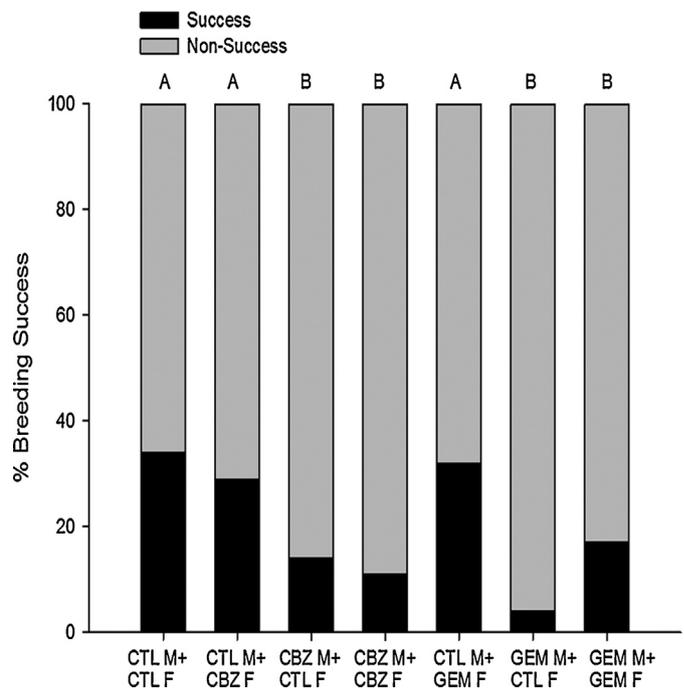
## 3. Results

### 3.1.1. Embryo production during pairwise and reciprocal crosses

Embryo production (i.e. viable embryos) was measured during 75 randomly paired breeding events for each within treatment group (i.e. both M and F from CTL, CBZ or GEM parents) and reciprocal cross (i.e. only one gender from exposed parents and the other from control parents); except those of F<sub>1</sub> CTL F + CBZ M and F<sub>1</sub> CTL F + GEM M. CBZ and GEM F<sub>1</sub> males were overly aggressive towards F<sub>1</sub> CTL females, repeatedly impacted the abdomen through snout or tail fin contact. After breeding, females were left with swollen and bruised bellies and often did not recover from these injuries. There was 38% mortality during these reciprocal crosses but mortality was 0% in the CTL pairwise crosses. Thus, we stopped the reciprocal cross matings of F<sub>1</sub> CTL F + CBZ M and F<sub>1</sub> CTL F + GEM M after 56 and 28 breeding events, respectively. We did not observe elevated aggression leading to an increased female mortality in the within treatment crosses.

There were significant differences in reproductive success in the within treatment crosses between control and both CBZ and GEM groups (Fig. 1). During matings involving F<sub>1</sub> CTL M + F<sub>1</sub> CTL F, a clutch was produced in 34% of pairs. Matings between F<sub>1</sub> CBZ M + F<sub>1</sub> CBZ F or F<sub>1</sub> GEM M + F<sub>1</sub> GEM F produced clutches in 11% and 17% of matings, respectively. Taking into account both the number and size of the clutches, the mean embryo production for each pairwise cross was determined and CBZ and GEM groups had lower fecundity than CTL animals (Fig. 2).

Reciprocal crosses were performed to isolate whether the decreased breeding with parental drug treatment was due to an effect on one or both genders. In these crosses, a F<sub>1</sub> CTL male was mated with a treated F<sub>1</sub> (CBZ or GEM) female or a treated F<sub>1</sub> (CBZ or GEM) male was mated with a F<sub>1</sub> CTL female. Breeding pairs involving CTL F<sub>1</sub> males had a similar fecundity to within CTL pairs (Fig. 2)



**Fig. 1.** Breeding success in F<sub>1</sub> zebrafish during pairwise and reciprocal matings. Male and female F<sub>1</sub> zebrafish were placed in pairwise tanks, within treatment and in reciprocal crosses. Pairs were given until 1.5 h after onset of light to breed; after which the presence or absence of a clutch was noted. Breeding success was based on 75 pairwise crosses for each within and across mating except for reciprocal crosses with CBZ and GEM males. CBZ and GEM males were aggressive and induced female mortality when paired with control females and only 55 and 28 reciprocal crosses were completed for CBZ male and control female and GEM male and control female groups, respectively. Significant differences were determined using a Mann–Whitney rank sums test. Letters denote significant differences from the within CTL pair.  $p \leq 0.05$ . CBZ is carbamazepine, GEM is gemfibrozil, CTL is control, M is male and F is female.

and there was no statistical difference between breeding success for F<sub>1</sub> CTL male + F<sub>1</sub> CTL female, F<sub>1</sub> CTL male + F<sub>1</sub> GEM female or F<sub>1</sub> CBZ female (Fig. 1). Pairs involving a F<sub>1</sub> CBZ or F<sub>1</sub> GEM males had significantly lower fecundity than those involving a F<sub>1</sub> CTL male (Fig. 2). Reciprocal crosses with either F<sub>1</sub> CBZ or F<sub>1</sub> GEM males mated with F<sub>1</sub> CTL females produced significantly fewer clutches (Fig. 1).

