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To cite this version:

Julie de Oliveira, Edith Chadili, Cyril Turies, François Brion, Xavier Cousin, et al.. A comparison of behavioral and reproductive parameters between wild-type, transgenic and mutant zebrafish: Could they all be considered the same “zebrafish” for reglementary assays on endocrine disruption?. Comparative Biochemistry and Physiology - Part C: Toxicology and Pharmacology, 2021, 239, pp.108879. 10.1016/j.cbpc.2020.108879 . hal-03183773

HAL Id: hal-03183773

https://hal.science/hal-03183773v1

Submitted on 30 Jun 2021

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A comparison of behavioral and reproductive parameters between wild-type, transgenic and mutant zebrafish: could they all be considered the same “zebrafish” for reglementary assays on endocrine disruption?

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Highlights:

- No critical difference in behavior (anxiety, sociality) between strains

- No significant difference in reproductive parameters between strains

- The transgene insertion and the mutations had no influence on the endpoints measured

- The use of transgenic zebrafish appears relevant for the study of EDC

Abstract

Transgenic zebrafish models are efficiently used to study the effects of endocrine disrupting chemicals (EDC); thereby informing on their mechanisms of action. However, given the reported differences between zebrafish strains at the genetical, physiological and behavioral levels; care should be taken before using these transgenic models for EDC testing. In the present study, we undertook a set of experiments in different transgenic and/or mutant zebrafish lines of interest for EDC testing: *casper, cyp19a1a-eGFP, cyp19a1a-eGFP-casper, cyp11c1-eGFP, cyp11c1-eGFP-casper*. Some behavioral traits, and some biochemical and reproductive physiological endpoints commonly used in EDC testing were assessed and compared to those obtained in WT AB zebrafish to ensure that transgene insertion and/or mutations do not negatively modify basal reproductive physiology or behavior of the fish.

Sociality was evaluated by monitoring the time spent near congeners in a shuttle box while anxiety was evaluated using the Novel tank diving test. No critical difference was observed between strains for either sociality or anxiety level. Concerning reproduction, no significant difference in the number of eggs laid per female, in the viability of eggs or in the female circulating VTG concentrations was noted between the 5 transgenic/mutants and the WT AB zebrafish studied.

In summary, the transgene insertion and the mutations had no influence on the endpoints measured suggesting these transgenic/mutant models can thus be relevant tools for EDC testing. More studies are needed to validate the use of these models to refine existing fish assays for EDC.

Keywords: Zebrafish, casper, transgenic, behavior, reproduction.

I. Introduction

Zebrafish (*Danio rerio*) is a cyprinid fish native from India and nearby countries (Engeszer *et al.* 2007) that is currently the most widely used aquatic organism in laboratories. Zebrafish success arises from its many advantages, notably a low cost of breeding, a short life cycle, a small size, transparent embryos and larvae and a fully sequenced genome. Therefore, this model species is used in several fields of biology, such as genetics (Kegel *et al.* 2019, Sabharwal *et al.* 2019), biology of development (Gore *et al.* 2018, Horsfield 2019), neurosciences (Joly 2017), biomedical research (Al-Samadi *et al.* 2019, Gaudenzi and Vitale 2019), and ecotoxicology (Almeida *et al.* 2019, Valadas *et al.* 2019).

Most of the scientific articles published on zebrafish do not refer to wild-caught strains (native) but rather to so-called “wild-type” (WT) zebrafish strains. This “wild-type” term covers several different strains used in laboratories including strains with no clear information on their genetic background (bought from commercial pet stores) to highly domesticated strains such as AB, Tübingen (TU), and other more recently domesticated as Wild India Kolkata (WIK) and TM1 for example. In addition, numerous strains of mutant zebrafish, such as *casper* (White *et al.* 2008), *leopard, longfin* (Haffter *et al.* 1996, Van Eeden *et al.* 1996) or *albinos* (Haffter *et al.* 1996, Kelsh *et al.* 1996), are being used for their particular features, mainly reduced pigmentation which improves transparency.

It is becoming increasingly obvious that all these zebrafish strains present differences at the genetical, physiological and behavioral levels. Regarding genetic (allelic richness, heterozygosity, nucleotide diversity), wild-caught zebrafish are more variable than the different laboratory strains compelling researchers to consider potential effects of genetic variation in experimental design and interpretation, especially for extrapolation of data to the population level (Guryev *et al.* 2006, Coe *et al.* 2009, Wilson *et al*. 2014, Balik-Meisner *et al.* 2018, Suurväli *et al.* 2019). Baseline mRNA expression profiles as well as baseline endocrine axis activity (cortisol secretion) also differ between zebrafish strains rendering reproducibility of studies in

zebrafish challenging (Drew *et al.* 2012, Gorissen *et al.* 2015, Van Den Bos *et al.* 2017, Holden and Brown 2018). Wild-type (AB) and mutant *casper* zebrafish also respond differently, *casper* being more resistant to fasting than AB (London and Volkoff 2019). In the end, while some authors report no behavioral difference between some zebrafish strains (WT, mutant, transgenic) (Snekser *et al.* 2006, Parker *et al.* 2013), a great number of studies argue for significant differences in diverse behaviors such as for example color conditioning, inhibitory avoidance, circadian rhythms, swimming capability, stress and anxiety or social dynamic, either between wild-caught and laboratory strains or between different laboratory strains (Egan *et al.* 2009, Vital and Martins 2011, Dereje *et al.* 2012, Drew *et al.* 2012, Vignet *et al.* 2013, Gorissen *et al.* 2015, Mustafa *et al.* 2019, Wakamatsu *et al.* 2019).

