Mysid crustaceans as standard models for the screening and testing of endocrine-disrupting chemicals

Running title: mysids as a standard screening and testing model

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Abstract

Investigative efforts into the potential endocrine-disrupting effects of chemicals have mainly concentrated on vertebrates, with significantly less attention paid to understanding potential endocrine disruption in the invertebrates. Given that invertebrates account for at least 95% of all known animal species and are critical to ecosystem structure and function, it remains essential to close this gap in knowledge and research. The lack of progress regarding endocrine disruption in invertebrates is still largely due to: (1) our ignorance of mode-of-action, physiological control, and hormone structure and function in invertebrates; (2) lack of a standardized invertebrate assay; (3) the irrelevance to most invertebrates of the proposed activity-based biological indicators for endocrine disruptor exposure (androgen, estrogen and thyroid); (4) limited field studies. Past and ongoing research efforts using the standard invertebrate toxicity test model, the mysid shrimp, have aimed at addressing some of these issues. The present review serves as an update to a previous publication on the use of mysid shrimp for the evaluation of endocrine disruptors (Verslycke et al., 2004a). It summarizes recent investigative efforts that have significantly advanced our understanding of invertebrate-specific endocrine toxicity, population modeling, field studies, and transgeneration standard test development using the mysid model.

Keywords: invertebrate, endocrine disruption, regulatory testing, standardization, *Americamysis bahia*

Introduction

At least 95% (and most likely 99+%, Dirzo and Raven, 2003) of all known animal species lack a backbone, and similar to vertebrates, they regulate physiological processes such as growth, reproduction, and development through hormone signaling. Most invertebrates, however, rely on an entirely different set of hormones than those commonly studied in vertebrate endocrine disruption studies, i.e., the estrogens, androgens, and thyroid hormones. Ecdysozoans account for more than 75% of all known animal species, including the crustaceans, and they rely largely on molting and juvenile hormones to regulate their physiology (Chang, 1993; deFur et al., 1999; Subramoniam, 2000). As such, vertebrate endocrine disruptors (EDs) may have effects in invertebrates other than those anticipated from knowledge of their action in vertebrates (OECD, 2006). An important change in the field of invertebrate endocrine disruption research since the publication by deFur et al. in 1999 has been a genuine acknowledgement of specific differences in hormone regulatory pathways between invertebrates and vertebrates. In our opinion, this has reduced efforts at overselling invertebrate models as well-established, highly standardized, and cheap alternatives to vertebrate test models. By recognizing the uniqueness of chemical signaling in invertebrates, we are moving toward a mechanistic understanding of endocrine toxicity and better ED screening and testing protocols that incorporate this understanding. Future progress in our understanding of invertebrate hormone regulation and its chemical disruption holds great promise given the recent explosion in genomic sequencing and its applications in physiology and toxicology, the latter research field recently referred to as ‘toxicogenomics’ or ‘ecotoxicogenomics’ (Snape et al., 2004).

A key issue that remains is the near absence of invertebrate models in regulatory ED screening and testing programs. While a wide range of invertebrate test models were recommended by experts in the field, including clams, worms, microcrustaceans, grass shrimp, insects, snails, and

echinoderms, among others (deFur et al., 1999), to date, crustaceans remain the almost exclusive invertebrate representatives in proposed regulatory programs for EDs. Crustacean models were generally chosen for their ecological relevance, the availability of well-developed test protocols, their established use in standard toxicity testing, and a comprehensive knowledge of arthropod hormonal systems (mostly derived from insects). Crustaceans will likely continue to dominate the ED regulatory arena, unless non-crustacean models are incorporated in the near future. Of the crustaceans, mysid shrimp have been proposed for the regulatory testing of EDs in the USA, Europe and Japan (deFur et al., 1999). Specifically, mysids were proposed for a two-generation reproductive/developmental toxicity test in the Tier 2 testing (*in vivo* testing) by EPA's Endocrine Disruptor Standardization and Validation Task Force (USEPA, 2002a). Unfortunately, no mysid (or other invertebrate) assay was included in the Tier 1 testing, and the endpoints suggested in the Tier 2 mysid assay encompass processes for which the hormonal regulation and their disruption are still poorly understood.

In 2004, we published a review that summarized the ecological importance of mysids in estuarine and marine ecosystems, their use in toxicity testing and environmental monitoring, and their endocrinology and important hormone-regulated processes to highlight their potential use in assessing environmental endocrine disruption (Verslycke et al., 2004a). The present manuscript serves as an update to the latter review with a focus on recent efforts that have significantly advanced our understanding of invertebrate-specific endocrine toxicity, population modeling, field studies, and transgeneration standard test development in mysids.

Mysid biology, ecology, phylogeny, and ecophysiology

Mysids are relatively small (1-2 cm on average) shrimp-like crustaceans that are characterized by a marsupium within which the entire larval development takes place (*cfr*. opossum shrimp).

Mysids are widespread over all continents and occur in various aquatic environments, including freshwater, groundwater, brackish, estuarine, coastal and oceanic habitats (Mauchline, 1980). Regarded as omnivores, mysids feed on phytoplankton, zooplankton, detritus, and carrion (Mauchline, 1980; Roast et al., 2004), and they form important links in the food webs of aquatic ecosystems (Mauchline, 1980; Mees and Jones, 1998).