For each pair, the clutch size (i.e. number of embryos produced in a single breeding event) was determined. Clutch sizes (Table 2) were highly variable and mean clutch sizes for matings with CTL F<sub>1</sub> males and females was  $102 \pm 72$  (mean  $\pm$  SD). There were no statistical differences in clutch size across the within treatment or reciprocal crosses. We conducted a power analysis using the variation in clutch sizes in our experiments and determined that 154 clutches would be needed in order to identify if there were differences in clutch size amongst treatment groups. Taking into consideration the breeding success in our crosses, 452 crosses would be required to produce this number of clutches in the crosses with CTL males and females.

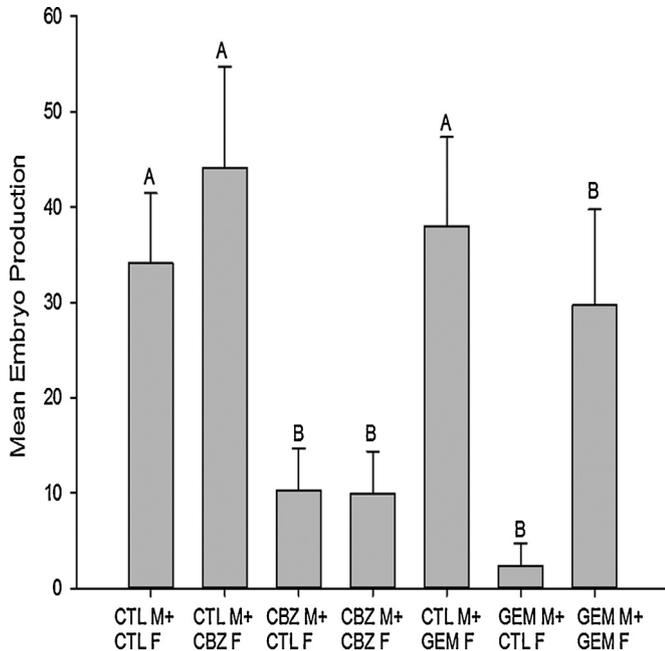
### 3.2. Courtship behaviours

As described above, zebrafish courtship is almost exclusively a male activity (Darrow and Harris, 2004). The male uses a series of distinct identifiable displays towards gravid females to lead them to a spawning site. All our control F<sub>1</sub> males courted in a typical zebrafish fashion beginning with an approach followed by leading and nudging the female and then performed several figure eight or circle displays with fins raised and then this series would culminate with a quiver, a behaviour linked to sperm release before the sequence would begin again (Spence et al.,

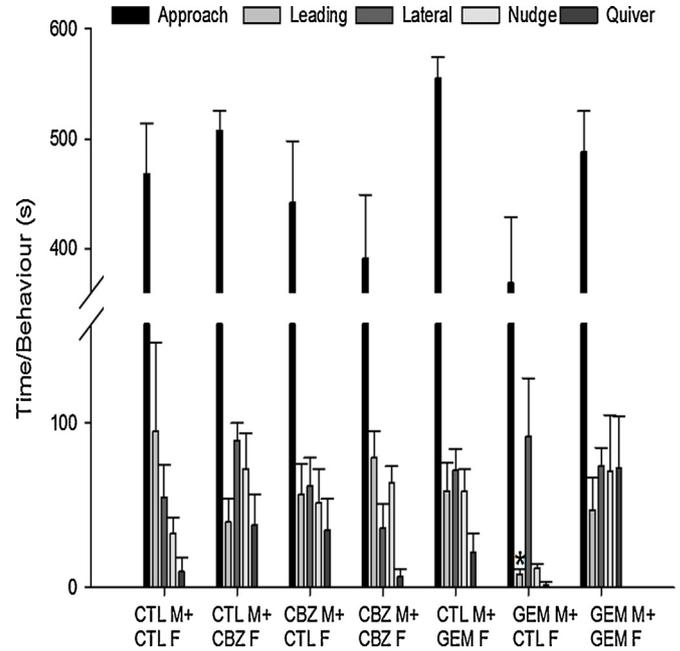
**Table 2**

Mean clutch size in F<sub>1</sub> zebrafish during pairwise and reciprocal matings. >Male and female F<sub>1</sub> zebrafish were placed in pairwise tanks, within treatment and in reciprocal crosses. Pairs were given until 1.5 h after onset of light to breed; after which the presence or absence of a clutch was noted. There was no variation amongst clutch size between pairs. CBZ is carbamazepine, GEM is gemfibrozil, CTL is control, M is male and F is female. The pairing of GEM M+CTL F produced a single clutch in all 75 breeding events, therefore the standard deviation (SD) is not available (NA).

	CTL M+CTL F	CTL M+CBZ F	CBZ M+CTL F	CBZ M+CBZ F	CTL M+GEM F	GEM M+CTL F	GEM M+GEM F
Mean Clutch ± SD	102 ± 72	155 ± 113	73 ± 58	93 ± 82	122 ± 108	68 ± NA	171 ± 144



**Fig. 2.** Mean embryo production in F<sub>1</sub> zebrafish during within treatment and reciprocal matings. Male and female F<sub>1</sub> zebrafish were placed in pairwise tanks, within treatment and in reciprocal crosses. Pairs were given until 1.5 h after onset of light to breed; after which embryos were collected and counted. Mean embryo production was based on 75 pairwise crosses for each within and across mating except for reciprocal crosses with CBZ and GEM males. CBZ and GEM males were aggressive and induced female mortality when paired with control females and only 55 and 28 reciprocal crosses were completed for CBZ male and control female and GEM male and control female groups, respectively. Significant differences were determined using a Mann–Whitney rank sums test. Letters denote significant differences from the within CTL pair.  $p \leq 0.05$ . CBZ is carbamazepine, GEM is gemfibrozil, CTL is control, M is male and F is female.



