Implementation of a functional endpoint to the zebrafish embryotoxicity test to evaluate craniofacial abnormalities.

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Abstract: The inclusion of a read-out to detect functional consequences of craniofacial alterations in the zebrafish embryotoxicity test will allow to evaluate these alterations which are difficult to assess morphologically, and to detect alterations in cranial nerves functions leading to impairment of jaw movements. In this study we have established an ingestion test in zebrafish larvae younger than 120 hpf. To overcome the challenge of evaluating larvae which still do not present independent feeding behaviour, we have tested the ability of 72, 96 or 102 hpf larvae to ingest food mixed with fluorescent microspheres under several conditions (dark/light, with/without shaking) to find the best experimental set-up for the test. We have included the investigation of two substances as potential positive controls: ketoconazole and tricaine. Ketoconazole 10 µM exposure during development produced significant embryotoxic effects including a characteristic craniofacial alteration pattern consisting in impaired development of brain, nasal cavity, mouth opening and jaw, as well as a significant decrease in food intake. Tricaine exposure at 380 µM during the food availability period significantly decreased the food intake. The method proposed will be a useful alternative tool to animal testing to detect compounds inducing adverse effects on craniofacial development.

Keywords: alternative methods; feeding; swallowing; jaw; cleft-palate; ingestion;

hpf: hours post fertilization; EW: OECD water; SC: solvent control; SEM: standard error of the mean.

1. Introduction

Craniofacial malformations are one of the most common congenital defects, being orofacial clefts the most frequent (Lowry, 1985; Martínez-Frías et al., 2010; Parker et al., 2010). The causes of these malformations, either isolated or syndromic, are mostly unknown (Martínez-Frías et al., 2010), therefore more research is needed to be able to prevent them. An alternative method to animal experimentation to easily screen for craniofacial malformations would be a valuable tool to reduce the costs, time and number of animals compared to the current *in vivo* testing strategies. However, the formation of the craniofacial area is such a complex process that most *in vitro* tests cannot represent it completely.

To this moment, the best alternative methods to reproduce craniofacial adverse effects observed *in vivo* were those based on whole organism models: the whole rodent embryo culture and the zebrafish embryo test (de Jong et al., 2011). In these tests the evaluation was mainly morphological based on different scoring systems (Beekhuijzen et al., 2015; Brown and Fabro, 1981; Piersma et al., 2004; Stephan, 1991; Van Maele-Fabry et al., 1990) and when more precision was needed a double staining of cartilage and bone was added to the zebrafish assay (Kimmel and Trammell, 1981; Walker and Kimmel, 2007). However, with these techniques it is difficult to assess how morphological alterations in early development relate to functional impairment. The assessment of functional impairment could aid in the interpretation of the results of these techniques, specially by clarifying the classification into variations or developmental toxicity, as in *in vivo* testing according to the OECD guideline 414 (OECD/OCDE, 2018, 2001). In the guideline, variations are defined as “structural changes considered to have little or no detrimental effect on the animal, may be transient and may occur relatively frequently in the control population” (OECD/OCDE, 2018, 2001), while developmental toxicity includes major manifestations as “death of the organism, structural abnormality, altered growth, and functional deficiency” (OECD/OCDE, 2018, 2001). Based on these definitions, if a functional deficiency could be observed in zebrafish embryos/larvae it would be clearly classified as a developmental toxicity effect and could not be misplaced as a variation. As mentioned, craniofacial alterations found in zebrafish do not allow yet to distinguish functional deficiencies as those findings on toxicity testing of rats and rabbit do after decades of discussion (Solecki et al., 2003, 2001). However, the use of zebrafish embryos opens the door to evaluate the inability to ingest food and therefore add a functional endpoint to evaluate craniofacial alterations.

Although it has been described that zebrafish do not present independent feeding until 120 hours post fertilization (hpf) and that they still relay on the given yolk, their digestive system is completely formed 24 h before (Strähle et al., 2012). Because 120 hpf is the time when protected life stages in animal welfare regulations start, our aim was to establish an ingestion test in zebrafish larvae younger than 120 hpf to be used as an alternative model in developmental toxicity. For that purpose, we adapted a method previously applied to 7-9 days post fertilization (dpf) larvae which uses food mixed with fluorescent microspheres (Field et al., 2009). The intention of establishing such an endpoint was to have a functional read-out of a complex behavior which includes identification of the food (visually or using chemosensation), capturing the food (opening and closing the mouth), and ingesting (swallowing) (Kalueff et al., 2013). This evaluation would be very useful to i) check if craniofacial morphological alterations have functional consequences, ii) evaluate in a more objective way craniofacial alterations which are difficult to score morphologically, iii) detect alterations in cranial nerves, which lead to impairment of jaw movements but with no musculoskeletal morphological effect.

