International harmonization in the development of the test guideline for endocrine disrupting chemicals

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Contents

- Testing methods for the endocrine disrupting chemicals
- Development of the fish testing
- Development of the Daphnia testing
- Problems and the future prospects

Testing methods for the endocrine disrupting chemicals (OECD,Japan)

Why we should develop the new tests methods for the endocrine disrupting chemicals?

- The current test guidelines are the acute /subacute toxicity tests.
- Because individual "life or death" is the endpoint by the current examination, we could not evaluate the "disrupting".
- It is difficult to know what will be at the next generation even if we examine the impact only for the first generation.
- There is no multi-generation method in the current OECD test guidelines.

⇩

Evaluate the impacts of the mating / breeding / the reproduction (number of eggs, fertility rate) !

Current OECD test guideline

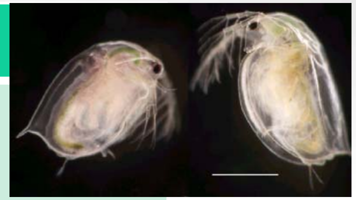
Fish

	F0					F1
	embryo	juvenile	young	adult	breeding	
203 204				death		
210	hatch/death/growth					
212	death/growth					
215			death/growth			

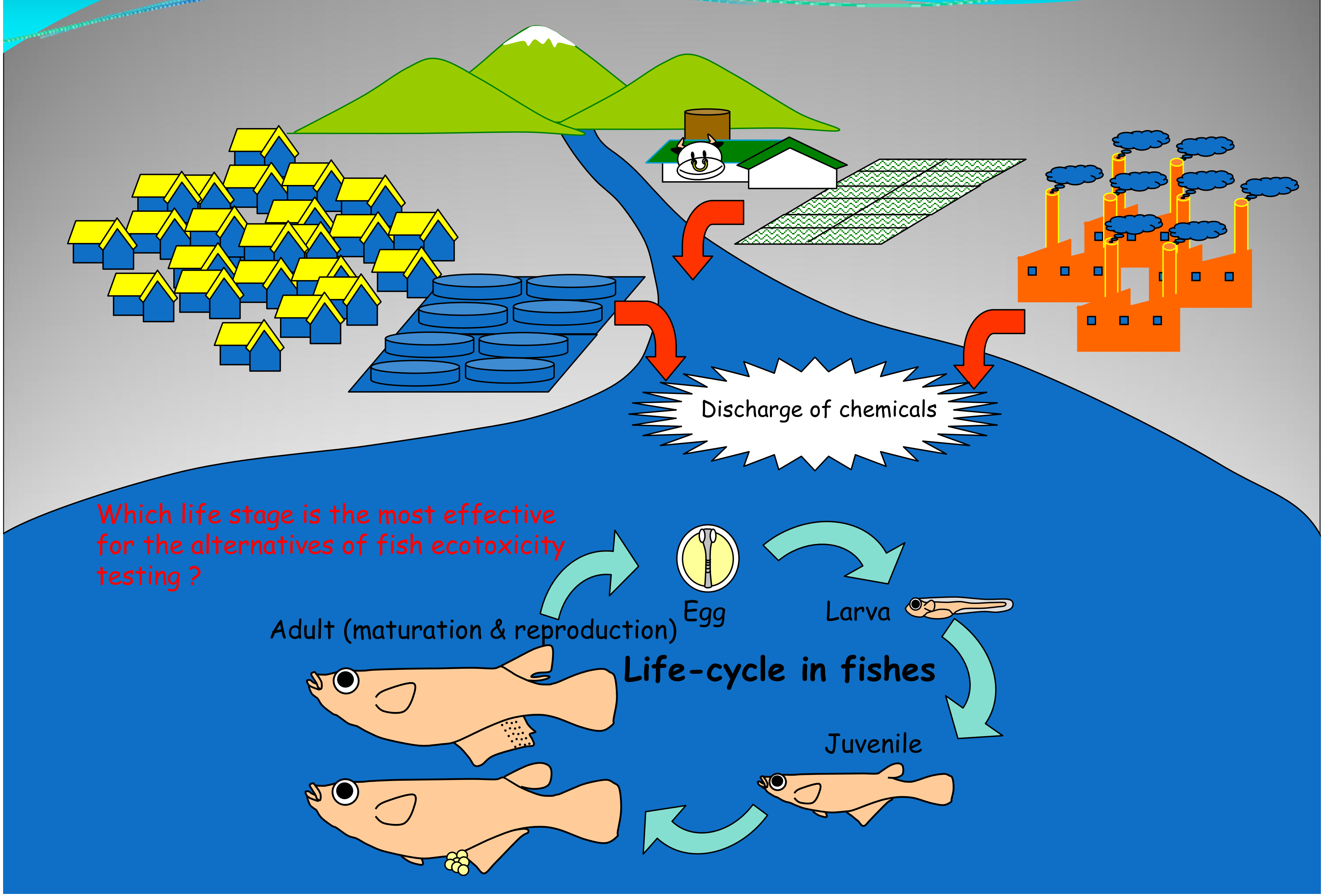
All tests begin with the fertilized! egg

Daphnia

	F0	F1
202	death	
211	Death/reproduction/juvenile death	



Issues in existing fish ecotoxicity testing



Endocrine Disrupters Testing and Assessment

－ Organaization chart －

EDTA

- Non-animal Testing
- Ecotoxicity Testing
- Mammalian Testing

- Fish Drafting Group
- Amphibian Expert Group
- Invertebrate Expert Group
- Gonad Histopathology e.t.c.

Secure the international consistency of the test !!

OECD-TG projects in progress now (Ecotoxicity Testing)

PJ No.	Comments	Project Lead
1	EDTA : New TG for Copepod Reproduction and Development	SWE
2.3	Avian Avoidance Studies	UK
2.4	2-Generation Avian Toxicity	US
.7	Fish Embryo Toxicity Test	GER
.8	EDTA : Enhanced Test Guideline 211: *Daphnia Magna* Reproduction Test	JPN
2.9	Collembola Reproduction Test	DK
12	EDTA : Development and Validation of a Fish Life-Cycle Test	US/GER /JPN
13	EDTA : New TG for Mysid Life-Cycle Toxicity Test	US
14	EDTA : Development and Validation of a New TG: Fish Sexual Development Test	DK
2.16	EDTA : Development of a DRP on Fish (Long Term) Testing	US
2.17	Avian Acute Toxicity	UK
18	EDTA : Development and Validation of a new TG: 21-Day Fish-Screening Test	OECD /JPN

PJ No.	Comments	Project Lead
2.19	EDTA : Development and Validation of Methods in Amphibians	US/ /J
2.20	New TG on Determination of Developmental Toxicity of a Test Chemical to Dipteran Dung Flies	EC
2.22	New TG on *Hypoaspis Aculeifer* Reproduction Test	NL
2.23	New GD on Application of the Step Down Approach (or Upper Threshold Concentration) as a Limit Test for Acute Fish Toxicity Testing	E
2.24	Update of TG 209: Activated Sludge Respiration Inhibition Test	UK
2.25	EDTA :New DRP on Molluscs Life-Cycle Toxicity Testing	UK/GER
2.26	EDTA : New TG: The 21-Day Female Stickleback endocrine screening assay	UK
2.27	EDTA : New TG: Fish Short Term Reproduction Assay	U
2.28	EDTA : New TG on a Chironomid Life-Cycle Toxicity Test	GER
2.29	New GD on Dung Beetles	EC
2.30	Fish Testing Framework	U

Development of the fish testing

Importance of medaka *Oryzias latipes* in chemical toxicity testing in East Asian countries

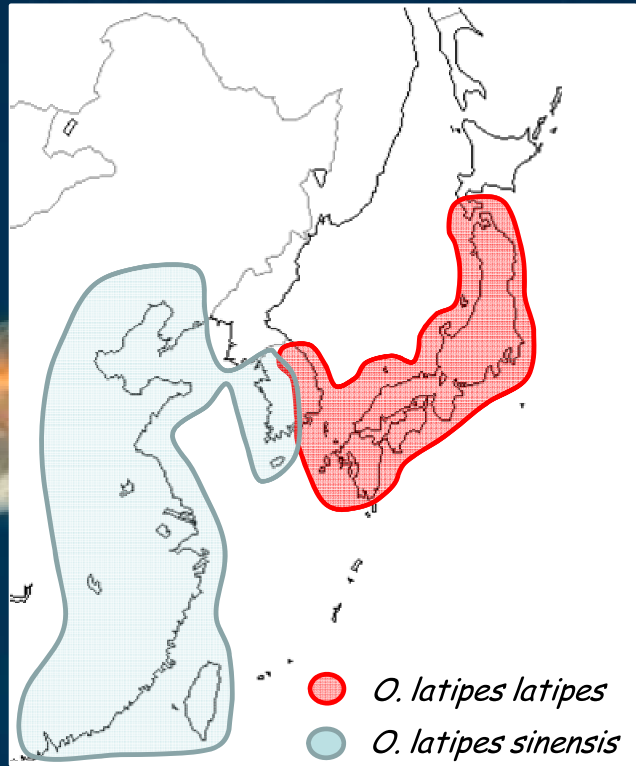
- Very common fish in fresh water area in East Asian countries
(Same genius Oryzias sps. are also in South-east asian countries (One of the dominant fish in western pacific freshwater environment))

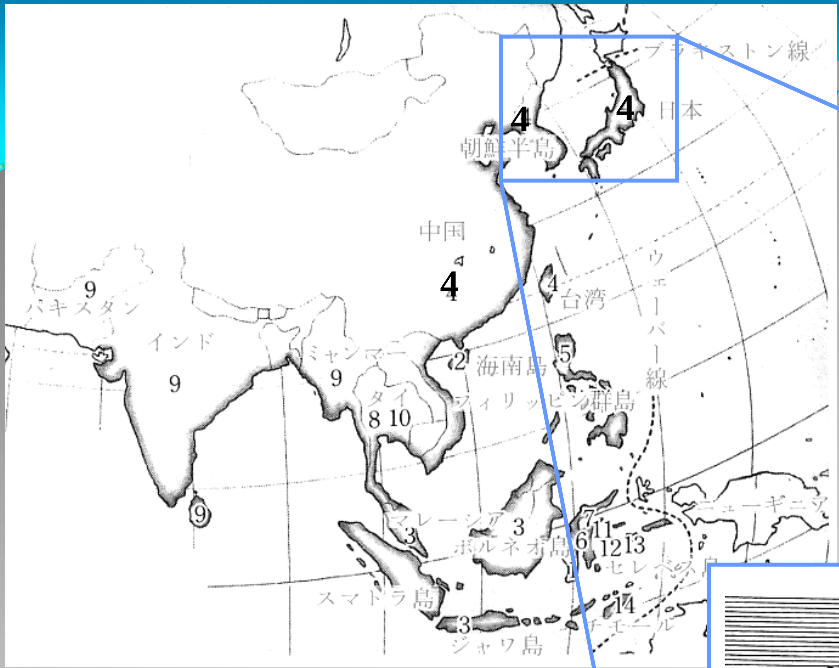
- The habitat close to human active environment
(Rice field, small canal around agriculture farm, and sub urban area)

- Small size and short term life-cycle, easy for fish culture, exposure testing and it is available to reduce the waste of test chemical.