**Fig. 3.** Duration of male courtship behaviours during F<sub>1</sub> mating. Male and female F<sub>1</sub> zebrafish were placed in pairwise tanks, within treatment and in reciprocal crosses and maintained in the dark with a tank divider. Courtship behaviours were recorded immediately after removing divider at first light on a Panasonic Lumix digital camera for 10 min. The videos were then analyzed for the amount of time (in seconds) characteristic male behaviours were displayed. The behaviours examined included approach, leading, lateral, nudge and quiver (Darrow and Harris, 2004). See Table 1 for a description and pictogram of the behaviours. A one-way ANOVA was conducted on log transformed data and significant differences between behaviours were across all treatments for individual behaviours only. When comparing across treatments, the asterisk denotes difference in that behaviour compared to that seen in the CTL crosses.  $p \leq 0.05$ . CBZ is carbamazepine, GEM is gemfibrozil, CTL is control, M is male and F is female.

2008; Stoltz and Neff, 2006). The most common display for all males was approach, followed by lateral displays, then leading behaviour, quivers were performed most infrequently (Fig. 4 and Table 3). Control F<sub>1</sub> males paired with exposed females had completely typical behaviour in terms of both duration and frequency of courtship behaviour. Interestingly, differences were observed when we considered the treated male crosses (Figs. 3 and 4). Gemfibrozil (GEM) exposed males paired with control females spent less time leading females but approached and quivered with them more frequently than control F<sub>1</sub> males (Fig. 4). Males in carbamazepine (CBZ) exposed pairs had more lateral displays but lower nudge rates compared to males in control pairings. No other behavioural differences were detected.

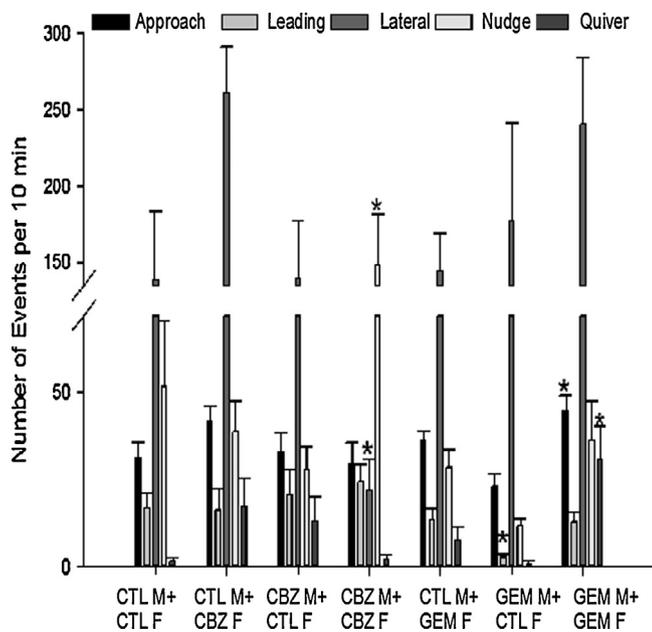
**Table 3**

Differences in duration and frequency of breeding behaviours within CTL pairs. Male and female F<sub>1</sub> zebrafish were placed in pairwise tanks maintained in the dark with a tank divider. See Table 1 for a description and pictogram of the behaviours. A one-way ANOVA was conducted on log transformed data and significant differences between behaviours were analyzed within the CTL pair. Behaviours that share a common letter are not statistically different from one another.  $p \leq 0.05$ . CTL is control, M is male and F is female. Data is represented as mean ± standard deviation (SD).

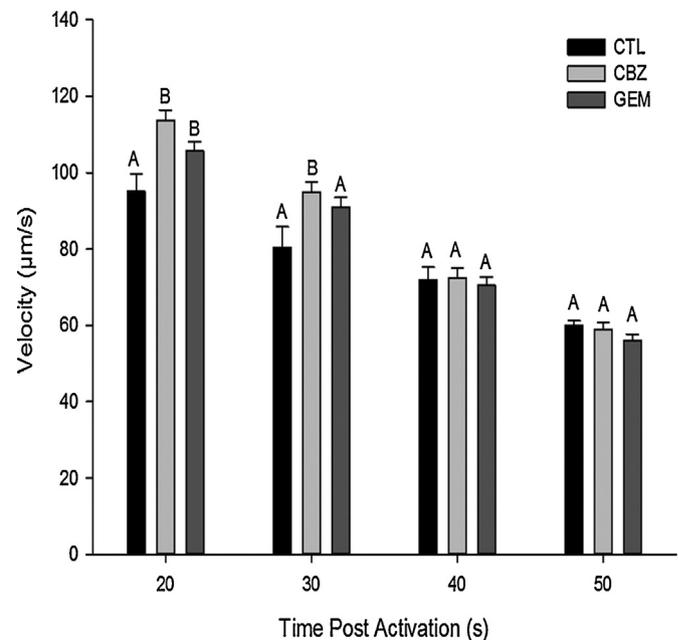
CTL M+CTL F	Approach	Leading	Lateral	Nudge	Quiver
Time	468 ± 144 <sup>A</sup>	96 ± 169 <sup>BC</sup>	55 ± 62 <sup>CD</sup>	32 ± 31 <sup>CD</sup>	9 ± 26 <sup>D</sup>
Frequency	31 ± 14 <sup>AC</sup>	17 ± 13 <sup>AB</sup>	138 ± 141 <sup>C</sup>	51 ± 59 <sup>AC</sup>	1.7 ± 3 <sup>D</sup>

