

*Critical Review*

**MYSID CRUSTACEANS AS POTENTIAL TEST ORGANISMS FOR THE EVALUATION OF ENVIRONMENTAL ENDOCRINE DISRUPTION: A REVIEW**

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**Abstract**—Anthropogenic chemicals that disrupt the hormonal systems (endocrine disruptors) of wildlife species recently have become a widely investigated and politically charged issue. Invertebrates account for roughly 95% of all animals, yet surprisingly little effort has been made to understand their value in signaling potential environmental endocrine disruption. This omission largely can be attributed to the high diversity of invertebrates and the shortage of fundamental knowledge of their endocrine systems. Insects and crustaceans are exceptions and, as such, appear to be excellent candidates for evaluating the environmental consequences of chemically induced endocrine disruption. Mysid shrimp (Crustacea: Mysidacea) may serve as a viable surrogate for many crustaceans and have been put forward as suitable test organisms for the evaluation of endocrine disruption by several researchers and regulatory bodies (e.g., the U.S. Environmental Protection Agency). Despite the long-standing use of mysids in toxicity testing, little information exists on their endocrinology, and few studies have focused on the potential of these animals for evaluating the effects of hormone-disrupting compounds. Therefore, the question remains as to whether the current standardized mysid endpoints can be used or adapted to detect endocrine disruption, or if new procedures must be developed, specifically directed at evaluating hormone-regulated endpoints in these animals. This review summarizes 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.

**Keywords**—Mysids Endocrine disruption Biomarkers Regulation Environmental monitoring

**INTRODUCTION**

Anthropogenic chemicals that disrupt the hormonal systems (endocrine disruptors) of wildlife species recently have become a widely investigated and politically charged issue [1–3]. Invertebrates account for roughly 95% of all animals [4], yet surprisingly little effort has been invested to understand their value in signaling potential environmental endocrine disruption [5–12]. Although growth, reproduction, development, and other aspects of invertebrate physiology are known to be under hormonal control, the endocrine systems and hormones produced and used in invertebrates are not directly analogous to those of vertebrates [13]. In invertebrates, the selection of suitable test methods and species for evaluating endocrine disruption is confounded by diversity. The use of a limited number of species as representative of this diversity is a naive approach destined to failure in the absence of suitable safeguards [2,9]. Hence, the key challenge for environmental assessment is to find invertebrate species, selected from multiple levels of ecosystem function, to efficiently monitor and evaluate the complexity of potential environmental effects of endocrine-disrupting chemicals at a reasonable financial cost [14].

Many anthropogenic pollutants have the world's oceans and seas as a final sink, and are carried there through riverine and estuarine conduits [12,15]. Estuaries are intrinsically and commercially important ecosystems and are amongst the first re-

cipients of endocrine disruptors in their seaward transport. Of the estuarine organisms that could be adversely affected by these compounds, crustaceans are good candidates for the study of potential impacts. Crustaceans are common in freshwater, estuaries, and shallow coastal waters and form vital links in aquatic food webs [16–21]. In addition, crustaceans are susceptible to the effects of endocrine disruptors [13]. An international Society of Environmental Toxicology and Chemistry workshop on endocrine disruption in invertebrates held in The Netherlands in 1998 [7] identified insects and crustaceans as potential organisms for evaluating chemically induced endocrine disruption by virtue of the wealth of information available on their endocrinology compared with other invertebrates [9,12,22–24].

Of the crustaceans, mysid shrimp have been put forward as suitable test organisms for the evaluation of endocrine disruption [7,9,25]. The U.S. Environmental Protection Agency established the Endocrine Disruptor Screening and Testing Standardization and Validation Task Force to coordinate and conduct the scientific and technical work necessary to validate the screens and tests recommended by the Endocrine Disruptor Screening and Testing Committee. The Standardization and Validation Task Force recommended a two-tiered approach for determining whether a chemical is an endocrine disruptor, and mysids were proposed as a suitable invertebrate assay in the tier 2 testing (in vivo testing) (<http://www.epa.gov/scipoly/oscpendo>) for a two-generation reproductive and developmental toxicity test. Recently, a draft review paper was com-

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piled on mysid life-cycle toxicity testing [13], and the two-generation mysid life-cycle assay was proposed to the Organization for Economic Cooperation and Development as a new Organization for Economic Cooperation and Development test guideline. Despite the long-standing use of mysids in toxicity testing, little information on their endocrinology has been published and few studies have focused on the potential of these animals for evaluating the effects of hormone-disrupting compounds. Therefore, the question remains as to whether the current standardized mysid endpoints can be used or adapted to detect endocrine disruption, or if new procedures must be developed, specifically directed at evaluating hormone-regulated endpoints in these animals.

This present review provides an overview of the available information on mysids relevant to the issue of endocrine disruption, including their ecological role in marine and estuarine ecosystems, their use in toxicity testing and environmental monitoring, and their endocrinology. A case is made for their potential use in assessing the environmental consequences of endocrine-disrupting chemicals.

#### MYSID BIOLOGY AND ECOLOGY

Mysids (Malacostraca: Peracarida: Mysidacea) are relatively small (with the majority of the species being between 5 and 25 mm in length), shrimplike crustaceans, often referred to as opossum shrimp because the oostegites form a ventral female marsupium for carrying the developing embryos. The latter feature distinguishes mysids from other shrimplike crustaceans. Mysids are identified from other peracarids (Amphipoda, Isopoda, Cumacea, and Tanaidacea) by the presence of a statocyst (containing large endogenous statoliths, the primary equilibrium organs for mysids) on the proximal part of the uropodal endopod. Mysids are distributed from 80°N to 80°S and occur in various aquatic environments, including freshwater, groundwater, brackish, estuarine, coastal, and oceanic habitats [26–28]. Mauchline and Murano [28] published a world list of mysids in 1977 (765 species distributed between ~ 120 genera); however, this number is ever increasing through improved sampling techniques and exploration of new habitats. The present count is more than 1,000 species belonging to approximately 160 genera (<http://crustacea.net/>). A comprehensive database on the world mysid fauna (Nemys, <http://intramar.ugent.be/nemys>), containing links to relevant information (i.e., taxonomical, morphological, ecological, biogeographic, literature, pictorial, and molecular information), on the species level presently is being constructed (T. Deprez, Ghent University, Section Marine Biology, Ghent, Belgium, personal communication).

