RESEARCH ARTICLE

Paternal exposure to a common herbicide alters the behavior and serotonergic system of zebrafish offspring

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Abstract

Increasingly, studies are revealing that endocrine disrupting chemicals (EDCs) can alter animal behavior. Early life exposure to EDCs may permanently alter phenotypes through to adulthood. In addition, the effects of EDCs may not be isolated to a single generation − offspring may indirectly be impacted, via non-genetic processes. Here, we analyzed the effects of paternal atrazine exposure on behavioral traits (distance moved, exploration, bottom-dwelling time, latency to enter the top zone, and interaction with a mirror) and whole-brain mRNA of genes involved in the serotonergic system regulation (*slc6a4a*, *slc6a4b*, *htr1Aa*, *htr1B*, *htr2B*) of zebrafish (*Danio rerio*). F0 male zebraFIsh were exposed to atrazine at 0.3, 3 or 30 part per billion (ppb) during early juvenile development, the behavior of F1 progeny was tested at adulthood, and the effect of 0.3 ppb atrazine treatment on mRNA transcription was quantified. Paternal exposure to atrazine significantly reduced interactions with a mirror (a proxy for aggression) and altered the latency to enter the top zone of a tank in unexposed F1 offspring. Bottom-dwelling time (a proxy for anxiety) also appeared to be somewhat affected, and activity (distance moved) was reduced in the context of aggression. *slc6a4a* and *htr1Aa* mRNA transcript levels were found to correlate positively with anxiety levels in controls, but we found that this relationship was disrupted in the 0.3 ppb atrazine treatment group. Overall, paternal atrazine exposure resulted in alterations across a variety of behavioral traits and showed signs of serotonergic system dysregulation, demonstrating intergenerational effects. Further research is needed to explore transgenerational effects on behavior and possible mechanisms underpinning behavioral effects.

Introduction

Exposure to endocrine and neuroendocrine disrupting chemicals (EDCs) are reported to induce a variety of aberrant behaviors (see reviews: [1,2]), particularly when individuals are exposed early in life [3–8]. EDCs interfere with the molecular mechanisms that underpin behavior, such as gene expression, hormone levels, neurotransmitter levels, and the molecular machinery that mediates between these inputs [9–15]. The effects of EDC exposures are

predominantly implicated in the disruption of typical reproductive behaviors (i.e. courtship and parental behavior; [2,3,16]), but non-reproductive behaviors, such as anxiety, aggression and risk-taking behavior also appear to be strongly affected [4,17–22,22–25]).

Increasingly, studies are revealing that the effects of EDCs may not be isolated to a single generation–offspring may indirectly be impacted through their parents via non-genetic processes (i.e. the transmission of parental phenotypic or environmental variation to offspring that do not stem from changes in DNA sequence; [4,6,25–29]). For example, female F3 descendants of mice embryonically exposed to the EDC, vinclozolin, prefer males without a history of exposure [27], and parental exposure to bisphenol A in medaka (*Oryzias latipes*) result in larval offspring with reduced locomotory behavior [30]. Maternal contribution to phenotypic variation via non-genetic inheritance is well documented (e.g. the transfer of maternal glucocorticoids to developing eggs [31–34] or maternal care; [35–40]). For instance, female sticklebacks (*Gasterosteus aculeatus*) exposed to predation risk produced eggs that were larger and had elevated cortisol levels, moreover, their juvenile offspring exhibited tighter shoaling behavior [33]. However, the understanding of the paternal contribution is more limited. Recent work is recognizing that sperm can be an important source contributing to non-genetic inheritance [41,42]. For example, offspring sired by zebrafish males experiencing increased sperm competition exhibit faster hatching rates, but reduced survival [43], and learned fear responses can be inherited via sperm in mice [41]. Furthermore, Anway et al. [44] found that transgenerational exposure of vinclozolin (through the male-line), results in increased incidents of infertility (up to four generations) in rats.

Non-genetic mechanisms are also hypothesized to contribute to animal behavior differences [45–47]. Thus, the behavioral phenotype of unexposed descendants, in combination with EDC exposure, may be detrimentally impacted. For instance, parental or transgenerational exposure to 17α-ethinylestradiol in zebrafish (*Danio rerio*) and guppies (*Poecilia reticulata*) results in heightened anxiety phenotypes in zebrafish F1 offspring and in F1 and F2 generations in guppies [4,25].

Here, we investigate whether exposure of zebrafish males to the herbicide, atrazine, during early juvenile development affects the behavioral traits of their unexposed F1 progeny at adulthood. Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a commonly used herbicide to control weeds in a variety of crops [48,49], but often leeches into the aquatic environment [35]. The maximum allowable atrazine concentrations in drinking water range from 0.1 to 5 ppb in most regions, particularly in Asia and the USA, though environmental concentrations of 30 ppb in ground and surface waters have been reported [50], and atrazine can sometimes be found in much higher concentrations, even over 300 ppb [51].

In addition, atrazine has been found to possess neuroendocrine disrupting properties [5,8,52–55]. The mechanism underlying atrazine neuroendocrine disruption still remains to be resolved, but is implicated in disrupting a variety of pathways including the hypothalamic-pituitary gonadal axis [56–59], monoaminergic systems within the central nervous system [5,54,60–62], alterations to the cyclic adenosine monophosphate (cAMP) dependent signaling pathway [63–67], as well as many epigenetic mechanisms, including microRNAs and expression of DNA methyltransferases (see [55] for more details; [7,68]). Previous research has demonstrated that atrazine exposure in fish can reduce mating behavior (male-male aggression in a courtship context; [3,68]) and alter some aspects of sociability [69], personality phenotypes in crayfish [70], increased anxiety and reduced spatial memory and learning in rodents [54,67,71], but to our knowledge, no study has yet explored how atrazine may affect behavioral traits of unexposed offspring. We hypothesized that if changes in progeny behavior (relative to controls) occurred, this would provide evidence that the herbicide atrazine has the capacity to influence behavior across generations, and these changes are likely to be attributed to a non-

genetic, paternal component of inheritance. Based on literature on zebrafish and other species (i.e. mouse and guppy), we predicted that exposure to increasing levels of atrazine increases bottom-dwelling time (a proxy for anxiety) and activity levels [5,60,72] and decreases latency to enter the top zone of a tank (a proxy for boldness), interaction with a mirror (a proxy for aggression) and exploration [3,68].