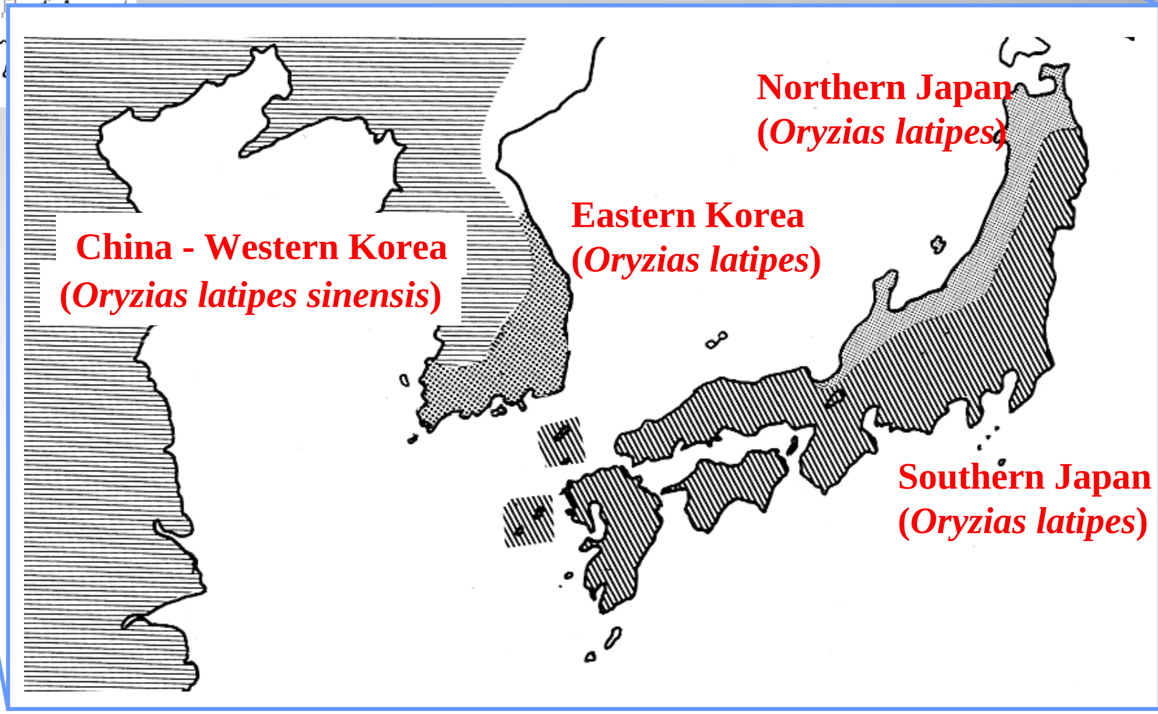
- Well-known and established the studies on development, sexual maturation, and reproduction

Distribution of medaka *Oryzias latipes* species in Eastern Asia





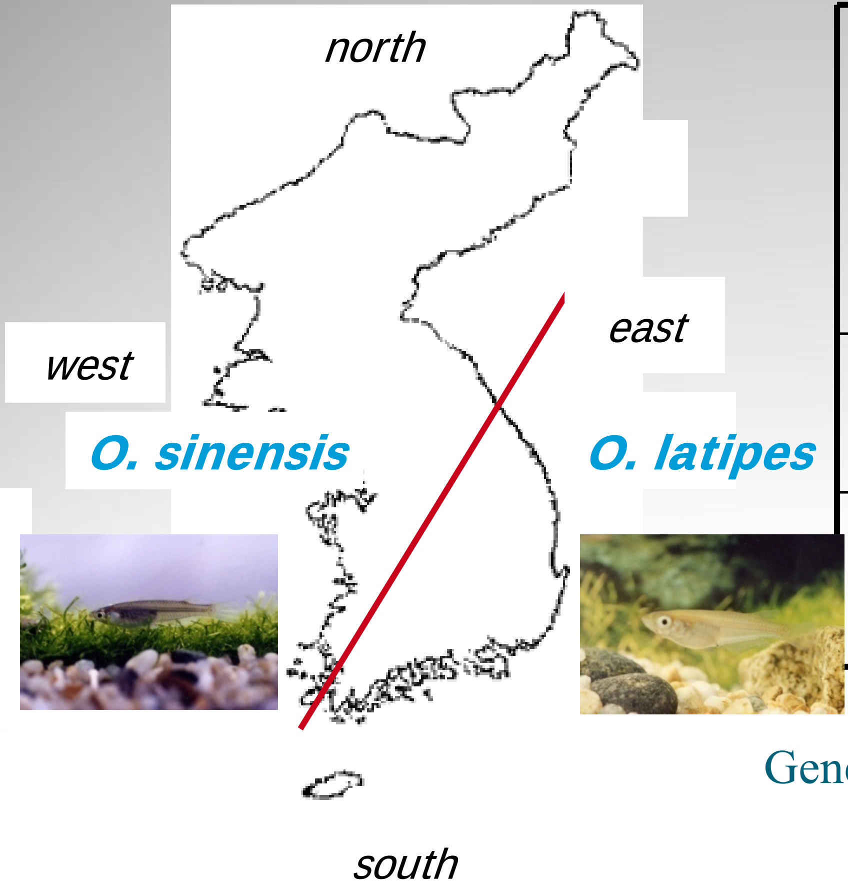
1 *Oryzias celebensis*
2 *Oryzias curvinotus*
3 *Oryziasjavanicus*
4 ***Oryzias latipes***
5 *Oryzias luzonensis*
6 *Oryzias marmoratus*
7 *Oryzias matanensis*
8 *Oryzias mekongensis*
9 *Oryzias melastigma*
10 *Oryzias minutillus*
11 *Oryzias nigrimas*
12 *Oryzias orthgnathus*
13 *Oryzias profundicola*
14 *Oryzias timorensis*



Geological Distribution
of Oryziidae

Oryzias latipes and sinensis
(Japanese medaka and Chinese medaka)

Both Species of Medaka are Living in Korean Freshwater
In Japan, only Latipes is habited, and In China, only sinensis is habited



Oryzias latipes called as (Japanese) Medaka * Same type as Medaka in Japan	*Oryzias sinensis* called as Chinese medaka * Same type as Medaka in China
Inhabit East Area in Korea	Inhabit West Area In Korea
Chromosome 2n=48	Chromosome 2n=46

Genetic homology is very height between two spieces

Oryzias latipes latipes
(Orange red)

Oryzias latipes sinensis
(obtained from NIER)

Oryzias latipes latipes
(Wild type)

primer (suitable for both of DMY and DMRT1)

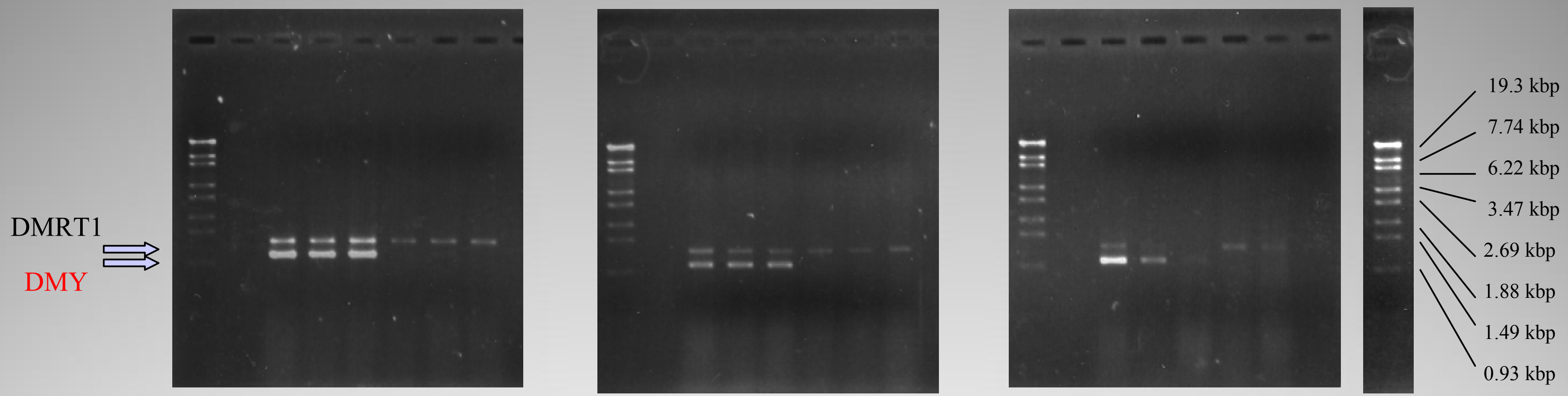
PG17.5	CCGGGTGCCCAAGTGCTCCCGCTG
PG17.6	GATCGTCCCTCCACAGAGAAGAGA

PCR condition

denature	: 94 degree, 2 min.	
denature	: 94 degree, 30 sec.	32 cycles
annealing	: 55 degree, 45 sec.	32 cycles
extension	: 72 degree, 2 min.	32 cycles
extension	: 72 degree, 2 min.	