3.2.1. Sperm velocity and morphology

Sperm velocity was measured over a 1 s duration at 20, 30, 40 and 50 s post activation (time after activating spermatozoa with tank water). In all treatment groups, mean sperm velocity decreased by approximately 40–50% over the 50 s (Fig. 5). At 20 and 30 s post activation, the sperm of CBZ F<sub>1</sub> males was significantly faster than sperm of control F<sub>1</sub> males (Fig. 5). GEM F<sub>1</sub> males had sperm that swam significantly faster than sperm from F<sub>1</sub> CTL males at 20 s post activation (Fig. 5). Interestingly, the faster sperm from F<sub>1</sub> CBZ males possessed significantly longer mid-pieces than those measured from F<sub>1</sub> CTL male sperm. However, the faster swimming sperm from F<sub>1</sub> GEM swam faster despite having significantly



**Fig. 4.** Frequency of male Courtship behaviours during F<sub>1</sub> mating. Male and female F<sub>1</sub> zebrafish were placed in pairwise tanks, within treatment and in reciprocal crosses and maintained in the dark with a tank divider. Courtship behaviours were recorded immediately after removing divider at first light on a Panasonic Lumix digital camera for 10 min. The videos were then analyzed for the number characteristic male behaviours displayed during the 10 min of breeding. The behaviours examined included approach, leading, lateral, nudge and quiver (Darrow and Harris, 2004). See Table 1 for a description and pictogram of the behaviours. A one-way ANOVA was conducted on log transformed data and significant differences between behaviours were across all treatments for individual behaviours only. When comparing across treatments, the asterisk denotes difference in that behaviour compared to that seen in the CTL crosses.  $p \leq 0.05$ . CBZ is carbamazepine, GEM is gemfibrozil, CTL is control, M is male and F is female.



**Fig. 5.** Sperm velocity in male F<sub>1</sub> zebrafish. Smooth path velocity ( $V_{AP}$ ) of sperm from F<sub>1</sub> males raised in clean water from control (CTL) parents or parents chronically exposed to carbamazepine (CBZ) or gemfibrozil (GEM) for 6 weeks. Sperm was removed from F<sub>1</sub> male zebrafish via abdominal massage and expelled on a glass, wetted slide. Sperm was then activated with system water (see Section 2), covered with a coverslip and recorded under 200 $\times$  magnification using Astro IICD recording software.  $V_{AP}$  was calculated for each male at 4 different 1 s intervals; 20, 30, 40 and 50 s using CEROS computer assisted sperm analysis system. Significant differences were determined using a Mann–Whitney rank sums test. Letters denote significant differences within each time interval.  $p \leq 0.05$ . CBZ is carbamazepine, GEM is gemfibrozil, and CTL is control.

shorter mid-piece than F<sub>1</sub> CTL sperm (Fig. 6). Other morphological differences were detected in the sperm of F<sub>1</sub> GEM and CBZ males. The sperm of F<sub>1</sub> GEM zebrafish had a significantly smaller head than sperm from control males, whereas CBZ F<sub>1</sub> zebrafish sperm had longer midpieces than F<sub>1</sub> CTL males (Fig. 6).

### 3.3. Histology

Male and female F<sub>1</sub> zebrafish, raised from parents chronically exposed to 10  $\mu\text{g L}^{-1}$  of CBZ and GEM were screened for histopathological damage in the kidneys, liver and reproductive organs. Animals from all treatments and sexes had no histological alterations in any organ examined (data not shown).

