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In the Next Issue

Animal Models of Addictions to Drugs and Other Controlled Substances

Volume 52, Number 3

Scientific Editors: Kimberlei Richardson and Gabrielle McLemore

Planned contents:

• Maladaptive Alcohol Drinking: Because of, or Despite, Stress? – Woody Hopf, Dennis Sparta, and Antonello Bonci
• Behavioral Abnormalities following Prenatal/Neonatal Nicotine Exposure in Rodents – Sonya K. Sobrian and R. Robert Holson
• Marijuana Dependence: Not Just Smoke and Mirrors – Divya Ramesh, Joel E. Schlosburg, Jason Wiebelhaus, and Aron H. Lichtman
• The Role of Narp in Limbic System Plasticity: Relevance to Drug Abuse – Irving M. Reti, Ashley Blouin, Paul F. Worley, Peter C. Holland, Alexander W. Johnson, and Jay M. Baraban
• Central Amygdala NMDA Receptors, Conditional Gene Deletion, and the Integrative Neuroscience of Learned Opioid Withdrawal Aversion – Michael Glass
• Cracking the Molecular Code of Cocaine Addiction – Serge Ahmed and Paul J. Kenny
• Changes in the Nucleus Accumbens in Response to Methamphetamine Neurotoxicity – Donald M. Kuhn, Mariana Angoa Pérez, and David M. Thomas
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All animals are equal, but some animals are more equal than others.

– George Orwell, Animal Farm

The above well-known quotation from Animal Farm in some ways illustrates humans’ ambivalent view of the relative status of invertebrates and vertebrates in a number of settings: in the research laboratory (differing legal protection), in wildlife conservation (e.g., the appeal of beetles vs. giant pandas), in the home (e.g., swatting flies vs. the humane disposal of mammalian vermin), and in cuisine (e.g., methods for cooking crustaceans vs. chicken). Even the terms “spineless” and “lacking a backbone” are used pejoratively.

Invertebrates are clearly considered by many to be at the “lower end” of a scale of creatures that puts humans at the extreme “upper end,” although the units on the y-axis of this graph are a matter for debate. And within the Vertebrate and Invertebrate subphyla, terminology implying some sort of ranking is often used even in academic publications—fish, which account for about half of vertebrate species, are referred to as “lower” vertebrates (e.g., Sneddon 2004) and Octopus vulgaris (a cephalopod) as an “advanced” invertebrate (e.g., Wells 1978). But Packard (1972) makes a compelling case that the “lower” vertebrate and “advanced” invertebrate are more than a match for each other in evolutionary competition.

The demarcation in the way invertebrates are viewed, and as a consequence treated, extends to the laboratory. While this issue of the ILAR Journal is very welcome and may mark a turning point, it is notable that this single issue covers about 95% of animal species (the more than 1 million invertebrate species) whereas previous issues have been devoted to amphibians, fish, and birds (ILAR 2007, 2009, 2010). This reflects the relative paucity of information in the area of invertebrate welfare and the relative youth of this topic as an area for research, with little study in major areas critical for research, such as criteria for general anesthesia.

Legislation also reflects the invertebrate/vertebrate divide. The United Kingdom enacted one of the first pieces of national legislation covering animal experimentation, the Cruelty to Animals Act of 1876 (Tansey 1998). This Act of Parliament permitted “the advancement of new discovery of physiological knowledge by experiments calculated to give pain” but it applied only to nonhuman vertebrates. It is not unreasonable to suggest that the exclusion of invertebrates indicates that, in contrast to vertebrates, they were considered to have a lesser (if any) perception of pain. Smyth (1978) comments that “invertebrates look far less like us,” so humans may find little to recognize in common with “us” in their appearance or behavior and therefore find it harder to empathize or connect at any level.

The 1876 UK legislation also reflects the species that were commonly used in physiological studies at the time (dogs, cats, rabbits, and frogs). In 1986 the legislation was revised and became the Animals (Scientific Procedures) Act; that revision did not include invertebrates but an amendment in 1993 gave O. vulgaris the same legal protection as vertebrates with respect to experimental procedures (however, to date there have been no studies on O. vulgaris under the authority of the Act).

In the European Union the October 2010 revised Directive (2010/63/EU)1 “on the protection of animals used for scientific purposes” covers “live cephalopods” under Article 1, 3b (however, decapod crustacea—e.g., crabs, lobsters—were included in drafts of the new EU legislation but not in the adopted directive). Member states are required to transpose it into national legislation by November 2012 and apply it by January 2013. Some of the challenges that will need to be addressed in the European Union to comply with this directive are considered in the articles in this issue.

Debating and solving the ethical and welfare issues that are often taken for granted when dealing with laboratory vertebrates in general and mammals in particular for “advanced invertebrates” will provide a useful learning process that will inform best practice when considering invertebrates that lack

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1Available online (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:EN:PDF); this and other websites cited in this Introduction were accessed between February 22 and April 11, 2011.
legal protection. The difficulties likely to be encountered should not be underestimated. Even Russell and Burch (1959, 6) in their classic Principles of Humane Experimental Technique avoided the issue: “The higher invertebrates perhaps deserve a review to themselves, but they raise many problems which would gravely complicate an account which can otherwise be quite general and confident.”

The papers in this special issue cover four major aspects of invertebrates in the laboratory: (1) the use of invertebrates in biomedical and related research, (2) the culture and maintenance of invertebrates, (3) evidence for pain and suffering and their alleviation, and (4) attitudes and their influence on regulation and oversight. Each of these will be reviewed briefly before concluding this introductory overview with some comments about future directions.

Use of Invertebrates in Biomedical and Related Research

…[T]he solution of a physiological or pathological problem often depends solely on the appropriate choice of the animal for the experiment so as to make the result clear and searching.

— Claude Bernard (1949/1865)

There is little doubt that invertebrate species have made major contributions to biomedical research even if judged only by their contribution to Nobel Prizes in the last 50 years, as illustrated in the following examples:

- *Caenorhabditis elegans*: genetic regulation of organ development and programmed cell death (Brenner, Horvitz, and Sulston; 2002);
- sea urchin and clam eggs: key regulators of the cell cycle (Hartwell, Hunt, and Nurse; 2001);
- *Aplysia*: signal transduction in the nervous system (Carlsson, Greengard, and Kandel; 2000);
- honeybee: organization of individual and social behavior patterns (von Frisch, Lorenz, and Tinbergen; 1973);
- squid: ionic mechanism involved in excitation and inhibition in the peripheral and central portions of the nerve cell membrane (Eccles, Hodgkin, and Huxley; 1963).

Wilson-Sanders (2011) provides a comprehensive overview of the diverse species of invertebrates used in biomedical and related research, with tables summarizing their utility in studies of developmental biology, genetics, and diseases.

While the emphasis is on *Drosophila melanogaster* and *C. elegans* primarily because of their tractability for genetic and molecular studies, it would be unwise to neglect the potential of the diversity of over a million species of invertebrates to contribute to biomedical research as model organisms to reveal fundamental biological processes including those involved in disease. Among the reasons to consider invertebrates for use in research are their

1. simpler system than that of vertebrates. This is the argument often applied to studying nervous systems (Usherwood and Newth 1975) such as that of *Aplysia californica* and using species such as the honeybee as behavioral models.
2. unique or larger structure than that of vertebrates. For example, the squid giant axon (originally mistaken for a blood vessel and rediscovered by Young in 1933) and giant synapse revealed fundamental insights into the operation of neurons (see Bullock and Horridge 1965 for references). The brain-controlled skin chromatophore system of cephalopods provides another example of a unique biological system (Hanlon and Messenger 2008).
3. properties not readily exhibited by vertebrates. Pupation in insects provided important insights into the *Hox* (homeotic) family of genes fundamental to the organization of body plans. The striking capacity for tissue regeneration is seen in many invertebrates but particularly in echinoderms, as exemplified by arm regeneration in brittlestars (*Amphiura filiformis*; Bannister et al. 2005) and regeneration of the gut in sea cucumbers (*Holothuria glaberrima*; Mashanov et al. 2010). It is likely that interest in these properties will increase because of the growth of research in tissue engineering for medical applications.

Several drivers are likely to increase the use of invertebrates in research, including the possibility of “replacing” vertebrate models with invertebrates (“relative substitution”; Russell and Burch 1959), although this requires an assessment of sentience so that an animal with “higher” sentience is replaced by one with “lower” (e.g., how would an ethical committee approach the replacement of a trout by an octopus to answer the same biomedical problem?); the relatively low cost of some invertebrates; and different regulatory and ethical considerations (but see below). However, the overriding justification should be a scientific one based on a “cost-benefit” analysis—that is, an assessment of the “cost” to the animal and the benefit, in the broadest sense, of doing the research.

Culture and Maintenance of Invertebrates

The use of invertebrates in research immediately raises the question of their sourcing and maintenance, as discussed by Smith and colleagues (2011). This is a crucially important aspect of the laboratory use of invertebrates as most research requires a supply of “standardized” animals throughout the year and journals require increasingly detailed information about the animals used in a reported study (see below). Sourcing and maintenance (including the correct environmental conditions to ensure optimal health, welfare, and, if required,
reproduction) may be relatively simple for *D. melanogaster* and *C. elegans*, but for marine species obtained from the wild they may be more of a challenge. Advances have been made in the culture of many aquatic invertebrates including cuttlefish (*Sepia officinalis*) and *S. pharaonis* and squid (*Sepioteuthis lessoniana*), but laboratory studies on *O. vulgaris* and *Eledone cirrhosa* usually involve wild-caught animals (e.g., Malham et al. 2002).

The inclusion of cephalopods in the revised EU legislation (as mentioned above) will necessitate the development of guidelines covering all aspects of their provision, maintenance, and welfare. Furthermore, bearing in mind the differences between the squid, cuttlefish, octopus, and nautilus, it is likely that each species will need its own set of guidelines. It will also be necessary to develop humane methods for handling (e.g., atraumatic weighing of cephalopods to monitor welfare may not be that simple and even in familiar mammalian species such as mice different handling techniques have a major impact on stress and anxiety; Hurst and West 2010) and for anesthesia and euthanasia, together with criteria for anesthesia and identification of pain and distress.

**Evidence of Pain and Suffering and Methods for Their Alleviation**

Elwood (2011), Crook and Walters (2011), and Cooper (2011) review different aspects of these difficult and somewhat controversial topics, an understanding of which is essential for both ensuring animal welfare during experimentation and minimizing suffering. Elwood (2011) and Crook and Walters (2011) draw attention to the difference between nociception and pain perception and the survival advantage of the ability to detect, avoid, and learn from noxious stimuli that have the potential to damage tissue. The general hierarchical organization of the central nervous system is well established in vertebrates, but it is appropriate to exercise caution about applying preconceived notions to invertebrate nervous systems.

**Defining Nociception and Pain**

Nociceptors can be identified by a growing range of molecular markers (e.g., those in the transient receptor potential cation channel subfamily V member 1 [TRPV1], or capsaicin, receptor family) and more classically by recording from the afferent nerve with careful characterization of stimulus intensity-response relationships. It is worth recalling that the behavioral consequences triggered by nociceptor activation are encoded in the primary afferent signal transmitted from the tissue to the central nervous system. In vertebrates responses to activation of nociceptors range from localized responses (e.g., edema, vasodilatation) in the affected tissue via axon collaterals if present, to reflex responses mediated via the spinal cord (e.g., limb withdrawal reflex, scratch reflex) and brainstem resulting in a coordinated response often involving several body systems. Complex endocrine responses to “stress” mediated via the hypothalamic-pituitary-adrenal axis require the more rostral projection of the information encoded in the nociceptive afferent, and conscious perception (feeling pain and the associated emotional aspects) requires projection to the thalamus and cerebral cortex.

Humans tend to think of the effects of noxious mechanical, thermal (hot and cold), and chemical stimuli (including pH) on the surface epithelium (“skin”), but both eating and breathing (air or water) can expose the “interior” of the animal to noxious stimuli that may evoke reflex response such as vomiting and coughing (both complex reflex motor responses mediated via the brain stem and widely present in vertebrates) to remove the irritant, and, in the case of ingested toxins, the unpleasant sensation of nausea (requiring cerebral cortical processing in humans) that is important in the genesis of learned aversions (Stern et al. 2011). Researchers have reported ejection of ingested toxic food in the sea anemone (*Lindquist and Hay 1995*) and gastropod *Pleurobrachia* (McClellan 1983) and of gastric contents in the squid *Sepioteuthis sepioidea* (Garcia-Franco 1992). Painful sensations can also arise from noxious stimulation of the viscera (e.g., gut pain) and could be as much a cause of reduced food intake in an invertebrate as in a vertebrate.

**Assessing and Treating Pain/Nociception in Invertebrates**

How can scientists determine whether invertebrates “feel” pain? In considering the issue of assessing pain in animals, Bateson (1991) wrote, “We may feel confident about a mammal or even a bird. But what about a locust or an octopus?” For vertebrates there is generally good knowledge of comparative brain neuroanatomy, allowing a degree of reverse engineering based on knowledge of human brain pain pathways to determine whether similar pathways exist in other vertebrates. Even among vertebrates, however, conclusions about “higher” brain functions based on comparative neuroanatomy have been challenged when considering whether fish feel pain (Rose 2002). Furthermore, as imaging techniques have improved it is becoming clear that even among mammals there are differences between primates and nonprimates in the brain pathways involved in processing information from nociceptors (e.g., Craig 2002). Thus investigators should not underestimate the difficulty in identifying functionally analogous pathways in invertebrates with fundamentally differently organized central nervous systems. It is worth recalling that until the late 1980s there was a common view that human neonates did not feel pain (Fitzgerald and McIntosh 1989).

Molecular, neurophysiological, and behavioral studies have provided evidence for responses to noxious mechanical stimuli in the hermit anemone (*Calliactis parasitica*) and the California sea slug and to noxious mechanical, thermal (heat), and chemical stimuli in the medicinal leech (*Hirudo medicinalis*), *D. melanogaster*, and *C. elegans* (St. John Smith and Lewin 2009 for review).
Behavioral studies provide important insights into the question of pain perception, but knowledge of the way information is processed is important and, together with molecular and physiological (biomarkers of stress such as heart rate, endocrine, and metabolic changes) studies, will be key to identifying endogenous pathways capable of modulating nociception, the transmitters of which provide targets for analgesics (e.g., opioids, cannabinoids, steroids).

Anesthetic and analgesic techniques for invertebrates are relatively poorly developed in contrast to those for vertebrates (and especially mammals), although as Cooper (2011) points out there may be more information in the world literature and a systematic approach is needed for collecting, assessing, and applying that information. There is certainly a need for some consensus on criteria for general (surgical) anesthesia in all invertebrates used in the laboratory and for decapod crustacea and molluscs in particular. Anesthesia has been used only for short-duration manipulation or surgical interventions. Techniques for sustained general anesthesia and maintenance of physiological systems would permit in vivo neurophysiological or functional brain imaging studies, and the lack of such methods limits knowledge of the central processing of nociceptive inputs, especially in cephalopods.3

Finally, although pain (usually assumed to be cutaneous) is perhaps the most common focus from a welfare perspective, animals are susceptible to numerous other unpleasant experiences such as anxiety, asphyxia, dyspnoea, fear, headache, itching, and photophobia. Researchers, animal care staff, and IACUC members should be mindful of all such stressors in considering the welfare of invertebrates in the laboratory.

Attitudes and Their Influence on Regulation and Oversight

“Slugs Displace Bunnies in the Lab” (Davis 2002). This headline accompanied a news article reporting the possibility of replacing the Draize test using rabbits with an irritancy test measuring defensive secretions from slug skin. Although many would view this as a positive development, a spokesman for the British Union for the Abolition of Vivisection is quoted as calling the use of any animal “morally unacceptable” (Davis 2002). This example illustrates the difficulty not only in making judgments about the use of animal in research but also particularly in determining the “relative positions” of vertebrates and invertebrates. This is a complex area and Mather (2011) tackles it by considering the philosophical basis for attitudes toward invertebrates. She describes the contractarian, utilitarian, and rights-based approaches, using diverse examples ranging from the treatment of invertebrates in the kitchen, commercial fisheries, public aquariums, and the laboratory. She accords decapod crustacea and cephalopods special consideration (as is the case in several other articles in this special issue). Mather (2011) concludes that “as invertebrates are better understood, people—whatever their value system—will come to appreciate and take better care of them.”

It is to be hoped that greater understanding will translate to the laboratory and this is likely to be facilitated by ethical review and regulatory frameworks, which are reviewed in the final article, by Harvey-Clark (2011). He presents two IACUC case studies that clearly reveal the particular challenges of research using invertebrates and then provides a useful resource to engage researchers and regulators in confronting some of the key issues.

Where Next?

Scientific journals have a key role to play in encouraging adoption of best practice in the welfare of animals used in research by ensuring that experiments on invertebrates are properly reported. The recently published “ARRIVE” guidelines for reporting animal research (Animal Research: Reporting In Vivo Experiments; Kilkenny et al. 2010, www.nc3rs.org.uk/ARRIVE) provide a clear list of the essential elements that need to be reported for in vivo experiments and, although primarily aimed at studies in vertebrates, can be readily adapted for use in invertebrates (especially decapod crustacea and cephalopods). Comprehensive reporting of methodological aspects is important to enable assessment of welfare and facilitate the systematic gathering of information on ethics, experimental design, housing, husbandry, and adverse events induced by experimental procedures. Many of these aspects are considered in the following articles and especially that by Harvey-Clark (2011).

There seems to be some agreement that in laboratory procedures involving invertebrates in general, and decapod crustacea and cephalopods in particular, the “precautionary principle” should operate at least until there is definitive evidence of their ability to suffer, recognizing that pain may be only one component of suffering. The agreed special position of cephalopods (particularly octopuses and cuttlefish) is already reflected in some national legislation (e.g., Canada). In the European Union it is likely that over the next 2 years such legislation will spur research to address a range of issues—from optimal anesthetic and handling protocols to recognition of signs of pain and distress analogous to those developed for mammals (e.g., Morton and Griffiths 1985)—as cephalopods, at least, will have the same legal protection as is afforded to vertebrates.

Understanding the functioning of phylogenetically ancient brains in highly evolved animals with a fundamentally different organization (at least anatomically) from vertebrates represents a major intellectual challenge and may also prompt reconsideration of some prevailing ideas of consciousness (see Edelman and Seth 2009 for discussion).

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3In the European Union it is likely that the forthcoming legislation will drive the development of a consensus view on many of these aspects for cephalopods.
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Invertebrate Models for Biomedical Research, Testing, and Education

Susan E. Wilson-Sanders

Abstract

Invertebrate animals have been used as medicinals for 4,000 years and have served as models for research and teaching since the late 1800s. Interest in invertebrate models has increased over the past several decades as the research community has responded to public concerns about the use of vertebrate animals in research. As a result, invertebrates are being evaluated and recognized as models for many diseases and conditions. Their use has led to discoveries in almost every area of biology and medicine—from embryonic development to aging processes. Species range from terrestrial invertebrates such as nematodes and insects to freshwater and marine life including planarians, crustaceans, molluscs, and many others. The most often used models are the fruit fly *Drosophila melanogaster* and the minuscule nematode *Caenorhabditis elegans*. Topics in this article are categorized by biologic system, process, or disease with discussion of associated invertebrate models. Sections on bioactive products discovered from invertebrates follow the models section, and the article concludes with uses of invertebrates in teaching. The models reviewed can serve as references for scientists, researchers, veterinarians, institutional animal care and use committees (IACUCs), and others interested in alternatives to vertebrate animals.

