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# STUDY OF INVERTEBRATE-SPECIFIC EFFECTS OF ENDOCRINE DISRUPTING CHEMICALS IN THE ESTUARINE MYSID *NEOMYSIS INTEGER* (LEACH, 1814)

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For the degree of Doctor (PhD) in Applied Biological Sciences

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Studie van invertebraat-specifieke effecten van endocrien-verstorende stoffen in de estuariene aasgarnaal *Neomysis integer* (Leach, 1814)

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# CHAPTER 5

## NON-TARGET EFFECTS OF METHOPRENE ON MOLTING IN *NEOMYSIS INTEGER*

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# CHAPTER 5

## NON-TARGET EFFECTS OF METHOPRENE ON MOLTING IN *NEOMYSIS INTEGER*

**ABSTRACT** ----------

Ecdysteroids, the molting hormones in crustaceans and other arthropods, play a crucial role in the control of growth, reproduction and embryogenesis of these organisms. Insecticides, such as methoprene - a juvenile hormone analog, are often designed to target specific endocrine-regulated functions such as molting and larval development.

The aim of this study was to examine the effects of methoprene on molting in a non-target species, i.e. the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea). Mysids have been proposed as standard test organisms for evaluating the endocrine disruptive effect of chemicals. Juveniles (< 24h) were exposed for 3 weeks to the nominal concentrations 0.01, 1 and 100 µg methoprene/l. Daily, present molts were checked and stored in 4% formaldehyde for subsequent growth measurements. Methoprene significantly delayed molting at 100 µg/l by decreasing the growth rate and increasing the intermolt period. This resulted in a decreased wet weight of the organism. The anti-ecdysteroidal properties of methoprene on mysid molting were also evaluated by determining the ability of exogenously administered 20-hydroxyecdysone, the active ecdysteroid in crustaceans, to protect against the observed methoprene effects. Co-exposure to 20-hydroxyecdysone did not mitigate methoprene effects on mysid molting. This study demonstrates the need for incorporating invertebrate-specific hormone-regulated endpoints in regulatory screening and testing programs for the detection of endocrine disruption caused by man-made chemicals.

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## 5.1. INTRODUCTION

It is increasingly recognized that the assessment of the ecological impact of potential endocrine disrupters relevant hormonal mechanisms for both invertebrates and vertebrates need to be studied. Invertebrates account for roughly 95% of all animals (Barnes, 1980), yet surprisingly little effort has been invested to understand their value in signaling potential environmental endocrine disruption. Since the hormones produced and used in invertebrates are different from those of vertebrates, it is essential to incorporate invertebrate-specific hormone-regulated endpoints in studies aimed at evaluating potential endocrine disruption.

Mysid crustaceans have been traditionally used in standard marine/estuarine toxicity testing because of their ecological importance, wide geographic distribution, year-round availability in the field, ease of transportation, ability to be cultured in the laboratory, and sensitivity to contaminants. In addition, mysids have been proposed as potential test organisms for the regulatory screening and testing of endocrine disruptors by several agencies such as USEPA, OECD and the Ministry of the Environment of Japan (Verslycke et al., 2004a).

Molting is regulated by a multihormonal system, but is under the immediate control of molt-promoting steroid hormones, the ecdysteroids, secreted by the Y-organ (Fig. 3.1). The Y-organ secretes ecdysone which upon release in the hemolymph is converted into active 20-hydroxyecdysone. Ecdysteroids also play a fundamental role in the control of reproduction and embryogenesis (Subramoniam, 2000). One major advantage of using ecdysteroid metabolism as an endpoint is that it provides a means for evaluating the impact of environmental chemicals on crustaceans (and potentially other arthropods); chemicals which may not necessarily affect vertebrates (Verslycke et al., 2004a). Juvenile hormones regulate metamorphosis and reproduction in insects. With the discovery of the chemical structure of insect juvenile hormone in 1967 (Roller et al., 1967), attempts were made to produce synthetic analogs for use as “third generation” insecticides (Williams, 1956). Methoprene is such an insecticide which acts as a juvenile hormone analog and disrupts normal development of insects by inhibiting developing pupae from molting and passing into the adult stage. Methoprene is one of the most widely used and succesful insect growth regulators. One of the main applications of methoprene is mosquito control. Methoprene can enter estuarine environments by either direct application for controling aquatic-borne pests or indirectly through land-drainage or erosion from adjacent pesticide-treated agricultural lands (Dhadialla et al, 1998; Retnakaran et al., 1985). Methoprene degrades rapidly in sunlight (Quistad et al., 1975) and in water (Schaefer and Dupras, 1973). Methoprene may have broken down during

