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

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CHAPTER 6

MARSUPIAL DEVELOPMENT OF *NEOMYSIS INTEGER* TO EVALUATE THE EFFECTS OF ENVIRONMENTAL CHEMICALS

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MARSUPIAL DEVELOPMENT OF *NEOMYSIS INTEGER* TO EVALUATE THE EFFECTS OF ENVIRONMENTAL CHEMICALS

ABSTRACT -----

Embryonic development is a crucial time window within an organism's life history. Relatively few studies have focused on understanding the potential effects of endocrine disruptors on embryogenesis in invertebrates. Mysids (Crustacea: Mysidacea) have been used extensively in regulatory toxicity testing and it is the only invertebrate model currently included in the USEPA's Endocrine Disruptor Screening and Testing Program. We developed a method to study mysid marsupial development of the embryos until the release of free-living juveniles. This method was used to evaluate the potential effects of the insecticide methoprene, a juvenile hormone analog, on mysid embryogenesis. Embryos were exposed to the nominal concentrations 0.01, 1 and 100 µg methoprene/l. Average percentage survival, hatching success, total development time and duration of each developmental stage were analyzed. Embryos exposed to 1 and 100 µg methoprene/l had a significantly lower hatching success and lower survival rates. Our study indicates that *in vitro* embryogenesis can be used as a valuable tool to study the effects of endocrine disruptors in mysids.

6.1. INTRODUCTION

The occurrence of endocrine disruptors in the environment and their potential effects on wildlife species is receiving increased public attention. Although invertebrates account for roughly 95% of all animals, little research has been performed to understand effects of endocrine disruptors on these organisms, compared to the vertebrates. Hormones involved in growth, development and reproduction differ between vertebrates and invertebrates (Chang,

1997b; Charmantier et al., 1997; Huberman, 2000; Hutchinson, 2002; Subramoniam, 2000; USEPA, 2002; Verslycke et al., 2004a). There is a need to develop sensitive and relevant assays that evaluate endocrine toxicity in invertebrates, based on their unique signaling pathways. Unfortunately, knowledge of endocrine-regulated processes and their potential disruption by chemicals in invertebrates is limited. The relatively large body of information on arthropod endocrinology makes insects and crustaceans good models for evaluating chemically-induced endocrine disruption. Recent studies have highlighted the need for more studies on the impact of endocrine disruptors on the reproduction and development of estuarine invertebrates (Lawrence and Poulter, 2001). Mysid crustaceans have been proposed by several regulatory bodies (e.g. USEPA, OECD) as suitable test organisms to evaluate the potential effects of environmental endocrine disruptors (Verslycke et al., 2004a).

Ecdysteroids (molting hormones) and juvenoids (juvenile hormones) represent two classes of hormones in arthropods that regulate many aspects of their development, growth and reproduction. However, the regulation, function and potential chemical disruption of ecdysteroid/juvenoid-regulated processes in crustaceans remains largely unknown. Increased understanding of the endocrine system of insects has led to the introduction of insecticides known as insect growth regulators, with the largest group being juvenile hormone analogues (Keeley et al., 1990; Riddiford, 1994). These insect growth regulators elicit highly specific effects on target insects based on their ability to interact with insect hormone receptors (Dhadialla et al., 1998). As ecdysteroid-like and juvenoid-like compounds function in crustaceans in a manner similar to that seen in insects, they may have a role in the regulation of crustacean reproduction and development (Charmantier et al., 1997; Laufer and Borst, 1988; Laufer et al., 1993; Walker et al., 2005). Consequently, chemicals with ecdysteroid/juvenoid activity are potentially adversely affecting susceptible non-target animals, such as crustaceans (OECD, 2005; Tuberty and McKenney, 2005).

