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The water flea *Daphnia magna* (Crustacea, Cladocera) as a test species for screening and evaluation of chemicals with endocrine disrupting effects on crustaceans

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Abstract The water flea *Daphnia magna* (Crustacea, Cladocera) is a cyclical parthenogen, which can reproduce both by parthenogenesis and by sexual reproduction. With its ease of handling in the laboratory, several testing methods using *D. magna* exist for regulatory toxicity testing. Recently, several studies revealed that one of the major hormone groups in insects and crustaceans, juvenile hormones, are involved in the shift of reproductive mode from parthenogenesis to sexual reproduction (production of male neonates). Using offspring sex ratio as a new endpoint has made it possible to identify chemicals with juvenile hormone-like effects on crustaceans. The testing method using *D. magna*, in which offspring sex ratio is incorporated as a new endpoint, is now being proposed to the OECD as an enhanced version of the existing OECD Test Guideline 211: *Daphnia magna* reproduction test. No other clear-cut endpoint for identifying juvenile-hormone disrupting effects has ever been found in crustaceans than the induction of male neonates production in cladocerans. In this regard, it is expected that testing methods using *D. magna* are suitable for screening and risk assessment of chemicals with juvenile-hormone disrupting effects.

Keywords Insect growth regulators · Juvenile hormones · Offspring sex ratio · OECD Test Guideline 211

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Introduction

Daphnia (Cladocera, Crustacea whose relatives include other arthropods such as lobsters and crabs), commonly called water fleas, are filter-feeding planktonic crustaceans and can be found in almost any permanent body of water. The most prominent external features of *Daphnia* are a single large compound eye and two pairs of highly branched antennae. The animal uses the antennae for locomotion. Its thorax and abdomen are protected by a transparent shell. Visible through the shell are several pairs of legs with setae (hairs). The moving legs generate a water current that directs protozoa, algae, bacteria, and organic detritus to the mouth.

In general, daphnids reproduce by parthenogenesis, in which females asexually produce genetically identical female offspring. Because the parthenogenetic reproduction system provides rapid expansion of daphnid populations, a daphnid population consists almost entirely of females when resources are abundant. It is known that daphnids switch their mode of reproduction from parthenogenesis to sexual reproduction in response to environmental changes. Environmental factors such as short day length, food depletion, and high population density are known to be keys for the initiation of sexual reproduction (Hobaek and Larsson 1990; Kleiven et al. 1992). After males are produced (for distinguishing males from females see Fig. 3) sexual reproduction can occur and resting eggs are produced that can withstand severe environmental conditions, such as freezing temperatures or drought. *Daphnia* occupies an important position in aquatic food webs because it is a major part of the diet of fish and invertebrate predators. It

also plays a major role in the control of water quality by feeding on algae (Dodson and Hanazato 1995).

Daphnia magna has been widely used as an experimental animal in aquatic environmental toxicity testing. Testing methodologies that use the water flea have been adopted by US-EPA, ASTM, and Environmental Canada including the test guidelines of the OECD (ASTM 1988, 2004; Environment Canada 1992; USEPA 2002; OECD 1984, 1998). Acute and chronic toxicity tests using *D. magna* are also used in the safety evaluation of chemicals in Japan, according to the amended Chemical Substances Control Law (National Institute of Technology and Evaluation in Japan 2004). Water fleas have a long history in aquatic toxicity testing for a number of reasons: their complete life is spent in water, they are prolific, easy to handle, and they have a comparatively short longevity. In addition, water fleas are known to be quite sensitive to many chemicals, including heavy metals.

Evidence of the disruption of endocrine mechanisms in cladocerans in the laboratory

Ecdysteroids and juvenile hormones, are a group of steroids and a group of sesquiterpenoids, respectively, that are major hormones in crustaceans (for a review see LeBlanc 2006). In some studies, exploration of endocrine disruption in cladoceran crustaceans has been conducted for the major hormones in insects and crustaceans and their analogs. Other studies focused on chemicals which have been suspected to have endocrine disrupting effects on vertebrates.

