FLUORESCING FISH: USE OF TRANSGENIC MEDAKA IN ESTROGEN DETECTION

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Submitted in partial fulfillment of the
requirements for Departmental Honors in
the Department of Biology
Texas Christian University
Fort Worth, Texas

May 8, 2023

FLUORESCING FISH: USE OF TRANSGENIC MEDAKA
IN ESTROGEN DETECTION

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ABSTRACT

In response to new legislation, there is a need to screen chemicals for endocrine disrupting activity. There are multiple methods approved for detecting endocrine disruption, but they are either too expensive, too time-consuming, or performed *in vitro* which limits their utility. The Rapid Estrogen ACTivity *In Vivo* (REACTIV) assay avoids these pitfalls as it is cheaper, quicker, and performed *in vivo*. For this method to be adopted by the Organization for Co-operation and Development (OECD), it must be proven to produce consistent results and avoid false positives. This project focuses on collecting data to determine the validity and reliability of this new assay by exposing transgenic Japanese medaka hatchlings to a variety of chemicals, including those that are inert or pro-estrogenic, for 24 hours. Following this exposure period, the level of fluorescence intensity in the livers of the hatchlings was measured and quantified. After statistical analysis was run, results were evaluated, and the level of fluorescence intensity matched the expected outcomes, indicating that the REACTIV assay is a valid method to detect endocrine disruption. Additionally, there were no false positive results in response to inert chemicals, giving evidence to the idea that the REACTIV assay does not produce false positives. The results from the Jeffries lab, along with the results from other laboratories around the world, have been compiled, and these data have been submitted to the OECD for further validation in hopes of adopting the REACTIV Assay for standard use in the field of toxicology.

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INTRODUCTION

In recent decades, with increasing global industrialization and subsequent pollution, there have been mounting concerns about the hazards posed by chemicals in the environment. Though a variety of dangerous compounds are present in the environment, reproductive endocrine disrupting chemicals (REDCs) are of particular concern because they affect the reproductive endocrine system, which is responsible for regulating the release of reproductive hormones and key aspects of sexual development and reproduction. In fact, REDCs have been shown to induce deleterious effects like abnormal sexual development[1] and decreased reproductive success[1] in fish. The conservation of the endocrine system across vertebrates suggests that other wildlife and humans could also be adversely affected by REDCs. Thus, there is a need to screen new and existing chemicals for reproductive endocrine disrupting activity.

To that end, international regulations such as the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) piece of legislation, require that potential REDCs be tested to uncover their hazardous properties[3]. However, the effects cannot be sufficiently determined using current *in vitro* (non-animal) testing methods as most *in vitro* methods are limited to detecting REDCs that bind directly to nuclear hormone receptors. Furthermore, existing *in vivo* (animal) testing methods are too time intensive, expensive, and involve the use of adult fish.

The current standard used to detect endocrine disruption is a 21-day fish assay that screens for estrogenic and androgenic activity, along with aromatase inhibition using zebrafish, fathead minnows, and Japanese medaka[5]. In this method, sexually-mature male and female fish are exposed to a chemical for 21 days, and their reproductive capacity and biomarker responses

(e.g., vitellogenin production, secondary sexual characteristics, etc.) are evaluated[5]. While this method reliably screens for REDCs, its major drawbacks are time and cost.

A rapid and cost-effective alternative, the EASZY Assay (Detection of Endocrine Active Substances, acting through estrogen receptors, using transgenic Zebrafish embryos), was recently approved to test for endocrine active substances using a transgenic line of zebrafish. This method is performed *in vivo* and has only a 96-hour exposure period. Here, activation of estrogen receptors induces expression of green fluorescent protein (GFP) which is driven by the cyp19a1b promoter; this allows for the detection of estrogenic activity[4]. While this method has been successfully validated, its utility is limited because the cyp19a1b promoter is expressed only in radial glial cells. Therefore, it can only detect REDCs that are able to pass the blood-brain barrier. Thus, there remains a need to develop and validate new *in vivo* methods that are faster, less expensive, and more applicable to a wide range of chemicals.