Mysids are peracarids (a large group of malacostracan crustaceans) whose systematics and phylogeny remain uncertain (Spears et al., 2005). Mysid phylogeny, particularly their phylogenetic relationship to amphipods and isopods, is subject of ongoing discussion (Remerie et al., 2004; Audzijonyte et al., 2005a). Presently, more than 1000 mysid species belonging to approximately 170 genera have been described. A comprehensive database on the world mysid fauna (NeMys), containing links to relevant information (i.e. taxonomical, morphological, ecological, biogeographic, literature, pictorial and molecular data) was constructed by T. Deprez (Ghent University, Belgium) and colleagues (online at www.nemys.ugent.be).

Mysids inhabit an extraordinary range of aquatic environments characterized by both slow and rapid environmental changes (Lejeusne and Chevaldonné, 2005), which requires a highly adaptive and flexible physiology. The influence of prevailing environmental variables (e.g. temperature, salinity, food quality and quantity, photoperiod) on growth, development, and reproduction, and their optimal range have to be known in order to develop optimal laboratory cultures and to differentiate between chemically induced variability and natural variability in toxicity testing. A significant amount of work has been done on the determination of optimal physiological salinity-temperature conditions for mysids (Verslycke et al., 2004a). For example, temperature and salinity were shown to interact and modify the reproductive capacity of *Americamysis bahia* (McKenney, 1996). Fockedey et al. (2005) recently described the effects of different environmentally-relevant salinity and temperature ranges on growth, survival, sexual development and maturity of *Neomysis*

integer over a full life cycle.

Mysids in standard toxicity testing and preferred species for endocrine disruptor testing

Chronic testing protocols for aquatic arthropod species have been developed by several regulatory agencies and include protocols for daphnids, chironomids, amphipods, copepods, and mysids. An excellent review by the OECD (Organization for Economic Co-operation and Development) was published in 2006 on candidate protocols for aquatic arthropod life cycle and two-generation toxicity testing. This review also summarizes strengths and weaknesses of several aquatic arthropod models for ED testing.

Mysids have been used in (regulatory) toxicity testing for more than two decades (Nimmo and Hamaker, 1982; Verslycke et al., 2004a). USEPA (US Environmental Protection Agency) and ASTM (American Society for Testing of Materials) have adopted the sub-tropical *Americamysis* (formerly *Mysidopsis*) *bahia* as a key testing species for coastal and estuarine monitoring, and standard guidelines for life-cycle toxicity testing with this species have been developed (USEPA, 1997, 2002b; ASTM, 1999; OECD, 2006). As such, there is a large amount of published toxicity data for *Americamysis* sp., but also a steadily growing amount of data on the sensitivity of other mysid species to toxicants. The available evidence suggests that mysids are generally more sensitive to toxic substances than many other standard toxicity test species (Hunt et al., 2002; Verslycke et al., 2003b). Toxicity test procedures have been published for *Neomysis mercedis, Mysidopsis intii*, *Holmesimysis costata*, *Americamysis bigelowi*, *Neomysis integer*, *Tenagomysis novaezealandiae*, *Praunus flexuosus*, *Neomysis americana*, *Mesopodopsis slabberi*, and *Neomysis awatschensis* (Table 1). In addition, methods for maintaining viable populations of different mysid species under laboratory conditions have been described by several researchers (Table 1).

In our opinion and in concurrence with a recently published review by OECD (2006), the

preferred mysid species for use in screening and testing of potential EDs should be *A. bahia*. The primary reasons for its selection over other mysid species are the high degree of standardization, its year-round availability, the availability of commercial cultures, the relative ease of maintenance and culture in the laboratory, and its well known biology and test requirements. Furthermore, the potential for full life-cycle (*A. bahia* has a life cycle of about 17-20 days) and multigenerational exposures are other important advantages (McKenney, 2005). Major disadvantages that preclude the general utility of *A. bahia* are a limited relevance for cold water and low salinity (including freshwater) systems. For the purpose of regulatory screening and testing of EDs, however, the advantages strongly outweigh the disadvantages. It should be noted that significant progress has been made in the development of novel invertebrate-specific endpoints for the evaluation of EDs in other mysid species, such as *N. integer* (Verslycke et al., 2004c; Ghekiere et al., 2005, 2006a, 2006b, 2006c). Ideally, these newly developed endpoints (discussed in more detail below) for endocrine toxicity should be validated with *A. bahia*, and potentially added to existing and proposed testing protocols. These validation studies should include interlaboratory comparisons, as well as the use of chemicals chosen for their known mode-of-action in arthropods, e.g., ecdysteroid (ant)agonist, juvenile hormone (ant)agonist (OECD, 2006).