Finally, during the last decades, due to the development of new genome editing technologies (Tol2 mediated transgenesis, bacterial artificial chromosome-based transgenesis, TALEs, CRISPR/Cas9…), a huge number of transgenic zebrafish models has emerged worldwide (Albadri *et al.* 2017, Kawahara 2017). Several of these transgenic models have been efficiently used to study the effects of chemicals, especially endocrine disrupting chemicals (EDC), in aquatic organisms (Gorelick and Halpern 2011, Brion *et al.* 2012, Gorelick *et al.* 2014, Hinfray *et al.* 2016, Hinfray *et al.* 2018, De Oliveira *et al.* 2020). Given the above-mentioned differences reported between zebrafish strains, care should be taken before using transgenic models for assessing the effects of EDC. Indeed, a transgene insertion and/or a mutation might modify reproductive physiology or behavior of the fish impeding their use for EDC testing or leading to misleading interpretation of the transgenic-based assays.

In that context, we thus undertook a set of experiments in different transgenic and/or mutant zebrafish lines of interest for EDC testing: *casper, cyp19a1a-eGFP, cyp19a1a-eGFP-casper, cyp11c1-eGFP, cyp11c1-eGFP-casp*er. Some behavioral traits as well as some critical biochemical and reproductive physiological endpoints that are commonly used in screening tests, notably in a regulatory perspective (e.g., OECD TG 229 and TG 230) were assessed and the acquired data were compared to those obtained in WT AB zebrafish.

2. Materials and Methods

All experiments conducted in this study were performed in agreement with the Directive 2010/63/EU.

2.1. Fish strains and maintenance

In the present study, six zebrafish strains were used. They are described in the Table 1. Zebrafish were raised in our laboratory facility at INERIS (Institut National de l'Environnement Industriel et des Risques, Verneuil-en-Halatte, France). They were maintained in 3.5 L aquaria in the same recirculation system (Zebtec, Tecniplast, Buguggiate, Italy) on a 14:10 light:dark cycle at a temperature of 27.0 ± 2.0°C. Fish were fed twice a day, the morning with flake food (SDS400, Scientific Fish Food) and the afternoon with newly hatched brine shrimp (Sep-Art, Ocean Nutrition Europe).

Table 1: Description of the six zebrafish models used in this study.

Zebrafish line	**Description**	**Number of generations in INERIS laboratory**	**Strains references**
Wild type AB	Laboratory strain of wild type zebrafish. The AB strain was obtained from Dr Rosa from ENS in 2002 and regularly breed with AB fish from ZIRC since 2010.	> 20	/
Casper	Zebrafish (AB genetic background) with a double mutation for *roy* and *nacre* genes, devoid of pigmentation	6	White *et al.* 2008
Cyp19a1a-eGFP	Transgenic zebrafish expressing the enhanced GFP under the control of the zebrafish *cyp19a1a* gene promoter (AB genetic background)	10	Hinfray *et al.* 2018 De Oliveira *et al.* 2020
Cyp19a1a-eGFP-casper	Breeding between the *casper* mutant and the *cyp19a1a-eGFP* transgenic strains	7	/
Cyp11c1-eGFP	Transgenic zebrafish expressing the enhanced GFP under the control of the zebrafish *cyp11c1* gene promoter (AB genetic background)	8	Supplementary data
Cyp11c1-eGFP-casper	Breeding between the *casper* mutant and the *cyp11c1-eGFP* transgenic strains	5	/

2.2. Behavioral challenges

Two behaviors were assessed in this study, i.e. anxiety and sociality, because of their potential influence on the reproductive success of fish. Behavioral experiments were conducted on sexually mature fish of 5 months old. A total of 12 individuals (6 males and 6 females) of each zebrafish strains were used. Behavioral experiments were performed in a dedicated room at 27±1°C, with a photoperiod synchronized with the rearing room. On the first day, at 16:00, the fish were placed in individual plastic aquariums containing one liter of system water, in the behavioral challenges room, for acclimation. On the second day, behavioral challenges took place between 9:00 and 18:00.

The two behavioral challenges were performed in the same aquarium starting with anxiety and finishing with sociality. Water was changed every 5 fish. The experimental device consisted of a Plexiglas® Novel tank (12 cm in height, 12 cm in width and 24 cm in length) joined on to a second Plexiglas® aquarium containing 6 congeners (3 males and 3 females; same phenotype as the experimental fish). This second aquarium is separated from the principal aquarium by a Plexiglas® barrier, preventing the exchange of water and other molecules between the two aquariums. The water of the “congeners” aquarium was changed regularly.

Anxiety was evaluated by performing a novel tank challenge. During this challenge, the second aquarium containing congeners is hidden by an automatic door. The acclimated fish is transferred to the Novel tank and activity recorded for 5 minutes as described by Vignet *et al.* (2013). For space occupancy analysis, the arena is virtually divided horizontally into two zones of equal volume. By analyzing the time spent in each zone, this test allows to determine the level of anxiety of each individual.

Once the anxiety challenge was over, the sociality assay began by the opening of the automatic door between the two aquariums. The test started once the door was open, and activity is recorded for 6 minutes. For this challenge based on procedure describe by Blaser and Gerlai (2006), the arena was virtually divided into three zones named according to the

proximity of the congeners as proximate, intermediate and distant zones, in order to determine in which zone the fish spends more time.

Videos of both tests were recorded with an analogue camera ICD-48E (Ikegami) and 2.7–13.5-mm lens (Fujinon) linked to a PC with an acquisition card and EthoVision XT 10.1 software (Noldus, The Netherlands). EthoVision software was also used for tracking and data extraction.

2.3. Monitoring of reproductive parameters

2.3.1. Female fecundity and viability of eggs

10 adult zebrafish (5 males and 5 females; 6-10 months old) of each line of interest, were randomly placed in aquariums containing 7 liters of water and aeration system (3-13 replicated aquarium per zebrafish line) in a continuous flow-through system with constant water renewing (about 10 times a day). Water quality was monitored every morning: temperature (27.1 ± 0.0°C), pH (7.9± 0.0), dissolved oxygen (96.9 ± 0.5 %), conductivity (377 ± 5 µS/cm), as well as nitrates, nitrites and ammonium concentrations (below detection limits). Photoperiod cycle was maintained to 14:10 hours light/dark. Fish were fed as described previously for regular rearing.