The Rapid Estrogen ACTivity In Vivo (REACTIV) assay screening method has been developed to detect the endocrine-disrupting capabilities of chemicals that have estrogenic activity or alter steroid hormone production[2]. It is unique in that it can detect estrogen receptor agonists and antagonists, as well as the disruption of the endocrine axis through the altered activity of aromatase or 5α-reductase[2]. In the assay, newly hatched transgenic Japanese medaka (*Oryzias latipes*) are exposed to a variety of REDCs within 24 hours of their hatch. These fish have been modified so that the binding of receptor alpha (ERα) to the choriogenin H promoter (an important egg-yolk protein) also induces expression of GFP. Thus, the estrogenic chemicals cause green fluorescence to appear in the livers of these transgenic medaka hatchlings.

Though initial evaluations of this assay have provided evidence of its utility, its widespread adoption as a routine screening assay requires that its reliability be validated. Thus,

the objective of this project was to validate the REACTIV Assay as a valid alternative for screening chemicals that may disrupt the reproductive endocrine signaling or steroidogenesis by demonstrating that it responds to a suite of chemicals in a predictable and reproducible fashion.

METHODS

Adult Larval Care

Fifty-five Japanese medaka (*Oryzias Latipes*) from Laboratorie WatchFrog (France) were housed in 30 L tanks once they matured past the juvenile stage. Each one of these tanks was filled with approximately 27 L of dechlorinated water and heated to around ~25.5 ^{o}C. Weekly, each tank was scrubbed to remove any algae or sediment that had built up on the sides or the bottom of the tank. Daily, a 1/3 water change was performed, temperatures were monitored, and any dead fish were removed and documented from each tank. Adult fish were fed a diet of Adult Tetramin ad libitum twice per day.

Egg Collection and Care

Eggs were collected by hand from each tank. Fish were transferred to a beaker, and each adult female was inspected for eggs. If present, eggs were carefully removed from the females and transferred to a 1 L beaker filled with ~800 mL of dechlorinated water. Eggs were sorted, cleaned, and stored in 300 mL crystallizing dishes ($n \leq 200$/dish) containing ~1 g/L of methylene blue. The eggs were cleaned daily to remove any dead/fungused eggs, and an 80% water change was performed daily. Upon hatching, the fish were used in the REACTIV assay.

Test Chemicals

The test chemicals used in this assay were testosterone (Tes), saccharin (Sac), estradiol (E2), and bisphenol A (BPA). All chemicals were provided by WatchFrog Laboratorie.

REACTIV Assay

Japanese medaka eggs that had been previously collected were monitored for hatching. As embryos hatched, they were removed and placed in the well of 6-well polystyrene plates at densities of 8 larvae/well.

The well plate included 5 wells of increasing concentrations of the test chemical. If enough embryos hatched ($n \geq 248$), a second test chemical was included in the same assay. This involved setting up a new well plate with increasing concentration of the chemical in question,

After the solutions were prepared and correctly placed in each well of each well plate and the fish were in each well, each plate was left in an incubator at a temperature of ~24.5^{o}C for 24 hours.

Chemical	**Concentrations Tested**
Estradiol (E2)	0, 24, 68, 135, 270, 540 (ng/L)
Bisphenol A (BPA)	0, 1, 2, 3, 4, 5 (mg/L)
Testosterone (Tes)	0, 3.7, 11, 33, 100, 300 (μg/L)
Saccharin (Sac)	0, 0.01, 0.1, 1, 10, 100 (mg/L)

Imaging

Once the 24-hour exposure period was complete, the well plates were removed from the incubator. The hatchlings were sequentially moved to petri dishes containing buffered MS222 to anesthetize them, allowing for easier positioning of the embryos for imaging. After ~30 seconds, the embryos were moved to a transparent well plate lid. Each hatchling was oriented using forceps and/or a micropipette so that each individual hatchling was lying on its back. The well plate was placed under the fluorescent microscope and an image was taken of each individual. The file of each image was labeled with the fish number and solution that each fish had been exposed too (e.g., SAC0.01-2 for the second fish exposed to 0.01 mg/L of Saccharin). Then, on

the computer, the image was sorted into the correct folder for later viewing and data analysis. Once each individual was imaged and file sorted, the hatchlings on the same well plate were returned to their well and later euthanized in MS222 upon completion of the imaging. This process was repeated for each well of all the different well plates.

