Organisation for Economic Co-operation and Development

ENV/CBC/MONO(2023)16

Unclassified English - Or. English

24 July 2023

ENVIRONMENT DIRECTORATE

CHEMICALS AND BIOTECHNOLOGY COMMITTEE

Validation of the Juvenile Medaka anti-androgen screening assay (JMASA)

Series on Testing and Assessment

No. 380

JT03523611

OECD Environment, Health and Safety Publications
Series on Testing & Assessment
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VALIDATION OF JUVENILE MEDAKA ANTI-ANDROGEN SCREENING ASSAY (JMASA)

February 2023

FOREWORD

This document contains the validation report for the Guidance Document (GD) on the Juvenile Medaka Anti-Androgen Screening Assay (JMASA). This validation report is the result of project 2.57 of the Test Guidelines Programme (TGP), led by Japan, which was included in the TGP workplan in 2016. The JMASA protocol and accompanying documents have been discussed in the Validation Management Group on Ecotoxicity testing (VMG-Eco) from 2016-2022, and subsequently reviewed in 2022. The document was subsequently approved by the WNT at its 35th meeting in April 2023, and the Chemicals and Biotechnology Committee agreed to its declassification on 20 June 2023.

This document is published under the responsibility of the Chemicals and Biotechnology Committee.

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Abbreviation and Definition

AFSS: Androgenized Female Stickleback Screen

AR: androgen receptor.

Body weight: fish wet weight in the condition of blotted dry.

DHT: 5α-dihydrotestosterone.

Dmy: sex determining gene of medaka; a Y-specific DM domain gene.

Dpf: days post fertilisation.

EDC: endocrine disrupting chemical.

ELISA: Enzyme-Linked Immunosorbent Assay.

Flow-through test: a test with continued flow of test solutions through the test system during the duration of exposure.

GD: guidance document.

ICP-MS: Inductively Coupled Plasma-Mass Spectrometry

JMASA: Juvenile Medaka Anti-Androgen Screening Assay.

LC_{50}: Median lethal concentration; the concentration of a test chemical that kills 50% of exposed test organisms within a given time period.

LOQ: limit of quantification.

Loading rate: the wet weight of fish per volume of water.

LOEC: Lowest observed effect concentration; the lowest tested concentration of a test chemical at which the chemical is observed to have a statistically significant effect (at $p < 0.05$) when compared with the control.

MCR: Mitsubishi Chemical Research Co.

MEOGRT: Medaka Extended One Generation Reproduction Test.

mRNA: messenger ribonucleic acid.

NIES: National Institute for Environmental Studies

NOEC: No observed effect concentration; the test concentration immediately below the LOEC.

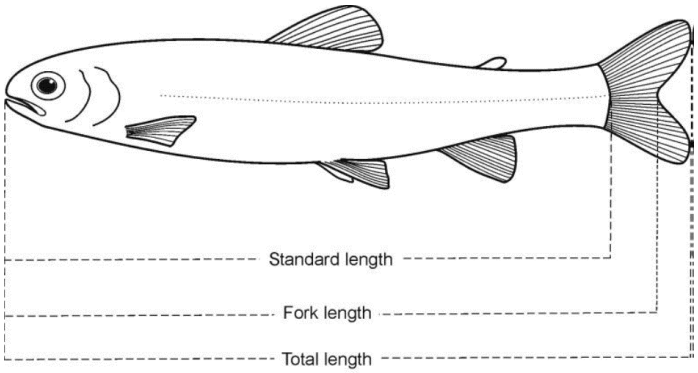
PCR: polymerase chain reaction.

RADAR: Rapid Androgen Disruption Activity Reporter

RT-PCR: Reverse Transcriptase Polymerase Chain-Reaction.

SSC: secondary sex characteristics.

Total length: refer to figure below.



TG: OECD guideline for the testing of chemicals.

U.S. EPA: United States Environmental Protection Agency.

VTG: Vitellogenin; a phospholipoglycoprotein precursor to egg yolk protein that normally occurs in sexually active females of all oviparous species.

Wpf: weeks post fertilisation.

Acknowledgement

This work is the collaborative effort of three laboratories which performed the experiments described here.

Prof. Taisen Iguchi (Yokohama City University) and Prof. Norihisa Tatarazako (Ehime University) coordinated the interlaboratory work. The following laboratories and their staff took part in the JMASA interlaboratory validation exercise:

- Institute of Environmental Ecology, IDEA Consultants Inc., Japan: Misa Toda, Yu Totsuka, Maki Sakurai, and Tetsuro Okamura performed the experiments, supervised by Yuta Onishi.
- Environmental, Health and Safety Assessment Center, Mitsubishi Chemical Research Co., Japan: Midori Mino, Junpei Morita, and Kazuyuki Niikura performed the experiments, supervised by Tatsuhiro Niino.
- Health and Environmental Risk Division, National Institute for Environmental Studies (NIES), Japan: Haruna Watanabe, Takafumi Horie, Ataru Nakamura, Ikumi Tamura, Hitomi Takanobu, Masaaki Koshio, Ayano Yagi, Yoko Shintaku and Takahiro Yamagishi performed the experiments, supervised by Norihisa Tatarazako and Hiroshi Yamamoto

1. Introduction

The overall objective of the validation exercise for the JMASA was to assess the reproducibility among three laboratories. This 28-day in vivo screening assay can identify the potency of endocrine disrupting chemicals (EDCs) with anti-androgenic activity in fish using Japanese (minami) medaka (*Oryzias latipes*). The concept of the JMASA is based on studies of papillary processes (papillae) on the anal fin, whose induction and development is regulated by the androgen receptor signaling pathway (1, 2). In Japanese medaka, papillary processes normally produced on the latter half of the anal fin are the main secondary sex characteristics (SSC) of males and can be a specific biomarker for androgens and anti-androgens.

In Japanese medaka, papillary processes normally appear in adult males but not in females. In males, papillary processes can be found on fin rays from the 2nd to the 8th or 10th from the posterior end of the anal fin depending on the strain and age, although papillae are rarely produced on the 1st fin ray (Figure 1: male anal fin, Figure 2: female anal fin).

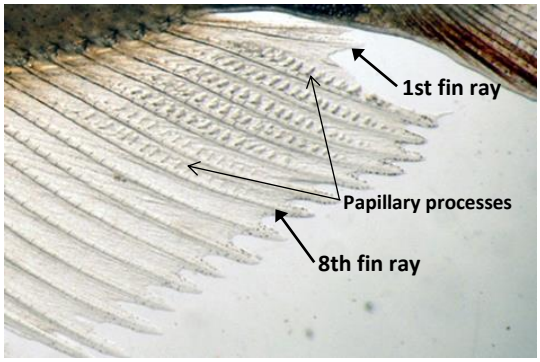


Figure 1. Anal fin of control male medaka.

Figure 2. Anal fin of control female medaka.

The current OECD Test Guidelines (TG), e.g., TG229 or TG230, provide a 21-day in vivo screening assay which can detect androgen antagonism in addition to other endocrine disrupting activities. However, the activity detected in these assays is not always specific to the identification of chemicals with androgen antagonism (3, 4, 5, 6). In addition, the GD No.148 provides the Androgenized Female Stickleback Screen (AFSS), a 21-day fish assay which assesses an androgen antagonism of a test chemical based on the reduction of spiggin in the kidneys of female sticklebacks in which the level of the specific biomarker protein (i.e., spiggin) is moderately induced by exposure to 5α-dihydrotestosterone (DHT) (7). Although the AFSS can specifically identify potential anti-androgenic chemicals, the assay may include some limitations: (a) the recommended test species, the three spined stickleback (*Gasterosteus aculeatus*), has not been adopted in current TGs for EDCs such as TG229, 230, 234 or 240 (3, 4, 8, 9); (b) the assay can identify the androgen antagonists which interact with androgen receptor, but may not be applicable to detection of the potential activities that interfere with steroid synthesis and metabolism, since androgenized females rather than intact males are used; and (c) the assay requires particular techniques and apparatus to maintain the same concentration of DHT in all test chambers over the test duration. More recently, to overcome these limitations with

considering the animal welfare, Rapid Androgen Disruption Activity Reporter (RADAR) assay was proposed and approved as TG No. 251 (10) in 2021, which was developed for the detection of androgen axis chemicals. It is performed in 6-well plate format and can serve as a quick screen for potential androgen axis disrupting chemicals using embryos/eleutheroembryos of *spg1-gfp* transgenic medaka, constructed with the promoter of the Spiggin 1 gene of the three spiced stickleback coupled to a reporter gene for Green Fluorescent Protein (GFP). This RADAR assay can mostly overcome the (a) and (b) above while the last (c) remained unresolved.

For eco-toxicity testing of EDCs, TG240, the Medaka Extended One Generation Reproduction Test (MEOGRT), which is defined as a test at Level 5 in the OECD's Conceptual Framework for the Testing and Assessment of Endocrine Disrupters (11) was adopted in 2015. In Japan's program on endocrine disruption "EXTEND2010" (currently updated as "EXTEND2016" and then, "EXTEND2022"), Japanese medaka is consistently used as a test species in Tier 1 screening (e.g., according to the TG229) and Tier 2 testing (e.g., according to the TG240). In this context, Japan started to develop and optimize the method of in vivo assay using juvenile Japanese medaka, which is applicable to the screening of anti-androgenic chemicals, in 2016.

In the JMASA, juvenile medaka (i.e., sexually immature medaka at 5-6 weeks post fertilisation (wpf)) are exposed to the test chemical during a limited part of their lifecycle (28 days) and the SSC (i.e., papillary processes on anal fin) are assessed at the termination of the exposure. The JMASA can detect both androgen receptor antagonistic and agonistic action in medaka: anti-androgens can be identified by an inhibition of SSC in genetic males and androgenic potency of test chemicals can be distinguished by induction of papillary processes in genetic females. When hepatic vitellogenin (VTG) levels (protein concentration or mRNA expression) are optionally measured, other potential activities of the test chemical (e.g., (anti-)estrogenicity or aromatase inhibition) can also be assessed. Other measurements including survival and growth (e.g., total length and body weight) are not considered as endpoints in the JMASA but are needed to confirm the statistical robustness of the assay and to identify toxic effects (i.e., not related to endocrine disruption) of the test chemical.

2. Principle of the test

The assay is initiated with juvenile medaka in which sex cannot be identified externally (i.e., papillary processes on the anal fin are not yet visible in males). At the termination of the assay, genetic sex is identified by the presence of the sex determining gene (i.e., *dmy* gene), for all surviving fish. Overviews of the relevant assay conditions are provided in Table 1. The assay is conducted using a range of test chemical exposure concentrations (normally three test concentrations are used), as well as a dilution water control and a solvent control (if needed). Four replicate vessels are used for each concentration including controls and each vessel contains 7 (or less) fish. The exposure is conducted for 28-days and sampling of fish is carried out at the end of this period. On sampling at day 28, all fish are killed humanely.