Condition for amplification of DMY and DMRT1

Orange red Japanese Chinese



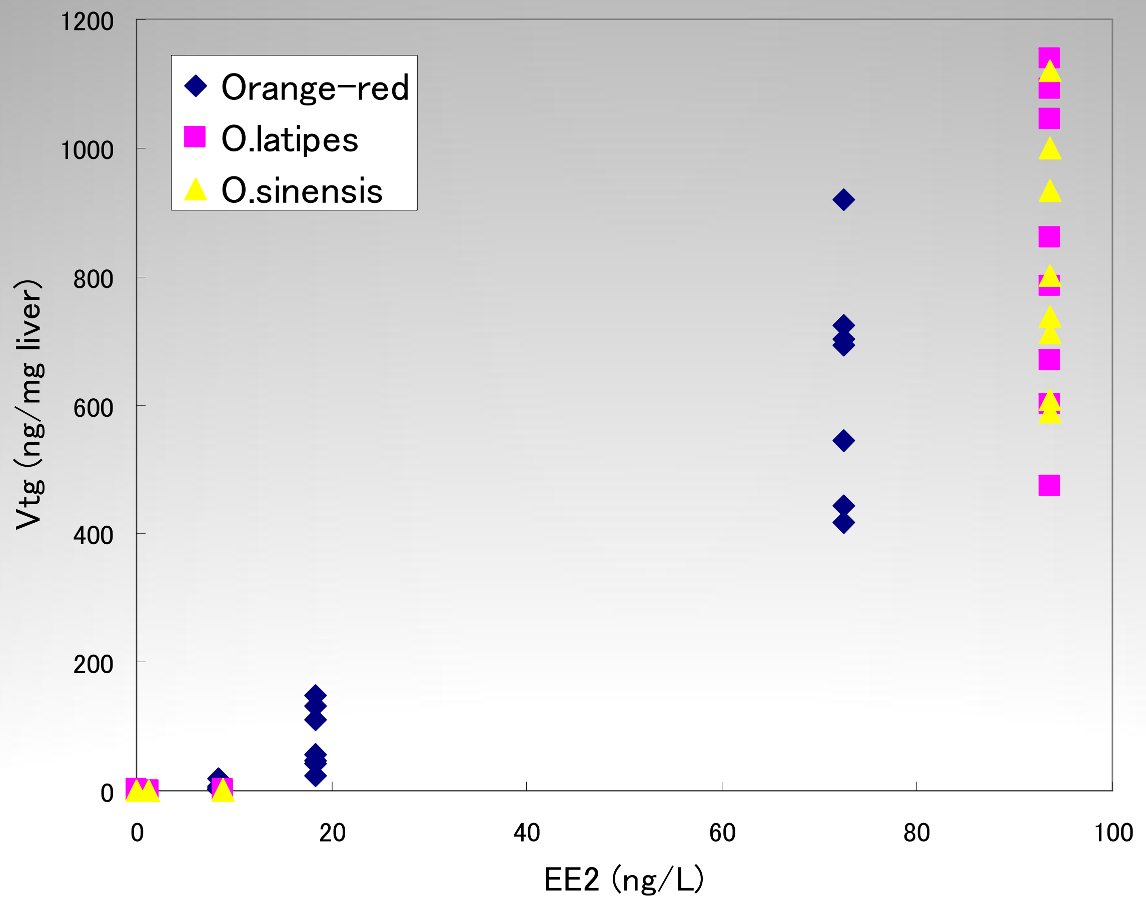
male female male female male female

Oryzias latipes (Orange red) *Oryzias latipes* (Wild) *Oryzias latipes* (obtained from NIER)

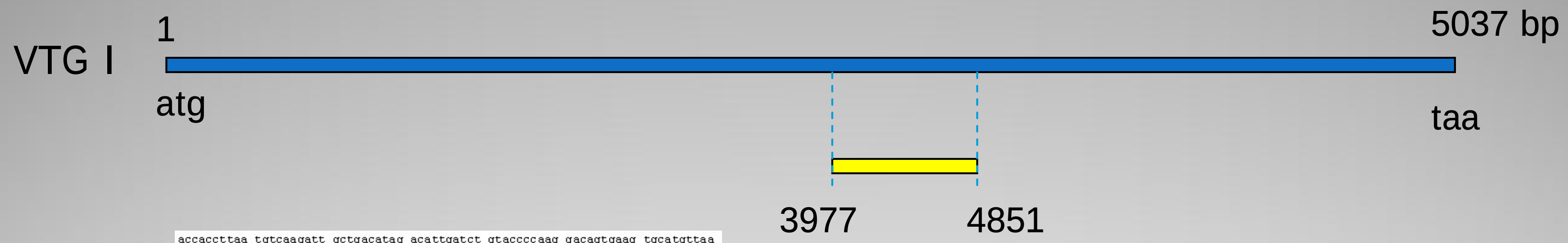
DMY and DMRT1 of individual type of Medaka

It is known that Mekong medaka and Celebes medaka have not DMY gene!!

Comparison of sensitivity for producing VTG with three species



Japanese medaka (*Oryzias latipes*)