In addition to behavioral outcomes, we also assess whether behavioral changes are underpinned by changes in the mRNA transcript of genes involved in regulating the serotonergic system, as recent transcriptomic studies from adult zebrafish embryonically exposed to atrazine have highlighted the serotonergic system as a potential target of developmental atrazine exposure [5,73]. As candidate genes, this study selected three serotonin (5-HT) receptor genes *htr1Aa*, *htr1B*, and *htr2B*, as well as the two 5-HT transporter genes *slc6a4a* and *slc6a4b* for their involvement in the regulation of the serotonergic system and in anxiety related behaviors [74–76]. Thus, for the second part of this experiment we specifically focus on bottom-dwelling time (anxiety) behavior. The three 5-HT receptors function by propagating neurotransmission via the relaying of 5-HT activity and are involved in controlling 5-HT release [77,78]. The two 5-HT transporters [79] function by regulating the strength and duration of neurotransmission [77]. Drugs that target the functional proteins and expression levels of these genes are implicated in anxiety-like behaviors [76]. Moreover, single nucleotide polymorphism of 5-HT transporters has been associated with behavioral differences in other taxa (e.g. [80–83]).

Material and methods

Experimental overview

Juvenile F0 zebrafish were exposed to atrazine at environmentally relevant concentrations of 0.3, 3 or 30 part per billion (ppb; [5,6,35]) for 10 days during sexual differentiation (27–37 days post fertilization; dpf). F1 offspring were produced from atrazine exposed and control males, via *in vitro* fertilization (IVF: modified from Johnson et al. [84]) with unexposed females, creating full-sibling families. F1 behavior (see below) was assayed at adulthood (behavior was also tested on the adult F0 fish [85]). Whole-brains of control and 0.3ppb fish were used to determine mRNA transcript number of candidate genes involved in the regulation of the serotonergic system (*slc6a4a*, *slc6a4b*, *htr1Aa*, *htr1B*, *htr2B)*. The 0.3ppb progeny were selected as we had previously found that bottom-dwelling time (anxiety) was significantly increased in the F0 0.3ppb treatment [85] and because 0.3 ppb represents a typical environmental concentration of atrazine exposure [35]. All experiments and handling of the animals were performed according to the New Zealand Animal Welfare Act and approved by the University of Otago Animal Ethics Committee.

Breeding and husbandry

Breeding, husbandry and atrazine exposure took place within the Otago Zebrafish Facility (OZF), a temperature-controlled facility with a 14h (0800–2200 h) dawn-dusk light cycle. All fish were housed in 3.5 L tanks (except during atrazine exposure) on a Tecniplast ZebTECH zebrafish housing system (Tecniplast). The conductivity, temperature and pH were maintained between 390–458 μS, 25.2–26.1˚C and 7–7.8 pH, respectively. Zebrafish were fed *ad libitum*, twice daily with dry food (ZM000-400, size-dependent) and once daily with live rotifer (*Brachionus* spp; 4–10 dpf) or live *Artemia* (*Artemia salina;* 10dpf and beyond).

F0 embryos were produced by group spawning wildtype AB zebrafish (24 females, aged ~10 months post fertilization; mpf and 34 males aged ~11 mpf) using a Tecniplast iSpawn Breeding System (Tecniplast) and exposed to atrazine at 27–37 days post fertilization (see below). Unexposed F1 progeny were then produced when F0 fish reached sexual maturity, ~3–4 months

post fertilization (106–141 dpf), using IVF (modified from Johnson et al. [84]) with untreated AB females (aged ~8 mpf). Three F0 males per treatment were randomly selected producing three full-sibling F1 families per treatment; 12 families in total). Both F0 and F1 embryos were incubated in petri dishes (90 mm diameter) with E3 media (Cold-Spring-Harbor-Protocols) at 28.4°C until 4dpf. After four days, hatched fry were moved into 3.5 L tanks with AquaOne, 5 parts per trillion Aquaria Salt solution (synthetic sea salt).

Atrazine exposure

A stock solution of atrazine (Sigma-Aldrich; CAS 1912-24-9; 5 mg in 200 mL) was prepared three days prior to exposures. At 27 dpf, F0 zebrafish fry (80 fry per treatment) were exposed to atrazine (at 0ppb 0.3ppb, 3ppb or 30ppb) in 500 mL of system water using 600 mL (80 mm diameter) glass beakers. The atrazine exposure regime lasted 10 days (26/02/17 through to the 8/03/17). This window corresponds with the first ~10 days of sexual differentiation in zebrafish in our OZF facility [86,87]. Fry were fed once a day during the exposure regime and water was changed daily (100% water change; from day 2 until day 10); thus, concentrations of atrazine were also renewed daily–the larvae were sieved, rinsed and placed in new beakers of the appropriate atrazine concentration. At the end of 10 days, fry were moved back onto the OZF system. While we did not analytically verify atrazine concentrations, the exposures were conducted in a closed system and this same exposure regime has been used in several studies [3,5,6,68] where the concentrations were verified. Furthermore, concentrations used by Shenoy [3,68] (1 ppb and 15 ppb) were found to decrease negligibly over 4–7 days without renewal in a closed system (to 0.26 ppb and 12.98–13.45 ppb, respectively). However, we recognize that there could be some error in using nominal (non-measured concentrations), though the concentrations will still vary by orders of magnitude.

F1 behavioral assays

F1 fish were exposed to a series of behavioral assays: a novel arena, a novel object and a mirror test, all in the same tanks [88–91]. Behavioral measurements were recorded during each test (see below for details about behavioral measures recorded and background of behavioral assays used) by live-tracking fish using EthoVision XT behavioral tracking software version 11.5 [92].

The novel arena, the novel object and the mirror tests were run consecutively in the same tank with each assay lasting 10 min (600 s) each, with three fish assayed simultaneously in three separate tanks (length = 30 cm, width = 15.5 cm, height = 27 cm filled with 8 L of system water to a depth of 19.5 cm; Fig A in S1 File). A flat mirror (vertical length = 19 cm, width = 15 cm) was fixed to the outside wall of each tank, which was covered with a removable, hard plastic opaque barrier during the novel arena and novel object tests. White film was fixed to the bottom and side of each tank wall to limit light and reflection during filming (except on the side where the mirror was placed). The back of the tank was fixed with white and opaque plastic. The novel object was an orange rubber bung (measuring 3.2 cm long, 4.3 cm wide at the bottom and 3.7 cm wide at the top) attached to plastic fishing wire hanging in the middle of the arena (at an approximate depth of 6.9 cm below the surface of the water). Tanks were lit 30cm above the tanks with one 240 V 48LED aluminum light strip and from behind using three Godox LED170 lights (31–35.5 cm away) to provide diffusing light to increase fish contrast during filming. Fish were filmed with a Basler acA1300-60/gc GigE camera with a 4.4–11 mm lens placed about 112 cm away from the row of tanks and live-tracked using EthoVision XT [92]. Live-tracking with EthoVision XT started 10–30 s into each trial (e.g., after the fish were placed in the novel arena, 10–30 s after the novel object was added and 10–30 s after the opaque barrier covering the mirror was removed).