Key Words: animal model; bioactive compound; instructional model; invertebrate

Introduction

History and Highlights of Invertebrate Use in Research

Invertebrate models of human genetics and disease first appeared in the scientific literature in the late 19th century. A search of the National Library of Medicine’s PubMed database for the dates 1800–2010 revealed a progressive increase in research involving invertebrates. During 1800–1900, three invertebrate articles appeared. William E. Castle, an animal geneticist, was one of the first researchers to publish studies based on invertebrates—he utilized the sea squirt (*Ciona intestinalis*) as his research model and published his dissertation on this species in 1896. The remaining two articles for the 19th century both appeared in the *Journal of Physiology*; the first reported on the presence of hematoporphyrin in the integument (*Munn 1886*), the second on respiratory exchange in marine invertebrates (*Vernon 1895*).

Research with invertebrates increasingly appeared in the scientific literature during the early 1900s, but it was not until the early 1940s that significant numbers of such papers were published. During the period of 1923–1943, 16 papers were based on invertebrate research as compared to nearly 14,000 during 1943–1963. Research with invertebrates showed further expansion in the 1960s, with over 40,000 papers on invertebrates published from 1963 to 1973. Research in the 21st century has continued to show the growing importance of invertebrates in biological and biomedical research: in 2008–2010 PubMed showed 44,000 papers that used invertebrate species as models for studies of genetics and disease and for drug development and testing.

Another mark of the importance of invertebrates to biomedical research is the number of Nobel Prizes awarded to researchers who have used them, whether as their primary model or one of several animal species. Since the first Nobel Prize for Medicine in 1901, 74 of the awards have been based on animal research, and 18 of these included invertebrate species. *Drosophila* was the model for Thomas H. M organ’s discoveries regarding the role of chromosomes in heredity; he was awarded the Nobel Prize for Medicine in 1933 and gave credit to Charles Woodworth and William Castle as pioneers of the *Drosophila* model. *Caenorhabditis elegans* genome was one of the first to be sequenced and the importance of this organism to scientific advancement is highlighted by three Nobel Prizes awarded in the 21st century. The first, in 2002, was awarded to Sydney Brenner, Robert Horvitz, and John Sulston for their work on similarities in genetic and molecular mechanisms of organ development and programmed cell death between humans and *C. elegans*. Andrew Fire and Craig Mello won the 2006 Nobel Prize for

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1Castle’s papers are available on the website of the American Philosophical Society (www.amphilsoc.org); this and other websites cited in this article were accessed between November 9, 2010, and April 12, 2011.
3Information about this and other Nobel Prize laureates discussed in this article is available online at http://nobelprize.org/nobel_prizes/medicine/laureates.
their studies with *C. elegans* illustrating the conservation of genes between species and elucidating the fundamental mechanisms of gene regulation (Nass et al. 2008). Most recently, the 2008 Nobel Prize for Chemistry was shared by Martin Chalfie, of Columbia University, for his work with *C. elegans* utilizing the green fluorescent protein (GFP) to facilitate his research on touch sensitivity and gene expression (Grandin 2009).

**Applications of Invertebrates in Research**

Biomedical research involving the use of animals has been the cornerstone of medical progress for the past several centuries, but ethical concerns about the use of vertebrates, which are more commonly understood to be sentient animals, have led researchers, veterinarians, and others in laboratory animal science to search for alternatives. Invertebrates can serve as replacements for their vertebrate counterparts in many areas of research, testing, and education.

A new area of focus for invertebrate research is drug development, including the discovery of bioactive products from both terrestrial and marine invertebrates. Invertebrates may also play a pivotal role in toxicity and efficacy testing of new pharmaceuticals for both human and animal diseases, sparing vertebrate animals from preliminary testing. As a result of animal rights proponents’ pressure on professional, preprofessional, and K–12 schools, the use of live vertebrate animals is rare in education. Invertebrates can serve as alternative teaching subjects, providing students with opportunities to observe behavior, anatomy, physiological principles, pathology, results of genetic manipulation, and mechanisms of drug actions.

To assist researchers, veterinarians, and institutional animal care and use committee (IACUC) members searching for models of specific conditions and diseases, this article is organized by biologic system, process, or disease with discussion of associated invertebrate models. Given the genetic and molecular basis of many of mechanisms and diseases, these topics are discussed in the systematic context with additional models found in the tables (which list models not discussed in the narrative, for reference and to illustrate the breadth of invertebrate use).

Because of their long prominence as research models, *Drosophila* and *C. elegans* are the first subjects of this discussion of invertebrate models.

**The Fly and the Worm**

*Drosophila melanogaster*

The fruit fly (*Drosophila melanogaster*) is one of the most studied organisms in the animal kingdom. Cytogenetic research has led to the complete mapping and sequencing of its chromosomes, enabling its use in an array of biological and biomedical investigations (Gilbert 2008). Thanks to the vast number of genetic and molecular tools available for use with *Drosophila*, coupled with similarities in development and behavior, the fruit fly has served as a unique and sensitive model for the study of human genetics and disease (Beckingham et al. 2005). The power of *Drosophila* genetic screens has led to the study of many mutant strains that have helped elucidate visual and behavioral pathways, embryonic patterning, and the development of models for numerous human diseases (Beckingham et al. 2005).

Several databases have been created to contain the genetic records for this tiny but exquisitely precious mine of information. A comparison of one of these databases against over 900 human disease genes (Drysdale and FlyBase Consortium 2008) showed that 714 human disease sequences matched to 548 *Drosophila* genes, of which 153 could be associated with mutant alleles—79 matched to human malignancies and 74 to human neurologic diseases (Reiter et al. 2001).

*Drosophila* modeling has also been used to express protein products found in human disease and to compare the resulting pathologic condition in the fly to the human counterpart (Beckingham et al. 2005). This approach has yielded positive results in three types of neurodegenerative disease: Alzheimer’s, Parkinson’s, and polyglutamine diseases such as Huntington’s chorea; for each, a fly model has been created and used in research (Bonini and Fortini 2003; Iijima et al. 2004; Iijima and Iijima-Ando 2008).

*Drosophila’s* current and potential uses are expected to transcend virtually every area of biologic study (Gilbert 2008) and to play a role in therapeutic trials for drug candidates targeting many human and animal diseases (Beckingham et al. 2005; Gilbert 2008). Beckingham and colleagues (2005), Gilbert (2008), and Korey (2007) provide excellent reviews of *Drosophila’s* uses in biomedical research.

**Caenorhabditis elegans**

*Caenorhabditis elegans* models have many advantages over vertebrate animals for use in biological and biomedical studies. These small worms are highly prolific reproducers with a short generation time, easily grown under laboratory conditions, and inexpensive to care for (Nass et al. 2008; Riddle 1997; Wood 1997). Additionally, *C. elegans* is anatomically simple and has a fully mapped nervous system (White et al. 1976). Humans and *C. elegans* have virtually the same number of genes, and there are many parallels in the ways that these divergent species operate on genetic and molecular levels. As a result, *C. elegans* has become an instrumental model for understanding the molecular mechanisms involved in many human diseases (Nass et al. 2008). It can serve as a model of both forward and reverse genetics, with mutants, transgenics, and knockouts easily created; and worms that express GFP enable in vivo observation of cellular and metabolic processes (Orgensen and Mångos 2002; Nass et al. 2008; Riddle 1997; Wicks et al. 2001).
**Caenorhabditis elegans** has also been used to study basic biological and physiological processes that are common to all animals (Strange 2007). It has served as a model for Parkinson’s, Alzheimer’s, and Huntington’s disease, diabetes, cancer, immune disorders, and the development and testing of therapeutic agents for these diseases (Artal-Sanz et al. 2006a; Faber et al. 1999; Link 2001; Nass et al. 2008; Pujol et al. 2008). *C. elegans* may someday be the model of choice for in vivo testing of new drugs, including high-throughput screening technologies (Silverman et al. 2009).

**Biological Models**

**Developmental Biology**

Invertebrates have been used in the study of embryology since the late 19th century, and several Nobel Prizes have been awarded to scientists who utilized invertebrates in their quest to understand developmental biology. Mchnikov published papers on the embryology of insects during the late 1860s and won the Nobel Prize in 1908. In 1995, the Nobel Prize for Medicine was awarded to three researchers who used *Drosophila* to evaluate genetic control of early embryonic development.

This section provides information on several of the main developmental models; Table 1 lists additional models.

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**Table 1 Selected invertebrate models of developmental biology**

<table>
<thead>
<tr>
<th>Model</th>
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<tr>
<td>Bone morphogenic proteins</td>
<td><em>Drosophila</em></td>
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<td>Calcium signaling</td>
<td><em>Asterinapectinifera</em></td>
<td>Santella et al. 2008</td>
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<td>Cilia regulation of development</td>
<td><em>Caenorhabditis elegans</em></td>
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<td>Developmental glycoobiology</td>
<td><em>Drosophila</em></td>
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</tr>
<tr>
<td>Formation of the nervous system</td>
<td><em>Drosophila</em></td>
<td>Kulesa et al. 2009; Quan and Hassan 2005</td>
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<tr>
<td>Gene regulatory networks</td>
<td><em>Echinoidea</em></td>
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<td>MicroRNA function in embryogenesis</td>
<td><em>C. elegans</em></td>
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<td>Nitrous oxide signaling during neural</td>
<td><em>Locusta, Schistocerca, Acheta, Manduca, Drosophila</em></td>
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<tr>
<td>development</td>
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<tr>
<td>Pattern signaling and retinal</td>
<td><em>Drosophila</em></td>
<td>Baker 2007; Buscarlet and Stifani 2007</td>
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<tr>
<td>development</td>
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<tr>
<td>Pituitary patterning</td>
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<td>Veitia and Salazar-Ciudad 2007</td>
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<td>Regulatory switches</td>
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<td>Semaphorin in developing nervous</td>
<td><em>Caelifera (grasshopper)</em></td>
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<tr>
<td>Tubulogenesis</td>
<td><em>Drosophila</em></td>
<td>Kerman et al. 2006</td>
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*aThese models are provided for reference; discussion of other models is provided in the text.*

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*A number of invertebrates have been used in developmental biology, but the two primary organisms are *Drosophila* and *C. elegans*, with *Drosophila* most often used. During the past 20-plus years, genetic studies using *Drosophila* have elucidated the regulatory mechanisms that control development of the embryo (Baker and Thummel 2007). Genetic screens in *Drosophila* have led to the discovery of the signaling pathways Notch, Wingless, and Hedgehog and furthered knowledge of vertebrate development and disease (Bier 2005). Genetic alteration of fly embryos has provided information about a variety of biological mechanisms (Shen et al. 2007). Planar cell polarity studies in *Drosophila* and other arthropod embryos have shown that coordination of cell polarization occurs in the development and function of many organs, particularly in epithelial cells such as the gut epithelium, which needs to move secretions (Simons and Mlodzik 2008). *Drosophila* models of planar cell polarity have focused on the development of hair patterns, the eye, and the cochlea and have supported comparisons to mammalian development pathways for each.

Because of the close similarities between *Drosophila* and vertebrate cardiogenesis and the conservation of key genes, the fly’s heart serves as an excellent model of cardiac development and disease (Medioni et al. 2009). Study of cardiac development in the fly has led to understanding of the
molecular and cellular mechanisms that underlie morphogenesis and has elucidated the genetic control of cardiac physiology (Medioni et al. 2009). The *Drosophila* heart model may also play a role in efforts to identify unknown genes and the regulatory networks that contribute to normal heart development and function (Tao and Schulz 2007).

Other aspects of *Drosophila* anatomy and function have proven useful in research. Because of the similarities between *Drosophila* and mammalian mechanisms of hematopoiesis, the fly serves as a model for vertebrate blood cell development (Crozatier and Meister 2007; Crozatier et al. 2007). The developing *Drosophila* eye has been used to study the Notch and tyrosine kinase signaling mechanisms, which direct cell fates during development; recruitment of factors (e.g., transcription factors for gene expression) is important in animal development (Voas and Rebay 2004). The fly’s excretory system has been used to study the development and differentiation of the renal system across species (Denholm and Skaer 2009).

In *C. elegans* the pharynx (foregut) has served as a key model for the study of general organ development, with high throughput genome scale analysis evaluating the involvement of genes and the regulatory networks that control gonadal development (Basson and Horvitz 1996). The gene *lin-9*, which affects signal transduction pathways that control gonadal development in the nematode, has counterparts in many species, making *C. elegans* a useful model for studies of developmental biology and intercellular signaling mechanisms (Bietel et al. 2000). Vulval development in *C. elegans* can be used as a model for understanding the roles of chromatin remodeling in multiple development pathways (Andersen et al. 2006). Additionally, through study of the proteins and genes involved in this development, scientists may be able to recognize how complex proteins could become targets for cancer therapy (Andersen et al. 2006). *C. elegans* has been the primary model used in high-throughput genome scale analysis evaluating the involvement of genes in tissue development (Ge et al. 2006).

**Other Invertebrate Species**

In recent years deuterostomes and other marine invertebrates, as well as insects, have been increasingly used both to understand the evolution of these organisms and to shed light on developmental processes in higher animals including humans (Arendt et al. 2008; Bicker 2005, 2007; Darling et al. 2005; Holland and Gibson-Brown 2003; Isbister and O’Connor 2000; Lowe 2008; Pourquie 2000; Swalla 2006; Wessel et al. 2010). Grasshoppers (*Dissosteira carolina* and others) have been used to study neural cell development, specifically, the genetics and development of axons through evaluation of grasshopper limb bud growth cones (Isbister and O’Connor 2000). The molecule nitric oxide (NO) is thought to play a role in the regulation of neuronal growth and migration, and gastropod molluscs and embryonic grasshoppers serve as models for elucidating NO signaling pathways (Bicker 2005, 2007).

For over a century the sea squirt (*Ciona intestinalis*) has been used as a model for studying animal development (Holland and Gibson-Brown 2003; Passamaneck and Di Gregorio 2005). This animal may also play an important role in studies to determine how to solve comprehensive gene networks in chordates and evaluate how these networks control development (Davidson 2007). The sea star, or starfish (*Asterias forbesii*), has been utilized in developmental research related to reproductive processes. Basic techniques (e.g., oocyte isolation, microinjection, and polymerase chain reaction) have been developed so that new researchers can easily work with these animals (Wessel et al. 2010). The genome of the starlet sea anemone (*Nematostella vectensis*) has been the focus of a genome project; as a result, this organism has been proposed as a model for molecular and evolutionary biology (Darling et al. 2005). Somitogenesis, a multistep patterning process in vertebrates, has been modeled in a variety of animal models from mice to protostomes and deuterostomes; research with invertebrates has indicated that the genetic machinery responsible for this segmentation is conserved throughout the animal kingdom (Pourquie 2000).

**Stem Cell Biology**

Stem cell biology has its roots in research performed with invertebrates: *Stammzelle*, the German word for stem cell, was created based on research using crustacean primordial germ cells in the 1890s by the German researcher Valentin Haecker (Kohlmaier and Edgar 2008). Invertebrates continue to be key models for understanding many of the characteristics and genetics of stem cells, including their pluripotency and ability to self-renew through proliferative growth (Kohlmaier and Edgar 2008).

The stem cell niche has been evaluated through study of *Drosophila* gonads. This research has furthered knowledge of the structure of the niche and its ability to produce signaling pathways, which lead to stem cell self-renewal (Lin 2002; Palasz and Kamiński 2009). The germline of *C. elegans*—specifically, its distal tip cell, the foundation for the animal’s stem cell niche—has also served as a model for stem cell biology (Byrd and Kimble 2009; Hubbard 2007). Using genetic analysis of *Drosophila* muscle and satellite stem cells, researchers have modeled the biology of vertebrate muscle stem cells (Figgec et al. 2007). Planarians, uniquely, regenerate from their stem cell system and thus serve as a model for gene and stem cell regulation (Agata 2003). Cnidarians (*Hydra, Nematostella*) have been used to study stem cell signaling pathways and other mechanisms of stem cell biology and function (Watanabe et al. 2009).

**Endocrine Function and Metabolism**

*Drosophila, C. elegans,* and marine invertebrates have been useful in the study of endocrine and metabolic diseases.
*Drosophila* is an increasingly common model for understanding metabolism across species boundaries (Baker and Thummel 2007). The ability to study the genetics of metabolic function in this small but sensitive model has provided insights into the central regulatory pathways of vertebrates.

Study of the fly has also elucidated the pathogenesis of human metabolic diseases such as diabetes and obesity. *Drosophila* has served as an excellent model for the study of diabetes, lipid metabolism, and other mechanisms of metabolism, including sterol adsorption and trafficking defects that occur in Niemann-Pick type C disease (Baker and Thummel 2007). Both diabetic and obese flies, as well as genetically “lean” and hypoglycemic phenotypes, have been created as models for human disease (Bharucha 2009). *Drosophila* is not suitable for the study of all aspects of human metabolic control; for example, the leptin signaling pathway is not present in the fly. But insulin signaling is very similar in flies and humans, making *Drosophila* an ideal model to study the ways insulin regulates metabolism (Teleman 2009).

Transgenic flies have been developed to study the molecular endocrinology of neuroendocrine signaling and control (Dow 2007). Insulin receptor–like signaling pathways in flies regulate a transcription factor known as DAF-16/FOXO, a “master regulator” of many biological mechanisms (Lin et al. 1997; Ogg et al. 1997). Both *Drosophila* and *C. elegans* can serve as models for clarifying the mechanisms of this transcription factor, which controls lifespan, metabolism, and stress responses, and, in the worm, regulates the dauer stage (when the animal goes into a state of hypometabolism) (Mukhopadhyay et al. 2006). *C. elegans’* dauer stage can be used to model protein targets in the stress responses of higher animals, and study of this stage may lead to recognition of therapeutic targets for human diseases such as ischemia, insulin resistance, neurodegenerative diseases, and cancer (Lant and Storey 2010).

*Caenorhabditis elegans* has shown promise as a model organism for studying AMPK (5′-AMP-activated protein kinase) signaling (Beale 2008), because it has demonstrated evidence of having AMPK pathways. AMPK is often called “the master metabolic switch” as it plays a key role in regulating metabolism, protein synthesis, and cell growth and in mediating the actions of hormones (Beale 2008). Research into the endocrine signaling pathways and hormone production control in *C. elegans* has yielded insights into similar pathways in humans (Beckstead and Thummel 2006).