Crustacean endocrinology

Crustaceans use a wide variety of hormones to regulate their growth, development, metabolism, and other physiological processes, similar to vertebrates. Crustaceans (and other arthropods) are the only invertebrates known to have true endocrine glands derived from epithelial tissue and functioning similar to vertebrate glands (deFur, 2004). Oehlmann and Schulte-Oehlmann (2003) and LeBlanc (this issue) published an overview of reported hormones and their known functions in crustaceans, including ecdysteroids, steroids, terpenoids (which include the juvenoids), and

neuropeptides. Ecdysteroids and juvenoids represent two classes of hormones in crustaceans that appear to play crucial roles in the regulation of many aspects of development, growth, and reproduction that are associated with androgens, estrogens and thyroid hormone in the vertebrates (Chang, 1993; deFur et al., 1999; Subramoniam, 2000). As such, any chemical that has the capacity to mimic or interfere with the action of these signaling molecules can be expected to cause significant physiological disruption. This is exemplified by the many effects on non-target arthropods of certain insect growth regulators (a class of pesticides) which are developed to intentionally mimic, block or otherwise interfere with insect ecdysteroid and juvenoid systems (insect growth regulator effects on non-target animals are reviewed by Oehlmann and Schulte-Oehlmann, 2003; Oetken et al., 2004; McKenney, 2005). Unfortunately, basic endocrinological studies using lower crustacean models, such as copepods, amphipods, and mysids, remain extremely limited. Consequently, most studies using these crustacean models continue to rely on the comparatively large body of information derived from studies with insects and higher (decapod) crustaceans.

An overview of potential endpoints to evaluate endocrine disruption

Mysids have been used as laboratory toxicity test models to evaluate a wide range of toxicant effects, such as effects on growth, swimming capacity, feeding behavior, molting, energy budget, reproduction, sexual maturity, steroid metabolism, and vitellogenesis. An overview of these studies (up until June 2003) was published by Verslycke et al. (2004a). Although many of the endpoints shown in Table 2 may indicate a response to an endocrine disruptor, most, if not all, also vary in response to exposure to other stressors and this is further confounded by the interrelatedness (i.e., non-independence) of some of these endpoints (USEPA, 2002a; OECD, 2006). The key to the interpretation and use of these endpoints as indicators of endocrine disruption will be to first

describe what constitutes a 'normal' unstressed response. While trivial, a 'disruption' can only be diagnosed if the normal state is known and this requires a thorough understanding of hormone-regulated processes in mysids, which at present, is still very limited. In addition, the interrelatedness of potential endpoints and differences in endpoint sensitivity underline the need to use multiple endpoints when evaluating endocrine toxicity. In the following paragraphs we present an updated review on existing and novel mysid endpoints that have been used in toxicity studies since the publication of our previous review (Verslycke et al., 2004a). A general overview of indicators of endocrine disruption in crustaceans is also given by LeBlanc in this issue.

Growth and molting

Crustacean growth is characterized by the periodic shedding (molting) and replacement of the exoskeleton. This process is regulated by a multihormonal system, but is under the immediate control of molt-promoting steroid hormones, the ecdysteroids. Ecdysteroids are secreted by ecdysial glands called the Y-organs (Huberman, 2000). During intermolt, Y-organ activity, and thus ecdysteroid release, is negatively controlled by the molt-inhibiting hormone which is produced by the X-organ-sinus gland complex (Zou, 2005). Over the past decade, our understanding of ecdysteroid regulatory action has significantly increased, largely driven by studies with insects for which full genome sequences are increasingly available (Lezzi et al., 1999; Bonneton et al., 2003). Genome sequencing projects are also underway for certain crustacean models (e.g., the cladoceran *Daphnia pulex*), and will hopefully expand over the next decade to include a number of invertebrate models commonly used in standard toxicity testing. In the meantime, based on sequence conservation between crustaceans and insects, recent studies have resulted in the cloning and characterization of a number of genes involved in hormone regulation in crustaceans. The ecdysteroid receptor complex was sequenced and characterized from the fiddler crab *Uca pugilator* and consists of an ecdysteroid receptor that heterodimerizes with a crustacean retinoid X receptor

(also ultraspiracle protein) (Chung et al., 1998). Sequencing efforts by Yokota et al. (2005) and Verslycke et al. (unpublished data) have resulted in cDNA sequences for the ecdysteroid receptor complex from the mysids *A. bahia* and *N. integer*, respectively. In addition, Yokota et al. (2005) developed an *in vitro* binding assay with the ecdysone receptor from *A. bahia* which holds promise as a rapid *in vitro* screen of chemical interaction with the mysid ecdysteroid receptor complex. Future molecular studies could focus on the phylogenetic relationships between mysid and other known ecdysteroid (and other hormone) receptors, functional divergence across species, hormone receptor expression during different life stages to establish function, and chemical interference at the transcriptional level. Such studies should lead to a better mechanistic understanding of hormone regulation in mysids and its potential disruption by chemicals.

Mysid growth has been measured as increase of dry weight or body length per time interval, in terms of intermolt period and growth factor, and has been modeled extensively (Mauchline, 1980, 1985; McKenney and Celestial, 1995; Gorokhova, 1998, 2002; Winkler and Greve, 2002). Exposure experiments have established mysid growth as a sensitive endpoint in toxicological testing (McKenney, 1994; McKenney and Celestial, 1996; Hunt et al., 1997). A recent study by Fockedey et al. (2005) described post-marsupial growth in *N. integer* from first day neonates until adulthood at eight environmentally relevant temperature-salinity conditions. Using a similar growth experiment as Fockedy et al. (2005), Ghekiere and co-authors (2006c) demonstrated that the insecticide methoprene significantly delayed molting in *N. integer* at 100 μg/l following a 3-week exposure. The mysid *in vivo* molting assay as described in the latter studies, while labor-intensive, allows for the *in vivo* evaluation of chemical interaction with mysid molting. Future exposure studies using this assay could be validated with chemicals that were demonstrated to interact with the mysid ecdysteroid receptor complex as determined by *in vitro* assays (e.g., Yokota et al., 2005). It should be noted that growth effects in mysids are likely to have important implications for

development, metamorphosis, and reproductive success since fecundity is related directly to female body size (Winkler and Greve, 2002). Considering the general importance of molting in the life of crustaceans and for the purpose of regulatory screening and testing, it seems necessary to develop screening assays that are useful to evaluate chemical interference with crustacean molting (Zou and Bonvillain, 2004). A major advantage of using molting as an endpoint is that it provides a means to evaluate the impact of EDs on crustaceans (and potentially other molting animals), that might be distinct from effects on vertebrates.