Image and Statistical Analysis

After completion of all the replicates of every test chemical, the images were run through a macro called FIJI. This program was able to look at each image taken of the fish and quantify the mean fluorescence and the total intensity of fluorescence in the liver of each fish. These values were then inputted into a single Excel file. After this process was complete, the data were run through JMP for further statistical analysis to determine the mean, standard deviation, and if there were unequal variances. A Kruskal-Wallis statistical test was done to determine if there were statistically significant differences between the different chemical concentration groups.

RESULTS AND DISCUSSION

Estradiol

Significant differences in the fluorescence intensity of medaka exposed to estradiol were detected (Kruskal-Wallis $p < 0.01$). The fluorescence intensity of medaka in the 0, 34, and 68 ng/L groups did not differ from one another, but significant and dose-dependent increases in fluorescence intensity were observed for medaka in the 135, 270, and 540 ng/L with significant differences being noted between each of these groups.

17β-Estradiol (E2) is an estrogen that is involved in a variety of functions including the maintenance of reproductive organs and the regulation of the cardiovascular and musculoskeletal systems[8]. To do this, E2 binds to estrogen receptors alpha or beta (ERα, ERβ) and then the E2-

bound estrogen receptors dimerize and act as transcription factors[6]. Previous studies featuring male Japenese medaka exposed to E2 or Orthoester-2k, a ligand that activates ERα, found increases in the mRNA levels of choriogenin H (an egg precursor protein) following exposures showing the choriogenin H expression is regulated by ERα[7]. Therefore, in this study, we expected E2 to bind to and activate ERα leading to the transcription of choriogenin H, and subsequently GFP. The results of this study are consistent with this prediction. Dose-dependent increases in fluorescence intensity were observed in response to E2. Because of this consistency, these trials give some evidence to the notion that the REACTIV assay is capable of measuring levels of endocrine disruption in response to pro-estrogenic chemicals.