Recently, several studies have demonstrated that production of male neonates in *D. magna* is induced by exposure to juvenile hormones of insects and crustaceans (Fig. 1a) or to pesticides designed as juvenile hormone mimics (Fig. 1b) (see Olmstead and LeBlanc 2002, 2003, Tatarazako et al. 2003; Oda et al. 2005b). Ten chemicals, all of which are juvenile hormones or JH analogs (depicted in Fig. 2), are now known to induce the production of male neonates in *D. magna* (male and female neonates are shown in Fig. 3). Induction of male neonates production is also reported for other cladoceran groups, including the genera *Moina* and *Ceriodaphnia* (Oda et al. 2005a). In the studies using 21-day reproduction tests based on the OECD Test Guideline 211 (OECD 1998), Tatarazako et al. (2003) and Oda et al. (2005b) found that most of these chemicals also reduce reproductive rate considerably (Fig. 4a, b). However, from Fig. 4b it is also evident that epofenonane deviates from this general observation, since it stimulates reproduction at concentrations around 30 μg/l, thereby generating an inverted U-shaped curve. The reason for this is still unknown. The LOECs for reduction in reproduction rate and induction of male neonates are shown in Table 1. Except for epofenonane, the LOEC for reproduction is lower than or at least equal to the LOEC for male offspring induction. This should be considered when these data are used for conducting an environmental risk assessment.

Interestingly, the effects of juvenile hormones and their analogs on daphnids, both as the change in offspring sex ratio and reduction in reproductive rate, disappear as soon as exposure is stopped. This indicates the reversibility of the effects of juvenile hormones and their analogs (Tatarazako et al. 2003, 2004). In addition, the stage of susceptibility to juvenile hormones and their analogs to induce male sex in neonates is estimated to be around the time when the eggs are in the ovary, before their release into the brood chamber (Olmstead and LeBlanc 2002; Tatarazako et al. 2004). Further, we confirmed that a 6-h exposure to a juvenoid insect growth regulator can induce production of male neonates in *D. magna* (Tatarazako et al. 2004).

Three chemicals, bisphenol A, *p*-nonylphenol, and 4-*tert*-octylphenol, which have been suspected to be estrogenically active in vertebrates (Ministry of the Environment in Japan 2004) were tested for induction of male neonates production in a 21-day reproduction test using *D. magna* (Tatarazako et al. 2004). These chemicals are known to be estrogenically active in fish, causing vitellogenin synthesis and feminization in appearance and in the gonad, i.e., development of female secondary sex characteristics and of ovotestis (Sohoni et al. 2001; Yokota et al. 2001; Seki et al. 2003; Tabata et al. 2003). Male neonates were not induced by exposure to these three chemicals in *D. magna*, suggesting that the chemicals have no juvenile hormone activity. Absence of male neonate induction with reduced reproductive rate at higher concentrations also suggests that production of male neonates is not induced by ''chemical stress'' which reduces reproductive rate.

Induction of male neonate production is, for most of the juvenile hormones and their analogs, accompanied by a considerable reduction in reproductive rate in *D. magna* (Fig. 4a, b; Tatarazako et al. 2004; Oda et al. 2005b). However, Oda et al. (2005b) reported that one of the juvenile hormone analogs, epofenonane, exceptionally induced male neonates with a small reduction in reproductive rate (see also Table 1). When daphnids were exposed to fenoxycarb for only 6 h, the sex ratio of neonates shifted to males, while the total number of neonates did not decrease (Tatarazako et al. 2004).