Energy metabolism

While typical and well-studied challenges to endogenous energy metabolism include environmental hypoxia, functional (internal) hypoxia, changing energetic requirements, disturbance to water balance/ion-homeostasis and changes in temperature (reviewed by Morris and Airries, 1998), exposure to toxicants will also result in an energetic challenge. Energy metabolism is hormonally controlled in crustaceans and therefore, by definition, sensitive to hormonal disruption. Alterations to the energy metabolism of mysids have been used successfully to indicate toxicant exposure (McKenney, 1998, Chin et al., 1998; Roast et al., 1999c; Verslycke et al., 2003c). Several methods for measuring such alterations have been used, such as O:N ratios (e.g., McKenney, 1998), C:N ratios (e.g., Gorokhova and Hansson, 2000), scope for growth (e.g., Roast et al., 1999c), and cellular energy allocation (e.g., Verslycke et al., 2004d).

Abiotic stress, including hypoxia, thermal stress, and salinity stress, as well as contaminant exposure have also been shown to affect glucose levels in several crustacean taxa via interference with the synthesis of a neuropeptide called the crustacean hyperglycemic hormone (CHH) (Fingerman et al., 1998; Chang, 2005). Immunoassays have been developed to measure CHH in several decapod crustaceans (Chang, 2005; Chung and Webster, 2005), but no such assays are available for mysids. Recent molecular studies (Chen et al., 2005) have demonstrated that the

amino acid sequence of CHH is highly homologous with the molt-inhibiting hormone (MIH), another product of the sinus glands in crustaceans, indicating possible involvement in the control of molting and reproduction (De Kleijn and Van Herp, 1998). While hormonal control of circulating glucose levels has been called one of the best studied subjects in the field of crustacean endocrinology (Medesani et al., 2004), its value as a biomarker of exposure to EDs in mysids needs development.

Despite having obvious biological relevance and being fairly easily extrapolated to higher levels of biological organization, the major disadvantage of endpoints related to energy metabolism is their difficulty in mechanistically distinguishing hormone-regulated responses from other toxic responses. While many studies contain background information on the biochemical composition (e.g., proteins, lipids, sterols) of mysids (reviewed by McKenney, 1999 and Verslycke et al., 2004a), information on neuropeptide and hormonal control of energy metabolism in mysids needs further development.

Steroid metabolism and cytochrome P450 (CYP)

Sex steroid (estrogens, androgens, and progestins) receptors were long believed to be vertebrate-specific novelties, based on their complete absence from the fully sequenced genomes of insects, nematodes and tunicates. However, recent studies identified estrogen receptor orthologs in the mollusk *Aplysia californica* and the cephalopod *Octopus vulgaris* (Thornton et al., 2003; Keay et al., 2006). These findings indicate that sex steroid receptors are far more ancient than previously thought, with their origin predating the protostome-deuterostome divergence. It also points to the potential presence of sex steroid receptor orthologs in all Bilateria, when not lost during evolution in a specific lineage. There is growing evidence suggesting the presence of a sex steroid-responding system in ecdysozoans and at least some crustaceans have retained features of estrogen-like signaling and of androgenic pathways including corresponding androgen binding sites

(reviewed by Köhler et al. in this issue).

Steroid metabolism and its disruption by chemicals have been studied in several crustaceans (Baldwin et al., 1998; Oberdörster et al., 1998; LeBlanc and McLachlan, 2000; Janer et al., 2005a, 2005b). Two recent comparative studies on androgen metabolism using gastropod, amphipod, and crustacean models demonstrated the ubiquity of some androgen biotransformation processes in invertebrates and also revealed interphyla differences in androgen metabolic pathways with different sensitivity of these pathways to some xenobiotics (Janer et al., 2005a, 2005b). Verslycke et al. (2002) reported testosterone metabolism and the presence of vertebrate-type steroids in *N. integer*, and demonstrated the presence of a complex steroid hydroxylase system consisting of different CYP isozymes. Since then, alterations in testosterone metabolism in *N. integer* following acute exposure to several chemicals have been reported, including tributyltin (Verslycke et al., 2003a), methoprene (Verslycke et al., 2004c), nonylphenol (Verslycke et al., 2004c), and benzo[a]pyrene (Poelmans et al., 2006).