After a 7-days acclimation period, reproduction monitoring began for a 15-days period. Eggs were collected every morning in rectangular glass containers, protected by a green mesh lid. Eggs were disinfected 5 minutes in water containing 0.1% commercial bleach (2.6% of sodium hypochlorite). In the early afternoon, eggs were then classified into 3 different categories: unfertilized, nonviable (not exceeding the 16-cells stage) and viable eggs using a SteREO Discovery.V8 stereomicroscope (Carl Zeiss, Germany). Finally, fertilization rate (number of fertilized eggs / total number of eggs) and viability rate (number of viable eggs / numbers of fertilized eggs) were calculated for each strain.

2.3.2. Fish sampling

After the breeding period, fish were euthanized with MS222 (200 mg/L), weighted and measured. 5 to 10 µl of blood of each individual was collected and transferred to tubes containing 1:10 v:v phosphate buffered saline (PBS), heparin (2000 U / ml), glycerol (20%) and PMSF (0.2 mM) and stored at -80 ° C for subsequent circulating vitellogenin concentration analysis. Gonads were then removed and weighed. The somatic mass (total fish body weight (mg) minus gonads weight (mg)) and the gonadosomatic index (GSI = gonad weight [mg] / body weight [mg] x 100) were determined.

2.3.3. Vitellogenin (VTG) ELISA

Circulating VTG concentrations were measured in whole blood by indirect competitive ELISA using a liquid handling robot Freedom EVO® (Tecan, Switzerland) according to the protocol described in De Oliveira *et al.* (2020). Briefly, whole blood samples were tested in 12 serial dilutions with a step of 3 (dilutions ranging from 1:500 to 1:88,573,500 for females and 1:100 to 1:17,714,700 for males). Zebrafish purified VTG was used as standard. The samples/standard dilutions were pre-incubated with the primary antibody (DR-264 zebrafish anti-VTG antibody, Biosense Laboratories, Norway; final dilution of 1:500 in PBS-BSA 1%) and deposited on a 384-wells plate, before addition of the secondary antibody (horseradish peroxidase goat anti-rabbit IgG; 1:2000 in PBS-BSA 1%). Once the enzymatic peroxidase reaction was stopped with the addition of phosphoric acid (1 M), the plates were read at 450 nm on a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (Biotek™, USA). The raw data were analyzed on the BioTek™ GEN5 software.

2.4. Statistical analysis

The data presented in this study are all expressed as mean ± standard error (SD). The normality of the data and the homogeneity of the variance were analyzed with Shapiro-Wilk and Levene's tests respectively. For non-parametric data, the Kruskal-Wallis and Wilcoxon-Mann-Whitney tests were performed. In the case of data following a normal distribution,

analysis of variance (ANOVA), followed by a post-hoc test (Tukey's HSD test), were carried out, to highlight the differences observed between the different zebrafish strains. All statistical analyzes were carried out at a 95% level of significance and significant differences between strains were indicated by different letters or asterisks on graphs.

3. Results

3.1. Fish survival and growth

No mortality was observed neither during the behavioral challenges nor during the monitoring of reproduction whatever the zebrafish line.

Physiological parameters are reported in Table 2 for the 6 zebrafish strains. Several differences in body weight and length were observed between the zebrafish strains as indicated by the different letters in the Table 2. One noticeable trend is that the body weights and lengths of *casper* strains, except the *cyp19a1a-eGFP-casper* strains, tend to be broadly smaller than those of *non-casper* strains, whatever the sex.

Comparison of GSI of all zebrafish strains showed that in females, no significant difference was observable while in males, the *cyp11c1-eGFP* zebrafish had a slightly higher GSI than all other zebrafish strains.

Table 2: Physiological parameters of zebrafish strains used in the present study. Data are represented as mean ± SD (n = 15 – 58 per groups). Different letters represent a significant difference between strains within a sex ($p < 0.05$).

Zebrafish strains	**Weight (mg)**		**Length (mm)**		**GSI**		**Age (month)**
	Females	**Males**	**Females**	**Males**	**Females**	**Males**	
wild type AB	490.5 ± 135.1 [ac]	396.2 ± 52.6 [abc]	35.2 ± 3.3 [a]	34.6 ± 2.8 [ab]	11.7 ± 5.2	1.0 ± 0.3 [a]	5 - 8
cyp19a1a-eGFP	497.5 ± 108.6 [a]	444.4 ± 87.3 [ad]	35.1 ± 3.9 [a]	35.1 ± 4.5 [ac]	11.5 ± 3.6	1.3 ± 0.3 [b]	5 - 9
cyp11c1-eGFP	549.3 ± 135.5 [a]	470.0 ± 58.2 [d]	37.0 ± 4.6 [a]	36.4 ± 4.1 [c]	11.3 ±3.4	2.1 ± 0.5 [d]	5 - 8
casper	382.2 ± 136.5 [b]	373.7 ± 58.6 [bc]	32.5 ± 3.4 [b]	33.4 ± 3.1 [b]	10.8 ± 3.2	1.5 ± 0.5 [bc]	5 - 9
cyp19a1a-eGFP casper	447.3 ± 102.4 [c]	419.1 ± 52.6 [acd]	34.3 ± 2.5 [ac]	33.8 ± 2.7 [ab]	11.9 ± 3.8	1.2 ± 0.6 [abc]	5 - 10
cyp11c1-eGFP casper	379.4 ± 125.0 [b]	365.2 ± 56.8 [b]	33.8 ± 3.1 [bc]	33.4 ± 2.7 [ab]	9.5 ± 2.9	1.7 ± 0.6 [c]	5 - 6

3.2. Fish behavior

3.2.1. Anxiety Test

Whatever the zebrafish line no statistical difference was found between male and female data, thus data from the two sexes were grouped. The results are presented in the Figure 1.