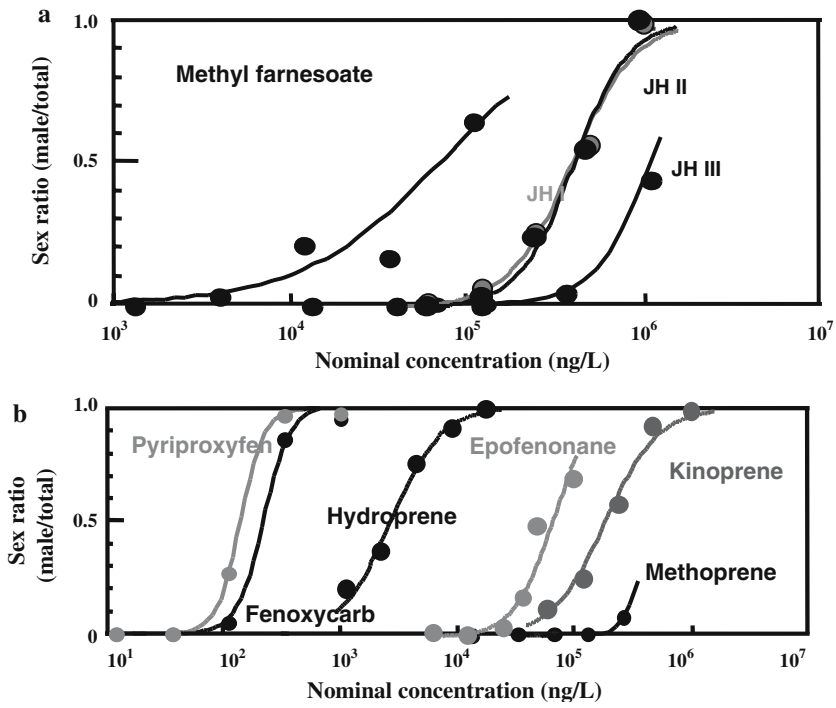

Fig. 1 Offspring sex ratio of *D. magna* exposed to juvenile hormones (**a**) and their analogs (**b**). Curves were fitted by logistic regression analysis. Regression coefficients for slopes were all statistically significant ($P < 0.01$)

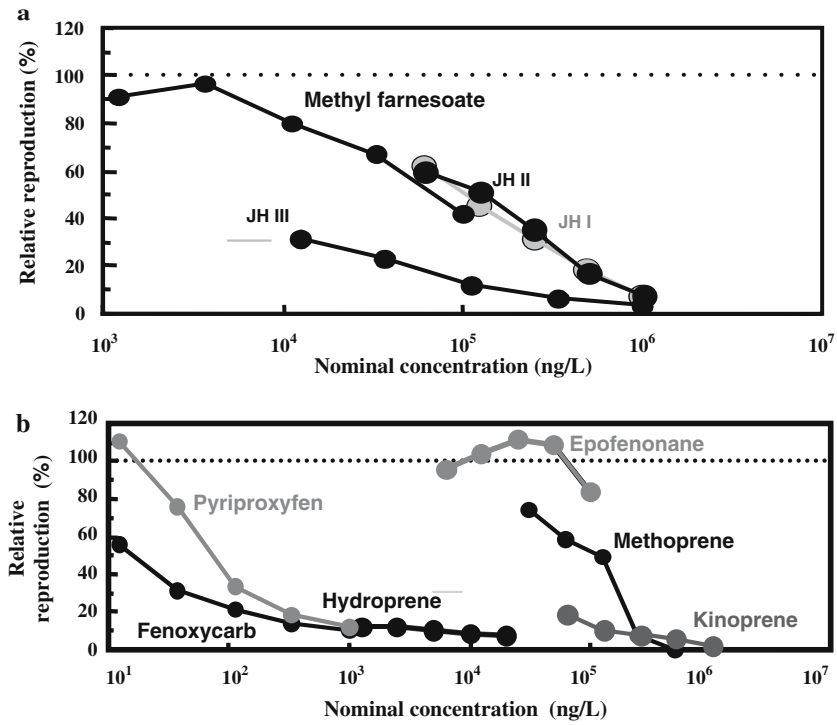

Fig. 2 Reproduction of *D. magna* exposed to juvenile hormones (**a**) and their analogs (**b**). Mean numbers of neonates produced per female in 21-day reproduction tests are represented as percentages relative to those of the control group in each test. The *dotted line* represents the mean number of neonates per female in the control group in each experiment

Thus, the toxicity represented as reduction in reproductive rate and male neonate induction may not be tightly associated with each other. This result also supports the idea that production of male neonates are induced not by ''chemical stress'' that causes reproductive toxicity, but by the endocrine disrupting effect of juvenile hormones and their analogs.