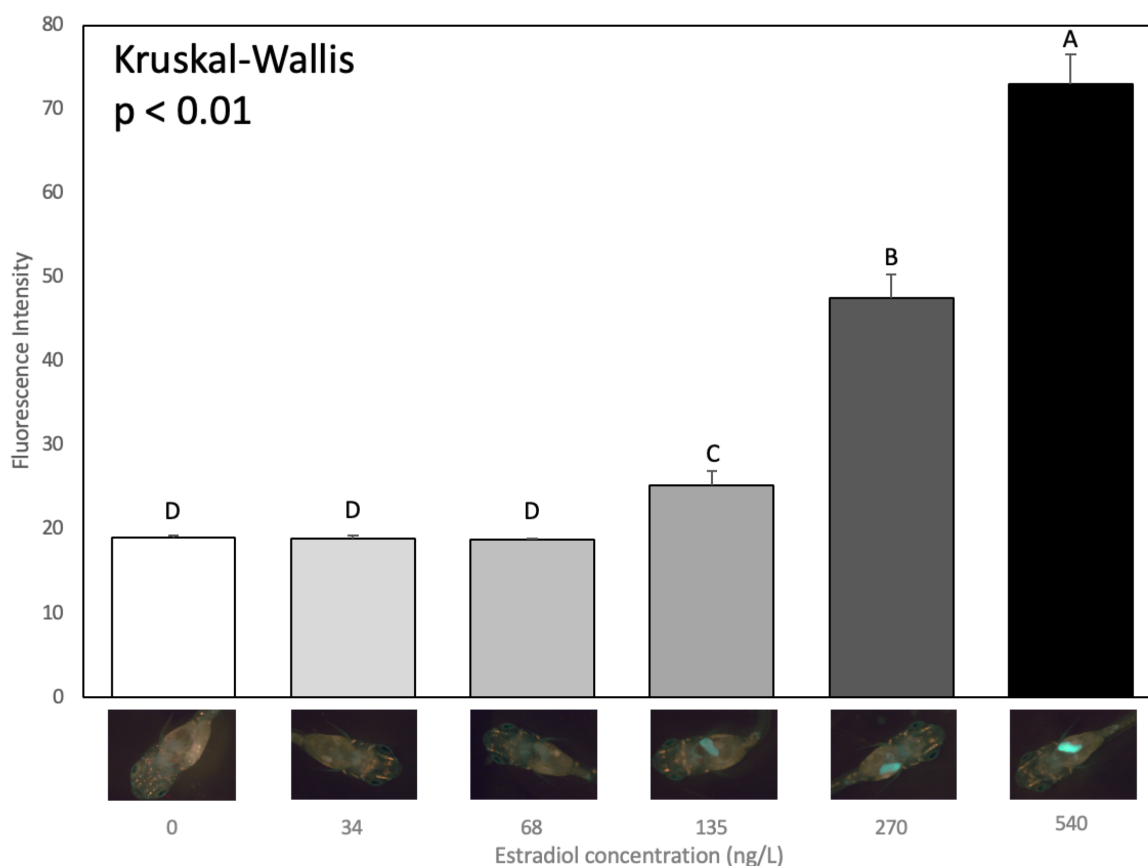

Figure 1. Mean fluorescent intensity values averaged across the three replicates of the REACTIV assay featuring exposures to estradiol. Error bars represent standard error and the letters above each bar indicate statistical significance between the groups. The representative

images below each bar serve as a visual aid in demonstrating the increase in fluorescence with increasing estradiol concentrations.

Bisphenol A

Similar to estradiol, significant differences in the fluorescence intensity of medaka exposed to BPA were detected (Kruskal-Wallis, $p < 0.01$). The fluorescence intensity of medaka in the 0, 1, and 2 mg/L groups did not differ from one another. However, significant dose-dependent increases in fluorescence intensity were observed in the 3, 4, and 5 mg/L, with significant differences occurring between the 3 mg/L group and the 5 mg/L group.

Bisphenol A is a compound that is used in large quantities to produce various polycarbonate plastics such as water bottles, eyewear, and shatterproof windows[10]. Bisphenol A (BPA) has many structural similarities with estradiol such as phenol groups and methyl groups; because of this likeness, BPA is capable of binding to estrogen receptor subtypes (ERα and ERβ) and activating them[9]. This is important because it means that in the case of these transgenic medaka, BPA is capable of inducing transcription of choriogenin H and GFP, leading to increased levels of fluorescence. The results indicate that with increasing concentrations of BPA (mg/L), there is an increasing level of fluorescence in the livers of the hatchlings. Overall, this matches the predicted effect of exposure to increasing levels of BPA and gives further evidence that the REACTIV assay provides valid and reliable results.