The *cyp11c1-eGFP* line spent significantly more time in the bottom zone than the WT AB, *casper* and *cyp11c1-eGFP-casper* strains. When this is analyzed by minutes using a repeated measures ANOVA there is only a trend ($p=0.055$) and post hoc pointed out some sporadic differences with lower time in top zone for *cyp11c1-eGFP* line during minutes 2 and 3 (data not shown). For all 6 strains, however, the time spent in the bottom zone was significantly greater, compared to the time spent in the top zone.

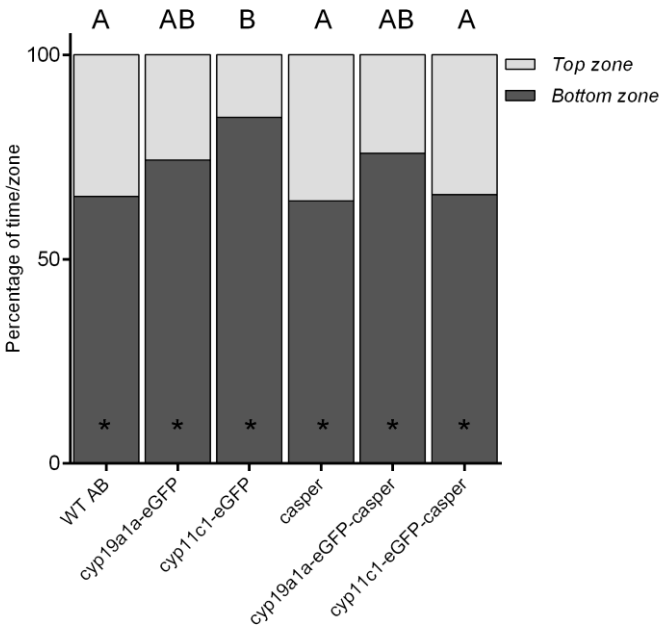

Figure 1: Novel tank assay (anxiety): time (seconds) spent in the top and bottom zone of the tank for the 6 zebrafish strains. The data are mean ± SD. Different letters represent significant differences between strains while asterisks represent differences between zones for each strain ($p < 0.05$, n = 12 per group).

3.2.2. Sociality test

Whatever the zebrafish line no statistical difference was found between male and female data, thus data from the two sexes were grouped. The results are presented in the Figure 2.

Whatever the area of the aquarium considered, no significant difference was shown between WT AB, *cyp19a1a-eGFP, casper* and *cyp19a1a-eGFP-casper*. The *cyp11c1-eGFP*, *casper, cyp19a1a-eGFP-casper* and *the cyp11c1-eGFP-casper* strains spent significantly more time in the intermediate zone as compared to the distant zone; whereas the 2 other zebrafish strains spent equal time in these two zones. For all the zebrafish strains however, the time spent in the proximate zone is significantly higher compared to the two other zones (between 72 and 93% of the time). Nevertheless, *cyp11c1-eGFP* strain spends overall a bit less time in the proximate zone compared to WT AB*, casper* and *cyp19a1a-eGFP-casper* strains. In addition, *cyp11c1-eGFP-casper* spends less time in the proximate zone compared to WT AB strain.

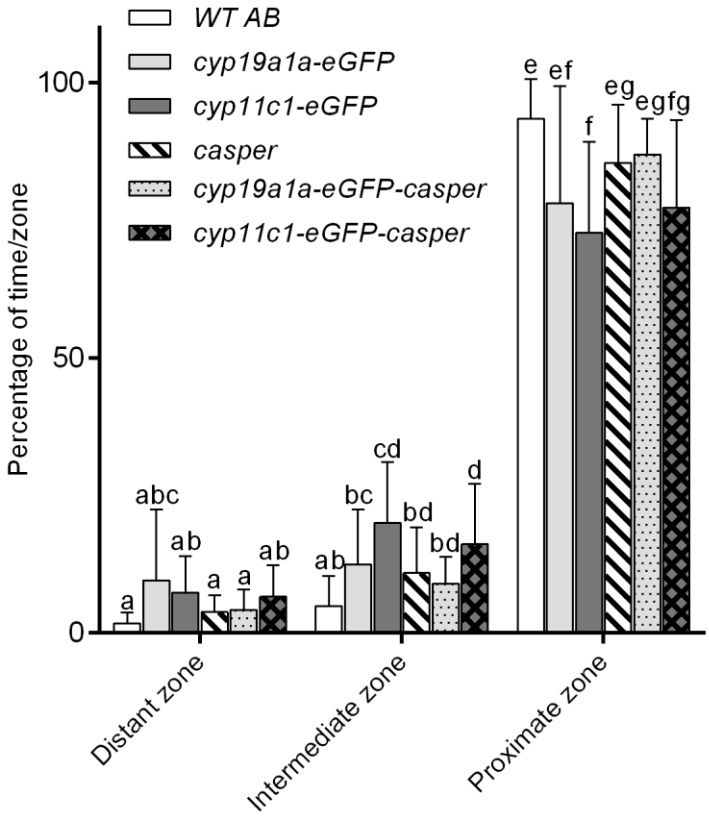

Figure 2: Sociality assay: time (seconds) spent in different zones of the aquarium (Distant, intermediate and proximate zone) for the 6 zebrafish strains. The data are mean ± SD. Different letters represent the significant differences between the different strains as well as between the different zones ($p < 0.05$, n = 10-11 per group).

3.3. Reproduction parameters

3.3.1. Monitoring of egg-laying

The daily monitoring of the number of eggs laid per female revealed no difference between the WT AB, the *cyp19a1a-eGFP* and the 3 *casper* strains (*casper*, *cyp11c1-GFP-casper* and *cyp19a1a-eGFP-casper*) (Figure 3A). However, the number of eggs laid per female per day is significantly greater for the *cyp11c1-GFP* line (28.7 ± 2.1 eggs/female/day) than for the other strains (< 24 eggs/female/day), exept WT AB and *cyp19a1a-eGFP-casper* line.