To overcome the challenge of using larvae which still do not present independent feeding behaviour, we have tested several conditions (dark/light, with/without shaking) in larvae of 72, 96 or 102 hpf to find the best experimental set-up to evaluate ingestion ability during these developmental stages. Besides, we have investigated two chemicals which are likely to affect ingestion of the zebrafish larvae by different mechanisms; ketoconazole most likely to interfere through craniofacial morphological alterations (Amaral and Nunes, 2008; Menegola et al., 2006; Nishikawa et al., 1984) and tricaine by suppressing movements needed for intake by blocking neuromuscular transmission (Attili and Hughes, 2014; Hedrick and Winmill, 2003; Zahl et al., 2012).

2. Materials and Methods

The determination of the concentration-effect relationship of ketoconazole on morphological endpoints and the implementation of functional endpoints in the zebrafish embryo assay were performed in two different facilities. The differences in the protocols were confirmed to have no impact on the comparability of results in both laboratories.

2.1. Adult zebrafish maintenance and egg production

In the laboratory at the University of Barcelona (laboratory A), maintenance of the adult colony of zebrafish was approved by the Ethics Committee for Animal Experimentation of the University of

Barcelona (CEEA), accepted by the Department of Environment and Housing of the Generalitat de Catalunya with the license number 334/18, and according to the Generalitat de Catalunya Decree 214/1997 of 30th of July, which regulates the use of animals for experimental and other scientific purposes. Adult zebrafish (*Danio rerio*) from BCN Piscicultra Iberica; Terrassa, Spain, were kept in tanks with a closed flow-through system in OECD water as specified in ISO 7346-1 and 7346-2 (ISO, 1996; 2 mM CaCl2·2H2O; 0.5 mM MgSO4 ·7H2O; 0.75 mM NaHCO3; 0.07 mM KCl). Animals were maintained at 26 ± 1 ºC on a 14 hours light and 10 hours dark cycle and were fed two times a day, once with brine shrimp and once with dry flake food. The day before the test, adult male and female were transferred to breeding tanks. Spawning was triggered once the light was turned on. Eggs harvested between 1 and 2 hours after spawning were collected, cleaned with OECD water diluted 1:5 and selected under a dissection stereomicroscope.

In the laboratories at the BASF SE (laboratory B) maintenance of the adult colony was approved by an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) certified laboratory. In-house bred wild type fish were kept in tanks with a single pass flow-through system in OECD water. Animals were maintained at 26 ± 1 ºC on a 16 hours light and 8 hours dark cycle and were fed two times a day, once with brine shrimp and once with dry flake food. The day before the test, adult male and female were transferred to breeding tanks in pairs. Eggs harvested between 1 and 2 hours after spawning were collected, cleaned with undiluted OECD water and selected under a dissection microscope.

2.2. Zebrafish embryo assay conditions

Only fertilized eggs demonstrating homogenous synchronous development (between 16- and 256-cell stage) were selected.

In the laboratory at the University of Barcelona (laboratory A) they were randomly distributed into 6-well plates (10 embryos/well), water was replaced by 5 mL of Danieau's solution 0.3X (17.4 mM NaCl; 0.23 mM KCl; 0.12 mM $MgSO_4 \cdot 7H_2O$; 0.18 mM $Ca(NO_3)_2$; 1.5 mM HEPES; pH 7,4) and plates were incubated at 26 ± 1 ºC. Danieau's solution was replaced (10 mL) after 24 h. In experiments with ketoconazole (Santa Cruz Biotechnology; ≥99% purity; CAS-No. 65277-42-1), wells were saturated with test solutions for 24 h prior to the start of the experiment, and test solutions were freshly prepared and replaced at 0 hpf and 24 hpf. 6-well plates were filled with 5, 5, and 10 ml solutions at -24, 0, and 24 hours, respectively.