Hedgehog signaling studies in *Drosophila*, nematodes, and mice have shown that this pathway inhibits the amount of fat in the body; thus, manipulation of the pathway may be useful in treating hyperlipidemia, obesity, and type 2 diabetes in humans (Suh et al. 2006, 2007). Studying the gene Adipose, Suh and colleagues (2007) determined that both mice (Adp) and flies (adp) heterozygous for the gene are obese and insulin-resistant, indicating that this gene has an antiadipogenic ability (Gilbert 2008). Other studies have used the fly as a model for human fatty liver disease; the fly oenocyte, which is comparable to the mammalian hepatocyte, participates in lipid metabolism by producing enzymes that lead to ketogenesis (Arguier and Leopold 2007; Downer 1985). In addition to serving as a model for lipid metabolism, the fly may be an effective model both in efforts to discover more genes involved in obesity and diabetes and in the screening of therapeutic agents developed for lipid-based diseases (Gilbert 2008).

Other endocrine models include the silkmoth and the sea squirt. The silkmoth (*Bombyx mori*) has an insulin-related peptide gene that is similar to human preproinsulin (Yoshida et al. 1998). The sea squirt (*Ciona intestinalis*) has many analogues to hormones (e.g., gonadotropin-releasing hormone, insulin, and insulin-like growth factor [IGF]) found in higher animals and thus may prove to be a useful model for understanding the function of these hormones and for the study of neuroendocrinology (Sherwood et al. 2006).

And *Drosophila* has insulinlike peptides that serve as hormones, neurotransmitters, and growth factors (Wu and Brown 2006).

**Immunology**

**Allorecognition and Adaptive Immune System**

Allorecognition and its molecular basis have been studied in ascidian urochordates such as the sea squirt and the star ascidian (*Botryllus schlosseri*) (Ben-Shlomo 2008). As they most likely share common ancestors with vertebrates, research into their patterns of self-recognition have provided insight into the development of the immune response of vertebrates.

Even though urochordates are not recognized to have an adaptive immune system, some genes in organisms such as *C. intestinalis* are related to those in vertebrates and give rise to adaptive immunity (Du Pasquier et al. 2004). Studies using urochordates and other invertebrate deuterostome model systems have provided information on mechanisms of antigen receptor diversification and immune system development relevant to vertebrates (Eason et al. 2004). Gene rearrangement studies in other species, such as lampreys and molluscs, have also provided information about differences and similarities between adaptive and innate immune systems (Flajnik and Du Pasquier 2004). Mckittrick and De Tomaso (2010) provide an excellent review of the molecular mechanisms of allorecognition in *B. schlosseri*.

Both colonial and solitary free-living reef corals such as *Fungia scutaria* show evidence of histocompatibility and allorecognition. Sea anemones from the order Actiniaria also have similar immune systems (Jokiel and Bigger 1994). These animals, along with *B. schlosseri*, can be used to study the evolution of the immune system and could serve as models for screening new therapeutics targeting cellular immunity and transplant rejection. The star ascidian has also been suggested to serve as a model for maternal-fetus allorecognition issues, as the organism has a natural killer (NK) cell similar to human uterine NK cells (Lightner et al. 2008).
Immunity and Response to Infection

Innate immunity is the invertebrate’s primary defense against infectious organisms, and this system has similarities to that of vertebrates (Mager et al. 1999). The fly’s sensing and signaling cascades during infection have stimulated the use of *Drosophila* as a model for innate immunity and response to infection (Feerrandon et al. 2007; Royet et al. 2005). Similarly, *C. elegans’* innate immunity has been used to study immune defense and the role of cellular stress in an organism’s response to infection (Millet and Ewbank 2004). The nematode has also been used to model the activation of genes in response to infection (Gravato-Nobre and Hodgkin 2005). *Octopus maya* has been suggested as a model for immune responsiveness because of its ability to become infected by pathogenic organisms (Van Heukelem 1977).

Macrophages

Invertebrates have played a key role in the history of the macrophage. The term “macrophage” (phagocyte) was coined by Ilya Illyich M echnikov, a comparative embryologist and winner of the Nobel Prize in 1908. He observed in starfish larvae a group of cells that had unusual characteristics—the ability to move in tissue: after introducing small rose thorns into the larvae, he noted the next morning that the thorns were surrounded by the mobile cells. He further studied this phenomenon using the freshwater flea (*Daphnia magna*), exposing it to fungal spores: the spores were attacked and isolated by the *Daphnia* macrophages.

Macrophage-like cells are present in many species of invertebrates. Often they originate from mesenchymal, endothelial, or fibroblastic cells that differentiate into phagocytes (Naito 2008). For example, *Hydra*, a member of the phylum Cnidaria, has cells with phagocytic capability that play a role in the animal’s ability to recognize “self” (Bosch and David 1986; K obyakawa and Koizumi 1997; Naito 2008) and, thus, make it a useful model of graft rejection. Molluscs also have cells that can act as macrophages, but the origin of these cells is mesenchymal, not hematopoietic, indicating divergent paths of differentiation (Naito 2008).

Neuroimmunology

Neuropeptides can transfer information from the nervous system to the immune system, perhaps serving as regulators of immune response (Stefano et al. 1991, 1996, 1998). Opioid peptides are present in the neural tissues of several molluscs, including the blue mussel (*Mytilus edulis*) (Stefano and Leung 1982) and the garden snail (*Helix aspersa*) (Marchand and Dubois 1986), and are involved in immune processes. These animals can thus serve as models to explore connections between the immune system and neural regulation (Liu 2008).

Infectious Disease

Viruses have been found in the genome of many species including invertebrates (Becker 2000). The gypsy element, which infects *Drosophila*, was the first retrovirus recognized in invertebrates and may be an ancestral precursor of vertebrate retroviruses (Pelisson et al. 2002). The gene *flamenco* modulates gypsy in *Drosophila*, and the interaction of the two genes has provided an excellent model for the study of the genetic relationships between virus and host (Bucheton 1995).

*Drosophila* has also served as a model for other host-parasite relationships—for example, as a host for the *Burkholderia cepacia* complex (Bcc), a group of bacteria that contribute to severe health risks among humans with cystic fibrosis (Castonguay-Vanier et al. 2010). Bcc studies performed in *Drosophila* showed similar virulence patterns to those observed in mammals, indicating that the fly is a useful alternative model for such studies (Castonguay-Vanier et al. 2010).

Virulence screening for agents with biological warfare potential may be possible in *Drosophila*. Recently, genomewide virulence screens were performed for *Francisella novicida* using *Drosophila* as the host. Researchers identified many similarities in gene function between flies and mammals but also found that a considerable number of the virulence factors that play a role in mammals do not in the insect model (Ahlund et al. 2010).

Pathogenic fungi have been studied in worms and insects. Several major fungal pathogens (e.g., *Aspergillus*, *Candida*, *Cryptococcus*) infect and kill roundworms, fruit flies, and wax moths. Because the genes that modulate virulence in these invertebrates are remarkably similar to those of humans, these three species have been evaluated as alternatives to mammalian models of fungal disease (Chamilos et al. 2007). Researchers have specifically used *C. elegans* to study bacterial and fungal virulence, pathogenicity, and mechanisms of host defense against invaders (Fuchs and M ylonakis 2006). Conservation of virulence mechanisms between roundworms, fruit flies, and wax moths and higher animals has led to the increased use of these and other invertebrates in virulence studies (O’Callaghan and Vergunst 2010).

Because of the similarities between human and invertebrate infections, insects are considered a model of choice for studying opportunistic microorganisms. Insect models can allow for rapid screening of potentially opportunistic infections while minimizing concerns about the ethics of vertebrate animal experimentation (Scully and Bidochna 2006). O’Callagan and Vergunst (2010) provide a review of the use of *Drosophila* and *C. elegans* as models for infectious disease.

Other invertebrates used to model infectious disease include *Daphnia* and marine shrimp species. *Daphnia* is recognized as a model system to study host-parasite interactions for diversity of parasites, from bacteria to helminths (Ebert 2008). Study of viral diseases in marine shrimp has
led to discovery of RNA interference-based therapies that may result in similar therapies for viral diseases of higher animals, including humans (Krishnan et al. 2009).

Memory, Learning, and Behavior

The California sea slug (Aplysia californica; Glanzman 2006, 2008, 2009), opalescent sea slug (Hermissenda crasicornis; Aikon 1987), and pond snail (Lymnaea stagnalis; Lukowiak et al. 1996)—all gastropod molluscs—have served as models in studies of neuronal mechanisms of learning and memory. Such studies have also used many cephalopod molluscs, especially the coleoid group, which includes octopuses, cuttlefishes, and squids.

The octopus has been an effective model in studies of behavioral communication. Octopuses use body color and tentacle positions to indicate their attitude toward approaching prey or other octopi (Pribram 1973)—their stance and color are comparable to the facial expressions and body language of monkeys and humans. Octopi have also proven useful in reversal learning experiments, with comparable results to similar studies in rats (Sutherland and Mackintosh 1971).

The chambered nautilus (Nautilus pompilius), one of the most ancient cephalopods, has been used in Pavlovian conditioning studies (Crook and Basil 2008), and Aplysia and H. crasicornis have been used to study classical and operant conditioning (Baxter and Byrne 2006). Studies in Aplysia have led to elucidation of the molecular mechanisms involved in all phases of implicit memory (Hawkins et al. 2006), and the mud flat crab (Chasmagnathus convexus) has served as a model in similar studies (Romano et al. 2006). Decapod crustaceans have been used to study aggressive behaviors (Barron and Robinson 2008).

Among insects, honeybees (Apis mellifera) have a long and rich history as research models—Aristotle studied them and recorded his observations of their behavior (Elekonich and Roberts 2005). More recently, von Frisch (1967) studied the bee’s behavior and communication through dance. Even though the brain of the honeybee is less than 1 cubic millimeter in diameter, it is very accessible for study and can serve as a model for many higher-order cognitive processes (Giurfa 2006, 2007). Investigators have studied learning, memory, and sensory processing by focusing on honeybee patterns of navigation and foraging, as bees follow several routes to and from their nest to their preferred blossoms, requiring them to recall memory sequences and respond to memory cues (Chittka et al. 1999; Collett 2005; Menzel 1999; M enzel and Giurfa 2006). Furthermore, the complete sequencing of the honeybee genome makes this tiny creature an excellent research model (M enzel et al. 2006). The study of gene expression and of the endocrine, metabolic, and neural physiology of bee colonies is revealing how these and other animals respond to their environment (Elekonich and Roberts 2005). Scientists are also studying neural mechanisms of reward reinforcement in honeybees (Gil et al. 2007).

A nts have been used to study the molecular genetics of social behavior and adaptation (Robinson et al. 1997) and to discover associative links between long-term memory and visual stimuli (Collett and Collett 2002). In the cricket (Gryllus bimaculatus), the sensory system and ability to respond to environmental stimuli have been compared to the complex responses of vertebrates to their environments (Jacobs et al. 2008). Drosophila has served as a model for olfactory learning and memory, partly because of the ability to chemically mutate genes in this organism (Glanzman 2005; Mc Guire et al. 2005). With the ability to manipulate Drosophila genes and to model aggressive behavior and its genetic basis (Robin et al. 2007), study of the fly may lead to understanding of the genetic and molecular basis of human emotions (Iliadi 2009). The fly also serves as a model of several human cognitive disorders and may be useful in the evaluation of drug therapies for them (Skoulakis and Grammenoudi 2006).

C. elegans has been the subject of studies on the behavior and genetics of habituation, the use of long- and short-term memory for learning (Gilles and Rankin 2009), and the neural and molecular mechanisms of behavior (Schaefer 2005; Sengupta and Samuel 2009). Its dauer stage serves as a model for the molecular mechanisms behind stress response behavior (Lant and Storey 2010).

Musculoskeletal Disease

The metabolism of proteins in the flight muscles of the tobacco hornworm (Manduca sexta) has been studied (Tischler et al. 1990), and the species has been used in space flight research to understand the effects of low gravity on muscles. Studies using both Drosophila and C. elegans have enhanced understanding of muscle formation and degeneration (Kim et al. 2008); for example, research with C. elegans contributed to knowledge of regulatory muscle proteins and the maintenance of muscle under certain physiological and pathological conditions (K im et al. 2008). Striated muscles of the leech (Pon tobdella maricata) have dystrophin-associated proteins that have striking similarities to those in humans; thus, leeches and other annelids may be useful in the study of interaction sites for muscular dystrophy-associated proteins (Royuela et al. 2001).

Neural and Neuromuscular Systems and Disease

Drosophila and C. elegans

The fruit fly and the nematode are the primary invertebrate models for many areas of neurobiological study.

Work with Drosophila has a long history and includes the cloning of the first potassium channel; demonstration of transient receptor potential (TRP) channels through cloning of trp; discovery of the genes responsible for the biological
clock; and studies of courtship behavior, sleep patterns, learning, alcoholism, and aggression (Foltenyi et al. 2007; Gilbert 2008; Grosjean et al. 2008; Vossall 2007). The fly serves as a model for many specific disorders. For example, there is a homologue or orthologue in Drosophila for most of the approximately 300 genes that participate in human retardation (Inlow and Restifo 2004), so the mysteries surrounding Fragile X retardation may be unraveled through study of the fruit fly model (Pan and Broadie 2007). Drosophila models will also play a vital role in the identification and evaluation of new therapies for neurological diseases, providing a preliminary animal model before the use of mammals (Marsh and Thompson 2004, 2006; Whitworth et al. 2006).

C. elegans is an important model for understanding the pathophysiology and molecular mechanisms of neurodegenerative diseases such as Alzheimer’s, Parkinson’s, and Huntington’s (Johnson et al. 2010; Troulinaki and Tavamarakis 2005).

**Tauopathies**

Drosophila and C. elegans have served as models for Alzheimer’s disease and other tauopathies (Crowther et al. 2005; Luheshi et al. 2007; Wheeler et al. 2010). Alzheimer’s is postulated to result from amyloid toxicity that initiates aggregation of proteins into amyloid fibrils (Luheshi et al. 2007). In the fly model, Crowther and Luheshi found that protein aggregation in fly brains leads to dysfunction of neurons and neuronal degeneration, which progresses to memory loss and shortened lifespan, hallmark symptoms of Alzheimer’s (Crowther et al. 2005; Luheshi et al. 2007). Transgenic C. elegans created to express human beta amyloid peptide (Abeta) develop intracellular deposits with the classical Alzheimer’s amyloid fibrillar component, indicating the usefulness of this organism in the study of Alzheimer’s (Link et al. 2001). The introduction of mutant human tau into Aplysia neurons grown in culture induced neuropathologic lesions typical of Alzheimer’s, indicating that Aplysia can serve as a model for this disease (Shemesh and Spiru 2010).

Drosophila is contributing to knowledge of Niemann-Pick type C (NPC), a tauopathy in which an overabundance of free cholesterol in the brain leads to neurodegeneration (Patterson 2003; Vance 2006). The pathogenesis of NPC is not well understood, but if either NPC1 or NPC2 is mutated in the human, NPC is likely to result (Patterson 2003). To determine whether the fly could serve as a model of NPC, researchers reviewed the fly database (flybase.org) and identified potential NPC models (Fluegel et al. 2006; Huang et al. 2005). As in humans, the presence of npc1 is necessary for sterol homeostasis; when it is mutated, the flies show molting defects. Study of Npc1a function in the fly has led researchers to hypothesize that NPC1 may play a vital role in the transport of sterol to the endoplasmic reticulum and mitochondria (Huang et al. 2005). Drosophila also contains a family of Npc2a genes whose mutation results in neurodegeneration (Huang et al. 2007). Similarities between NPC in flies and humans suggest that the fruit fly can also serve as a therapeutic test model for this devastating disease (Gilbert 2008).

**Parkinson’s, Huntington’s, and Other Neurological Diseases**

The discovery of Drosophila homologues of Parkinson’s-associated loci has resulted in the use of pink1 and parkin fly mutants as models for Parkinson’s disease (PD) (Greene et al. 2003; Laurent 1999; Pesah et al. 2004). PD has also been studied using C. elegans, which has not only dopamine neurons, receptors, transporters, and the enzymes that catabolize dopamine (Nass et al. 2002, 2008; Nass and Blakey 2003; Sulston et al. 1975; Wintle and Van Tol 2001), but also orthologues of most of the human Parkinson’s genes (Nass et al. 2008). As a result, various genetic manipulations with worms have produced models of the genetics and molecular pathways of Parkinson’s. For example, C. elegans has been used in human gene expression assays to identify genes associated with PD. The genes are overexpressed in dopamine neurons; if they are PD-associated, the worms show signs of neurodegeneration (Berkowitz et al. 2008). Mitochondrial dysfunction plays a role in the pathology of PD (Korey 2007), and work with the C. elegans model has contributed to understanding of intercompartmental proteostasis and its role in cellular function (Kirstein-Miles and Morimoto 2010). Both Drosophila and C. elegans have been proposed as alternatives to vertebrate animals in the screening of drugs with therapeutic potential for PD (Plenaar et al. 2010).

Drosophila models show promise for elucidating many other neurodegenerative diseases including Huntington’s (HD) (Gilbert 2008) and neuronal ceroid lipofuscinoses (NCL), neurodegenerative disorders associated with accumulations of cellular material and the formation of inclusions in lysosomes (Korey 2007). Research using Drosophila homologues for HD and NCL has assisted efforts to understand the pathogenesis of both diseases (Korey 2007).

Fly homologues have also been found for several hereditary spastic paraplegias (HSPs), disorders that exhibit symptoms such as progressive weakness of the legs due to axonal degeneration (Korey 2007). For example, Kennedy’s disease results in progressive muscle atrophy and weakness in males and is caused by an androgen receptor (AR) mutation. Research on flies on which an AR-like gene is overexpressed in neuromuscular junctions in lysosomes (Korey 2007). Research using Drosophila homologues for HD and NCL has assisted efforts to understand the pathogenesis of both diseases (Korey 2007).

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Transgenic Drosophila models exist for Alzheimer’s, Fragile X, HD, Kennedy’s, Machado-Joseph, NCL, spinocerebellar ataxia, and spinal and bulbar muscular atrophy (Celotto and Palladino 2005). An unusual use of transgenic technology in flies has been the production of a transgenic, prion-induced neurodegenerative disease in Drosophila. The
resulting “mad fly” disease, characterized by locomotor dysfunction and shortened lifespan, is similar to Gerstmann-Sträussler-Scheinker syndrome, an inherited prion disease in humans (Chandran and Lewis 2007).

Human mitochondrial encephalomyopathy disorders include neuropathy, ataxia, retinitis pigmentosa, Leigh syndrome, and familial bilateral striatal necrosis, all of which have components of neurological and muscular dysfunction coupled with tissue degeneration (Korey 2007). A number of fly mutants have phenotypes that can be directly related to the symptoms observed in this complex set of disorders. For example, flies with mt:ATPase6 mutation show shortened lifespan, progressive degeneration of flight muscles, and neural dysfunction (Celotto et al. 2006). Indicative of even closer correlation with the human disease, these animals have mitochondrial dysfunction and reduced ATP (adenosine triphosphate) production, both of which characterize the human disease.

Recent reviews of Drosophila models of neurodegenerative diseases are available (Lu 2009; Lu and Vogel 2009).