A recent study by McKenney Jr. (2005) found that juvenile mysids released by adults exposed to the juvenile-hormone analog phenoxy carb and reared through maturation without further exposure produced fewer young and had altered sex ratios. This study indicates that pesticides with ecdysteroid/juvenoid activity, may be acting like other EDCs with exposure during developmental periods (in this case during ovarian, embryonic and larval development) producing irreversible reproductive dysfunction in adults. In addition, we have demonstrated the ability of a number of insecticides to disrupt hormone-regulated processes in mysids at very low concentrations, i.e. vitellogenesis (Chapter 4), molting (Chapter 5), and steroid and energy metabolism (Verslycke et al., 2004c). To further explore the potential effects of EDCs

on ecdysteroid/juvenoid-regulated processes in mysids, we developed a mysid embryogenesis assay. In this study, we validated our embryogenesis assay through an exposure experiment with the mysid *Neomysis integer* and the juvenile-hormone analog methoprene. Methoprene is an insect growth regulator that is generally used to control mosquitos. 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 (Celestial and McKenney, 1994; DeFur et al., 1999; McKenney and Celestial, 1996; McKenney and Matthews, 1990; Mu and LeBlanc, 2004; Olmstead and LeBlanc, 2001; Peterson et al., 2001, Templeton and Laufer, 1983; Walker et al., 2005).

Neomysis integer, like all mysids, carries its embryos in a marsupium where the entire embryonic development takes place from oviposition to the release of free-living juveniles (Wittmann, 1984). Unfortunately, studying the embryonic development *in vivo* is difficult due to the semi-transparent oostegites (Fockedey, personal observation) and requires anaesthetization (Irvine et al., 1995). Recently, Fockedey et al. (2005a) developed a methodology to study the *in vitro* embryogenesis of *N. integer* and evaluated the combined effects of temperature and salinity on mysid embryogenesis. A few studies on *in vitro* embryogenesis in mysids and the effects of temperature and salinity were previously published by Greenwood et al. (Greenwood et al., 1989), Johnston et al. (Johnston et al., 1997) and Wortham-Neal and Price (Wortham-Neal and Price, 2002). In addition, some studies have evaluated the effects of endocrine disruptors on embryonic development in other crustaceans (Kast-Hutchenson et al., 2001; Lawrence and Poulter, 2001; LeBlanc et al., 2000). To date, no studies have evaluated embryonic development in mysids as a tool to study the potential effects of endocrine disruptors.

6.2. MATERIAL AND METHODS

6.2.1. Chemicals

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

6.2.2. Experimental animals

The mysid crustacean, *Neomysis integer*, was collected from the dock B3 in the harbor of Antwerp (Belgium), situated at the right bank of the river Scheldt. The dock B3 is in open connection with the river Scheldt through the Berendrecht and Zandvliet sluices. Animals are extracted over a 1x1 mm sieve. Salinity and temperature conditions during the sampling period (weekly from 30th March to 16th April 2004) were 5 psu and 11°C on average. *N. integer* were collected with a handnet (2x2 mm mesh size) and transported within 2 hours after sampling in 15-L bins containing environmental water. The animals were kept in a 16°C climate room for a maximum of 7 days at a concentration of \pm 50 ind/L and at a salinity of 5 psu (made from artificial seawater, Instant Ocean®, Aquarium Systems, France). They were fed *ad libitum* with <24h old *Artemia* nauplii and water was replaced every 2-3 days.

6.2.3. Test design

Non-gravid females with well developed ovaries were selected and placed with 2 adult males in a 400 ml glass beakers filled with 350 ml artificial seawater (5 psu) to allow fertilization. The ovary is situated in the posterior dorsal lateral regions of the thorax and can be easily observed through the carapax (Fig. 6.1). Mature males were distinguished by their elongated 4th pleopods that are stretched to the end of the last abdominal segment (Fig. 1.5). A 12h light: 12h dark photoperiod was used and the water temperature was maintained at 16°C. Daily, excess food (<24h old *Artemia* nauplii), faeces, molts and dead animals were removed. Dead individuals were replaced by new animals and 80% of the medium was renewed and fresh food was added. Mating takes place at night (Mauchline, 1980) and coincides with the molting of the female (Wittmann, 1984). Upon fertilization, gravid females were placed in individual beakers for two more days before removal of their embryos from the marsupium on

day three. Before day three, the embryos are too fragile and removal of the embryos causes damage. Non-fertilized embryos disintegrate within 24h and are not included in the test. After decapitation of the gravid females, the embryos were removed with a fine spatula while submerged in artificial seawater medium (15 psu, 15°C).