Table 1. Test conditions for Juvenile Medaka anti-Androgen Screening Assay

1.	Test species (recommended)	Japanese medaka (*Oryzias latipes*)
2.	Test type	Flow-through test
3.	Water temperature	25 ± 1 ^{o}C (the recommended mean temperature throughout the test in each tank is 25 ± 1 ^{o}C)
4.	Illumination	Fluorescent bulbs (wide spectrum)
5.	Illumination level	10-20 µE/m^2/s, 540-1000 lux, or 50-100 ft-c
6.	Photoperiod	12-16 hours light, 12-8 hours dark
7.	Loading rate	<5 g/L
8.	Test chamber size	Minimum of 1.8 L
9.	Volume exchanges of test solutions	Minimum of 5 daily
10.	Age of test organisms at initiation	35-42 dpf
11.	Number of organisms per replicate	7 fish/replicate tank (recommended)

12. Number of treatments	3 test chemical treatments plus appropriate control(s)
13. Number of replicates per treatment	4 replicates per treatment for test chemical and control (minimum)
14. Number of organisms per test	Minimum 112 fish (minimum 140 fish, if solvent control is used)
15. Feeding regime	Live brine shrimp (*Artemia* spp.) *nauplii*, supplemented with a commercially available flake food if necessary, two or three times daily, *ad libitum*
16. Aeration	None unless dissolved oxygen falls below 60 % ASV
17. Dilution water	Clean surface, well or reconstituted water or dechlorinated tap water.
18. Chemical Exposure duration	28 days (pre-exposure period is not required)
19. Biological endpoints	Survival; abnormal response (e.g., in behavior and appearances), secondary sex characteristics (number of papillary processes); vitellogenin (VTG protein or *vtg* mRNA, as an optional endpoint)
20. Test acceptability criteria	Mean mortality of ≤10% in the controls; the concentrations of the test chemical in each test vessel are satisfactorily maintained within ±20% of the mean measured value in the treatment group; dissolved oxygen of ≥60% ASV; water temperature of 25±1 ^{o}C throughout the test and ±1 °C between test vessels at any one time; the number of males per tank should be at least one in at least three out of four replicates

Measurements in the course of the exposure include indications of general toxicity (i.e., mortality, abnormal behavior and growth (e.g., total length and body weight)), as well as the endpoints (SSC and VTG (optional)) to evaluate (potential) interaction with the endocrine system. All endpoints are analyzed in the context of determination of the genetic sex of the individuals. The number of papillary processes on the anal fin serves for the detection of androgen receptor antagonists and agonists. Reduction of the number of papillary processes in genetic males has been demonstrated following exposure to androgen antagonists (2, 12). Also, it has been well documented that the detection of androgen agonists is possible based on the appearance of SSC in females (5, 12, 13). If the VTG levels in males and females are measured, potential (anti-)estrogenic activity and/or aromatase inhibition can also be identified (4).

3. Overview of the Validation Study

3.1Specific goals

1) Phase 1 validation study was conducted only in two laboratories with positive (flutamide, vinclozolin, and fenitrothion) and negative (cromolyn) chemicals, and phase 2 with the third laboratory.
2) In addition to the anti-androgenic chemicals, the chemicals with other mode of actions (i.e., estrogen, androgen, and steroidogenesis inhibitors) are examined in this protocol.
3) Applicability to the different Japanese medaka strain was investigated in addition to clarify the relationship between the age, the growth (body weight and length) and the secondary sexual characteristics.
4) Performance was compared between the laboratories. Reliability, reproducibility across laboratories, and sensitivity of the assay were determined.

3.2Overview of the Test Conditions

All the experiments carried out by three laboratories were performed using Japanese (minami) medaka strain bred in their laboratory. For the interlaboratory validation, all participating laboratories (NIES, IDEA, and Mitsubishi Chemical Research) were asked to test three expected anti-androgenic chemicals and one expected inert chemical. These are shown in Table 2 below.

Table 2. List of chemicals asked for all three laboratories to test.

	Test Chemical	Mode of Action
Anti-androgenic	Flutamide	AR antagonist
	Vinclozolin	Metabolites are AR antagonists
	Fenitrothion	AR antagonist
Expected Inert	Cromolyn	Mast cell stabilizer

Additional expected anti-androgenic chemicals, the chemicals with the other mode of action(s), and expected inert chemicals were also examined in NIES or IDEA. These are shown in Table 3 below.

Table 3. List of chemicals optionally used in the validation test.

	Test Chemical	Mode of Action
Anti-androgenic	Manneb	Expected AR antagonist
	Linuron	Inhibition of steroidogenesis, AR antagonist
	Cyproteron acetate	AR antagonist

Estrogen	17β-estradiol	ER agonist ligands
	estrone	ER agonist ligands
Androgen	Trenbolon	AR agonist
Steroidogenesis Inhibitor	Ketoconazole	Expected steroidogenesis inhibitor
	Tebuconazole	Expected steroidogenesis inhibitor
Expected Inert	Zinc Chloride	Unknown
	SDS	Unknown

In all three laboratories, the flow-through system was conducted based on the draft protocol. Based on the growth curve, the age of test organisms at initiation was 35 dpf at NIES and Mitsubishi Chemical Research and 42 dpf at IDEA. 5 L chamber was used for all the test chemicals except for vinclozolin; 1.8 L chamber was used to save the amount of chemical used and to minimize the frequency of preparing the stock solution. The overview of the testing conditions which might affect the results are summarized in Table 4.

Table 4. Overview of key testing conditions in three laboratories participated the interlaboratory ring test.

	NIES	IDEA	Mitsubishi Chemical Research
Test organism	*Oryzias latipes* (NIES-R strain)	*Oryzias latipes* (NIES-R strain)	*Oryzias latipes* (NIES-R strain)
Dilution water	Dechlorinated tap water (Tsukuba, Japan)	Dechlorinated tap water (Yaizu, Japan)	Dechlorinated tap water (Yokohama, Japan)
Feeding (mg dry weight/fish/day)	*Artemia* sp. nauplii 15% of estimated body weight of fish	*Artemia* sp. Nauplii sufficient amount to maintain body condition.	*Artemia* sp. nauplii 9.5 (0-7 d) 15.9 (8-28 d)
Age of test organisms at initiation	35 dpf	42 dpf	35 dpf
Chamber size	5 L (1.8 L for vinclozolin)	2.5 L	5 L (1.8 L for vinclozolin)
Appearance of flow-through system			

In NIES, the relationship between age, growth (weight and length), and secondary sexual characteristics (first appearance of papillary processes) was investigated for two strains, NIES-R and SKR. The former one has been bred in NIES for more than 20 years while the latter has been bred for three years in NIES after sampled in Sakura City, Chiba, Japan. The overview of

these two strains is shown in Table 5.

Table 5. Overview of two strains used for drawing growth curve.

	NIES-R	SKR
Species name	*Oryzias latipes* (Japanese medaka)	*Oryzias latipes* (Japanese medaka)
Appearance		
Origin	Unknown	Sakura, Chiba, Japan
Size (Total body length)	3.2-3.4 cm	2.7-2.9 cm
Hatching days	7-8 days after fertilization	10-11 days after fertilization
First observation of papillary process	Approx. 45 days post hatching Approx. 53 days post fertilization	No data

3.3Statistical Analysis

If the data (normally, replicate means) are assumed to be a monotone trend (in an increase or a decrease), it is recommended to analyse the data by a trend-based step-down methods (e.g., step-down Williams test, Jonckheere-Terpstra test). To assess monotonicity, a visual check from a scatter plot can be used, although data should preferably be evaluated by using linear and quadratic contrasts. If the data are non-monotonic, a multiple comparison test, such as the Dunnett test (parametric data) or Mann-Whitney test according to Holm (non-parametric data), should be performed.

As a preliminary, the data should be assessed for normality (e.g., using the Shapiro-Wilk test) and homogeneity of the variance (e.g., using Levene's test) among the treatment groups where parametric methods (e.g., Williams test, Dunnett test) are employed for analysis. Where the assumption of normality or variance homogeneity is not met, data transformation, to achieve these requirements, can be sought or non-parametric methods employed.

It is important that the strongest, valid tests are employed for statistical analysis of the endpoints. For example, the power properties of the step-down Jonckheere-Terpstra test are very similar to those of the step-down Williams test, when the data are normally distributed with homogeneous variances, and are superior to Williams when those conditions are violated. On the other hand, for datasets with few replicates, the power properties of the Mann-Whitney and Dunn tests are worse, sometimes much worse, that those of Dunnett's test.

An example of statistical flow chart for biological data (e.g., SSC, VTG) from JMASA provides below.

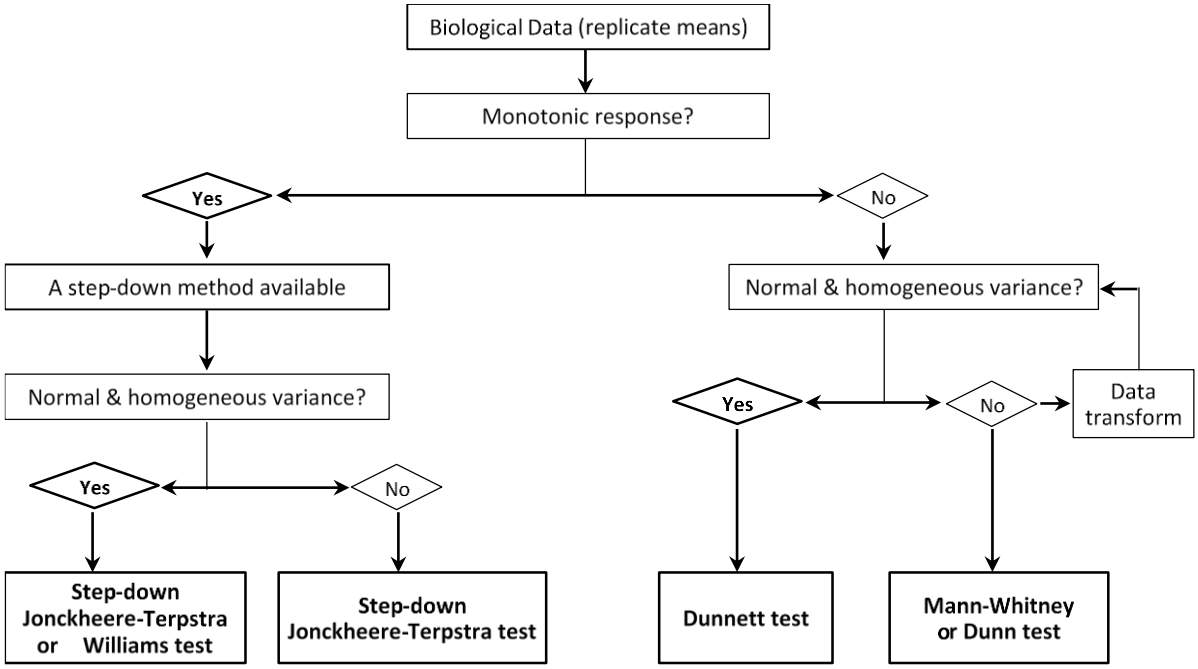


Figure 3. An example of statistical flow chart

4. Results and Discussion

4.1Results of expected anti-androgenic chemicals

4.1.1 Vinclozolin

Figure 4 shows the results of vinclozolin, a well-known AR antagonist, in three laboratories. The top subfigures (a, b, and c) show the number of papillary processes in each concentration, the middle subfigures (d, e, and f) show the relationship between the body length and the number of papillary processes to possibly discriminate the growth inhibition-driven decrease in papillary processes. The bottom subfigures (g, h, and i) show the liver vitellogenin (VTG) concentration in genetic males and females in each concentration.