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gaaaatgagg agggagtttc tgtgtttgca tccagccatg ggcttcatga agtctatgtt aacaagacct
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attttaccag cagaaagctg caaagacaac tctgagtgcc gcatgaagta tgaatccatc cagctggaga
agaaggttaa tgtccatggt caagactcca catgcttctc t
```

Korean wild medaka (*Oryzias sinensis*)

Partial VTG I cDNA (815bp)

Amino acids (translated) identity = 96%

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tggggcattg attgcaagca gtacaacgct tcaatcatcg ctgaaactgg tctggtcgga aaagaacctg
cagctcgatt gaaaatgacc tgggaaagac tccctgaggg cttgaaaaga tacgccaagt gggcttctga
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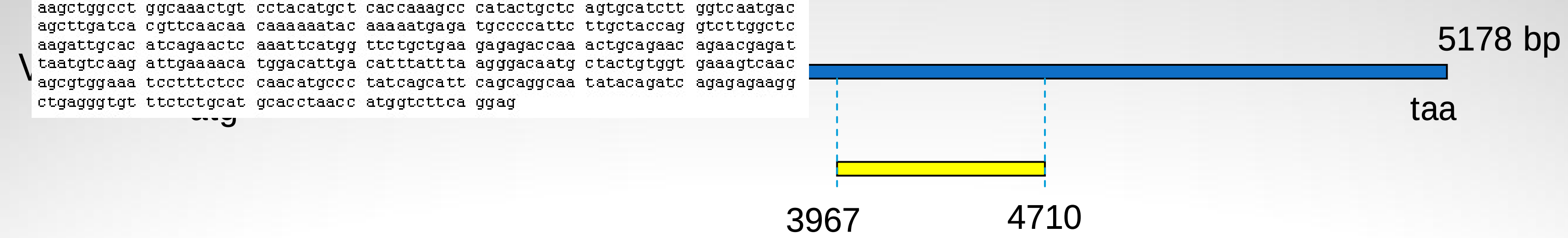


Korean wild medaka (*Oryzias sinensis*)

Partial VTG II cDNA (744bp)

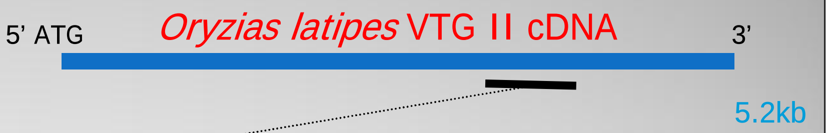
Amino acids (translated) identity = 96%

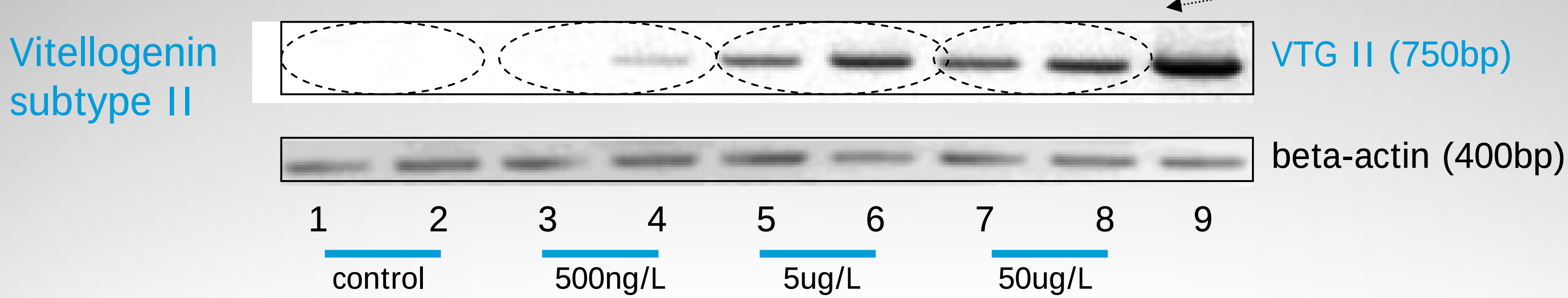
VTG mRNA Expression in Liver

Reverse Transcription - PCR

-Vitellogenin mRNA expression in the liver of ***Oryzias sinensis*** by 17 α -ethinylestradiol (7 day-exposure)

- RT-PCR was performed for 34 cycles





1, 3, 5, 7	*Oryzias latipes*
2, 3, 4, 8, 10	*Oryzias sinensis*

This data were clarified by a study of NIER(Korea)

Fish ecotoxicity testing: Current Status in medaka

< Popular test methods>

Acute toxicity test: OECD TG203,

Exposure stage and period: Adult, 96 hours

Endpoint: 96 h LC50,

Early life-stage toxicity test: OECD TG210,

Exposure stage and period: Egg, Larva and Juvenile, 38days in medaka (10days for egg, 28 days for larva and juvenile)

End point: LC50 at end of test, LOEC on growth at end of test

< Test methods for EDCs>

Life-cycle test: Partial-life, full-life, Multi-generation, etc.

Exposure stage and period: Egg, Larva, Juvenile, and Adult, over 70days in medaka

End point: LOEC on growth , sexual maturation, sex change and reproduction at end of test

Comparison of test stage between full-life cycle test(FLCT) and 2-generation test(2GT)

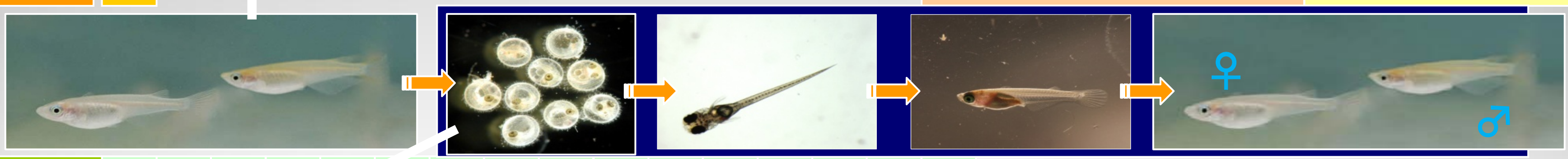
※As for Medaka 2-generation test protocol, US.EPA cooperates with Japan and considers by standardization.

copulation

※focus to maternal transfer

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
2GT	F0	1	2	3	4	5																					
		breeding																									
	F1			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15									
				hatch/growth										breeding													
	F2															1	2	3	4	5	6	7	8	9	10	11	12
																hatch/growth							breeding				
FLCT	F0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15											
		hatch/growth										breeding															
	F1															1	2	3	4	5	6	7	8	9	10		
																hatch/growth											

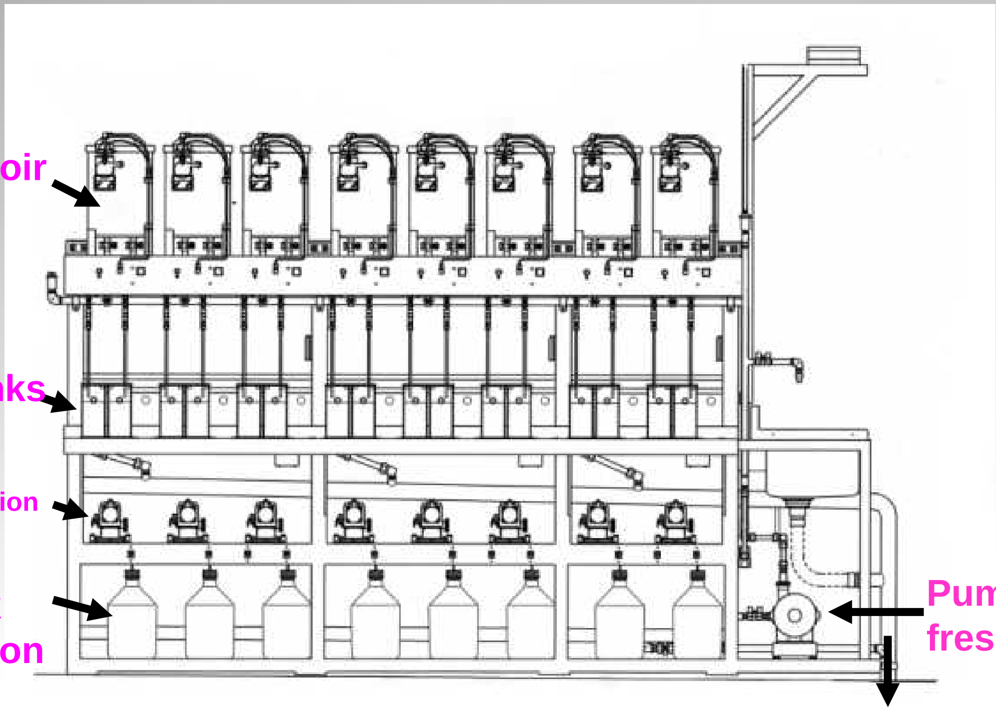
period(week): 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26

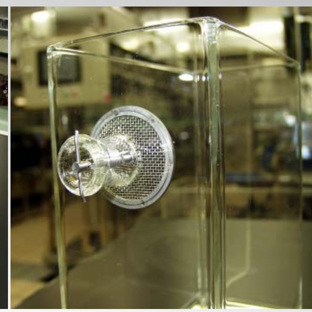


※Japan developed this method of testing originally. We submitted a protocol to OECD.

Exposure system, facility

Continuous exposure using
flow-through exposure system





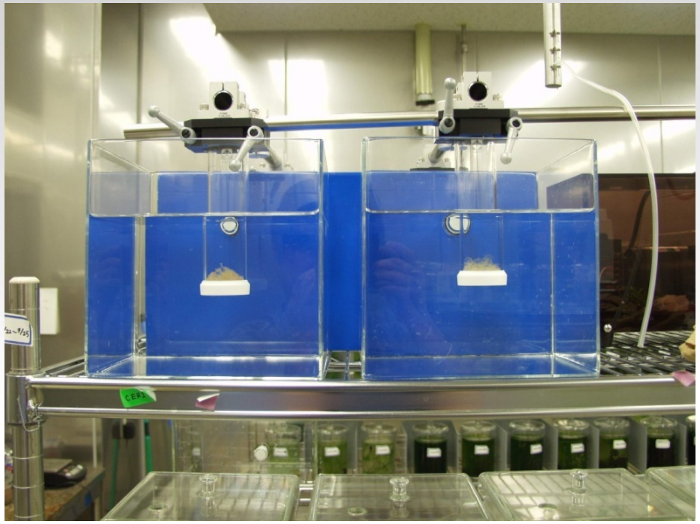