the bioassay, but methoprene breakdown products are also known to be bioactive (Harmon et al., 1995; LaClair et al., 1998). It was beyond the scope of this study to determine whether the effects observed were mediated by methoprene itself or by its breakdown products such as methoprenic acid. The use of methoprene at recommended application rates is expected to result in environmental concentrations of ~10 µg/l (Ingersoll et al., 1999). Methoprene concentrations in natural water of the US ranged from 0.39 to 8.8 µg/l (Knuth, 1989), which is in the concentration range where laboratory effects were observed on endocrine regulated processes in crustaceans (McKenney and Celestrial, 1996; McKenney and Matthews, 1990; Peterson et al., 2001). However, USEPA has not reported any specific ecological effects indicating a significant risk associated with methoprene (USEPA, 2001).

Similarities between the endocrinology of molting in crustaceans and insects led to the discovery of a crustacean analog (methyl farnesoate, the unepoxidated form of juvenile hormone III) to the insect juvenile hormone. Figure 5.1 represents the chemical structures of juvenile hormone III, methyl farnesoate and methoprene.

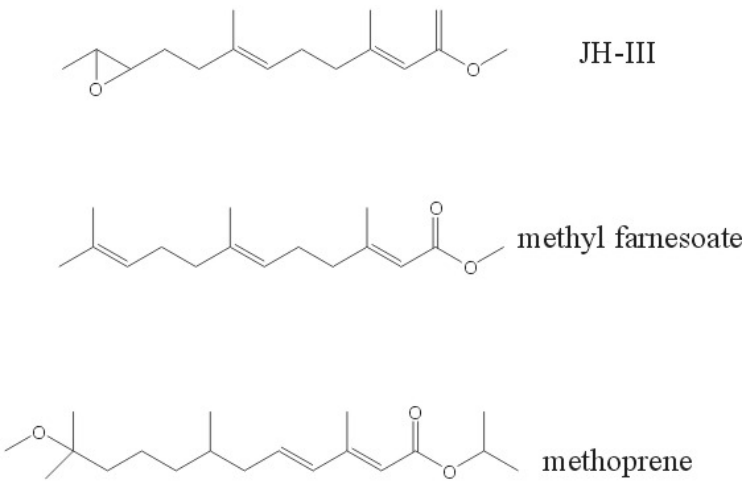


**Figure 5.1:** Chemical structures of juvenile hormone III (JH-III) present in insects, methyl farnesoate in crustaceans and the juvenile hormone analog methoprene.

We previously developed assays to evaluate chemical effects on steroid and energy metabolism in *Neomysis integer* (Verslycke et al., 2002; Verslycke and Janssen, 2002). The purpose of this research is to evaluate molting of *N. integer* as invertebrate-specific endpoint. To this end, we exposed *N. integer* to the test compound methoprene. Methoprene has been shown to reduce mysid fecundity (McKenney and Celestrial, 1996), interfere with juvenile crustacean development (Celestrial and McKenney, 1994; McKenney and Matthews, 1990;

Olmstead and LeBlanc, 2001; Templeton and Laufer, 1983) and act as an anti-ecdysteroid in daphnids (Mu and LeBlanc, 2004). In a recent study, Mu and LeBlanc (2004) demonstrated that juvenile hormones - and their chemical analogues - interfere with normal ecdysteroid signaling in daphnids, probably via a receptor-based process. Although other crustaceans most likely have similar cross talk between juvenoid and ecdysteroid signaling pathways, this has not been studied yet in mysids. We performed a co-exposure using the juvenile hormone analog methoprene and the active ecdysteroid 20-hydroxyecdsyone.