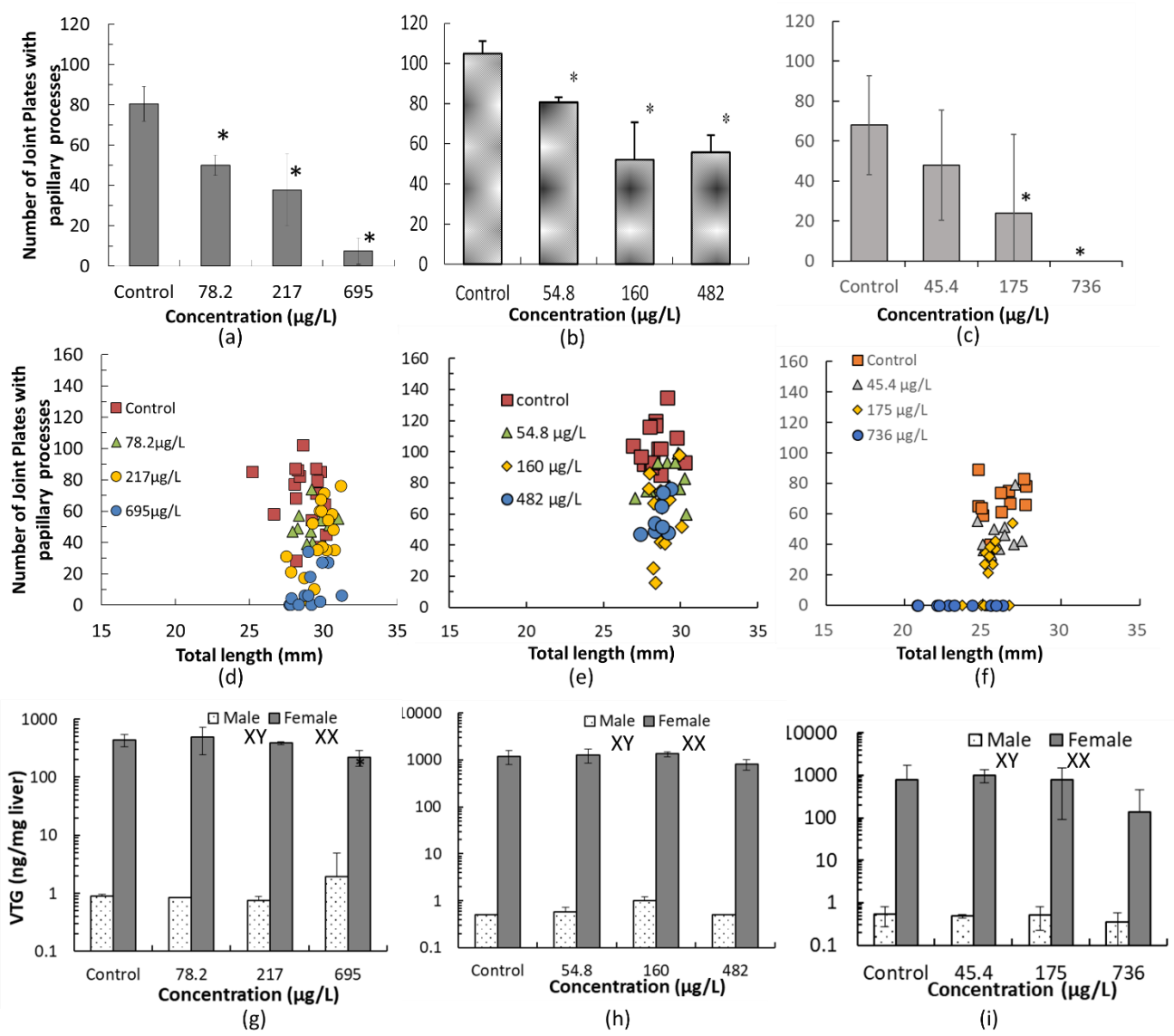


Figure 4. Results of interlaboratory studies for vinclozolin: Concentration-Response Relationship in the number of papillary processes in (a) NIES, (b) IDEA, and (c) Mitsubishi Chemical Research (MCR); Relationship between total length and the number of papillary processes in (d) NIES, (e) IDEA, and (f) MCR; liver VTG concentration in both genetic males and females in each concentration in (g) NIES, (h) IDEA, and (i) MCR (*: $p<0.05$).

As can be seen, similar significant decreasing trend in the number of joint plates with papillary processes (PPs) was found in all the three laboratories as the increase of the aqueous concentration of vinclozolin, and the results agreed well each other although the slight difference in LOEC was observed (78.2, 54.8 and 175 µg/L in NIES, IDEA, and MCR, respectively). The slight growth inhibition was observed at the highest concentration in MCR (Figure 4f) while no apparent growth inhibition was observed in the lower concentration with the statistically significant decrease observed in the number of PPs (175 µg/L). The significant growth inhibition was observed even at the highest concentration neither in NIES (Figure 4d) nor in IDEA (Figure 4e). The liver VTG concentration did not change significantly even at higher concentrations.

4.1.2 Flutamide

Figure 5 shows the results of flutamide, another well-known AR antagonist, in three laboratories. As with vinclozolin, the concentration-response relationship for the number of PPs are shown in Figure 5a to 5c, while the relationship between the body length and the number of PPs (growth inhibition) was plotted in Figure 5d to 5f. The liver VTG concentration both in genetic male (XY) and female (XX) is shown as bar graph in Figure 5g to 5i.

The significant decreasing trend of PPs similar to vinclozolin was found in all the three laboratories as the increase of the aqueous concentration of flutamide and the results agreed well each other although the slight difference in LOEC was observed, as 271, 497, and 243 µg/L in NIES, IDEA, and MCR, respectively. As with vinclozolin, slight growth inhibition was observed at highest concentration (936 µg/L) in MCR but no apparent growth inhibition was observed at the lower concentrations with statistically significant decrease of PPs were observed (Figure 5c). The significant growth inhibition was observed neither in NIES nor in IDEA. Since the concentration level of growth inhibition was well above the LOEC for the number of PPs even in MCR, and the anti-androgenic effects were observed by the decrease (delay) in the formation of PPs for flutamide. The liver VTG concentration did not change significantly in all three laboratories.

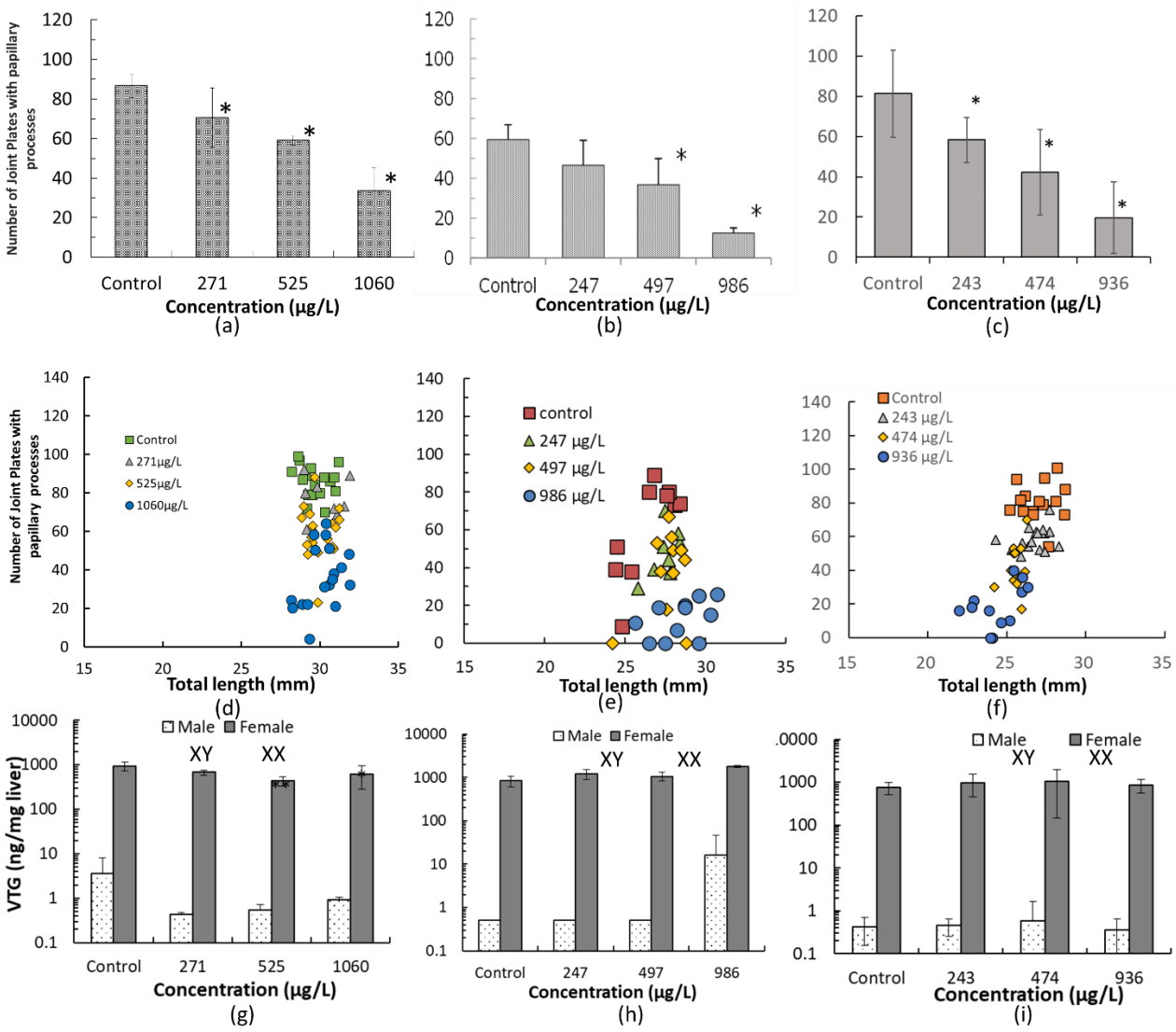


Figure 5. Results of interlaboratory studies for flutamide: Concentration-Response Relationship in (a) NIES, (b) IDEA, and (c) Mitsubishi Chemical Research (MCR); Relationship between total length and number of papillary processes in (d), NIES, (e) IDEA, and (f) MCR; liver VTG concentration in both genetic males and females in (g) NIES, (h) IDEA, and (i) MCR (*: $p<0.05$; *: $p<0.01$).