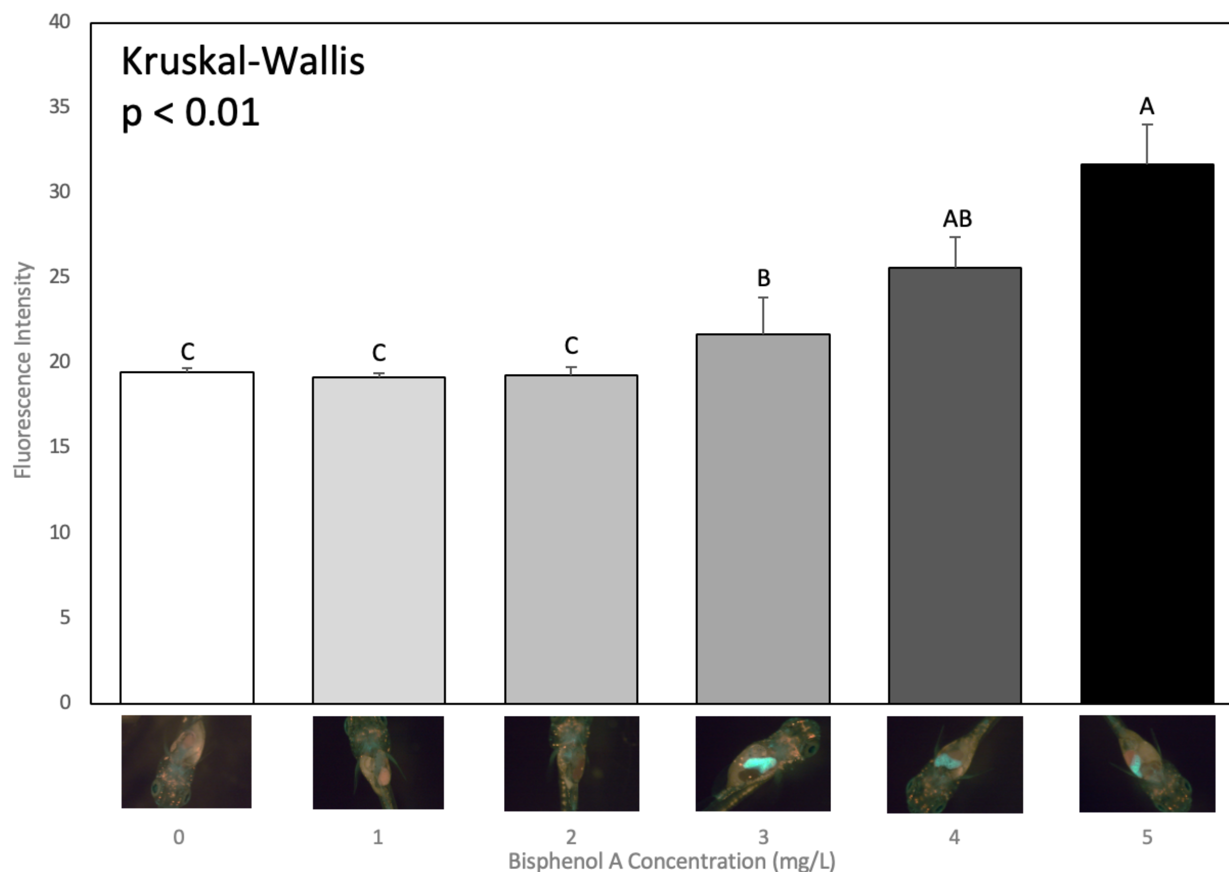

Figure 2. Mean fluorescent intensity values averaged across the three replicates of the REACTIV assay featuring exposures to bisphenol A (BPA). Error bars represent standard error and the letters above each bar indicate statistical significance between the groups. The representative images below each bar serve as a visual aid in demonstrating the increase in fluorescence with increasing estradiol concentrations.

Saccharin

Unlike the previous chemicals, significant differences in the fluorescence intensity of medaka exposed to saccharin were not detected (ANOVA $p > 0.05$). There is an “A” above each bar because the test groups were not statistically different. The fluorescence intensity of medaka in the 0, 1, 2, 3, 4, and 5 mg/L groups did not differ from one another, illustrating that there was no dose-dependent increase in fluorescence intensity.

Saccharin is an artificial sweetener used in various consumer products such as chewing gum, jelly, and candy[11]. It does not act as an estrogen mimic and thus does not bind to ERα in the hepatocytes of Japanese medaka. Because of this, saccharin was not expected to induce transcription of choriogenin H and GFP. Furthermore, despite increasing concentration of saccharin, it is expected that the levels of fluorescence should remain the same. Statistical analysis shows that there were no statistically significant differences between the groups despite the increasing concentrations of saccharin. Because, saccharin plays no part in activating nor inhibiting this pathway, such a result was expected. Here, a false positive would mean that one of the groups had a statistically significant difference in terms of fluorescence intensity when compared to the other groups. This did not occur, and the findings provide evidence that the REACTIV assay does not detect false positive results. Despite the increasing concentrations of saccharin, the levels of fluorescence were not statistically different from the control group (0 mg/L saccharin).