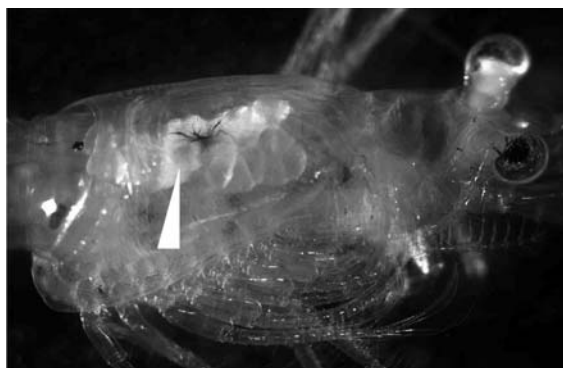


Figure 6.1: Ovary of *Neomysis integer* (Fockedey et al., 2005a). The ripe ovary fills the posterior dorsal lateral regions of the thorax (white arrow).

Using a glass pipette, the embryos were individually transferred at random to each of the wells of a 12-cell plate containing 4 ml of the different exposure concentrations of methoprene (dilution water is artificial seawater of 15 psu). Embryos were exposed to 0-0.01-1-100 μg methoprene/l. All concentrations reported in this study are nominal, based on dilutions of the stock solutions. Twelve replicates per concentration and at least 7 embryos per replica were used. Multiwells were placed on an orbital shaker (80 rpm) and covered from the light. Daily, survival, developmental stage and hatching were recorded, dead embryos were removed, and 75% of the medium was replaced.

6.2.4. Description of the embryology

The intra-marsupial development of *Neomysis integer* was divided into 3 substages in the present study, while generally for mysids a subdivision into 3 to 12 substages is common (de Kruijf, 1977; Mauchline, 1973; Wittmann, 1981b). Table 6.1 and Figure 6.2 summarize the terminology used by the different authors, including the one used in the present study, and applied to the observed morphology in the intra-marsupial development of *Neomysis integer* (with supporting pictures).

The early embryos (stage I) are spherical or sub-spherical (Fig. 6.2a). Rudiments of antennae and abdomen are developing (Fig. 6.2b) and observable under low magnification (25x) as a lighter coloured disk. The abdominal rudiment is ventrally bent and develops anteriorly towards the cephalic appendix. Stage I ends with the hatching from the egg membrane by puncturing it with the developing abdomen. The shed egg membrane quickly disintegrates, but is sometimes visible in the wells.

