Validating the Poeciliopsis animal model: Effects of perinatal fluoxetine exposure on mother and offspring of live bearing fish with and without placentation

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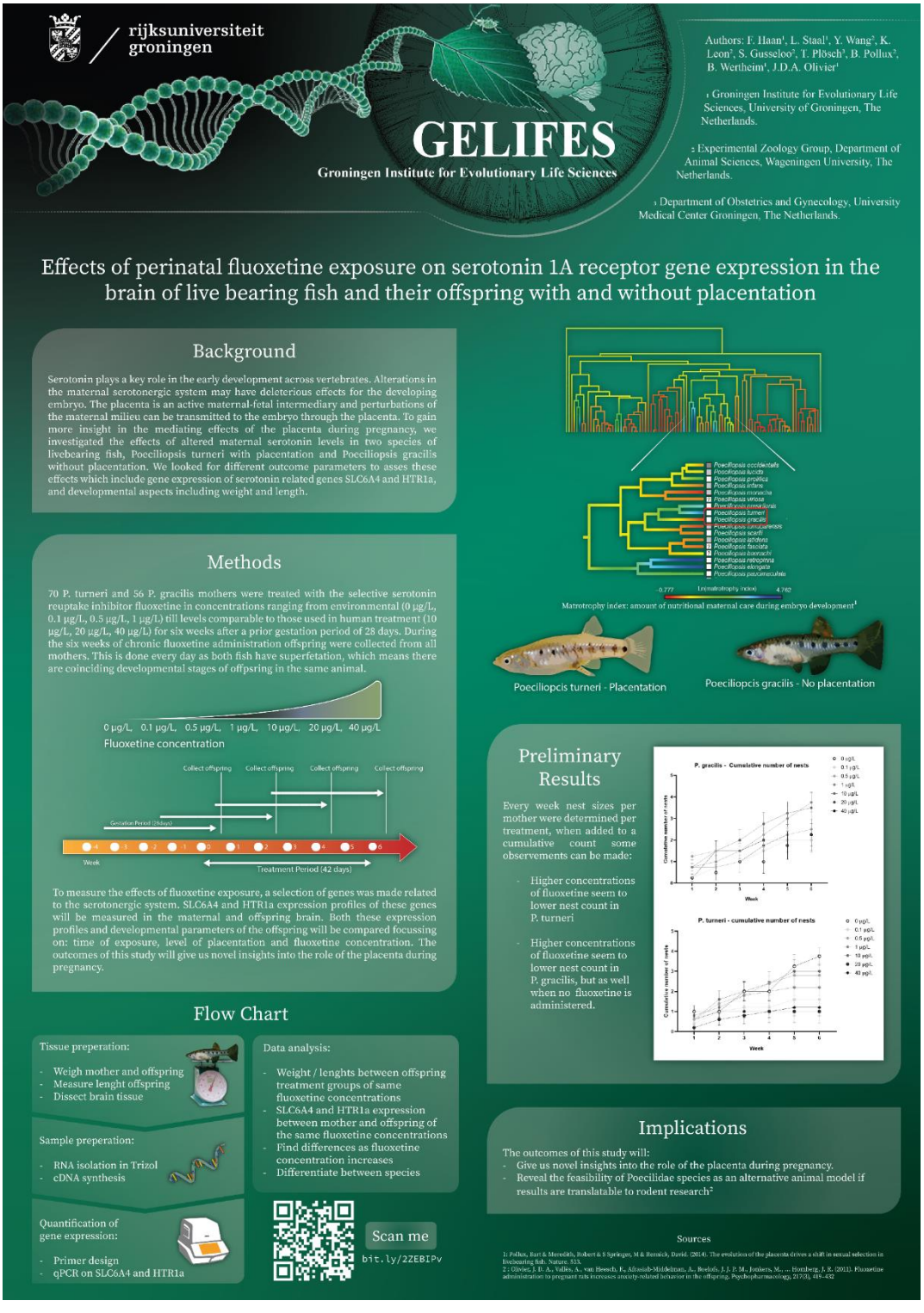


Contents

Abstract

Serotonin plays a vital role in the early development across vertebrates. Alterations in the maternal serotonergic system may have deleterious effects for the developing embryo. The placenta is an active maternal-foetal intermediary and perturbations of the maternal milieu can be transmitted to the foetus through the placenta. To gain more insight in the mediating effects of the placenta during pregnancy, we investigated the impact of altered maternal serotonin levels in two species of live-bearing fish, Poeciliopsis gracilis without a placenta and Poeciliopsis turneri with a placenta.

Both species mothers were treated with a selective serotonin reuptake inhibitor fluoxetine in concentrations ranging from environmental (0 µg/L, 0.1 µg/L, 0.5 µg/L, 1 µg/L) to human treatment levels (10 µg/L, 20 µg/L, 40 µg/L) for six weeks after a prior gestation period of 28 days. During the six weeks of chronic fluoxetine administration offspring were collected daily. It was observed that the weights of both species' mothers were not affected by the fluoxetine treatment. P. gracilis offspring length was changed in an inverted-u shape where the middle range of fluoxetine concentration resulted in longer offspring than those exposed to the lowest and highest tested concentrations. P. turneri offspring was observed to be affected by fluoxetine in a dose-responsive matter where more fluoxetine resulted in a smaller offspring length. P. gracilis offspring were found to be heavier regardless of the concentration. P. turneri offspring became shorter as fluoxetine concentration increased.

Results suggest that the Poeciliopsis model is a viable candidate since P. turneri offspring results are comparable to those seen in humans and rodents. An apparent difference arose between placentation and no placentation, meaning the placenta likely has a role during the fluoxetine exposed pregnancy. In the future looking at genes which relate to the serotonin and neurological system, more insights into how the offspring are affected can be revealed and can offer more considered choices for pregnant mothers who are experiencing depressive symptoms.

Introduction

Serotonin plays a vital role in the early development across vertebrates including humans. However, up to 20% of all mothers find that their serotonin levels are imbalanced during pregnancy (Ryan et al. 2005). In a minority of the cases, four to eight per cent, it can be described as major depression (Kim et al. 2015; Melville et al. 2010). In these cases, antidepressants are often prescribed to treat depressive symptoms. Across Europe, roughly 3% of the pregnant woman are treated with antidepressants, and in the United States, it is as much as 13% (Cooper et al. 2007; Hayes et al. 2012; Kieler et al. 2012; El Marroun et al. 2012). The most common antidepressants prescribed are serotonin selective reuptake inhibitors (SSRIs) (NAMI 2017). SSRIs are currently considered safe for consumption and have a high efficacy to relieve depressive symptoms (Barbey et al. 1998). Fluoxetine, also known as Prozac, is the most commonly used and belongs to the selective serotonin reuptake inhibitor (SSRI) category (Bairy et al. 2007).

Rising evidence suggests that the use of fluoxetine during pregnancy can have a long-lasting negative impact on juveniles. Side effects reported include heightened levels of internalising behaviour (Hanley et al. 2015), including anxiety and depression, but also externalising behaviour (Oberlander et al. 2010), like aggression. Sleep behaviour in young infants is altered as well, where a more substantial amount of rapid-eye-movement sleep was observed (Zeskind et al. 2004). Recent evidence also shows that prenatal exposure to SSRIs increases the risk of malformations and developing autism spectrum disorder (Andalib et al. 2017; Ban et al. 2014; Gentile 2015; Tohru Kobayashi et al. 2016; Womersley et al. 2017). Previous works in rodents have observed negative changes in the offspring after pre- and postnatal SSRI treatment both in affective and social behaviour. When prenatally exposed to SSRIs, it was found that rat offspring were less engaged in social play behaviour, and had more anxiety-like behaviour (Olivier et al. 2011). Postnatally exposed neonate rats also had altered behavioural effects. These include reduced play behaviour, sensorimotor reaction times and lower performance in sexual behaviour, which seems analogous to autism spectrum disorder (Rodriguez-Porcel et al. 2011).

Since fluoxetine is among the most commonly prescribed SSRIs, it is vital to elucidate the mechanism behind possible side effects of fluoxetine administration during pregnancy. Fluoxetine's mechanism of action acts by inhibiting the reuptake of released serotonin in the pre-synapse, the available serotonin in the synaptic cleft increases as a result of this (Benfield et al. 1986). Serotonin acts as a crucial neurotrophic factor for the developing brain. It has an essential role in the early development of neuronal branching, differentiation, migration, mitogenesis, synaptogenesis, terminal sprouting, preventing apoptotic cell death, proliferation and more (Azmitia 2001; Vitalis et al. 2003). During the early development of the foetus, the embryo is nourished via the placenta of the mother. One might expect that the placenta acts as a barrier to fluoxetine for the offspring. However, the characteristics of SSRIs, being small, lipophilic, and non-ionized enables passive diffusion through the lipid-soluble membrane of the placenta into the intrauterine environment (Ewing et al. 2015). It was found that 60 to 70 per cent of the administered fluoxetine ends up in the offspring's circulation (Heikkinen et al. 2002; Hendrick et al. 2003; Rampono et al. 2009; Sit et al. 2011).

How the placenta plays a mediating role in prenatal fluoxetine exposure is not known yet. The Poeciliidae family offers an excellent opportunity to study the role of the placenta during prenatal SSRI exposure. This is because the family of Poeciliidae consists of closely related species, all with different levels of placentation. For this study, Poeciliopsis turneri, which has a sophisticated level of placentation, and Poeciliopsis gracilis, which has a minimal amount of placentation (Figure 1), were chosen due to these differences. Next to the possibility of studying the placenta's mediating influences during prenatal fluoxetine exposure, the possible side effects themselves can also be studied.