Classically, EDs have been viewed as exerting their effects exclusively by genomic actions, acting as steroid (ant)agonists by binding to the receptor. As well as acting as exogenous steroid mimics, however, EDs may alter the synthesis or availability of endogenous hormones (Waring and Harris, 2005). These chemicals often interfere with the microsomal P-450 monooxygenase system, also called the mixed-function oxygenase (MFO) system. Studies over the last 30 years have established the important role of CYPs in the biotransformation of xenobiotics and endogenous compounds (such as ecdysteroids) in crustaceans (for a review on crustacean CYPs, refer to James and Boyle, 1998). While attention on CYP in aquatic invertebrates has focused primarily on its application in pollution monitoring, the basic characteristics, such as levels, activities, structure and regulation, are relatively unknown in most crustaceans (Solé and Livingstone, 2005). In insect models such as *Drosophila melanogaster* and *Anopheles gambiae*, genome sequencing has recently

allowed the identification of all putative CYPs (Tijet et al., 2001; Ranson et al., 2002) with two families (CYP4 and CYP6) being the most diverse. Few studies have dealt with CYP identification and phylogeny in crustaceans, but the CYP4 family also appears very diverse, and CYP4 expression in daphnids was shown to be related to environmental xenobiotic concentrations (David et al., 2003). Similar molecular approaches should result in insights into the function and expression of CYPs in other crustaceans in the context of endocrine disruption. In addition, *in vivo* metabolic studies with different substrates, such as the testosterone studies discussed above, could provide valuable tools for evaluating the effects of toxicant exposure, particularly when these can be linked with effects on higher levels of biological organization. From the *in vivo* testosterone metabolism studies with *N. integer* by Verslycke et al. (2002, 2003a, 2004c), there is sufficient information to suggest that mysids can be useful models to evaluate potential chemical interaction with CYP-mediated endogenous and exogenous metabolism.

Vitellogenesis, marsupial development and reproductive measurements

Despite the well-established use of mysid reproductive endpoints such as fecundity, egg development time, and time to first brood release in standard toxicity testing, little information exists on the hormonal regulation and basic biology of these processes (Verslycke et al., 2004a). Hormonal control of vitellogenesis in crustaceans is closely linked with the molt cycle (Chang, 1993; deFur et al., 1999). The production of vitellin is under direct control of peptide hormones like the 'vitellogenesis-inhibiting hormone' (VIH) and the 'vitellogenesis-stimulating hormone' (VSH). The complex hormonal regulation of vitellogenesis makes it an excellent model for studying mechanisms of hormone signaling at the cellular and molecular level (Tuberty et al., 2002). An increasing number of studies have evaluated endocrine toxicity to vitellogenesis in crustaceans (Lee and Noone, 1995; Volz and Chandler, 2004; Sanders et al., 2005). Furthermore, vitellin was recently purified from the mysid *N. integer* (Ghekiere et al., 2004) and subsequently a

quantitative enzyme-linked immunosorbent assay was developed (Ghekiere et al., 2005). In a recent study, gravid mysids were acutely (96h) exposed to methoprene, nonylphenol, and estrone (Ghekiere et al., 2006b). Methoprene exposure did not significantly affect mysid vitellogenesis, whereas exposure to nonylphenol significantly induced vitellin levels in the lowest exposure concentration (0.01 µg/l) with no effects at higher test concentrations. Estrone significantly decreased vitellin levels at the highest test concentration (1 µg/l). While these results demonstrate that mysid vitellogenesis can be chemically disrupted, difficulties in the interpretation of the observed chemical-specific and concentration-specific responses highlight the need for a better understanding of hormone regulation of mysid vitellogenesis. Determining sequences of genes involved in mysid vitellogenesis and measuring their expression could lead to a better mechanistic insight. In general, an improved understanding of the role of the different neuropeptides, and the mandibular organ control over molting and vitellogenesis require further study to effectively use mysids and other model crustaceans for ED testing in the future (USEPA, 2002a).

In mysids, embryonic and post-embryonic development occurs in the female marsupium and includes several stages from oviposition to the juvenile stage (Mauchline, 1980; Wittmann, 1984; Greenwood et al., 1989). Recently, protocols were developed to evaluate *in vitro* marsupial development in *A. bahia* (Wortham and Price, 2002) and *N. integer* (Fockedey et al., 2006) and early development in *N. integer* was used to evaluate the effects of methoprene (Ghekiere et al., 2006a). *N. integer* embryos exposed to 1 and 100 µg methoprene/l had a significantly lower hatching success and lower survival compared to control animals. Similar to the popular use of early-life stage testing using vertebrate models, this assay might provide an opportunity to test the potential effects of chemicals on mysid embryogenesis in the future.

There are several other measures of reproductive performance that can be used to assess the sublethal response of mysids. For example, sexual maturity (Khan et al., 1992), intersexuality and

sex determination (McKenney, 2005), time to first brood release (McKenney, 1996), time required for egg development (Gentile et al., 1983), fecundity (Gentile et al., 1982), and alterations in reproductive characteristics in populations have all been used as endpoints (Raimondo and McKenney, 2005a).

Population dynamics

Determining mysid population-level effects that result from altered physiological (growth, metabolism) and organism-level (survival, reproduction) endpoints is critical to assessing the risk of EDs to long-term mysid population viability. Unfortunately, long-term population field studies are typically not feasible, and it is generally difficult to isolate the effects of a toxicant on a field population that is also exposed to natural environmental variation. Population models are useful tools for linking organism-level effects with population-level responses and afford the opportunity to integrate probabilistic approaches into ecological risk assessments (Raimondo and McKenney 2005b).