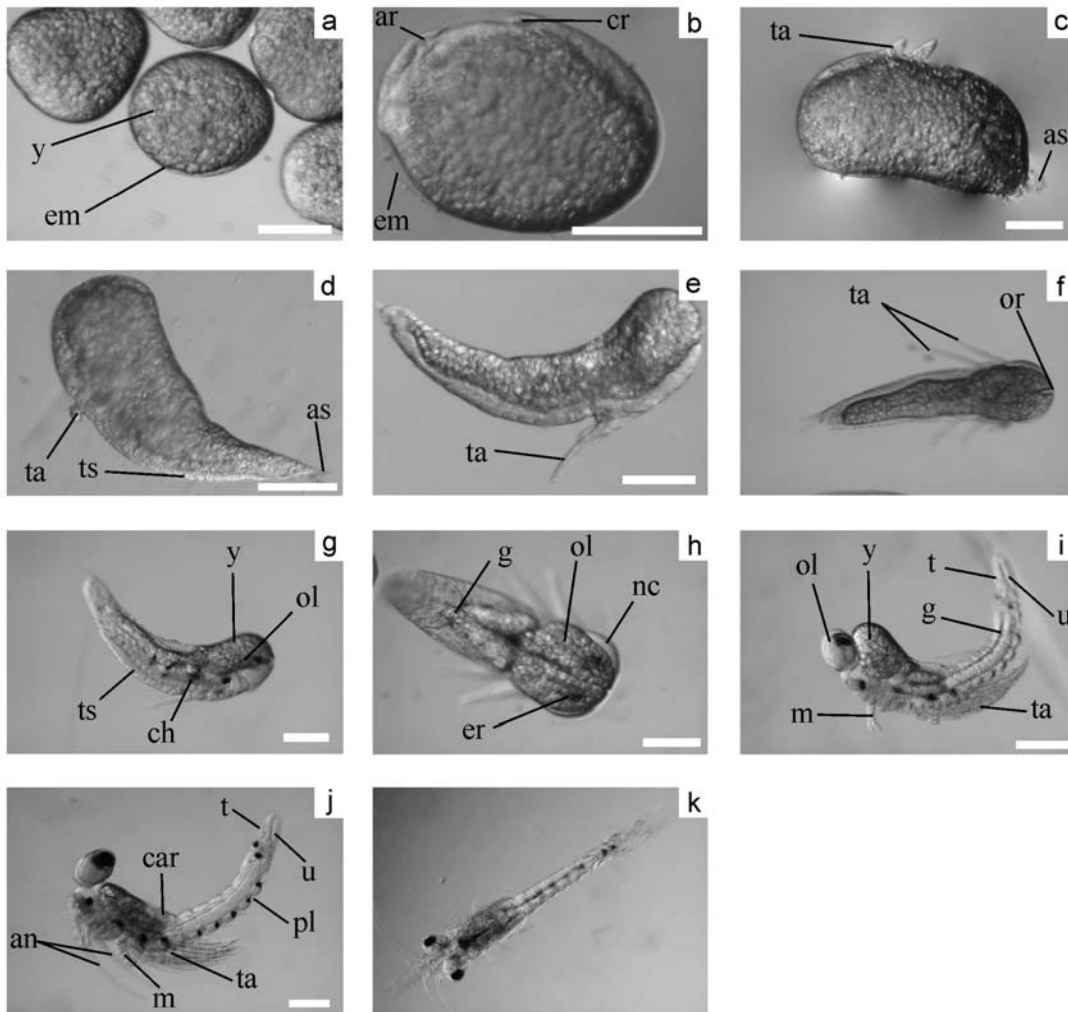


Figure 6.2: Intra-marsupial development of *Neomysis integer* (Fockedey et al., 2005a): stage I (a,b), stage II (c-h), stage III (i,j) and the free-living juvenile (k). (an: antennae; ar: abdominal rudiment; as: abdominal setae; car: carapace; c: naupliar cuticle; cr: cephalic rudiment; ch: thoracic chromatophore; er: eye rudiment; em: egg membrane; g: gut; m: mouth parts; nc: naupliar cuticle; or: optic rudiment; ol: optic lobe; pl: pleopods; t: telson; ta: thoracic appendages; ts: thoracic segmentation; u: uropods; y: yolk granules). Scale bar = 250 μ m.

Table 6.1: Morphological and activity characteristics of the intra-marsupial development of *Neomysis integer* (Fockedey et al., 2005).

Morphology	Yolk	Activity	PRESENT STUDY		
			Mauchline(1973)	De Kruif (1977)	Wittmann (1981)
<ul style="list-style-type: none"> • Egg-like, (sub)spherical (Figure 2a); first 2 days in 2 packages within a tertiary egg membrane 	Yolk granules spread all over embryo	Inactive	stage I = 'egg'	stage I	E1 – half E5
<ul style="list-style-type: none"> • Later: cephalic and abdominal rudiments developing (Figure 2b) 					
<ul style="list-style-type: none"> • Shedding of egg membrane 					
<ul style="list-style-type: none"> • Comma-shaped habitus with rudimentary pointed abdomen clearly distinguished from rounded anterior; appearance of two pair of rudimentary thoracic appendages and abdominal setae (Figure 2c) 	Yolk granules homogeneously spread all over embryo	Inactive	stage II = naupliar stage = 'eyeless' larva	stage II stage III stage IV	Half E5 + N1 to N4
<ul style="list-style-type: none"> • Later: the beginning of abdominal segmentation, without appendages (Figure 2d) 					
<ul style="list-style-type: none"> • Further extension of the body and elongation thoracic appendages (Figure 2e); appearance of cleft at optic rudiment (Figure 2f) 	Yolk migrates dorsally	Inactive			
<ul style="list-style-type: none"> • Development of head; optic lobes with pigmented eye rudiments; rudiments of telson and uropods visible; further segmentation of abdomen; brown chromatophores appearing laterally (Figure 2g and 2h) 	Yolk diminishes and migrates dorsally in the anterior part	Rhythmic contractions of the gut and beating of the heart		stage V	
<ul style="list-style-type: none"> • Moulting from naupliar cuticle 					
<ul style="list-style-type: none"> • Distinct eye projections; development of uropods and pleopods; developing 8 thoracic appendages, mouthparts and antennae; developing carapace and elongated abdomen (Figure 2i and 2j) 	Yolk disappears	Very active flexing and stretching of the body; moving of the appendages	stage III = post-naupliar stage = 'eyed' larva	stage VI	P1 – P3
<ul style="list-style-type: none"> • Moults 					
<ul style="list-style-type: none"> • All (except sexual) characteristics similar to adult (Figure 2k) 	No yolk left; actively feeding	Freely swimming	Juvenile	Juvenile	Juvenile