4.1.3 Fenitrothion

Figure 6 shows the results of fenitrothion, another well-known AR antagonist, in three laboratories. As with vinclozolin and flutamide, the concentration-response relationship for the number of PPs are shown in Figure 6a to 6c, while the relationship between the body length and the number of PPs (growth inhibition) was plotted in Figure 6d to 6f. The liver VTG concentration both in genetic male (XY) and female (XX) is shown as bar graph in Figure 6g to 6i.

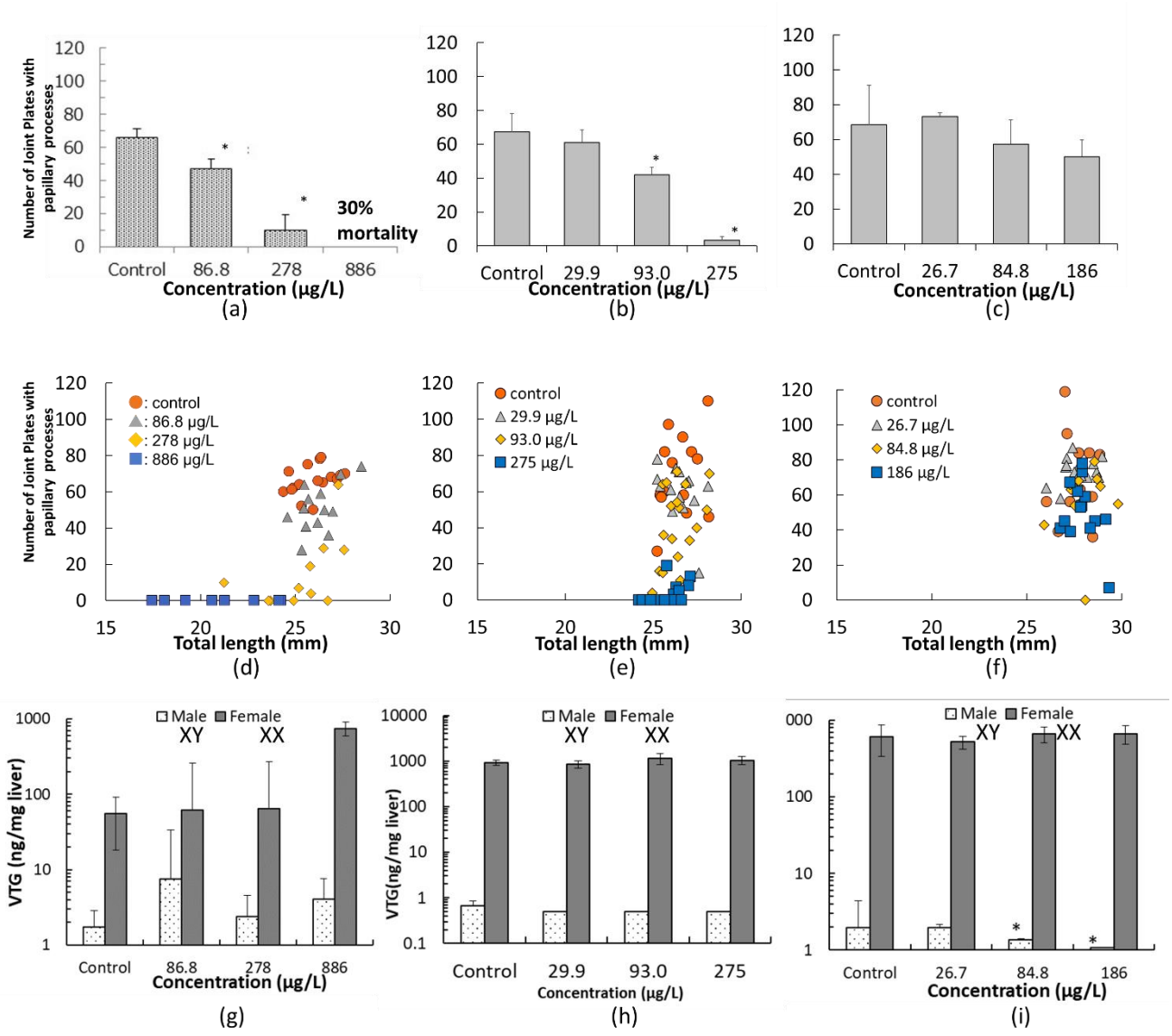


Figure 6. Results of interlaboratory studies for fenitrothion: Concentration-Response Relationship in (a) NIES, (b) IDEA, and (c) Mitsubishi Chemical Research (MCR); Relationship between total length and number of papillary processes in (d) NIES, (e) IDEA, and (f) MCR;

liver VTG concentration in both genetic males and females in (g) NIES, (h) IDEA, and (i) MCR (*: $p<0.05$).

As can be seen, similar decreasing trend was found in all the three laboratories as the increase of the aqueous concentration of fenitrothion while no statistically significant decrease at the highest concentration was observed in MCR (Figure 6c). At the highest concentration (886 μg/L) in NIES, 30% mortality and the significant growth inhibition was observed in this concentration (Figure 6d). The relatively higher variation in the control (and the highest concentrations) and the slightly low concentration at the highest concentration (186 μg/L) compared to the other two laboratories might be the cause of the difference. Therefore, the special attention is necessary to set the proper concentration to detect the anti-androgenic activity and distinguish from the general toxicity such as mortality and growth inhibition effects. No statistically significant difference was observed in liver VTG level except for the higher concentrations in MCR where very slight decrease was observed near the lower limit of quantification around 1 ng/mg liver.
In NIES, we also conducted the JMASA using a different strain (SKR, Table 4) and compared the results with our NIES-R strain. Figure 7 shows the results of fenitrothion with SKR and NIES-R. As can be seen, the decreasing trend was evident as the increase of the fenitrothion concentration for both strains, which suggest the applicability of JMASA to wide range of strains, once the relationship between the age and growth including SSC is revealed for the strain with the breeding condition in each laboratory. While light growth inhibition was observed at the highest concentration in both strains, the statistically significant decrease in the number of PPs was observed at lower concentrations (104 and 86.8 μg/L in SKR and NIES-R, respectively). Again, slight decrease in liver VTG was observed in SKR (Figure 7e) but this is close the level of lower limit of quantification for VTG measurement using ELISA kit.

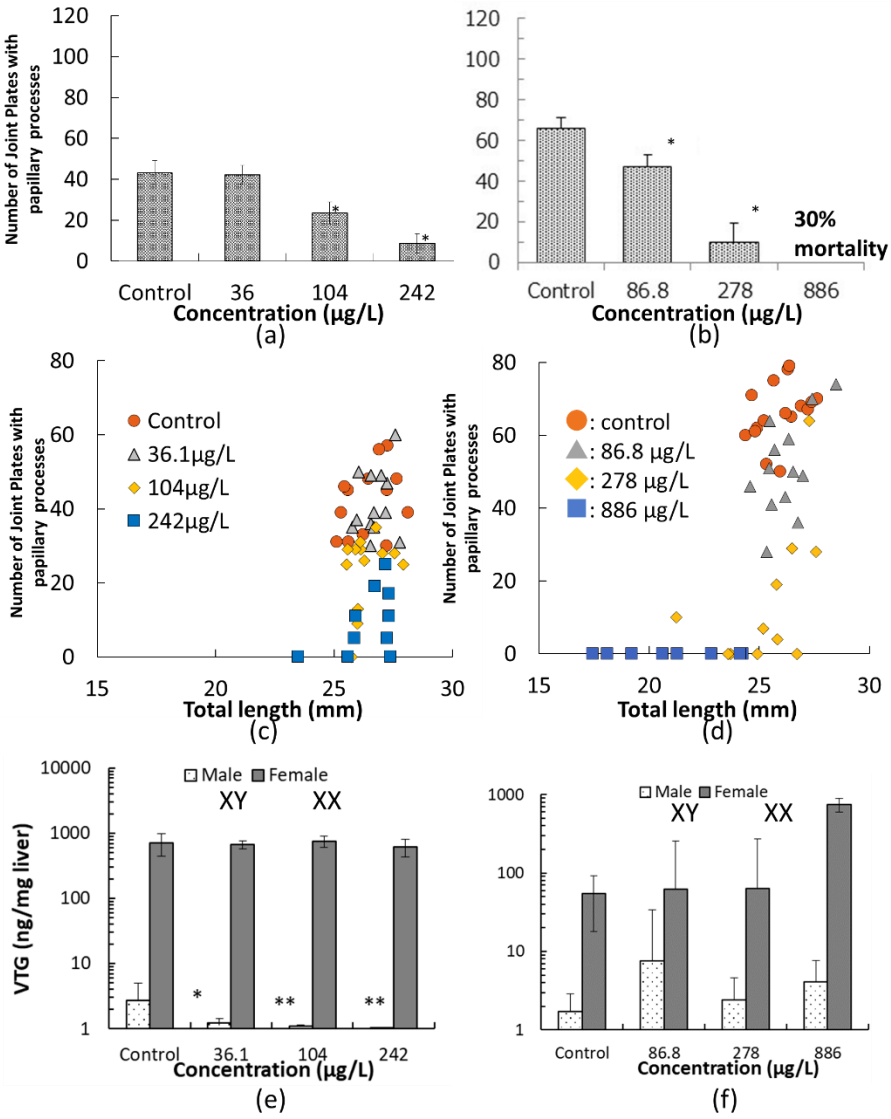


Figure 7. Results of fenitrothion: Concentration-Response Relationship of (a) SKR strain and (b) NIES-R; Relationship between total length and number of papillary processes in (c) SKR and (d) NIES-R; liver VTG concentration in both genetic males and females in (e) SKR and (f) NIES-R. (*: $p<0.05$; **: $p<0.01$)

4.1.4 Cyproteron acetate

Figure 8 shows the results of cyproterone (or chlormadinone) acetate, another well-known AR antagonist, used as anti-androgen or progestin medication, conducted only in IDEA. Slight decrease in the number of PPs with the increase of the cyproterone concentration was observed, and the statistically significant decrease was observed at the highest concentration (33.4 µg/L) while no significant growth inhibition was observed in all the concentrations. In addition, significant change in hepatic VTG concentration was observed neither in XY male nor in XX female.