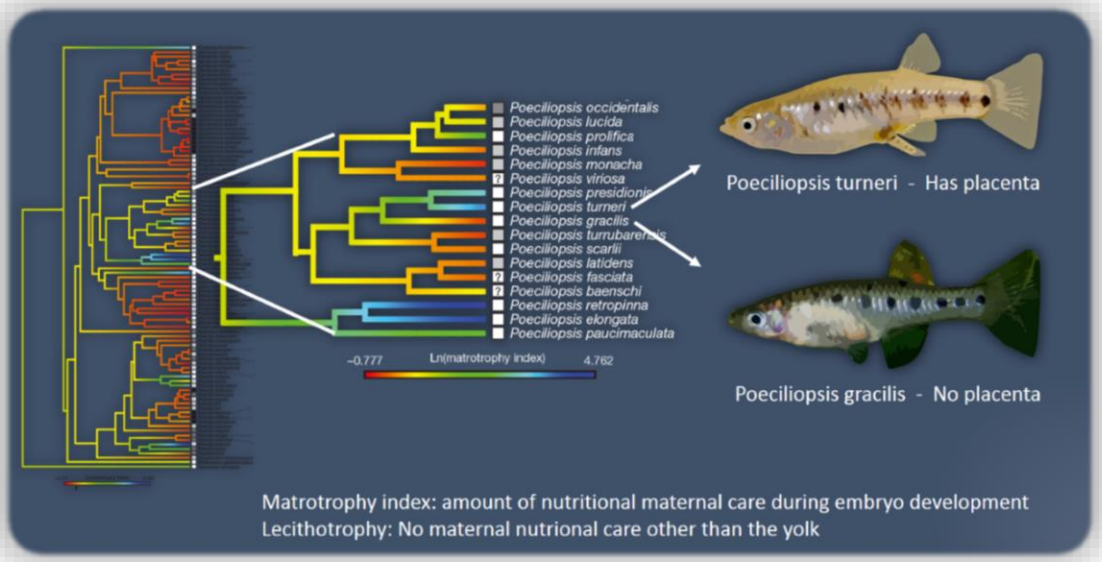


Figure 1: Amount of matrotrophy in Poeciliidae family
The matrotrophy index displays the amount of maternal care during embryo development. A higher matrotrophy index indicates more maternal care during embryo development, a lower index indicates low to no maternal nutritional care during development other than yolk. P. turneri and P. gracilis were selected for their close genetic resemblance and oppositions on the matrotrophy scale. (Figure adapted from Pollux et al. 2014)

To research the serotonergic system in these animal models, it is evident to establish if the Poeciliidae has one. Indirect evidence suggests that the Poeciliidae family does have a serotonergic system similar to those in rats and humans. According to Lillesaar 2011, many similarities are present between vertebrate species; these include proteins and transporters, raphe neurons and their projections, serotonin and its influence on behaviour, and responses from drugs which are known to affect serotonin (5-HT). Also, serotonergic markers tryptophan hydroxylase 1, aromatic L-amino acid decarboxylase, serotonin transporter (SERT) and monoamine oxidase (MAO) are observed in fish. With fluorescence imaging, Antri et al. 2006; Carrera et al. 2008; Kaslin and Panula 2001 saw that serotonergic neurons are present in some brain areas including the hypothalamus, raphe nuclei and spinal cord in the lamprey, dogfish and zebrafish. Cachat et al. 2010; Gabriel et al. 2009; Norton and Bally-Cuif 2010 also showed that behavioural deviations occur when the serotonin balance is altered in the zebrafish. Some of these changes include locomotion, aggression, fear and anxiety.

More indirect evidence for a serotonergic system in fish arises when looking for drug targets. According to Gunnarsson 2008, most biological targets are conserved amongst vertebrate species. From ~1400 drug targets tested, including the serotonin transporter, the Gasterosteus aculeatus (stickleback) and Danio rerio (zebrafish), have an overlapping range from ~900-~1200 targets (Figure 2-A). These species are therefore more similar to the human than most other vertebrates concerning the similarity of conserved drug targets. Conserved drug targets ranked on gene ontology shows the two species of interest have a high percentage of similarity when compared with other vertebrate species (Figure 2-B). High similarity in drug targets does not mean that the serotonin system as a whole and its function is conserved in fish, but it is promising nonetheless.

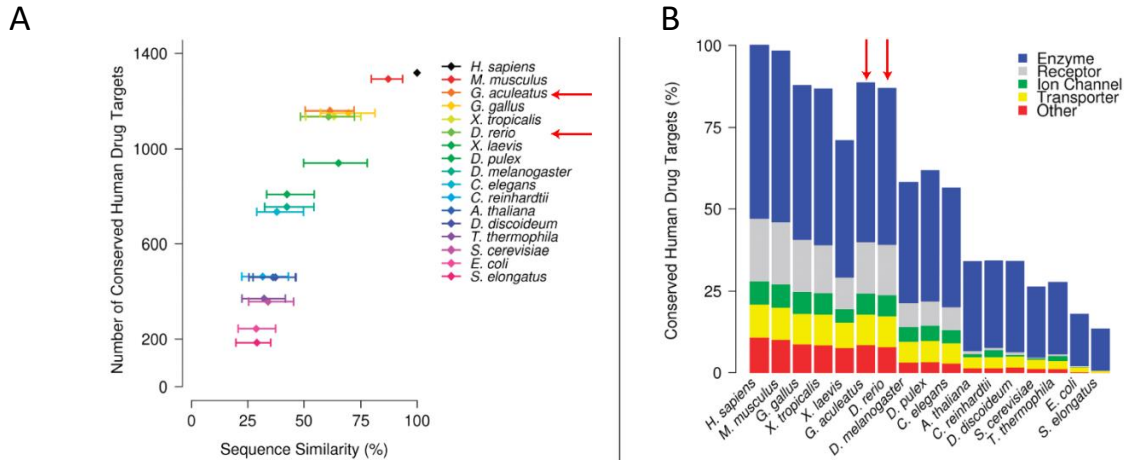
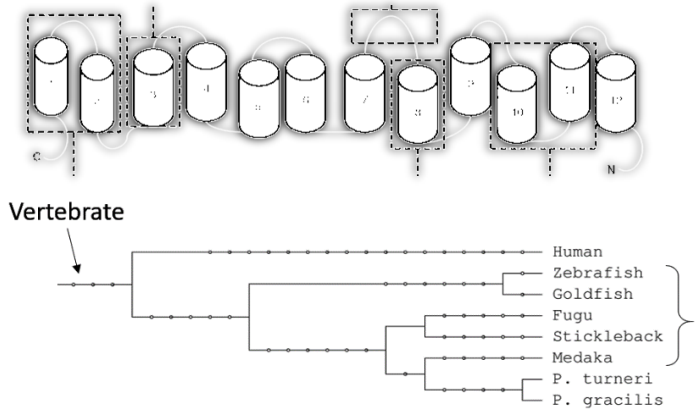


Figure 2: Similarity between conserved human drug targets compared between investigated vertebrate species

Left: Sequence similarity of human drug target genes in multiple vertebrates. G. aculeatus and D. rerio are among the most similar of compared vertebrates (~900 till ~1200 targets). Right: Percentage of conserved drug target functions in multiple vertebrates, ranked on GO categories. After M. musculus the most similarity is seen in G. aculeatus and D. rerio. (Gunnarsson 2008)

Also, when looking at the serotonin receptor, indirect evidence can be found which supports the idea of fish having a serotonin system. Mennigen et al. 2011 studied the serotonin receptor gene (SLC6A4) in vertebrates. Amino acids of the human SLC6A4 gene were compared to known amino acid sequences from vertebrates, including two zebrafish, a goldfish, stickleback, fugu, and a medaka (Figure 3). Comparison of the whole SLC6A4 gene showed that 69% of the human gene is identical to that of the researched fish. Furthermore, when comparing binding locations which SSRIs are known to bind on, 95% of the SLC6A4 gene in fish overlap with that of the human. This high similarity suggests that SSRIs used for human consumption should also bind well to the serotonin transporters in fish and likely elicits similar developmental and behavioural effects.



Serotonin Transporter Sequence Identity at the Amino Acid Level, Comparing Total Sequence Identity With Identity in Amino Acid Residues Involved in SSRI Recognition

Vertebrate	Conserved AA compared to human Slc6a4 (average %)	Conserved AA with known function in SSRI binding compared to human Slc6a4 (average %)
Mammals ($n = 2$)	89	94
Fish ($n = 5$)	69	95
Birds ($n = 3$)	80	75
Reptiles ($n = 1$)	85	89
Amphibians ($n = 1$)	72	76
Invertebrates ($n = 3$)	55	35

Figure 3: Serotonin transporter sequence similarity between the human SLC6A4 gene and known SERT transporter sequences in fish and other vertebrates

Compared fish species share a 95% coverage of amino acids with known function of SSRI binding sites of those seen in humans. Top left: schematic overview of human SERT transporter SSRI known binding locations (Mennigen et al. 2011).

With all these findings combined, we hypothesise that the Poeciliidae animal model will be an adequate model to study effects of fluoxetine treatment during pregnancy. Further confirmation on the Poeciliidae animal model's validity will be assessed by comparing effects induced by fluoxetine. These effects include weight, length, and gene expression deviations. Fluctuations in weight of the Poeciliidae mothers will be assessed. In a large human study, it was shown that mothers who take fluoxetine during their pregnancy tended to lose weight (Michelson, Amsterdam, Quitkin, et al. 1999). This weight change was also observed in rodents by Houwing et al. 2019. Rat mothers exposed to fluoxetine had a significantly lower body weight than their control counterparts during and after pregnancy. Following this study, we hypothesise that mothers from both P. turneri and P. gracilis will have the same effect and lose weight as a result of fluoxetine exposure during pregnancy.

P. turneri and P. gracilis offspring length and weight will also be measured to compare with rodent studies. In these rodent studies, it was observed by Houwing et al. 2019 that litter weight was effected when healthy mothers were exposed to fluoxetine during their pregnancy. Due to these findings, we hypothesise that offspring weight from fluoxetine exposed P. turneri will also be affected in the same manner. Zebrafish embryos which were exposed to the tricyclic antidepressant amitriptyline had a significant decrease in length (Yang et al. 2014). Amitriptyline has the same mechanism of action by inhibiting the reuptake of serotonin. Thus, we hypothesise that offspring length of P. turneri will be affected in a similar way to that seen in zebrafish, and consequently, the weight will also be reduced.

For P. gracilis, we expect that the offspring is shielded from fluoxetine exposure since all nutrients are stored in the yolk at the very beginning of development. After prolonged fluoxetine exposure, when mothers have had the opportunity to build up fluoxetine in their tissue, there is a possibility that stored fluoxetine is transferred into the yolk and would allow for exposure to the offspring. Overall, due to not exchanging nutrients with the offspring during the development, it is expected that P. gracilis offspring is not or insignificantly affected in weight and length.

Alterations in serotonergic and neurotrophic gene expression in the brain will also be studied to clarify if fluoxetine exposure can cause changes to gene expression levels. Genes of interest with a significant role in the serotonergic system, as well as genes which play a vital role in neurogenesis were selected (Table 1). These genes were also compared in sequence similarity to that of the human to verify to what degree the selected genes were comparable (Supplementary Table 1). By comparing the gene expression levels between P. turneri and P. gracilis, placentation and no placentation respectively, it is possible to identify the mediating role of the placenta. But also, it will be apparent if fluoxetine can disturb the natural gene expression levels in the brains of developing offspring.

Expectations are less straightforward as length and weight since genes may be part of complicated cascades. Selected genes of interest Netrin-1 and Pet-1 are attractive candidates due to their function in serotonergic neuron development, however, to the best of our knowledge, there is no existing literature which describes the effects of fluoxetine on these genes. With this research, we hope to shed light on changes in these expressions. Chronic exposure to fluoxetine might increase the gene expression of SLC6A4 since the brain is trying to compensate for an increased amount of serotonin between synapses. This idea is also supported by findings of Baudry et al. 2010 as a result of amplified noncoding RNA targeting SERT expression. Over sensitisation of serotonin receptors might also decrease the amount of HTR1a/b expression. MAO-A, a degradation enzyme which catalyses the oxidation of amines such as serotonin (Tipton et al. 2004), can be expected to become more active to reach homeostasis. Conversely, Song et al. 2015 saw that fluoxetine increased the amount of NGF expression. According to McGeary et al. 2011, an increase in NGF leads to a decrease in expression of HTR3a and MAO-A, which would conflict with the expectation of maintaining homeostasis by upregulating HTR1a/b and MAO-A. BDNF expression levels have been studied widely already. After

both short- and long-term exposure to fluoxetine, upregulation of BDNF was measured. Upregulation of BDNF is due to the activation of 5-HT4 receptors leading to activation of the CREB pathway stimulating expression of BDNF (Pascual-Brazo et al. 2012). Therefore, we expect to see similar results. Lastly, Fatemi et al. 2009 saw that fluoxetine exposure increases the levels of Reelin in rats. Following this study, we expect to see the same result.