The stage II larvae are dorsally bent and have a comma-like appearance. Initially, a rudimentary abdomen with a clear distinction between the rounded anterior and the pointed posterior of the larva can be observed together with two thoracic appendages (Fig. 6.2c). In a later phase, the abdomen shows the clear beginning of segmentation, however, without any appearance of appendages (Fig. 6.2d). Later on, the body is further extended and the thoracic appendages more elongated (Fig. 6.2e). The larvae have globules of the yolk protein vitellin within their tissues. These globules are homogeneously distributed throughout the body in stage I embryos and early stage II larvae, but as the yolk volume decreases relative to the body volume, the yolk becomes more concentrated in the anterior dorsal regions at the end of stage II. Dorsally the optical rudiment is visible as an anterior cleft (Fig. 6.2f). As the larva grows, the naupliar cuticle is stretched and the uropods and telson are formed. Eight abdominal segments are clearly visible. Lateral chromatophores appear, mainly in the anterior part (Fig. 6.2g). The optical lobes are visible with pigmented eye rudiments (Fig. 6.2h). A rhythmic beating of the heart and contractions of the gut are visible. The naupliar stage II terminates with the moulting from the naupliar cuticle.

The post-naupliar stage III larvae (Fig. 6.2i) have stalked eyes, a developed telson and uropods without lith in the statocyst of the inner ramus. The thoracic appendages, mouth parts and antennae are developing. All over the body, darkly pigmented chromatophores appear. Near the end of this stage a carapace can be observed (Fig. 6.2j). The larvae are very actively moving by a longitudinal dorsal flexing and stretching of the body. Also an active rhythmic moving of the thoracic appendages is observed. Stage III terminates in a moult, leading to free-living young juveniles (Fig. 6.2k) that are, except for the sexual characteristics, morphologically similar to the adults. The gradually disintegrating yolk is completely consumed.

6.2.5. 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).

6.3. RESULTS

6.3.1. Survival

The percentage embryo survival/day was calculated for each of the exposure concentrations: 0, 0.01, 1 and 100 μg methoprene/l (Fig. 6.3). The highest mortality generally occurred within the first 6 days of embryonic development, i.e. during stage I. Survival did not change from day 7 until day 12, however, survival was lower in organisms exposed to 1 and 100 μg methoprene/l. From day 13 onwards, mysid survival was affected in all methoprene exposures. Average daily survival was $69.4 \pm 22.0\%$ and $70.8 \pm 15.3\%$ for the control and exposure to 0.01 μg methoprene/l, respectively. Exposure to 1 and 100 μg methoprene/l resulted in average daily survival of $55.4 \pm 19.0\%$ and $56.4 \pm 17.9\%$, respectively. Although average survival was affected in a concentration-dependent way, this effects was not significant (one-way ANOVA between the control and any of the treatments ($p=0.090$)). Major hatching occurred on day 15. Due to this hatching we observed a higher variation of survival % on day 16.