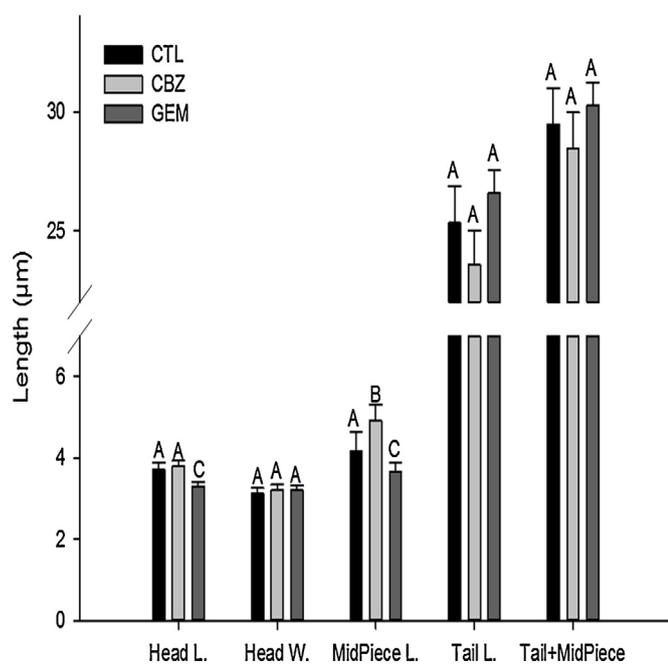
## 4. Discussion

Exposure of adult zebrafish to 10  $\mu\text{g L}^{-1}$  CBZ and GEM caused a significant decline in fecundity (i.e. total embryos produced) with 6 weeks exposure (data not shown). This agrees with our previous research, where 6 weeks exposure to GEM and CBZ at 10 and 0.5  $\mu\text{g L}^{-1}$  reduced fecundity compared to unexposed fish (Galus et al., 2013b). In that study, CBZ and GEM not only reduced reproduction but exposed female zebrafish had elevated numbers of atretic oocytes and increased TUNEL positive staining, which indicated elevated apoptosis, in the ovaries (Galus et al., 2013b). This research suggested that CBZ and GEM had a direct effect on oocyte production (Galus et al., 2013b). Zebrafish exposed to 0.5 and 10  $\mu\text{g L}^{-1}$  CBZ had decreased 11-ketotestosterone (11-KT) levels suggesting altered sex steroid hormones levels (Galus et al., 2013b). Thus, exposure of adult fish appeared to impact zebrafish

reproduction with direct effects on gonadal function and steroid levels (Galus et al., 2013b).

In our previous studies, parental exposure of zebrafish to either 0.5 and 10  $\mu\text{g L}^{-1}$  CBZ and GEM did not impact abnormality, mortality or hatching rate of the offspring during embryogenesis (up to 96 hpf), when reared in clean water after parental exposure (Galus et al., 2013b). In agreement with that data, we did not observe any alterations in abnormality, mortality or hatching rate of the offspring from parents exposed to 10  $\mu\text{g L}^{-1}$  CBZ or GEM, when reared in clean water (data not shown). In our current study, we continued to rear the F<sub>1</sub> offspring in clean water to sexual maturity to assess whether parental exposure impacted reproduction in F<sub>1</sub> offspring at sexual maturity.

To our knowledge, this is the first study to assess whether parental exposure to pharmaceuticals was sufficient to cause reproductive impacts in F<sub>1</sub> offspring that were not directly exposed to the compounds in any fish species. Traditional life cycle assessments either evoke a cradle-to-grave or cradle-to-gate approach (EPA, 1993). In both schemes, exposure is often from the onset of fertilization until terminal sampling (full life cycle) or until 24 h past sexual differentiation (partial life cycle) (EPA, 1993). Generally, life cycle tests are conducted to evaluate potential chronic effects of chemicals on fish populations (Benoit, 1982). This study (Benoit, 1982), and other life cycle assessments, gives emphasis to potential population effects, mainly focusing on impacts relating to survival, developmental and reproduction, with the end goal often to determine a margin of safety or the No Observed Effect Concentration (NOEC). These kinds of studies often start exposure with F<sub>0</sub> (fertilized embryos) and end with F<sub>1</sub> fry or juveniles. Full life cycle assessments that aim to explain multi- or trans-generational effects typically expose and assess offspring for several generations (>F<sub>2</sub>) (EPA, 1993; OECD, 2002). In these kinds of tests, exposure



**Fig. 6.** Sperm Morphology in male  $F_1$  zebrafish. Sperm head length (L) and width (W.), midpiece and flagella length of  $F_1$  zebrafish males raised in clean water, from control (CTL) parents or parents chronically exposed to carbamazepine (CBZ) or gemfibrozil (GEM) for 6 weeks. Sperm was removed from male  $F_1$  zebrafish via abdominal massage and fixed in 10% neutral buffered formalin. 10  $\mu$ l of spermatozoa was diluted 1:1 with distilled water and placed on a glass microscope slide and screened at 400 $\times$  magnification using a Prosilica EC-650 digital camera mounted on a Leica DMBL microscope and analyzed under phase contrast. Images of 25 spermatozoa per male were taken and measured to the nearest 0.1  $\mu$ m using ImageJ. Significant differences were determined using a Mann–Whitney rank sums test. Letters denote significant differences with each time interval.  $p \leq 0.05$ . CBZ is carbamazepine, GEM is gemfibrozil, and CTL is control.

starts with reproducing  $F_0$  and continues until the  $F_2$  are past sexual maturity (OECD, 2002). With multi-generational assessments, exposure impacts to the parents are recorded, however the focus is on effects with developmental and sexual differentiation to help understand how exposure can lead to multi-generational impacts (EPA, 1993). We have tested direct exposure of both CBZ and GEM on zebrafish embryogenesis and found that exposure to 0.5 and 10  $\mu$ g  $L^{-1}$  GEM and 0.5  $\mu$ g  $L^{-1}$  CBZ caused increased mortality, but did not affect abnormality rates (Galus et al., 2013b). We have not reared these animals past 96 h post fertilization and cannot comment on any persistent impacts from direct embryonic exposure. Parrott and Bennie (2009) however did conduct a full life cycle assessment of 0.01 to 1  $\mu$ g  $L^{-1}$  of naproxen, gemfibrozil, diclofenac, ibuprophen, triclosan, salicylic acid, and acetaminophen in fathead minnow (*Pimephales promelas*) and observed no impacts in  $F_1$  or  $F_2$  offspring in that species.