In general, mysids are regarded as omnivores and feed on phytoplankton, zooplankton, and organic detritus [26,27,29–31]. Pelagic forms filter particles during swimming, whereas benthic species have been observed actively hunting and grabbing small particles [27]. Mysids form important links in the food webs of aquatic ecosystems and often feed selectively for size or species (or both) of prey [26,32]. Consequently, they have the potential for structuring zooplankton communities [33,34] and influencing the structure of phytoplankton, tychoplankton, and meiofaunal communities [32,35–41]. Most mysids utilize organic detritus to a considerable extent and are capable of remineralizing a substantial portion of the nonrefractory detritus suspended in the water column or buried in the surface sediments [29,37,42,43]. Mysid size is intermediate between mesozooplanktonic ( $\mu\text{m}$ ) and endobenthic or epi-

benthic (cm) prey items, and mysids often progressively replace copepods in the diet of many postlarval and juvenile commercial fish species [19,26,44,45]. In addition, mysids may serve as prey for larger crustaceans, marine mammals, or wading birds [26,27,32,46–48].

Estuarine mysids have a flexible physiology that responds to a host of dynamically changing environmental variables, characteristic of the complex chemistry of estuaries. Temperature and salinity are the dominant ecological variables, and may act either singly or in combination to modify the physiological and ecological properties of estuarine organisms as well as responses to xenobiotic exposure. Therefore, empirical determination of the optimal salinity and temperature conditions of estuarine mysids is essential for the development of optimum laboratory culture of these organisms and their use in toxicity and hazard assessment. For example, the optimal salinity and temperature conditions for growth of *Americamysis bahia* (formerly *Mysidopsis bahia*) through its entire life cycle [49] are correlated with resistance patterns to these dominant environmental variables [50] and distribution of this species in estuaries. Moreover, temperature and salinity interact to modify the reproductive capacity of this species [51].

#### MYSIDS AND TOXICOLOGY

Mysids are sensitive to some chemical contaminants at environmentally relevant concentrations and have been used in regulatory toxicity testing for more than 20 years [15,43,52–66]. The U.S. Environmental Protection Agency and the American Society for Testing and Materials both have adopted the subtropical *A. bahia* as a key testing species for coastal and estuarine monitoring, and standard guides for conducting life-cycle toxicity tests with this species have been developed [13,67–72]. Although a relatively large amount of published toxicity data is available for *Americamysis* species, relatively limited data are available on the sensitivity of other mysid species to toxicants [64]. However, the available evidence suggests that mysids are generally more sensitive to toxic substances than many other test species [43,73–75]. Toxicity test procedures have been published for *Neomysis mercedis* [52,76], *Mysidopsis intii* [59], *Holmesimysis costata* [62], *Americamysis bigelowi* [77], *Neomysis integer* [64,75,78], *Tenagomysis novae-zealandiae* [79], *Praunus flexuosus* [80,81], *Neomysis americana* [82,83], and *Neomysis awatshensis* [84] (Table 1). In addition, methods for maintaining viable populations of different mysid species under laboratory conditions have been described by several researchers [46,59,75,79,85–89]. Recently, a strong correlation was reported between the toxic response of daphnids and mysids ( $R^2 = 0.941$ ,  $n = 28$ ; 96-h median lethal concentrations for *A. bahia* and 48-h median lethal concentrations for *Daphnia magna*) for pesticides and organics, emphasizing the use of mysids in future toxicity testing [90].

Mysids have been used successfully to measure various sublethal toxicant effects, such as growth, swimming capability, feeding behavior, molting, energy budget, reproduction, sexual maturity, and vitellogenesis (described in detail in the following paragraphs and summarized in Table 2). Also, field studies and caging experiments with mysids have been published [17,91–94].

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 sensi-

Table 1. Candidate mysid test species for toxicity testing with details on their natural habitat and culturing

Species name	Distribution	Habitat description	Commercial culture	Culture protocol
<i>Americamysis bahia</i> (= <i>Mysidopsis bahia</i> )	Coastal estuaries and embayments ranging from the Gulf of Mexico to Narragansett (RI, USA) [250]	Marine (>15‰), <20–34°C [49–51]	Yes	[46,89,251]
<i>Americamysis bigelowi</i> (= <i>Mysidopsis bigelowi</i> )	Eastern coast of the USA from MA (Georges Bank) to FL often together with <i>A. bahia</i> [250]	Marine (30–35‰), 2–30°C	No	[46]
<i>Americamysis almyra</i> (= <i>Mysidopsis almyra</i> )	Eastern coast of the USA, inshore waters along the entire coast of Gulf of Mexico and northward along Atlantic coast to Patuxent River (MD) [250]	Marine (10–20‰), >20°C	Yes	[85,88,252]
<i>Holmesimysis costata</i> (= <i>Acanthomysis sculpia</i> )	Principal species of the genus, from southern California (USA) to British Columbia (Canada) [122, 253]	Marine, planktonic, lives within surface canopy of kelp	No, field-collected animals available [253]	[122,253]
<i>Mysidopsis intii</i>	Eastern Pacific from South America to the southern California coast of the USA [59, 250]	Marine, epibenthic, optimal temperature 20–22°C, optimal salinity 28–35‰	No	[59]
<i>Mysis mixta</i>	Eastern (from White Sea to Iceland) and Western (Greenland coastal waters down to Cape Cod) Atlantic regions [26]	Brackish, low salinity, cold water	No	[114]
<i>Neomysis awatschensis</i>	Pacific coast of Japan, Korea, and USA [115]	Marine, estuarine	No	[115]
<i>Neomysis mercedis</i>	Northeastern Pacific coast of the USA (southern Alaska to Gaviota Bay, CA) [52]	Freshwater, estuaries, and coastal lakes, planktonic/epibenthic, euryhaline (<0.5 to >25‰), 6–22°C	No	[52]
<i>Neomysis integer</i>	Northern European estuaries and coastal waters; oligohaline and freshwater lakes [20, 43]	Marine, estuarine, freshwater, hyperbenthic, euryhaline (<0.5 to >25‰), cold water (<20°C) [116]	No	[75]
<i>Praunus flexuosus</i>	Northern European coastal waters	Hyperbenthic/planktonic, euryhaline, eurytherm [254, 255]	No	[123]

tivity to contaminants, mysids are appropriate toxicity test organisms.