Other Invertebrate Models

Because of their giant axons, fibers, and synapses, octopi and squid are often used as research and teaching models for neurobiology (Grant et al. 2006; Van Heukelem 1977). The Yucatan octopus (O. maya), which can easily be grown under laboratory conditions, is used as both a teaching and research model for comparative psychology and neurobiology (Van Heukelem 1977). Additionally, octopi and squids are excellent models for neural electrophysiology, neurochemistry, and neurosecretion (Packard 1972; Sanders et al. 1975; Young 1967, 1971). Research utilizing the long-finned squid Loligo pealei in nerve conduction studies garnered Andrew Huxley and Alan Hodgkin the 1963 Nobel Prize for Medicine. Because of its giant fiber system, Loligo has also been proposed as a model for neurodegeneration and dementia (Grant et al. 2006). The somatogastric nervous system of decapod crustaceans (e.g., lobsters, crayfish, and crabs) can be used for modeling the neuromodulator actions of vertebrates (Stein 2009).

The brain of freshwater planarians (Platyhelminthes) may appear to be very simple in comparison to that of humans, but these organisms have many neural genes and transcription factors that are homologous to those that cause pathology in humans. Furthermore, planarians are known for their ability to regenerate—even their central nervous system (CNS)—and researchers using gene silencing techniques hope to elucidate the molecular mechanisms and genes that enable the animal’s regeneration (Cebria 2007). Understanding this process may yield important information for treating human CNS injuries and disease.

The highly sensitive auditory system of the cricket can be an effective model in studies of the development of dendrites and their response to injury (Horch et al. 2009). The cricket has also contributed to the understanding of adult neurogenesis (the production of new neurons throughout life), which occurs in most species including humans (Cayre et al. 2007).

Pathophysiology

Aging and Healthspan

C. elegans has been used to study genetic regulation of lifespan. Some C. elegans are endowed with multiple copies of the gene sir-2.1 (counterparts occur in humans), which works in combination with the transcription factor DAF-16 to produce greater longevity. The longevity is based on 14-3-3 proteins, which activate a pathway that increases resistance to both oxidative and genotoxic stress (Berdichevsky et al. 2006). Evolution of lifespan and the biology of aging have also been studied extensively in Drosophila (Grotewiel et al. 2005).

Other models have been established in Drosophila and C. elegans to evaluate insulin/IGF-1 signaling (IIS) pathways, the role of the intestine in germline signaling, and the ablation of germline precursors in longevity—all of which are involved in the regulation of lifespan (Mukhopadhyay and Tissenbaum 2007). In one study, germline precursors were ablated in C. elegans, which lived up to 60% longer than their unaltered counterparts (Hsin and Kenyon 1999). Because the IIS pathway participates in regulating lifespan in both invertebrates and mammals, Drosophila and C. elegans are effective models to study this pathway and the mechanisms through which it can increase lifespan (Giannakou and Partridge 2007; Piper et al. 2008). The Yucatan octopus has also been suggested as a model for healthspan and the effects of aging (Van Heukelem 1977).

Many studies using invertebrates have focused on the beneficial effects of reduced caloric intake on lifespan (Kennedy et al. 2007; Masoro 2005). Similar studies in mice, flies, and monkeys have shown rejuvenation of the immune system when the animals were subjected to caloric restriction (Nikolich-Zugich and Messaoudi 2005), thus providing the opportunity for extended life. Additional models of aging and lifespan are shown in Table 2.

Apoptosis

The word apoptosis translated from the Greek means “falling or dropping off” and was used by the Greeks to refer to petals and leaves falling from flowers and trees (Collins English Dictionary 2009). It was not until 1842 that the German scientist Carl Vogt described the modern-day pathological process of apoptosis after studying the tadpole of the midwife toad Alytes obstetricans (Vogt 1842). The term then fell into disuse until 1965, when John Foxton Ross Kerr of Australia recognized apoptosis, as distinguished from traumatic cell death, while performing electron microscopy of rat liver cells affected by acute injury (Kerr 1965). Kerr’s
later work with colleagues Jeffrey Searle, Andrew Wyllie, and Alastair Currie furthered understanding of the role of apoptosis in both normal and disease processes (Kerr et al. 1972; O’Rourke and Ellem 2000).

Subsequent study of C. elegans was pivotal in the quest for understanding the genetics of apoptosis. The 2002 Nobel Prize in Medicine was awarded to three researchers—Sydney Brenner, H. Robert Horvitz, and John E. Sulston—for their work on genetic regulation of organ development and programmed cell death, for which their primary model was C. elegans. They were also able to determine that similar genes are present in humans to control apoptosis.

Pathways for apoptosis are conserved throughout most of the animal kingdom, from invertebrates to humans, but C. elegans and Drosophila remain models of choice, especially for genetic, biochemical, and molecular-mechanistic studies (Bao et al. 2005; He et al. 2009; Xu et al. 2009). Moths are also useful in the study of apoptosis and in particular programmed cell death in skeletal muscles (Schwartz 2008).

Tumor necrosis factor (TNF), a cytokine involved in programmed cell death, has functional analogies in the earthworm Eisenia fetida. The worm counterpart to mammalian TNF, while not homologous, has similarities in function based in lecithinlike activity/domains (Beschin et al. 2004), so the earthworm may be a useful model for studying apoptosis.

**Cancer**

Drosophila and C. elegans are long-established models for the study of neoplastic diseases. Because the pathways of

<table>
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<th>Table 2 Other disease modelsa</th>
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<tr>
<td><strong>Model</strong></td>
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<tr>
<td>Age-related cardiac disease</td>
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<tr>
<td>Angiogenesis/vasculogenesis</td>
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<td>Axon guidance regulators</td>
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<td>Caloric restriction/diet</td>
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<td>Ehlers-Danlos syndrome</td>
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<td>Epilepsy</td>
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<td>Freeze tolerance</td>
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<td>Mitochondria-associated</td>
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<td>diseases</td>
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<td>Postural control</td>
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<td>Sleep regulation</td>
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<td>Stem cell roles in cancer</td>
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<td>Testicular cancer</td>
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<td>Tumor metastasis</td>
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<tr>
<td>Tumor suppression</td>
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<td>Wound healing</td>
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aThese models are provided for reference; discussion of other models is provided in the text.
gene functions in the two species have many similarities to those in humans, the study of these organisms has provided much insight into tumorigenesis in both humans and animals (Gateff and Schneiderman 1967, 1969; Gilbert 2008; Kirienko et al. 2010; Saito and van den Heuvel 2002).

Genetics. Many genetic mutations in the fly lead to uncontrolled cell division, neoplasia, and death (Gateff and Schneiderman 1967, 1969; Gilbert 2008). As a result, *Drosophila* is a useful model for gene regulation, particularly tumor suppressor genes (TSGs) and oncogenes (Brumby and Richardson 2005; Gilbert 2008; Menut et al. 2007). Similarly, *C. elegans* is also an excellent model for cancer including the study of apoptosis, cell cycle progression, growth factor signaling, genome stability, and mechanisms of invasion and metastasis (Kirienko et al. 2010). Studies with *C. elegans* have provided important clues about the function of homologous oncogenes and TSGs in humans. Genomewide RNA interference screens in *C. elegans* have facilitated the identification of new cancer gene candidates and how they function in the pathogenesis of cancer (Poulin et al. 2004).

Understanding how multiple genes function together to create a cascade of events provides opportunities for identifying therapeutic agents that can target genes contributing to cancer. *C. elegans* thus may serve as a key model in screening potential cancer therapeutic agents (Saito and van den Heuvel 2002).

A specific genetic screen has been developed to delineate neoplastic TSGs that provide control over cell polarity and proliferation (Menut et al. 2007). Hippo, a kinase, modulates a chain of events that lead to expression of genes involved in cell proliferation and growth regulation (Badouel et al. 2009). Study of the Hippo pathway in flies has identified close similarities to the same pathway in mammalian species (Butitta and Edgar 2007). In flies, mutations in *hippo* can lead to epithelial cell proliferation in several tissues—and play a role in managing apoptosis—so cancer researchers can add *Drosophila* to their arsenal as a model for studying control of cell proliferation (Gilbert 2008). Both *Drosophila* and *C. elegans* have also served as models for evaluating the role of FOXO genes in cancer; FOXO factors affect many physiological processes, including differentiation of cells, tumor suppression, cell-cycle arrest, and apoptosis (Ardon 2008).

**RUNX** (runt-related) genes in humans regulate a number of processes—control of cellular proliferation, maintenance of stem cells, development of specific cell lineages, and regulation of cell differentiation; disruption of the genes leads to pathology and often cancer. RUNX transcription factors have been studied in *Drosophila, C. elegans,* and the purple sea urchin (*Strongylocentrotus purpuratus*) (Braun and Woollard 2009).

*Drosophila* is also being used to evaluate the roles of axon guidance genes, such as *Netrin* and *Deleted in Colorectal Cancer* (*dcr*), for which the human counterparts are implicated in causative mechanisms of human cancer. These models may prove useful in developing cancer therapeutic agents (Duman-Scheel 2009).

Table 3 shows other invertebrate models for genetic studies.

**Molecular and Cellular Biology.** A symmetric cell division (the generation two different daughter cells from a single cell) plays a role in the development of cancer. The mechanisms of such division and its potential to lead to tumorigenesis have been modeled in both *C. elegans* and *Drosophila* (Chartier et al. 2010), but the embryo of *C. elegans* has served as the primary model for understanding asymmetric cell division in cancer—in particular, determining stem cell function and tumorigenesis in humans (Hyenne et al. 2010).

Molecular control of cell migration and tumor metastasis has been modeled in *Drosophila* (Jang et al. 2007). For example, a model for ovarian cancer metastasis has been evaluated in *Drosophila* based on the migration of ovarian border cells (Naora and M ontell 2005). Human ovarian cells and fly border cells have many similarities: both are controlled by steroid hormones; during development, each can show characteristics of epithelial and mesenchymal cells; and both can migrate to other sites to form cell nests (Gilbert 2008; Naora and M ontell 2005). Several genes and proteins have been identified in border cell migration in flies. The proteins, which also occur in women, have been tested in vitro on ovarian cancer cells and the cytopathology observed. Unlike cancer cells, border cell migration represents a normal process in flies—it ends without harm to the animal (Gilbert 2008; Naora and M ontell 2005). Further studies in flies may assist in determining the factors that turn off cell migration and may lead to an understanding of ovarian cancer metastasis and, ultimately, methods to prevent it. Border cell migration may also serve as a biological assay for the development of treatments for ovarian cancer (Gilles 2008).

*Drosophila* has also been used to understand the concept of cell competition, whereby cells of different genotypes are located next to each other and compete for proliferative advantage. In cancer, the abnormal cells dominate the normal; thus, cell competition may play a role in cancer development (Baker and Li 2008). Planarians also have found a niche in cancer research. These tiny organisms have been studied to understand the molecular biology and genetics of cancer as well as the possible role of regeneration in causing or curing cancer (Oviedo and Beane 2009).

Table 2 lists additional cancer models.

**Substance Abuse**

*Drosophila* and *C. elegans* have been used to study the genetic and behavioral mechanisms of cocaine, alcohol, and nicotine addiction (Schafer 2004; Wolf and Heberlein 2003). In addition, the pond snail has been used to study cocaine addiction and therapies to treat or prevent it; changes in learning and memory in the snail can be easily evaluated and demonstration of impairment is readily observed (Carter et al. 2006). The honeybee is another model for studying cocaine addiction. Bees fed low levels of cocaine show altered patterns in their foraging dance and removal of cocaine from their diet results in withdrawal effects (Barron et al. 2009).
Ethanol response has been studied in several invertebrates. Research on ethanol sensitivity and tolerance in *Drosophila* revealed genes that are directly linked to the behavioral responses of the inebriated flies (Berger et al. 2008). Work with intoxicated *Drosophila* has shed light on the specific neurons that mediate observed behaviors (Scholz 2009). The honeybee has also been proposed as a model of alcoholism and its effects. After consuming concentrations of ethanol up to 20% of their diet (Abramson et al. 2000), the honeybee’s behavior changes in ways similar to those observed in vertebrates, with effects on locomotion and learning. The social bee may also be appropriate to study alcohol influence on language, social interaction, development, and learning (Aramson et al. 2000). *C. elegans* has also served as a model of alcoholism (Dolganic and Szabo 2009).

### Table 3 Genetic models

<table>
<thead>
<tr>
<th>Model</th>
<th>Species used</th>
<th>References</th>
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<tbody>
<tr>
<td>Behavior</td>
<td>Drosophila</td>
<td>Jasinska and Freimer 2009; Mackay and Anholt 2007</td>
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<tr>
<td>Chromosome speciation</td>
<td>Drosophila, mosquitoes</td>
<td>Ayala and Coluzzi 2005</td>
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<tr>
<td>Cocaine-related behaviors</td>
<td>Drosophila</td>
<td>Heberlein et al. 2009</td>
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<tr>
<td>Complex traits</td>
<td>Drosophila</td>
<td>Mackay and Anholt 2006</td>
</tr>
<tr>
<td>CT/CGRP b</td>
<td>Pecten maximus, Haliotis tuberculata, Crassostrea gigas, Drosophila</td>
<td>Lafont et al. 2007</td>
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<tr>
<td>Gene perturbations</td>
<td>Caenorhabditis elegans</td>
<td>Borgwardt 2008</td>
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<tr>
<td>Gene regulation</td>
<td>C. elegans, Drosophila</td>
<td>Ercan and Lieb 2009; Large and Mathies 2007; Mendjan and Akhtar 2007</td>
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<tr>
<td>Genetic interaction networks</td>
<td>C. elegans</td>
<td>Lehner 2007</td>
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<tr>
<td>Meiosis</td>
<td>C. elegans</td>
<td>Colaiaioco 2006; Schwarzstein et al. 2010</td>
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<tr>
<td>Mitochondrial DNA</td>
<td>Mytilus edulis, M. galloprovincialis, Venerupis philippinarum, Lampsiilis, Inversidens japonensis</td>
<td>Breton et al. 2007</td>
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<tr>
<td>Muscle development</td>
<td>Drosophila</td>
<td>Maqbool and Jagla 2007</td>
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<tr>
<td>Myoblast fusion</td>
<td>Drosophila</td>
<td>Richardson et al. 2008</td>
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<td>Noncoding RNA</td>
<td>Drosophila</td>
<td>Deng and Meller 2006</td>
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<td>p53</td>
<td>C. elegans, Drosophila</td>
<td>Lu and Abrams 2006</td>
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<tr>
<td>RNA silencing</td>
<td>Drosophila</td>
<td>Kavi et al. 2005</td>
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<td>Sleep</td>
<td>C. elegans, Drosophila</td>
<td>Andreic et al. 2008</td>
</tr>
<tr>
<td>Spindle assembly and regulation</td>
<td>Drosophila</td>
<td>Buchman and Tsai 2007; Doubilet and McKim 2007</td>
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<tr>
<td>Telomere protection</td>
<td>Drosophila</td>
<td>Cenci et al. 2005</td>
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<tr>
<td>Transposable elements</td>
<td>Drosophila</td>
<td>Le Rouzic and Deceliere 2005</td>
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<tr>
<td>Tumor susceptibility gene (TSG) 101</td>
<td>Drosophila</td>
<td>Herz and Bergmann 2009</td>
</tr>
<tr>
<td>Wnt</td>
<td>Drosophila</td>
<td>Bejsovec 2006</td>
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*a* These models are provided for reference; discussion of other models is provided in the text.

*b* CT/CGRP, calcitonin/calcitonin gene-related peptide

### Toxicology

Pharmaceutical research requires the detection of adverse reactions to new drugs as early in development as possible. A number of in vitro tests are available (e.g., cell culture, tissue slice) but do not always translate to animal model systems or relate to clinical experience. Invertebrates can be used as models for many toxicological studies and can bridge the gap between in vitro models and vertebrate animal studies. Close similarities to vertebrate response, rapid reproduction rate, cheap cost, and ease of housing and care have made invertebrates key organisms in toxicologic screens (Avanessian et al. 2009). The literature shows numerous examples of the use of invertebrates in toxicity evaluations; the most commonly used organisms are *C. elegans*, *Drosophila*, and the water flea.
The nematode has proven to be an excellent model for use in toxicology studies, drug development, and research on environmental toxicology due to its mapped genome and simple nervous system (Williams et al. 2000). Because of its genetic and cellular similarities to humans, C. elegans is an important model for high-throughput screening of therapeutic agents for human diseases (Nass et al. 2008). It can be used in both LD50 (lethal dose, 50%) and behavioral paradigms (D’Hawan et al. 1999), and it shows results comparable to those of mouse systems (Williams et al. 2000). The worm has been used for evaluations of genetic and environmental toxicology, neurotoxicology, and high-throughput experiments to screen for molecular and genetic targets of chemical toxicity. Neurotoxicity can also be modeled in Drosophila and various species of cockroaches (Peterson et al. 2008). All of these organisms provide simple and inexpensive alternatives to mammals for evaluating the toxicity of new pharmaceuticals (Artal-Sanz et al. 2006; Leung et al. 2008).

Both the sludge worm (Tubifex tubifex) and D. magna can also be used as models in preliminary toxicology screening, including LD50 evaluations (Devillers and Devillers 2009). Because of Daphnia’s sensitivity to toxicants, it is also used in water quality monitoring to identify the presence of contaminants (Martins et al. 2007). In addition, the mussel has been used to study organophosphate toxicity and proposed as an additional biomarker for pollution (Brown et al. 2000). Drosophila has proved to be a useful model in drug-feeding experiments to evaluate new antiepileptic drugs (Ackermann et al. 2008). Drug delivery, however, is a major challenge when using Drosophila as it is very difficult to standardize the amount of drug consumed in a fly’s diet (Avanesian et al. 2009); other options, such as microinjection in the abdomen, have been proposed (Dzitoyeva et al. 2003). In spite of the drug delivery challenge, a number of studies have used Drosophila in toxicological evaluations (Ackermann et al. 2008; Avanesian et al. 2009; Gupta et al. 2007b; Patnaik and Tripathy 1992).

Endocrine-active chemicals are present in the environment and some therapeutic agents have endocrine effects; in both cases, these compounds disrupt normal endocrine function in mammals. Changes in sperm counts, breast cancer, congenital abnormalities of the genitalia, and other pathologic conditions in humans are linked to these chemicals and therapeutic agents. Invertebrates can be used to study endocrine effects of drugs and environmental contaminants (Avanesian et al. 2009; Duft et al. 2007; Gupta et al. 2007b; Patnaik and Tripathy 1992; Tatarazako and Oda 2007). For example, researchers have observed adverse reproductive effects in Drosophila adults and cell lines exposed to a variety of insecticides (Gupta et al. 2007b; Patnaik and Tripathy 1992). Similarly, Avanesian and colleagues (2009) studied methotrexate toxicity in flies and found ovarian impairment comparable to that observed in mammalian models. The freshwater mud snail (Potamopyrgus antipodarum) and Daphnia have also served as models for the assessment of environmental chemicals (Duft et al. 2007; Tatarazako and Oda 2007). Many gender-related differences in toxicant effects have been noted in humans, and these effects can be modeled in many different invertebrates including insects, nematodes, crustaceans, molluscs, corals, and echinoderms (McClellan-Green et al. 2007).