The *casper* strains (*casper, cyp11c1-GFP-casper* and *cyp19a1a-eGFP-casper*) appeared to lay broadly fewer eggs/female/day compared to the other strains (Figure 3A). These *casper* fish were also smaller and thinner than the *non-casper* strains (Table 2). A linear

correlation between the mean number of eggs laid per female per day for each aquarium and the mean somatic mass of the fish of the aquarium was thus established (Figure 3B; Pearson's test: cor=0.66, $p<0.01$). This correlation allowed normalization of the fecundity data (Figure 3A) by the mean somatic mass of the fish. After normalization, no significant difference in the "fecundity" was observable between the 6 strains (Figure 3C), indicating that differences in the number of eggs laid per female per day were mainly due to differences in the body-mass of females.

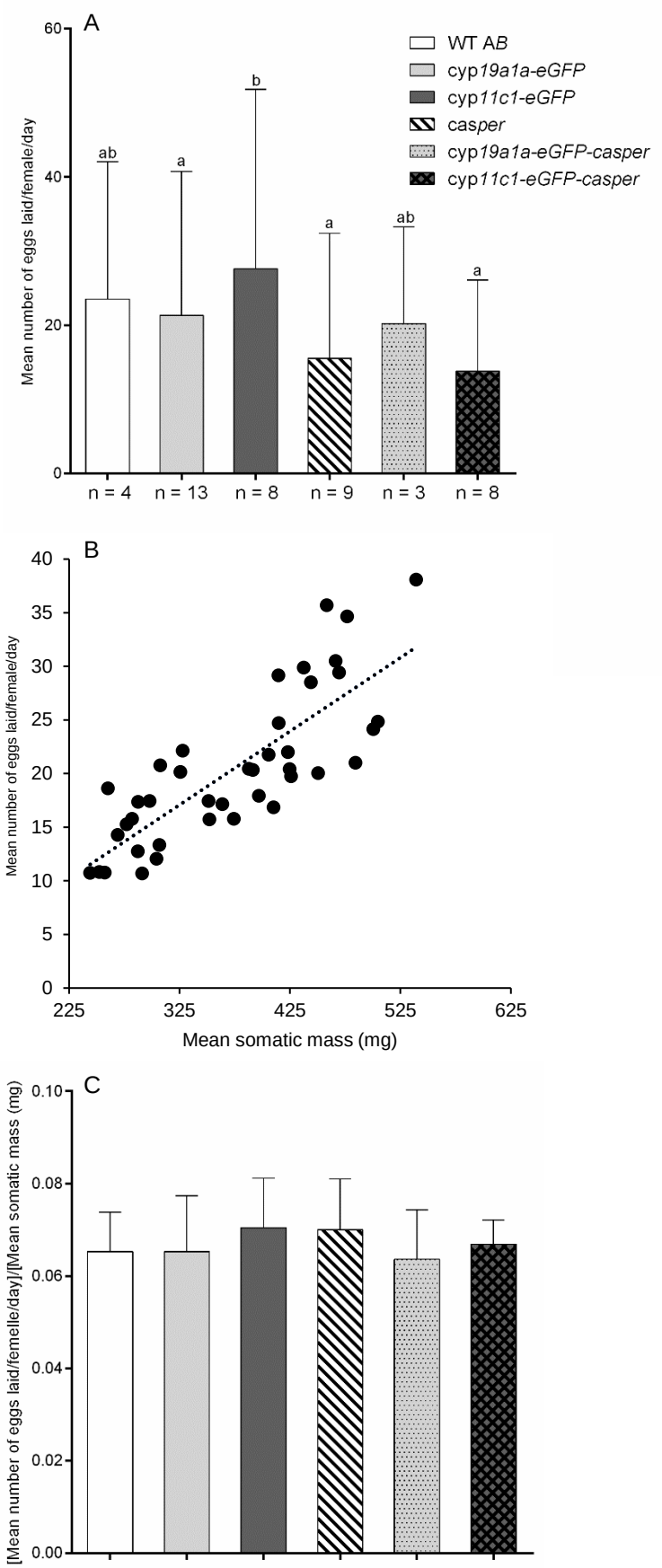

Figure 3: Reproductive parameters of the fish of the 6 strains. (A) Mean number of eggs laid/female/day for the 6 strains of zebrafish. Data are mean ± SD (n = 3 – 13 replicate tanks). Different letters represent

significant difference between strains ($p<0.05$). (B) Correlation between the mean number of eggs laid per female per day and the mean somatic mass of females (per aquarium) (Pearson's correlation test, cor = 0.66, $p<0.01$). (C) Mean fecundity normalized with mean somatic mass of each aquarium for all zebrafish strains. Data are mean ± SD.

3.3.2. Viability of eggs

Fertilization success showed no significant difference between the different zebrafish strains (data not shown). Likewise, viability of eggs after the first sorting (about 4 to 8 hpf) was equivalent for the 6 strains of zebrafish (Figure 4).