It is possible to keep the concentration of test chemical with constantly mixing of stock solution and fresh water.

Glass tank

Live food

Artemia salina

Hatching machine



Breeding system of Medaka

SPEED'98 < May 1998～ >
Strategic Programs on Environmental Endocrine Disrupters

Evaluation of 69 Chemicals in the list Under the Framework of SPEED'98

69 → 66 chemicals → 36 ***Tests Completed (2006)***

69 → 3 *Potential Hazardous Aspects other than Endocrine Disruption*

66 → 30

30 → *Not Measured* → 3 ***Not confirmed to be in Actual Use in Japan***

30 → *Measured* → 11 ***Not Detected in Japan***

30 → *Detected* → 16

16 → 2 ***No Reliable Data***

16 → 14 ***Evaluation of Reliability of the Data***

14 → 2 ***Unintentional Products etc***

14 → 12 ***Pesticides***

Evaluated chemicals In SPEED'98

Material	VTG	PLCT	FLCT	Results	Notes
Tributyltin	CERI	CERI			
Octyl phenol	CERI	CERI	CERI	Testis-ova	
Nonyl phenol	CERI	CERI	CERI	Testis-ova	
Di-n-butyl phthalate	CERI	CERI	CERI	Testis-ova	
Octachlorostyrene	METO	CERI			
Benzophenone	METO	CERI			
Dicyclohexyl phthalate	METO	CERI			
Di-(2-ethylhexyl)phthalate	METO	CERI			
Triphenyltin	*NIES*	*NIES*			
Butyl benzyl phthalate	*NIES*	*NIES*			
Diethyl phthalate	*NIES*	*NIES*			
Diethylhexyl adipate	*NIES*	CERI			
Pentyl phenol	CERI	CERI	CERI		
Pentachlorophenol	*NIES*	*NIES*			
Amitrole	*NIES*	*NIES*			
Bisphenol A	METO	*NIES*	*NIES*	Testis-ova	
2,4-Dichlorophenol	*NIES*	*NIES*			
4-Nitrotoluene	METO	CERI			
Dipentyl phthalate	METO	CERI			
Dihexyl phthalate	METO	CERI			
Dipropyl phthalate	METO	CERI			
Hexachlorobenzene (HCB)	CERI	CERI			POPs
Hexachlorocyclohexane	METO	CERI			
Chlordane	METO	CERI			POPs
trans-Nonachlor	METO	CERI			
o,p-DDT	CERI	CERI			POPs
p,p-DDT	*NIES*	*NIES*			POPs
p,p-DDE	*NIES*	*NIES*			
p,p-DDD	*NIES*	*NIES*			
Aldrin	*NIES*	*NIES*			POPs
Endrin	*NIES*	*NIES*			POPs
Dieldrin	*NIES*	*NIES*			POPs
Heptachlor	METO	CERI			POPs
Mirex	METO	CERI			POPs
Kelthane	CERI	CERI			
Malathion	METO	CERI			
Permethrin	CERI	CERI			
17β-Estradiol		*NIES*	CERI	Testis-ova	
17α-Ethinyl Estradiol	CERI	*NIES&CERI*	*NIES*	Testis-ova	
Metyl testosterone	CERI		CERI		
Fultamide	CERI		CERI		

Yellow	before2001
Purple	2001
Green	2002
Light blue	2003
Red	2004

Results of EDCs with Medaka test

(Nonyl phenol(NP),4-t-Octyl phenol(OP), Bisphenol A(BPA), o,p'-DDT)

Testing Methods	Endopoints	LOEC (μg/L)			
		NP	OP	BPA	DDT
Estrogen receptor binding assay	Binding to receptor	+	+	+	+
Vitellogenin assay	Vitellogenin induction	22.5	64.1	334	1.50
Partial life-cycle assay	Vitellogenin induction	11.6	11.4	890	3.36
	Testis-ova development	11.6	23.7	890	0.195
Full life-cycle assay	Vitellogenin induction	-	9.92	>1179	0.522
	Testis-ova development	17.7	30.4	1179	0.522
	Fertility	(17.7)	82.3	>1179	0.522
	NOEC	6.08	9.92	247	0.145

Detailed results of the fish testing

Chemical	Vitellogenin Assay	Partial Life Cycle (extended early life-stage)	Full Life Cycle
17β-Estradiol	↑ ♂ 14 & 21-d 22ng/L	↓ BL & BW 71 ng/L; ↑ testis-ova ♂ 24 ng/L; ↑ LSI ♀ 2.5 & ♂ 7.5 ng/L; ↑ VTG ♂ 0.9 & ♀ 24 ng/L	**F_0** ↓ fecundity 28 ng/L; ↓ egg fertilization 8.7 ng/L; ↑ testis-ova ♂ 28 ng/L; ↑ LSI ♂ & ♀ 28 ng/L; ↑ VTG ♂ 8.7 ng/L **F_1** ↑ body length 8.7 ng/L; no male or female VTG change at 8.7 ng/L