#### CANDIDATE MYSID TEST SPECIES FOR ENDOCRINE-DISRUPTION RESEARCH

General selection criteria for the most appropriate mysid species for toxicological testing are given by Nimmo and Hamaker [15] and Roast et al. [43]. These criteria include available when required; already adapted to laboratory conditions, eliminating an (expensive) conditioning phase; collection for laboratory testing will not decimate field populations (or destroy habitat during collection); easily transported; life history is short, making it possible to study the effects of a pollutant on various aspects of reproduction; diet is known and readily controlled; and ecologically important. In addition, the important characteristics for the selection of a suitable test species for identifying the effects of endocrine disruption in the environment are given by DeFur et al. [7] and include primary mode of reproduction, culture in the laboratory, generation time, size, knowledge of endocrinology, and standard methods available. Some attributes described in the latter publication (e.g., mode of reproduction or knowledge of endocrinology) do not allow for discrimination among candidate mysid species. A very useful document in this context is a draft review paper on life-cycle toxicity testing with mysids in which sev-

eral species (*A. bahia*, *Americamysis almyra*, *A. bigelowi*, *H. costata*, *M. intii*, *N. mercedis*, and *N. integer*) are considered for their potential utility in endocrine-disruption testing [13]. From this review, it may be concluded that, although *A. bahia* has many strengths, limited ecological relevance for high-latitude and low-saline systems preclude its general utility. However, given the high degree of standardization in *A. bahia*, progress in development of standardized test protocols for endocrine-disruption testing should be fastest in this species. Table 1 summarizes the distribution, habitat description, and available culture protocols for other candidate mysid test species.

#### MYSID ENDOCRINOLOGY AND HORMONE-REGULATED ENDPOINTS

The use of hormones to regulate biological processes is a strategy common to vertebrates and invertebrates. Although the endocrine systems of invertebrates regulate many of the same processes in vertebrates (development, growth, and reproduction), some endocrine-regulated processes are unique to specific groups of invertebrates. For example, molting, diapause, and limb regeneration are endocrine-regulated processes associated with some invertebrate groups that are rare or absent among vertebrates [7].

Most of the current knowledge of crustacean endocrinology

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

Endpoint	Use in mysids	References/comments
Survival (acute)	<i>Americamysis bahia</i> (S) <sup>a</sup> <i>Americamysis bigelowi</i> (S) <i>Americamysis almyra</i> (S) <i>Holmesimysis costata</i> (S) <i>Neomysis mercedis</i> (S) <i>Mysidopsis intii</i> <i>Neomysis integer</i> Other species	[69,253] [69] [69] [62,67,253] [52,67,76] [55,59] [53,64,75,78] [79,80–84]
Life-cycle testing	<i>A. bahia</i> (S) <i>A. bigelowi</i> (S), <i>A. almyra</i> (S) <i>H. costata</i> (S) <i>M. intii</i> <i>N. integer</i>	[68,70,72,253] [68] [56,68,71] [55,59] [160]
Two-generation testing	<i>A. bahia</i> , <i>A. bigelowi</i> , <i>A. almyra</i> (S in prep) <i>M. intii</i> <i>H. costata</i> , <i>N. mercedis</i> , <i>N. integer</i>	[13,219,256] Method should be developed Probably impractical because of long generation time
Fecundity (brood size)	<i>A. bahia</i> , <i>A. bigelowi</i> , <i>A. almyra</i> (S) <i>H. costata</i> (S) <i>N. mercedis</i> <i>M. intii</i> <i>N. integer</i> , <i>Praunus flexuosus</i> <i>Mysis mixta</i> <i>Neomysis awatschensis</i>	[68,70] [71,122] [257] [55,59] [123,258] [114] [115]
Embryonic development	<i>A. bahia</i> <i>Mesopodopsis slabberi</i>	[205] [208]
Sexual maturity	<i>A. bahia</i> (S)	[51,58,60,68,218]
Time to first brood release	<i>H. costata</i>	[122]
Egg development time	<i>N. integer</i> , <i>P. flexuosus</i> <i>M. mixta</i> <i>M. intii</i>	[123] [114] [55,59]
Sex ratio and intersexuality	<i>A. bahia</i> (S) <i>N. integer</i>	[68,219,253] [223–225]
Growth, biomass	<i>A. bahia</i> , <i>A. bigelowi</i> , <i>A. almyra</i> (S) <i>H. costata</i> (S) <i>N. mercedis</i> <i>M. intii</i> <i>M. mixta</i> <i>N. integer</i> <i>N. awatschensis</i> <i>P. flexuosus</i> <i>Tenagomysis novae-zealandiae</i> Other species	[68,70] [56,71,122] [52] [55,59] [124] [107,116] [84,115,150,151] [114] [79] [117,119–121,132]
Molt time and success	<i>A. bahia</i> <i>N. integer</i> <i>M. mixta</i> <i>Siriella armata</i> <i>N. awatschensis</i>	[259] [111,140] [140] [138] [115]
Energy metabolism	<i>A. bahia</i>	[63]
O:N ratio, C:N ratio	<i>N. mercedis</i>	[260]
Respiration	<i>N. integer</i>	[153,155,164–167,169–175,178–181]