## 5.2. MATERIAL AND METHODS

### *5.2.1. Chemicals*

Methoprene (CAS # 40596-69-8) and 20-hydroxyecdysone were obtained from Sigma-Aldrich (Bornem, Belgium). Stock solutions of methoprene and 20-hydroxyecdysone were prepared in absolute ethanol and stored in a dark refrigerator. The ethanol concentration in the solvent control and in the different test concentrations was 0.01%.

### *5.2.2. Test organisms*

The mysid crustacean, *Neomysis integer*, was collected by handnet in the Braakman, a brackish water (10 psu) near the Schelde estuary in Hoek (The Netherlands). The mysids were cultured in the laboratory as described in Chapter 2 (§ 2.2.1.).

### *5.2.3. Chronic toxicity test*

Gravid females were collected from the culture and individually transferred to aquaria. The aquaria were examined daily for newly released juveniles. Juveniles <24h old were placed individually in 80 ml glass recipients containing 50 ml of the desired test concentration at a salinity of 5 psu and a temperature of 15°C. The juveniles were randomly distributed between the different test vessels containing 0-0.01-1-100 µg methoprene/l and 100 µg methoprene/l + 0, 24, 77, 240 mg/l 20-hydroxyecdysone (=0, 0.05, 0.16, 0.5 µM 20-hydroxyecdysone). These concentrations are based on previous studies with *N. integer* (Verslycke et al., 2004c) and *Daphnia magna* (Mu and LeBlanc, 2002). All concentrations reported in this study are nominal, based on dilutions of the stock solutions. Exposure lasted 5 molts (~3 weeks) and 15

replicates per concentration were used. Exposure solutions were renewed every 48h and juveniles were fed daily with 24- to 48h-old *Artemia* nauplii *ad libitum*. Daily, dead food was removed and molts were stored in 4% formaldehyde for subsequent growth measurements.

### *5.2.4. Growth and Molting*

Toxicological endpoints include time (days) between two successive molts (intermolt period; IMP), and length incresase (growth rate, µm/day) during IMPs. The standard length of *N. integer* or the distance from the base of the eyestalks to the posterior end of the last abdominal segment (Fig. 5.2), cannot be measured directly on the exuvia since the molt is too fragile and easily brakes during manipulation. Therefore, well-defined rigid parts of the molts were measured using conventional light microscopy (Fig. 5.2). Preferably, the length of the exopodites of the uropod (EXO) were used. The standard length (SL) can subsequently be calculated from the exopodite length (EXO) using the linear regression: SL (mm)= 1.085566 + 4.081793 * EXO(mm); $R^2$= 0.9569, n=97 (Fockedey et al., 2005b).

### *5.2.5. Statistics*

All data were checked for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene's test respectively, with an $\alpha = 0.05$. The effect of the treatment was tested for significance using a one-way analysis of variance (Dunnett's test; Statistica™, Statsoft, Tulsa, OK, USA). All box-plots were created with Statistica™ and show the mean (small square), standard error (box), and the standard deviation (whisker).

## 5.3. RESULTS

In a preliminary study, we exposed subadults (average length 7 mm) to the test compound methoprene (0.01, 1, 100 µg/l) over the course of 5 molts (data not shown). Because of the high individual varability in mysid subadult intermolt period (IMP) and growth rate (GR), we decided to work with freshly released juveniles (<24h) to minimize individual variability. The duration of the first intermolt stage was equal for all animals of the same brood and occured 3 to 4 days after release from the marsupium (Fockedey et al., 2005b). Animals of the same brood were randomly distributed over the different exposure treatments which significantly

decreased the individual varability of the IMP and GR as compared to the preliminary study with subadults.