In the laboratories at the BASF SE (laboratory B) eggs were randomly distributed in 24-well plates (1 embryo/well), water was replaced by 1 ml of OECD water, and replaced (1 ml) after 24 h. In experiments with ketoconazole (Sigma-Aldrich; ≥99% purity; CAS-No. 65277-42-1), wells were saturated with test solutions for 24 h prior to the start of the experiment, and test solutions were freshly prepared and replaced at 0 hpf and 24 hpf. 24-well plates were filled with 1 ml at the same time points of culture.

For the determination of concentration-effect relationship of ketoconazole in the concentration range from 0.1 to 33 µM in laboratory B three independent experiments aiming to use 12 embryos per test group (finally resulting in sample size of 9 to 12 embryos per test group depending on availability of suitable eggs) were performed. Two control groups were included each time 1) OECD water (embryonic water - EW) and 2) vehicle control (0.5% DMSO, solvent control - SC). Morphological evaluation of the embryos was performed at 120 hpf under a dissection microscope using a morphological score with 39 parameters (Supplementary Table 1) including two criteria for mortality (coagulation and/or heartbeat arrest), 13 parameters for general embryotoxicity and 24 parameters for specific embryotoxicity (Flick et al., 2017). For the evaluation the zebrafish were in tricaine narcosis.

2.3. Fluorescent tracer

The fluorescent tracer was prepared as described in Field et al., (2009). Briefly, 100 mg of powdered larval feed (Zebrafeed by Sparos <100 µm) were mixed on a watch glass with 150 µL of yellow-green 2 µm fluorescent polystyrene microspheres (Invitrogen by Termo Fisher Scientific, FluoSpheres TM carboxylate, 2.0 µm, yellow-green (505/515), Ref. F8827) and 50 µL of deionized water. Ingredients were stirred until a paste was formed and it was spread as a thin layer. The mixture was left overnight in the dark at room temperature to dry, and then was scraped from the glass and grinded between two pieces of weighing paper to powder. The fluorescent tracer was stored in a glass recipient at room temperature protected from the light.

2.4. Food administration

At 72 hpf, 96 hpf or 102 hpf, approximately 2 mg of tracer was added to each well of a 6-well plate. The larvae were left at 26 ± 1 ºC in the constant-temperature room with the tracer for 3 h. Depending on the testing conditions (A to F) plates were stirred every 30 minutes (160-170 rpm, 10

s). After these 3 h, medium with tracer was removed and replaced with 5 ml of Danieau's solution 0.3X. Afterwards, larvae were transferred to a new well of another 6-well plate with new Danieau's 0.3X (5 mL). Larvae were placed individually on wells of 24-well plates and anesthetized by adding 650 µL tricaine 0.1% (ethyl 3-aminobenzoate methanesulfonate salt, by Sigma Aldrich, ≥98 % purity, CAS-No. 886-86-2) per well. Larvae were analyzed using an inverted fluorescent microscope (Nikon eclipse TS100) for presence/absence of fluorescence in the gastrointestinal tract (no quantification of the amount ingested was intended). It was easily distinguishable the background fluorescence of the yolk (see picture of condition A in Figure 1) from the food fluorescence in the gastrointestinal track (see picture of conditions C and E in Figure 1). Pictures of representative larvae were taken from different body parts using the same conditions for fluorescent channel exposure (200 ms) and subsequently joining together the fragments with Adobe Photoshop CC®.

Tricaine (also known as 3-aminobenzoic acid ethyl ester or MS-222), the general anaesthetic used in fishes to suppress movement (Attili and Hughes, 2014; Hedrick and Winmill, 2003; Zahl et al., 2012), was selected as a compound to affect the 'food ingestion' endpoint. The conditions for this experimental group were established as follows: zebrafish embryos were grown under control conditions until 102 hpf. Fifteen minutes before adding the fluorescent food, medium was replaced by tricaine 380 µM in Danieau's solution 0.3X. Fluorescent food was then added to the wells for 3 h under light and shaking conditions.

2.5. Statistics

Statistical analysis was performed with GraphPad Prism software v7. Statistical comparisons were made with two-tailed t-test, Fisher's exact test or one-way ANOVA as indicated in each graph or table. Significance threshold was established at $p \leq 0.05$.

3. Results

3.1. Establishment of experimental conditions

To establish the feeding test in larvae under 120 hpf, several conditions were tested. Starting conditions were based on a previous publication of Field et al., (2009), where 7 dpf larvae were fed food mixed with fluorescent microspheres, but some adaptations were made: only larvae between 3 and 4.5 dpf were used, food availability period was extended from 2 to 3 hours, larvae were visualized

immediately after removing the food instead of waiting 3, 6, 12 or 24 h, and light/dark and/or shaking conditions were applied during the feeding time.