Environmental impacts on the lysosomal-autophagic system have been studied in bivalve molluscs (Moure et al. 2006). Environmental metal contamination can be monitored in various insects, as metal accumulates in chitinous exoskeleton and is incorporated into insects’ internal issues (Hare 1992). Additionally, many aquatic species serve as biomarkers for organic xenobiotic and metal contamination in both fresh and saltwater (Rainbow 2007; Raisuddin et al. 2007; Sarkar et al. 2006).

“The Five Senses”

Hearing

The Johnston’s organ, located in the antenna of Drosophila, is the counterpart of the mammalian ear. Similarities in the genes involved in development of the hearing structures of flies and mammals have led to speculation that study of fly hearing may provide insights into deafness in humans (Boekhoff-Falk 2005). The role and function of TRP channels, present in both flies and vertebrates, have been evaluated in flies to provide a model system to study vertebrate inner ear disorders that affect hearing and balance (Cujungco et al. 2007). The creation of mechanical models has enabled modeling of auditory transducer dynamics, which will be used to test auditory performance in both vertebrates and Drosophila (Nadrowski and Gopfert 2009).

Olfaction

There are many similarities—structural, functional, and physiologic—between the olfactory systems of vertebrates and insects (Kay and Stopfer 2006), making insects excellent models for the study of olfaction. Vertebrates use their noses to smell, whereas insects have antennae; however, both structures have specialized epithelium lined with ciliated olfactory receptor neurons (ORNs) that respond to odors (Hallem et al. 2006; Kay and Stopfer 2006; Malnic et al. 1999; Rospars et al. 2003). From the ORNs, both vertebrates and insects send neural processes to the brain—the insect’s antennal lobe and the vertebrate’s olfactory bulb (Kay and Stopfer 2006; Laurent 1999). Investigators have studied insect olfactory systems to understand interactions between animals and their environment (de Bruyne and Baker 2008). Other studies have focused on odor processing and the capacity to detect odor blends using moths and honeybees (Lei and Vickers 2008), and C. elegans and Drosophila have been models in olfactory signaling research (Kaupp 2010; Nakagawa and Vosshall 2009).

Decapod crustaceans can serve as olfactory system models because the cellular and morphological organization of their olfactory system has many similarities to the human
olfactory pathway. In many decapod crustaceans, neurogenesis occurs in the olfactory system throughout life, so these animals are being used to study lifelong neurogenesis, with the aim of understanding how it occurs (Sandeman and Sandeman 2003; Schmidt 2007). This research may elucidate methods to stimulate neurogenesis in humans to treat neurological diseases or injuries.

All organisms are exposed to an enormous diversity of chemicals in the environment, and understanding the way the nervous system recognizes and responds to these chemical signals is challenging. The chemosensory system of insects has become an important area of research, and Drosophila is the primary model under study (Benton 2008). Crustaceans are also effective models for the study of chemoreception, which is based in their olfactory system (Derby and Sorensen 2008).

Taste and Satiation

Researchers study Drosophila to understand taste perception and the neural circuits (in particular one called hugin) that affect feeding behavior. An organism’s decision, whether human or fly, is basically to eat or not to eat; the mechanisms in the brain that prompt this decision are similar (Amrein and Thorne 2005; Melcher et al. 2007).

Touch and Temperature Sensing

Responses to mechanical forces, including touch, are poorly understood at the molecular level. Touch-sensitive mutants of C. elegans have been created and the defective genes studied to help identify sensory components that affect the cells that sense gentle touch (Bounoutas and Chalfie 2007).

All animals have the ability to detect changes in environmental temperature based on the presence of neuronal and molecular substrates that affect thermosensation. The three primary models for studies of thermosensation are the mouse, C. elegans, and Drosophila (McKemy 2007). Investigators have also studied thermosensing in C. elegans to understand the molecular and cellular basis for neural plasticity (Mori et al. 2007).

Vision

In the 1930s, horseshoe crabs (Limulus polyphemus) were used by Haldan K. Hartline and C. H. Graham as their model for studying the optic nerve, and in 1967 Hartline, Ragnar Granit, and George Wald were awarded the Nobel Prize in Medicine for their research into visual processes of the eye. Hartline’s Limulus model continues to serve as an excellent model for vision research because of its complex ocular-neural network. Limuli have large, easily accessible retinal neurons, allowing for electrophysiological study. Study of the horseshoe crab has provided insight into the operation of human vision, particularly adaptation to light and lateral inhibition (Liu and Passaglia 2009).

Octopuses have also been used in vision research. Their sucker chemotactile systems have been compared to the mammalian eye (Packard 1972; Sanders et al. 1975; Young 1967, 1971).

Other Models

Space Biology

Invertebrates are useful in studying the effects of ionizing radiation, both on earth and in space. C. elegans has been effectively used in microbeam irradiation studies to evaluate bystander effects (Bertucci et al. 2009), to study the long-term effects of radiation exposure in space travel, and to evaluate the gravitational effects of space travel on muscle gene expression (Zhao et al. 2005). It has also been used to study natural space radiation exposure, and the resulting mutants have been genetically evaluated (Nelson et al. 1994). Tardigrades (water bears, moss piglets) have also served as models for open space research, because they have the ability to survive desiccation, extreme cold, and radiation, all of which occur in space. Additional invertebrate species showing potential for use in outer space biological experiments are the sleeping chironomid (Polypedilum vanderplanki), the brine shrimp (Artemia salina and A. franciscana), and several types of rotifers, which are commonly called “wheel animals” (Jonsson 2007).

Symbiosis

Throughout nature, symbiotic relationships between organisms contribute to survival and the ability to flourish. Even humans are dependent on lowly microorganisms, which contribute to nutrition and defense. The relationship between host and microbe has been modeled through invertebrates, which have a diverse set of associated microorganisms (Chaston and Goodrich-Blair 2010). The primary model systems in such research are insects and nematodes. The former include various species of termites (Hongoh et al. 2005; Okhuma 2008; Yang et al. 2005), the honeycomb moth (Galleria mellonella; Gouge and Snyder 2006; Walsh and Webster 2003), the tobacco hornworm (Manduca sexta; van der Hoeven et al. 2008), the Asian gypsy moth (Lymantria dispar; Brenderick et al. 2004), and Drosophila (Ryu et al. 2008). The nematode Steinernema carpocapsae and its association with the bacterium Xenorhabdus nematophilus have been used as a model for symbiotic relationships (Goodrich-Blair 2007).

Bioactive Products

Biomaterials and Biomimetics

Baculovirus

Baculovirus-insect cell expression systems have enabled the production of recombinant proteins for use in research
Bioluminescence, the ability of a living organism to produce light, results from a biochemical reaction where oxidation of luciferin, a substrate, occurs through the action of the enzyme luciferase (Day et al. 2004). Many organisms can produce bioluminescence, but the animal most studied and used for biomedical research applications is the firefly or lightning beetle (Photinus pyralis). Light in beetles is produced in “lanterns,” organs containing photocytes that are layered between two rows of cells. Uric acid crystals in the cell layers reflect the light produced by the photocytes (Fraga 2008; Hastings and Wilson 1976; Hastings 1983, 1989a,b). During the late 1800s, Raphael Dubois, a French physiologist, studied the biochemical properties of bioluminescence in these insects and created a luminescent solution by crushing their abdomens and mixing the crushed organs in cold water (Harvey 1957; McCapra 1982).

Luciferases have been incorporated into many in vitro molecular assays to allow evaluation of gene expression in transformed cell lines (Contag et al. 1998, 2000; de Wet et al. 1987; Lim et al. 2009; Sherf and Wood 1994; Takakuwa et al. 1997; Wood et al. 1989; Wood 1995; Zhang et al. 1994, 2008). In addition to the firefly and other beetles, luciferases have been isolated from the sea pansy (Renilla reniformis) and jellyfish (Aequorea victoria) and used as reporters in mammalian cells and in other types of animal studies (Contag et al. 2000). Cloning of image reporters, such as the luc gene, is allowing researchers to study transcriptional regulation, signal transduction, protein-protein interactions, tumor transformation, cell trafficking, and targeted drug actions in living animals without invasive techniques (Gross and Piwnica-Worms 2005).

Silk Products

For centuries silk fibers were the primary source of suture materials, but in recent years synthetic materials have dominated the market. Studies of silkworm fibers have shown biocompatibility between silk and the commonly used biomaterials polyactic acid and collagen (Altman et al. 2003). As a result, silk and silklike fibrous proteins from the silkworm (B. mori), the golden orb web spider (Nephila clavipes), diadem spider (Araneus diadematus), and other insects are being considered for use in biomedical applications such as tissue scaffolding for joint repair (Altman et al. 2003). The design of silk-inspired polymers and proteins and their uses in bioengineering and biotechnology are reviewed by Hardy and Scheibel (2009).

Biomimetics

Biomimetics is the study of a living organism to create a device, either medical or nonmedical, by applying information gained from the organism. Invertebrates have been useful models in this area of applied research. For example, the study of marine ragworms (Nereis virens and N. diversicolor) supported the development of a new endoscope based on the ragworm’s ability to move in slippery substrates, similar to mucus in the gastrointestinal tract (Hesselberg 2007).

Drug Discovery

Invertebrates have been used for many centuries for their medicinal properties. In the western hemisphere, the leech (Hirudo medicinalis) was used for bloodletting of patients with many different disorders and maggots were (and sometimes still are) used for cleaning wounds. In the East, the Chinese valued the sea cucumber for its ability to cure many human diseases (Kelly 2005).

A wide range of invertebrate species—from insects to marine life—serve as reservoirs of bioactive compounds, but marine life accounts for the largest number: over 14,000 pharmacologically active compounds have been identified from marine plants and animals (Adrian 2007), 961 of them in 2007 alone (Blunt et al. 2009). Bis(indole) and tris(indole) alkaloids are among the most commonly isolated compounds and show high biological activity, with potential as pharmaceutical agents (Gupta et al. 2007a). These alkaloids have a wide range of effects including antimicrobial (bacterial, fungal, viral), antiparallel, antitumor activity, anti-inflammatory, antiproliferative, and antiserotonin activity, and RNA and DNA synthesis inhibition. In addition to the discovery of pharmacologically active compounds, invertebrates such as snails and sea anemones are being used in the creation of antisera used in humans (Redwan 2009). Excellent reviews are available of the many species studied, compounds identified, and their biological activities (Blunt et al. 2009; Gupta et al. 2007a; Kelly 2005).

While marine invertebrates are proving to be outstanding sources of pharmaceuticals, the harvesting of marine life, especially by third-world countries to sell species to pharmaceutical companies, is decimating native populations. There is significant concern among conservationists that such over-exploitation of marine animals is going to result in the extinction of many (Duckworth et al. 2003; Lawrence et al. 2010). Fortunately, many countries are initiating aquaculture programs for the cultivation of marine invertebrates to spare wild populations while providing new means of revenue for their societies (Kelly 2005). As research and development with bioactive compounds move forward, global measures to ensure species protection and welfare will be critical.

Anticoagulants

Tick anticoagulant peptid and other natural anticoagulants have been isolated from hematophagous invertebrates and
testing of these products in mammalian animal models of thrombosis and atherosclerosis has shown that they have potent anticoagulant properties (Fioravanti et al. 1993; Ragosta et al. 1994; Schaffer et al. 1991; Schwartz et al. 1996; Sitko et al. 1992). Ticks and similar organisms may thus serve as sources of natural inhibitors in the design of improved anticoagulants (Corral-Rodríguez et al. 2009) and antithrombotics (Koh and Kini 2009). Evaluation of hirudin, a natural thrombin inhibitor present in the blood-sucking leech, for use in the management of thromboembolic diseases (M arkwardt 2002) has resulted in the creation of two recombinant versions of hirudin (Lepirudin and Desirudin) that are now on the market for use in humans. Lepirudin has found a second niche as an effective treatment for angina (Redwan 2009).

Sulfated fucans and galactans (homopolysaccharides) isolated from marine invertebrates have powerful natural pharmacological actions that can be therapeutically effective in humans (Pomin 2009) as anticoagulants (Farias et al. 2000; Mourao and Pereira 1999; Mourao and Pereira 1999; Mourao 2004; Pereira et al. 1999), antithrombotics (Berteau and M ulloy 2003; Mourao and Pereira 1999; Mourao 2004), and anti-inflammatories (Berteau and M ulloy 2003). The main source organisms for fucans and galactans are the sea cucumber, sea urchin, and ascidians (Pomin 2009).

**Antimicrobials**

In 2005, Salzet reported the identification of over 30 neuropeptide-derived antimicrobials from such diverse invertebrate species as shrimp, fly maggots, mosquitoes, scorpions, horse-shoe crabs, sea cucumbers, and numerous other marine invertebrates (Lawrence et al. 2010; Salzet 2005). Chemokine binding proteins (CBPs), lectins that have been isolated from the sea worms *Chaetopterus variopedatus* and *Laxus oneistus*, have potential as antivirals as research has shown that they can inhibit HIV infection in cells and prevent virus transmission from infected to unaffected T cells (Balzarini 2006). HIV-inhibitory compounds have also been isolated from soft corals (*Lobophytum sp.*) from the Philippines (Rashid et al. 2000). Sulfated fucans and galactans from marine invertebrates can also serve as antivirals (Harrop et al. 1992).

Other antimicrobial peptides isolated from invertebrates have other bioactive properties (B ulet et al. 2004). Cecropin A, identified in the silkworm (*Hyalophora cecropia*) and the mosquito (*Anopheles gambiae*), has antibacterial and antifungal properties and lyses yeast cells. Stomoxyn, from the stable fly (*Stomoxys calcitrans*), is toxic to bacteria and fungi and has lytic effects on trypanosomes. Similar peptides have been isolated from fire ants (*Pachycondylas goeldii*), termites (*P. spiniger*), and the spiders *Oxyopes kitabensis* and *Cupiennius salei*. Two bioactive peptides with multiple uses have been identified in the stalked sea squirt (*Styela clava*) (B ulet et al. 2004).

**Cancer**

Over the past 10 years, a number of compounds originating from marine invertebrates have entered preclinical and clinical development as cancer therapeutics (A drian 2007; J imeno 2002). C-nucleosides from the Caribbean sponge (*e.g.*, *Cryptotheca crypta*) served as the chemical model for synthesis of Cytarabine, which is used in the treatment of leukemia and lymphoma (Schwartsmann et al. 2003). Compounds in clinical development include idemimins, *Kahalalide F*, hemiasterlin, dolastatins, camadotin, soblidotin, bryostatins, ecteinascidin-743, and aplidine (Rawat et al. 2006; Schwartsmann et al. 2003). Many of these compounds are still being evaluated clinically; others (*e.g.*, bryostatin 1) have been removed from trials because of severe side effects (Singh et al. 2008). Bonnard and colleagues (2010) recently reported the discovery of antitumor promoters in two types of Comorian soft corals; they are under investigation for potential use in cancer therapy. Sulfated fucans and galactans from marine invertebrates have also been recognized to have antitumour metastatic properties (C oombe et al. 1987).

**Immune Protectors**

Parasite colonization can provide protection from immuno-mediated diseases. Studies in mice colonized with helminths showed that the animals were protected when challenged with colitis, asthma, encephalitis, and diabetes (Elliott et al. 2007). Similarly, clinical trials have shown that helminth exposure can reduce the symptoms of ulcerative colitis and Crohn’s disease in human patients by altering immune response. The effects on the immune response may result from the induction of regulatory T cell activity (W einstock et al. 2005). Induced helminth infections may some day be used to treat many inflammatory and immune-mediated diseases (Mck ay 2009).

**Pain**

Cone snail (*Conus* sp.) venom, known as conotoxin, can alleviate pain and prevent or treat epilepsy. Based on the number of species of cone snails and the number of conopeptides each can produce, it is expected that 70,000 different conotoxins will be characterized and tested for therapeutic potential (Ekberg et al. 2008). Evaluation of conotoxins in mammalian animal models has led to numerous applications, including use as antinociceptives, antiepileptics, neuroprotectives, and cardioprotectives. Conopeptides may also find use as therapeutic agents for cancer and neuromuscular and psychiatric disorders (Han et al. 2008). The drug Prialt, a derivative of conotoxin, has been approved for and is being used to treat intractable pain (Lee et al. 2010); it has a potency 800 times that of morphine (Xia et al. 2010).

**Sunscreens and Antioxidants**

A wide variety of marine organisms can be used to obtain effective sunscreens and antioxidants. Mycosporinelike amino acids (MAAs), prevalent in corals, have the ability to
absorb UV rays and serve as natural sunscreens in marine animals (Dunlap et al. 1999); synthetic sunscreens have been developed based on these MAA s (Dunlap et al. 1995, 1999; Karentz et al. 1991).

Marine invertebrates, either independently or in symbiotic relationship with bacteria, also produce potent antioxidants (Dunlap et al. 1999). These antioxidants have potential biomedical applications, ranging from use as food supplements to cosmetic additives and chemopreventives in oxidative stress-related disease.

Harvest of Bioactive Products from Sponges

Marine sponges contain a circular proteoglycan called “spongican” that is involved in species-specific cell adhesion, resulting in cell aggregation (Fernandez-Busquets and Burger 2003). There are many similarities between the responses of cell aggregation in sponges and processes in humans. For example, both human platelets and sponge cells respond similarly to stimuli that either inhibit or accelerate aggregation (Philip et al. 1992). Because of the cell-aggregation response, sponges can be used to study both inflammation and anti-inflammatory compounds and may serve as models in anti-inflammatory drug development (Dunham et al. 1985).

Spongicans may also be useful in the study and treatment of specific human diseases; for example, a derivative of spongican has been shown to block replication of HIV, indicating potential as a treatment for HIV infection (MacKenzie et al. 2000). Sponges may also serve as a model to evaluate treatment for Alzheimer’s and other amyloid disorders, as spongicans affect amyloid fibrils by causing aggregation, thus helping block the characteristic lesions of Alzheimer’s (Mclaurin et al. 1999).

Spongion is a protein that makes up the fibrous skeleton of sponges and may have potential uses in the treatment of osteoarthritis and other degenerative bone diseases (Kim et al. 2009). Spiculogenesis creates sponge skeleton and has been studied to find new approaches to treat dental and bone disease. It is initiated by the enzyme silicatein, beginning the process of creating silica nanoparticles that fuse in layers around a central protein filament of silicatein and silintaphin-1, which serves as the scaffolding protein (Muller et al. 2009). This process has led to the development of synthetic biomaterials containing recombinant silicatein and silintaphin-1, which have been used to induce biosilica-mediated regeneration for tooth and bone defects. The assembly of silica nanoparticles by the action of silicatein and silintaphin-1 results in the synthesis of light waves; these nanoparticles could serve as an alternative to fiber optics in biomedical applications (Muller et al. 2009).