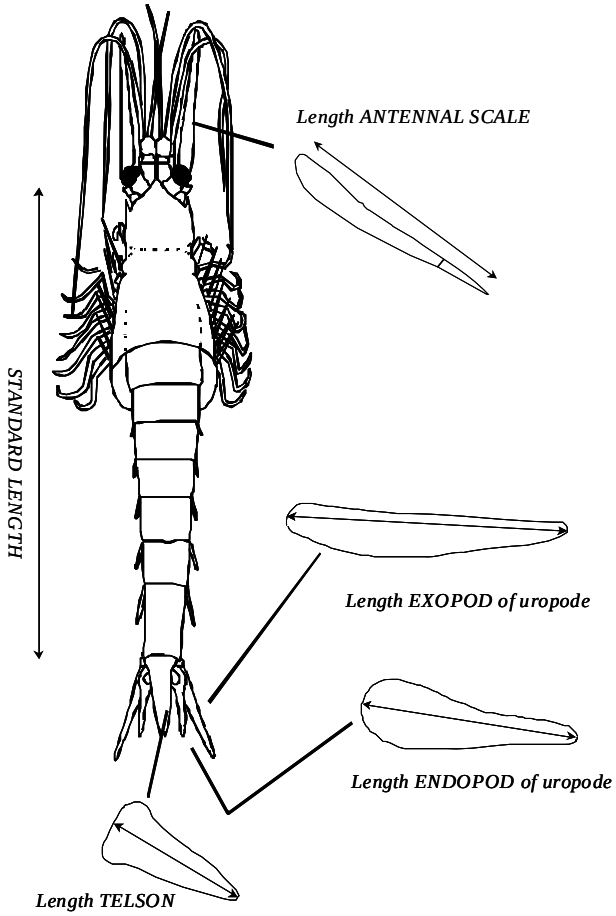


**Figure 5.2:** Schematic representation of *Neomysis integer* with indication of the rigid parts of the molts measured in order to calculate the standard length: length of antennal scale, length of endopod and exopod of the uropod and telson length (Fockedey, 2005).

### *5.3.1. Effect of methoprene on mysid intermolt period (IMP)*

Figure 5.3A shows the effect of methoprene on the IMP during five successive molts. Generally, the growth of *N. integer* is characterized by successively increasing IMPs (Fockedey et al., 2005b). In the controls, the first IMP (1-2) takes 3.4 ± 0.63 days on average, whereas the last IMP (4-5) takes about 4.8 ± 1.12 days. Except for IMP(4-5), all the IMPs were significantly longer in the highest exposure concentration (100 µg methoprene/l)

compared to the respective controls. Although the first three IMPs appeared to be longer in the 1 µg methoprene/l treatment, these differences were not statistically significant. Only the third IMP (3-4) was significantly longer in *N. integer* exposed to 0.01 µg methoprene/l.