Although the mechanisms are not clear, the results of these studies thus suggest that juvenile hormones play a key role in the production of male offspring or in the switching of the reproductive system from parthenogenetic to sexual reproduction, in cladoceran crustaceans in which cyclical parthenogenesis occurs (Hebert 1987).

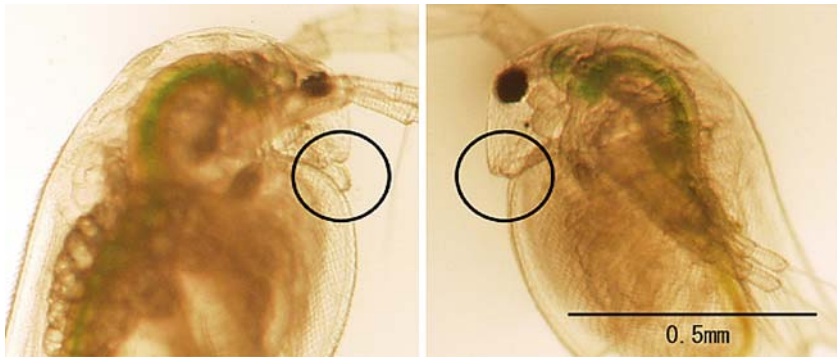

Fig. 3 Male (*left*) and female (*right*) *D. magna* aged ca. 24 h. Males can be distinguished from females by the length and morphology of the first antennae as shown in the *circles* (after Tatarazako et al. 2004)

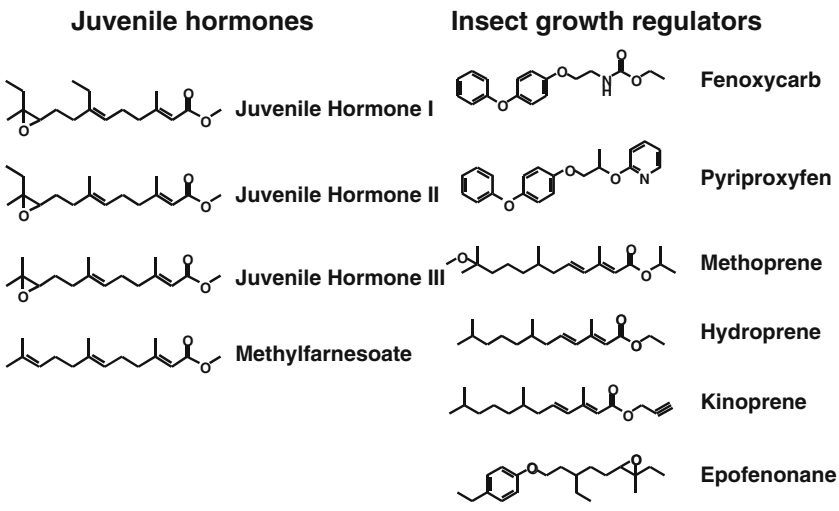

Fig. 4 Chemical structures of juvenile hormones and their analogs (juvenoid insect growth regulators)

Reproducibility of experimental data

Induced production of male neonates by exposure to the juvenile hormone analog fenoxycarb has been reported for four other species in the family Daphniidae as mentioned above (Oda et al. 2005a). Juvenile hormone is probably playing a role in switching the mode of reproduction from parthenogenesis to sexual reproduction. In this sense, it is suggested that induction of male neonates production by exposure to fenoxycarb and other juvenoids occurs not only in the family Daphniidae, but also across the wider taxonomical group, that is, in the order Cladocera, where most species have environmental sex determination systems with cyclic parthenogenesis (Hebert 1987).

On the other hand, Oda et al. (2006) reported the inter-strain difference in sensitivity to juvenile hormone analogs in three genetically distinct strains of *D. magna* (Figs. 5, 6). The difference is both in median effective concentration (EC_{50}) for induction of male neonates production and in the EC_{50} for reproduction. In using offspring sex ratio of *D. magna* as an endpoint for identifying chemicals with juvenile hormone-like effects on crustaceans, it is difficult to detect changes in the offspring sex ratio when the toxicity of the applied test chemicals strongly reduces the number of neonates. The same problem was also found in the OECD pre-validation test, in which seven strains of *D. magna* maintained in six countries throughout the world were studied for their sensitivity to a juvenile hormone analog (fenoxycarb) and a general toxicant (3,4-dichloroaniline) at the National Institute for Environmental Studies in Japan (unpublished data).