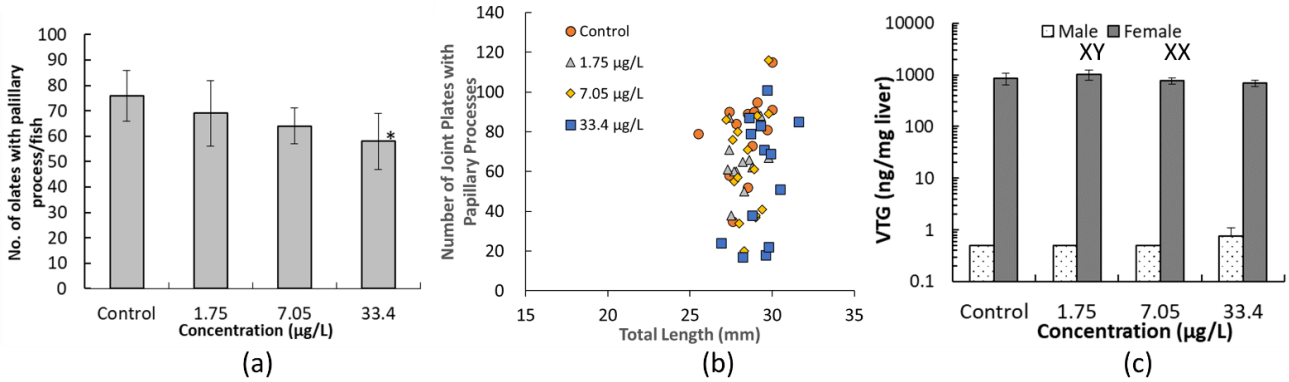


Figure 8. Results of cyproterone acetate in IDEA: (a) Concentration-Response Relationship, (b) Relationship between total length and number of papillary processes (c) liver VTG concentration in both genetic males and females. (*: $p<0.05$)

4.1.5 Linuron

Figure 9 shows the results of linuron, a suspected AR antagonist, in two laboratories. JMASA was found to be unable to detect anti-androgenic activity from linuron in both laboratories. while AFSS and RADAR with spiggin of stickleback could detect positive effect from this chemical (7, 14). We did not observe the anti-androgenic actions in the co-exposure of a competitive androgen, 11-ketotestosterone (at 50 nM) using the reporter gene assay with Japanese medaka ARβ, at the level below cytotoxicity was observed (10^{-4}M). Thus, the results of JMASA in this validation study did not contradict with that of reporter gene assay, we think that the anti-androgenic activity of linuron in medaka through AR is negative and is not considered as false negative in EXTEND program (15) with literature review on medaka and other rodents although the anti-androgenic activity on three spine stickleback is evident.

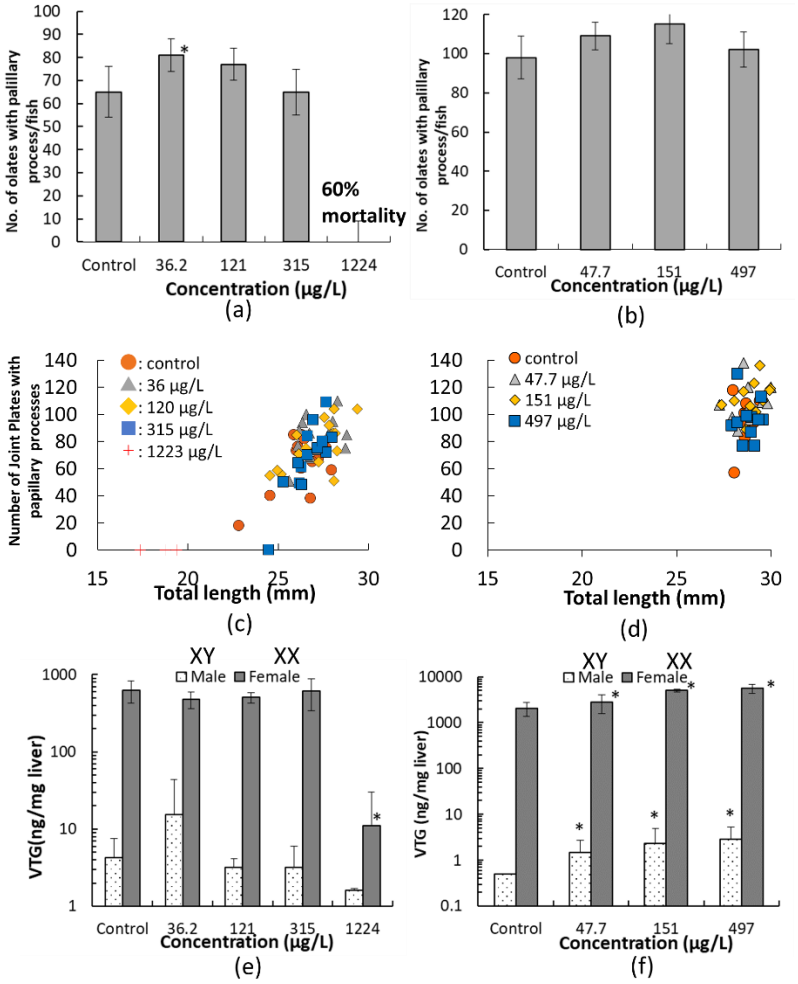


Figure 9. Results of interlaboratory studies for linuron: Concentration-Response Relationship in (a) NIES, and (b) IDEA; Relationship between total length and number of papillary processes in (c) NIES and (e) IDEA; liver VTG concentration in both genetic males and females in (e) NIES and (f) IDEA. (*: $p<0.05$)

4.1.6 Manneb

Figure 10 shows results of manneb, a herbicide and a suspected AR antagonist based on in vitro reporter-gene assay in the framework of EXTEND2016 (15) under Ministry of the Environment, Japan and was investigated in NIES. We found 68% mortality at the highest concentration (40 µg/L nominal) and apparent growth inhibition was observed in addition to the lethal effects. No significant decrease in the number of PPs at the lower concentrations. Additionally, we found unstable aqueous concentration even with the flow-through system to renew the stock solution twice a day. Thus, the total concentration was monitored using ICP-MS and found that the total Mn concentration is in the range between 80 and 120% of the nominal.

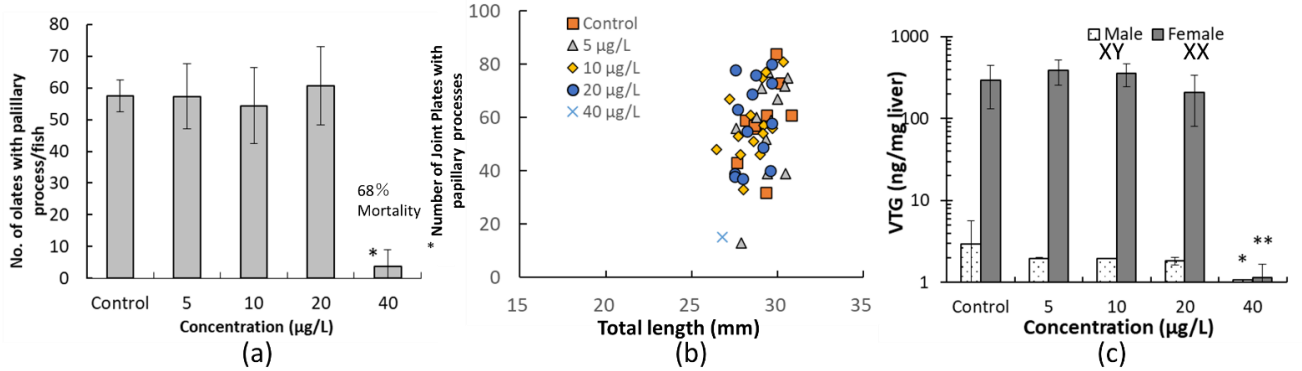


Figure 10. Results of manneb in NIES: (a) Concentration-Response Relationship, (b) Relationship between total length and number of papillary processes (c) liver VTG concentration in both genetic males and females. (*: p<0.05)

4.2Results of the chemicals with the other mode of actions

4.2.1 17β-Estradiol

Figure 11 shows the results of 17β-estradiol, a natural estrogen and is also considered as an AR antagonist. As can be seen, the number of joint plates with papillary processes significantly decreased at the highest concentration (34.8 ng/L) while no significant growth inhibition was observed (Figure 11b). At the highest concentration, hepatic VTG concentration significantly increased for both genetic male and female, which suggests both estrogenic and anti-androgenic activity was detected in this test although these two actions cannot be clearly distinguished.

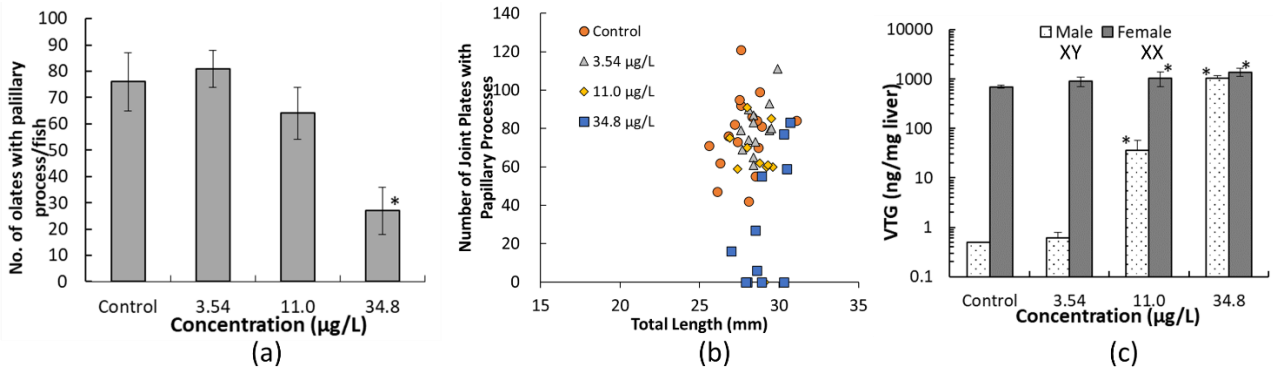


Figure 11. Results of 17β-estradiol in IDEA: (a) Number of plates with papillary processes at each concentration, (b) Relationship between total length and number of papillary processes, and (c) liver VTG concentration in both genetic males and females. (*: p<0.05).

4.2.2 Estrone

Figure 12 shows the results of estrone, another natural estrogen and is also considered as an AR antagonist. As with 17β-estradiol, the number of joint plates with papillary processes significantly decreased in all the concentrations above 79.9 ng/L while no significant growth inhibition was observed except for the higher concentrations (901 and 3050 ng/L, Figure 12b). In all the concentrations, hepatic VTG concentration significantly increased for both genetic male

and female, which again suggests both estrogenic and anti-androgenic activity was detected in this test although these two actions cannot be clearly distinguished. The fish short-term reproduction assay (OECD TG No. 229) was conducted under EXTEND2016 program and the LOEC was reported to be 272 ng/L for VTG and 1009 ng/L for reproduction (16), and the LOEC for PPs (79.9 ng/L) is even lower than these values as with the LOEC for liver VTG was also 79.9 ng/L.