Most traditional life cycle assessments provide continuous exposure of organisms throughout the experiment, typically from the onset of fertilization. Our methods differed from conventional life cycle assessment as the  $F_0$  fish were adults and were exposed for only 4–6 weeks,  $F_1$  offspring were immediately collected, and once the embryos were removed from the parental tank, the offspring were only held in clean water. Therefore the  $F_1$  offspring in our study would have been exposed to aqueous GEM or CBZ for a maximum of 1.5 h; enough time for the embryo to progress to the 16-cell stage maximum and on average embryonic exposure was less than 1.5 h. There are three ways by which drug exposure may have impacted the  $F_1$  offspring. First, the parental exposure may have altered the quality of egg and/or sperm. Second, maternal deposition of drug may have resulted in exposure during

embryogenesis. Third, embryos may have taken up aqueous drugs (while in the egg trap) immediately after being laid (but prior to the trap being removed from the parental tank). Although no uptake data is available for embryonic fish pertaining to CBZ and GEM, given the aqueous drug concentration, their bioconcentration factors and the small exposure window, we estimate that only a fraction of the compound would have been able to enter the embryo, and any effects are most likely attributable to parental exposures alone or due to maternal deposition in the egg. Maternal deposition of contaminants into eggs is known for lipophilic contaminants such as polychlorinated biphenyls (Westerlund et al., 2000) and organochlorines (Fisk and Johnston, 1998; Russell et al., 1998) however there are no studies that examine the deposition of pharmaceuticals into fish eggs. Both GEM and CBZ are lipophilic compounds, therefore it is possible that they could be deposited into the oocyte during vitellogenesis and production of yolk. However, as we did not measure GEM or CBZ concentration in the embryos, this route of embryonic exposure cannot be ruled out.

#### 4.1.1. Breeding success and embryo production

Our strategy for assessing reproductive effects in  $F_1$  offspring was to first cross animals within treatment groups, i.e. male and female from control treatment or male and female from a single parental drug (CBZ or GEM) treatment. Out of 75 pairwise mating events, the reproductive success of the CTL pair was 34% (Fig. 1). Zebrafish are asynchronous batch spawners, where an individual female does not spawn on daily basis, even if conditions are favorable (Breder and Rosen, 1966). On average, a female will spawn once every 1.9 days and even though the spawning cycle may be routine, the clutch size can be highly variable ranging from 1 to 200 embryos (Eaton and Farley, 1974). In order to ensure our fish were in optimal breeding conditions, both males and females were fed to satiation three times a day with both commercial flake and live (artemia) foods, as well as separated from the opposite sex before the breeding trials to allow investment of nutrients to sperm and ovarian growth. Food quantity and quality is highly important in the reproductive output of fish (Bagenal, 1969). We typically fed 3 times a day but have a reduced feeding schedule (twice a day) on weekends. Fish in our facility breed less frequently at the beginning of the week (unpublished data), and we assume this is due to lowered weekend feedings. In this study, we completed pairwise breedings every day of the week and included days that were likely impacted by our feeding schedule to ensure we could complete large numbers of pairs within a reasonable time frame. Pairwise breeding success in our facility in unexposed, adult animals is typically 35–48%, and seems to depend on the batch and age of fish, days of the week we breed, and feeding schedule (unpublished data, J.Y. Wilson). Fish whose parents were exposed to CBZ and GEM had significantly lower breeding success (Fig. 1) and fecundity (Fig. 2), compared to controls. In all pairwise and reciprocal matings involving  $F_1$  males from exposed parents, reproductive success was below 15% (Fig. 1) and mean fecundity was reduced by at least 50% (Fig. 2) when compared to the control pairs. Yet, the reciprocal crosses involving control males with females whose parents had drug exposure were not different from unexposed parents control crosses, in terms of either breeding success (Fig. 1) or fecundity (Fig. 2). The consistent low level of reproductive success and mean embryo production, in matings involving males from exposed parents but not females from exposed parents, suggest that parental exposure impacted males more than females.

The clutch of a female zebrafish can be highly variable ranging from 1 to 200 embryos (Eaton and Farley, 1974). Mean clutch sizes were not different between offspring from exposed parents and control offspring with unexposed parents (Table 2). However

given the small number of clutches produced from the crosses involving males from treatment groups, there was too little statistical power needed to distinguish any potential effects. Based on the variance in clutch size observed in our experiment, a power analysis was conducted and revealed that 154 clutches would have been needed in order to uncover differences across treatment groups. Given rather low reproductive success observed in this study (a maximum around 30%), we would have needed to conduct at least 452 crosses to produce 154 clutches. It is therefore extraordinarily difficult to assess impacts on clutch size and clutch size is likely a difficult endpoint in zebrafish with reduced fecundity.

Interestingly, in the F<sub>1</sub> GEM M + CTL F reciprocal cross, the GEM male was only able to stimulate the female to successfully produce a clutch once out of 28 attempted mating events. In this treatment, along with CBZ male + CTL female, we had to terminate our pairwise matings prior to completing the 75 crosses achieved in other treatment groups due to an increase in male aggression that resulted in serious female injury and mortality. Mate choice amongst female zebrafish may be linked to olfactory cues as Gerlach (2006) has shown that females exposed to male pheromones produce 17% more viable embryos than unexposed females. Although the exact pheromones involved are unknown, the male derived F prostaglandin is known to stimulate follicular ripeness in goldfish (Sorensen et al., 1995) and other teleosts (Goetz and Garczynski, 1997). In order for successful reproduction to occur, both males and females need to find a sexually mature individual of the opposite sex possibly through the use of pheromonal cues, and a sequence of courtship displays must be performed to ensure successful clutch production (Gerlach, 2006). Considering the increased aggression identified with the males from the treatment groups and the fact that courtship behaviour is primarily completed by male zebrafish, courtship or breeding behaviour was investigated.