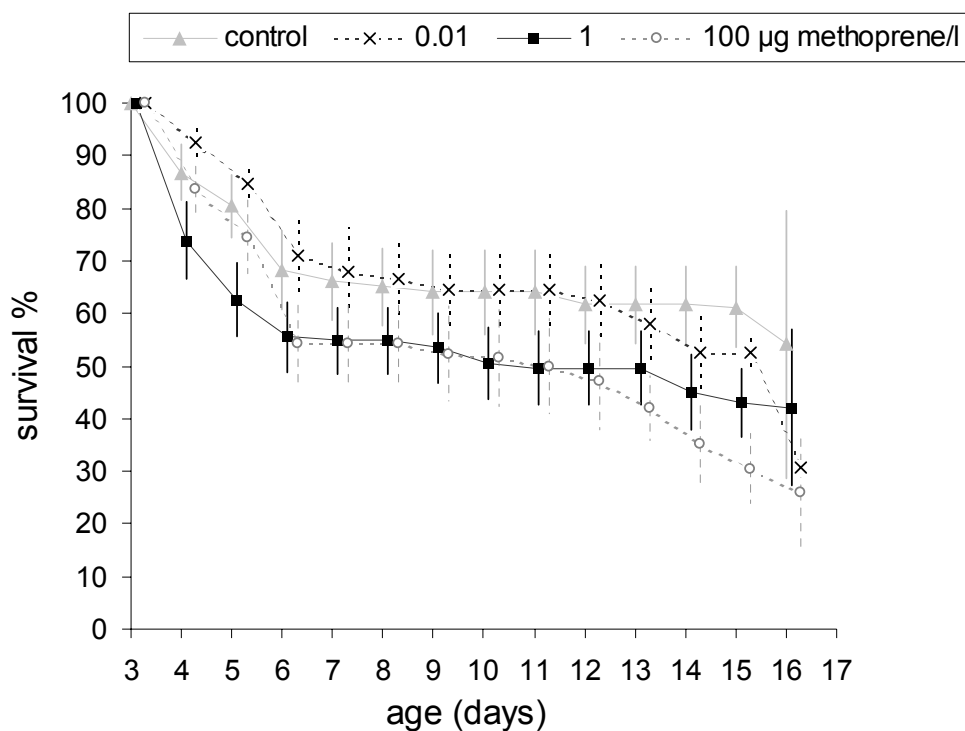


Figure 6.3: Percentage survival of the embryos exposed to 0.01, 1 and 100 μg methoprene/l.

6.3.2. Duration of the different developmental stages

Figure 6.4 shows the duration of the different developmental stages of the embryos exposed to methoprene. The total length of *N. integer* embryonic development was about 15 days and this length was not significantly different between treatments. However, significant effects were seen between treatments on stage-specific length. The duration of the stage I was between 4 and 5 days and not significantly different between treatments. Stage II embryos had a development time between 6 and 7 days and embryos exposed to 0.01, 1 and 100 μg methoprene/L had a significantly longer development time than that of control embryos. Stage III embryos had a development time between 3 and 4 days and embryos exposed to 0.01 μg /L methoprene had a significantly shorter development time than control animals.