Zebrafish larvae open the mouth first at 72 hpf (Kimmel et al., 1995), so this was the point chosen for the initial test condition (A). However, at this early developmental stage almost no larvae ingested food (Figure 1, condition A; mean ± SEM= 3% ± 3). As liver, pancreas and gut are formed at 76 hpf and anus opens at 96 hpf (Field et al., 2003a, 2003b; Strähle et al., 2012), we waited until the whole digestive system was formed (96 hpf) for condition B. At this point, approximately 50% of larvae were ingesting fluorescent food (mean ± SEM= 48% ± 11) which represented a significant increase from condition A. Considering that larvae at these stages are mainly inactive and they only start to actively swim freely after 120 hpf (Strähle et al., 2012), and also that in locomotion tests, darkness is described to increase locomotion (MacPhail et al., 2009; McDougall et al., 2016), we introduced two modifications to the protocol: shaking of plates every 30 minutes (C and D) and/or feeding under dark conditions (D). Besides, the number of larvae tested per group was increased from 10 to 20. The inclusion of shaking every 30 minutes significantly increased the percentage of larvae ingesting compared to condition B, but only under light conditions (Figure 1, condition C; mean ± SEM= 76% ± 5). To finally check if the conditions could still be improved without exceeding the 120 hpf, we tested the ingestion of 102 hpf larvae, again under light and dark conditions, and including shaking in both cases. At 102 hpf under light and shaking conditions the highest percentage with the lowest SEM was obtained (mean ± SEM= 89% ± 0.3). At this time-point, again, the percentage of larvae ingesting was lower under dark conditions. In view of these results, condition E was selected for further experiments.