Table 1: Selected genes of interest related to the serotonergic and neurotrophic system and their function

SLC6A4: Codes for the serotonin transporter which is involved in the regulation of available serotonin signalling. The serotonin transporter has the function to reuptake serotonin from the synaptic cleft for re-use or recycling (Coleman et al. 2016).	**Pet-1 (FEV):** Critical for the development of serotonergic neurons. Knockout of Pet-1 gene induces a loss of roughly 80% of serotonergic neurons (Tomohiro Kobayashi et al. 2008).
HTR1a: Responsible for the release of serotonin and is located on the pre- / post-synapse.	**HTR1b:** Responsible for the release of serotonin and is located on the pre- / post-synapse.
MAO-A: Catalyzes oxidation (degradation) of amines such as 5-HT, norepinephrine and epinephrine (Tipton et al. 2004).	**PGF:** Plays an important role in angiogenesis and vasculogenesis. Has a key role in embryogenesis (Chau et al. 2017).
NGF: Involved in neuronal differentiation, proliferation and survival (Carvalho et al. 2011). Mediates development and maintenance of sympathetic and sensory nervous systems (Einarsdottir et al. 2004).	**BDNF:** Important for survival and differentiation of neurons in the peripheral and central nervous system. Involved in axonal growth and pathfinding (Hofer et al. 1990). Mediates synaptic plasticity of the adult brain in a large number of areas in the CNS (Linnarsson et al. 1997).
Reelin: Important in layering neurons inside the cerebral cortex, modulates migration via cell-cell interactions, responsible for synaptic plasticity after the brain has finished developing (Bosch et al. 2016).	**Netrin-1:** Contributes to forming patterns and guiding axons (Gingrich et al. 2017).

A valid Poeciliidae model will offer the opportunity to study more side effects of fluoxetine, and possibly more SSRIs, on prenatally exposed offspring. The Poeciliidae animal model will also be able to give a better understanding of the placenta during pregnancy. Benefits of this new animal model compared to rodents are more efficient housing and handling of the animals. This also allows for a higher number of animals per experiment when compared to rodent studies. Further research could be focused on finding methods of stopping fluoxetine from entering the intrauterine environment. It is important to stress that this research is performed in healthy fish, whereas in the real situation, mothers suffer from depression, so translational aspects remain questionable to a certain degree.

Material and Methods

Fish Housing

Two animal models with different Poeciliidae species, Poeciliopsis turneri and Poeciliopsis gracilis were used to assess the possible effects of fluoxetine in offspring during pregnancy. Both species are viviparous (offspring develops inside the mother); however, P. turneri has a complex placenta, where P. gracilis has not. The complex placenta allows P. turneri to exchange nutrients between mother and offspring, P. gracilis has no nutrient exchange but provides this at the start of pregnancy in the yolk. Also, both species are superfetatious, meaning continuous conception during pregnancy, and thus offspring being born throughout the whole experiment.

Initial experiments were performed at the research facility 'Carus', at the University of Wageningen, The Netherlands. During tests, the animals were held in 10L tanks, at a constant temperature of 25°C ± 1, under a light-dark cycle of 12:12 hours. Temperature and water volume were kept at a steady level to prevent significant environmental changes. Animals were fed with Salt Lake Aquafeed Premium Artemia Cysts at 08:00 and fish flakes at 16:00. Before fluoxetine treatment started the animals were bred and standardised for age, length and other related characteristics.

An offspring of n=14 was desired for both P. turneri and P. gracilis species per dosage group. A higher amount of P. turneri mothers were used due to a lower expected clutch length (1-3) compared to P. gracilis (±30). In total, 56 P. gracilis mothers (8 per dose * 7 doses), and 70 P. turneri (10 per dose * 7 doses) were used. First, mothers were randomly assigned to a dosage group. Then, mothers of the same dosage group and species were housed in a tank as pairs.

Fluoxetine concentrations

A total of seven fluoxetine concentrations were prepared in the water of the tanks: 0 µg/L, 0.1 µg/L, 0.5 µg/L, 1 µg/L, 10 µg/L, 20 µg/L, 40 µg/L. Low concentrations, 0 µg/L till 1 µg/L, represent those which are found in nature, high concentrations, 10 µg/L till 40 µg/L, represent those similar to human therapeutic levels (Weinberger et al. 2014). Concentrations were made with fluoxetine pills acquired from Eli Lilly and Company (The Netherlands) and prepared in the laboratory of Groningen University, The Netherlands. Stock solutions of 0 µg/mL, 10 µg/mL, 100 µg/mL and 200 µg/mL fluoxetine in sterile water were made and kept at 4°C during the experiment. Fluoxetine was administered daily between 09:15 and 10:15 (adjusted for daylight savings) to the tanks individually.

Drainage of the water is filtered in a large tank, which is shared with other tanks of the same dosage group, and sent back to the individual tanks. Water samples of the tank were taken 30 minutes before and after fluoxetine administration to check if the desired fluoxetine concentrations were reached. This was done daily during the first week, after the first-week fluoxetine concentrations were checked weekly.

Offspring collection

During the six weeks of treatment, offspring that was born was collected daily. Due to the superfetatious nature of both species we were able to collect offspring that was exposed to fluoxetine during different time windows of gestation (Figure 4). Collection of offspring was performed before administration of fluoxetine with a small fishnet. Fish were directly sacrificed in MS222 and transferred to an Eppendorf cup filled with RNAlater. Cups were stored overnight at 4°C. Afterwards, excess

RNAlater was pipetted out and bodies were stored at -20°C. The number of offspring found both dead and alive were registered daily. After six weeks all mothers were sacrificed in MS222, length and wet body weight were measured to 0.01 mm and 0.01 g precision respectively three times and averaged.

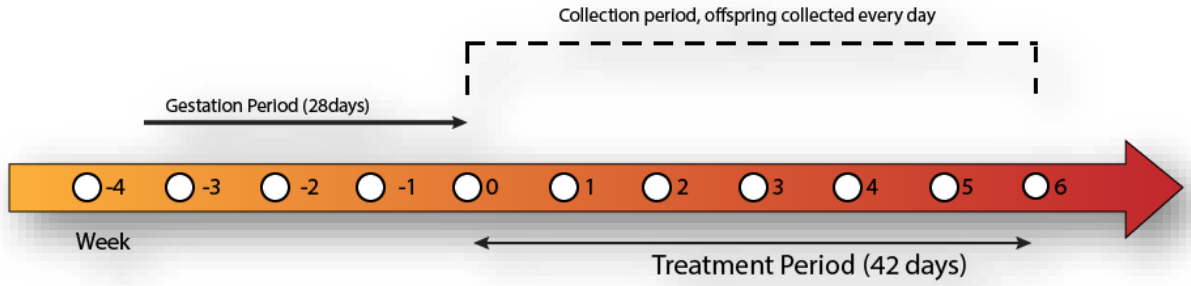


Figure 4: Schematic overview of fluoxetine treatment and offspring collection

P. turneri and P. gracilis mothers were first housed with males for four weeks as a gestation period. After four weeks males were removed and fluoxetine was introduced specific per dosage group. Due to both species being superfetatious, offspring were collected every day of the whole treatment period and directly sacrificed in MS222 and stored at -80° in RNAlater. After 42 days all mothers were sacrificed in MS222

Placenta dissection

Sacrificed mothers were dissected in order to obtain the placentas and embryos. Females were first transferred to an RNAlater filled petri dish before dissecting. Mothers were dissected from the anal fin and cut from the belly to the operculum (Figure 5-1). Then, a cut was made from the anal fin around the side of the fish up to the gills (Figure 5-2). Finally, the body cavity was opened from the side (Figure 5-3), and the ovaries were taken out. The ovaries were stored in an Eppendorf cup filled with RNAlater on ice. After the removal of the ovaries, the females head was cut, and both parts of the body were stored separately in a 12 ml Sterile Conical Centrifuge Tube filled with RNAlater on ice. All samples were then stored overnight at 4°C. Excess RNAlater was pipetted out and stored at -20°C.

Figure 5: Placenta dissection method. Source: Yuji Wang

1: Cut from anal fin to operculum
2: Cut from anal fin to gills
3: Body cavity opened via the side

This research was approved by CCD and Institutional Animal Care and Use permits

Mother and offspring measurements and brain dissection

The mother's and offspring bodies dry weight were weighed on a digital scale. The offspring were also measured for standard (from anterior to beginning of the tail fin) and total length (from anterior till posterior). For dissection of the mothers, the head was cut off with dissection scissors and transferred to a PBS petri dish cooled with dry ice from underneath. The offspring bodies were kept intact while dissecting. Dissected brains were brought over to an RNAse free 2.0ml Eppendorf cup filled with 500µL TRIzol™ Reagent (Invitrogen™) and a TissueLyser steel bead and ready for sample preparation and qPCR.

Brain dissection of Poeciliidae mother

With forceps, the soft tissue on the ventral side was removed until the skull bone was reached. The head was spread open by pushing on the cartilage bone of the eye sockets after the hard tissue around the eyes was pried away. In between the eye sockets, soft tissue was torn away until the optic nerves were visible. The optic nerves were cut with scissors, and the eyes were removed. Left and right lateral sides of the skull were pinched, close to the optic nerve endings, and carefully pulled away from each other to free the brain from the skull. A detailed video guide is available online (Haan, 2019).

Brain dissection of Poeciliidae offspring

First, the dorsal side of the head was torn open with precise forceps. After removing skull tissue, the brain is already visible and removed by first pulling on the medulla. Then, forceps were pushed under the brain and held by the optic nerves. By pulling on the optic nerve, the whole brain was freed from the further remaining intact body.

Sample preparation and qPCR

Total RNA was isolated from dissected brain tissue from both mother and offspring to assess gene expression of the 5-HT1a receptor. Tissue was homogenized in a TissueLyser II (Qiagen) on 30Hz for four minutes. Total RNA was isolated from the homogenized samples following the 'TRIzol Reagent User Guide' (ThermoFisher, Pub. No. MAN0001271). RNA concentrations and purity were measured using a NanoDrop 2000c (ThermoFisher). Next, RNA samples were cleaned from genomic DNA using DNase I Amplification Grade (ThermoFisher) and converted to cDNA in equal amounts using RevertAid H minus Reverse transcriptase and Oligo(dT) 18 primers (ThermoFisher).

Amplification through qPCR was performed with PowerUp SYBR Green Master Mix (AppliedBiosystems), primers used for amplification were designed in Geneious (Biomatters Ltd) using Primer3. For each reaction, 10 ng of cDNA and 500 nM of forward and reverse primer were used. Sequences of the target gene 5-HT1a, Beta-Actin (housekeeping gene), and qPCR schedule are displayed in table 3 and 4.