Sponges of the genus Spongisorites contain several bis(indole) alkaloids that have potent antifungal properties and produce moderate cytotoxicity in cancer cell lines (Oh et al. 2006). Stevensine, an alkaloid metabolite with antitumor properties, is produced by the sponge Axinella corrugata, which is being laboratory raised to create sufficient quantities of Stevensine without affecting natural-living specimens (Duckworth et al. 2003).

Invertebrate Models in Teaching

For most of the history of biological, medical, veterinary, and agricultural teaching, vertebrate species have been the models of choice because of their close similarities to humans and other target species. For example, veterinary colleges have used vertebrates to help students understand physiology, pharmacology, and pathology and practice surgery and clinical care. Similarly, colleges of medicine have used turtles, rats, dogs, and other vertebrates to teach medical students the fundamentals of physiology, pharmacology, and other aspects of medicine. But fewer vertebrate animals are being used in teaching as pressure from animal rights activists has led many medical and veterinary programs to reduce or even eliminate the use of animals, despite students’ expressed concerns about the lack of hands-on experience with living animals.

In K–12 teaching, many animals commonly used in the past have been extensively harvested or, among wild populations, decimated by disease. Reduced numbers of some species have led to the animals’ being listed as endangered, which means that these animals cannot be taken from the wild. As a result of all of these factors, fewer vertebrate animals are available or used for study by K–12, college, and professional students.

Invertebrate species can serve as substitutes for vertebrates in some, but not all, educational experiences for students. The wide range of animals available for study, the vast populations available either in the wild or from laboratory suppliers, their similarities to (and differences from) vertebrates, the ease of keeping them, and the low cost of acquisition and maintenance—all contribute to their value as experimental subjects (Deyrup-Olsen and Linder 1991).

A number of books and scientific articles document the varied uses of invertebrates in teaching; Deyrup-Olsen and Linder (1991) provide a concise review of the uses of invertebrates in teaching physiology. Additionally, a number of examples of the use of invertebrates in teaching K–12 and college-level students are available on the Internet. Following are some of the many examples of the use of invertebrates in teaching (with websites as available; also see Smith 2011, in this issue):

- In lieu of the frog sciatic nerve preparation for demonstrating action potentials, recordings from cockroaches (Periplaneta sp.) can be substituted.
- Crayfish (Procambarus sp. and P. fastastacus sp.) are effective for modeling the effects of changing environmental temperature on metabolic rate (Casterlin and Reynolds 1977).
- Circulation and the heart can be observed in Mercenaria clams (Florey 1968a; Greenberg 1965) and crayfish (Florey 1968b).
• *Aplysia* is an effective model for teaching physiology and neuroscience.

• Mussels are useful for demonstrating muscle function (Hoyle 1968).

• Mussels and clam gills can be used to study the function of cilia, including the effects of environmental toxicants on cilia (Deyrup-Olsen and Linder 1991; Hoar and Hickman 1983).

• The tobacco hornworm (*Manduca sexta*) can be used to observe the active transport of ions in the midgut, serving as an alternative to frog skin preparations (Deyrup-Olsen and Linder 1991).

• Bryn Mawr College has developed the “Serendip” program (http://serendip.brynmawr.edu), providing a hands-on guide to teaching middle and high school biology. The classes include Invertebrate Diversity, for which students purchase earthworms, snails, and arthropods from pet stores and use them for class activities.

• Oklahoma State University offers a class called Laboratory of Comparative Psychology and Behavioral Biology, using honeybees, houseflies, carpenter ants, and crabs, to teach students about the neuronal mechanisms of learning and memory (http://psychology.okstate.edu/faculty/abramson).

• The University of Southern California teaches a month-long short course in Antarctica, where students study invertebrates to learn about the adaptations of animals, including humans, in extreme environments (http://antarctica.usc.edu).

• Lafayette College has included invertebrate research opportunities for undergraduates in its curriculum for a number of years (Sherma and Fried 1987).

• Johns Hopkins University School of Medicine uses *C. elegans* as a molecular model to teach undergraduate students about congenital myasthenic syndromes (Kaas et al. 2010).

• And the University of Arizona offers research projects for students to study biological processes and disease using *C. elegans*, black flies, mosquitoes, cockroaches, shrimp, and others.

Because of the growing culture of invertebrates for food, medicinal purposes, and pets, a number of veterinary colleges offer invertebrate classes to teach students how to provide care for invertebrates (e.g., North Carolina State University’s Invertebrate Medicine; www.cvm.ncsu.edu/content/invert.html). In addition, veterinary schools are teaching conservation medicine (Veterinary Examiner 2009), particularly targeting marine life. Marine invertebrates, so necessary for their biologically active properties, will open new avenues of practice for veterinarians, allowing the veterinary profession to provide additional contributions to environmental protection and animal welfare.

Many of the invertebrate species and models discussed in this article have also been used in teaching to help students learn about basic biological processes. Advanced students can also use invertebrates to learn about genetics, developmental biology, cancer, and other areas of study. However, there remain teaching activities, such as learning and practicing surgical techniques or working with livestock in animal science or wildlife biology curricula, for which vertebrate animals will remain the models of choice.

**Conclusions**

There is a long and illustrious history of invertebrates as models for research, testing, and education. Use of *Drosophila* for genetic studies was established in the early 20th century, and the fly has since become one of the most prolific models for mammalian disease. Similarly, *C. elegans* paralleled with mammalian genetics and molecular biology have made the worm a vital model for understanding the molecular mechanisms involved in disease. While *Drosophila* and *C. elegans* remain the two most studied organisms, there is no paucity of information about other invertebrates. Many terrestrial and marine organisms serve as models for human disease and provide nonvertebrate alternatives for preliminary toxicology and efficacy studies, as evidenced by the examples in this article and its extensive bibliography, coupled with the 300,000-plus articles on invertebrate use available through PubMed and other databases.

Similarly, invertebrates have many uses in education, offering a wealth of educational opportunities throughout the spectrum from K–12 classes to professional and graduate curriculums without having to rely on vertebrate species. However, vertebrate animals in teaching are still necessary, so that students at all levels can learn from hands-on encounters with the animals they will work with during their careers or with which they will share their lives as companions and friends. And professional students must have the opportunity to observe and practice techniques in living animals so that they can ethically practice their professions—for the welfare of both humans and animals.

For all uses of animals, IACUCs, veterinarians, members of the scientific community, governmental regulators, and the public must balance ethical concerns about the use of animals with the needs of society. There is also an ethical responsibility to question the use of higher animals—in research, testing, and teaching programs and in the evaluation of protocols. Invertebrate models provide rich opportunities for learning about and practicing ethical animal care and use by meeting the first and most critical principle of Russell and Burch’s Three Rs—replacement.

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Abstract

Invertebrate species have been used for many years in the laboratory and teaching environment. We discuss some of the most commonly maintained invertebrates—the nematode (Caenorhabditis elegans), the California sea hare (Aplysia californica), the fruit fly (Drosophila melanogaster), terrestrial hermit crabs, the horseshoe crab (Limulus polyphemus), and cephalopods—and briefly describe general techniques for culturing them in captivity. The aim of this article is to give potential users an idea of the materials, methods, and effort required to maintain each type of organism in a laboratory or classroom setting.

Key Words: Aplysia; Caenorhabditis elegans; cephalopod; fruit fly (Drosophila melanogaster); hermit crab; horseshoe crab (Limulus polyphemus); invertebrate care; invertebrate culture

Introduction

Numerous species of invertebrates are used in a variety of research, teaching, and public display activities. These species have been adapted for captivity over the years for their aesthetic value, ease of culturing, and utility for a multitude of teaching and research purposes.

In the research laboratory, invertebrates have served as models for cancer, aging, immunology, endocrinology, toxicology, developmental biology, tissue regeneration, genetics, molecular biology, learning and memory, and biomimetics (Wilson-Sanders 2011, in this issue). In the primary and secondary school classroom they have been used to demonstrate comparative anatomy, developmental biology, genetics, behavior, evolution, ecology, and conservation. Public aquariums have displayed numerous species of invertebrates—sponges, coral, anemones, octopus, jellyfish, starfish, sea urchins, and crustaceans—and zoos have built ant colonies, bee hives, and butterfly houses and assembled diverse insect and arachnid collections.

In this article we discuss some of the commonly maintained invertebrate species—the nematode (Caenorhabditis elegans), the California sea hare (Aplysia californica), the fruit fly (Drosophila melanogaster), terrestrial hermit crabs (Coenobita spp.), the horseshoe crab (Limulus polyphemus), and cephalopods (octopuses, squid, and cuttlefish)—and describe general techniques for culturing and maintaining them in captivity. As this is only a brief introduction to these animals, readers are encouraged to acquire more detailed information by both consulting the literature cited in this review and conducting a thorough reference search for the latest information about these species and their culture and maintenance.

Caenorhabditis elegans

Caenorhabditis elegans is a free-living nematode that has been used extensively for research in developmental biology, neurobiology, behavioral biology, and cell death (Hope 1999; Riddle et al. 1997; Wood 1988) and, because it is transparent, in anatomical studies. The genome of C. elegans was the first to be completely sequenced for a multicellular organism. Because the nematode has only 959 cells and the position of each cell is consistent from worm to worm, researchers now know specifically which genes encode the development of each individual cell (Brenner 1974; Strange 2006) and how alteration of a gene will affect a particular cell’s development (Hall and Altun 2007).

Anatomy and Biology

Caenorhabditis elegans is an unsegmented nematode that is about 1 mm in length. Its life cycle is completed in about 5 days at room temperature (22–23°C) if sufficient food is available. The worms are either male or, more often, hermaphroditic and able to self-fertilize (Byerly et al. 1976; Stiernagle 1999). The offspring of self-fertilized worms are usually genetically identical to each other and to the parent. The parent nematode
lays eggs that hatch in about 12 hours and go through four larval stages (L1-L4) before becoming adults. However, if food is scarce, the L2 larvae enter a dauer stage, an inactive alternative form that can live without eating or reproducing. The organism can survive in this stage up to several months until food becomes available, when it enters the L4 larval stage for up to 15 days before becoming an adult.

Culturing *C. elegans*

The preferred food source for monoxenically culturing *C. elegans* is a bacterial lawn of *Escherichia coli* OP50 (Stiernagle 2006). Five ml of Luria-Bertani broth (10 g tryptone, 5 g yeast extract, and 10 g sodium chloride in 1 L of distilled water, dH2O) is inoculated with a single colony of *E. coli* OP50 and incubated for 1 to 2 days at room temperature without aeration (i.e., without shaking). A small sample (100 µl) of this culture is then spread onto a standard (60 mm) agar plate of nematode growth medium, taking care not to spread the bacteria all the way to the edges of the plate (Stiernagle 2006); the remainder of the liquid culture can be used for the next 1 to 2 weeks if stored at 4°C. Plates are incubated for 1 to 2 days until a bacterial lawn is visible; lawns can be stored at 4°C up to 1 week, but must be warmed to room temperature before use. Bacterial lawns are then inoculated with *C. elegans* by using a sterile needle or platinum wire to transfer 10 worms from an old plate, placing the worms near the center of the new plate. Worms can also be transferred by cutting a small chunk of agar from an old plate (using a sterile spatula) and placing it near the center of the new plate. This “chunking” method can transfer hundreds of worms and is commonly used to maintain a worm colony (Figure 1).

Plates with worms are maintained at between 16°C and 25°C and observed daily. Worms are transferred to new plates when the bacterial lawn has been consumed, usually about every 3 to 5 days depending on temperature (the higher the temperature the faster the lawn consumption). A recently developed chemically defined medium (*C. elegans* Maintenance Medium, CeM-1) eliminates the need for chemical chemicals. This plate from a previous transfer of *C. elegans* to the plate. (Photograph courtesy of the laboratory of Diya Banerjee, Virginia Tech)

**Health Challenges and Treatment**

Bacterial and fungal contaminants are the only potential problems with maintaining stocks of *C. elegans*. Contamination does not usually harm the worms but it can make it difficult to score phenotypes and complete transfers. Fungal contamination can be removed by repeated transfer of worms to fresh *E. coli* OP50 lawns, and bacterial contamination can be removed by treating the plate with a 5% sodium hypochlorite solution (Stiernagle 2006), which kills the bacteria on the plate without affecting worms in the egg stage. After transfer, the eggs are washed with dH2O and seeded onto a fresh lawn of *E. coli* OP50. Contaminated plates, plates whose lawns have been consumed, and plates with surplus worms should be placed in a biohazard bag and autoclaved for disposal.

**Aplysia californica**

The California sea hare (*Aplysia californica*) is an opisthobranch mollusc in the order A. The organism’s large and relatively few neurons facilitate the study of neuronal architecture, physiology, and control of instinctive and learned behaviors. Research on *Aplysia* has also led to a greater understanding of memory formation and developmental biology.2

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1Abbreviations that appear >3x throughout this article: dH2O, distilled water; ppt, parts per thousand

2Unless otherwise indicated, information presented here is from the University of Miami/Natural Resource for *Aplysia* at the Rosenstiel School of Marine and Atmospheric Science; available online (http://aplysia. miami.edu). This and other websites cited in this article were accessed on March 1, 2011.
Anatomy and Biology

*Aplysia californica* is an herbivorous marine gastropod that is found naturally in the Pacific Ocean off the coast of California. Unlike other gastropods, it does not have a large external shell but only a small vestigial shell that protects the heart and other internal organs; this lack of an obvious shell gives the animal a slug-like appearance. *Aplysia* has two large ventrally placed tentacles and, on top of the anterior portion of its head, two sensory tentacles called rhinophores, which have tactile and chemoreceptor functions.

*Aplysia* breathes by directing water over the gills using modified flaps, called parapodia, on its back or mantle. After the water has passed over the gills, it is expelled through a posteriorly directed tube called a siphon. When disturbed, the animal retracts its siphon into the mantle cavity, but can modify this behavior through experience. *Aplysia* also has an ink gland just under the shell and releases a purple ink when threatened. The mottled reddish-brown color of *A. californica* and the color of its ink are derived from pigments in the algae the animal consumes.

*Aplysia* is hermaphroditic but does not self-fertilize. Under natural conditions it alternates being male and female on different days during the spring and summer mating season. The animal lays several thousand eggs in long string masses about an hour after mating. The eggs hatch 7 to 8 days later (Hirscha and Peretza 1984; Kriegstein et al. 1974) and enter the initial free-swimming veliger phase for about 12 days, during which the veligers become larvae. The larval phase lasts until 30 days posthatch, and then the animal enters the juvenile phase. *Aplysia* reach adulthood at about 94 days and can live for an additional 6 to 10 months (Hirscha and Peretza 1984; Kriegstein et al. 1974). Adults weigh 500–1000 g (Gerdes and Fieber 2006; Michael C. Schmale, Rosenstiel School of Marine and Atmospheric Science, University of Miami, personal communication, November 24, 2010).

Housing and Diet

*Aplysia* may be housed in glass, fiberglass, or plastic tanks that provide for ease of cleaning and adequate circulation and/or filtration of water (Figure 2). Water quality is critical for maintaining healthy cultures of *Aplysia*. They require either natural seawater or artificial seawater at a salinity of 30 to 36 parts per thousand (ppt1) and a water temperature of 13–16°C. The pH of the water should be between 8.0 and 8.6 as is typical for most marine environments, and the oxygen content should be at least 95% or 8–9 mg/l.

*Aplysia californica* should be fed to satiation every 3 days. Its recommended diet in captivity is marine macroalgae, principally red algae (e.g., *Gracilaria* spp., *Agardhiella* spp., and *Laurencia* spp.), which must be cultured separately from the *Aplysia*. Red algae are grown in nutrient (nitrate and phosphate)-supplemented seawater at room temperature with aeration and either direct natural light or full-spectrum fluorescent light (Schmale, personal communication, November 24, 2010).3

*Drosophila melanogaster*

The fruit fly (*Drosophila melanogaster*) has been used as a model research organism for almost a century (Ashburn et al. 2005; Lachaise 1988; Rubin 2000), originally in studies of genetic inheritance. Today it remains the classic organism for genetic research, but more recent studies have focused on molecular and developmental biology, especially embryonic development although there is also interest in the development of adult structures in the pupal stage (Bate and Arias 1993; Goldstein and Fyrberg 1994; Markow and O’Grady 2006).

Anatomy and Biology

*Drosophila*, like other insects, have an exoskeleton to protect their internal body parts, and their bodies are divided into three segments: the head, the thorax, and the abdomen. The head comprises compound eyes, a mouth, antennae, and ocelli. The thorax is the middle segment, where the wings and legs are attached, and the rearmost part of the fly is the abdomen. The body is covered with small hairs, called sensilla, which act as sense organs and provide information about touch, taste, smell, and sound to the fly’s nervous system.

The typical life cycle of *Drosophila* lasts about 12 to 14 days (Matthews 1994). Flies are fertile and begin mating

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3 As distinct from the other species discussed in this article, the scientific literature does not include reports of diseases or treatments for *Aplysia*. 
within 12 hours of eclosure (emergence of an adult insect from a pupal case). Females can store sperm deposited internally by multiple males. Fertilized eggs are deposited directly on a food source. Within a day, they hatch and the larva emerges. The first instar larva stage lasts about 24 hours, then the organism molts to a second instar larva and, after about a day, the third instar larva stage, which lasts about 3 days, after which the larva molts and forms a pupa. The pupal stage lasts 5 to 7 days, during which the larva undergoes metamorphosis to become an adult fly.

*Drosophila* are sexually dimorphic: females (about 2½ mm in length) tend to be larger than males and have a pointed, banded abdomen; the abdomen of the male is more rounded and generally darker than that of the female. Males also have a darkened area on their front legs called the sex comb. Virgin females are generally larger than mature females.

**Housing and Diet**

*Drosophila* are typically maintained in commercially available fly culture vials or small glass jars containing a commercial fly growth medium (Figure 3). The medium is mixed with tap water and allowed to solidify and dry before flies are added to the vial and the mouth of the container closed with a cotton or foam plug (Dahmann 2010). Flies are incubated at room temperature (22–23°C) and then transferred as necessary, for example to new vials containing fresh medium as the old medium is consumed or in the case of overcrowding by adult flies.

**Culturing and Genetic Breeding**

One method of propagating *Drosophila* is to transfer adult flies from the old vial to a new one as the food source is consumed. Gently tapping the old vial causes the flies to fall to the culture medium at the bottom, at which point the foam plug is removed and the new vial quickly placed so that the two vials open into each other. Gentle tapping will prompt the flies to fall from the old vial into the new. When all flies are in the new vial, the two vials are separated and a foam plug is quickly placed into the new vial.

If it is necessary to separate adult flies based on sex or phenotypic characteristics, they must first be anesthetized, using a commercially available anesthetic (e.g., chloroform or ether), CO₂, or chilling (Dahmann 2010). They can then be separated using a dissecting microscope and transferred onto a white card or into a Petri plate for observation and separation. Chilled flies must be kept on a chilled glass Petri plate (on ice) or they will start to wake up. A small paintbrush is an effective tool for gently separating the flies, after which the desired populations (usually 5 to 10 flies) can be placed for mating in vials containing fresh medium.