Table 2. Continued

Endpoint	Use in mysids	References/comments
Biochemical composition	<i>M. mixta</i> <i>N. awatschensis</i> <i>P. flexuosus</i> <i>Leptomysis lingvura</i> <i>Mysis relicta</i> <i>Gastrosaccus brevifissura</i>	[114,182] [150–152] [81] [54] [176,261] [163]
Ecdysteroid metabolism	<i>A. bahia</i> <i>N. integer</i> <i>S. armata</i>	S.R. Tuherty <sup>b</sup> and C.L. McKenney, <sup>c</sup> unpublished data A. Ghekiere et al., <sup>d</sup> unpublished data [139]
Steroid metabolism	<i>N. integer</i>	[162,185,198,199]
P450 enzymes	<i>N. integer</i>	[162,185,198]
Vitellogenesis	<i>A. bahia</i> <i>S. armata</i> <i>N. integer</i>	[215] [139] A. Ghekiere et al., <sup>d</sup> unpublished data
Osmoregulation	<i>A. bahia</i> <i>N. integer</i> <i>P. flexuosus</i> Other species	[259,262,263] [264] [265] [248]
Morphology, histology	<i>A. bahia</i> , <i>A. bigelowi</i> <i>N. integer</i>	[77] [225]
Swimming behavior	<i>N. integer</i>	[43,226–230,232]
Feeding behavior	<i>A. bahia</i> <i>M. mixta</i>	[231,241] [239,242]
Other behavioral endpoints	Mating, grooming, swarming, burrowing ability, predator-prey dynamics	[233–238,243–247]

<sup>a</sup> S = method is standardized.<sup>b</sup> Center for Environmental Diagnostics and Bioremediation, University of West Florida, Pensacola, FL, USA.<sup>c</sup> U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Gulf Breeze, FL, USA.<sup>d</sup> Laboratory of Environmental Toxicology and Aquatic Ecology, Ghent University, Ghent, Belgium.

is based upon studies with decapods such as crabs, lobsters, crayfish, and shrimp, and has been reviewed previously [7,95–100]. The main biological processes, such as growth, molting, and reproduction, are cyclic and fairly well understood in benthic and terrestrial malacostracans, such as decapods, isopods, and amphipods [22,101,102]. These biological processes are regulated by a complex endocrine system [96,101]. Basically, inputs from the environment are integrated by the central nervous system; neurotransmitters and neuromodulators govern the release of neuropeptides, which control the production of hormones by the endocrine glands [103]. The main crustacean endocrine centers include the Y-organ, mandibular organ, androgenic gland, X-organ, and sinus gland [7,97]. Unfortunately, endocrine glands or sites of hormone production in mysids are largely undescribed.

The effects of organic and inorganic contaminants on crustacean functions regulated by hormones are being investigated with increasing frequency and several show promise as biomarkers of environmental contamination and endocrine disruption [7,8,104,105]. Unfortunately, relatively few data are available on the hormonal control of biological processes in mysids. Having said that, certain endpoints relevant to the testing of suspected endocrine disruptors, such as survival, fecundity, sexual maturation, and biomass increase, are already standardized procedures for some mysids, such as *A. bahia*, *A. bigelowi* (partly), *A. almyra*, *H. costata*, and *N. mercedis*, and many other endpoints or species are promising [7]. The use of potential mysid hormone-regulated endpoints as biomarkers of exposure or effects of endocrine disruptors are discussed in detail in the following paragraphs and are summarized in Table 2. Although many, if not all, of these endpoints may indicate a response to an endocrine disruptor, most also vary in response to exposure to other stressors and this is further confounded by the interrelatedness (i.e., nonindependence) of some of these endpoints [13]. The key to the interpretation of these endpoints as indicators of endocrine disruption will be to create for each species a large database of what constitutes the normal unstressed response, and what constitutes a normal reference site or population when working under field conditions.

#### Growth and molting

Most commonly, growth is measured either by increases of dry weight or body length per time interval [59,106–108] and, for crustaceans, is often expressed in terms of intermolt period and growth factor (percentage increase in body size at the molt) [26]. Growth curves (such as the von Bertalanffy equation) can be fitted to the growth data [20,109,110], allowing comparisons of the different growth parameters between treatments. Although several studies have focused on growth and molting in mysids under natural conditions [49,107,111–123], exposure experiments also have confirmed the sensitivity of these endpoints in toxicology [52,56,61,84,92,106,108,124–131]. For mysids, reduced growth is the most common sublethal response to toxicant exposure and this has important implications for reproductive success because fecundity is related directly to female body size [20,84,118,123]. In crustaceans, significant growth occurs only as a result of molting; therefore, disruption of molting may result in alterations in growth [13,132].

Ecdysteroids (the molting hormones in crustaceans) also function in the control of reproduction and embryogenesis [97,133]; therefore, the crustacean molt cycle has profound

effects on many aspects of organismal function, including physiology, behavior, and changes in biochemical composition [134,135]. Molting is regulated by a multihormonal system but is under the immediate control of molt-promoting steroid hormones (ecdysteroids) secreted by an ecdysial gland, called the Y-organ (the homologue of the prothoracic gland in insects [103,136]. The Y-organ secretes ecdysone, which, on release in the hemolymph, is converted into active 20-hydroxyecdysone (synonyms: crustecdysone and ecdysterone). Circulating titers of 20-hydroxyecdysone vary impressively during the molt cycle [103,134]. The Y-organ produces two other ecdysteroids, 3-dehydroecdysone and 25-deoxyecdysone, with the latter forming the immediate precursor to the active ponasterone A [133]. More studies have been done on the effects of contaminants on molting and limb generation than on any other hormone-mediated process in crustaceans [8,135,137].