In total, 190 F1 fish were assayed (control males = 21, females = 28; 0.3ppb males = 22, females = 24; 3ppb males = 18, females = 30; 30ppb males = 18, females = 29), however, there were eight instances during the novel object assay where tracking was unable to be accurately established due to experimental error. These data (three controls (two males, one female), one 0.3ppb male, three 3ppb fish (one male and two females) and one 30ppb female) were therefore excluded from subsequent analyses on behavioral measures recorded during the novel object test.

Background information of assays and behavioral measures recorded

The novel arena test is routinely used to measure anxiety, activity, and exploration (predominately after chemical exposures in fish; [88,93]). Zebrafish, when exposed to an unfamiliar environment (i.e. the novel arena), tend to dive to the bottom of the test arena and tend to remain in the lowest portion (bottom-dwelling behavior), avoiding the higher portions of the water column, and then the fish usually begin to explore the other areas of the tank after a few minutes [88,89]. Thus, we measured anxiety in the novel arena by the amount of total time spent (s) in the bottom zone (i.e. the total time spent in the lowest four zones superimposed within EthoVision XT; Fig A in S1 File). Exploration was measured by the standard deviation of time spent (s) in each of 12 zones (Fig A in S1 File) during the novel arena test (i.e. an explorative fish would have a standard deviation closer to zero, meaning a fish spent an equal amount of time in all areas of the tank, a high standard deviation indicates the fish was less explorative, preferring to spend the majority of the assay in a few areas of the tank [91]). We measured the latency (s) to enter the top zone (taken as the first moment that a fish entered the top portion of the tank; i.e. the first time a fish entered into one of the four highest zones superimposed within EthoVision XT).

The novel object test was considered a complementary assay to the novel arena test. When exposed to a novel object, zebrafish show a tendency to avoid the foreign object, exhibit bottom-dwelling behavior, and after a certain amount of time, the zebrafish should begin to inspect the object [91,94,95]. We measured the amount of time spent in the bottom zone (s) throughout the assay and novel object approach (1), approached the novel object (entered the zone surrounding the novel object), or (0), did not approach the novel object. (Fig A in S1 File). A zebrafish approaching the novel object (orange rubber stopper) within 1–1.5 body lengths is considered a measure of boldness and is interpreted as a predator inspection behavior [89,91,94–96].

Finally, the mirror test is typically used in assessing an individual's level of aggression (e.g. [90,97,98]. Zebrafish are unable to recognize their reflection and are thought to perceive their mirror image as an intruder [90,99]. The time spent (s) interacting with the mirror (within the mirror zone; Fig A in S1 File) was recorded. Across all three assays, activity was measured by the total distance moved (cm).

Previous work in our lab has shown that the repeatability (the intra-class correlation values, i.e. animal personality) of zebrafish behavior (for the behavioral measures described above) is dependent on an initial exposure to the assay regime, i.e., repeatability was low between the initial test and additional tests, but repeatability was high from the second assay onwards [91]. Considering these findings, an 'assay experience' was provided (i.e. fish were exposed to the assay regime twice, but the data presented is that of the second assay trials). The assay regime proper was conducted 7 days after the assay experience. The repeatability of behavior exhibited during the mirror tests (i.e. time spent interacting with the mirror) does not appear to be dependent on an initial exposure [85], but it was included to maintain consistency with the

other behavioral tests. Because fish were not individually marked, we were unable to establish changes in behavior of individuals between trials.

F1 brain collection

Immediately after phenotyping (the second trials i.e. the assay regime proper) whole-brain tissue was collected from the two males and two females (per each family of the control and 0.3ppb treatment) that spent the most and the least amount of time at the bottom zone during the novel arena assay (a high and low anxiety phenotype). We selected the most and least anxious fish to capture a wide range of behavioral expression and potentially any underlying signal in mRNA gene expression. Once selected, fish were then euthanized and dissected in 1X PBS (total number of fish dissected = 24 fish per treatment). There were two instances in the control group (two males) where we were unable to extract brain tissue. Once extracted, whole-brain tissue was immediately stored in 50μL of RNAlater (Invitrogen) and subsequently stored at −30˚C. The 0.3ppb treatment was chosen for comparison against controls as it represents a typical environmentally relevant level of atrazine exposure [35] and because we previously found that F0 fish exposed to the 0.3ppb atrazine treatment exhibited significant increases in bottom-dwelling [85].

Quantitative real-time PCR (qPCR)

RNA was extracted from whole-brain tissue using a modified protocol from the Norgen Biotek RNA kit and using TRIzol reagent (Ambion) for initial homogenization. Extracted RNA was purified using Turbo DNase (Invitrogen). The RNA was reverse transcribed to obtain cDNA using 400 ng of RNA and a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The parameters for reverse transcription (following the manufacture's protocol) consisted of one cycle of 25˚C for 10 min, 37˚C for 120 min and 85˚C for 5 min (ThermoFisher).

Whole-brain mRNA expression of genes encoding for *slc6a4a*, *slc6a4b*, *htr1Aa*, *htr1B*, and *htr2B* (Table A in S1 File) was conducted using QuantStudio 5 (Thermofisher). The total reaction volume was 10 μL, containing 5 μL SYBR green (Takara), 0.5 μL (10 μM) forward primer, 0.5 μL (10 μM) reverse primer, 2.8 μL MilliQ water, 0.2 μL ROX and 1 μL (10 ng/μL) of cDNA. For each primer, a gradient temperature assessment was carried out from 60–64˚C in order to find the optimal annealing temperature. Each cycle of qPCR was 95˚C for 2 min, 95˚C for 5 s, optimal annealing temperature of gene primer for 10 s, 72˚C for 5 s, 95˚C for 1 min, 55˚C for 30 s, 95˚C for 30 s, for 40 cycles per plate. qPCR standards for each gene were made using pooled cDNA (of all 46 samples; 22 controls and 24 0.3ppb treatment offspring) and serially diluted (6X) at a ratio of 1:3 dilution. Efficiency of standards for all qPCR runs was between 95–105%. *b-actin* was validated to be a suitable reference gene.