Genetic crosses require the collection of virgin females (which must be collected within 8 hours of eclosure), which are anesthetized and placed in separate vials for 2 to 3 days of observation to verify their virginity (virgin females can lay eggs but the eggs will not be fertile and will not hatch). The virgins are then placed in a fresh vial with an equal number of males and allowed to mate. Breeding flies are removed from the vial when the larvae begin to pupate. The adult offspring can then be anesthetized, examined, and enumerated using a dissecting microscope. Surplus flies can be disposed of by first anesthetizing them and then placing them in a jar or vial containing 70% ethyl alcohol, isopropyl alcohol, or mineral oil.

**Health Challenges and Treatment**

There are few health challenges associated with *Drosophila* cultures. Contamination with bacteria or fungus and infestation with mites are the most common problems. Bacterial and fungal contaminations are prevented or eliminated by using only freshly made fly medium and by frequently transferring flies to clean vials containing fresh food. Bacteria can also be eliminated by adding a small amount (1%) of a 1:100 penicillin-streptomycin solution to the surface of the food and allowing it to dry completely before flies are added. Mites are difficult to eradicate; in most cases cultures contaminated with mites must be destroyed to eliminate the infestation.

**Hermit Crabs**

Hermit crabs have been used for scientific research in the fields of behavior, competition, and population studies, but they are more often purchased as pets or display animals for school classrooms (Fox 2000).

4Detailed information about *Drosophila* culture media is available from the Harvard Medical School *Drosophila* RNAi Screening Center (www.flyrnai.org/DRSC-PRC.html).
There are two broad categories of hermit crabs: marine (or aquatic) and terrestrial (Giwojna 2009; Nash 1976). There are four families of aquatic hermit crabs: Diogenidae, Paguridae, Pagicolidae, and Pylojacquesidae; and terrestrial hermit crabs are classified into two families: Coenobitidae and Parapaguridae. Of the more than 600 species of hermit crabs, most are marine species; the most commonly maintained are species of Clibanatus, Paguristes, and Calcinus. There is only one known species of true freshwater hermit crab, Clibanarius fonticola, which lives in a small lake on Espiritu Santo Island, Vanuatu, in the South Pacific Ocean. Land hermit crabs are more commonly used as laboratory animals or kept as pets than marine hermit crabs, and the most common types are various species of Coenobita (e.g., C. clypeatus, C. compressus, and C. rugosus).

Anatomy and Biology

Hermit crabs are not true crabs, as they lack a complete exoskeleton. Only the front portion of the animal's body is covered with an exoskeleton; the rearmost portion is soft tissue that is protected by a shell. Similar to other crabs, hermit crabs have five pairs of legs, one of which has a large pinching claw. The innermost fifth pair of legs is small and serves to help keep the crab inside its borrowed shell. Hermit crabs molt their exoskeletons periodically as they develop, and move to successively larger shells for protection of the soft body as they grow in size.

Hermit crabs are most active nocturnally. They are also social animals, preferring to be in groups of three or more rather than singly housed. They live for up to 30 years in their natural habitats and as long as 15 years in captivity.

Terrestrial Hermit Crabs

Housing and Husbandry

Both tropical and subtropical land hermit crabs are commonly kept in a glass aquarium with a ventilated lid and a substrate of sand, wood chips, or coconut fiber (Figure 4). The substrate should be deep enough that the crab can bury itself when it needs to molt.

Land crabs require water, and their habitat (“crabitat”) should contain two nonmetallic water dishes, one with fresh, chlorine-free water and the other with saltwater. The water dishes should be deep enough for the crab to bathe but not so deep that it cannot get back out of the dish, and the water in each bowl should be changed daily. The habitat should be cleaned out weekly to remove waste and any food that the crabs may have hidden, and dishes and “toys” should be cleaned and rinsed well to completely remove any detergent residue.

Land hermit crabs are omnivores and scavengers, and may be fed fruits, vegetables, and meats as well as commercially available pelleted food. Fruits and vegetables should be washed thoroughly to remove any pesticide or detergent residue. Hermit crabs may also occasionally eat their own exoskeleton after shedding.

Temperature and humidity levels are critical (Provenzano 1962). Land hermit crabs have modified gills that allow them to breathe air, but these gills must be kept moist. Humidity in the crabitat should be between 70% and 80%. It should never be allowed to drop below 70%, and above 90% it can cause unacceptable bacterial and fungal growth. A natural sponge in a dish of freshwater can help to maintain humidity. Temperature can be maintained with an undertank heater.

Hermit crabs like “toys” such as driftwood and stones; items made of metal should be avoided. Empty shells of varying sizes should be provided for the crabs to use after molting.

Health Challenges and Treatment

One of the primary problems with land hermit crabs is suboptimal husbandry. Environmental stressors such as incorrect temperature or humidity can cause a crab to discard its shell and go naked, rendering it vulnerable to injury and infection. Crabs may also lose limbs due to aggression from tank mates, traumatic injuries, or improper environmental conditions, but with proper husbandry the limbs generally regenerate when the crab molts.

Of particular concern is infestation by various species of mites. To prevent or remove them, the aquarium and substrate should be thoroughly cleaned and sterilized on a regular basis (e.g., once a week). Infestations of dust (Dermatophagoides sp.), grain (Acarus sp.), and house (Liponyssoides sp.) mites can be prevented by removing uneaten food daily and by cleaning the tank weekly. Substrate and pieces of wood may be baked in an oven to sterilize them, and a small vacuum should be used to remove all sand and grit from the corners of the habitat.

Infestation by fungus gnats (Bradysia spp.) can also be a problem for terrestrial hermit crabs. In addition to thorough cleaning of the habitat and replacement of the substrate, fresh gnat traps made with a small jar containing apple cider vinegar or a similar solution can be added to the crabitat.
There are also predatory mites, *Hypoaspis* sp. (Cloyd 2010), that attack gnats and other mites and are effective for eradicating such infestations on hermit crabs.

Marine Hermit Crabs

Marine hermit crabs breathe through gills and require a saltwater aquarium (Bookhout 1964; Dawirs 1979; Harms 1992; Roberts 1971; Young and Hazlett 1978). The salinity of the water should be between 30 and 40 ppt, with a temperature of 22–28°C. The aquarium should be large enough that the crabs have space to roam, and there should also be rocks or corals for the crabs to climb. As with land hermit crabs, extra shells of various sizes should be provided to accommodate molting.

Aquatic species are omnivores and scavengers and will eat detritus, algae, shed exoskeletons, and dead organic matter. There are also several commercially available pelleted foods for marine hermit crabs.

Horseshoe Crab (*Limulus polyphemus*)

Horseshoe crabs have survived essentially unchanged for more than 200 million years, with fossil records of members of the family Limulidae dating back as far as 500 million years (Shuster et al. 2003; Walls et al. 2002). They are the closest living relatives of the ancient trilobites and are more closely related to modern-day scorpions and spiders than to true crabs. Today only four species of horseshoe crabs remain in various regions of the world. The “American” horseshoe crab, *Limulus polyphemus*, occupies the Atlantic coast of North America from Maine to the Yucatan peninsula (Shuster 1990). The other three species—*Tachypleus tridentatus*, *T. gigas*, and *Carcinoscorpius rotundicauda*—are found in coastal waters of Asia from India to Japan to the Philippine Islands (Shuster 1990). Because limited information is available about the culture of these latter species, the remainder of this section concerns the North American species of horseshoe crab.

*Limulus polyphemus* is a unique marine invertebrate with multiple commercial uses. Once an important resource for fertilizer and livestock feed, the horseshoe crab is now a bait in commercial whelk and eel fisheries; a common exhibit animal in public aquaria and classrooms to teach conservation and environmental issues; a laboratory research animal model to study the embryology, physiology, and function of marine invertebrates; and the primary source of *Limulus* amebocyte lysate, which is widely used to detect endotoxins on or in medical devices, implants, and vaccines (Berkson and Shuster 1999; Botton and Ropes 1987; Walls et al. 2002).

Anatomy and Biology

The body of the horseshoe crab is dorsoventrally flattened and divided into three sections: a frontal prosoma (cephalothorax) with an anterior flange; a hindbody opisthosoma (abdomen), and a posterior telson (tail). The dorsal surface of the prosoma has a pair of laterally located compound eyes, a centrally located ocellus, a raised anterior-to-posterior midline keel, and a hinge connecting the prosoma and opisthosoma. The opisthosoma protects the soft internal organs and book gills, and the animal uses the highly mobile telson to right itself. Ventrally the horseshoe crab has a single, anterior pair of modified chelicera, followed by five pairs of segmented legs, and then posteriorly the brachial appendages. The latter are attached to the underside of the opisthosoma and bear five pairs of book gills that contain the gill leaflets used for respiration, osmoregulation, and propulsion during swimming. Like most invertebrates, the horseshoe crab has an open circulatory system with hemolymph containing a copper-based hemocyanin for oxygen transport (Boiling et al. 1976).

When horseshoe crabs in the wild reach sexual maturity they mate during an annual spring migration to inshore spawning areas. Females may lay multiple clusters of 20,000 to 30,000 eggs on each spawning visit to the beach, after which multiple males pass over the clusters and release sperm to fertilize the eggs. Horseshoe crabs also readily spawn in the laboratory—females release eggs on the bottom of the tank and, as in the wild, males externally fertilize the eggs (Brown and Clapper 1981; French 1979; Gonzalez-Uríbe et al. 1991).

The small, green, round eggs have a relatively opaque shell (chorion) and take approximately 1 month to hatch at room temperature. A day or two before hatching, the outer shell splits open, revealing a transparent membrane surrounding the developing larva. First-stage larvae resemble adult horseshoe crabs except for the lack of a telson, which shows up after the first molt. In the wild, juvenile horseshoe crabs molt several times the first year and thereafter once annually, approximately 16 to 18 times over the next 10 to 12 years as the animal eventually reaches sexual maturity. (In the laboratory, where nutrition can be optimized and water temperatures can be held constant or elevated above natural cyclic temperatures, juvenile horseshoe crabs may have a higher number of molts annually.) The lifespan of horseshoe crabs is not well documented, but it is estimated at 18 to 22 years (Shuster et al. 2003).

During the molting process, the horseshoe crab’s external carapace splits along the cranial peripheral margin of the prosoma, allowing the soft-bodied crab to crawl out between the dorsal and ventral portions of the old outer shell. Until the new shell hardens, the horseshoe crab is susceptible to predation by horseshoe crabs and other marine species.

It is not possible to morphologically distinguish the sexes until the horseshoe crab reaches the final (terminal) molt. Sexually mature males are generally smaller than adult females and are readily identifiable by their first pair of legs, which are modified during the last molt into large bulbous claspers for grasping the female’s opisthosoma during mating.

Husbandry

Horseshoe crabs have been maintained in captivity in a wide range of systems from small glass aquaria to large fiberglass...
Horseshoe crabs in captivity. Note the filtration unit at the far end of the fiberglass holding tank, the submersible pump in the tank for circulating water through the filter, and the plastic screening on the bottom of the tank to assist the horseshoe crab in righting itself (originally published in Smith and Berkson 2005).

Figure 5 A simple recirculating aquaculture system for maintaining horseshoe crabs (Limulus polyphemus) in captivity.

Horseshoe crabs are extremely tolerant of a wide range of environmental conditions, but it is nonetheless important to conduct adequate water testing to evaluate various water quality parameters on a regular basis (weekly or more often depending on the number of horseshoe crabs or other biomass being maintained). Horseshoe crabs have been reported to exist in natural waters ranging in temperatures from −5°C to 35°C, and they can tolerate salinities ranging from 5 ppt to 35 ppt, although larval stages do not survive well at low salinities (Nolan and Smith 2009; Smith 2006; Smith and Berkson 2005). In the laboratory, juvenile and adult horseshoe crabs have been maintained long-term with water temperatures between 15°C and 21°C and salinity around 27 ppt (Brown and Clapper 1981; French 1979; Gonzalez-Urri et al. 1991; Laughlin 1982; Smith and Berkson 2005).

Both natural seawater and commercially prepared synthetic marine salts can be effective, but with a natural marine water source appropriate disinfection is necessary before use to reduce the risk of introducing any potential infectious or parasitic pathogens into the system. With recirculation systems, 25–30% of the marine water should be replaced every 3 to 4 weeks to reduce the amount of accumulated nitrates and to replenish any ions or minerals removed from the water by the crab’s osmoregulatory processes (Nolan and Smith 2009; Smith 2006).

Aeration of the system is important for both the animal’s respiration and the nitrification process of the holding system. Most aquaculture systems use water movement or supplemental air stones to ensure adequate aeration. Horseshoe crabs in the wild feed on a variety of bivalve molluscs, marine snails, marine worms, and other benthic invertebrates. In captivity, larval horseshoe crabs can be fed newly hatched live brine shrimp, and juveniles and adults are commonly fed dead fish, squid, small crabs, clams, and frozen brine shrimp (Botton 1984; Smith and Berkson 2005). The horseshoe crab also readily consumes commercially prepared artificial shrimp and fish diets but their nutritional value for the horseshoe crab is not known (Smith 2006).

Health Challenges and Treatment

There are only a few descriptions of the diseases and syndromes that affect horseshoe crabs. Noninfectious challenges in captive horseshoe crabs include water quality problems (e.g., ammonia toxicity, pH extremes, gas supersaturation, and high turbidity), developmental problems (e.g., molting problems of the shell, legs, or telson), and traumatic injuries caused during collection, transport, or overcrowding during captivity (Nolan and Smith 2009; Smith 2006). Physical trauma can result in puncture wounds, crushing, and fractures of the exoskeleton. Hemorrhage from traumatic lesions often appears significant but is rarely fatal.

Infectious challenges include algae, fungus, colonial and filamentous cyanobacteria, Gram-negative bacteria, and a variety of parasites (Smith 2006; Nolan and Smith 2009). Lesions of the shell due to external pathogens are probably the most common problem seen in horseshoe crabs and are usually evident in discoloration or erosion of the carapace. Algal, fungal, and bacterial infections may colonize and penetrate the carapace, eyes, and gill surfaces and go on to become systemic, involving the deeper tissues of the organs, gills, and circulatory sinuses and resulting in extensive tissue necrosis and death. Specific bacteria species isolated from external lesions of the horseshoe crab include Oscillatoria, Leucothrix, Vibrio, Flavobacterium, Pseudomonas, and Pasteurella.

In addition, a variety of internal and external parasites have been reported, including protozoans, a larval digenetic...
trematode, a few nematodes, and several turbellarid worms. Probably the most significant parasites are the turbellarid worms, which reside between the gill leaflets, on the ventral surface of the carapace, or on the appendages. These ectoparasites lay stalked cocoons on the external surfaces of the gill leaflets, causing superficial lesions on the surface of the gill tissue.

The most noticeable organisms are the ectocommensals—bryozoans, sponges, barnacles, blue mussels, lady slippers, snails, oysters, and whelks—that frequently occur on the external surfaces of the exoskeleton but seldom cause any harm to the horseshoe crab. Freshwater baths (3–12 min), acetic acid baths (3–5% for up to 1 hour), and formalin baths (1–1.5 ppt for up to 12 hours) have been used to remove external parasites and ectocommensals from the carapace of the horseshoe crab (Bullis 1994; Landy and Leibovitz 1983; Nolan and Smith 2009).

The pharmacokinetic profiles of oxytetracycline and itraconazole have been reported in the American horseshoe crab (for a full discussion of anesthesia and analgesia in invertebrates, see Cooper 2011, in this issue). A single oral or intravenous (i.v.) dose of oxytetracycline (25 mg/kg or 50 mg/kg) yielded pharmacokinetic data suggesting that i.v. would be the route of choice for continued maintenance of drug-serum concentrations (Nolan et al. 2007). Data from a study involving i.v. administration of a single dose of itraconazole at 10 mg/kg suggested that such a dose would be necessary every 24 hours to maintain an effective treatment level (Allender et al. 2008). Fluconazole, injected i.v. into the cardiac sinus at a dose of 2 mg/kg body weight every 4 days for 6 treatments, has been reported for the treatment of *Aspergillus niger* fungal infections (Tim Tristan, Texas State A quarantine, personal communication, November 22, 2010).

Unfortunately, most treatments for bacterial or fungal infections are generally ineffective, as the horseshoe crabs become lethargic and anorectic before eventually dying from the infection. Humane and rapid euthanasia is possible with the injection of 1 to 2 ml of pentobarbital solution directly into the dorsal cardiac sinus (Smith 2006).

### Cephalopods

Cephalopods (the name originates from the Greek meaning “head foot”) are an ancient group of animals that has been developing through the ages with many earlier species now extinct. They have significant commercial value both for human and animal consumption and for basic and biomedical research. Research has largely focused on neurobiology and behavior, with studies on sensory perception, angular acceleration, vision, central and peripheral nerve conduction, neurotransmitters, and cells associated with the skin function.3 Cephalopods have been successfully maintained and cultured for over 30 years at the National Resource Center for Cephalopods (NRCC), a laboratory supported by the National Institutes of Health’s National Center for Research Resources. The squid (*Sepioteuthis lessoniana*) has been cultured through seven successive generations (Walsh et al. 2002); the pharaoh cuttlefish (*Sepia pharaonis*) through five consecutive generations in the laboratory (Minton et al. 2001) using closed, recirculating water filtration systems; and *S. officinalis* through seven generations in a recirculating marine system (Forsythe et al. 1994). However, as explained under Husbandry below, cephalopods are not the easiest of animals to maintain and care for unless the user is both knowledgeable about species-specific aquatic environment needs and experienced with water quality parameters essential for marine invertebrates.

Scientists believe ancestors of modern cephalopods (subclass Coleoidea) diverged from the primitive externally shelled Nautiloidea very early, perhaps in the Ordovician period some 438 million years ago, before the existence of mammals and fish on this planet. Cephalopods were once one of the dominant life forms in the world’s oceans, but today there are only about 800 living species.4 The class Cephalopoda (phylum Mollusca) has two very distinct sub-classes: Coleoidea (octopuses, squid, and cuttlefish) and Nautiloidea (nautilus). This section focuses on octopuses, squid, and cuttlefish (nautilus can be raised in captivity but the author [JMS] is most familiar with the Coleoidea).

### Anatomy and Biology

Cephalopods have some unique anatomical features and should be considered highly specialized animals that have successfully evolved through the ages. Cuttlefish, octopuses, and squids are semelparous, meaning they grow rapidly to sexual maturity, spawn once, and die (Hanlon 1987). The animals’ lifespan in laboratories, depending on temperature and nutrition, is usually 1 year (the nautilus, in contrast, may live up to 20 years; Scimeca 2006). Cephalopods (excluding the nautilus) are primarily top predators and very active in hunting their prey. They are not good community tank inhabitants and the various species are best housed separately.