Mysid models have been developed to link impairments of vital rates, such as survival and reproduction, to long-term mysid population viability (Kuhn et al., 2000, 2001; Raimondo and McKenney, 2005a, 2005b, 2006). Raimondo and McKenney (2005a, 2005b) developed matrix population models from survival and reproduction measured in life table response experiments for population-level risk assessments of methoprene and thiobencarb exposure on *A. bahia*. In addition to determining critical exposure levels at which populations decline in deterministic systems, these models defined the parameters responsible (i.e. reduced reproduction) for the observed effect. Risk assessment of thiobencarb exposure included probabilistic assessment of mysid population viability under various environmental conditions and management scenarios (Raimondo and McKenney, 2005b). Elasticity analyses performed with these models determined that survival of intermediate life stages (approximately 10-16 days old) was the most critical vital rate to mysid population

growth rate and impairments to it would have the most dramatic effect on growth rate (Raimondo and McKenney, 2005a, 2005b).

Mysid models developed for six toxicants showed that despite the relative importance of survival to population growth rate, mysid populations would also be significantly impacted by sublethal effects. In this analysis, reduced reproduction was the primary contributor to reduced population growth rate for most concentrations, emphasizing the importance of altered reproduction in mysid population-level risk assessment (Raimondo and McKenney, 2006).

Previous population modeling efforts by Kuhn et al. (2000, 2001) were aimed at evaluating the ecological relevance of mysid bioassays and population models. Kuhn et al (2000) found high correlations of 96h-LC50 values and toxicant concentrations where populations would begin to decline. These results have also been observed in later mysid population studies by Raimondo and McKenney (2006). Kuhn et al. (2001) confirmed that mysid models generated from laboratory-derived survival and reproduction could predict population projections of mysids in laboratory conditions. The mysid models described here are useful tools for linking the organism-level responses to population viability, yet future research that may provide information to incorporate density dependence and environmental stochasticity into mysid models will improve probabilistic risk assessment of mysid populations.

Morphology and histology

Morphological and histological changes resulting from exposure to EDs have been documented in fish, amphibians, reptiles, mammals, and arthropods (e.g., Gross et al., 2001; Vandenbergh et al., 2003; Schirling et al., 2006). In mysids, morphology and histology have not been considered widely as a measurable endpoint in toxicological studies. Gentile et al. (1982) reported morphological aberrations at the onset of sexual maturity in *A. bahia* and *A. bigelowi* exposed to cadmium in the laboratory. In addition, field observations of intersexuality and variable telson

morphology were reported in *N. integer* from different European estuaries and the Baltic (Mees et al., 1995 and references therein). Remerie et al. (2005) recently used morphology to differentiate geographically separated populations of *N. integer* and *M. slabberi*. Using the mysid marsupial development assay developed by Fockedey et al. (2006), it would be possible to create a library of normal and abnormal development for future reference in ED studies.

To the best of our knowledge, the only histological study using a mysid model, is a recent study by Sardo et al. (2005a) evaluating the effects of 3,4-dichloroaniline on *M. slabberi*. The latter study demonstrated that mysid muscular tissue, cuticular lens, and gonads were clearly affected by the test compound.

Behavioral and other endpoints

Disruption of mysid swimming behavior has been used as an endpoint to evaluate the effects of sublethal exposure to chlorpyrifos and cadmium (Roast et al., 2001, 2002). Other behavioral responses that have been measured in mysids include feeding activity (Engstrom et al., 2001), grooming behavior (Acosta and Poirrier, 1992), burrowing ability (Nel et al., 1999), eye spectral sensitivity (Audzijonyte et al., 2005b), and predator/prey dynamics (Rademacker and Kils, 1996). A few studies have been published on the interaction between osmoregulation and chemical exposure in mysids (e.g., Wildgust and Jones, 1998; Kline and Stekoll, 2000). Other hormonal responses and disturbances in crustaceans, such as color changes, retinal pigments and limb regeneration are discussed in a review by Fingerman et al. (1998), however, the use of these endpoints in mysids awaits further study.

Life-cycle and transgenerational studies

Mysids offer clear advantages over other invertebrate models for chronic and life-cycle studies which were previously reviewed in Verslycke et al. (2004a). For *A. bahia*, standard life cycle test

protocols have been developed, applied and evaluated (e.g., McKenney and Celestial, 1996; USEPA, 1997; ASTM, 1999). Longer-term studies spanning critical life stages over multiple generations can identify and characterize possible latent or cumulative adverse effects occurring in an organism's life history (McKenney, 2005). For this purpose, a transgeneration mysid toxicity protocol was developed which extends the single life-cycle test such that juveniles, released by adults exposed since <24h-old juveniles, are reared through maturation without further exposure. Survival, growth, development and reproduction of *A. bahia* were monitored through an entire life-cycle exposure to fenoxycarb and during the second generation without additional exposure (McKenney, 2005). Neither maturation time, sex determination nor young production was significantly altered during the life-cycle exposure. However, second-generation adults, exposed to fenoxycarb only as developing embryos and juveniles, produced fewer young (at 6 μg fenoxycarb/l) and contained significantly fewer males (at 1 μg fenoxycarb/l). These findings strongly suggest the need for, at least, two-generational exposure protocols to adequately predict the potential chronic impact of EDs (OECD, 2006). Interestingly, exposure to fenoxycarb and other juvenile hormone analogs stimulates the production of only male offspring at low nanomolar concentrations in certain cladoceran species (Oda et al., 2005a, 2005b). These apparent differences in the sex determining role of juvenoids between mysids and cladocerans underline the need for caution against extensive extrapolations of observations among different crustacean classes (see also LeBlanc, this issue).