Table 3:qPCR gene of interest and reference gene sequence

Gene	Sequence	Product Length
5-HT1a	5′-TTCATTGTGGCGCTGGTTCT-3′	89
	3′-GAGTAGCCCAGCCAGTTGAT-5′	
Beta-Actin	5′- GCGACCTCACAGACTACCTC-3′	88
	3′- ATGTCACGCACGATTTCCCT-5′	

Table 4:qPCR cycle conditions

Step	Temperature	Duration	Cycles
UDG activation	50°C	2 minutes	Hold
Dual-Lock DNA polymerase	95°C	2 minutes	Hold
Denature	95°C	15 seconds	40
Anneal/Extend	60°C	1 minute	

Step	Ramp Rate	Temperature	Time
1	1.6°C/s	95°C	15 seconds
2	1.6°C/s	60°C	1 minute
3	0,5°C/s	95°C	15 seconds

Statistical Analysis

Mother and offspring weight and length were first analysed for normal distribution with a D'Agostino & Pearson test. If groups were normally distributed, a one-way ANOVA was performed. If there was a significant difference between groups, a post hoc Tukey's multiple comparisons test was used. In the case of non-normally distributed data, a Kruskal-Wallis (KW) test was performed with Dunn's multiple comparisons as a post hoc test. In the case of normal distribution in groups, but standard deviations were not assumed as equal a Brown-Forsythe and Welch ANOVA was done with Dunnett's T3 multiple comparisons as post hoc. Dosage groups were also analysed on different phases of the experiment. Week one and two are represented as phase one, week three and four as phase two, and week five and six as phase three. Phase separated groups were internally tested with a one-way ANOVA if normally distributed, or an unpaired t-test if not. When standard deviations were not assumed equal Welch's t-test was performed. Error bars represent standard error of mean. P-values ≤ 0.05 were considered as significant. All statistical analyses were performed in Graphpad Prism 8.

Results

In this study, we looked at the effects of the SSRI fluoxetine during pregnancy in P. turneri and P. gracilis mothers and offspring. In earlier research it was found that P. turneri had a noticeable decrease in cumulative amount of offspring born in higher fluoxetine concentrations, in P. gracilis this decrease was also observed but less severe (Supplementary table 2). To follow up on these results the length and body weight of both mothers and offspring were measured. Pilots on HTR1a gene expression were also performed.

Mothers Weight

In the analysis of P. turneri mothers, 10 and 20 µg/L were significantly different (Figure 6-A). In the analysis of P. gracilis mothers, groups which do not share the same letter differ significantly from each other with $p<0.05$ (Figure 6-B). p-values per comparison are displayed in supplementary table 3.

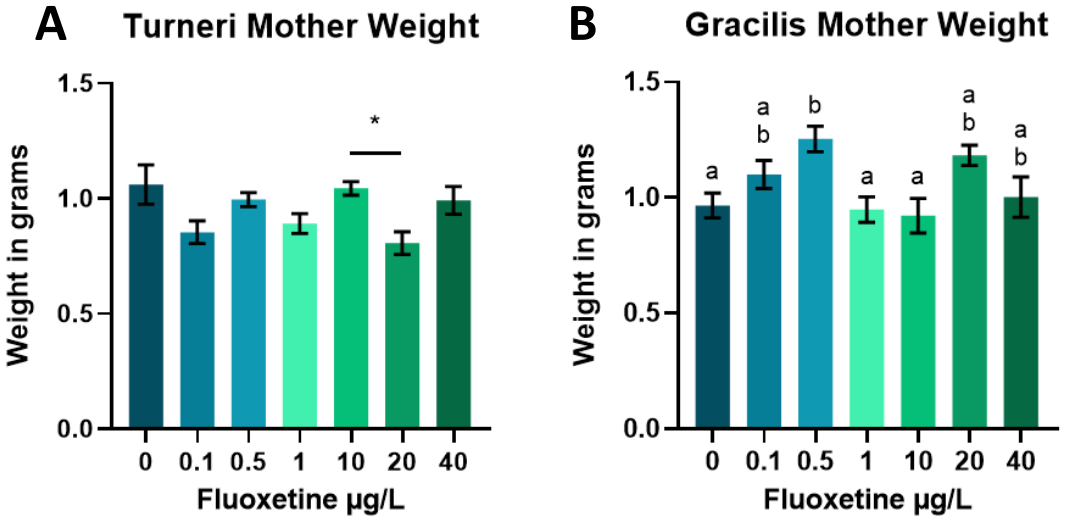


Figure 6: Weight of P. turneri and P. gracilis mothers after 6 weeks of fluoxetine treatments

Left: P. turneri weight. KW ANOVA revealed significance between groups ($H_{(19.42)}$; p=0.0035). DMC follow-up revealed significant difference between 10 and 20 µg/L (p=0.0079). Right: P. gracilis weight. One way ANOVA revealed a significant effect of fluoxetine at for all dosages [$F_{(6,101)}$ = 3.862; p=0.0016]. Tukey's multiple comparisons (TMC) revealed a significant difference between groups. Groups which do not share the same letter differ significantly with p<0.05 (Supplementary Table 3).

Offspring Weight

For P. turneri and P. gracilis offspring weight, groups which do not share the same letter differ significantly from each other with $p<0.05$ (Figure 7), p-values per combination is displayed in supplementary table 4.

For each fluoxetine dosage group of offspring weight, the exposure periods were compared. Each exposure period consisted of two weeks of fluoxetine treatment, divided into phase one: week 1-2, phase two: week 3-4, and phase three: week 5-6.

For P. turneri offspring, significant differences were found in dosage groups 0.5, and 20 µg/L (Figure 8-A). For 0.5 µg/L, phase one and three were significantly higher than two. For 20 µg/L, phase one was significantly higher than two. For P. gracilis offspring, significant differences were found in dosage groups 0.1, 1, 10, 20, and 40 µg/L (Figure 8-B). For 0.1 µg/L, weight was significantly higher in phase three compared to phase two. For 1µg/L, offspring were found to be lower in weight in phase two compared to one. For 10µg/L, offspring weighted more born in phase two compared to phase one and

three. For 20 µg/L, a slight increase in weight was observed in phase two compared to phase one. For 40 µg/L, a significant drop in weight was observed in phase two compared to phase one.

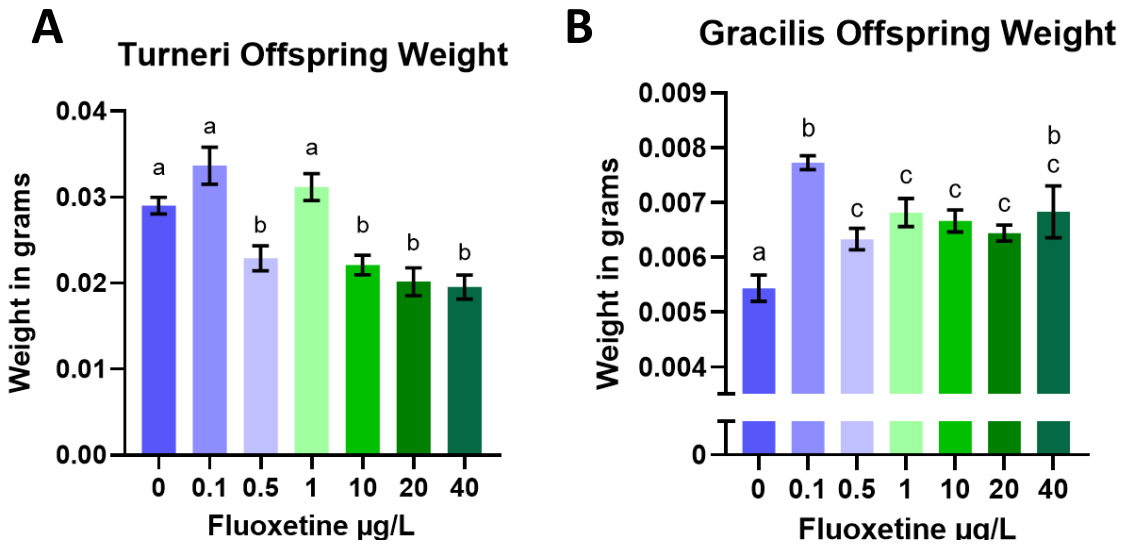


Figure 7: Weight of P. turneri and P. gracilis offspring after fluoxetine exposure

Left: P. turneri offspring weight. One-way ANOVA revealed a significant difference between different fluoxetine dosage groups [$F_{(6,164)} = 11.80$; $p<0.0001$]
Right: P. gracilis offspring weight. One-way ANOVA revealed a significant difference between different dosage groups at $p < 0.05$ [$F_{(6,208)} = 10.34$; $p<0.0001$] (Figure 7-2). Further analysis with TMC revealed significant differences between fluoxetine exposure groups in both P. turneri and P. gracilis offspring. Groups which do not share the same letter are significantly different for $p<0.05$ (Supplementary table 4)

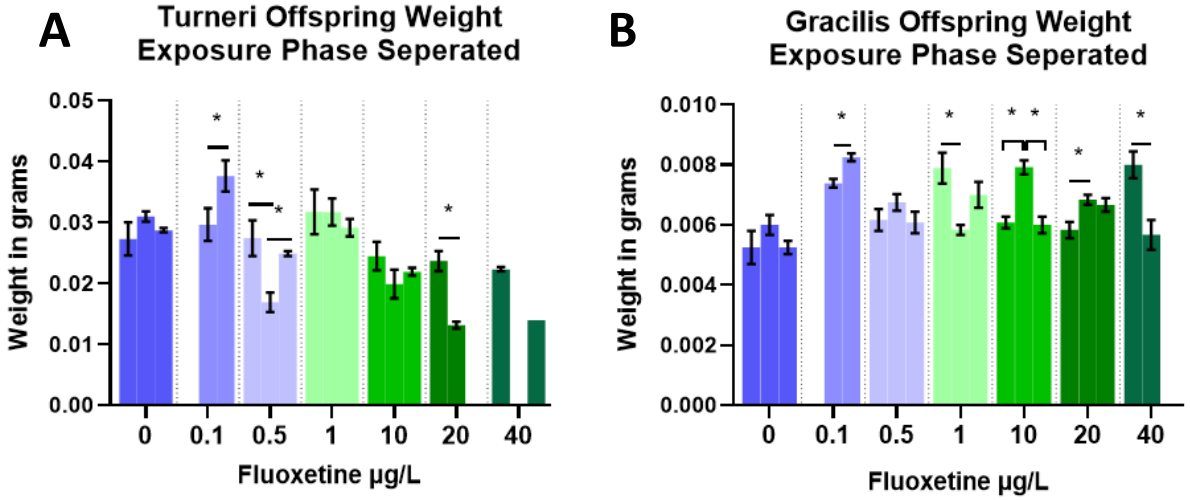


Figure 8: Phase separated weight of P. Turneri and P. Gracilis offspring after fluoxetine exposure

Left: P. turneri offspring weight phase separated. Significant differences were found in dosage groups 0.5, and 20 µg/L. For 0.5 µg/L, a KW ANOVA revealed a significant difference between phases (H(9.883); p=0.0071). DMC revealed a significant difference between phase one and two (p=0.0359), and between phase two and three (p=0.0136). For 20 µg/L, an unpaired t-test revealed a significant difference between phase one and two (p=0.0005).
Right: P. gracilis offspring weight phase separated. Significant differences were found in dosage groups 0.1, 1, 10, 20, and 40 µg/L. For 0.1 µg/L, a Kolmogorov-Smirnov (KS) test revealed a significant difference between phase two and three (p=0.0033). For 1µg/L, a one-way ANOVA revealed a significant difference between phases [F2,30) = 7.202; p=0.0028). TMC revealed a significant difference between phase one and two (p=0.0022). For 10µg/L, a KW ANOVA revealed a significant difference between phases (H(19.69); p<0.0001). DMC revealed a significant difference between phase one and two (p=0.0004) and phase two and three (p=0.0003). For 20 µg/L, a KW ANOVA revealed a significant difference between phases (H(7.616); p=0.0222). DMC revealed a significant difference between phase one and two (p=0.0245). For 40 µg/L, an unpaired t-test revealed a significant difference between phase one and two (p=0.0057).