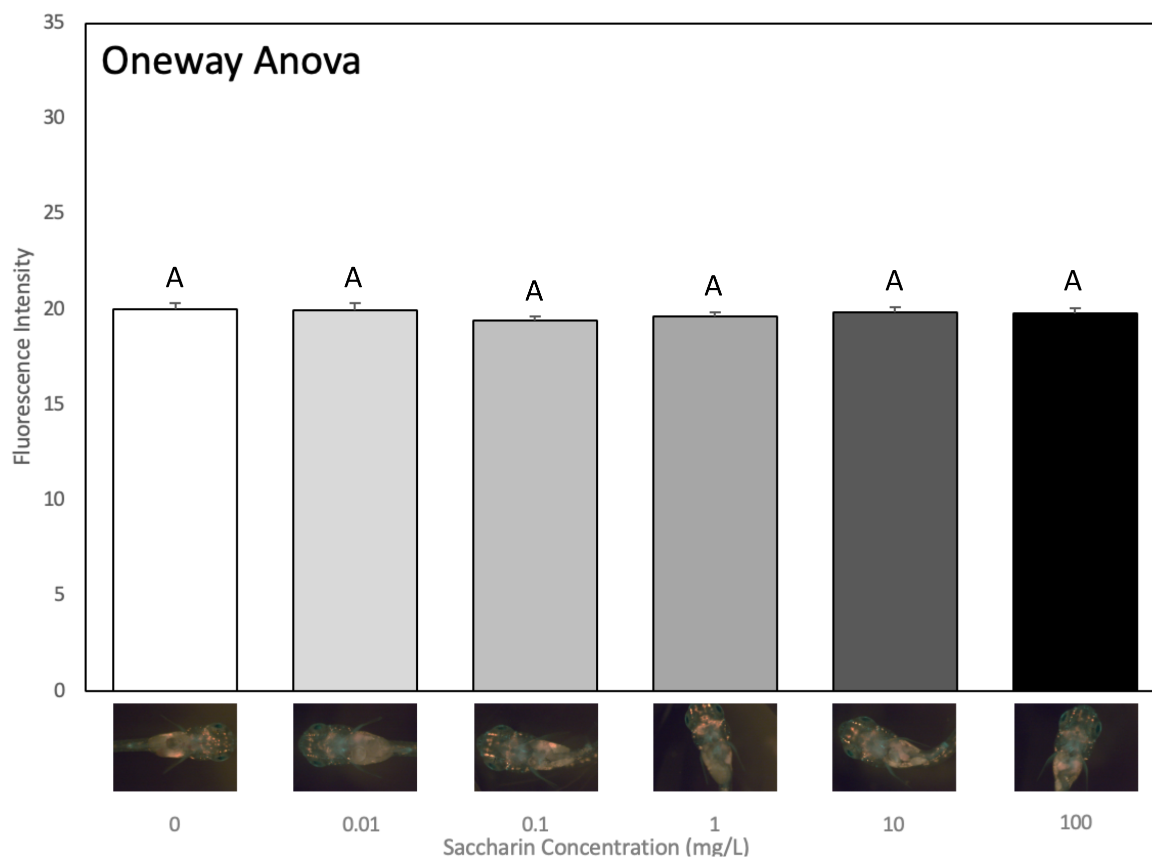

Figure 3. Mean fluorescent intensity values averaged across the three replicates of the REACTIV assay featuring exposures to saccharin. Error bars represent standard error and the letters above each bar indicate statistical significance between the groups. The representative images below each bar serve as a visual aid in demonstrating the increase in fluorescence with increasing estradiol concentrations.

Testosterone

Significant differences in the fluorescence intensity of medaka exposed to testosterone were detected (Kruskal-Wallis $p < 0.01$). The fluorescence intensity of medaka in the lowest 5 concentrations (0, 3.7, 11, 33, and 100 μg/L) were not statistically significant from each other. However, a dose-dependent increase in fluorescence intensity was observed in the exposure with the highest concentration group (300 μg/L) showing a significant difference from the lowest three concentrations.