Ethinyl Estradiol	↑ ♂ 21-d 15 ng/L ↑ ♀ 21-d 61 ng/L	↑ GSI ♂ 93 ng/L; ↑ testis-ova ♂ 25 ng/L; ↑ LSI ♂ 25 ng/L; ↑ VTG ♂ 25 & ♀ 93 ng/L	**F_0** ↑ time to hatching 94 ng/L; ↑ testis-ova ♂ 29 ng/L; ↓ egg fertilization 9.3 ng/L; ↑ VTG ♂ 9.3 ng/L **F_1** ↑ VTG ♂ as low as 1 ng/L
Bisphenol A	↑ ♂ 14-d 72 µg/L and 21-d 334 µg/L	↑ hatching time 4,410 µg/L; ↑ testis-ova ♂ 890 µg/L; ↑ LSI ♀ 2,120 & ♂ 4,410 µg/L; ↑ VTG ♂ 470 & ♀ 2,120 µg/L	**F_0** ↑ mortality1,185 µg/L; ↑ LSI ♂ 1,185 ng/L **F_1** ↑ body weight & length 1,185 µg/L; ↑ testis-ova ♂ (2/10) 1,185 µg/L; ↑ VTG ♂ 1,185 µg/L
p,p'-DDE	↑ ♂ 14 & 21-d 54 µg/L	↑ time to hatching & mortality 32 µg/L; ↑ testis-ova ♂ 32 µg/L; erratic LSI; ↑ VTG ♂ 32 & ♀ 111 µg/L	In progress

Reference

Comparison of test condition with other test species

	Fathead minnow (*Pimephales promelas*)	Medaka (*Oryzias latipes*)	Zebrafish (*Danio rerio*)
Test type	Flow-through	Flow-through	Flow-through
Water temperature	25 ± 2℃	24± 2℃	26 ± 2℃
Photoperiod (dawn /dusk transitions are optional, however not considered necessary)	16 h light, 8 h dark	16 h light, 8 h dark	16 h light, 8 h dark
Test chamber size	10 L (minimum)	2 L (minimum)	4 L (minimum)
Age of test organisms	Reproductively mature fish (not senescent)	Reproductively mature fish (not senescent)	Reproductively mature fish (not senescent)
Wet weight of adult fish (g)	Females: 1.5 ± 20% Males: 2.5 ± 20%	Females: 0.35 ± 20% Males: 0.35 ± 20%	Females: 0.65 ± 20% Males: 0.5 ± 20%

Our suggestion

- We Japan propose new testing method using the Medaka for OECD.
- We have a lot of know-how about the examination of the Medaka fish.
- In addition, we have a lot of data of the Medaka about the endocrine disrupting chemicals(SPEED'98).
- There is two kinds of Medaka in China, Korea, Japan, but the property/sensitivity are very similar.
- Joint ownership of data and the know-how of the Medaka is possible around the 3 countries.
- We (Asian scientists) will cooperate in future and should increase more information about EDCs based on it.

Development of the Daphnia testing

Different Endocrine Systems Between Vertebrates and Invertebrates

Vertebrates (e.g., Mammals)	Invertebrates (e.g., Arthropods)
Androgen Estrogen	Ecdysteroid Juvenile hormone
DES EE2, etc.	Insect Growth Regulators (IGRs)
Nonylphenol Bisphenol A, etc.	?

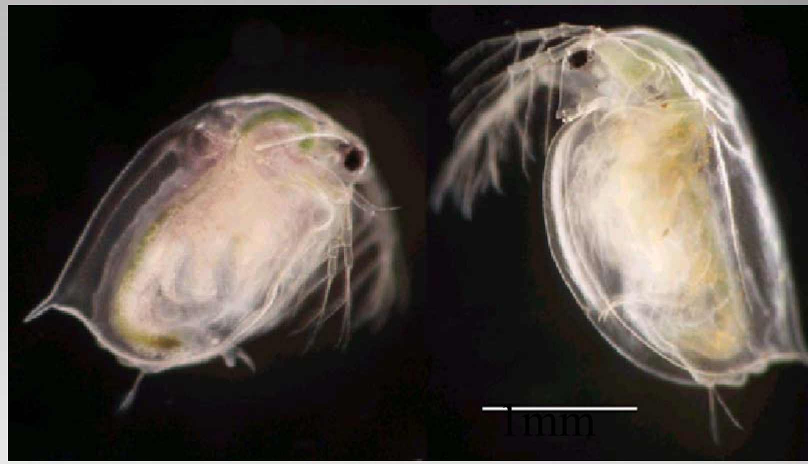
Endocrine System in Invertebrates (e.g., Arthropods)

- **Endogenous hormones**
  - **Ecdysteroids**
  - **Juvenile hormones**
- **Hormone mimics**
  - **Insect growth regulators (pesticides)**

Crustacean Reproduction Toxicity Tests in OECD

- Draft DRP on mysid life cycle toxicity test
  (USA)
- Draft proposal on a new test guideline copepod development and reproduction test
  (Sweden)
- *Daphnia magna* reproduction test
  (TG 211; OECD 1998)
  - Proposal for enhanced TG 211 for endocrine disrupting chemicals from Japan

Daphnia magna



- Kingdom Animalia
  - –Phylum Arthropoda
    - •Class Crustacea

Cyclic Parthenogen

–Order Cladocera

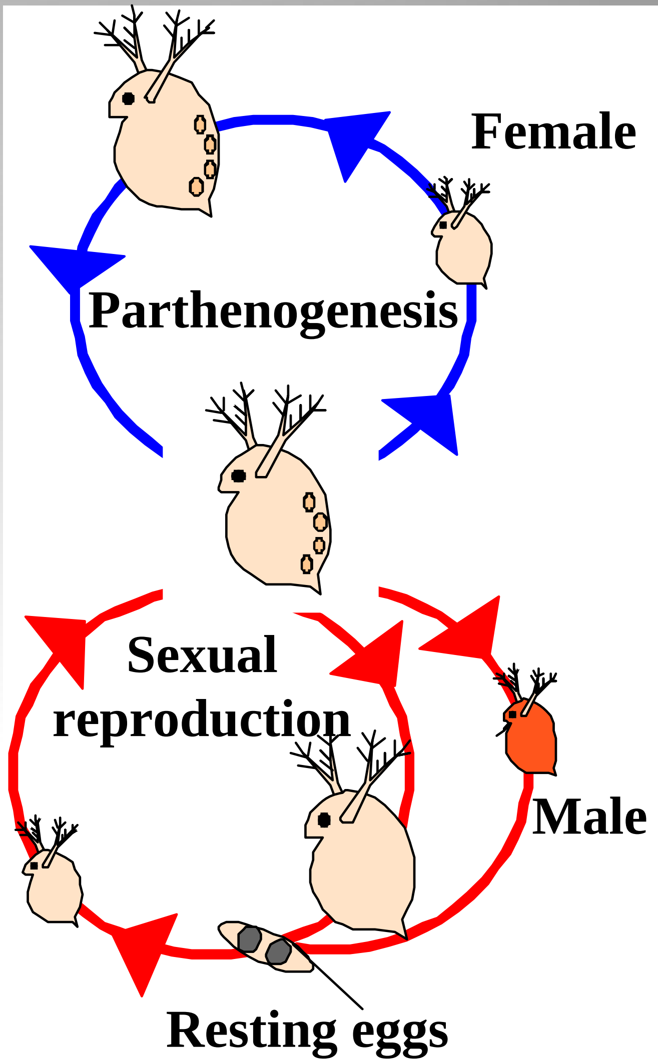
»Family Daphniidae

- Genus *Daphnia*

Species *Daphnia magna*