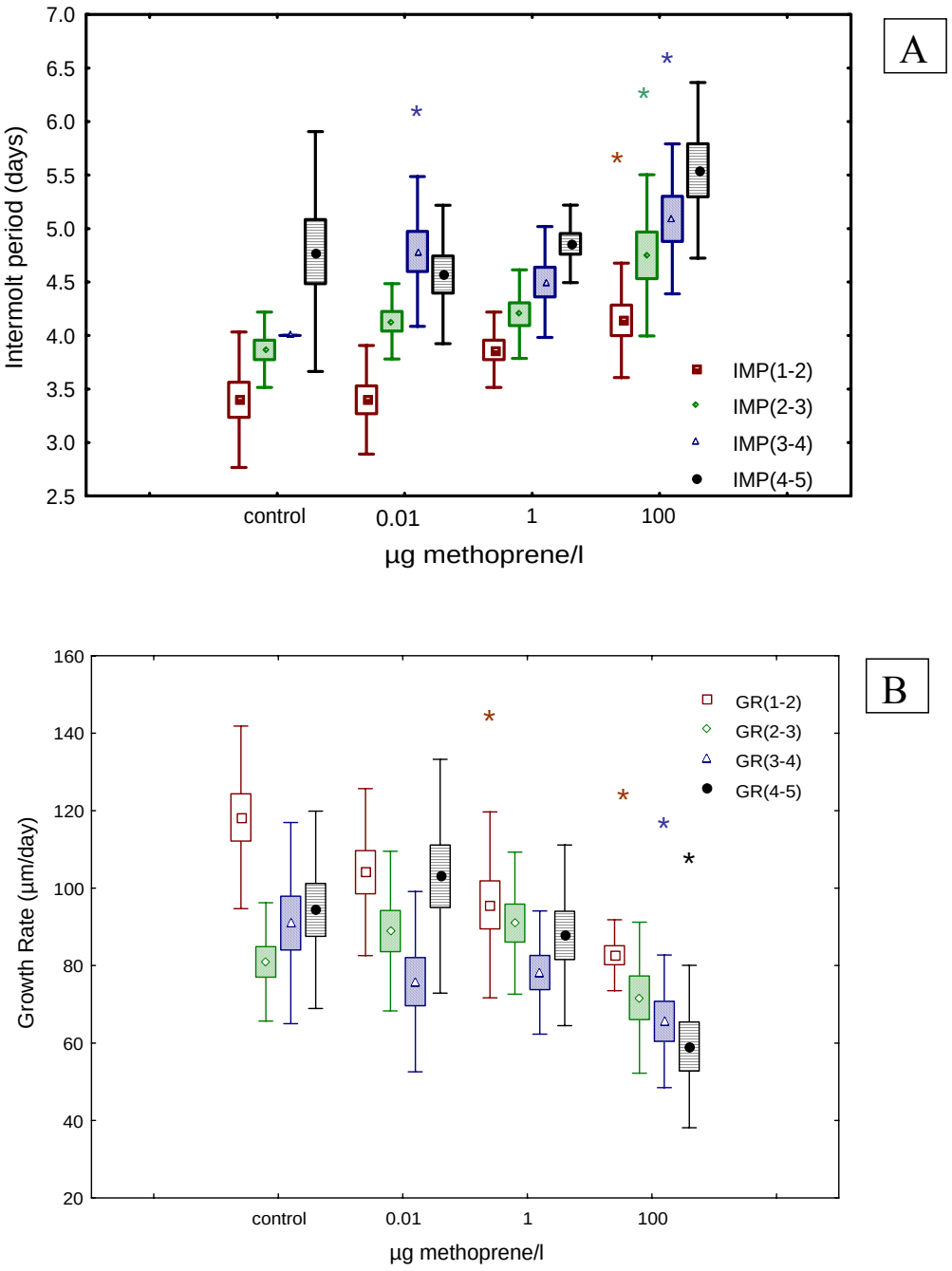


**Figure 5.3:** Effect of methoprene on A) intermolt periods (IMP) and B) growth rates on five successive molts of *Neomysis integer*. * significantly different from control (Dunnett's; $p<0.05$).

*5.3.2. Effect of methoprene on mysid growth rate*

Figure 5.3B shows the effect of methoprene on mysid growth rate during the first five molts. Generally, mysid growth rate is highest during the first molt GR (1-2) and subsequently decreases (Fockedey et al., 2005b). Significant effects were seen on mysid growth rate of juveniles exposed to 100 µg methoprene/l for all molts (GR (1-2), (3-4), (4-5)), except the second GR (2-3). Exposure to 1 µg methoprene/l reduced the growth rate at the first molt only. When growth rate is calculated as total growth (µm) over the total exposure time (day), a significant decrease is found in the 100 µg methoprene/l treatment (data not shown).

*5.3.3. Effect of methoprene on mysid wet weight*

After the fifth molt, all organisms were weighed. Figure 5.4 shows the effect of methoprene on mysid wet weight. There was a significant decrease in wet weight at the highest exposure concentration compared to control animals. The average wet weight of control organisms was 1.43 ± 0.32 mg, almost double of organisms in the 100 µg methoprene/l treatment (average wet weight of 0.75 ± 0.17 mg).