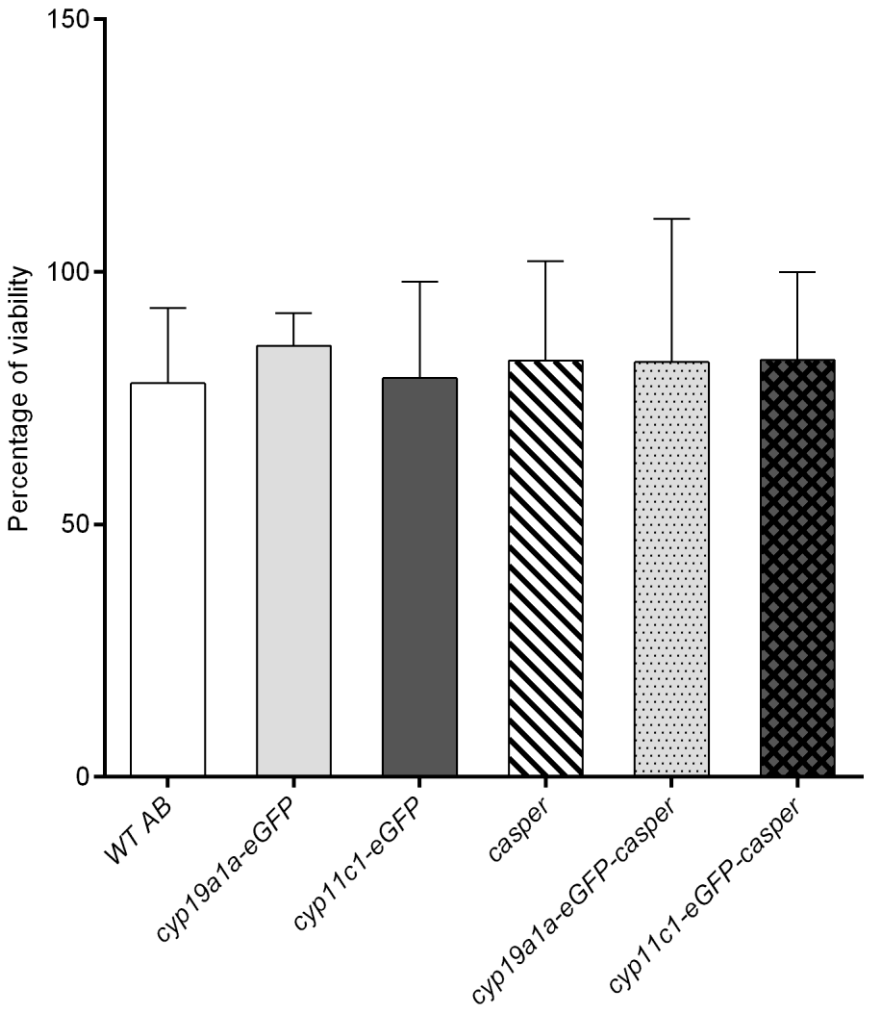

Figure 4: Viability of the eggs after the first sorting for the 6 zebrafish strains. The data are means ± SD (n= 3 – 13).

3.3.3. Circulating VTG concentrations

Circulating VTG concentrations were assayed on the whole blood of females and males of each zebrafish strain (Figure 5). For females, no significant difference was highlighted between the 6 strains. For males, the VTG concentrations of all strains were not significantly different, except for the *cyp19a1a-eGFP-casper* strain which has slightly higher VTG levels compared to the other strains. In addition, levels of circulating VTG concentrations were significantly lower for males compared to females of each strain.

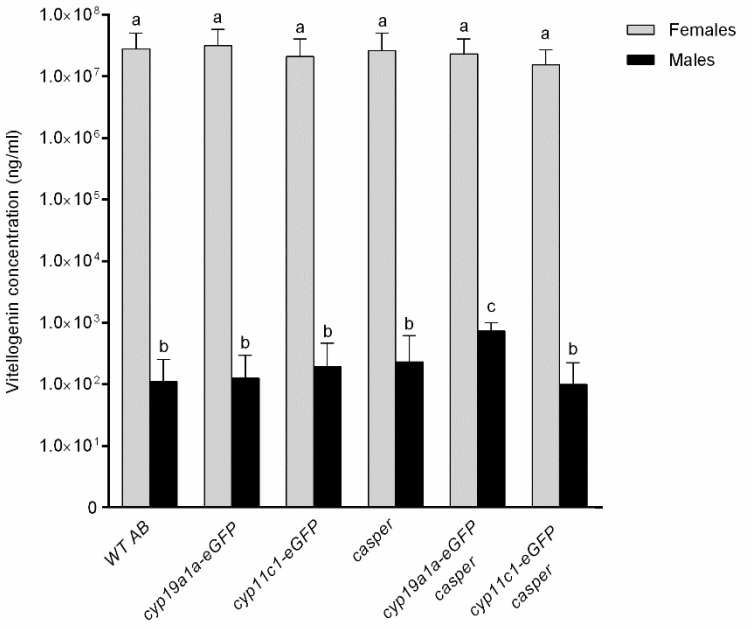

Figure 5: Circulating vitellogenin concentrations in females and males of the 6 zebrafish strains. Data are mean ± SD. Different letters represent significant differences between the different strains as well as between the sexes ($p < 0.05$, n = 15-60 per group).

4. Discussion

Differences between zebrafish strains at the genetic, physiologic and behavioral levels can lead to biased interpretations of experimental results (Coe *et al*. 2009, Drew *et al.* 2012, London and Volkoff 2019). Therefore, a strong knowledge of biological models is important for their optimal use, especially for regulatory assays. In the present study, we thus characterized two behaviors potentially influencing reproduction (anxiety and sociality) and reproductive parameters (fecundity, spawn characteristics and circulating VTG concentrations) in 5 different

strains of zebrafish (*casper, cyp19a1a-eGFP, cyp19a1a-eGFP-casper, cyp11c1-eGFP, cyp11c1-eGFP-casper*) in comparison with WT AB, to evaluate how differences in genetic background or transgene insertion could have modified some basal physiology traits and if this could have consequences on their suitability for EDCs regulatory tests.