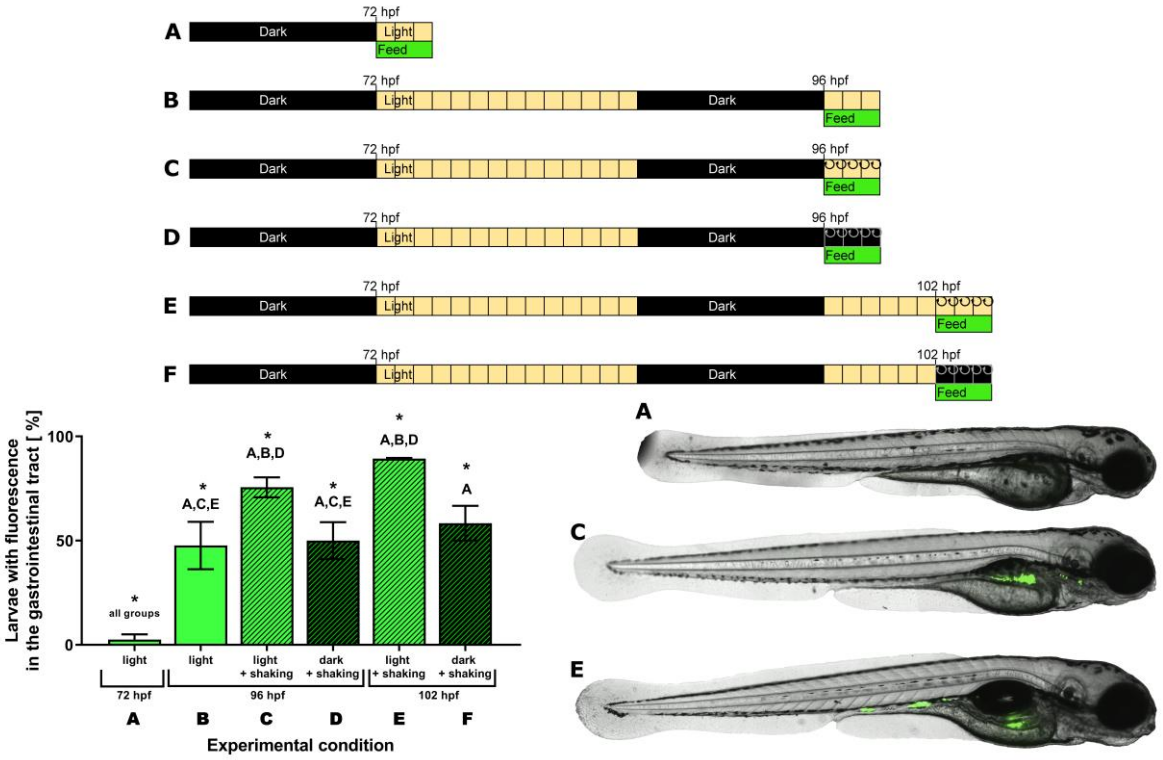

Figure 1. Summary of timelines of the six different experimental conditions assayed to establish the ingestion test. Shaking of 6-well plates every 30 minutes is indicated with circular arrows. Graph represents the percentage of larvae with fluorescence in the gastrointestinal tract for all conditions tested. Results presented as mean ± SEM of at least three independent experiments with 10 (A and B) or 20 (C to F) larvae per group and experiment. *: $p \leq 0.05$ versus the group indicated in letters underneath by ANOVA and Holm-Sidak test. Representative pictures of a larvae from each age tested: A (72 hpf) showing no fluorescence; C (96 hpf) showing fluorescence in the oesophagus and the stomach; and E (102 hpf) with fluorescence in the stomach, intestine and outside the cloaca.

3.2. Establishment of positive controls

Following the recommendations for developing alternative methods for the screening and prioritization of chemicals (Crofton and Mundy, 2011), two substances considered as potential positive controls of the test. Two positive controls reliably and consistently altering the end-point by two different known mechanisms were established.

3.2.1. Induction of craniofacial malformations with ketoconazole.

Ketoconazole was selected as potential endpoint-specific control for the induction of craniofacial alterations because this azole-derivative is known to induce craniofacial malformations in rats *in vivo*, including induction of cleft palate (Amaral and Nunes, 2008; Nishikawa et al., 1984) and in rat embryos *in vitro*, including abnormalities in branchial arches (Menegola et al., 2006).

A broad concentration range study was conducted in the laboratories at the BASF SE (laboratory B) to evaluate developmental toxicity of ketoconazole in zebrafish embryos. At 120 hpf there was no mortality or alterations higher than 10% in any parameter of the morphological score in any control group (control and solvent control) demonstrating the validity of the three performed experiments. After exposure to increasing concentrations of ketoconazole between 0.1 and 3 µM the results were comparable to the solvent control in mortality and general as well as specific embryotoxicity. At 10 µM the incidence of mortality was still comparable to controls, but the percentages of general and specific embryotoxicity were significantly increased (mean ± SEM= 97.3% ± 2.7 and 58.3% ± 29.3, respectively; Figure 2). As ketoconazole exposure at 33 µM was lethal to all embryos in all experiments, no further assessment of morphological alterations at this concentration is given. Summarizing these results of laboratory B, 10 µM exposure represents the lowest observed adverse effect concentration (LOAEC) of ketoconazole based on the findings in the morphological score for general and specific embryotoxicity (Figure 2).

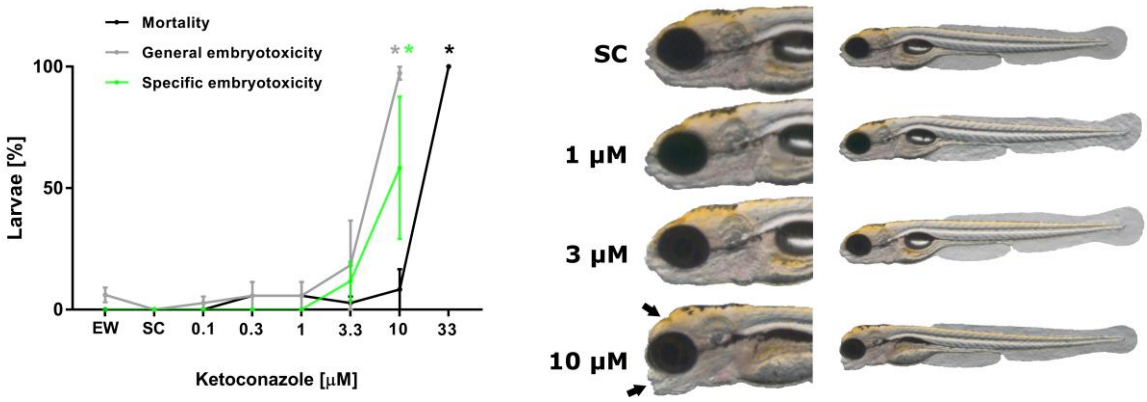

Figure 2. Graph summarizing the percentages of larvae presenting: specific embryotoxicity (green), general embryotoxicity (gray) and mortality (black) as mean ± SEM of three independent experiments performed in laboratory B for increasing concentrations of ketoconazole. Statistical analysis performed with one-way ANOVA and statistical significance (*) defined as $p \leq 0.05$ versus solvent control. EW: embryo water; SC: solvent control (0.5% DMSO). Representative pictures of 120 hpf larvae from solvent control, 1, 3 and 10 µM groups, with magnification of the craniofacial area of the same larvae, and black arrows indicating the specific embryotoxic effects detected in the craniofacial area.