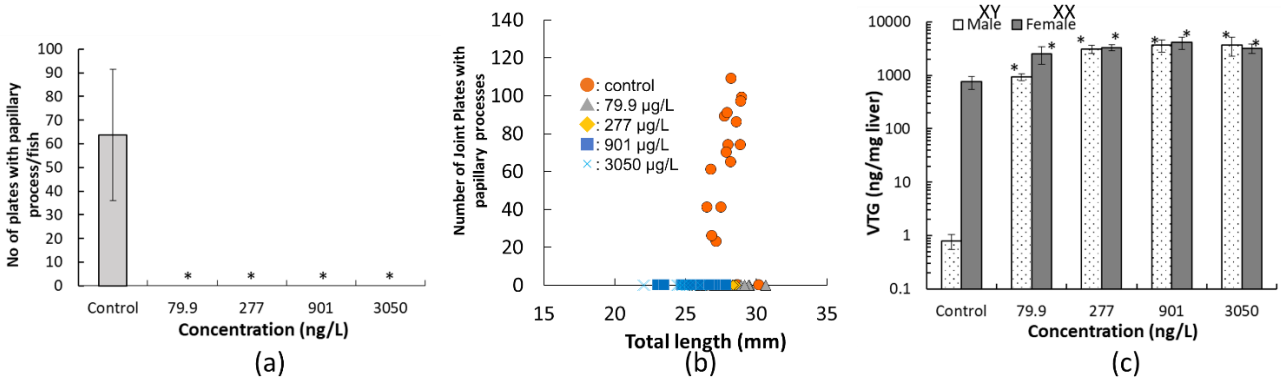


Figure 12. Results of estrone in NIES: (a) Number of plates with papillary processes at each concentration, (b) Relationship between total length and number of papillary processes, and (c) liver VTG concentration in both genetic males and females. (*: p<0.05).

4.2.3 Trenbolone

Figure 13 shows the results of trenbolone, an androgen agonist. In contrast to the two estrogens and androgen antagonists, PPs formed in genetic female while no significant change in the number of PPs was found for genetic males (Figurer 13a). As for hepatic VTG concentration, the significant decrease was observed for genetic females (Figure 13c). These results suggest that this method could detect androgen agonist in addition to the chemicals with anti-androgenic activity.

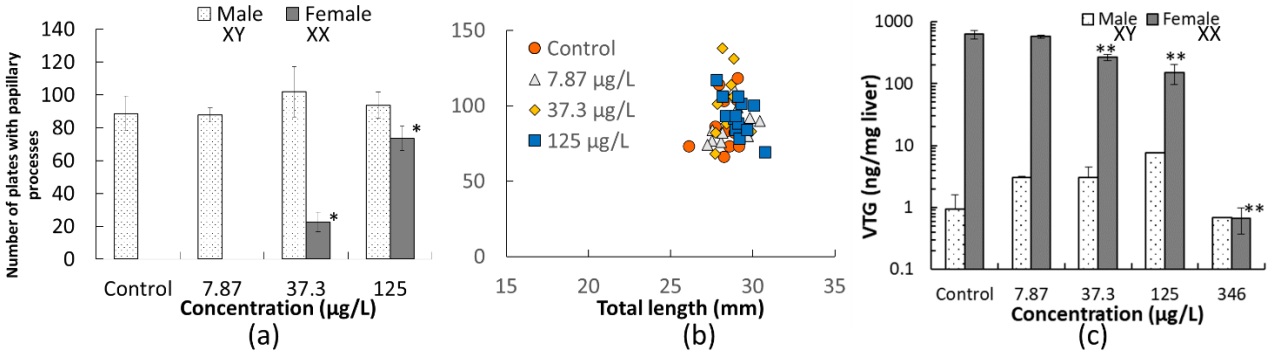


Figure 13. Results of trenbolone in NIES: (a) Number of plates with papillary processes at each concentration in both genetic males and females, (b) Relationship between total length and number of papillary processes (genetic males), and (c) liver VTG concentration in both genetic males and females. (*: p<0.05; **: p<0.01)

4.2.4 Ketoconazole

Figure 14 shows the results of ketoconazole, an antifungal agent and suspected as a steroidogenesis inhibitor. As with androgen antagonists, the number of PPs decreased with the increase of ketoconazole concentration (Figure 14a). At 885 and 413 µg/L, slight growth inhibition was observed while no growth inhibition was evident at 159 µg/L (Figure 14b). The hepatic VTG in genetic female decreased at the higher concentration with the concentration-dependent manner while the VTG did not increased in genetic male. These results suggest the inhibition of steroidogenesis led the formation of androgens and the inhibition of the androgen-axis actions (the formation of estrogens from androgens by aromatase). Thus, this method may possibly detect a steroidogenesis inhibitor with the measurement of VTG concentrations.

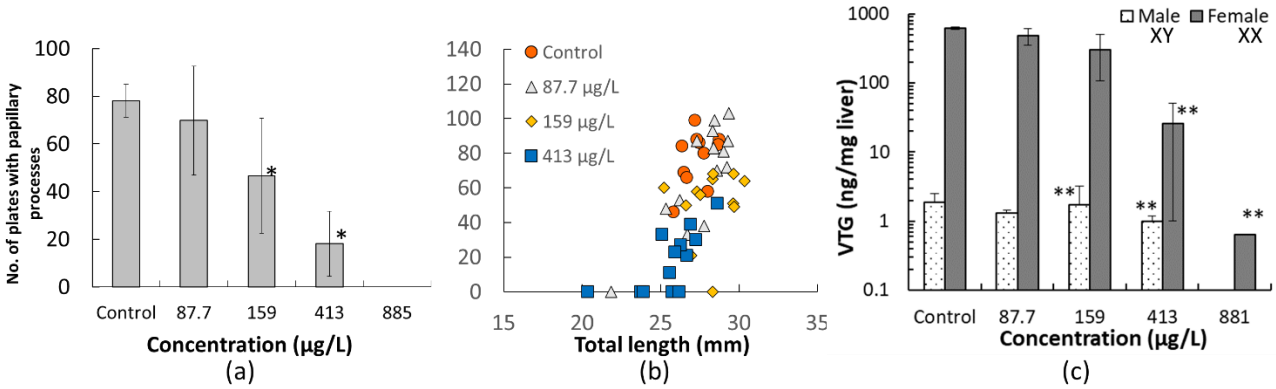


Figure 14. Results of ketoconazole in IDEA: (a) Number of plates with papillary processes at each concentration, (b) Relationship between total length and number of papillary processes, and (c) liver VTG concentration in both genetic males and females. (*: $p<0.05$; ** $p<0.01$).

4.2.5 Fluconazole

Figure 15 shows the results of fluconazole, another antifungal agent and is unknown to be a steroidogenesis inhibitor and used as an antifungal drug. Since fluconazole is highly insoluble in water, DMF was used as a solvent and both solvent and non-solvent controls were prepared. In contrast to ketoconazole, the number of PPs did not change significantly even at the highest concentration (Figure 15a). In this method, no effect on androgen-axis was observed for fluconazole even at the highest concentration (8.80 µg/L). The liver VTG concentration in genetic male decreased but the concentration in the controls was low and the decrease was considered as biologically significant. Ketoconazole and fluconazole are both in the category of azole antifungal agents to inhibit the steroidogenesis from lanosterol to ergosterol but their structures are different. Further research should be conducted to clarify the steroidogenesis inhibition potency of these azoles based on their structural differences (quantitative structure-activity relationship: QSAR) to inhibit the formation of androgens/estrogens from cholesterol.

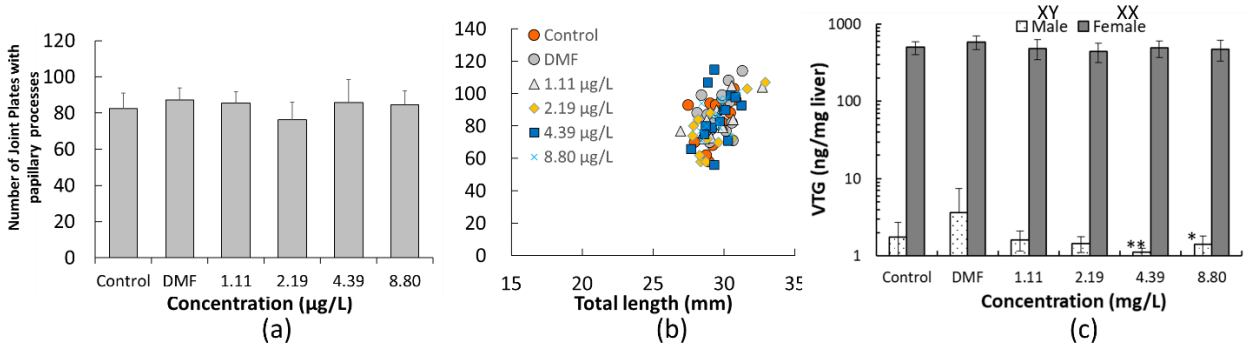


Figure 15. Results of fluconazole in NIES: (a) Number of plates with papillary processes at each concentration, (b) Relationship between total length and number of papillary processes, and (c) liver VTG concentration in both genetic males and females. (*: $p<0.05$; ** $p<0.01$)

4.3Results of expected inert chemicals

4.3.1 Cromolyn

Figure 16 shows the results of cromolyn, expected inert chemicals. While no significant change in the number of PPs were observed for IDEA (Figure 16b) and Mitsubishi Chemical Research (Figure 16c) while slight decrease was found in NIES and the decrease was statistically different (Figure 16a). In this JMASA protocol, four tanks were prepared with seven unmatured fish before SSC. In the experiment in NIES, accidentally only one genetically male (and six genetically females) was observed in two tanks and the number of PPs of these two were much lower than the others. The statistical analysis was conducted based on tank average, which might cause this unexpected detection. Most of the cases, the number of males is two or more but there might be a limited case, accidentally. If the sex ratio is assumed as 1:1, the probability of no male is $1/2^7$=0.78% and that of only one male is $7/2^7$=5.47%. Thus, the sex ratio should be carefully reviewed in such extreme cases. To avoid these problems, the genetic sex of each fish might be pre-checked by the DMY analysis of the eggshells. No growth inhibition was observed in all three laboratories even at the highest concentration (Figure 16d, e, and f) and the change in the liver VTG was not evident except for the slight increase in genetic females in IDEA (Figure 16h) and slight decrease in genetic males in MCR (Figure 16i).