#### 4.2. Courtship behaviour

Courtship behaviour amongst zebrafish is primarily conducted by males alone who display actively to stimulate the female to release a clutch (Darrow and Harris, 2004). Females will accept or decline the male courtship display but do not appear to alter their behaviour or produce any courtship behaviour of their own (Darrow and Harris, 2004). There are five characteristic behaviours previously identified in courtship and reproduction of zebrafish; approach, leading, lateral, nudge and quiver (Darrow and Harris, 2004). Male zebrafish perform this series of identifiable behaviours often starting with an interaction display (approach) and ending with quiver; as this behaviour is linked to release of sperm (Darrow and Harris, 2004). It is not well known if the behaviours must occur in a specific order (Darrow and Harris, 2004). We did notice that control F<sub>1</sub> males always initiated courtship by approaching and then leading the female, before engaging in physical contact behaviours such as nudges. During pairwise and reciprocal crosses, males were analyzed for both the duration (total time spent; Fig. 3 and Table 3) and frequency (number of individual displays; Fig. 4 and Table 3) of the five characteristic male behaviours in the initial 10 min after first light. Darrow and Harris (2004) have extensively examined zebrafish courtship displays and have shown that egg laying peaks occur within the first 10 min of courtship and infrequently beyond 30 min. Although zebrafish can engage in courtship behaviours and clutch laying beyond the first 10 min, these activities sharply decline at 30 min post first light, and occur seldom beyond 70 min (Darrow and Harris, 2004). By recording the most robust time in which zebrafish are actively courting, we should have captured most of the breeding displays that would occur between pairs.

While courtship displays have been qualitatively described, to our knowledge, no study has documented the frequency or duration of specific behaviours during courtship. Yet, this is clearly needed to determine how exposure/treatment affects courtship behaviour. Males spent significantly more time approaching the female than any other behaviour (Table 3); control males had a lower frequency of quiver compared to any other behaviour (Table 3). While quantitative studies of courtship behaviour are not established for zebrafish, the characteristic courtship behaviours in other fish species have been well documented (Breder and Rosen, 1966; Cole and Smith, 1987; Darrow and Harris, 2004). Based on the qualitative research in zebrafish and quantitative research in other fish species, we can conclude that the control animals within our study were displaying typical courtship behaviours.

Successful reproduction requires a suite of behaviours and interactions ranging from spawning site selection, to courtship and post natal investment (Potts, 1984). Toxicant exposure could potentially interfere with the order or timing of appropriate reproductive behaviours and disrupt mating. In our study, GEM males were observed to spend less time in leading, but invested a higher frequency in approached and quivered at higher frequencies compared to control pairings. CBZ males performed more lateral displays, but fewer nudges when compared to control males. Similar effects have been reported in Japanese medaka (*Oryzias latipes*) that were exposed to 25 µg L<sup>-1</sup> of 4-*tert*-octyphenol until 6 months post fertilization and showed a decreased frequency of approach and leading (Gray et al., 1999). Spawning goldfish (*Carassius auratus*) (Bjerselius et al., 2001) and adult Japanese medaka (Oshima et al., 2003) exposed to 1 µg L<sup>-1</sup> of 17β-estradiol for 28 days, both showed reduced frequency of leading and nudge. Long term exposure to estrogens has the ability to alter circulating levels of other hormones, including those which are key for normal reproduction (Nash et al., 2004). Bell (2001) examined the effects of estrogenic compounds on the courtship behaviour of stickleback (*Gasterosteus aculeatus*) and found a positive correlation between courtship behaviour and breeding success. Collectively, these studies suggest that estrogenic compounds can impact normal courtship behaviour; and in our study the pharmaceuticals CBZ and GEM appeared to impact male courtship in F<sub>1</sub> offspring. Interestingly, CBZ has been shown to alter hormone levels in adult fish (Galus et al., 2013b; Li et al., 2010). Although the exact mechanism impacting the duration or frequency of spawning behaviours observed in our study is unknown, it is possible that altered steroidogenesis was the mechanism. We did not have sufficient numbers of animals to verify if parental exposure altered hormone levels in F<sub>1</sub> offspring.

##### 4.2.1. Sperm morphology and velocity

The relationship between spermatozoa form and function has been heavily debated and studied in a broad range of species (Gage et al., 2002; Malo et al., 2006; Parker, 1993; Snook, 2005). Some of the above studies suggest that the morphology of the sperm is not always indicative of its velocity or swimming performance. However, sperm morphology and velocity have been shown to be good indicators of male fertilization rates (Casselman et al., 2006). The sperm of GEM F<sub>1</sub> males was found to have a shorter head and mid-piece length (Fig. 5), whereas CBZ F<sub>1</sub> males had a larger mid-piece when compared to sperm from control males (Fig. 5). We also analyzed sperm velocity and both GEM and CBZ sperm swam faster at 20 s post activation; CBZ was also faster 30 s post activation compared to CTLs (Fig. 6). Despite there being conflicting points of view whether sperm form is linked to function, the changes observed in GEM and CBZ F<sub>1</sub> morphology could explain the alterations observed in velocity.