Looking in detail at the individual parameters of the morphological score (Table 1) representing general embryotoxicity, a significant increase in pericard oedema was found at 3.3 µM. However, this finding was not observed at 10 µM and there was no concentration-dependency, so it was considered to be spontaneous and not related to the treatment. The 10 µM concentration, significantly increased parameters related with general embryotoxicity like yolk sac still present and overall degeneration. For those parameters related with specific embryotoxicity, at 3.3 µM four out of 24 parameters were slightly increased, but since the incidences were below or only slightly above 10%, and were not significant, these findings were considered spontaneous and comparable to the controls. At 10 µM, however, the same four parameters: brain impairment, nasal cavity impairment, mouth opening impairment and jaw impairment were all significantly increased. The combination of these manifestations represents a pattern of craniofacial alteration in the zebrafish. Since the increase in the other 20 of 24 parameters for specific embryotoxicity was not significant (very low percentages), it was concluded that this craniofacial altered pattern is characteristic for ketoconazole in the zebrafish embryo assay.

Classification of findings	**Parameter of morphological score**			**Ketoconazole [µM]**					
		EW	**SC**	**0.1**	**0.3**	**1**	**3.3**	**10**	**33**
Mortality	coagulated	0%	0%	0%	6%	6%	3%	8%	100%*
General embryotoxicity	yolk still present	3%	3%	0%	0%	0%	0%	58%*	
	overall degenerated	0%	0%	0%	0%	0%	0%	33%*	
	unhatched	3%	0%	0%	6%	6%	0%	13%	
	pericard oedema	0%	0%	3%	0%	0%	18%*	0%	
Specific craniofacial embryotoxicity	brain impairment	0%	3%	0%	0%	0%	12%	58%*	
	nasal cavity impairment	0%	3%	0%	0%	0%	9%	39%*	

mouth opening impairment	0%	3%	0%	0%	0%	6%	39%*
jaw impairment	0%	3%	0%	0%	0%	9%	58%*

Table 1. Summary of the percentages of larvae per test group with alterations (expressed as frequencies) corresponding to each parameter evaluated in the morphological score and classified as mortality, general embryotoxicity or specific craniofacial toxicity. Results obtained in laboratory B. Statistical analysis performed with Fisher's exact test and statistical significance (*) defined as p≤0.05 versus solvent control. EW: embryo water; SC: solvent control (0.5% DMSO).

From these results, the 10 µM concentration was preliminarily selected to be tested in laboratory A (University of Barcelona) as a potential positive control for the ingestion test. A first range finding experiment with four concentrations of ketoconazole (1, 5, 10 and 20 µM) was performed to test the inter-laboratory reproducibility of the effective concentrations (Supplementary Table 2). After 72h of exposure, at 20 µM of ketoconazole 100% of mortality was found while at 5 µM no craniofacial adverse effects were observed (n=1). Therefore, the 10 µM concentration was finally selected and zebrafish embryos were grown under exposure to this concentration of ketoconazole until 102 hpf, when fluorescent food was added to the wells for 3 h under light and shaking conditions. As expected, 10 µM ketoconazole exposure induced a significant increase in craniofacial alterations (mean ± SEM= 40.7% ± 5.4) without significantly increasing mortality (mean ± SEM= 8.3% ± 3.3). Moreover, a significantly lower percentage of larvae with fluorescence presence in the gastrointestinal tract was observed at this concentration (Figure 3; mean ± SEM= 52.1% ± 13.5).

3.2.2. Inhibition of neuromuscular transmission with tricaine.

Tricaine exposure during the food availability time, completely reduced the number of larvae with fluorescence presence in the gastrointestinal tract (mean ± SEM= 1.7% ± 1.7; Figure 3).