Molt staging, based on changes in the integument, has been developed for various crustaceans and is generally divided into four major periods: postmolt, intermolt, premolt, and molt (ecdysis). Mysid molt stages have been described for *Siriella armata* [138,139], *Mysis mixta* [140], and *N. integer* [140]. In mysids, ecdysis is instantaneous, with the entire carapace lifting up and the mysid sliding out of the old cuticle while swimming. For female mysids, integumental development during molt preparation, marsupial brood development, and development of new eggs in the ovary are synchronized, facilitating molt staging [101]. To date, only one study has quantified ecdysteroid titers during the mysid molt cycle and this study was with *S. armata* [101]. However, both ecdysone and 20-OH ecdysone have been identified in *N. integer* (A. Ghekiere et al., Laboratory of Environmental Toxicology and Aquatic Ecology, Ghent University, Ghent, Belgium, unpublished data) and *A. bahia* (S.R. Tuberty, Center for Environmental Diagnostics and Bioremediation, University of West Florida, Pensacola, FL, USA, and C.L. McKenney, Jr., U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Gulf Breeze, FL, unpublished data).

As mentioned previously, molting is controlled by ecdysteroids [136]. Ecdysteroids control the activity of specific genes at the transcriptional level by interacting with the intracellular ecdysteroid receptor [103,135,141]. In arthropods, the ecdysone receptor is in the same gene family as the vertebrate thyroid receptor but, interestingly, steroidal estrogens do not agonize or antagonize the ecdysteroid receptor [142]. Evidence exists [142] that some nonsteroidal environmental estrogens are ecdysteroid antagonists (e.g., lindane, bisphenol A, diethylphthalate, and *p,p'*-DDT). In addition, several classes of phytochemicals antagonize ecdysone activity [137]. The apparent ubiquity of the antiecdysteroidal activity of environmental chemicals necessitates investigation into their potential effects on crustaceans [143,144]. Many pesticides, generally classed as insect growth regulators, function as ecdysone agonists [7,11]. As suggested by Zou and Fingerman [136], future investigations of molting in crustaceans should have two emphases, one examining interactions between potential endocrine disruptors and the ecdysone receptor, and one focusing on the possible impairment of ecdysteroidogenesis by these agents. In vitro assays can determine quickly whether a chemical has (anti)ecdysteroidal activity [145,146]. Given the current methods for quantifying ecdysteroids by using immunoassay [139,144,147] and the available methods for molt staging [138,140], it should be possible to evaluate the potential in-

teraction of these chemicals with the process of molting in mysids.

Because ecdysteroids are used as major endocrine-signaling molecules in crustaceans [7], and little is known of their other functions, it may be expected that a chemical with (anti)ecdysteroidal activity also will affect other hormone-regulated processes in crustaceans. Support for this hypothesis is provided by Mu and LeBlanc [144], who demonstrated that the fungicide fenarimol altered embryo development in daphnids by interfering with ecdysteroid metabolism. However, one major advantage of using ecdysteroid metabolism as an endpoint is that it provides a means of evaluating the impact of environmental chemicals on crustaceans (and potentially other arthropods), while not necessarily affecting vertebrates. Because (anti)ecdysteroidal activity has been proven in vitro for certain chemicals [145], and disruption of molting has been observed as a result of chemical exposure, these chemicals should be tested in exposures with mysids. In these exposures, endpoints such as intermolt period, growth, morphological aberrations, ecdysone titers, protein concentrations, integument development, as well as related endpoints such as vitellogenesis, should detect in vivo effects of chemicals on ecdysteroid metabolism and molting in mysids. In vitro assays should aid the mechanistic understanding of chemical action to better allow distinction between endocrine-specific and pharmacological effects.

#### Energy metabolism

Biomarkers linked with physiological energetics provide information on key processes in the organism's energy acquisition and expenditure, and possibly also elucidate the mode of action of the toxicant. Under normal conditions, specific amounts of energy are allocated to basal metabolism, growth, and reproduction and therefore, theoretically, changes in metabolic turnover and specific allocations should be linked to effects at higher levels of ecological organization [148]. A large body of information is available on the neuroendocrine pathways of physiological regulation in macrocrustaceans. Although typical and well-studied challenges to endogenous energy metabolism include environmental hypoxia, functional (internal) hypoxia, changing energetic requirements, disturbance to water balance and ion homeostasis and changes in temperature (for review, refer to Morris and Airriess [149]), exposure to toxicants also will result in an energetic challenge. Because energetic processes are hormone-regulated, they are, by definition, sensitive to hormone disruption and several measurements of energy reserves and consumption may serve as useful biomarkers of endocrine-disrupting substances in crustaceans [11]. However, once again, the utility of bioenergetic endpoints will depend strongly on establishment of a consensus database of what the normal bioenergetic state is for mysids.

Alterations to the energy metabolism of mysids have been used successfully as an indicator of stress to toxicant exposure in *A. bahia* [63,125,126,129,130], *P. flexuosus* [81], *N. awatschenis* [150–152], and *N. integer* [153–156]. In *A. bahia*, *P. flexuosus*, and *N. awatschenis*, weight-specific respiration, ammonia excretion rates, and oxygen to nitrogen ratios have been measured after toxicant exposure. In *N. integer*, Roast et al. [153] used scope for growth [157], whereas Verslycke and Janssen [155] and Verslycke et al. [156] used the cellular energy allocation assay [158]. Both methods are promising and were recently validated in *N. integer* after exposure to the

pesticide chlorpyrifos (T.A. Verslycke et al., unpublished data). The cellular energy allocation assay also was recently validated in the field (Scheldt estuary, The Netherlands) [94]. The ecological relevance and utility of short-term bioindicators of metabolic processes in *A. bahia* have been demonstrated after chronic exposure to pesticides [63,125,126,129,130]. In these studies, pesticide-exposed juvenile mysids had a greater reliance on the more energy-rich lipid substrates during maturation to support elevated metabolic demands, resulting in less lipid material available for gamete production and reduced reproductive success. Unexposed mysids shift toward more proteinaceous substrates during maturation, as demonstrated for *A. bahia* [63] and *N. integer* [155,159]. These changes in metabolic substrate usage can be measured by monitoring the oxygen to nitrogen ratio [125,160], the lipid and protein content [155], or the carbon to nitrogen ratio of the test organism [114]. On the other hand, hyperglycemia is a common response to environmental or functional hypoxia and contaminant exposure in numerous decapods, and it is thought to be triggered by the action of crustacean hyperglycemic hormone on various target tissues [8,149]. The amino acid sequence of crustacean hyperglycemic hormone is highly homologous with that of the molt-inhibiting hormone, another product of the sinus glands in crustaceans, indicating possible involvement in the control of molting and reproduction [149]. Several investigators have examined the effects of metals and organic contaminants on blood glucose concentrations and crustacean hyperglycemic hormone titers in crustaceans [8]. Changes in blood glucose levels in mysids exposed to potential endocrine disruptors may indicate disruption of hormonal activity other than that associated with molting or reproduction [13].