Offspring length

P. turneri and P. gracilis offspring standard and total length were analysed, and groups which do not share the same letter are significantly different with a significance level of $p<0.05$ (Figure 9). For P. gracilis standard length, a 20µg/L concentration resulted in a shorter length in comparison to 1 µg/L, this was also true for 40µg/L. Multiple comparison p-values of P. turneri are shown in supplementary table 5 and p-values of P. gracilis are shown in supplementary table 6.

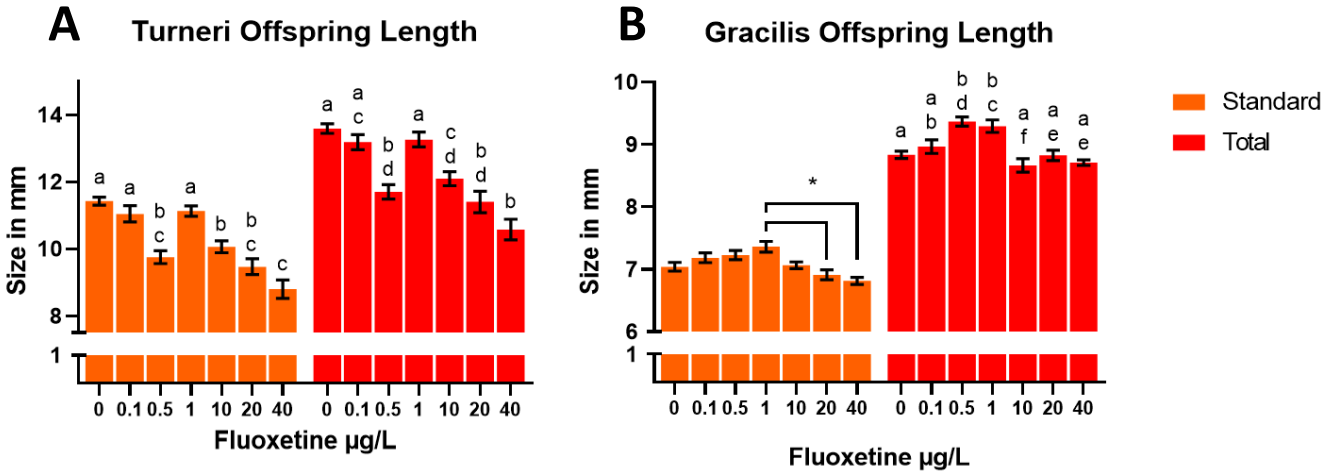


Figure 9: Standard and total length of P. turneri and P. gracilis offspring after fluoxetine exposure

Left: P. turneri offspring standard and total length. Both standard and total length were found to be significantly different by a one-way ANOVA [F(5, 129) = 27.10; p<0.0001] and [F(6, 164) = 19.00; p < 0.0001] respectively. TMC revealed further significant differences between all group combinations in standard and total length. Groups which do not share the same letter are significantly different with a significance level of p<0.05 (Supplementary Table 5).
Right: P. gracilis offspring standard and total length. Both standard and total length were found to be significantly different by a KW ANOVA, (H(24.96); p=0.0003) and (H(44.32); p<0.0001) respectively. Between standard and total length groups a DMC revealed further significant differences between all group combinations of standard and total length. For standard length groups, a significant difference was found between 1 and 20µg/L (p=0.0073), and between 1 and 40µg/L (p=0.0048). For total length, groups which do not share the same letter are significantly different with a significance level of p<0.05 (Supplementary table 6)

For each dosage group of P. turneri and P. gracilis offspring length, the exposure periods were compared. Each exposure period consisted of two weeks of fluoxetine treatment, divided into phase one: week 1-2, phase two: week 3-4, and phase three: week 5-6.

For P. turneri standard length, significant differences between phases were found in dosage groups 0.1, 1, 10, and 20µg/L fluoxetine (Figure 10-A). In group 0.1 µg/L, a longer exposure resulted in a higher length. In group 1 µg/L, it was found that the length of the offspring was successively lower with a higher exposure period. In group 10 µg/L, the second phase of offspring was found to be shorter in standard length. The same observation in group 10 µg/L was also made in group 20 µg/L

For P. turneri total length, significant differences between phases were found in dosage groups 0.1, 0.5, 1, and 20 µg/L fluoxetine (Figure 10-B). In group 0.1 µg/L a slight increase in length was observed as a result of a longer exposure period. In group 0.5 µg/, offspring were found to be shorter in phase two compared to phase one. Group 1 µg/L phase three offspring were slightly shorter than phase two. Offspring of group 20 µg/L were longer in phase one than in phase two.

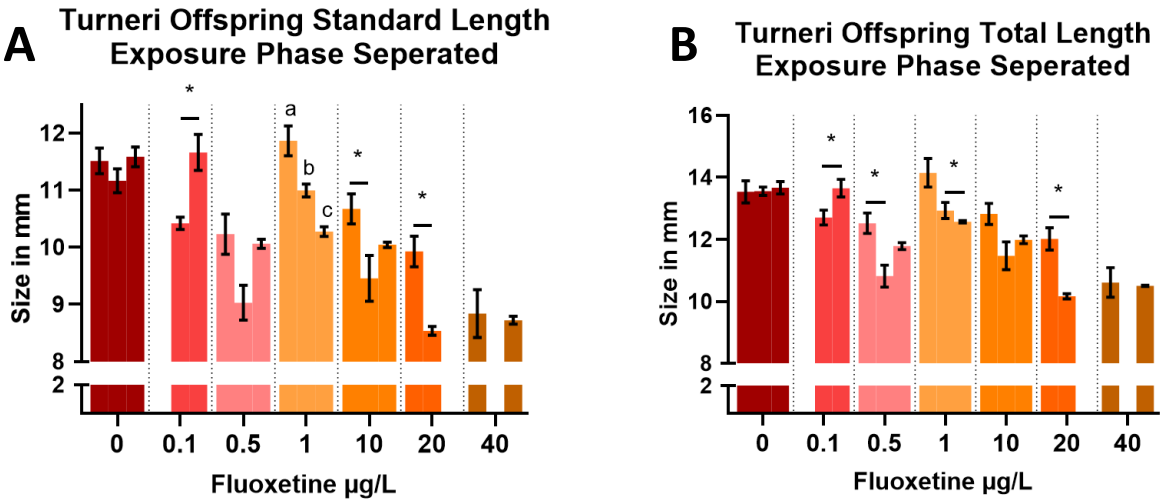


Figure 10: Phase separated standard and total length of P. turneri offspring after fluoxetine exposure

Left: P. turneri standard length. Significant differences between phases were found in dosage groups 0.1, 1, 10, and 20µg/L fluoxetine. In group 0.1 µg/L, Welch's t-test revealed a significant difference between phase two and three (p=0.0094). In group 1 µg/L, a one way ANOVA revealed significant differences between groups (F(2,24) = 16.67; p<0.0001), phases which do not share the same letter have a significant difference between them. In group 10 µg/L, a one way ANOVA revealed significant differences between groups (F(2,33) = 4.780; p=0.015), TMC showed a significant difference between phase one and two (p=0.011). In group 20 µg/L, a KS test revealed a significant difference between phase one and two (p=0.0001).
Right: P. turneri total length. Significant differences between phases were found in dosage groups 0.1, 0.5, 1, and 20 µg/L fluoxetine. In group 0.1 µg/L, an unpaired t-test revealed a significant difference between phase two and three (p=0.0285). In group 0.5 µg/L, a KW ANOVA showed significant differences between phases (p=0.044). DMC showed a significant difference between phase one and two (p=0.0393). In group 1 µg/L, Welch's ANOVA revealed a significant difference between phases (p=0.0116). Dunnett's T3 multiple comparisons (DTMC) found a significant difference between phase two and three (p=0.0249). In group 20 µg/L, a KS test revealed a significant difference between phase one and two (p=0.0001).

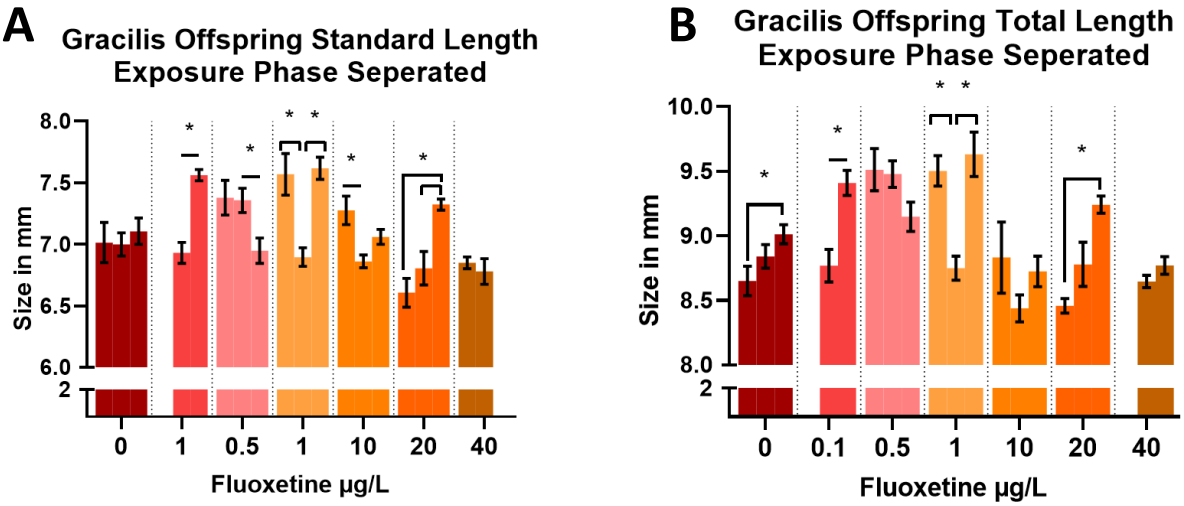


Figure 11: Phase separated standard and total length of P. Gracilis Offspring after fluoxetine exposure