A validation test (ring test) for an enhanced version of the *Daphnia magna* reproduction test (Enhanced Test Guideline 211) proposed for the purpose of testing endocrine disrupting effects of chemicals on crustaceans is planned for 2006 at the OECD using a common strain of *D. magna*. The results of the ring test would make it possible to discuss the reproducibility of the testing method using a common strain of *D. magna* and to clarify the limitations of the test.

Table 1 LOEC values (in ng/l) for reduction in reproduction rate and induction of male neonates (after Tatarazako et al. 2003; Oda et al. 2005b)

Chemical	LOEC (ng/l)	
	Reduction in reproductive rate	Induction of male neonates[a]
JH I	63,000	63,000
JH II	63,000	63,000
JH III	10,000	330,000
Methylfarnesoate	3,700	3,700
Fenoxycarb	12	100
Pyriproxyfen	63	100
Methoprene	31,000	130,000
Hydroprene[b]	63	130
Epofenonane	100,000	6,300
Kinoprene	6,300	63,000

[a] Since the LOEC for induction of male neonates varies, depending on the number of neonates observed, the lowest concentration at which male neonates were first observed, was adopted as the LOEC

[b] Values for hydroprene were derived from the results of three experiments conducted with different concentration ranges

Comparison with other species

Many invertebrate toxicity test protocols are used in regulatory toxicity testing (OECD 1998). However, there are few test protocols designed to assess the effects of endocrine disruption. An enhanced version of the existing test guideline 211 "*Daphnia magna* reproduction test" is one of them. The primary objective of the original test guideline 211 is to assess the effect of chemicals on the reproductive output of *D. magna*. Thereby, the effect relates to reproductive toxicity in general and is not specifically focused on endocrine disrupting effects. The enhanced TG 211 indicates the influence of an endocrine disruptor by adding sex ratio of offspring to the endpoints of the original test guideline.

Two other test guidelines have been proposed as testing methods for endocrine disrupting chemicals in crustaceans, as well as for toxicity tests, i.e., mysid life cycle toxicity test by the United States (see Verslycke et al.2006) and the copepod development and reproduction test by Sweden (see Kusk and Wollenberger 2006). These crustaceans are not decapods (shrimps and crabs) for which the endocrinology is best studied among the crustaceans. In decapods, ecdysteroids and juvenile hormones are known to be two major hormone groups. On the other hand, the endocrine systems of cladocerans, mysids, and copepods are not so well understood compared to that of decapods. To develop these above-mentioned testing methods for endocrine disrupting chemicals in crustaceans, it is necessary to explore appropriate endpoints in these test animals for evaluation of endocrine disruption.

The growth, reproduction, development, and other aspects of crustacean physiology and life cycle are regulated by the endocrine system. For endocrine disrupting effects of juvenile hormones in cladocerans (e.g., *Daphnia*) the effects can be detected as the start of male neonates production. This is a strong point to emphasize. Offspring sex ratio can serve as a clear-cut endpoint for identifying chemicals with juvenile hormone-like activity. On the other hand, species used in the other two testing methods, mysids and copepods, do not reproduce by cyclical parthenogenesis nor have environmental sex determination. Concentration-dependent change in sex ratio of neonates or of test animals by juvenile hormones or their analogs has not been reported for these crustaceans. However, in