Statistical analysis

All statistical analyses were conducted in R, version 3.5.0 [100]. All analyses on behavior described below contained the two main predictors, treatment (i.e. controls, 0.3ppb, 3ppb, 30ppb) and sex (male and female) and an interaction term between treatment and sex. The interaction term was removed if non-significant and the data were re-analyzed without it. All behavioral measures were analyzed individually with linear mixed effects models (LMMs: the time spent in the bottom zone of the novel arena/ novel object test, exploration in the novel arena, time spent interacting with the mirror and the total distance moved in each assay) or with generalized linear mixed effects models (GLMMs: the latency to enter the top zone of the novel arena using a gamma error structure (log-link function), and approaching or not approaching the novel object using a binomial error structure (logit-link function)), using the

package '*lme4*' version 1.1–1.3 [101]. The assumptions of a GLMM with a gamma error structure are that zeros are unable to be analyzed (therefore if a fish was in the top zone at the start of the trial; it was given a latency of 0.1 s, if the fish never entered the top zone; it was given a latency of 600 s (the total duration of the assay). In all models, family identity was included as a random effect to account for genetic influences.

To test if an bottom-dwelling time (a proxy for anxiety) was underpinned by mRNA transcript number and whether or not paternal atrazine exposure altered the relationship of mRNA transcripts between treatments, we ran several univariate LMMs for each candidate gene. As the response we used the anxiety measure, time spent in the bottom zone (of the novel arena). As predictors, we used treatment group (control and 0.3ppb), relative mRNA of the candidate genes (each candidate gene was normalized by *b-actin* expression and centered using the '*scale*' function) and an interaction term between treatment group and relative mRNA. Family ID was used as a random effect to account for genetic differences. The interaction term was removed if non-significant and models were re-analyzed. The effects of paternal atrazine exposure on transcript number alone were additionally assessed using linear mixed effect model with treatment, sex and an interaction term. Family ID was used as a random effect and the interaction term was removed if non-significant and models were reanalyzed without it (results are presented in supplementary material).

Significant (or marginally non-significant) differences are presented in text with estimates and their 95% confidence intervals (CI). Confidence intervals were calculated using the '*confint*' function. For reference, 95% CI of estimates that do not include zero are statistically significant. The full model outputs, including parameter coefficients (back transformed where appropriate), parameter standard errors (SE), test statistics and p-values are reported in the supplementary material (Tables B, C and D in S1 File). P-values were calculated using the '*lmerTest*' package [102]. Descriptive statistics presented in the results text are ranges and/or means ± SE.

Results

Effect of paternal atrazine exposure on F1 behavioral traits

Bottom-dwelling time. The time spent in the bottom zone in both the novel arena and novel object assay by F1 zebrafish ranged the full length of the assays from 0–600 s (Fig 1A and Fig 1B). We found no significant difference between controls and atrazine treatments in the time spent in the bottom zone during the novel arena test, though, visually, controls tended to spend less time in the bottom zone on average (Fig 1A). In the novel object test, however, F1 fish paternally exposed to 0.3ppb atrazine spent significantly more time in the bottom zone compared to F1 controls (Est. 180.19 s [13.18, 347.20 95% CI). Additionally, a marginally non-significant increase in bottom-dwelling time was observed in fish from the 3ppb treatment (Est. 163.90 s [−2.92, 330.71 95% CI]), but no significant differences were observed in offspring from the 30ppb treatment compared to controls (Fig 1B). Male fish spent less time in the bottom zone than females across both tests (novel arena test Est. −86.78 s [−128.30, −45.25 95% CI]; novel object test Est. −78.44 s [−137.31, −19.57 95% CI]) and averaged 253 ± 18 s and 355 ± 25 s in the bottom zone of the novel arena and novel object assay, respectively, whereas females spent an average of 344 ± 17 s and 437 ± 22 s, respectively.

Latency to enter the top zone. Paternal atrazine exposure significantly increased the latency to enter the top zone of the novel arena in all F1 atrazine treatment groups compared to controls (0.3ppb Est. 6.16 s [2.01, 18.73 95% CI]; 3ppb Est. 4.07 s [1.34, 12.30 95% CI]; 30ppb Est. 3.71 s [1.22, 11.25 95% CI]; Fig 1C). In addition, male fish, regardless of treatment, took significantly less time to enter the top zone (Est. −2.3 s [−2.32, −2.27 95% CI]), taking on
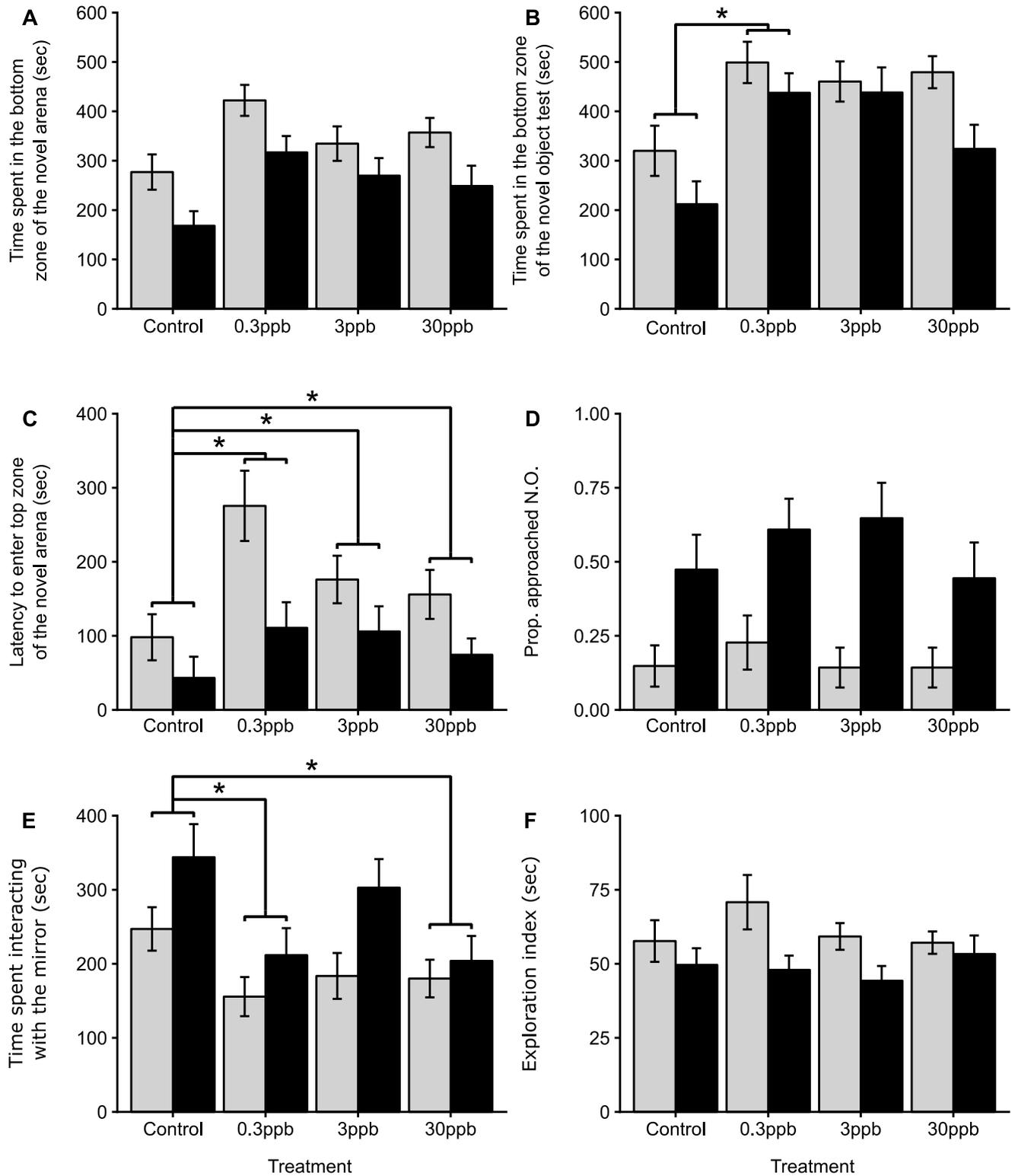
Fig 1. The effects of paternal atrazine exposure (at control [0ppb], 0.3ppb, 3ppb and 30ppb) on F1 behavioral traits. (A) the time spent in the bottom zone (s) during the novel arena test, (B) time spent in the bottom zone (s) during the novel object test, (C) the latency to enter the top zone (s) during the novel arena test, (D) the proportion of fish that approached the novel object test; (E) the time spent interacting with the mirror (s) and (F) exploration (s). Bars represent means, with error bars representing standard errors of the mean. An asterisk indicates a significant difference between controls and an atrazine treatment (p <0.05). For each sex and treatment, sample sizes ranged from n = 18–30. Total sample size for F1s were n = 190, with n = 3 families per treatment. See methods for sample sizes.