Testosterone is an aromatizable androgen and is typically called the "male sex hormone"[12]. While testosterone cannot bind directly to these estrogen receptors, it is biotransformed by the enzyme aromatase into E2 which can then subsequently bind to ERα or Erβ, activating transcription of choriogenin H and GFP[12].Thus, it is expected that with exposure to increasing concentrations of testosterone, there will be a greater concentration of E2; and thus, a higher level of fluorescence intensity will be observed. The results back up this prediction and indicate that there are higher levels of fluorescence in the livers of hatchlings exposed to higher concentrations of testosterone. This gives further evidence that the REACTIV assay provides valid results which match the expected outcome. Furthermore, this ability to measure endocrine disruption even when biotransformation occurs is sometimes not possible in certain *in vitro* assays, thereby offering the REACTIV assay a unique advantage in comparison with these methods[13].

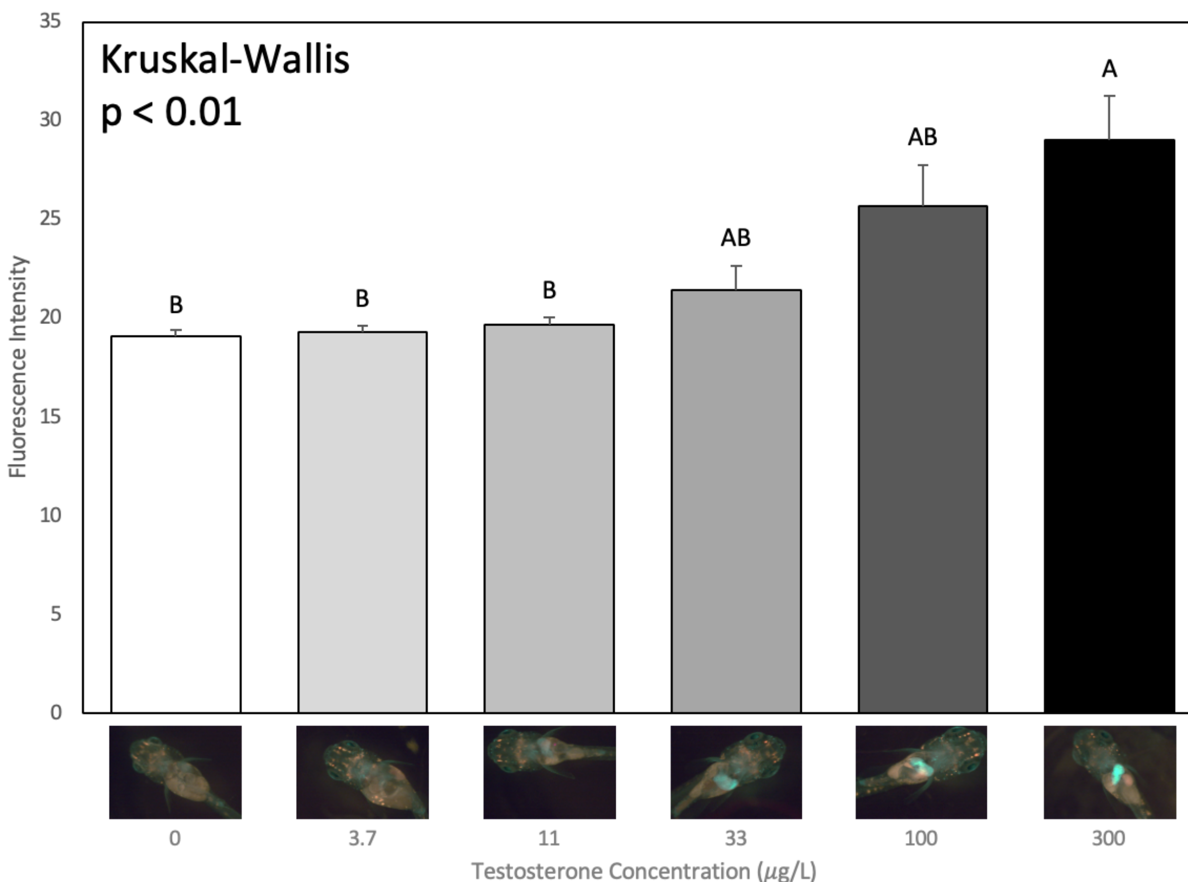

Figure 4. Mean fluorescent intensity values averaged across the three replicates of the REACTIV assay featuring exposures to testosterone. Error bars represent standard error and the letters above each bar indicate statistical significance between the groups. The representative images below each bar serve as a visual aid in demonstrating the increase in fluorescence with increasing estradiol concentrations.