The methods described above are transferred easily to other mysid species. Endpoints related to energetic processes are relatively easy to measure, but a better and holistic understanding of the role of the different hormones involved in energy metabolism, such as crustacean hyperglycemic hormone, is needed to evaluate the potential impact of hormone-mimicking substances on mysids. In this context, new immunoassays for determination of circulating hormones in the hemolymph, such as crustacean hyperglycemic hormone, are promising [161].

Other endpoints related to metabolism in mysids have been studied. High acetylcholinesterase activity in *Siriella clausi*, indicating a high metabolic rate, identified this mysid as particularly suited for research based on biomarkers in the marine environment [17]. In addition, *N. integer* has been used as a viable alternative model for the partial replacement of vertebrate animals in metabolic studies with illegal growth promoters and veterinary drugs [162]. Finally, respiratory responses have been studied in mysids in relation to a variable environment and toxic exposure [63,81,84,115,125,160,163–165].

Although undoubtedly having environmental 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 explaining hormone-regulated responses as can be expected from exposure to endocrine disruptors. Many abiotic and toxic stressors affect the energy metabolic processes of organisms [114,127,152–156,164], while not necessarily being related to disruption in normal hormonal regulation. Therefore, the successful use of biomarkers for the evaluation of endocrine disruption will be limited by the amount of background data on



natural variation and normal levels of the endpoints in question, and also by the fundamental understanding of the toxicant action at the (sub)cellular level. In this regard, many studies containing background information on the biochemical composition (e.g., proteins, lipids, and sterols) of mysids have been published [166–182], but information on neuropeptide and hormonal levels in mysids needs development.

#### *Steroid metabolism and cytochrome P450*

Pollutants may exert reproductive effects through interference with normal steroid metabolism [183–187]. For invertebrates, several studies have focused on pollutant-induced alterations in steroid metabolism. These chemicals often interfere with the microsomal P450 monooxygenase system, also called the mixed-function oxygenase system. The mixed-function oxygenase system is involved not only in the metabolism of organic toxicants but also in steroid metabolism; consequently, induction or inhibition of the mixed-function oxygenase system also may have repercussions for the hormonal control of reproduction. In sea stars, a linkage was demonstrated between impaired reproductive success, pollution-modulated endocrine function, and induction of the mixed-function oxygenase system [187]. In gastropods, much work on steroid metabolism has been initiated by the observation of tributyltin-induced imposex, a state of pseudohermaphroditism in which females exhibit functional secondary male characteristics. Although the underlying mechanism by which tributyltin causes imposex in gastropods has not been elucidated conclusively, the weight of evidence is in favor of the cytochrome P450-dependent aromatase inhibition hypothesis [187–192].

Alterations in steroid metabolism have been studied in *D. magna* [186,193–197] and in the blue crab *Callinectes sapidus* [191]. In daphnids, changes in steroid metabolism could provide an early indication of potential reproductive toxicity after sublethal exposure to suspected endocrine disruptors [186,193,194,197]. Verslycke et al. [185] 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 P450 isozymes. The remarkable diversity of testosterone hydroxylation exhibited should stimulate further studies on the induction, stereospecificity, and regulation of the enzyme systems of *N. integer* and other mysids. More recently, alterations in the phase I and II testosterone metabolism in *N. integer* after acute exposure to tributyltin have been demonstrated [198]. In addition, metabolic studies with *N. integer* have been used recently in exposure experiments with other chemicals, such as nonylphenol and methoprene, and also in the field [199].

The presence of sex hormones has been suggested in many, if not all, arthropods [183]. Vertebrate-type steroids (such as 17 $\beta$ -estradiol, testosterone, and progesterone) have been measured in several malacostracan crustaceans [7,184]. Although the lack of a role for vertebrate sex steroid hormones in arthropods has been highlighted [7,183], fragmented evidence suggests that some of these compounds may function as hormones in crustaceans [7,143,185]. Endogenous androgens may be the precursors for other hormones; therefore, exposure to exogenous androgens (or androgen mimics) could elicit activity through receptors other than the androgen receptor. Although this has not been determined in crustaceans, Verslycke et al. [185] found evidence of a sex-specific production of androgens, such as testosterone and androstenedione, in *N. integer*. Similarly, LeBlanc and McLachlan [200] reported var-

ious rates of testosterone conversion to androstenedione in daphnids. Future studies are needed to reveal if these conversions are affected by age, gender, reproductive state, or changes in the abiotic environment. Note that an androgen receptor has not been found or cloned in crustaceans. Therefore, identification and characterization of the androgen receptor should be a priority for research to explore the usefulness of sex steroids for evaluating endocrine disruption in crustaceans and other invertebrates.