Reproductive System and Sex Determination in Cladocerans

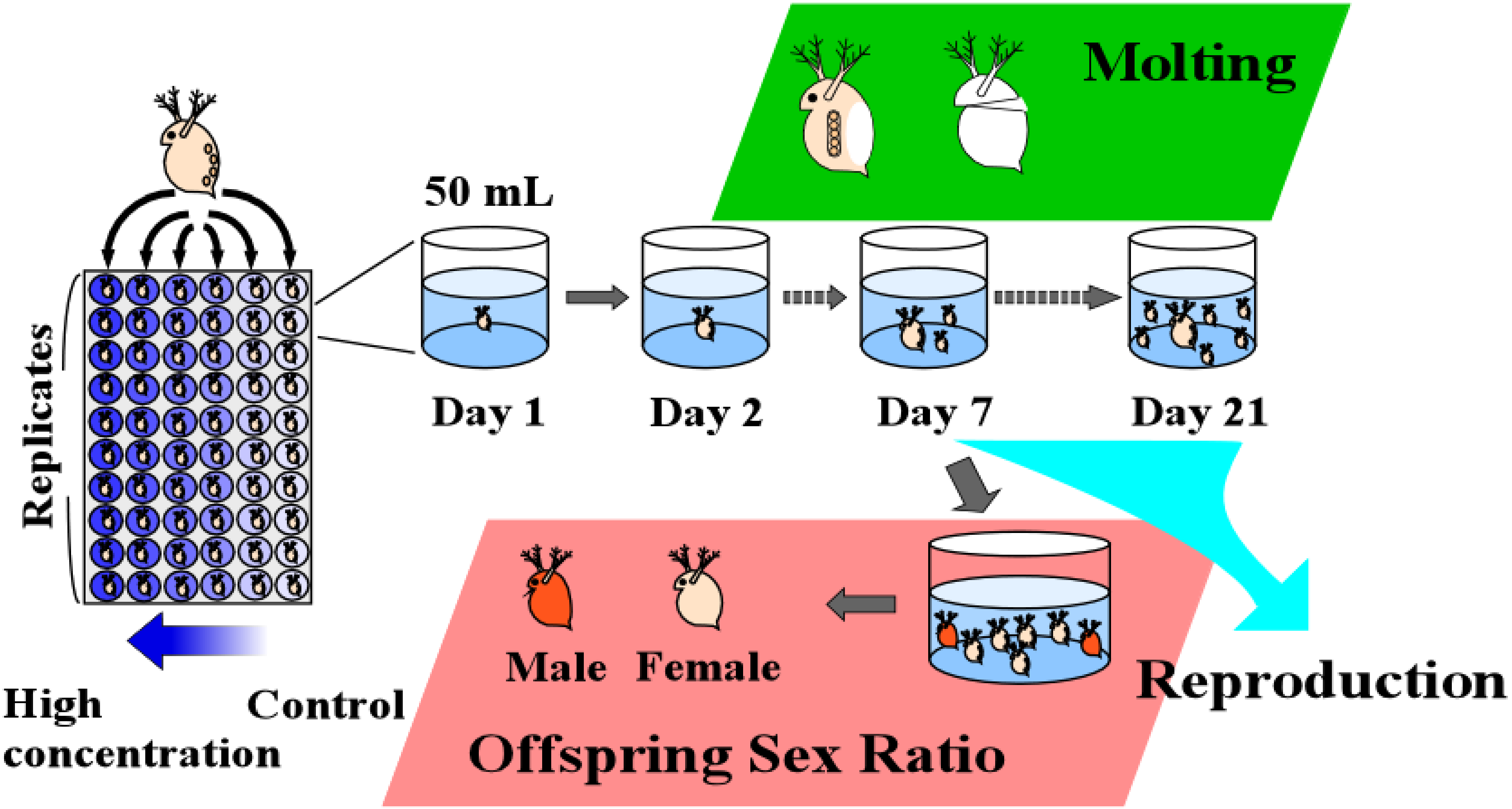
- Cyclic parthenogenesis
- Environmental sex determination
- Emergence of males and initiation of sexual reproduction by
  - -Short photoperiod
  - -Low food concentration
  - -High population density, etc.



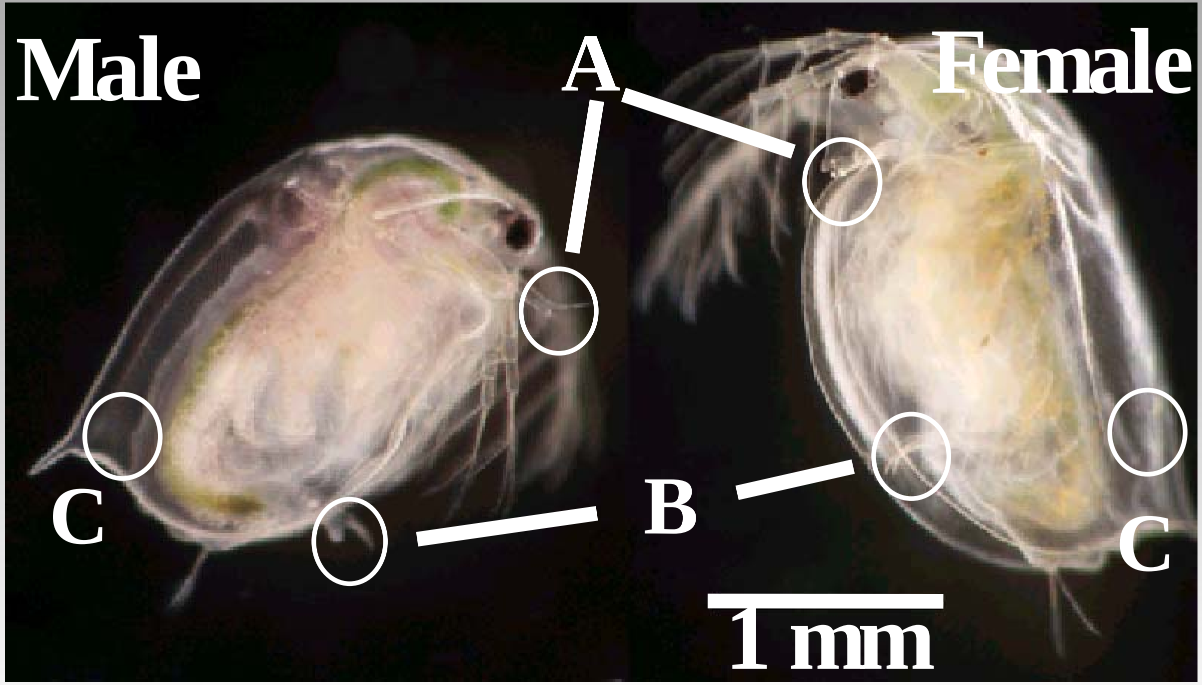
Experimental Design

Based on OECD TG 211

***Daphnia magna* reproduction test**



Sex Differentiation



A: 1st antenna

B: post abdominal claw

C: abdominal process

Juvenile Hormones and Their Analogs

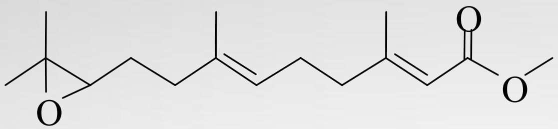
- **Juvenile hormones**
  - JH I
  - JH II
  - JH III
  - Methyl farnesoate

- **Insect growth regulators**
  - Fenoxycarb
  - Pyriproxyfen
  - Methoprene
  - Kinoprene
  - Epofenonane
  - Hydroprene

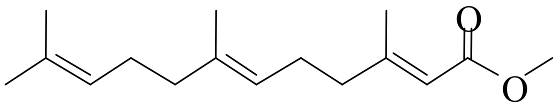
Juvenile Hormones and Their Analogs

Juvenile hormones

juvenile hormone III

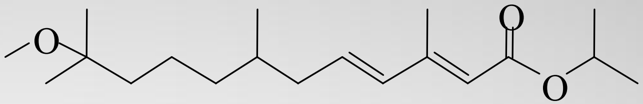


methylfarnesoate

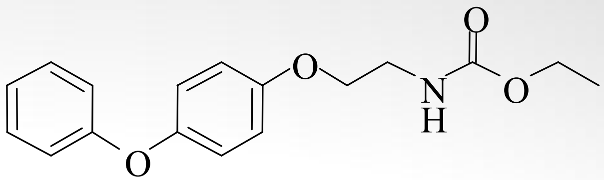


Juvenile hormone analogs (Insect Growth Regulators; IGRs)

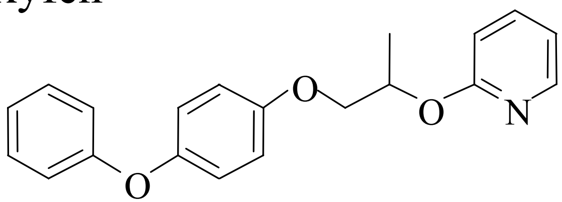
methoprene



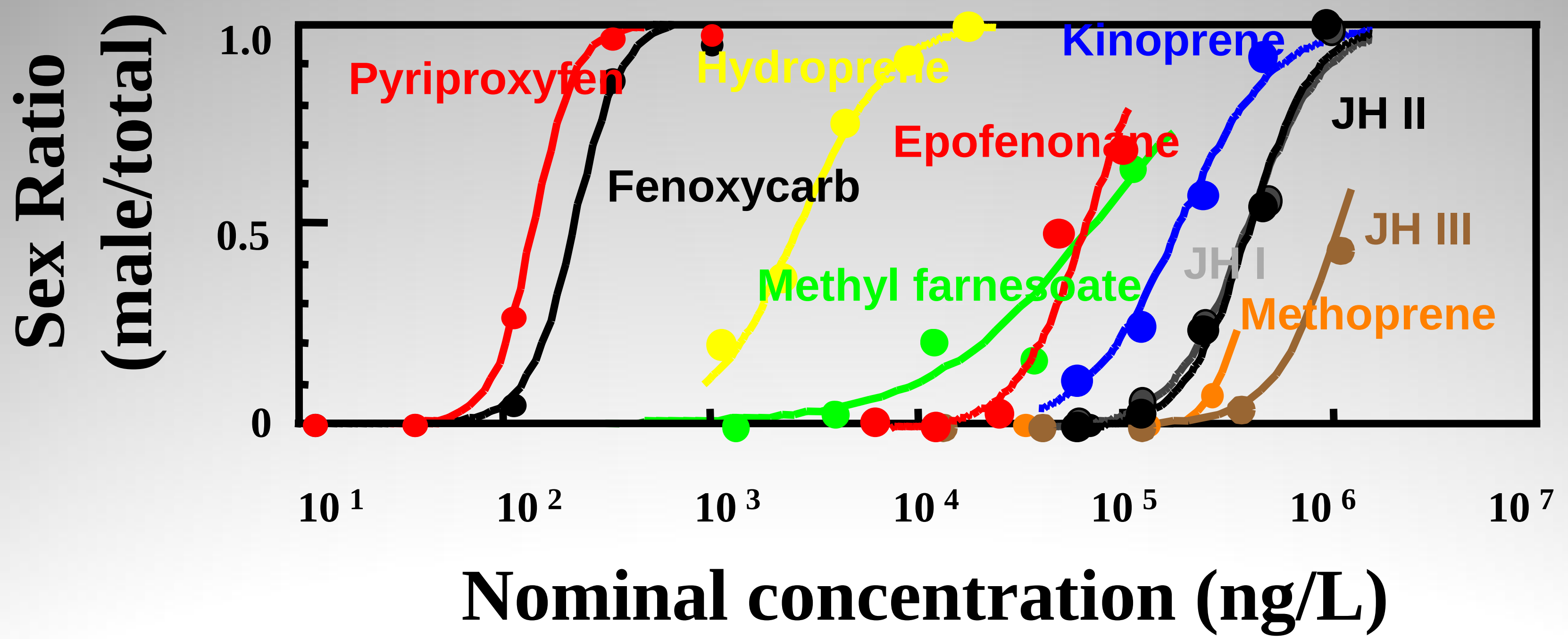
fenoxycarb



pyriproxyfen



Offspring Sex Ratio

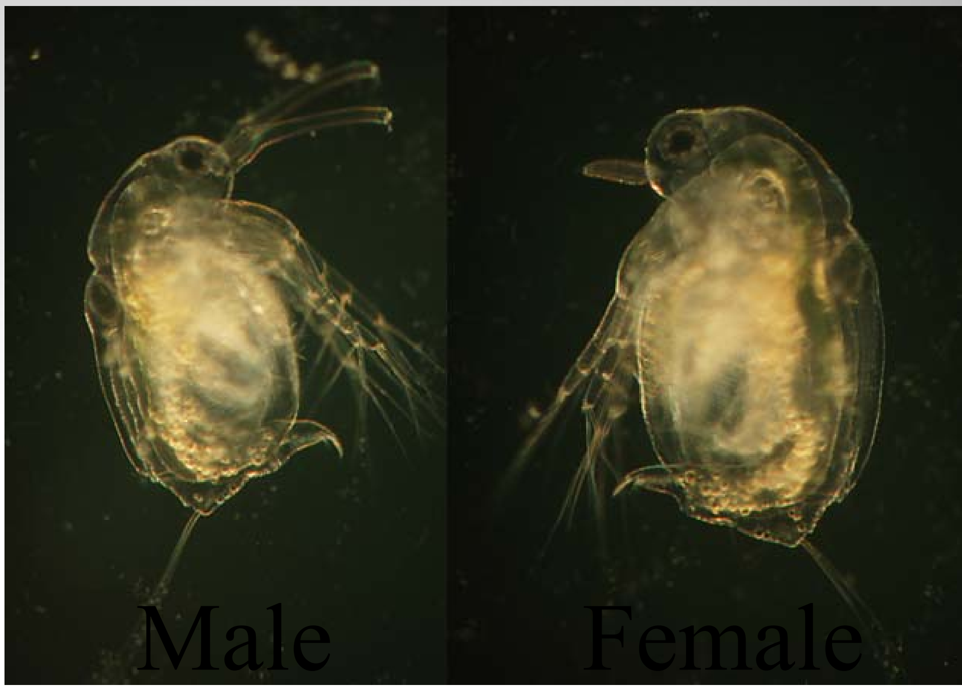


Male Neonates Induction in Different Taxonomical Groups

Other Daphnids in the Genus *Moina*

M. macrocopa

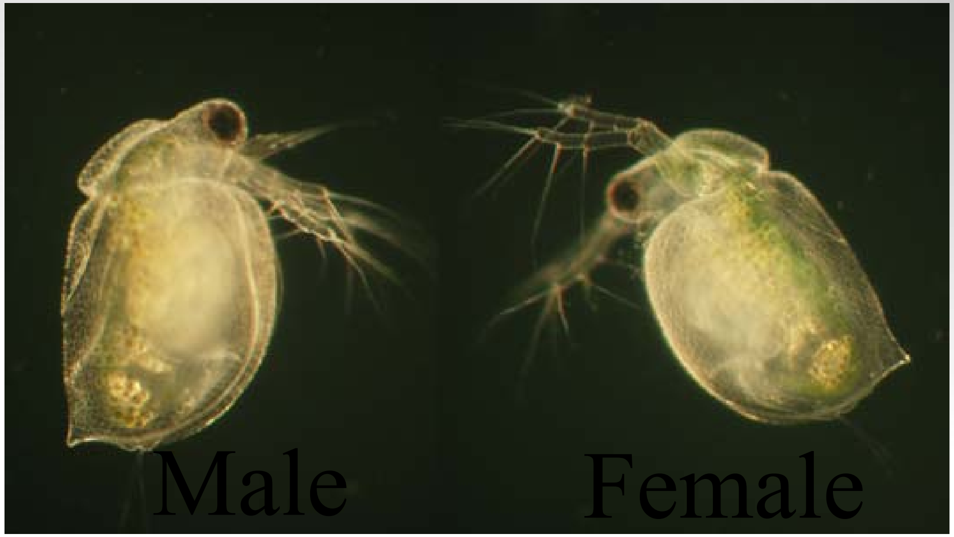
M. micrura



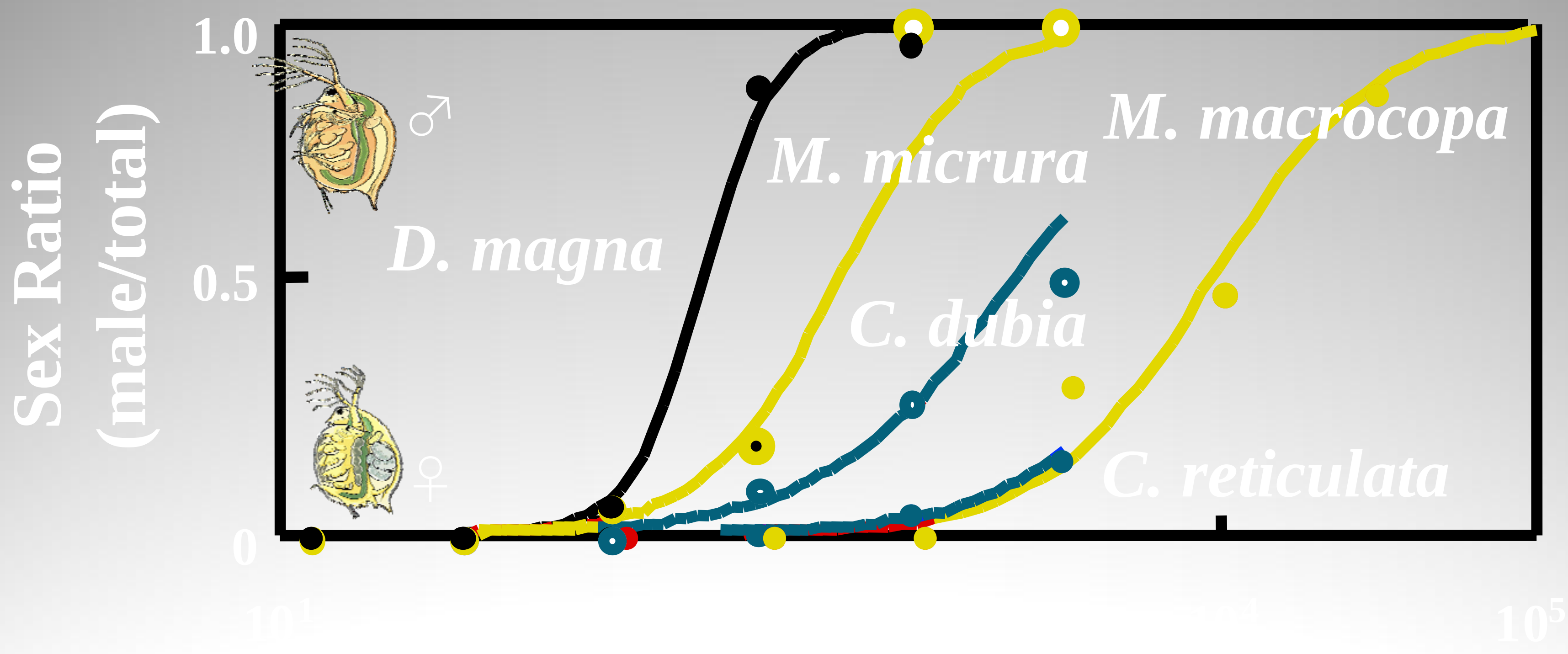
Other Daphnids in the Genus *Ceriodaphnia*

C. dubia

C. reticulata



Change in Offspring Sex Ratio Exposed to Fenoxycarb



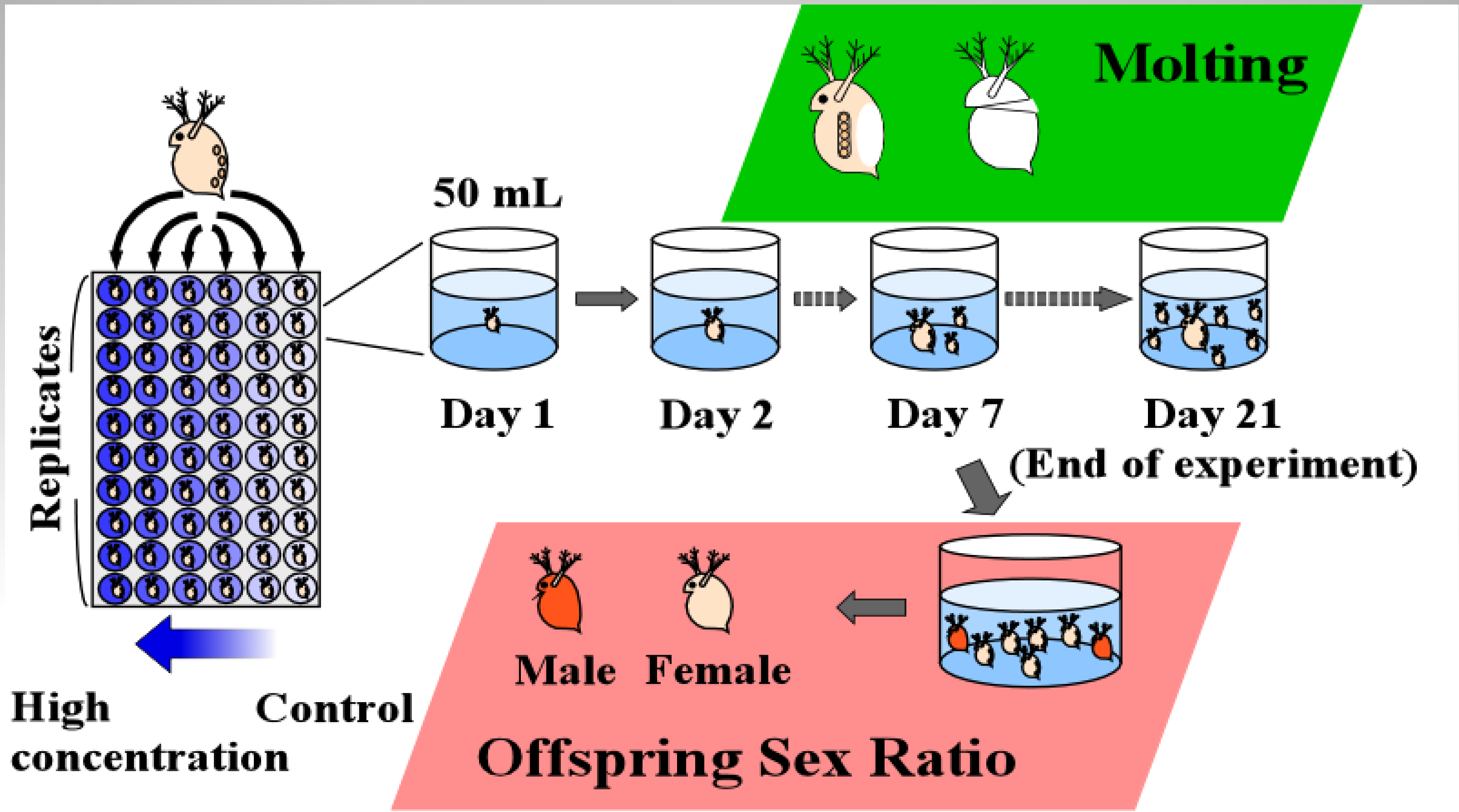
JH analogs increase male production in cladoceran

Switching from asexual to sexual cycle can be a marker for endocrine disruption

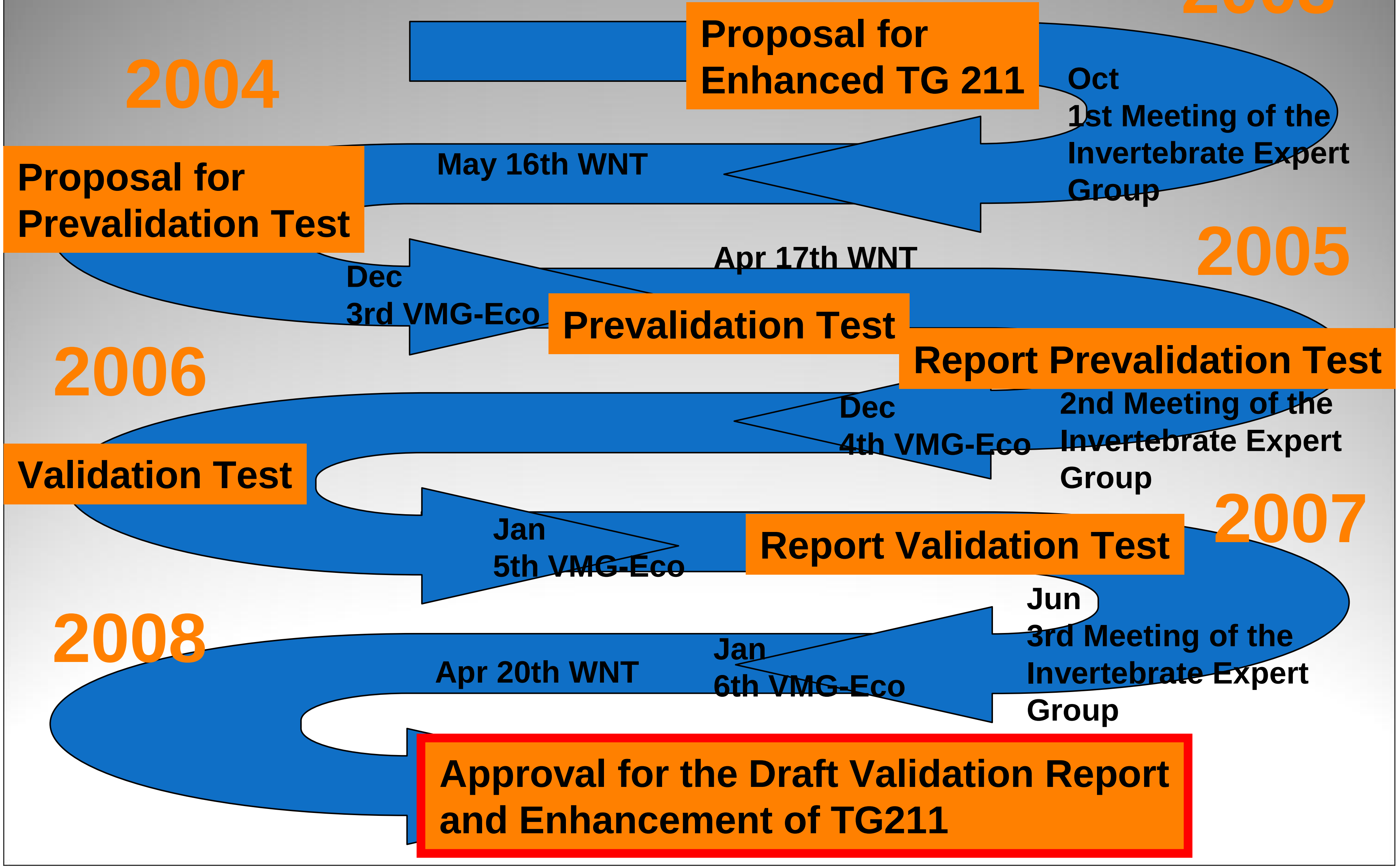
Experimental Design EnhancedTG211

Based on OECD TG 211

Daphnia magna reproduction test



Process before being authorized, OECD Enhanced TG 211



collaborated organizations at ring test

1. Finnish Environment Institute, Finland
2. French National Institute for Industrial Environment and Risks (INERIS), France
3. Laboratoire Ecotoxicité et Santé Environnementale (ESE) Equipe CNRS UMR, France
4. Aachen University, Germany
5. Bayer CropScience AG, Germany
6. Institute for Biological Analysis and Consulting (IBACON), Germany
7. UMWELTBUNDESAMT (UBA), Germany
8. Laboratory of Hydrobiology, Hungary
9. National Institute of Health, Italy
10. Agricultural Chemicals Inspection Station (ACIS), Japan
11. Kureha Special Laboratory, Co., Ltd., Japan
12. National Institute for Environmental Studies (NIES), Japan

Screening system of Endocrine disruptor for invertebrate

Potential Endocrine Disruptors

For Insects

(Nijhout, 1994)

Metamorphosis

Polyphenism

Caste differentiation in social insects

Regulation of behavior in honeybee colonies

Larval and adult diapause regulation

Vitellogenin synthesis

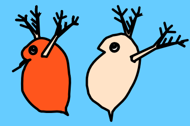
Ovarian development

Unknown effects to non-target species other than Cladocerans

For Daphnia

Enhanced TG 211

Cladocerans



Male neonates production

JH Active!

Not JH Active!