average 84 ± 16 s, whereas females took an average of 171 ± 18 s to enter. There was no difference between treatments in the proportion of fish that approached the novel object (Fig 1D), but male fish were far more likely to approach than female fish (Est. 0.87 [0.76, 0.93 95% CI]), with on average 54% of males that approached compared to 16% of females (averaged across all treatment groups).

Interaction with a mirror. The time that F1 fish spent interacting with the mirror was significantly lower in the 0.3ppb treatment and 30ppb atrazine treatment compared to controls (0.3ppb Est. −110.63 s [−45.91, −3.35 95% CI]; 30ppb Est. −95.98 s [−31.72, −2.93 95% CI]; Fig 1E). Additionally, F1 fish from the 3ppb treatment exhibited a marginally non-significant decrease compared to control fish (3ppb Est. −56.32 s [−120.25, 7.61 95% CI]; Fig 1E). In general, male fish spent significantly more time engaging with the mirror than females (Est. 74.42 s [27.96, 120.89 95% CI]), spending an average of 264 ± 20 s interacting, whereas females spent on average 193 ± 14 s.

Exploration. Across all trials, the exploration index ranged from 15–177 s with a mean of 56 ± 2 s. There was no difference in exploratory behavior amongst the offspring of atrazine treated males compared to the offspring of control males (Fig 1F); however, male F1s were more exploratory than female F1s (Est. −12.43 s [−20.77, −4.09 95% CI]) and had an exploratory index mean of 49 ± 3 s, whereas females had a mean of 61 ± 3 s.

Activity. During both the novel arena and novel object test, no significant differences in activity were observed across any of the treatment groups compared to controls (Fig 2A and Fig 2B). In contrast, offspring from the 0.3ppb and 30ppb treatment groups travelled significantly less than offspring from control males during the mirror test (0.3ppb Est. −110.63 cm [−175.35, −45.91 95% CI] and 30ppb Est. −95.98 cm [−160.24, −31.72 95% CI]; Fig 2C). Offspring from the 3ppb treatment also travelled less than control offspring, but this observation was marginally non-significant (Est. −56.32 cm [−120.25, 7.61 95% CI]). Male offspring were more active than female offspring, and travelled a greater total distance during the novel arena test (Est. 1255.64 cm [892.14, 1619.14 95% CI]), the novel object test (Est. 1257.18 cm [917.76, 1596.60 95% CI]) and during the mirror test (Est. 689.30 [393.78, 984.89 95% CI]). Overall, males travelled on average 4044 ± 170 cm, 2660 ± 167 cm and 2569 ± 115 cm, in the novel arena test, novel object test and the mirror test, respectively. Whereas females travelled 2780 ± 97 cm, 1381 ± 82 cm and 1881 ± 99, in the same tests.