Studies over the last 30 years have established the important role of cytochrome P450 in the biotransformation of xenobiotics and endogenous compounds (such as ecdysteroids) in crustaceans (for a review on crustacean P450, refer to James and Boyle [184]). Although no structural information on cytochrome P450 in crustaceans is available, it is clear that they are involved in several steps in the biosynthesis of ecdysteroids and other physiologically important substrates in crustaceans [201]. More studies are needed to understand the effects, if any, of various classes of environmental and other chemicals that are known modulators of cytochrome P450 expression or activity. New molecular tools, such as primer-based reverse transcription-polymerase chain reaction procedures and expression of P450s in heterologous systems, should result in better insights into the function and expression of P450s in the context of endocrine disruption. In addition, in vivo metabolic studies with different substrates (testosterone and ecdysone) will provide valuable tools for evaluating the effects of toxicant exposure, particularly when linked with effects on higher levels of biological organization. Although information on the identity of P450s and their functional role in mysids is, to our knowledge, nonexistent, mysids should be a good model to study these mechanisms. From the preliminary studies with *N. integer* by Verslycke et al. [185,198,199], sufficient information is available to suggest that mysids have an enzymatic biotransformation system that rivals that of other invertebrates and vertebrates. Metabolic studies with physiologically relevant substrates that also measure hormone-regulated effects at a higher level of biological organization (i.e., reproductive success) would be valuable in evaluation of environmental endocrine disruption.

#### *Reproduction and vitellogenesis*

Although the main neurosecretory centers and the sinus gland in mysids resemble those from decapods, sexual differentiation in juveniles and mysid reproduction are more like those of amphipods and isopods and are strictly linked to the molt cycle [101]. In mysids, embryonic and postembryonic development occurs in the female marsupium and includes five consecutive stages from oviposition to the juvenile stage [26,118,202–205]. Juveniles are liberated immediately before ecdysis of the mother, shortly after which she lays a new batch of eggs in the marsupium. A secondary vitellogenic cycle starts for a new batch of oocytes on the second day of the molt cycle. Secondary vitellogenesis is not only cyclical, as in other crustaceans [206], but also strictly linked to the molt cycle, offering an example of the type 2 pattern (e.g., Amphipoda, Isopoda, and Decapoda) for the regulation of simultaneous gonadal and somatic growth in crustaceans [206,207]. Cuzin-Roudy and Saleuddin [101] published an excellent review on the use of the mysid *S. armata* as a biological model for the study of hormonal control of molt and reproduction, which should be extended for other mysid species. In addition, Wortham and Price [205] and Greenwood et al. [208] published studies on



the in vitro culture of mysid marsupial developmental stages at different temperatures. These assays should be evaluated further as a means of detecting effects of contaminants on marsupial development in mysids.

In general, few studies have been conducted on the effects of contaminants on gonadal maturation of crustaceans [8]; however, much attention has been given recently to vitellogenin, the precursor to the yolk protein vitellin in egg-laying invertebrates and vertebrates, as an indicator of exposure to estrogenic xenobiotics [5,209–216]. Control of vitellogenesis is being studied intensively because yolk is an excellent model for studying mechanisms of hormonal control at the cellular and molecular levels [5,215]. To assess the potential adverse effects of xenobiotics on crustacean reproduction, it is important to measure accurately vitellogenin and vitellin in crustacean models (an overview of crustacean species from which vitellin, vitellogenin, or lipovitellin has been isolated or partially characterized is given by Tuberty et al. [215]). Recently, a quantitative enzyme-linked immunosorbent assay was developed for the mysid *A. bahia* by using polyclonal antisera [215]. In addition, studies are under way to characterize and purify vitellin of the mysid *N. integer* (A. Ghekiere et al., Laboratory of Environmental Toxicology and Aquatic Ecology, Ghent University, Ghent, Belgium, unpublished data). Future laboratory and field studies with mysids are needed to evaluate the use of these immunoassays for investigating effects of xenobiotics on crustacean vitellogenesis. A good example of this is given by Oberdörster et al. [213,214], who reported the effects of chronic pyrene exposure on molting and reproduction assessed in the grass shrimp *Palaemonetes pugio* by using a monoclonal enzyme-linked immunosorbent assay for vitellin. Other studies also have found an impact of xenoestrogens on the production of crustacean proteins (e.g., vitellin and cypris major protein), which are thought to be under estrogen control [5,217]. Future work on sequence determination of vitellogenic genes and their hormonal activity will provide interesting insight into the vitellogenic process in mysids. Study of genomic and nongenomic effects of ecysteroids on ovarian maturation is another potential area of work. Synergistic and antagonistic actions of the different neuropeptides, and the mandibular organ control over molting and reproduction, are other areas requiring further study as a basis for use of crustaceans for endocrine-disruption testing in the future [13].

#### Life-cycle testing and population and field studies

Despite superficial resemblance to decapod shrimps, mysids are more closely related to amphipods and isopods, and are grouped together in the superorder Peracarida. All three orders are good candidates for toxicological testing, and amphipods and mysids are used routinely. However, for endocrine-disruption testing, especially for life-cycle tests, mysids offer clear advantages over amphipods. Most marine amphipods used in toxicological testing must be collected from their natural habitats before use in tests. Although they can be held for a few weeks before testing, they generally are not cultured for tests. Conversely, several mysid species have been cultured in the laboratory and used in life-cycle tests [13]. Several measures of reproductive performance can be used to assess sublethal response in life-cycle testing, including sexual maturity, the time to first brood release, the time required for egg development (and its separate phases), fecundity, brood success, and alterations in reproductive characteristics in popu-

lations [11,58,60,63,108,125,126,128,130,218,219] (Table 2). Inhibited reproduction is the most sensitive, sublethal population response of *A. bahia* chronically exposed to pesticides [63]. Numerous studies have described the use of reproductive endpoints in mysids after toxic exposure and changes in the abiotic environment [15,123,131,220]. Although standard chronic assays, including reproductive endpoints, are described for *A. bahia*, these should be applicable to other mysids, although the longer life cycle in other species may restrict their use in routine testing.