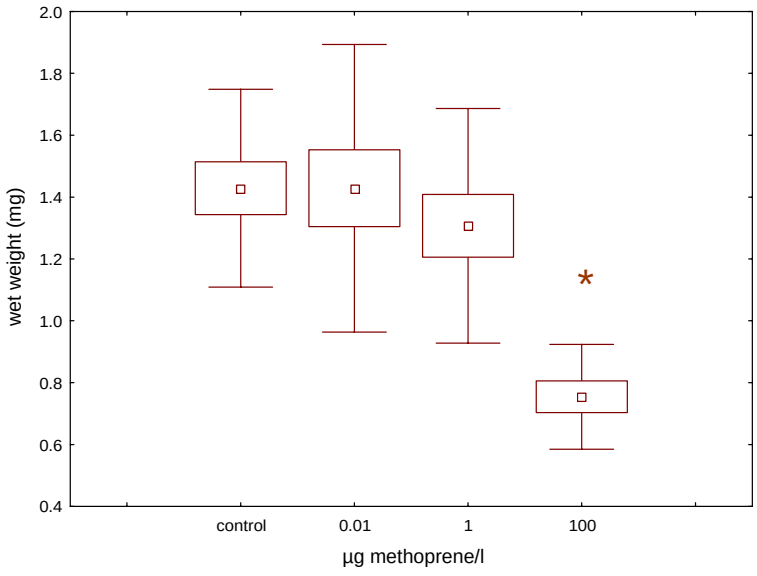


**Figure 5.4:** Wet weigth of *Neomysis integer* after the fifth molt, following exposure to methoprene.

*5.3.4. Combined effects of methoprene and 20-hydroxyecdysone*

At 100 µg/l methoprene significantly reduced mysid growth rate by delaying the IMPs (Fig. 5.3). To further investigate the anti-ecdysteroidal effects of methoprene, mysids were co-exposed to the active ecdysteroid, 20-hydroxyecdysone, to establish whether the observed methoprene effect (IMP delay and decreased growth rate) could be mitigated. Figure 5.5 shows the growth rate expressed as total growth (µm) during the total exposure time (day) to 100 µg methoprene/l and increasing concentrations of 20-hydroxyecdysone (0.05, 0.16 and 0.5 µM 20E). 20-hydroxyecdysone did not mitigate the putative anti-ecdysteroidal effects on growth rate caused by methoprene. The effects of methoprene on mysid growth reduction were confirmed in this second study.