Left: P. gracilis standard length phase separated. Significant differences between phases were found in dosage groups 0.1, 0.5, 1, 10, and 20µg/L fluoxetine. In group 0.1 µg/L, a KS test revealed a significant difference between phase two and three (p<0.0001). In group 0.5 µg/L, a KW ANOVA revealed a significant difference between phases (p=0.0199). DMC revealed a significant difference between phase two and three (p=0.0325). In group 1 µg/L, Welch's ANOVA revealed a significant difference between phases (p<0.001). DTMC revealed a significant difference between phases one and two (p=0.0071) and between phase two and three (p<0.0001). In group 10 µg/L, Welch's ANOVA revealed significant differences between phases (p=0.0062), DTMC revealed a significant difference between phase one and two (p=0.0153). In group 20 µg/L, Welch's ANOVA revealed a significant difference between phases (p<0.0001), DTMC revealed a significant difference between phase one and three (p=0.0002) and between phase two and three (p=0.0093).
Right: P. gracilis total length phase separated. Significant differences between phases were found in dosage groups 0, 0.1, 1, and 20µg/L fluoxetine (Figure 11-2). In group 0 µg/L, a one-way ANOVA revealed significant differences between phases [F2,33) = 3.694; p=0.0357], TMC revealed a significant difference between phase one and thee (p=0.0274). For group 0.1 µg/L, Welch's t-test revealed a significant difference between phase two and three (p=0.0005). For group 1 µg/L, a one-way ANOVA revealed significant differences between phases [F2,33) = 12.97; p<0.0001], TMC revealed significant differences between phase one and two (p=0.0009) and between phase two and three (p=0.0001). In group 20 µg/L, Welch's ANOVA revealed significant differences between phases (p<0.0001), DTMC revealed a significant difference between phase one and three (p<0.0001).

For P. gracilis standard length, significant differences between phases were found in dosage groups 0.1, 0.5, 1, 10, and 20µg/L fluoxetine (Figure 11-A). In group 0.1 µg/L, a longer exposure resulted in a longer length of offspring. In group 0.5 µg/L phase three offspring were shorter than those in phase two. In group 1 µg/L phases phase offspring were found to be shorter than phase one and three. In group 10 µg/L phase two was had shorter offspring than phase one. Finally, in group 20 µg/L, offspring from phase three were longer in comparison to both phase one and two.

For P. gracilis total length, significant differences between phases were found in dosage groups 0, 0.1, 1, and 20µg/L fluoxetine (Figure 11-B). In the control group 0 µg/L the offspring were longer in phase three than in phase one. In group 0.1 µg/L a longer exposure period resulted in a longer offspring. In group 1 µg/L the total length was lower in phase two than both phase one and three. Group 20 µg/L the offspring were longer in total length in phase three when compared to phase one.

Optimisation of qPCR

Genes related to the serotonergic system were selected (Table 1). HTR1a was chosen as the target gene to optimise the qPCR process since this gene was assumed to be one of the affected targets of fluoxetine.

In pilot one, recommended settings for a fast cycle and a total of 10 µL reaction volume were used. Amplification in negative controls in two samples, high standard deviation in replicate groups in eight samples, failure in the exponential algorithm in one sample, and multiple melting curve peaks in eight samples were observed. Next to that, early (cycle two-four) amplification was observed in 30 samples.

In pilot two, a slower ramp speed was selected instead of fast. Amplification in negative controls in two samples, a high standard deviation in replicate group in 12 samples, no amplification two samples, an outlier in a replicate group in one sample, failure in exponential algorithm in five samples, multiple melting curve peaks in eight samples, and early (cycle 2-4) amplification in 49 samples were observed.

In pilot three, new cDNA was made by vortexing well after DNAseI got added to the RNA samples. A high deviation between replicates in 27 samples, no amplification in 5 samples, failure in the exponential algorithm in 10 samples, multiple melting curves in 14 samples, and early (cycle two to four) amplification in 46 samples was observed. Product was placed on an agar gel to check for primer dimers and presence of DNA. Bands were seen on expected height, also in the negative control. No primer dimers were present. Smears were present in lanes where early amplification was observed (data not shown).

In pilot four, cDNA was remade by vortexing after every step of the protocol. In pilot four, the DNAseI was vortexed, in comparison of pilot three where this did not happen, before adding to the RNA samples. Also, all primers were diluted again from the stock on an RNA workbench instead of a DNA workbench. Two DNA dilutions were made for samples: 1x and 10x diluted. The PCR cycle was set to a fast ramp speed. There was amplification in all six negative controls (Figure 12-A), early (cycle two to four) amplification in one sample (Figure 12-B), and multiple melting curves in three samples (Figure 12-C) was observed. Also, a curving structure between cycle 6 and 18 appeared (Figure 12-D). Dilutions of 1x to 10x had a shift of ~15 cycles. A summary of all pilots is written in Table 4.

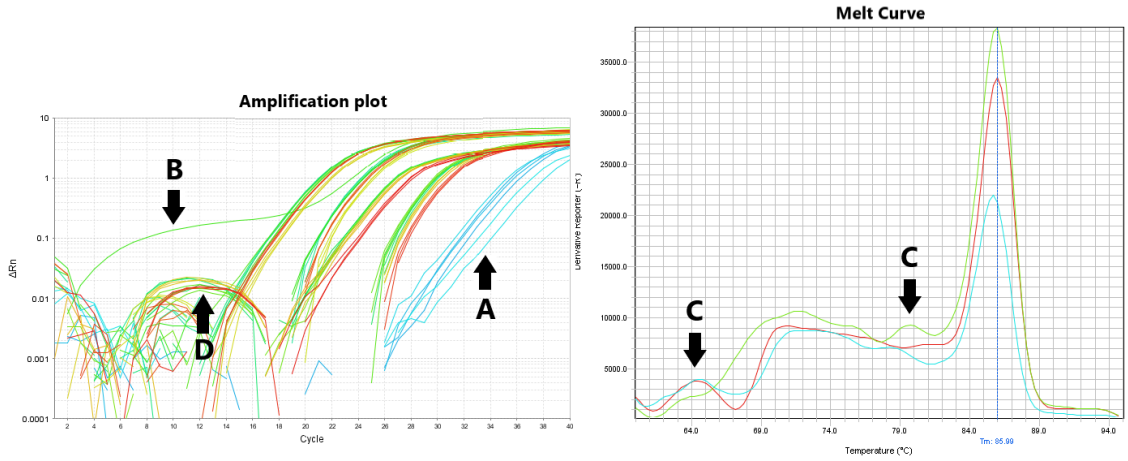


Figure 12: Overview of qPCR results of pilot four: Amplification plot (left) – Melt Curve (right)

A (left): Amplification in negative control. B (left): Early amplification from ~cycle 2, gain in amplification around cycle ~20. C (right): Multiple Tm peaks indicating non-pure product. D (left): Abnormal curving structure. Shift of ~10 cycles can be seen in both gene of interest (HTR1a) and housekeeping gene (beta-actin).

Table 4: Summary Pilots

Pilot	Characteristics	Observation
1	Recommended settings (Pub. No. 100031508) for a fast cycle and a total of 10 µL reaction volume. 54 samples used, including six negatives. Gene of interest: HTR1a, housekeeping gene: beta-actin.	- Negative control amplification: 2 - High deviation in replicates: 8 - Failure in exponential algorithm: 1 - Multiple melting curve peaks: 30 - Too early amplification: 30
2	Standard ramp speed instead of fast. 90 samples used, including six negatives. Gene of interest: HTR1a, housekeeping gene: beta-actin	- Negative control amplification: 2 - High deviation in replicates: 12 - Outlier in replicate group: 1 - Failure in exponential algorithm: 5 - Multiple melting curve peaks: 8 - Too earlier amplification: 49
3	New cDNA made by vortexing after DNAseI got added to RNA sample. 90 samples used, including six negatives. Gene of interest: HTR1a, housekeeping gene: beta-actin	- High deviation in replicates: 27 - No measurable amplification: 5 - Failure in exponential algorithm: 10 - Multiple melting curve peaks: 14 - Too early amplification: 46
4	Fast ramp speed. New cDNA made, vortexed during all of only vortexing after adding DNAseI. New primers made at RNA work bench instead of DNA work bench. Dilutions: 1x and 10x. Gene of interest: HTR1a, housekeeping gene: beta-actin	- Negative control amplification: 6 - Multiple melting curve peaks: 3 - Too early amplification: 1 - Curving structure between cycle 6 and 18 - Dilutions of 1x to 10x resulting in 15 cycle shift

Discussion

In this study we aimed to validate the Poeciliidae animal model. The Poeciliidae model consists of two viviparous superfetatious species, P. turneri with a complex amount of placentation, and P. gracilis with a low amount of placentation. Due to this unique combination of species, the role of the placenta during fluoxetine treatment could also be assessed. We studied this by treating pregnant Poeciliopsis females with varying amounts of fluoxetine concentrations, 0, 0.1, 0.5, 1, 10, 20, and 40 µg/L, for a period of six weeks. After this treatment period, the weight and length of offspring, and the weight of mothers were measured. Also, the measurements of HTR1a receptor expression in offspring were optimised (supplementary 7).

Acute fluoxetine exposure does not affect weight of mothers

No significant pattern was found in the bodyweight of both Poeciliidae females. In P. turneri, none of the fluoxetine groups differed significantly from the control. In P. gracilis, the group exposed to 0.5 µg/L fluoxetine was the only observed group to be significantly different from the control and weighed more. It was expected that weight would decline as a result of increased fluoxetine exposure. This has been shown in rodents by Houwing et al. 2019, where pregnant rats were exposed to fluoxetine and were observed to be lighter during and after pregnancy. In humans, it was also shown that acute treatment with fluoxetine leads to a decrease in weight (Michelson et al. 1999).

According to Scabia et al. 2018, fluoxetine affects the energy balance and leptin sensitivity via BDNF. It has been shown that BDNF is upregulated by fluoxetine during both short- and long-term exposure due to activation of the CREB pathway (Pascual-Brazo et al. 2012). Leptin, which regulates appetite, can signal to the brain to stop eating. Following an increase in sensitivity to leptin, a decrease in weight can be expected. However, to the best of our knowledge, the presence of a leptin gene was not found in all available annotated genomes in the Poeciliidae family present on NCBI. It would be interesting to find out if the Poeciliidae has a different form of digestive system as it not affected by the weight loss generally seen during fluoxetine treatment. Another reason for the absence of the expected decrease in weight could be that the BDNF levels of the Poeciliopsis species react differently than what was previously hypothesised. In future research, the expression levels of BDNF will be assessed in both the P. turneri and P. gracilis and can reveal more answers.