The life history of *A. bahia* is very amenable to demographic modeling because of rapid growth, early sexual differentiation (at 14 d) and reproduction (commencing around 17 d), and frequency of brood production (average of five to seven per female) over the full life span of 90 d [221,222]. These endpoints provide useful information for predicting population-level effects of reproductive toxicants. However, further validation is needed in multigenerational laboratory studies as well as incorporation of other population growth parameters such as density dependence, predation, migration, and competition, before conclusions can be formulated that are relevant for natural environmental conditions. Preliminary transgenerational responses of *A. bahia* to a pesticide acting as a juvenile hormone agonist have been reported [219]. Survival, growth, development, and reproduction of this estuarine mysid were monitored through an entire life-cycle exposure to fenoxycarb and during the second generation without additional exposure. Juvenile mysid growth, and carbon and nitrogen accumulation, as well as mysid survival through the first brood production, were significantly affected by fenoxycarb. On the other hand, maturation time, sex determination, and young production were not significantly altered during the life-cycle exposure. However, second-generation adults, exposed to fenoxycarb only as developing embryos and juveniles, produced fewer young and contained significantly fewer males. These results demonstrate clearly the need for transgenerational studies with mysids to fully understand the potential chronic impact of endocrine disruptors.

Detailed information and the short life cycle of *A. bahia* clearly favor the use of this species in the initial development and further validation of population models based on reproductive endpoints. A concise draft of a detailed review paper has been produced by U.S. Environmental Protection Agency [13] on a recommended protocol and additional data needs for a two-generation life-cycle test with *A. bahia* in the context of endocrine disruptors. In this review, the following endpoints and their preferred methods for quantification are given: survival, molting frequency, reproduction (sexual maturity, time to first brood release, brood size, and number of offspring produced), metabolic disruption, disruption in steroid metabolism, vitellogenin induction, cytochrome P450 levels, and blood glucose levels. Table 2 summarizes the potential endpoints for evaluating environmental endocrine disruption in mysids.

The use of mysids in field studies has been extremely limited. McKenney et al. [92] and Clark et al. [91] performed experiments with caged mysids to evaluate the lethal and sublethal responses of *A. bahia* during field applications of fenitrothion, an organophosphate insecticide. To our knowledge, these are the only published studies on in situ exposures with caged mysids. In addition, studies that have investigated biomarker responses in field-exposed mysids also are very limited

[17,94]. Clearly, field validation of the biomarkers described in this review is a strong research need for the future.

#### *Morphology and histology*

Morphological changes resulting from exposure to contaminants have been documented for many taxa, including arthropods, but have not been considered widely in mysid toxicological studies as a measurable endpoint [13]. Gentile et al. [77] 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 Sea [223–225]. Most of the telson differences may be explained by regeneration of parts damaged by predation and cannot be related directly to physiological perturbations during molting. Still, a genetic or epigenetic basis cannot be ruled out completely [225]. The degree of fluctuating asymmetry in mysids has been proposed as a quantifiable measure of morphological aberrations and is thought to arise from environmental or genetic stress during development [13]. Because the results from earlier studies on morphological aberrations could not give a clear mechanistic explanation for the observed effects, preliminary studies examining different potential characteristics would first have to be performed in mysids, before further considering this endpoint.

#### *Behavioral and other endpoints*

Disruption of mysid swimming and position maintenance behavior has been investigated in laboratory studies with *N. integer* exposed to sublethal concentrations of chlorpyrifos (an organophosphorous pesticide) and cadmium [226–230]. Although the mode of action of the toxicant on swimming remains unknown, the authors speculated that the disruption in chlorpyrifos-exposed mysids was probably due to the inhibitory action on acetylcholinesterase. In addition, Cripe et al. [231] reported a reduction in the maximum sustained swimming speed of *A. bahia* after exposure to sublethal levels of two pesticides. Other authors have investigated the swimming behavior of mysids in laboratory or field studies [232–238]. For mysids, disruption of swimming and swarming behavior may lead to increased predation or displacement from optimum sites in the estuary [43].

Other behavioral responses that have been measured in mysids include feeding activity [39,239–242], grooming behavior [243], burrowing ability [244] and predator–prey dynamics [245]. The use of behavioral responses as a monitoring tool, however, has little utility unless behavioral changes are understood within an ecological context; that is, how well the patterns are understood within the context of an animal's natural life habits and ecological requirements [246] and if the changes can be related clearly to internal residue levels or environmental levels of specific contaminants [247].

Several studies have been published on osmotic regulation in mysids (Webb et al. [248] and references therein) and the interaction between osmoregulation and chemical exposure [66,249]. Other hormonal responses and disturbances in crustaceans, such as color changes (one of the earliest studied phenomena that provided definite proof of a hormone-mediated process in a crustacean), retinal pigments, and limb regeneration are discussed in a review by Fingerman et al. [8]. However, the use of these endpoints in mysids awaits further study.

#### CONCLUSION

This review clearly demonstrates the ecological relevance and the potential use of mysid shrimp as test species for the evaluation of environmental endocrine disruption and as a potential surrogate for many other crustaceans. The highly standardized use of mysids in toxicity testing is an important advantage and research should be directed at evaluating the current standardized endpoints, such as survival, growth, and reproduction, preferably through an entire life cycle, with a number of endocrine disruptors. In this context, a number of reference chemicals, chosen for their possible mode of action (i.e., ecdysone agonist, estrogen antagonist, juvenile hormone agonists, and others) was proposed by DeFur et al. [7] for evaluating relative endpoint sensitivity to potential endocrine-disrupting compounds. In addition, evidence of transgenerational effects has been published and presently a two-generation life-cycle protocol is being investigated with the standard species *A. bahia*. However, an extensive list of nonstandardized endpoints has been published and should be investigated further. Some of these endpoints, such as disruption of ecdysteroid metabolism and embryonic development, might differentiate for invertebrate-specific effects of chemicals. The selection of which mysid species to use will be a balance of its ecological relevance and its ease of use for measuring the selected endpoints. Clearly, the amount of available information and the relatively short life cycle of *A. bahia* favor the use of this species, but its narrow salinity and temperature range limit its use in colder water or low-salinity testing. Various other mysid species are proposed in this review, together with a list of potential endpoints to evaluate the effects of endocrine disruptors in these animals. These should stimulate the scientific community to explore further the use of mysid shrimp as an invertebrate model for the evaluation of environmental endocrine disruption.

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