Zebrafish, like many other fish species, display anxiety responses when they face changes in their environment. In this study, the anxiety of each strain was assessed using the Novel tank assay in which zebrafish dive directly and stay at the bottom of the novel aquarium to seek protection until they feel safe enough to explore their new environment (Gerlai *et al.* 2000, Levin *et al.* 2007, Egan *et al.* 2009). Our results showed that all the strains spend significantly more time in the lower part of the device rather than in the upper part, a well-known behavior called "bottom-dwelling". Indeed, fish spend usually 70 to 85% of the first minute of a test in the lower part of the tank (Levin *et al.* 2007, Bencan *et al.* 2009). Later on, this percentage decreases, however the time spent in the bottom zone remains more important (Mezzomo *et al.* 2016, Vossen *et al.* 2020). The bottom diving preference of zebrafish was shown to be motivated by avoiding the surface instead of approaching the bottom substrate (Blaser and Goldsteinholm 2012). Indeed, the surface could embody either a danger (predation, manipulator nearby...) or simply a source of aversion for fish moved to a new environment. Although the 6 strains used in our study exhibited similar anxiety behavior, the *cyp11c1-eGFP* strain spent more time at the bottom than the WT AB, the *casper* and the *cyp11c1-eGFP-casper* strains, suggesting a higher level of anxiety. This characteristic of the *cyp11c1-eGFP* strain does not seem to come from the transgene insertion since the *cyp11c1-eGFP-casper* strain did not elicit differences from the WT AB. Even if a strain difference could not be excluded, as already observed for *leopard* and *albino* for example (Egan *et al.* 2009), the time spent by the *cyp11c1-eGFP* zebrafish in the top zone remained within the variability observed for different wild-type and transgenic zebrafish (few seconds to 100 s) (Egan *et al.* 2009, Grossman *et al.* 2010, Grossman *et al.* 2011, Maximino *et al.* 2013, Quadros *et al.* 2019).

In zebrafish, social behavior has been shown to be of crucial importance to adaptation, reproductive success, predator avoidance or foraging success (Hoare *et al.* 2000, Krause *et al.* 2000, Miller and Gerlai 2011). In this study, the 5 transgenic and/or mutant zebrafish strains exerted the same behavior as WT AB zebrafish in sociality assays. Whatever the sex and the strain, each fish spent more time in the area close to the congeners, consistent with the known literature on the subject. Indeed, it has been shown that zebrafish, whether wild-type, mutant or transgenic, is a species with a natural preference for congeners under neutral conditions (Snekser *et al.* 2006, 2010, Saverino and Gerlai 2008, Parker *et al.* 2013, Araujo-Silva and Pinheiro-da-Silva 2018, Fontana *et al.* 2018, Fernandes *et al.* 2019).

As regards reproductive parameters, neither the viability of eggs laid per female nor the concentrations of VTG, an estrogen-regulated protein synthesized by female liver, were significantly different between the 6 strains of zebrafish. In the literature, eggs viability varies from 70 to 95% for WT AB zebrafish, which is consistent with the range of values reported in the present study for the 6 strains (Kanuga *et al.* 2011, Han *et al.* 2013, Cardoso *et al.* 2017). Likewise, circulating VTG concentrations measured in females agreed with the concentrations reported in the literature that show a high inter-study and inter-individual variability. Indeed, whole blood VTG concentrations in female zebrafish range from 0.1 mg / ml to 10 mg / ml (Nash *et al.* 2004, OECD 2006). In males of all strains, measured circulating VTG concentrations are consistent with the literature data for WT zebrafish (Brion *et al*. 2002, OECD 2006). Although the *cyp19a1a-eGFP-casper* strain had slightly higher levels of VTG than the other five strains, concentrations were still less than 1000 ng/ml, which will not preclude the use of this strain to detect VTG inductions by (xeno-)estrogens (OECD 2006).

The fecundity data of the 6 zebrafish strains were also consistent with data from the literature reporting number of eggs laid per female per day ranging from 19 to 85 (Hua *et al.* 2016, Xu *et al.* 2017, Liang *et al.* 2018). In zebrafish, egg output is highly variable and could be affected by reproduction conditions such as sex-ratio, density, fish age or size, or temperature (Spence and Smith 2005, Paull *et al.* 2008, Uusi-Heikkilä *et al.* 2010, Kanuga *et*

al. 2011). Concerning the 6 zebrafish strains, female fecundity data revealed some differences with the *cyp11c1-eGFP* zebrafish producing significantly more eggs per female per day compared to most of the other strains studied. These different fecundity levels seem to be largely explained by weight variations between the strains, with a correlation between somatic mass and average number of eggs laid per female per day explaining 66% of the differences in fecundity. Therefore, once normalized by the average somatic mass of each aquarium, the fecundity data were no longer different between the 6 zebrafish strains. Moreover, in the present study, even if not always statistically significant, the *casper* strains tend towards smaller clutch of eggs, maybe because they also seem to be shorter and thinner than the non *casper* strains. Recently, it has been shown that *casper* and WT AB zebrafish display strain variations in some mechanisms regulating feeding, at least in part due to differences in the melanocortin system and the melanin pathway, that may explain the differences in size and weight between the two strains (London and Volkoff 2019).

In summary, not critical difference in the behavioral (sociability and anxiety) and reproductive physiology (fecundity, eggs viability and VTG concentrations) was found between the 5 mutant/transgenic zebrafish lines and the wild-type AB. The absence of difference shows that the transgene insertion and the mutations had no influence on the endpoints measured whatever their organizational level (biochemical (VTG), physiological (fecundity) or behavioral). This suggests that these transgenic/mutant models can be pertinently used in laboratory tests assessing the endocrine properties of chemicals such as the OECD TG 229 and TG 230 (OECD, 2009; 2012), without compromising the basal expression of key endpoints. In that respect, the use of transgenic models instead of WT appears relevant as they provide additional information on the chemicals' mechanism of action on the tissue-specific expression of the transgene, as previously demonstrated (De Oliveira et al, 2020). However, more studies are needed to validate the use of these models to refine existing fish assays for EDCs.

Declaration of Competing Interest

The authors declare there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgements

This work was supported by the French National Research Program for Environmental and Occupational Health of ANSES [AIDEZ project n°EST-2016/1/189] and by the French Ministère de la Transition Ecologique et Solidaire [Programme 190 ECOT].

The authors wish to acknowledge the UMS AMAGEN for the production of the F1 generation of the *cyp19a1a-eGFP* and *cyp11c1-eGFP* zebrafish lines; Emmanuelle Maillot-Maréchal for her help in fish sampling, Olivier Palluel for VTG measurements and Benjamin Piccini for the maintenance of the zebrafish.