The spermatozoa of CBZ males possessed a larger midpiece (Fig. 5) than that of CTL sperm. The midpiece is the region of sperm which is rich in mitochondria and produces all the ATP needed

for spermatozoa propulsion (Ruiz-Pesini et al., 1998). It is plausible that with a larger midpiece, there was a higher abundance of mitochondria which in turn were able to produce a higher proportion of ATP resulting in the increased swimming speed observed over CTL sperm (Fig. 6). In a comparative study between the sperm of many species of New World blackbirds, it was found that spermatozoa with a larger midpiece had a higher swimming velocity over those with a smaller midpiece (Lüpold et al., 2009). To our knowledge, the impacts of CBZ exposure on fish sperm have not been studied before. Humans treated with therapeutic doses of CBZ show similar changes in sperm morphology (Isojärvi et al., 2004) and velocity (Pack, 2005) to those that were observed in our study.

Spermatozoa of GEM males had a shorter midpiece along with smaller head length than CTL animals (Fig. 5). As discussed above, the midpiece is the portion of the sperm which contains the energy to propel the sperm forward. Although GEM sperm had a shorter midpiece than CTL sperm, they possessed a higher swimming velocity at 20 s post activation (Fig. 6). In theory, it may seem counterintuitive for a sperm with a reduced midpiece to outperform a sperm with a larger midpiece, however GEM sperm also possessed a smaller head length than that of CTL sperm (Fig. 5). It is plausible that the morphological changes to midpiece, coupled with those of the head, could have reduced the surface area of the spermatozoa, in turn reducing the resistance associated with swimming and therefore contributed to faster swimming speed observed (Figs. 5 and 6). Although there are no studies which definitively correlate multiple changes in sperm morphology to swimming speed, it has been shown that a smaller head:flagellum ratio results in an overall increase in sperm velocity amongst passerine birds (Lüpold et al., 2009). Work with clofibrac acid, a drug with a similar mode of action to GEM, has shown that exposure of fathead minnows to 1000 µg L<sup>-1</sup> reduces sperm count and velocity (Runnalls et al., 2007). Much like clofibrac acid, GEM modulates androgens, and spermatogenesis is a heavily androgen dependant process (Schulz et al., 2001).

Alterations in sperm morphology and velocity may suggest impacts on embryo fertilization rate as well, yet our data does not necessarily suggest this. We did not see an increase in unfertilized embryos in crosses with CBZ or GEM F<sub>1</sub> males. Indeed, most of the embryos were fertilized. Warner (1997) suggests that small fish species do not release excessive amounts of sperm during courtship, therefore any alterations in sperm quality or quantity could directly impact fertilization rates. In our study, breeding success was lowered in those crosses with CBZ and GEM F<sub>1</sub> males but fertilization rates were not altered. It may be that larger numbers of clutches are needed to document subtle differences in fertilization success.

Although the exact mechanism describing how parental exposure can impact the physiology in offspring is unknown, it is possible that chronic pharmaceutical exposure may result in permanent changes to the target cells, allowing the effects of the drug to persist long after the exposure has stopped (Csoka and Szyf, 2009). Maternal deposition into the egg (Fisk and Johnston, 1998; Russell et al., 1998) is a possible mechanism for ensuring persistent exposure; after the source contaminant has been removed. Transcriptional changes (Nakari and Erkomaa, 2003), or DNA methylation (Mhanni and McGowan, 2004), as a result of the embryonic exposure or during gamete production are possible mechanisms that parental exposure could result in F<sub>1</sub> offspring effects. However, which process and which target cells are impacted are dependent on mechanism of action of the specific pharmaceutical. We have not yet assessed transcriptional changes in zebrafish embryos or parental gamete cells, after CBZ and GEM exposure. Transcriptomic studies will be necessary to understand if transcriptional changes are present and persistent to sexual maturity in F<sub>1</sub> offspring.

## 5. Conclusion

The results of this study demonstrate that chronic exposure of adult zebrafish to GEM and CBZ is sufficient to reduce breeding success and fecundity, alter courtship behaviours and sperm morphology and velocity in F<sub>1</sub> offspring reared in clean water. Thus, parental exposure of fish to pharmaceuticals may be sufficient to cause reproductive effects even if offspring are not environmentally exposed to the compound themselves. F<sub>1</sub> offspring from exposed parents showed reduced reproductive output but this effect appeared to be primarily related to males. Females from exposed parents were successful at breeding with control males. Male courtship displays were impacted by parental exposure to both CBZ and GEM, suggesting that males from exposed parents were not able to successfully court a female. Finally, sperm morphology and velocity was impacted by parental exposure to CBZ and GEM. Although we are unable to describe the mechanism of action behind these effects, we have shown that chronic parental exposure is sufficient enough to impact reproductive systems in offspring. Future research is now needed to explore hormone levels in F<sub>1</sub> offspring and to examine gene expression changes in brains and gonads of male F<sub>1</sub> offspring. Such results will help elucidate the mechanism by which parental GEM and CBZ exposures selectively impact male offspring.

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