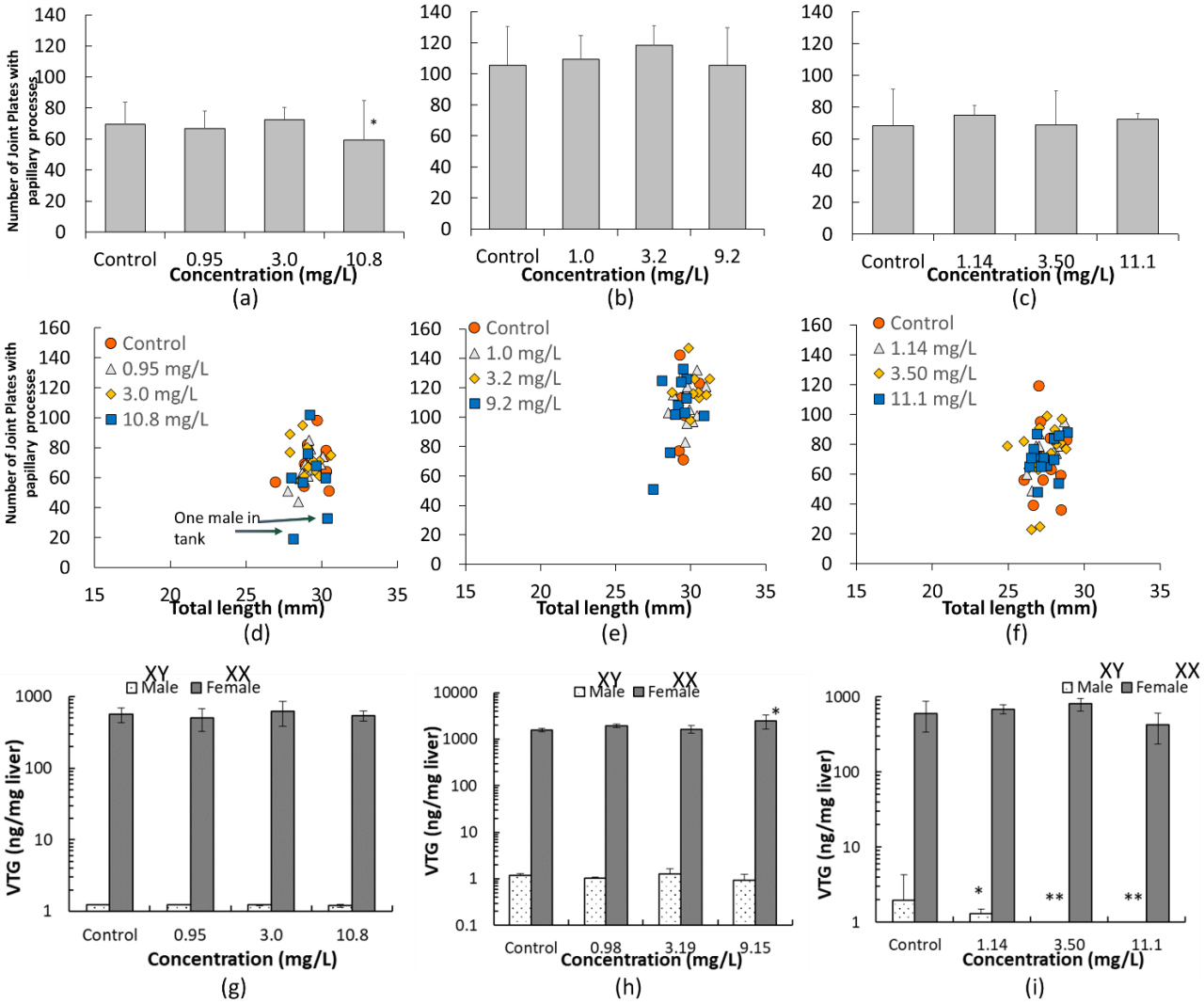


Figure 16. Results of interlaboratory studies for cromolyn sodium salt: Concentration-Response Relationship in (a)NIES, (b)IDEA, and (c) Mitsubishi Chemical Research (MCR); Relationship between total length and number of papillary processes in (d) NIES, (e) IDEA, and (f) MCR; liver VTG concentration in both genetic males and females in (g) NIES, (h) IDEA, and (i) MCR. (*: $p<0.05$; **: $p<0.01$)

4.3.2 Zinc chloride

Figure 17 shows the results of zinc chloride, another expected inert chemicals. Slight increase in the number of PPs was observed at the higher concentration. The growth seemed to be slightly promoted at the higher concentration or a slight growth delay was observed in the control with average number of PPs at 52 (Figure 17b). No significant change in liver VTG was observed in Figure 17c.

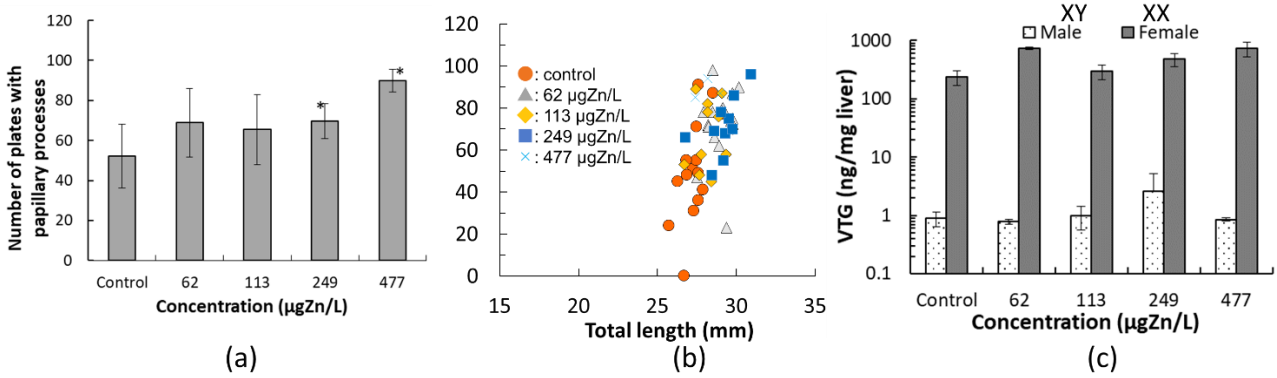


Figure 17. Results of zinc chloride in NIES: (a) Number of plates with papillary processes at each concentration, (b) Relationship between total length and number of papillary processes, and (c) liver VTG concentration in both genetic males and females. (*: p<0.05).

4.3.3 SDS

Figure 18 shows the results of sodium dodecyl sulfate, an anionic surfactant, expected inert chemicals. Slight growth inhibition was observed at the highest concentration (9.51 mg/L, Figure 18b) while slight increase in the number of PPs was observed for the lower concentrations (0.827 and 2.67 mg/L, Figure 18a). The slight decrease was observed for the liver VTG in genetic female (Figure 18c).

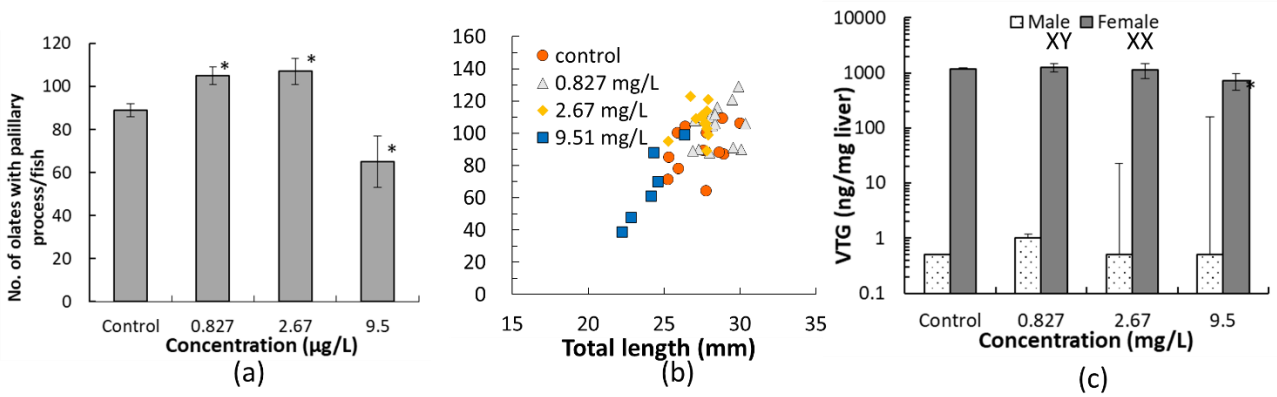


Figure 18. Results of SDS in IDEA: (a) Number of plates with papillary processes at each concentration, (b) Relationship between total length and number of papillary processes, and (c) liver VTG concentration in both genetic males and females.. (*: p<0.05).

4.4Growth curve of two Japanese medaka strain

As presented above, the growth curve (relationship between total length, age and the numbers of PPs) of Japanese medaka is highly depending on the strain and breeding conditions. Figure 19 shows the growth curve of two Japanese medaka strains (NIES-R and SKR) based on total length and fresh body weight. As can be seen, NIES-R grows faster than SKR. The hatching was also found to take longer for SKR (11 days) compared to NIES-R (7 days). The first observation of PPs was 45 dpf (38 dph) for NIES-R and 53 dpf (42 dph) for SKR while total length became asymptotic and plateau at 34 mm for NIES-R and 28 mm for SKR around 90 dpf. Despite the

difference in the growth speed, the SSC seems to be within approximately one week. Consequently, we decided to slightly modify the age of initiating the test to be 35-42 dpf, approximately 10 days before the first observation of papillary processes. Again, we strongly recommend to those who are planning to conduct the JMASA to carefully observe the growth curve in their strain and breeding condition to confirm that the age of initiating the test is approximately 10 days before the first observation of papillary processes.

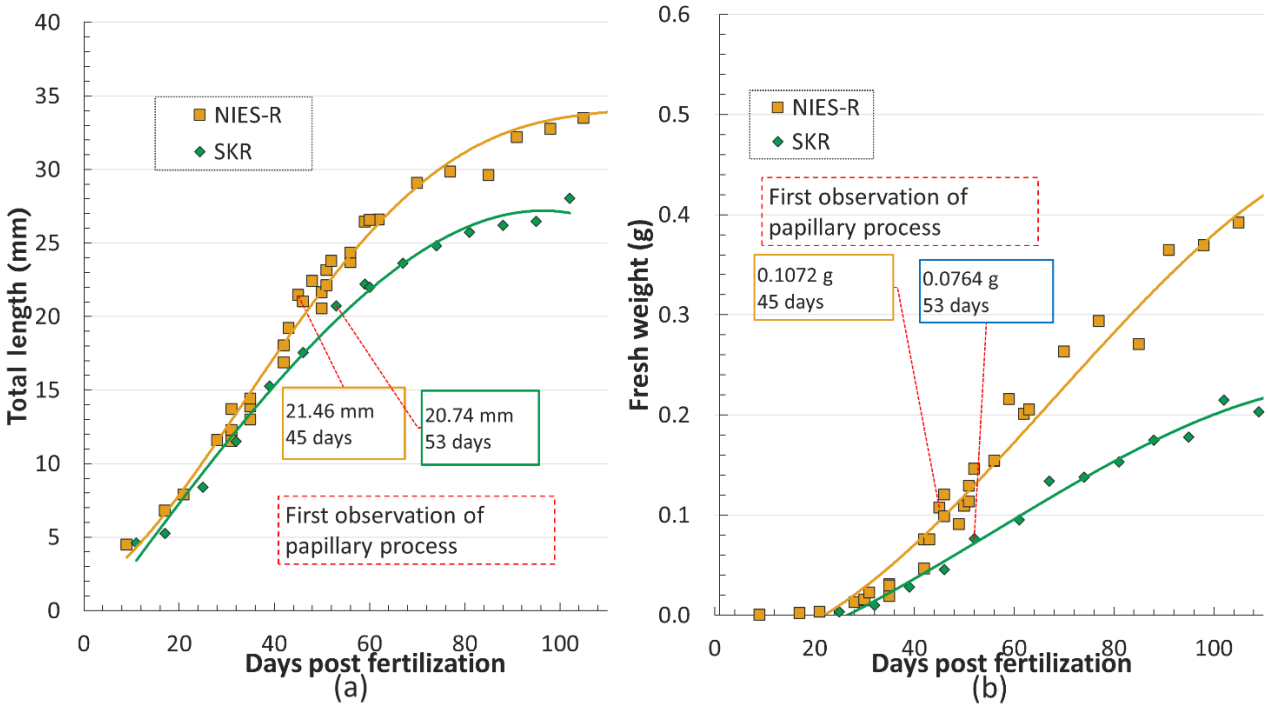


Figure 19. Growth curve of two Japanese medaka strains (NIES-R and SKR) in NIES with the first observation of secondary sex characteristics (papillary processes) based on (a) total length and (b) fresh weight.