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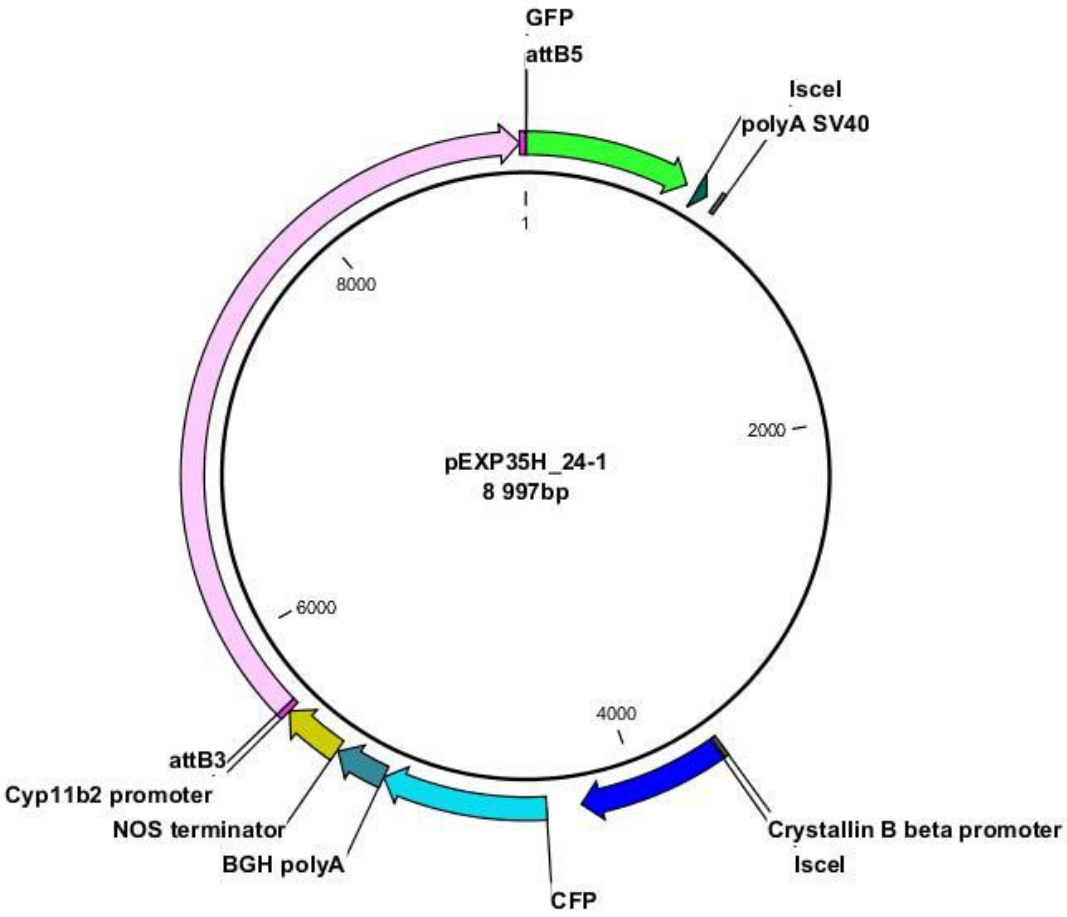
Supplementary data

The *cyp11c1-eGFP* transgenic line was created from a wild-type AB zebrafish by the UMS AMAGEN. Methods for the transgenic vector design, the PCR amplification of the promoter and the generation of transgenic zebrafish have been described in Hinfray *et al.* (2018). The sequence and the important features of the transgenesis vector are provided below.

Name: pEXP35H(24-1)
Type: Expression Vector
Size: 8997 bp
Selectable makers: Ampicillin
Bacterial strain Top10
Comments Zebrafish selectable marker: expression of CFP in the lens from 48 hours onward



pEXP35H_24-1 map

Features

name : Enhanced Green Fluorescent Protein
type : Open Reading Frame
subtype : NA
origin : Aequorea victoria
start : 4
end : 723
complementary : no
comments : genetically Enginereed. also known as GFP-mut1. Excitation maximum = 488 nm; emission maximum = 507 nm.

name : polyA SV40
type : terminator of transcription
subtype : polyadenylation signal
origin : SV40 virus
start : 773
end : 823
complementary : no
comments : none

name : IsceI
type : cleavage site
subtype : meganuclease
origin : S. cerevisiae
start : 871
end : 888
complementary : yes
comments : none

name : IsceI
type : cleavage site
subtype : meganuclease
origin : S. cerevisiae
start : 3599
end : 3616
complementary : no
comments : none

name : beta crystallin promoter
type : promoter
subtype : NA
origin : Danio rerio
start : 3625
end : 4259
complementary : no
comments : zebrafish Crystallin beta B promoter from zebrafish genomic DNA using Forward primer:CbBFA: AGAGCTCGAGCAATTGGTGCTATCATCATGACAAAGAG and Reverse primer: CbBR: AGAGAGTTTAAACTACAGCTGTGCTGGGAAGGCTG

name : Cyan Fluorescent Protein
type : Open Reading Frame
subtype : NA
origin : Aequorea victoria
start : 4410
end : 5129
complementary : no
comments : genetically engineered. Cyan derivative of the classical GFP.
excitation max = 433 nm (+ minor peak = 453 nm);
emission max = 475 nm (+ minor peak = 501 nm).
Sequence origin : Parinov & Emylanov

name : NOS (nopaline synthetase) terminator
type : terminator of transcription
subtype : NA
origin : Agrobacterium tumefaciens
start : 5361
end : 5626
complementary : no
comments : none

name : attB3
type : recombination site
subtype : Gateway
origin : ND
start : 5628
end : 5652
complementary : no
comments : none

name : cyp11b2 promoter
type : promoter
subtype : insert
origin : Danio rerio
start : 5653
end : 8972
complementary : no
comments : none

name : attB5
type : recombination site
subtype : Gateway
origin : ND
start : 8973
end : 8997
complementary : yes
comments : none

Fasta sequence

>pEXP35H_24-1

ACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGAC
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