Effects of atrazine on mRNA transcript number

Paternal atrazine exposure significantly altered the relationship between the time spent in the bottom zone of the novel arena (bottom-dwelling) and *slc6a4a* mRNA transcript number (Est. −191.46 s [−355.69, −27.23 95% CI]; Fig 3A). Bottom-dwelling behavior amongst F1 controls increased positively with increased *slc6a4a* mRNA transcript number whereas the opposite pattern was observed amongst F1 fish paternally exposed to 0.3ppb atrazine (Fig 3A). In contrast, no significant relationship was observed between bottom-dwelling and *slc6a4b* mRNA transcript number (Fig 3B). The relationship between bottom-dwelling behavior and *htr1Aa* mRNA was significantly altered between treatment groups (Est. −260.37 s [−465.39, −55.35 95% CI]; Fig 3C). *htr1Aa* mRNA transcript levels of control F1 fish increased with increased time spent in the bottom zone, whereas transcript levels of atrazine treated F1s decreased with
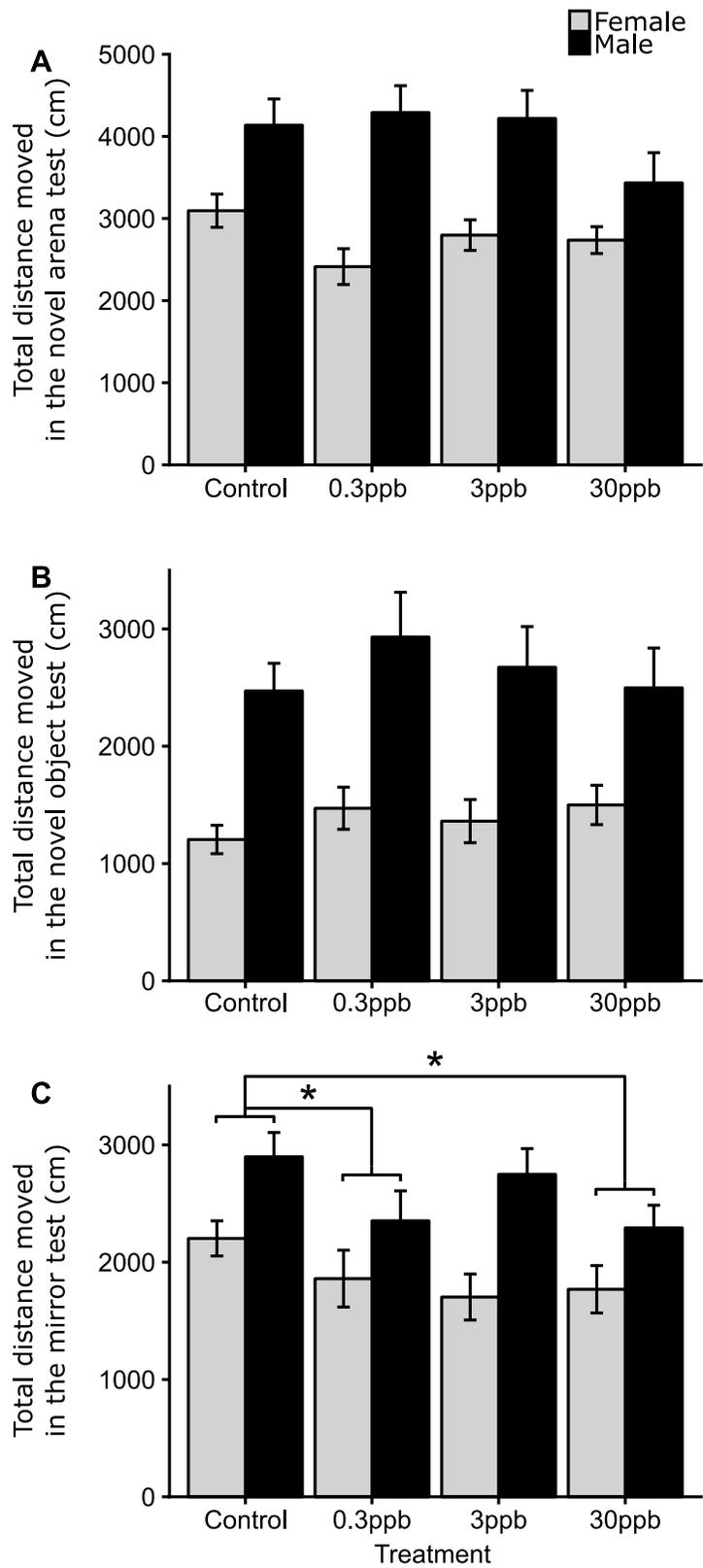
Fig 2. The effects of paternal atrazine exposure (at control [0ppb], 0.3ppb, 3ppb and 30ppb) on F1 activity, measured by the total distance moved (cm). (A) during the novel arena test, (B) the novel object test, (C) and during the mirror test. Bars represent means, with error bars representing standard errors of the mean. An asterisk indicates a significant difference between controls and an atrazine treatment (p <0.05). For each sex and treatment, sample sizes ranged from n = 18–30. Total sample size for F1s were n = 190, with n = 3 families per treatment. See methods for sample sizes.

increased time spent in the bottom zone (Fig 3C). There was no significant relationship between bottom-dwelling in the novel arena and either *htr1B* and *htr2B* mRNA transcript number (Fig 3D and Fig 3E, respectively).

Discussion

We show that paternal exposure to the herbicide atrazine, during juvenile development, significantly influences a variety of behavioral traits of unexposed F1 offspring at adulthood (with many effects occurring in a non-dose dependent manner), including: bottom-dwelling time (at 0.3ppb, albeit non-significantly in the novel arena test), latency to enter the top zone of the arena (at 0.3ppb, 3ppb and 30ppb), interactions with a mirror (at 0.3ppb and 30ppb) and activity (at 0.3ppb and 30ppb during the aggression assay). Importantly, these effects are observed at environmentally relevant concentrations (0.3ppb is a typical low dose and 30ppb is a typical high dose likely to be encountered in the environment [35]). This study also shows that paternal atrazine exposure at 0.3ppb is involved in the disruption of some aspects of the serotonergic system, in particular, paternal atrazine exposure appears to have altered the trend between time spent in the bottom zone (a measure of anxiety) and *slc6a4a* mRNA and *htr1Aa* mRNA transcript number between treatments. Together, these results suggest that behavioral changes and underlying mRNA transcript difference might be transferred transgenerationally.

Effects of paternal atrazine on F1 behavior

The trend of increased time spent in the bottom zone in the novel arena (at 0.3ppb) and the significant increase observed in time spent in the bottom zone of the novel object test (at 0.3ppb) suggest changes in anxiety due to low-dose paternal atrazine exposure. These differences are in line with previous work in fishes [103] and in rodents [54,60,104]. But the lack of substantial differences, when compared to the previous studies, may be due to the lower concentrations used and the intergenerational nature of this study design, as the aforementioned studies (exposed at concentrations up to 3000ppb in fish and up to 250 mg/kg in rodents) were either direct or prenatal exposures.