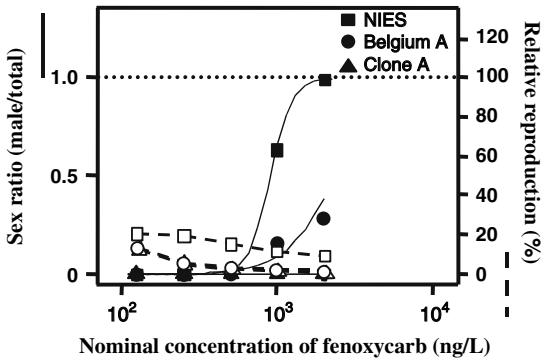

Fig. 5 Comparison of the responses of three clones after 21 days fenoxycarb treatment. Sex ratios of neonates (*closed symbols*, *solid line*) and mean total number of neonates per female (*open symbols*, *dotted line*), represented as values relative to those of the control group in each experiment. Curves for sex ratio were fitted by logistic regression (after Oda et al. 2006)

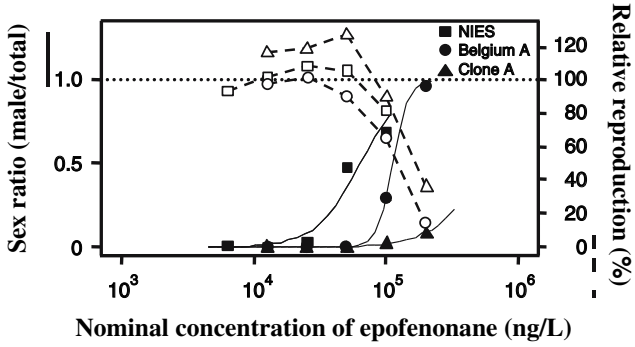

Fig. 6 Comparison of the responses of three clones after 21 days epofenonane treatment. Sex ratios of neonates (*closed symbols*, *solid line*) and mean total number of neonates per female (*open symbols*, *dotted line*), represented as values relative to those of the control group in each experiment. Curves for sex ratio were fitted by logistic regression (after Oda et al. 2006)

mysids, less males occurred in the second generation after exposure to 1 μg/l fenoxycarb (discussed in Verslycke et al. 2006). In most cases, reduction in reproduction rate is adopted as an endpoint in these crustaceans. Therefore, it is difficult to distinguish between general toxicity and endocrine disruption as the main causes of reduced reproductive rate.

Unlike cladocerans, it is hard to differentiate clearly between endocrine disrupting effects and toxicity of chemicals with juvenile hormone-like effects in the testing methods using mysids or copepods due to the lack of clear-cut endpoints. Testing methods that use *D. magna* are thus suitable for screening of juvenile-hormone disrupting effects.

Since ecdysteroids are critical to normal molting in crustaceans, exposure of crustaceans to chemicals with ecdysteroidal or anti-ecdysteroidal activity could interfere with normal molting. The effects of chemicals with ecdysteroid or anti-ecdysteroid activity can be detected through changes in molt frequency. Baldwin et al. (2001) exposed *D. magna* to the molting hormones 20-hydroxyecdysone or ponasterone A for 21 days. Reproductive rates were reduced at the highest exposure concentrations for both chemicals. Test animals also suffered premature death associated with incomplete loss of the exoskeleton. Exposure of daphnids to chemicals with anti-ecdysteroidal activity has been shown to delay molting and interfere with normal embryo development (Mu and LeBlanc 2002a, b). In mysids and copepods, molt interference has not been reported by the exposure to ecdysteroids or anti-ecdysteroids. Other endpoints such as mortality or reproduction are reported to be sensitive to ecdysteroid exposure in copepods (Hutchinson et al. 1999). The mortality caused by ecdysteroid exposure may be because of incomplete molting. Since all crustaceans molt to grow, direct observation of molt inhibition, the molting intervals, or number of molting during a test can also be used as endpoints to identify chemicals with ecdysteroid or anti-ecdysteroid-like effects.

Conclusions

The induction of male offspring in *D. magna* has proven to be a highly specific endpoint for the detection of juvenile hormone like activity of chemicals, which are as such endocrine disruptors for arthropods. However, for the detection of (anti-)ecdysteroid effects of chemicals, *Daphnia* might be less suitable as a test organism compared to other crustaceans, e.g., mysids (see Verslycke et al. 2006) or copepods (see Kusk and Wollenberger 2006). Since the endocrine systems of many invertebrate groups are still not fully characterized or even largely unknown, the detection of an endocrine disruptor is best conducted with more than one species, so that these may complement each other.

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