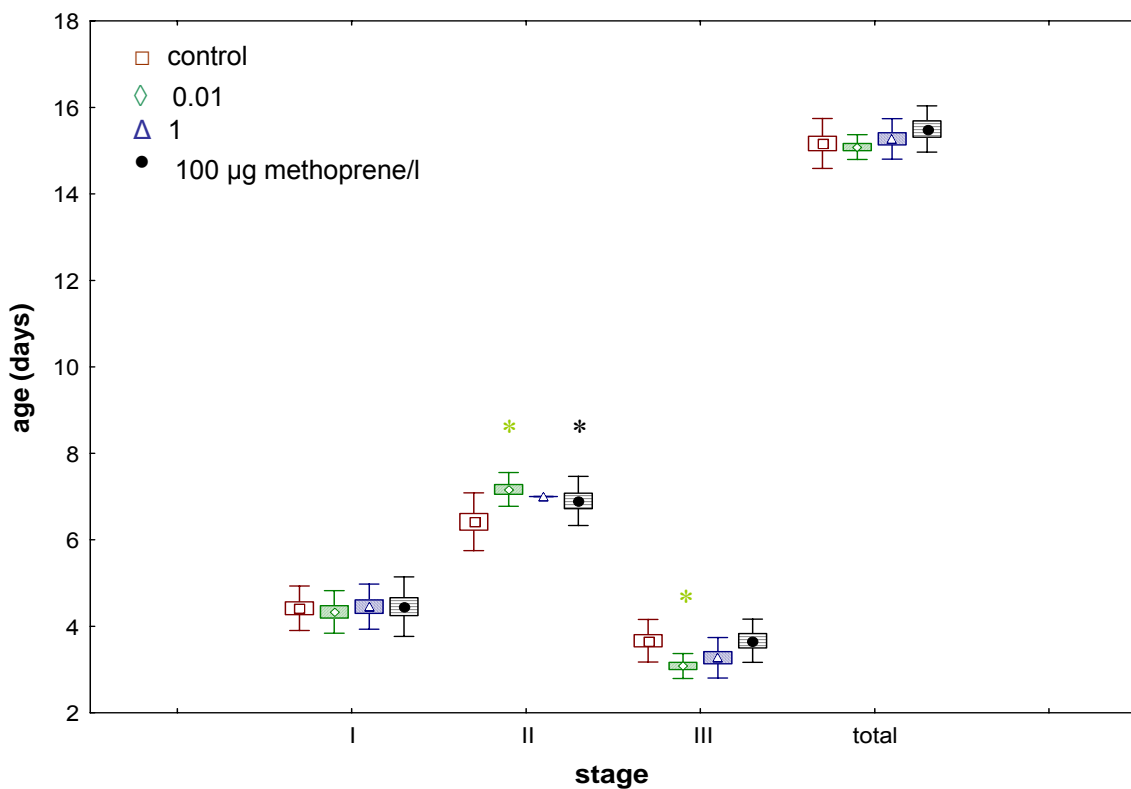


Figure 6.4: Duration of the different developmental stages of embryos (days) exposed to 0.01, 1 and 100 μg methoprene/l. Total= duration of the total development time. (Anova, Dunnett; * $p < 0.05$, significance from control)

6.3.3. Hatching

The most obvious effects of methoprene were seen on mysid embryonic hatching success. The average hatching percentages were 59.7 ± 26.0 %, 47.8 ± 20.6 %, 40.2 ± 23.8 % and 23.3 ± 21.8 % for the control and 0.01, 1 and 100 μg methoprene/l treatments, respectively (Fig. 6.5). Exposure to 1 and 100 μg methoprene/l resulted in significantly less embryos that hatched compared to the control.

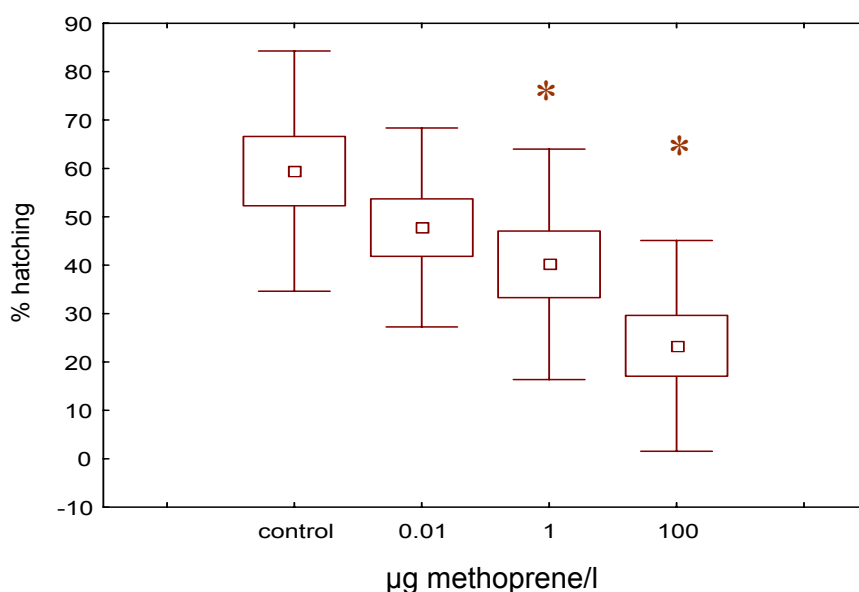


Figure 6.5: Percentage hatching of embryos exposed to 0.01, 1 and 100 μg methoprene/l. (Anova, Dunnett; * $p < 0.05$, significance from control)

6.4. DISCUSSION

Ongoing studies in our laboratory are aimed at understanding the regulatory role of ecdysteroids and juvenoids in mysids, and how this regulation can be chemically disrupted. We have developed several new assays that quantify processes controlled by ecdysteroids and juvenoids in mysids. More specifically, we have developed a quantitative mysid vitellogenesis assay (Chapter 3), as well as a mysid *in vivo* assay to quantify effects on molting (Chapter 5). Using these assays, we were able to demonstrate that the juvenile hormone analogue methoprene significantly affects mysid vitellogenesis and molting

(Chapters 4, 5). In the present study, we describe marsupial development in mysids as a research tool to evaluate potential endocrine toxicity on embryonic development. Given the known sensitivity of this particular life stage in arthropods and many other animals, we expect mysid embryonic development to be a particularly sensitive life stage for the effects of endocrine disruptors. Very few studies have evaluated the effect of endocrine disruptors on the *in vitro* embryogenesis of crustaceans. Indeed, only for the amphipod *Chaetogammarus marinus* (Lawrence and Poulter, 2001) and the cladoceran *Daphnia magna* (Kast-Hutchenson et al., 2001; LeBlanc et al., 2000) have this type of studies been reported.