A mysid transgeneration test protocol has been submitted to OECD as a proposed guideline for further development, validation, and acceptance (OECD, 2006). The proposed guideline also allows for the exposure of the second generation to the test compound. While the guideline is specific to *A. bahia*, it should be modifiable to suit other mysid species. A detailed description of the method was published by OECD (2006) and includes recommended test species, gaps in current knowledge, and necessary complementary studies. Primary endpoints in the two-generation mysid

assay are survival, growth (change in dry weight), and reproduction (time to sexual maturity, time to first brood release, total number of offspring, sex ratio, percentage of females that are reproductively active). Optional biochemical endpoints are metabolic disruption (O:N ratio), steroid metabolism, vitellogenin levels, cytochrome P450 levels, and blood glucose levels (as discussed in the previous paragraphs of this review). According to the OECD document, the goal of designing and conducting detailed chronic toxicity tests with mysids would be to determine whether specific endpoint responses can be determined for different classes of compounds that affect ecdysteroids, juvenile hormone analogs, vertebrate androgens, vertebrate estrogens, or other hormonal axes. As the document further points out, biochemical studies must be included into the experimental regime to help verify that the observed endpoints resulted from disturbance of hormone systems. In this perspective, some of the endpoints that are mentioned in the present review (e.g., mysid vitellogenesis assay, mysid ecdysteroid receptor binding assay) should be valuable tools to getting mechanistic information. In our opinion and from a risk assessment point-of-view, the distinction between endocrine or metabolic toxicity based on sound mechanistic evidence is not crucial and probably less important than identifying the most sensitive endpoints that can lead to long-term impacts at the population level.

Field studies

The use of mysids in field studies has been very limited. McKenney et al. (1985) and Clark et al. (1986) performed experiments with caged mysids to evaluate the lethal and sublethal responses of *A. bahia* during field applications of fenthion, an organophosphate insecticide. In addition, studies have used a biomarker approach in field-exposed mysids (Fossi et a., 2001; Verslycke et al., 2004b; Ghekiere, 2006). ENDIS-RISKS (www.vliz.be/projects/endis) is a four-year project that has focused on evaluating the incidence, distribution and potential effects of a wide range of EDs on the

resident mysid population of the Scheldt estuary (Belgium, The Netherlands). So far, this project has demonstrated significant endogenous levels of organotins, surfactants, and flame retardants in mysids (Verslycke et al., 2005), as well as estrogens and some pesticides (Noppe et al., 2006; Noppe et al., unpublished data). In addition, vitellogenesis (Ghekiere, 2006), growth, steroid and energy metabolism (Verslycke, 2003) were measured in the resident mysid population of this estuary and significant effects were observed along the pollution gradient.

Conclusions

The large diversity of forms, life histories, and biology in the invertebrates can not be used as an excuse for a near exclusionary focus on mammalian models in the current and proposed regulatory programs for ED screening and testing. The long standing use of mysids in toxicity testing and the proposed inclusion of mysid models in regulatory screening programs for EDs has led to a recent increase in studies on the potential effects of several xenobiotics (notably certain insect growth regulators) on several established and novel endpoints. Some of these newer endpoints are promising as they might lead to a better understanding of hormonal control of physiological processes such as vitellogenesis, molting, and early development in mysids. While our understanding of hormone regulation in mysids is still very limited, it is probably no worse than for most other invertebrate models, with the notable exception of some insect and decapod crustacean models. It can be expected that future progress in our understanding of hormone function in the invertebrates will be expedited with the rapidly increasing availability of genomic sequence data. Given the established use of mysids in regulatory toxicity testing programs worldwide, a mysid genome project would be extremely useful. In addition, new efforts in standard protocol development, driven by the observation of multi-generational effects in mysids, has led to the proposal of a two-generation mysid life cycle test which will be validated over the next couple of

years. One large omission in current regulatory screening and testing programs for EDs is the absence of assays that evaluate invertebrate-specific endocrine toxicity. Given the accumulating data on the effects of certain insect growth regulators and other chemicals on invertebrate ecdysteroid and juvenoid hormone regulated pathways, the current approach risks significantly underestimating the true impact of these chemicals on our ecosystems. The mysid model offers important opportunities for identifying EDs that may affect important economic and ecological resources, as well as provide insights into invertebrate-specific endocrine toxicity. Furthermore, it is very likely that continued fundamental investigation of the mysid endocrine system will lead to important structural and functional insights that will be needed to understand the uniqueness of, as well as commonalities between mysids and other invertebrates, and mysids and vertebrates.

Acknowledgement

Tim Verslycke was supported by a Fellowship of the Belgian American Educational Foundation.