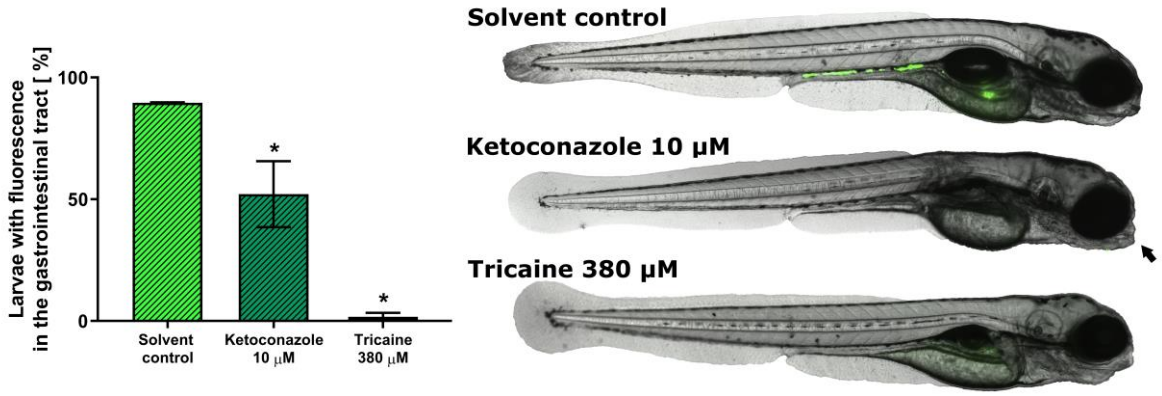

Figure 3. Graph representing the percentage of larvae with fluorescence in the gastrointestinal tract for the solvent control and two tested substances: ketoconazole 10 µM and tricaine 380 µM. Results presented as mean ± SEM of three independent experiments with 20 larvae per group and experiment. *: $p \leq 0.05$ versus control by two-tailed t-test. Representative pictures of a larvae from each condition tested: Solvent Control (0.1% DMSO) showing fluorescence in the stomach and intestine; ketoconazole 10 µM with craniofacial alterations (black arrow) and no fluorescence; tricaine 380 µM without food fluorescence in the gastrointestinal tract and showing the variability in natural fluorescence of the yolk sac area.

4. Discussion

This study presents the methodology to implement the functional endpoint 'food ingestion' to the zebrafish embryotoxicity test to add the final adverse outcome 'food ingestion impairment' to the evaluation of craniofacial abnormalities. It is important to remark that the endpoint is evaluated before 120 hpf and can therefore be included as part of a battery of alternative tests to animal experimentation according to the animal welfare law in most regions.

Previous evaluations on food intake behavior of zebrafish larvae already determined that they are not able to feed independently until 120 hpf. Belanger et al., (2010) evaluated their intake abilities at 96 hpf and observed that 0% of larvae ingested food, while at 120 hpf 75 to 85% did. These findings could seem contradictory with our results at 96 hpf, but this is not strictly true, as several conditions were changed in order to increase the chances of food intake observation. While the study of Belanger et al., (2010) aimed at recreating conditions similar to the natural environment of zebrafish to assess the development of their feeding skills, our aim was to create an artificial situation favoring the

maximum food intake. In their study, food was available for only 1 h while in our protocol it was available for 3 h. Their food were fluorescent protozoa or protozoa mixed with fluorescent microspheres while ours was processed food with fluorescent microspheres. Maybe ingestion of alive and moving food requires more coordination of movements for pray tracking and capturing than non-moving food. Besides, their embryos were grown at 25 ± 1 ºC and ours at 26 ± 1ºC which could explain a slightly faster development under our conditions. But the critical difference was the addition of shaking every 30 minutes during the 3 h of availability of food. The inclusion of this step in the methodology forced the larvae to swim and significantly increased the chances of food intake observation, while in other studies, larvae probably remained immobile during the short time of food exposure (Strähle et al., 2012). Our work shows that parameters like developmental stage, duration of food availability, kind of food, light intensity and forced swimming conditions have a clear influence on food intake behavior before 120 hpf. It is important to remark that none of the conditions tested achieved a 100% of larvae ingesting food in any of the experiments, indicating that at the tested time-points, even under the most favorable conditions, not all larvae have developed the ability to ingest food. Although the yolk of the zebrafish embryos was significantly decreased during the culture, it was still present at 102 hpf ensuring the nutrition supply.