Nijhout, H.F., 1994. Insect Hormones. Princeton University Press, New Jersey.

Publications in Refereed Journals and Proceedings

Tatarazako, N., Oda, S., Sonobe, H., Watanabe, H., Morita, M., and Iguchi, T., 2002. Insecticides for juvenile hormone agonists exert the influence on the occurrence of the male daphnid. Proc. Jpn. Soc. Comp. Endocrinol., 17: 87.

Tatarazako, N., Oda, S., Watanabe, H., Morita, M., Iguchi, T., 2003. Juvenile hormone agonists affect the occurrence of male *Daphnia*. Chemosphere 53, 827–833.

Tatarazako, N., Oda, S., Abe, R., Morita, M., Iguchi, T., 2004. Development of a screening method for endocrine disruptors in crustaceans using *Daphnia magna* (Cladocera, Crustacea). Environmental Science 17, 439-449 (in Japanese with English abstract).

Oda, S., Tatarazako, N., Watanabe, H., Morita, M., Iguchi, T., 2005. Production of male neonates in *Daphnia magna* (Cladocera, Crustacea) exposed to juvenile hormones and their analogs. Chemosphere 61, 1168-1174.

Oda, S., Tatarazako, N., Watanabe, H., Morita, M., Iguchi, T., 2005. Production of male neonates in four cladoceran species exposed to a juvenile hormone analog, fenoxycarb. Chemosphere 60, 74-78.

Watanabe, H., Tatarazako, N., Oda, S., Nishide, H., Uchiyama, I., Morita M., Iguchi, T., 2005. Analysis of expressed sequence tags of the water flea, *Daphnia magna*. Genome 48: 606-609.

Oda, S., Tatarazako, N., Watanabe, H., Morita, M., Iguchi, T., 2006. Genetic differences in the production of male neonates in *Daphnia magna* exposed to juvenile hormone analogs. Chemosphere 63, 1477-1484.

Tatarazako, N. and Oda, S., 2007. The water flea *Daphnia magna* (Crustacea, Cladocera) as a test species for screening and evaluation of chemicals with endocrine disrupting effects on crustaceans. Ecotoxicology, 16: 197-203.

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Watanabe, H., Takahashi, E., Nakamura, Y., Oda, S., Tatarazako, N., and Iguchi, T., 2007. Development of a *Daphnia magna* DNA microarray for evaluating the toxicity of environmental chemicals. Environmental Toxicology and Chemistry, 26: 669-676.

Oda, S., Tatarazako, N., Dorgerloh, M., Johnson, R., Kusk, K. O., Leverett, D., Marchini, S., Nakari, T., Williams, T., Iguchi, T., 2007. Strain difference in sensitivity to 3,4-dichloroaniline and insect growth regulator, fenoxycarb, in *Daphnia magna*. Ecotoxicology and Environmental Safety, 67: 399-405.

Kato, Y., Kobayashi, K., Oda, S., Colbourne, J. K., Tatarazako, N., Watanabe, H., Iguchi, T., 2008. Molecular cloning and sexually dimorphic expression of DM-domain genes in *Daphnia magna*. Genomics, 91: 94-101.

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Problems and the future prospects

There are few study examples that paid the attention to "impact to the Southeastern Asian ecosystem" by chemicals

It may be necessary to build a tool generalizing data affecting Southeastern Asian ecological effects

The scientist will have to cooperate in future to protect a Southeastern Asian ecosystem.

It is preferable to use common technique and organism.

In China, Korea and Japan , it is possible to cooperate.