In the large human cohort of Michelson et al. 1999, 882 patients were exposed to fluoxetine and were observed to have a small but significant decrease in weight in the first 12 weeks. At a mean of 76,2 kg among all woman, only a ~1% significant decrease in mean weight was observed. Compared to the pregnant Poeicliidae, which sometimes differ ~20% in mean weight, it would be better to follow individual non-pregnant mothers to see if Poeciliidae are affected in weight or to have a larger pool of mothers to make a conclusion if the Poeciliidae model is comparable to rodents and humans in regard to weight change as result of fluoxetine treatment.

Fluoxetine induces length alterations in offspring

In P. gracilis offspring, it was found that an increase in fluoxetine concentration did not develop into a significant increase in length. We expected to see a shielding effect on the offspring since P. gracilis mothers provide all the nutrition in the yolk and to see less of this shielding effect at later phases of the experiment since the fluoxetine takes its time to reach the yolk. However, for standard length, group 20 and 40 µg/L was significantly lower than 1 µg/L, but not different from the control group. There tends to be an inversed u-shape, but this shape is not supported by significance. For total length, a slight inversed u-shape is noticeable where 0.1, 0.5 and 1 µg/L are higher than the control and 10, 20, and 40 µg/L.

This pattern might come forth due to how fluoxetine treatment affects P. gracilis. Research in Pimephales promelas (fathead minnow), and in Betta splendens (fighting fish), observed that low concentrations of 0.75 and 3 µg/L fluoxetine resulted in a decrease in predator avoidance and aggressive behaviour respectively (Lynn et al. 2007; Painter et al. 2009). But at higher fluoxetine concentrations, it has been shown in goldfish exposed to 54 µg/L fluoxetine that cortisol-releasing-hormone was increased and feeding was decreased (Mennigen et al. 2010). The same effect on cortisol has also been demonstrated in Oncorhynchus mykiss (rainbow trout), 54 µg/L fluoxetine exposure resulted in an increase in plasma cortisol (Mennigen et al. 2011). Although more evidence exists to support the finding that high fluoxetine concentrations result in higher cortisol levels, the opposite is seen as well. Zebrafish exposed to 100 µg/L of fluoxetine had a decrease in cortisol and matching anxiolytic behaviour (Egan et al. 2009).

If these fish species are comparable to the Poeciliidae, low concentrations of fluoxetine could lead to tranquil mothers interpreting the environment as safe, resulting in more nutrients going into the yolk. High concentrations of fluoxetine lead to an activated stress-axis and less feeding resulting in less nutrition provided in the yolk and thus smaller offspring. To support this theory, it is important to look for cortisol levels and related behavioural tests like predator avoidance in Poeciliidae mothers.

In P. turneri offspring, it was found that in a dose-responsive manner increased fluoxetine concentrations lead to a decreasing trend in standard and total length. This result was expected since earlier research done in zebrafish with comparable antidepressant amitriptyline also observed a decrease in offspring length when mothers were treated during pregnancy (Yang et al. 2014). Although amitriptyline is a tricyclic antidepressant, it has the same mechanism of action as fluoxetine. Also, the fluoxetine which is taken up by the mothers results in an increase in serotonin plasma concentration. Since serotonin has a vasoconstrictive ability, it is possible that the placenta is able to offer less post-fertilization provisioning resulting in a lower weight and length (Taniguchi et al. 1994). The same tranquil and stressed effect at low and high fluoxetine concentrations could be mentioned for P. turneri as well. However, an increase in length is not observed for the lower concentration, which could be due to the vasoconstrictive characteristics of serotonin being dominant over the post-fertilization provisioning increase.

Differences between exposure periods of the offspring during gestation were also analysed to see if fluoxetine posed a different effect on offspring over time. Phases were divided into phase one (week one and two), phase two (week three and four), and finally phase three (week five and six).

P. turneri offspring were found to be longer after a longer exposure time at a low concentration for both standard and total length. At higher concentration, the reverse was observed. Expectations were to see a more significant effect of fluoxetine at later phases since it is known that SSRIs take a few weeks to take effect (Taylor et al. 2006). More dominantly, we expect to see a more prominent effect at later phases because the offspring have been exposed to fluoxetine for a longer period. However, this expected pattern is not convincing as results only converge with the hypothesis for some concentration. Also, significant differences between phases were only commonly seen between two out of three with no apparent pattern. Future research might benefit from increasing offspring sample length to evade large SEMs which are present in current data sets.

For P. gracilis standard length, significant differences between phases were found in groups 0.1, 0.5, 1, 10, 20 µg/L fluoxetine. For total length, significant differences were found between phases in groups 0, 0.1, 1, and 20 µg/L fluoxetine. We expected to see close to no effect at early phases, and small effects at later phases. For standard and total length, no clear patterns emerge, which is probably due

to sample size being too low. Also, there is already a significant increase in total length seen for the 0 µg/L control group, which is an interesting observation since no changes over time were expected.

Fluoxetine induces weight alterations in offspring

In P. turneri offspring, it was found that an increase in fluoxetine concentration leads to a decrease in body weight. This was expected as this effect on offspring was also seen in rats and zebrafish (Houwing et al. 2019; Yang et al. 2014). This result converges with results observed for length, as it would make sense that a smaller organism is also a lighter organism.

In P. gracilis offspring, it was found that fluoxetine exposure leads to an increase in weight, but this effect was not observed in a dose-dependent manner. We hypothesised that, due to P. gracilis providing all nutrients in the yolk, the offspring would be shielded from the fluoxetine exchange between their mothers resulting in no effects observed in early phases. The shielding effect could be less in later phases as the mothers have had time to take up fluoxetine and transfer this to the yolk. This effect was not seen as any concentration used resulted in a significant increase in weight unrelated to phase. The length of P. gracilis offspring tends to follow an inversed-u shape; it is unclear why the weight change does not follow the same pattern.

To get a better insight at what's going on during the development of P. gracilis we would look into the content of the yolk to see whether or not fluoxetine reaches the offspring or if the length and weight changes are actually due to changes in the mother and not the child. In future research, we will also focus on the gene expression in this offspring which will tell more about changes caused by the fluoxetine treatment. Behavioural experiments will also be performed to see how the offspring reacts on a behavioural level.

Placentation complexity affects the response to fluoxetine exposure

In P. turneri offspring, we found a dose-dependent correlation between fluoxetine exposure and resulting body weight and body length. For P. gracilis offspring, an inversed u-shape tends to be created when increasing fluoxetine concentrations, and weight of the offspring got increased for all used concentrations. Also, P. turneri had a noticeable decrease in the cumulative amount of offspring born in higher fluoxetine concentrations. In P. gracilis, this decrease was also observed but less severe. These differences in results suggest that the placenta has a mediating role during fluoxetine treatment in pregnant Poeciliidae.

How the placenta plays a role is unknown, and it will take more research to find out how offspring are affected by the fluoxetine treatment. First, it is essential to know whether the fluoxetine was taken up by the mothers. This can be done by analysing the tissue of the mother for the presence of fluoxetine, as it is known that over time this antidepressant is able to be stored in various tissues like the bile, liver, lung, kidney and more (Johnson et al. 2007). Next, it is essential to know if the fluoxetine reaches the offspring. In humans, it has been found that 60-70% of the administered fluoxetine is found back in the offspring in its metabolised form (Heikkinen et al. 2002; Hendrick et al. 2003; Rampono et al. 2009; Sit et al. 2011). With Liquid chromatography-mass spectrometry, it is possible to image fluoxetine and norfluoxetine in the offspring's tissue to see if the drug got transported from the mother. Also, staining the brain might reveal where precisely the fluoxetine is most present if present at all. For P. gracilis, which has a no placentation, it would be interesting to see if the offspring is also affected by fluoxetine since there is no nutrient exchange during pregnancy. This can be done by analysing the yolk of the eggs for the presence of fluoxetine as previously mentioned.

Conclusion

In the current study, we have looked at the effects fluoxetine can have on viviparous superfetatious P. turneri and P. gracilis pregnant females and offspring. We have done this by treating pregnant mothers with varying doses of fluoxetine (0, 0.1, 0.5, 1, 10, 20, 40 µg/L) for six weeks and collected the offspring each day of the week during the treatment period. We chose two closely related species which have a high amount of placentation (P. turneri), and no placentation (P. gracilis). Bodyweight of both mothers and offspring, and standard and total length of the offspring were measured to find morphological changes after fluoxetine exposure. Also, we looked at the gene expression of HTR1a, which transcribes the crucial 5-HT1a receptor necessary for serotonin signalling. However, the gene expression essay is still in its optimisation phase. By comparing results between P. turneri which has a nutrient exchange between offspring and mother during pregnancy, and P. gracilis which provides all nutrients needed in a yolk at the beginning of the pregnancy, we aimed to find an answer as to how the placenta plays a role during the exposure of fluoxetine. With these results, we would also be able to verify if the Poeciliopsis is a reliable and translatable animal model for future similar research.

We hypothesized to see a decrease in weight of the mothers as this is seen in rats and humans (Houwing et al. 2019; Michelson et al. 1999). However, it was observed that the weights of both species mothers were not affected by the fluoxetine treatment. This result might have to do with the way fluoxetine can affect weight. Fluoxetine is able to affect the energy balance and leptin sensitivity through BDNF (Pascual-Brazo et al. 2012). To the best of our knowledge, the leptin gene is not present in the Poeciliopsis genome which could explain no effect on weight. BDNF could also be differently affected than previously tested species and thus not affect leptin sensitivity in case the leptin system is present in Poeciliopsis. Lastly, observed results could be due to too high standard deviations within data sets, making weight changes un-observable.

It was expected to see a drop in P. gracilis offspring length as this effect was seen as a result of antidepressant amitriptyline affecting the SERT in zebrafish (Yang et al. 2014). This specific effect was not observed. However, there was a tendency present of an inversed u-shape in both standard and total length. This effect could be explained by mothers experiencing the environment as harmless at low concentrations due to the effect fluoxetine has on mood and behaviour, and stressful at very high concentrations due to a raised stress-axis (Lynn et al. 2007; Mennigen et al. 2010, 2011; Painter et al. 2009). P. turneri offspring was hypothesized, like P. gracilis, to be smaller than its control counterparts. This expectation was met by observing a dose-response curve where more fluoxetine resulted in a lower offspring standard and total length. Fluoxetine has a vasoconstrictive ability which might lower post-fertilization provisioning (Taniguchi et al. 1994).

P. turneri body weight was expected to be reduced as a result of fluoxetine treatment as this has also been seen in rats and zebrafish (Houwing et al. 2019; Yang et al. 2014). Results converge with this expectation; weight was observed to be lower, which makes sense as a lower length results in a lower weight. For P. gracilis, the unexpected result was found that the weight of the offspring went up for all fluoxetine concentrations. How the offspring of P. gracilis became heavier but not bigger in standard and total length is not clear yet, and more research is needed to elucidate this curious finding.