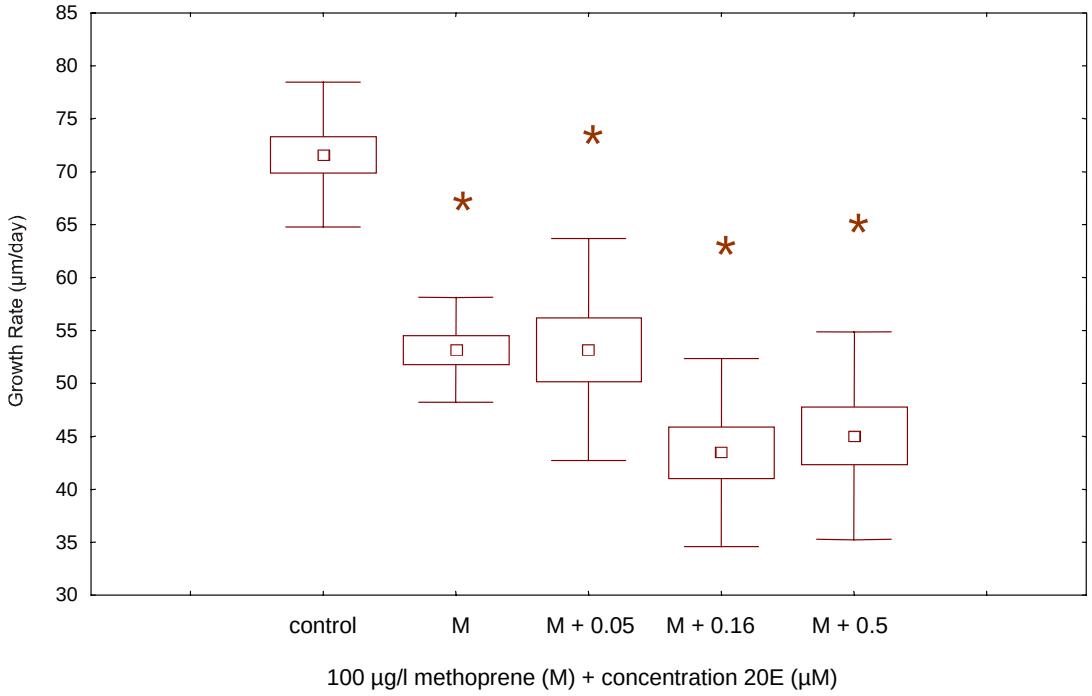


**Figure 5.5:** Growth rate expressed as total growth over the total exposure time (in µm.day$^{-1}$), following exposure to 100 µg methoprene/l (M) spiked with increased concentrations of 20-hydroxyecdysone (0.05, 0.16 and 0.5 µM 20E).

## 5.4. DISCUSSION

Ecdysteroids (molting hormones) and juvenoids (juvenile hormones) represent two classes of hormones in arthropods that regulate many aspects of their development, growth, and reproduction. Therefore, chemicals that disrupt normal ecdysteroid/juvenoid signaling could have profound effects on many aspects of invertebrate function. During their development, insects undergo changes at specific times (such as pupation) which are mediated by endogenous hormones. The active molting hormone 20-hydroxyecdysone, triggers larva-to-larva molts as long as the juvenile hormone is present. In its absence, ecdysone promotes the pupa-to-adult molt. Thus, juvenile hormone present at specific times during insect development leads to normal metamorphosis, however, if present at other times it will lead to morphogenetic abnormalities. This is the basic theory behind the use of methoprene and other juvenile hormone analogues (e.g. pyriproxyfen and fenoxycarb) as insect growth regulators (Dhadialla et al., 1998; Hoffmann and Lorenz, 1998). Methoprene is therefore not directly toxic to insects, but as it disrupts the development of the insect it causes death or reproductive failure at a specific time during the insect life-cycle.

A large portion of the aquatic fauna are crustaceans, making the group important for assessing the non-target effects of many pesticides - such as the mosquitocidal agent methoprene - that end up in aquatic ecosystems (McKenney and Celestrial, 1996; Olmstead and LeBlanc, 2001; Peterson et al., 2001; Templeton and Laufer, 1983). As the potential invertebrate-specific endocrine-disruptive effects of chemicals to non-target organisms are presently not specifically addressed in regulatory screening and testing programs, this could lead to significant underestimations of the actual environmental risk of these chemicals.

While growth through molting of *Neomysis integer* has been described in the laboratory (Astthorsson and Ralph, 1984; Fockedey et al., 2005b; Winkler and Greve, 2002), its disruption by chemicals through specific hormone-regulated mechanisms has not been studied. Methoprene effects on growth of *N. integer* were observed after the first molt, which should therefore allow the use of shorter exposure periods in future studies. Methoprene is acutely toxic (96h) to *Neomysis integer* at 320 µg/l (Verslycke et al., 2004c) and to *Americamysis bahia* at 125 µg/l (McKenney and Celestrial, 1996). McKenney and Celestrial (1996) examined the influence of methoprene on survival, growth and reproduction of *A. bahia* during a complete life cycle, from one-day-old juvenile through juvenile growth and maturation and production of young as an adult. The most sensitive response was a significant reduction in the number of young produced per female at concentrations ≥ 2 µg/l. The mysids

weighed significantly less at exposure concentration of 62 µg methoprene/l as compared to the controls, which is in the same range as what we found in this study (*N. integer* weighed significantly less at 100 µg/l). Our results also corroborate effect concentrations reported for other non-target crustaceans. Methoprene significantly reduced completion of larval metamorphosis in the estuarine grass shrimp *Palaemonetes pugio* at a concentration of 100 µg/l (McKenney and Matthews, 1990). Methoprene adversely affected molting and reproduction in *Daphnia magna* at concentrations higher than 30 nM (~10 µg/l) (Olmstead and LeBlanc, 2001). Recently, we found that methoprene adversely affects the energy and steroid metabolism of *N. integer* at 100 µg/l (Verslycke et al., 2004c). Recently, we also tested the effect of ecdysone agonists, the bisacylhydrazines tebufenozide, halofenozide and methoxyfenozide on the molting of the non-target organism *Neomysis integer* and found that halofenozide and tebufenozide inhibited growth at 1mg/l and 0.1 mg/l, respectively (Soin et al., in preparation).