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Table 1. Mysid species that have been used in toxicity testing (redrafted after Verslycke et al., 2004a)

Species name	Distribution	Habitat description	Commercial culture	Culture and test protocols
Americamysis bahia (= *Mysidopsis bahia*)	Coastal estuaries and embayments ranging from the Gulf of Mexico to Narragansett (RI)	Marine (>15‰), <20-34°C	yes	Ward, 1984; Lussier et al., 1988; Nimmo et al., 1991
Americamysis bigelowi (= *Mysidopsis bigelowi*)	Eastern coast of the USA from MA (Georges Bank) to FL, often together with *A. bahia*	Marine (30-35‰), 2-30 °C	no	Lussier et al., 1988
Americamysis almyra (= *Mysidopsis almyra*)	Eastern coast of the USA, inshore waters along the entire coast of Gulf of Mexico and northward along Atlantic coast to Patapsco River (MD)	Marine (10-20‰), >20°C	yes	Domingues et al., 1999; Reitsema and Neff, 1980
Holmesimysis costata (= *Acanthomysis sculpta*)	Principal species of the genus, from Southern California to British Columbia	Marine, planktonic, lives within surface canopy of kelp	no	USEPA, 1995b; Martin et al., 1989; Hunt et al., 1997
Mesopodopsis slabberi	NE-Atlantic, from Scandinavia to W-Africa, including North Sea; Mediterranean; Black Sea; Sierra Leone Estuary; Suez Canal; NE- to South Africa	Euryhaline, superbenthic	no	Sardo et al., 2005b
Mysidopsis intii	Eastern pacific from South-America to the southern California coast of the USA	Marine, epibenthic, optimal temperature 20-22 °C, optimal salinity 28-35‰	no	Harmon and Langdon, 1996; Langdon et al., 1996, USEPA, 2002b
Mysis mixta	Eastern (from White Sea to Iceland) and Western (Greenland coastal waters down to Cape Cod) Atlantic regions	Brackish, low salinity, coldwater	no	Gorokhova, 1998; Gorokhova and Hansson, 2000
Neomysis integer	North-European estuaries and coastal waters, oligohaline and freshwater lakes	Marine, estuarine, freshwater, hyperbenthic, coldwater (< 20°C)	no	Roast et al., 1999a; Verslycke et al., 2003b
Neomysis americana	Western Atlantic from Florida to Newfoundland, and also South America	Sandy bottoms at depths of 0-240 m, 10-32‰, 20-25°C	no	Smith and Hargreaves, 1984; USEPA, 2002b
Neomysis awatschensis	Pacific coast of Japan, Korea and USA	Marine, estuarine	no	Yan et al., 2003
Neomysis mercedis	North-Eastern pacific coast of the USA (southern Alaska to Goviota Bay, CA)	Freshwater, estuaries, coastal lakes, planktonic/epibenthic, 6-22 °C	no	Brandt et al., 1993
Praunus flexuosus	North-European coastal waters	Hyperbenthic/planktonic, euryhaline, eurytherm	no	Garnacho et al., 2001; Winkler and Greve, 2002
Tenagomysis novaezealandiae	New Zealands's North and South Islands	Marine	no	Nipper and Williams, 1997

Table 2. List of potential endpoints for evaluating the effects of endocrine disruptors and their use in mysids

Endpoint	Standard or selected references[a]
Survival[b]	ASTM, 1998; 1999; 2002; USEPA, 1997
Growth, Biomass	ASTM, 1999; USEPA, 1995a, 1995b
Molt time; Molt success	Gorokhova, 2002; De Lisle and Roberts, 1994; Cuzin-Roudy and Saleuddin, 1989; Fockedey et al., 2006; Ghekiere et al., 2006a
Energy metabolism; O:N ratio, C:N ratio Respiration, Biochemical composition, Crustacean hyperglycemic hormone	McKenney 1998, 1999; Roast et al., 1999b; Verslycke et al., 2003c, 2004d; OECD, 2006; Chang, 2005
Embryonic development	Greenwood et al., 1989; Wortham and Price, 2002; Fockedey et al., 2006; Ghekiere et al., 2006a
Sexual maturatity, Time to first brood release, Egg development time	ASTM, 1999; OECD, 2006
Sex ratio and intersexuality	ASTM, 1999; OECD, 2006
Fecundity (brood size)	ASTM, 1999; USEPA, 1995a, 1995b; OECD, 2006
Vitellogenesis	Tuberty et al., 2002; Ghekiere et al., 2004, 2005, 2006c
Population dynamics	Kuhn et al., 2000, 2001; Raimondo and McKenney, 2005a, 2005b, 2006
Ecdysteroid metabolism, Ecdysteroid receptor interaction	Cuzin-Roudy and Saleuddin, 1989; Yokota et al., 2005; Verslycke et al. (unpublished data)
Steroid metabolism	Verslycke et al., 2002, 2003a, 2004c
P450 enzymes	James and Boyle, 1998; Verslycke et al., 2002, 2003a, 2004c
Osmoregulation	De Lisle and Roberts, 1994; Webb et al., 1997
Morphology, histology	Mees et al., 1995; Remerie et al., 2005; Sardo et al., 2005a
Swimming behavior	Roast et al., 2001, 2002
Feeding behavior	Roast et al., 2004
Other behavioral endpoints: mating, grooming, swarming, burrowing ability, predator/prey dynamics	Acosta and Poirrier, 1992; Ritz and Metillo, 1998; Rademacher and Kils, 1996; Nel et al., 1999; Linden et al., 2003; Viitasalo and Viitasalo, 2004; Audzijonyte et al., 2005b

[a] for an extended list of references of endpoints that have been measured in *A. bahia* and other mysids, we refer to the table published in Verslycke et al. (2004a)

[b] although survival is not a specific endpoint for endocrine toxicity, it is listed here for completion as most chemical effect evaluations start with determining acute or chronic lethal toxicity