This is the first research described in Poeciliopsis that looks at the effect of fluoxetine treatment during pregnancy on offspring. One of the objectives was to validate if the Poeciliopsis is a suitable animal model for researching antidepressive drugs and if results are translatable to those of rodents and humans. Although there are still important questions to be answered, results suggest that the Poeciliopsis model is a viable candidate since P. turneri offspring results are comparable to those seen

in humans and rodents. An apparent difference arose between placentation and no placentation meaning the placenta likely has a role during the fluoxetine exposed pregnancy and letting the offspring be affected by the antidepressant. The next goal is to confirm if the fluoxetine reached to the offspring by doing fluorescent imaging and analysing the tissue of both mother and offspring. Also, it is critical to elucidate whether the P. gracilis mothers transfer fluoxetine into the yolk, which might affect the development of the offspring. By looking at selected genes of interest which relate to the serotonin and neurological system (Table 1), we can reveal more insights into how the offspring are affected. Insights into changes which arise from fluoxetine exposure during pregnancy can offer more considered choices for pregnant mothers who are experiencing depressive symptoms. By preventing adverse outcomes of such antidepressive treatments, if proven, unnecessary harm to the offspring can be prevented on the long-term.

Citations

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Supplementary data

Supplementary 1: *Amino acid sequences from Homo sapiens were gathered from Uniprot and compared through BLAST against the specific taxa mentioned. Protein names and functions from the fish species were predicted by 'The NCBI Eukaryotic Genome Annotation Pipeline', although it is assumable that found comparisons are accurate, there is a possibility of false positives due to the annotation service. Overall, these BLAST results suggest that the Poeciliidae family are similar, to a certain degree, to that of the mouse and human when comparing selected genes related to the serotonergic system.*

Supplementary 1: BLAST similarities between human, rat and Poeciliidae family genes of interest

Included Poeciliidae family species are: Poecilia fermosa, Poecilia latipinna, Poecilia Mexicana, Poecilia reticulata, Xiphophorus maculatus and Gambusia. G. affinis lacked gene annotations and showed up with protein names 'hypothetical protein', however, similarity percentages were similarly to other Poeciliidae species which makes these hypothetical proteins assumable to hold the same name and function. R. norvegicus has the highest similarity between amino acid sequences of genes of interest. Similarity between fish species range between 58% and 88%

Human Gene	Compared organism	Similarity
SLC6A4	R. norvegicus	90%
	P. fermosa	77%
	P. latipinna	77%
	P. mexicana	77%
	G. affinis	76%
	P. reticulata	72%
HTR1a	Rattus norvegicus	90%
	P. fermosa	72%
	P. reticulata	72%
	G. affinis	72%
	X. maculatus	71%
Pet-1 (FEV)	R. norvegicus	96%
	P. mexicana	61%
	P. reticulata	61%
	P. formosa	61%
	X. maculatus	61%
	G. affinis	62%
NGF	R. norvegicus	86%
	X. maculatus	59%
	P. reticulata	58%
	P. fermosa	58%
	G. affinis	54%
PGF	G. affinis	67%
	R. norvegicus	65%
	P. reticulata	65%
	X. maculatus	59%
	P. formosa	58%
	P. latipinna	58%

Human Gene	Compared organism	Similarity
MAO-A	R. norvegicus	88%
	P. formosa	68%
	P. mexicana	68%
	P. reticulata	68%
	P. latipinna	68%
	G. affinis	68%
BDNF	R. norvegicus	97%
	X. maculatus	72%
	P. fermosa	71%
	P. reticulata	71%
	P. mexicana	71%
	P. latipinna	71%
Reelin	R. norvegicus	95%
	P. latipinna	70%
	P. mexicana	69%
	P. fermosa	69%
	P. reticulata	69%
Netrin-1	R. norvegicus	99%
	P. latipinna	88%
	P. formosa	88%
	P. reiculata	88%
	X. maculatus	86%
	G. affinis	86%

Supplementary 2: *Cumulative amount of offspring from P. turneri and P. gracilis separated in phases. Concentration values are represented as µg/L fluoxetine.*

P. turneri

µg/L	Phase 1	Phase 2	Phase 3
0	10	9	15
0.1	8	5	2
0.5	12	11	4
1	7	7	2
10	14	6	6
20	5	2	
40	7		1

P. gracilis

µg/L	Phase 1	Phase 2	Phase 3
0	8	9	36
0.1	28	29	34
0.5	29	16	6
1	32	22	20
10	34	36	20
20	43	30	5
40	15	30	

Supplementary 3: *Tukey's Multiple comparisons of P. Gracilis Mother weight after six weeks of fluoxetine exposure. Groups values are represented as µg/L fluoxetine. Not mentioned combinations were non-significant.*

P. gracilis		P Value
0 vs. 0.5	*	0.0345
0.5 vs. 1	*	0.0123
0.5 vs. 10	**	0.0079

Supplementary 4: *Tukey's Multiple comparisons of weight of P. turneri and P. gracilis offspring weight after fluoxetine exposure. Group values are represented as µg/L fluoxetine. Not mentioned combinations were non-significant.*

P. turneri		P Value
0 vs. 0.5	**	0.0084
0 vs. 10	**	0.0011
0 vs. 20	***	0.0005
0 vs. 40	**	0.0083
0.1 vs. 0.5	***	0.0003
0.1 vs. 10	****	<0.0001
0.1 vs. 20	****	<0.0001
0.1 vs. 40	***	0.0003
0.5 vs. 1	***	0.0003
1 vs. 10	****	<0.0001
1 vs. 20	****	<0.0001
1 vs. 40	***	0.0007

P. gracilis		P Value
0 vs. 0.1	****	< 0.0001
0 vs. 0.5	*	0.0347
0 vs. 1	***	0.0001
0 vs. 10	***	0.0006
0 vs. 20	*	0.0104
0 vs. 40	*	0.011
0.1 vs. 0.5	****	< 0.0001
0.1 vs. 1	*	0.0401
0.1 vs. 10	**	0.0064
0.1 vs. 20	***	0.0004

Supplementary 5: *Tukey's Multiple Comparisons of standard and total length of P. turneri offspring after fluoxetine exposure. Group values are represented as µg/L fluoxetine. Not mentioned combinations were non-significant.*

P. turneri standard		P Value
0 vs. 0.5	****	<0.0001
0 vs. 20	****	<0.0001
0 vs. 40	****	<0.0001
0.1 vs. 0.5	***	0.0005
0.1 vs. 20	****	<0.0001
0.1 vs. 40	****	<0.0001
0.5 vs. 1	****	<0.0001
1 vs. 20	****	<0.0001
1 vs. 40	****	<0.0001

P. turneri total		P Value
0 vs. 0.5	****	<0.0001
0 vs. 10	****	<0.0001
0 vs. 20	****	<0.0001
0 vs. 40	****	<0.0001
0.1 vs. 0.5	**	0.0027
0.1 vs. 20	***	0.0007
0.1 vs. 40	****	<0.0001
0.5 vs. 1	****	<0.0001
1 vs. 10	**	0.0015
1 vs. 20	****	<0.0001
1 vs. 40	****	<0.0001
10 vs. 40	**	0.0072

Supplementary 6: *Dunns's Multiple Comparisons of standard and total length of P. gracilis offspring after fluoxetine exposure. Group values are represented as µg/L fluoxetine. Not mentioned combinations were non-significant.*

P. gracilis standard		P Value
1 vs. 20	**	0.0073
1 vs. 40	**	0.0048

P. gracilis total		P Value
0 vs. 0.5	**	0.0013
0 vs. 1	*	0.0362
0.5 vs. 10	****	<0.0001
0.5 vs. 20	***	0.001
0.5 vs. 40	**	0.0036
1 vs. 10	***	0.0003
1 vs. 20	*	0.0287
1 vs. 40	*	0.0382

Supplementary 7: *Optimisation of qPCR discussion*

Box 1. Optimisation of qPCR

Gene expression in P. turneri and P. gracilis is not commonly analysed and posed a challenge to set up during the experiment. Four custom HTR1a primer sets for P. turneri and P. gracilis were first created and tested at different concentration combinations to analyse product and presence of primer dimers. The four sets of primers were created from a fragment of the whole genome sequence of mentioned species. The amino acid sequence is not publicly available and was delivered by collaborators in Wageningen. Created primer pairs did not cross over an intron, as the gene of interest's sequence did not contain an annotation of this. All sets of primers were tested on genomic DNA extracted from the hind fin of the mothers and tested in an ordinary PCR machine to find the optimal primer pair. Each set of primers worked well regarding expected band height and absence of primer dimers (data not shown). Experiments were continued with the primer pair which resulted in the cleanest looking bands (Table 3).

For pilot one, qPCR standard cycle condition settings (Pub. No. 100031508) were followed as recommended by 'applied biosystems', manufacturer of used SYBR Green Master Mix to use as a base to start from. Fast cycle was selected and recommended by experienced colleagues to evade primers binding to off-target sequences. The low reaction volume of 10 µL was selected to save costs. Results were non-optimal and contained amplification in negative controls, high standard deviations in replicate groups, multiple melting curve peaks, and early amplification. These results suggest that contamination of samples occurred since negative control samples, which should not have contained any amino acids, contained amplification. Contamination could also have been the case for early amplification. Due to seeing high deviation in replicate groups as well, it was concluded that results were because of human error during the preparation of the qPCR plate.

For pilot two, a standard ramp speed was selected to make sure ramp speed was not the cause of non-optimal results. The qPCR plate was set up like pilot one, this time making sure no human errors were introduced. Results were again non-optimal containing amplification in negative controls, a lot of high deviations in replicate groups, early replication, and multiple melting curves. Human error was eliminated during the preparation of the qPCR plate, meaning the cause of non-optimal results lied in the presence of a contamination. To eliminate the source of contamination cDNA was remade, but this time making sure DNAseI was well mixed with RNA samples by vortexing. Propper vortexing was not done before as it was not considered important for the process.

For pilot three, new cDNA was used. However, results came back non-optimal again with a large number of samples with high deviation in replicates, multiple melting curves, and early amplification. This time however, no amplification was measured in negative controls. Products of pilot two and three were placed on an agar gel to verify the source of contamination which was still present after making new cDNA. The agar gel revealed smears in lanes of samples which had early amplification and a visible band around 100 bp for the negative control. We found that although samples were well mixed after adding DNAseI, DNAseI should have been vortexed as well before adding to RNA samples. DNAseI is delivered in a highly viscous mixture and is thus not easily homogenised. cDNA was made again but this time vortexing during every step of the process ensuring that no traces of DNA were left in the RNA samples. Also, primers were diluted again, this time on the RNA bench, from stock to also avoid contamination coming from here.

For pilot four, new cDNA and new primers were used, and a fast cycle speed was selected again due to fast cycle speed being more recommended over standard. Results showed amplification in most negative controls, however, in only one sample early amplification was observed and multiple melting curves in only three. These results meant that the contamination was almost removed. Finally, it was realised that primers were initially diluted at a DNA bench which might have contaminated the initial stock. Also, it was concluded that a high probability existed of a contaminated lab space. The latter was derived from non-related experiments where qPCR also resulted in non-optimal results. Only after plates being initially prepared in another lab room, and only brought back to add cDNA sample resulted in optimal results it was concluded that the workspace must be contaminated.

For future experiments, every chemical and mixture was thrown away and re-ordered to ensure no contaminations.