The marsupial development of 16 mysid species has been previously described (Greenwood et al., 1989; Johnston et al., 1997; Wortham-Neal and Price, 2002). The opaque marsupium of the living female makes it difficult to study embryonic development in mysids (Fockedey et al., 2005a). However, mysid embryos can be removed from the marsupium to allow for an easy assay to evaluate mysid embryonic development. In a previous study, we evaluated the effects of salinity and temperature on the *in vitro* embryogenesis of *Neomysis integer* to determine the optimal abiotic conditions for our assay: a salinity of 15 psu and a temperature of 15°C (Fockedey et al., 2005a). These conditions were used in the present exposure study with the juvenoid methoprene.

Percentage survival, stage duration and hatching were all easily quantified and were affected at different concentrations by methoprene in *N. integer*. In short, methoprene had no effect on the duration of the first stage, prolonged the second stage and shortened the last stage. Interestingly, the mysid embryos molt after stage II, so a possible explanation for the prolonged second stage is that methoprene interferes with the process of molting/metamorphosis in the mysid embryo. An increase in larval development time is a common sublethal response in crustacean larvae exposed to juvenoids (Celestial and McKenney, 1994; McKenney and Celestial, 1996; McKenney and Matthews, 1990; McKenney et al., 2004). In a recent study, we evaluated the effects of methoprene on the molting success of juvenile *N. integer* (< 24h old) through five successive molts. Methoprene delayed juvenile molting at 100 µg/l demonstrating that this chemical can interfere with normal molt success (Chapter 5). Similarly, Lawrence and Poulter (2001) reported that copper, pentachlorophenol, and benzo[a]pyrene only affected specific embryonic stages of the amphipod *C. marinus*. Indeed, stage I was not affected, but stages II to IV -in which the embryo undergoes development of e.g. the germinal disc, dorsal organ rudiments, eye and heart- were all prolonged in the toxicant exposures. Stage V was generally shortened in these studies. These and a growing number of studies are adding to the body of evidence that

juvenoids can cross communicate with the ecdysteroid pathway in ecdysozoans (animals that molt) (Mu and LeBlanc, 2004; Tuberty and McKenney, 2005). The mechanisms involved in the disruption of ecdysteroid/juvenoid signaling in crustaceans remain to be discovered. It is likely that ecdysteroid/juvenoid disruption in crustaceans is caused, at least to a certain extent, by the interaction of insect growth regulators with the nuclear receptors for the endogenous ecdysteroids/juvenoids.

A study by McKenney and Celestial (1996) in which *Americamysis bahia* were exposed during a complete life cycle to methoprene, showed that fecundity was significantly altered at concentrations ≥ 2 $\mu\text{g/l}$. They reported a lower number of young produced per female. This corroborates our findings that methoprene causes lower hatching rates at 1 and 100 $\mu\text{g/L}$ compared to the control. Other studies have focused on comparing responses between crustacean embryos and larvae to juvenoids (McKenney et al., 2004). Concentrations of methoprene ≥ 8 $\mu\text{g/l}$ resulted in significant mortality in larval grass shrimp *Palaemonetes pugio* (McKenney and Celestial, 1993), whereas embryos successfully hatched under exposure to 1 mg/l methoprene (Wirth et al., 2001). While these studies demonstrate the differential sensitivity between life stages in one species, it also shows significant differences in sensitivity to juvenoids between crustacean species. We found that methoprene is acutely toxic to juvenile *N. integer* at 320 $\mu\text{g/l}$ (96h-LC50) (Verslycke et al., 2004c), whereas hatching in this study was affected at 1 $\mu\text{g/l}$.

Finally, we also measured the length of the embryos during this exposure experiment (data not shown). We found no differences in length of the embryos exposed to the different methoprene concentrations.

In conclusion, the juvenoid methoprene is capable of interfering with many aspects of mysid growth, development and reproduction. In the present study, we described the effects of this chemical on embryonic development in *N. integer*. The mysid embryogenesis assay provides a novel and interesting addition to existing and proposed assays for endocrine disruptor testing with mysids. In chronic studies (partial or full life cycle, multigenerational), this assay would focus on the effects during embryonic development and how these are correlated with effects in later life. Such studies would provide important insights into critical time-windows of exposure and the chemical mode-of-action. Finally, mysid embryos are easily recorded through image documentation, and a library of normal and abnormal development could be produced for future reference in toxicity studies.