The present study demonstrates that methoprene significantly affects mysid molting and growth at sublethal concentrations. However, previously reported methoprene effect on mysid reproduction were noted at lower concentrations (McKenney and Celestrial, 1996). As juvenoids and ecdysteroids play a crucial role in the regulation of mysid growth, reproduction and development, comparative approaches that look at a range of ecdysteroid/juvenoid regulated processes in crustaceans should be informative in selecting which endpoints are most sensitive. In addition, measuring the hormones and receptors involved in mysid ecdysteroid/juvenoid signaling will provide insights into the mode-of-action of juvenile hormone analogues and other pesticides in non-target arthropods and how this compares to what is known in insects. In an effort to improve our understanding of ecdysteroid/juvenoid signaling in mysids, we have recently developed assays to study mysid vitellogenesis (Ghekiere et al, 2005; Chapter 3), embryonic development (Fockedey et al., 2005a), ecdysteroid receptor interaction (Verslycke, personal communication) and are validating these assays in exposure studies with methoprene and other pesticides (Chapters 4,6).

Although the ecdysteroid hormone 20-hydroxyecdysone acts as a EcR ligand and activates transcription through EcR/USP heterodimers, the activity of juvenoids and juvenile hormone-analogs such as methoprene remains unclear. Recently, Maki and co-workers (Maki et al., 2004) have demonstrated that JH III- and methoprenic acid-bound USP markedly repressed ecdysone-dependent EcR transcription.

In the second part of the present study, we evaluated the anti-ecdysteroidal activity of methoprene in *N. integer* by exogenously administrating 20-hydroxyecdysone. We found no

mitigation of the inhibiting effect on growth. This could indicate that methoprene did not exert its effect through ecdysteroid receptor antagonism. Mu and LeBlanc (2002) demonstrated that testosterone had an anti-ecdysteroidal activity in *Daphnia magna* by delaying the molt frequency and this effect was mitigated by co-exposure to 20-hydroxyecdysone. They proposed ecdysteroid receptor antagonism as one possible mechanism by which testosterone caused these effects. We previously tested the effect of 20-hydroxyecdysone on molting of *N. integer* and found no effects on the molting frequency. Raising the concentration to $10^{-5}$ M was associated with premature death caused by incomplete ecdysis. These results correlate with the findings of Baldwin and co-workers (Baldwin et al., 2001). Although recent studies with daphnids indicate that juvenoids modulate ecdysteroid signaling through a mechanism that may involve reduced availability of the receptor partner protein ultraspiracle (the ecdysone receptor is functional only as a heterodimer with ultraspiracle), the exact mechanism of action of juvenoids and methoprene remains unclear (Mu and LeBlanc, 2004). In this respect, the increasing availability of sequences for the different receptors involved in crustacean ecdysteroid/juvenoid signaling may be very valuable. Recently, the EcR and USP has been isolated from the fiddler crab *Uca pugilator* and the mysid *Americamysis bahia* (Chung et al., 1998, Yokota et al., 2005). The deduced amino acid sequences of both EcR and USP share 40-60% homology with insect counterparts.

The endocrine system of an invertebrate differs from that of a vertebrate organism both in the type of endocrine glands present and in the chemical structure of specific hormones that are produced. As such, assessing the impact of endocrine disrupting chemicals on invertebrates, requires an approach that is specifically directed at invertebrates. In this context, we are exploring a range of endocrine-regulated processes in invertebrates that could be specifically disrupted by chemicals. This approach should lead to both a better understanding of hormone regulation and its disruption by chemicals in invertebrates.