Paternal exposure to atrazine significantly increased the latency to enter the top zone of the novel arena at all concentrations tested (0.3ppb, 3ppb and 30ppb), with effects appearing to be more pronounced in females than males. These behavioral differences indicate a non-monotonic response, with the greatest increase observed in offspring of parents exposed to the lowest concentration. Previous work suggests that the latency to enter the top zone is often taken as a measure of anxiety-like behavior (e.g. [4,25,88,89,93]). In an ecological setting, entering the top portion of the water column where little vegetation occurs likely carries a higher risk of avian predation and may increase conspicuousness of the individual to fish predators [105–107]. When placed in a novel environment (such as in the novel arena test) or confronted by an avian predator simulation above the tank (e.g. a bird silhouette or a black dot increasing in size, simulating an approaching bird), defensive behaviors such as bottom-dwelling are triggered, and then over time, the fish will begin to explore the higher portions of the tank [88,93,108,109]. If the highest portion of the tank is perceived to carry a higher risk by the
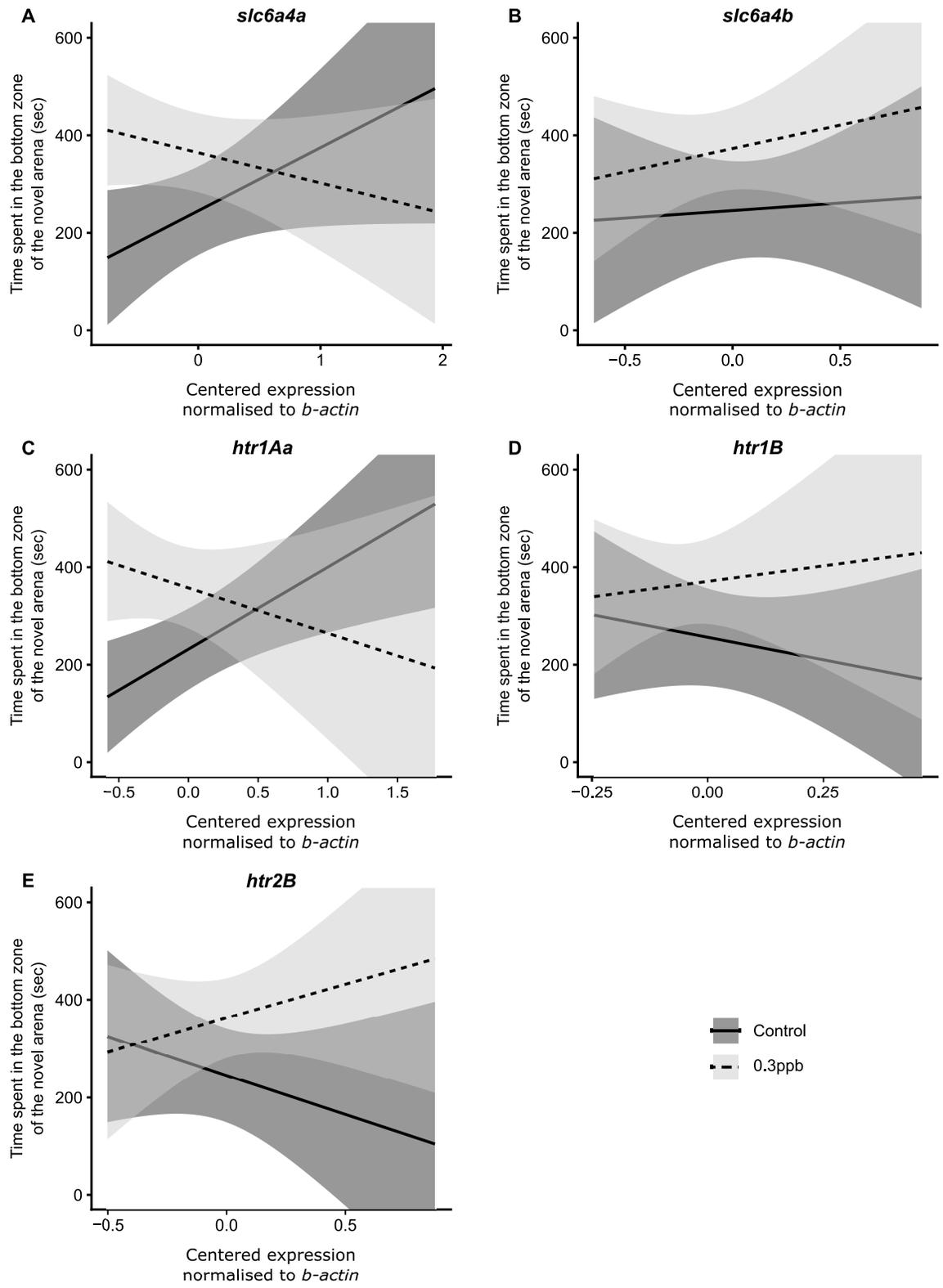
Fig 3. The relationship between time spent in the bottom zone of the novel arena (s) and relative mRNA levels of candidate genes. (A) *slc6a4a*, (B) *slc6a4b*, (C) *htr1Aa*, (D) *htr1B* and (E) *htr2B* of F1 offspring from control (solid line) and 0.3ppb atrazine treated (dashed line) males, normalized to b-actin and then centered (from whole-brain samples) and their 95% CI (shaded areas). Sample sizes for control offspring were n = 22 (males = 10; females = 12; progeny per family = 6–8; families n = 3), and for 0.3ppb offspring were n = 24 (males = 12; females = 12; progeny per family = 8; families n = 3).

zebrafish, then changes in latency observed from atrazine treated offspring might reflect a distortion in an individual's ability to either assess risk, or a change in propensity to take risks, regardless of the risk entailed. Risk assessment of the environment is crucial during foraging and exploration [110]. Individuals that under assess risk, or are generally bolder, have increased susceptibility to predation [111,112]. Whereas over assessment or more anxious individuals may have increased long-term survival, but may have costs on other functions, such as the ability to forage which may then have negative consequences on growth and reproduction [113,114].

Paternal atrazine exposure was found to significantly decrease interactions with a mirror (a proxy for aggression) in male and female offspring from the 0.3ppb and 30ppb treatment. The lack of a significant decrease in aggression in the 3ppb treatment offspring (though the decrease was marginally non-significant) may be due to the non-monotonic nature of atrazine as found in other studies [3,115–118]. For instance, Shenoy et al. [3,68] found that male guppies prenatally exposed to 1 ppb were consistently less aggressive (within a mating context) than those prenatally exposed to 15 ppb. Changes in activity levels during the mirror test (but not in the other two behavioral assays) might also suggest that zebrafish are less willing to engage in aggressive bouts or perhaps are warier to engage, as has been found previously in male-male interactions in guppies [68]. Reductions in aggression are in line with similar studies measuring male-male aggression and male sexual displays after direct atrazine exposure [3,52,53,68]. In the wild, aggression in zebrafish is important for establishing dominance hierarchies, enabling the monopolization of foraging resources and the maintenance of territories for spawning sites [119]. In addition, more aggressive zebrafish males exhibit higher reproductive success [98]. The consequences of reduced aggression may therefore limit the ability to compete for access to foraging sites and limit the ability to maintain territories. Aggression is also important in females, as either sex can establish a dominance hierarchy [119,120].

The behavioral measures tested here are known to exhibit long-term repeatability in zebrafish, using the same setting and testing regime [91]. Thus, it may be possible that atrazine has the capacity to affect animal personality traits in zebrafish as it has been shown in crayfish [70]. Some EDCs such as 17α-ethinylestradiol have been shown to disrupt individual consistency (e.g. [18–21]) and behavioral responses between individuals as a result of EDC exposure (including atrazine) can be personality dependent [70]. Hence, the behavioral data shown here may not capture the full extent to which atrazine is capable of altering animal behavior and highlights that further work is needed to investigate if atrazine exposure in fish shares similar endpoints.