CONCLUSION

The results of the E2, BPA, and testosterone exposures demonstrate that there is increased fluorescence with exposure to increasing concentrations of these compounds, matching the molecular mechanism of E2 or BPA binding to ERα and inducing transcription of choriogenin H and GFP in the transgenic medaka embryos. Overall, the preliminary results of the levels of fluorescence due to exposure to saccharin, estradiol, bisphenol A, and testosterone indicate that the REACTIV assay is a reliable screening method to test for endocrine disruption using

transgenic Japanese medaka. Furthermore, the data from the saccharin exposures demonstrated that this assay does not detect false positives. Additionally, the results from the testosterone trials indicate that the REACTIV assay is able to detect endocrine disruption even when the test chemical is being biotransformed. This preliminary data along with those collected from other labs around the world involved in this project have been sent to the OECD for further validation in hopes that the REACTIV assay will become adopted as standard practice in the field of aquatic toxicology in the future. While this study evaluated four chemicals, future studies should examine other compounds to provide further evidence that the REACTIV assay produces consistent results and avoids false positives.

REFERENCES

1. (Interference of endocrine disrupting chemical with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish - Ksenia Cheschenko, Farzad Pakdel, Helmut Segner, Olivier Kah
2. 2020, REACTIV Assay Interlaboratory Study Document
3. REACH Regulations; https://ec.europa.eu/environment/chemicals/reach/reach_en.htm
4. OECD (2021), *Test No. 250: EASZY assay - Detection of Endocrine Active Substances, acting through estrogen receptors, using transgenic tg(cyp19a1b:GFP) Zebrafish embrYos*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/0a39b48b-en.
5. OECD (2009), *Test No. 230: 21-day Fish Assay: A Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264076228-en.
6. Yaşar, P., Ayaz, G., User, S. D., Güpür, G., & Muyan, M. (2016, December 5). *Molecular mechanism of estrogen-estrogen receptor signaling*. Reproductive medicine and biology. Retrieved March 21, 2023, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5715874/
7. N;, Y. A. K. K. A. K. T. (2014, December 10). *Induction of the estrogen-responsive genes encoding choriogenin H and L in the liver of male medaka (oryzias latipes) upon exposure to estrogen receptor subtype-selective ligands*. Journal of applied toxicology : JAT. Retrieved March 21, 2023, from https://pubmed.ncbi.nlm.nih.gov/25501297/
8. JC;, G. C. J. T. W. S. C. H. (n.d.). *Production and actions of Estrogens*. The New England journal of medicine. Retrieved March 21, 2023, from https://pubmed.ncbi.nlm.nih.gov/11821512/

9. Acconcia, F., Pallottini, V., & Marino, M. (2015, October 7). *Molecular mechanisms of action of BPA*. Dose-response : a publication of International Hormesis Society. Retrieved March 21, 2023, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4679188/
10. U.S. Department of Health and Human Services. (n.d.). *Bisphenol A (BPA)*. National Institute of Environmental Health Sciences. Retrieved March 21, 2023, from https://www.niehs.nih.gov/health/topics/agents/sya-bpa/index.cfm
11. Brown, M. J. (2019, October 7). *Saccharine: What is it, safety, and more*. Healthline. Retrieved March 21, 2023, from https://www.healthline.com/nutrition/saccharin-good-or-bad
12. Schulster M, Bernie AM, Ramasamy R. The role of estradiol in male reproductive function. Asian J Androl. 2016 May-Jun;18(3):435-40. doi: 10.4103/1008-682X.173932. PMID: 26908066; PMCID: PMC4854098.
13. Rehberger, K., Kropf, C., & Segner, H. (2018). In vitro or not in vitro: A short journey through a long history. *Environmental Sciences Europe*, *30*(1). https://doi.org/10.1186/s12302-018-0151-3