5. Validation of the number of replicates

5.1Mean number of joint plates with papillary processes

The histogram of the mean number of joint plates with papillary processes per fish in genetic males in the controls in 24 tests presented in Chapter 4 is shown in Figure 20. As can be seen from Figure 20, the mean number of joint plates with PPs per fish ranged from 53 to 105 (except for the case of SKR strain, where we observed 43), and the 20 out of 23 tests exceeded 60 (87%) while 3 tests were in the range between 50 and 60.

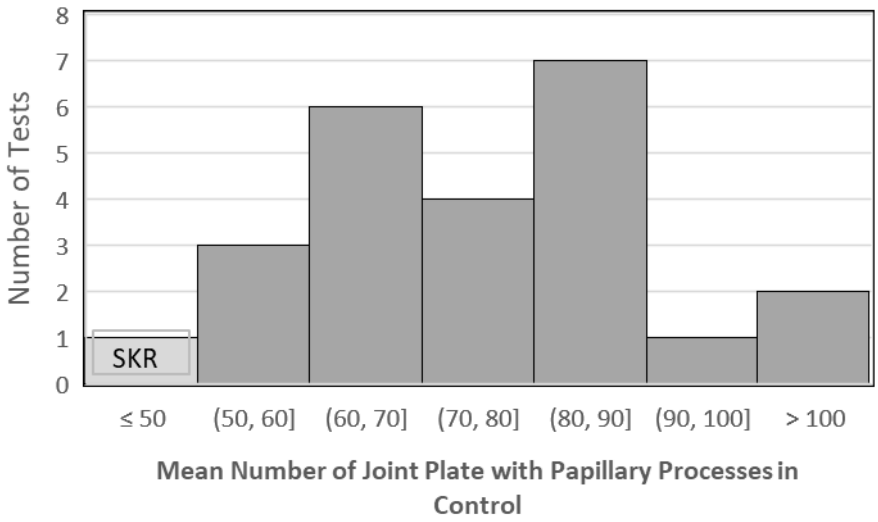


Figure 20. Histogram of the mean number of joint plates with papillary processes per fish in genetic males

5.2Number of males in replicates

The number of genetic males in seven fish in replicates is highly dependent on sex ratio. We simulated the case of male ratio of 30, 40, and 50% and predicted the probability of the number of males in each replicate and shown in Table 6. In addition, the probability of the number of replicates with no male (or one male) was also estimated and shown in Table 7. As can be seen from Table 6, the probability of 0 male in replicate increases with the decrease of male ratio, and it becomes 2.80% and 8.24% in the case of 40% and 30% male, respectively. The probability of less than 3 (2, 1 or 0) replicates with one or more males in each replicate also increases with the

decrease of male ratio and becomes 0.45% (=0.44+0.00085+6.1x10^{-5}) and 3.7% (=3.43+0.21+0.0046) and in the case of 40% and 30% male, respectively.
These simulations suggest the importance of sex ratio of male and the ratio should be at least 40% or higher to be valid tests with at least three replicates with one or more males. In the case of only one male in three replicates with the other one replicate with no male is also shown in brackets in Table 7. This percentage is 0.50% in the case of 30% male ratio but it decreases down to 0.025% in the case of 40% male ratio.

Table 6. Estimated probability of number of males per replicates in the case of male ratio of 30, 40, and 50%.

Sex ratio / Estimated Number of Males per Replicate	Male 30%: Female 70%	Male 40%: Female 60%	Male 50%: Female 50%
0	8.24%	2.80%	0.78%
1	24.71%	13.06%	5.47%
2	31.77%	26.13%	16.41%
3	22.69%	29.03%	27.34%
4	9.72%	19.35%	27.34%
5	2.50%	7.74%	16.41%
6	0.36%	1.72%	5.47%
7	0.02%	0.16%	0.78%

Table 7. Estimated probability of replicates with no (or one) male in the case of male ratio of 30, 40, and 50%.

Sex ratio / Number of Replicates with one or more males	Male 30%: Female 70%	Male 40%: Female 60%	Male 50%: Female 50%
0	0.0046%	6.1x10^{-5}%	3.7x10^{-7}%
1	0.21%	0.0085%	0.00019%
2	3.43%	0.44%	0.036%
3	25.45% (all three replicates with one male: 0.50%)	10.28% (all three replicates with one male: 0.025%)	3.05% (all three replicates with one male: 0.00051%)
4	70.91%	89.26%	96.91%

The numbers of males per replicates in the controls in the interlaboratory ring tests was also recorded and the histogram is shown in Figure 21. The mean number of males in the replicates was 3.24, while the total sex ratio of males in the ring tests was 46.4% and the number of replicates with zero male in controls was observed only in one replicate (out of 100 controls=1.0%) and close to 0.78% in Table 6 for male 50%:female 505, which is also observed in one test (out of 24 tests=4.2%) and close to 3.05% of 3 replicates with one or more males in the case of male ratio of 50% in Table 7.

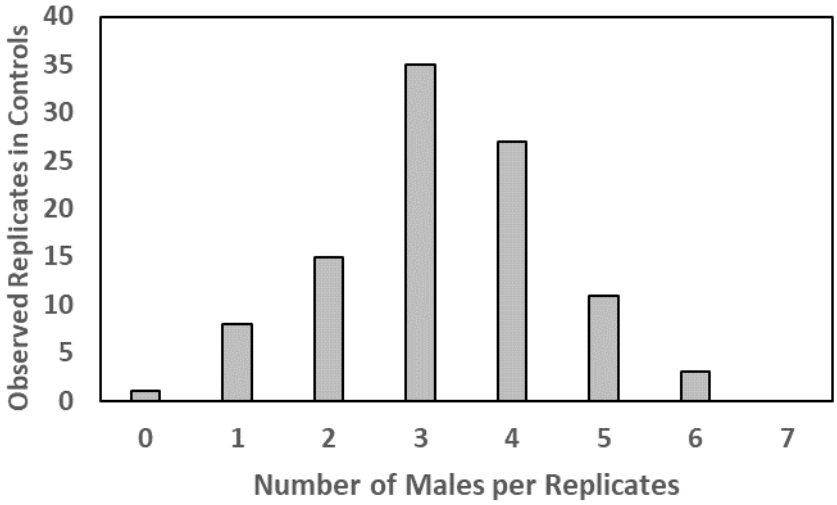


Figure 21. Observed number of replicates with different number of males per replicates in the controls in the interlaboratory ring test.

6. Conclusions

The interlaboratory validation exercise of JMASA demonstrated that the assay provides almost the expected results with the expected anti-androgens (vinclozolin, flutamide, and fenitrothion) while no anti-androgenic effect was detected for linuron, considered as positive based on AFSS and RADAR. The negative results in JMASA does not contradict with reporter gene assay with medaka ARβ. JMASA assay can be used to detect ER and AR agonists and could be used to detect steroidogenesis inhibitors if liver VTGs were measured optionally.
Overall, the data generated in the three laboratories matched the expected response profiles and the test chemicals were almost correctly classified as anti-androgens in each laboratory.

7. References

(1) Ogino Y, Hirakawa I, Inohaya K, Sumiya E, Miyagawa S, Denslow N, Yamada G, Tatarazako N, Iguchi T. (2014). Bmp7 and Lef1 are the downstream effectors of androgen signalling in androgen-induced sex characteristics development in medaka. Endocrinology. 155(2), 449-62.

(2) Nakamura, A., Takanobu, H., Tamura, I., Yamamuro, M., Iguchi, T. and Tatarazako, N. (2014). Verification of responses of Japanese medaka (*Oryzias latipes*) to antiandrogens, vinclozolin and flutamide, in short-term assays. Journal of Applied Toxicology, 34, 545-553.

(3) OECD (2012), Test No. 229: Fish Short Term Reproduction Assay, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264185265-en.

(4) 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.

(5) OECD (2006) Report of the Initial Work Towards the Validation of the 21-Day Fish Screening Assay for the Detection of Endocrine active Substances (Phase 1A). Series on Testing and Assessment No. 60, ENV/JM/MONO(2006)27, OECD, Paris.

(6) OECD (2006) Report of the Initial Work Towards the Validation of the 21-Day Fish Screening Assay for the Detection of Endocrine active Substances (Phase 1B). Series on Testing and Assessment No. 61, ENV/JM/MONO(2006)29, OECD, Paris.

(7) OECD (2011) Guidance Document on the Androgenised Female Stickleback Screen. Series on Testing and Assessment, No. 148, ENV/JM/MONO(2011)29, OECD, Paris.

(8) OECD (2011), Test No. 234: Fish Sexual Development Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264122369-en.

(9) OECD (2015), Test No. 240: Medaka Extended One Generation Reproduction Test (MEOGRT), OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264242258-en.

(10) OECD (2022), Test No. 251: Rapid Androgen Disruption Activity Reporter (RADAR) assay, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/da264d82-en.

(11) OECD (2018), Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption, OECD Series on Testing and Assessment, No. 150, OECD Publishing, Paris, https://doi.org/10.1787/9789264304741-en.

(12) U.S. Environmental Protection Agency. (2013) Validation of the Medaka Multigeneration Test: Integrated Summary Report. http://www.epa.gov/scipoly/sap/meetings/2013/062513meeting.html.

(13) Asai, T., Senou, H., Hosoya, K. (2011) *Oryzias sakaizumii*, a new ricefish from northern Japan (Teleostei: Adrianichthyidae). Ichthyological Exploration of Freshwaters, 22 (4): 289-299.

(14) OECD (2022) Validation of the Rapid Androgen Disruption Activity (RADAR) assay for the detection of androgen axis active substances, described in OECD Test Guideline, Series on Testing and Assessment No. 353, ENV/CBC/MONO(2022)9, OECD, Paris.

(15) Ministry of the Environment Japan (2022): Future actions on the endocrine disrupting chemicals – EXTEND2022 – (https://www.env.go.jp/content/000081844.pdf) (in Japanese, English version will be available soon)
(16) Kawashima, Y., Onishi, Y., Tatarazako, N., Yamamoto, H., Koshio, M., Oka, T., Horie, Y., Watanabe, H., Nakamoto, T., Ishikawa, H., Sato, T., Yamazaki, K., Iguchi, T. (2021): Summary of 17 chemicals evaluated by OECD TG229 using Japanese Medaka, *Oryzias latipes* in EXTEND 2016, J. Appl, Toxicol., (https://doi.org/10.1002/jat.4255)