From all the conditions tested in this study, 102 hpf was selected as the best time-point to perform the test because it had the highest percentage of larvae ingesting food and the lowest variability among experiments. However, depending on the desired dynamic range of the test, e.g. to detect an increase in the % of larvae ingesting food after developmental exposure to a compound, 96 hpf could also be a valid time-point. The disadvantage of the 96 hpf time-point is a high variability among different experiments that could fail to detect small increases/decreases in the percentage of larvae ingesting food or to identify the effects of compounds which induce alterations with high variability in results too.

To prove that following the proposed methodology it is possible to detect decreases in the evaluated endpoint, we investigated two chemicals acting by different mechanisms. The first substance tested was ketoconazole, an azole-derivative that induces craniofacial malformations in rats *in vivo* (Amaral and Nunes, 2008; Nishikawa et al., 1984) and *in vitro* in the post-implantation whole embryo culture (Menegola et al., 2006). Our evaluation of the adverse effects of ketoconazole detected a characteristic pattern of craniofacial malformations at 10 µM consisting in alterations of brain, nasal cavity, mouth opening and jaw. In previous studies in rat embryos, the NOAEL was

established at 5 µM, the first significant increase in the percentage of malformed embryos was detected at 10 µM and approximately 70% of embryos presented branchial arch malformations at 50 µM (Menegola et al., 2006). Therefore, it can be concluded that ketoconazole produces craniofacial adverse effects in a similar concentration range in zebrafish and in rat embryos *in vitro*. To the best of our knowledge, this is the first time that craniofacial adverse effects of ketoconazole have been reproduced in zebrafish embryos. Ketoconazole was previously investigated using zebrafish in the US EPA ToxCast program (Truong et al., 2014). However, in this screening program, which includes more than 1000 compounds, no detailed concentration ranges of effective concentrations could be investigated for all test compounds to identify the specific morphological pattern detectable around the LOAEC for each test substance. The investigated concentration range by ToxCast (0.064 - 64 µM overlaps with our concentration range tested (0.1 – 33 µM) but our smaller dilution factor of 3 instead of 10 offered a more precise detection of the LOAEC.

The characterization of the developmental adverse effects of ketoconazole in zebrafish has been performed in a different lab than the food intake assessment. However, the concentration-effect relationship determined for ketoconazole in both laboratories was comparable demonstrating the robustness of the zebrafish embryo assay. The differences in the protocol, mentioned in material and methods, including different zebrafish strains, culture vessels, light/dark periods, mating procedure, etc., did not bias the comparability of results in case of ketoconazole testing.

Food intake in embryos developmentally exposed to ketoconazole 10 µM was significantly decreased compared to solvent control (mean ± SEM= 52.1% ± 13.5). Although at 10 µM exposure general embryotoxicity was also observed in 97% of embryos (Figure 2, gray line), still approximately 50% of them were ingesting food which indicates that, in this particular case, this general embryotoxicity was not an impairment for food ingestion. This is important, as the aim of the test is to detect functional consequences of specific craniofacial adverse effects. A limitation of the test is that in case of severe general retardation of development in the embryos, a decrease in the percentage of larvae ingesting food would be expected. It is therefore strongly recommended to register general parameters of development like yolk sac size to detect general embryotoxicity (Supplementary Table 1) to improve the selectivity of the endpoint. Nevertheless, small retardations do not suppose an impairment for feeding under the conditions established, as already shown in this study. Accordingly to this evaluation, ketoconazole 10 µM exposure during development was selected as positive control for the test.

The second test substance selected was tricaine at concentration 380 µM. In this case, there was no developmental exposure to the compound, but just exposure from 15 minutes before and during the 3 h of food availability. The percentage of larvae ingesting food was significantly decreased. This shows that the endpoint is not only altered when embryos have craniofacial malformations, but also when zebrafishes cannot coordinate craniofacial movements. This condition was therefore selected as a suitable positive control to inhibit the active and coordinated facial movements needed for ingestion, e.g. moving the jaw to open and close the mouth or swallowing. As an active behavior is required for feeding, the test could also be used in the future to evaluate developmental neurotoxicity, as changes in feeding behavior can reflect changes in swimming/lethargy status as well as problems in more refined coordination.

Considering all results presented, we can confirm that the method proposed enables the implementation of the assessment of the adverse outcome “impaired food intake ability” in zebrafish larvae younger than 120 hpf. This new end-point will be a useful alternative tool to animal testing to detect compounds inducing adverse effects on craniofacial development.

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