Effects of paternal atrazine on mRNA transcription in F1 brains

Paternal atrazine exposure appears to have altered the relationship between *slc6a4a* and *htr1Aa* mRNA and time spent in the bottom zone (a measure of anxiety in zebrafish), suggesting possible serotonergic dysregulation (Fig 3). In both genes, mRNA transcript was found to increase positively with the amount of time spent in the bottom zone amongst controls, whereas amongst fish that were paternally exposed to 0.3ppb atrazine, decreased mRNA transcript was associated with reduced time spent in the bottom zone. In addition, it appears that the relationship between *hrt2B* mRNA and bottom-dwelling behavior between treatments

followed a similar pattern to the one found at *slc6a4a* and *htr1Aa* mRNA, but high individual variation likely precluded the detection of a significant interactive effect. The results of this study support previous research associating atrazine exposure with disruption of some aspects of the serotonergic system [5,54,60,61]. For example, adult mice exposed to atrazine (at 3mg/kg) during gestation and lactation, via atrazine in the mothers drinking water, exhibited decreased 5-HT levels in the striatum (and females additionally exhibited decreases in the perirhinal cortex [60]. Another study found disparities in the metabolome (the entire metabolite profile) of atrazine exposed male mice, including tryptophan (precursor to 5-HT) and other metabolites important in normal 5-HT function (e.g. linoleic acid and α- linolenic acid), at concentrations >5mg/kg [61]. At higher doses, (>125 mg/kg; but not at concentrations tested between 0–25 mg/kg) atrazine increased the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA) levels and altered 5-HT turnover in the brain [54].

Previous research has found that bottom-dwelling in the novel arena test is inversely correlated with 5-HT levels and drugs targeted to interfere with *slc6a4a* and *htr1Aa* protein products can alter this behavior [76,121]. Indeed, blocking 5-HT re-uptake with fluoxetine treatment, thereby increasing 5-HT accumulation in the synaptic cleft, produces an anxiolytic effect in the novel arena test [76]. Furthermore, blocking 5-HT from binding to HT1A receptor (HTR1AR) on post synaptic membranes, using busporine (an agonist of the pre-synapse and antagonist on the post synapse) or WAY 100 635 (antagonist at both pre and post synapse), is associated with producing an anxiolytic effect in zebrafish [76]. The up-regulation of *slc6a4a*, *htr1Aa* and *htr1B* mRNA transcripts by the element selenium (in the dietary form of selenomethionine) is associated with increased bottom-dwelling behavior in zebrafish [122]. These results support the hypothesis that *slc6a4a* and *htr1Aa* (and potentially *htr1B*) gene expression are associated with anxiety in zebrafish.

While this study did not test for the effects of paternal atrazine exposure over developmental stages, it is possible that *slc6a4a* and *htr1Aa* mRNA disruption were present over ontogeny. Proper serotonergic function is critical during early life [123]. Indeed, disruption of HT1AR during adolescence in mice is also enough to sustain increased anxiety levels through to adulthood [74]. Likewise, HT1AR knockout mice exhibit increased anxiety at adulthood [124] and it appears that retaining proper function of HT1AR during development is critical for normal anxiety behavior as an adult [75]. Future studies could examine ontogenic features of atrazine exposure, anxiety and their relationship to *slc6a4a* and *htr1Aa* mRNA transcript levels.

The lack of a relationship at *slc6a4b* and *htr2B* mRNA transcript with bottom-dwelling might suggest that these genes are not as strongly associated with anxiety. The serotonergic system is a complex system that appears to govern a variety of behaviors other than anxiety such as aggression for which *slc6a4b* and *htr2B* mRNA transcripts and their protein products may be more involved in [9,125,126]. However, our results display a high level of variation, so it is possible that we did not have enough power to detect such an effect. Furthermore, while we did not find any significant interactive effect, we found it interesting that there was relatively minimal difference in the expression of bottom-dwelling at low transcript levels at *htr1B* and *htr2B* (and to some extent at *slc6a4b*) between treatments, but as mRNA transcript levels increased, the difference in bottom-dwelling behavior became more contrasting between the controls and treatment (Fig 3). In contrast, the expression of bottom-dwelling at low *slc6a4a* and *htr1Aa* mRNA transcript levels was disparate between treatments.

Possible mechanisms underlying effects of atrazine on F1 progeny

We observed intergenerational effects of atrazine exposure that can only be attributable to the sperm of the exposed males, suggesting an underlying epigenetic mechanism. Epigenetic

effects (i.e. DNA methylation, non-coding RNAs and histone modifications) are increasingly being studied [28,31,32,34,127–129] and it is known that exposure to strong environmental stressors (especially during early life) may leave epigenetic marks that, in turn, permanently alter the phenotype of the adult (see reviews; [128,130]). Studies suggest that the paternal DNA methylome is inherited in an unchanged state in zebrafish [131,132], as opposed to mammals (e.g. in humans and mice), where upon fertilization, both the maternal and paternal methylomes are erased and 're-programmed', with only a small percentage (~5%) thought to escape this process [133–135]. Thus, in zebrafish, there is greater potential for transfer of environmental specific information. However, an epigenetic inheritance explanation requires transgenerational observations, i.e. the effect must be observed in the first generation where the germ line was not developmentally exposed [136,137], which would be the F2 generation in fish. However, previous research has shown that atrazine (at 25 mg/kg BW/day and 100 mg/kg/day) can induce transgenerational effects of disease in mice [138,139] and produce transgenerational reproductive defects (at 5ppb and 50ppb) in medaka [140]. Additionally, it appears that disease phenotypes can be exacerbated with each generation [139], suggesting that transgenerational effects on behavior are likely.

Conclusions

In conclusion, we found that several aspects of progeny behavior were altered by paternal exposure to environmentally relevant concentrations of atrazine, thus representing intergenerational effects on behavior, though many of the effects on behavior were non dose-dependent. Moreover, some aspects of the serotonergic system were disrupted in the offspring, though given the high variation, further research is needed. Overall, these results add to the ecological consequences of environmental contaminants, most importantly, that further research may reveal that effects may be further propagated down the germ line.

Supporting information

S1 File. Supporting information. Details of supplementary methods including ethovision setup used for behavioral testing (Fig A in S1 File), primers used (Table A in S1 File), as well as supplementary results including treatment effects on transcript number (Fig B in S1 File) and model outputs (Tables B, C and D in S1 File).
(DOCX)

Acknowledgments

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