Most cephalopods have only a shell remnant that is either greatly modified or completely absent. In the subclass Coleoidea, the shell is internal and reduced in size. In the order Sepioida (the cuttlefishes and bottle-tailed squids), the calcareous chambered shell is present internally, functioning as a buoyancy organ. Representatives of this subclass include *Sepia* and *Euprymna*; *S. officinalis* is the common or European cuttlefish. In the order Teuthoida (the shallow-water and oceanic squids), the shell is reduced to a chitinous “pen” (or gladus) that lies dorsally in the body. The body is elongated and usually finned, with eight suckered arms and two long tentacles; the Atlantic brief squid (*Loliguncula brevis*) belongs to this group. The order Octopoda

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3Information Resources on Amphibians, Fish and Reptiles Used in Biomedical Research; available online (www.nal.usda.gov/awic/pubs/amphib.htm).

4Information from the Cephalopod Page (www.thecephalopodpage.org).
Creating an environment suitable for cephalopods is essential to successful culture. The temperature range for temperate species of cephalopods is 15–22°C and for tropical species 25–32°C. In general, salinity should be 27–36 ppt; pH 7.7–8.2, ammonia and nitrites <0.1 mg/l, and nitrates <20.0 mg/l (Hanlon 1987; Lee et al. 1994a; Oestmann et al. 1997; Sherrill et al. 2000). Water outside these parameters will likely cause cephalopods to become stressed and result in greater susceptibility to disease. Therefore, routine monitoring of water quality cannot be overemphasized.

Water should be conditioned and adjusted for the correct salinity, and, in the case of open water systems, filtered and contaminants removed. Care should be taken when using potable municipal water, which may be treated with potentially toxic chlorines and chloramines. Evaluating makeup water is essential for the culture of cephalopods and sending water out for analysis for metals, organics, hardness, and possible toxins is well worth the time and effort. Such analysis enables more precise adjustment of the animal’s holding water and thus results in a suitable environment that both supports the animal’s health and minimizes variability in research outcomes.

After analysis of the makeup water, water filtration should occur as follows: from the holding tank water passes through (1) a protein skimmer (or foam fractionator) that strips dissolved organic compounds (this equipment is essential as cephalopods produce and often expel abundant ink when alarmed or startled); (2) a mechanical filter that removes particles down to 100 µm; (3) a high-grade activated carbon and a biological filter, where ammonia is eventually broken down to nitrites and nitrates; and (4) a UV sterilization unit before the water is returned to the animal holding.

Figure 6 Numerous octopuses (Octopus bimaculoides) in a fiberglass tank with short lengths of PVC piping for housing and enrichment. Note the live shrimp (upper left) provided for food.

Husbandry

Creating a laboratory environment for cephalopods that promotes species-typical behavior, reproduction, and optimal health conditions is challenging. There is a wide variety of seawater systems suitable for maintaining these animals in captivity and there is considerable variation in their design to accommodate laboratory location and species (Figure 6). Tanks and holding systems are highly variable and adaptable; both open and closed seawater systems are available. Piping should be of PVC, not copper, which is toxic to all cephalopods. With any type of support system, close monitoring is necessary to maintain optimal water quality because animal densities in captivity are often higher than those in the wild.

There is a wide range of suitable housing composition and substrates depending on the proposed use (e.g., culture or display) (Forsythe and Hanlon 1980; Forsythe et al. 1991; Hanlon and Forsythe 1985; Yang et al. 1989), and several companies custom design and produce tanks to suit different needs. Fiberglass composite works well and tanks can be fitted with viewing windows (acrylic is strong but scratches fairly easily). Large animal water troughs have also been modified for use with cephalopods. Tanks with exposed metal should be avoided.

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Health Challenges and Treatment

Most health problems in cephalopods result from either poor water quality or traumatic injuries. Wild-caught animals are particularly prone to physical and mechanical damage. As mentioned above, the epidermal microvillous skin layer is easily injured, and cuts and abrasions of the skin from nets and handling are a frequent cause of morbidity and eventual mortality. Captive cuttlefish can jet across the tank and damage the skin, eventually leading to septicemia and death (Scimeca and Oestmann 1995). Mantle lesions are typically preceded by abnormal swimming behavior and commonly lead to secondary bacterial infections that affect multiple organ systems and rapidly lead to death (Sherrill et al. 2000). Furthermore, if the force of the animals’ propulsion against the side of the tank is great, as is often the case with cuttlefish, the result may be fractures of the internal cuttlebone.

Bacterial infections and septicemias in cephalopods are commonly reported to be caused by various species of the bacterium *Vibrio* (Hanlon and Forsythe 1990; Scimeca 2006). Common infections secondary to abrasions or traumatic skin lesions have been summarized in different cephalopod species (Scimeca 2006).

Cannibalism is well documented in captive octopuses, cuttlefish, and squids (Budelmann 2010). Stacking densities and limited food supplies have been suggested as possible etiologies for this problem. Autophagy has been reported in *Octopus vulgaris* and there is some evidence that it may be due to a released substance or possibly a virus or bacteria (Budelmann 2010).

Various anesthetic compounds are effective for cephalopods; as with other animals, the age, sex, size, and species should be considered closely before performing anesthesia. Magnesium chloride (MgCl₂) is the anesthetic agent of choice for cephalopods because it is easy to obtain, inexpensive, stable, nontoxic, and easy to prepare (Messinger et al. 1985). A standard solution of 75 g of MgCl₂ dissolved in 1 L of dH₂O and mixed with 1 L of seawater has worked well (Scimeca and Forsythe 1999). This one to one (1:1) mixture can be used for surgical anesthesia and some invasive clinical procedures, a 1:3 or 1:4 dilution is adequate for handling and examinations, and a 1:10 dilution induces mild sedation for stress reduction and shipping.

Preliminary evaluation of MgCl₂ in five different genera of cephalopods showed effective anesthesia in all individuals (Scimeca 2006). The site of action for MgCl₂ is thought to be the central nervous system because stimulation of the fin nerve in anesthetized cuttlefish elicits a motor response. Thus, it is believed that MgCl₂ works at the postsynaptic membrane of the nerve-muscle junction in crustaceans and vertebrates (Scimeca 2006).

The MgCl₂ preparation can also produce a surgical plane of anesthesia in octopuses with the addition of an ethanol “push” consisting of a 1% solution (by volume) of ethanol (e.g., 10 ml of ethanol is added to 1 L of the 1:1 seawater/MgCl₂ mixture). And researchers have described the use of ethanol for anesthetic induction and maintenance during an excisional biopsy and surgical repair of a mycotic skin lesion on a European cuttlefish (Harms et al. 2006). The authors reported an induction time of less than 1 minute with 3% ethanol (30 ml/l) and successful anesthetic maintenance at 1.5% ethanol in seawater.

Benzocaine has been used in the giant Pacific octopus for effective anesthesia at concentrations above 1,000 mg/l and also as a compound for euthanasia. This method of euthanasia was reported to be a relatively rapid and humane method at 3,500 mg/l compared to other euthanasia practices (Barord and Christie 2007).

Treatment with antibiotics such as enrofloxacin (Baytril), gentocin, nitrofurin, and metronidazole has been reported (for a review of suggested dosages and routes of administration, Scimeca 2006). Because cephalopod skin is just 1 cell layer thick and the animals have a large surface area, care in the dosage and time of exposure for water baths is essential—it is easy to overdose these animals with water bath antibiotics. An alternative technique used with cuttlefish involves injecting live shrimp with 10 mg/kg enrofloxacin and immediately feeding this medicated food to the cuttlefish. If the cuttlefish are not eating, i.v. injection using a 25 g or smaller needle of 5 mg/kg enrofloxacin into the muscular portion of the cephalic vein every 8 to 12 hours is also effective (Gore et al. 2005).

As is true for all species of laboratory animals, training of staff and research personnel is essential for successfully maintaining cephalopods in the research laboratory. Appropriate care and husbandry are imperative, as is clinical observation, especially given the semelparous life cycle challenges in the management of cephalopods’ 12- to 14-month lifespan (Sherrill et al. 2000). Experience in the necropsy and pathology of cephalopods is also a key component to success when problems arise related to colony health.
Invertebrates have been used for many years in various research, teaching, and public display settings. As with other laboratory animals, the proper culturing and care of invertebrates in captivity entails specific housing, environmental, nutritional, and management requirements. A thorough investigation of these requirements is always advisable together with implementation of the specific recommendations to ensure the humane care and welfare of these organisms.

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Abstract

The use of invertebrates in research laboratories, classroom teaching, and public displays has greatly increased over the past 20 years, accompanied by a corresponding increase in the amount of online information and literature about invertebrates. This brief overview of Internet resources is intended to aid both novice and experienced individuals in the search for such information.

Key Words: annelid; arthropod; cephalopod; Internet resource; invertebrate; mollusc; platyhelminth

Invertebrates consist of over 30 phyla of organisms and account for more than 95% of all animal species on this planet. They are extremely diverse organisms, ranging from simple sponges to complex cephalopods, and have played a major role in exhibits or displays at most aquaria, zoos, and nature centers worldwide for over a century. Invertebrates have also been used for a variety of research purposes, from basic biological studies to investigations of mechanisms of cell signaling to experimental models of cancer and human disease. In the classroom, invertebrates have been used for teaching zoology, anatomy, physiology, embryology, genetics, husbandry, and conservation to primary, secondary, and college students.

The popular use of invertebrates coupled with the emergence of the Internet has spawned an array of online resources for invertebrate species. Conservation of invertebrate groups such as marine corals and freshwater molluscs is a major theme of a number of websites, but only a few websites convey serious consideration of the welfare of invertebrates. As concern increases for the care, welfare, and enrichment of invertebrates cultured and/or maintained in captivity, this will undoubtedly change in the near future.

The following compilation of Internet links is designed to be a practical resource for all who use and care for invertebrates, from high school biology teachers to experienced researchers. It is not meant to be an exhaustive listing as the exponential expansion of the World Wide Web simply does not allow the publication of such a document. Instead, this resource should be used as a guide for locating preliminary information, beginning with established organizations that have an interest in expanding knowledge about invertebrates and/or ensuring their appropriate care and use in the laboratory, classroom, or display environment.

The list is organized by general phylum and selected species, with a descriptive title, web address, and brief synopsis provided for each website. Specific information about taxonomy, natural history, husbandry, culture, welfare, anatomy and histology, physiology, ontogeny, genetics, conservation, toxicology, educational resources, listservs, and databases must be derived from each group or species link.

The following list includes only those links that are considered to be of substantive value, and not sites of primarily commercial interest. However, due to the ever-changing world of the Internet, with new websites springing up every day and other sites no longer functional, the accuracy and consistency of the links cannot be guaranteed. In light of the massive amount of information about invertebrates on the Internet, it is possible that some useful websites or links are inadvertently missing from this compilation.

General Information

The Invertebrate Phyla – Earthlife.net
www.earthlife.net/inverts/an-phyla.html
Brief overview of all invertebrate phyla with links to information on specific groups.

Invertebrate – Wikipedia
http://en.wikipedia.org/wiki/Invertebrate
Foundational information on the classification, phyla, and life history of invertebrates.

The Invertebrate Animals
http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/I/Invertebrates.html
Introductory information on the origin and evolution of invertebrates with descriptions of major taxa.

Invertebrates – Animal Kingdom
http://animalkingdom.net/category/invertebrates/
General website on invertebrates with links to other websites for specific groups.

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1 All the websites listed here were accessed on March 11, 2011.
The Tree of Life Web Project
http://tolweb.org/tree/phylogeny.html
General information about the biodiversity, characteristics, and phylogeny of different groups of invertebrate organisms.

Invertebrate Zoology Online – Lander University
http://webs.lander.edu/rsfox/invertebrates/
A anatomical descriptions, systematics, and references for over 100 invertebrate species used in zoology teaching and research.

Invertebrates – Biology4kids.com
www.biology4kids.com/files/invert_main.html
Educational resource on invertebrate classification for primary and secondary teachers and students.

Invertebrate Lesson Plans – Discovery Education
www.discoveryeducation.com/teachers/free-lesson-plans/invertebrates.cfm
Lesson plans, materials, suggested readings, and links for teaching students in grades 6-8 about invertebrates.

Dissections Online – University of Buffalo
http://ublib.buffalo.edu/libraries/asi/guides/bio/dissections.html
Links to dissection guides for a variety of invertebrates (earthworm, crayfish, grasshopper, tick, roundworm, starfish, clam, squid, and sponge).

Invertebrate Printouts – Enchanted Learning.com
www.enchantedlearning.com/subjects/invertebrates/index.shtml
Information and printouts on invertebrates for primary and secondary education.

Invertebrates – Electronic Zoo/NetVet
http://netvet.wustl.edu/invert.htm
Links relating to Drosophila, bees, arthropods, worms, and crustaceans.

Riverwatch – Aquatic Invertebrates
www.riverwatch.ab.ca/how_to_monitor/macronvertebrates.cfm
Information on collecting, identification, behavior, habitat preference, and pollution tolerance of aquatic invertebrates.

Marine Species Identification Portal – ETI Bioinformatics in the KeyToNature program
http://species-identification.org/index.php
Information, images, and links for numerous species of marine invertebrates.

Invertebrates – Smithsonian Institute National Zoological Park
http://nationalzoo.si.edu/Animals/Invertebrates/default.cfm
Interactive resource for general information on invertebrates.

Invertebrate Zoology – Bishop Museum
www.bishopmuseum.org/research/natsci/invert/
Catalogue of invertebrate specimens and images from the Hawaiian and Pacific areas including a comprehensive checklist of all marine invertebrates native to the area.

Invertebrates – Australian Museum
http://australianmuseum.net.au/invertebrates.html
Catalogue of the museum’s invertebrate collections, which primarily focus on crustaceans, worms, insects, spiders, and molluscs.

GBR Explorer – ReefEd
www.reefed.edu.au/home/explorer
Interactive online guide to the invertebrates of the Great Barrier Reef.

Invertebrates as Indicators – US Environmental Protection Agency
www.epa.gov/bioiweb1/html/invertebrate.html
Information about invertebrates as biological indicators of watershed health.

Information Resources on the Care and Use of Invertebrates – US Department of Agriculture
Online compilation of references related to the care and use of invertebrates in the laboratory, classroom, or display environment (AWIC Resource Series No. 8, 2000).

Invertebrate Link – Joint Committee for the Conservation of British Invertebrates
www.royensoc.co.uk/InvLink/Index.html
Forum to advance the conservation of invertebrates in the United Kingdom through the exchange of information among organizations and statutory bodies for the development of strategies, policies, and best practice guidelines.

BugLife – The Invertebrate Conservation Trust
www.buglife.org.uk/
Information about the conservation of European invertebrates.

Basic Information on the Major Invertebrate Phyla

Porifera (Sponges)

Introduction to Porifera – University of California Museum of Paleontology
www.ucmp.berkeley.edu/porifera/porifera.html
Introduction to the simplest phyla of invertebrates, with examples of sponge types.

World Porifera Database
www.marinespecies.org/porifera/
Database of sponges worldwide and part of the global initiative of the World Register of Marine Species (WoRMS) to register all marine organisms.

Sponges of Britain and Ireland
www.habitas.org.uk/marinelife/sponge_guide/

**Palaeos Metazoa: Porifera**
www.palaeos.com/Invertebrates/Porifera/Porifera.htm
Introduction to the taxonomic anatomy of sponges.

**Sponge Barcoding Project**
www.spongebarcoding.org/
Database that compares DNA signature sequences to conventional morphological taxonomic characters of sponges.

**Cnidaria (Corals, Anemones, Box Jellies, Hydroids, and Jellyfish)**

*Introduction to Cnidaria – University of California Museum of Paleontology*
www.ucmp.berkeley.edu/cnidaria/cnidaria.html
Introduction to Cnidarians including basic characteristics and examples of this phylum.

**The Hydrozoan Society**
www.ucmp.berkeley.edu/agc/HS/
List of scientists working on hydrozoans and links to other websites on hydrozoans.

**The Hydrozoan Directory – Natural History Museum, Geneva**
www.ville-ge.ch/mhng/hydrozoa/hydrozoa-directory.htm
Introduction to hydrozoans.

**Synopsis of the Medusae of the World – National Marine Biological Laboratory, Plymouth, UK**
www.mba.ac.uk/nmbl/publications/jmba_40/jmba_40.htm
Links to a PDF collection describing the medusae of the world.

**The Scyphozoan – University of California, Merced**
http://thescyphozoan.ucmerced.edu/tSA ug07.html
Resource describing biodiversity in the Scyphozoa (true jellyfish) including their behavior, biogeography, ecology, and evolution.

**Biogeoinformatics of Hexacorals – Kansas Geological Survey**
www.kgs.ku.edu/Hhexacoral/
Resource for methods and links related to the taxonomy, biogeography, habitat characteristics, and environmental correlates of Hexacorallia (corals and sea anemones) and allied taxa.

**Hexacorallians of the World – Kansas Geological Survey**
http://hercules.kgs.ku.edu/hexacoral/anemone2/index.cfm
Bibliographic references and a synonymy list for each taxon, published distribution of each species, and images of many species.

**Octocoral Research Center – California Academy of Sciences**
Information about soft corals, gorgonians, and sea pens, with references, links, and images.

**ReefBase – The WorldFish Center**
www.reefbase.org/
Official database of the Global Coral Reef Monitoring Network (GCRMN) and the International Coral Reef Action Network (ICRAN), with consolidated knowledge about coral reefs to facilitate analyses and monitoring of coral reef health, understanding of the relationship between human activities and coral reefs, enhancement of the quality of life of reef-dependent people, and informed decisions about coral reef use and management.

**Coral Reef Information System – US National Oceanic and Atmospheric Administration**
http://coris.noaa.gov/
Information from NOAA about coral reef research, monitoring, and management activities, with emphasis on the US states, territories, and remote island areas.

**Coral Reef Conservation Program – US National Oceanic and Atmospheric Administration**
http://coralreef.noaa.gov/
Expertise and information from a wide array of NOAA programs and offices to reduce harm to and restore the health of coral reefs, including deep-sea corals, by addressing national threats and local management priorities through conservation activities.

**EnviroLink – International Coral Reef Information Network**
www.envirolink.org/resource.html?itemid=200211111503070.477551&catid=3
General information about coral reefs, with coral reef outreach tools and resources such as fact sheets, a searchable directory of over 600 coral reef organizations, images, and a list of teacher’s resources.

**CORAL – Coral Reef Alliance**
www.coral.org/
Educational resources to help local communities become environmental stewards for coral reefs.

**National Coral Reef Institute (NCR1) – Nova Southeastern University Oceanographic Center**
www.nova.edu/ocean/ncri/
Information on the assessment, monitoring, and restoration of coral reefs through research and education.

**Remote Sensing of Coral Reefs – US National Aeronautics and Space Administration**
http://oceancolor.gsfc.nasa.gov/SeaWiFS/reefs/
Remotely sensed observations for mapping of coral reefs around the globe.
Jellyfish – Wikipedia
General information on the anatomy, life cycle, and classification of freshwater and marine jellyfish.

Jellywatch
www.jellywatch.org/
Reports of sightings of jellyfish and other marine life.

Freshwater Jellyfish
http://freshwaterjellyfish.org/
General information on the species, distribution, natural history, and life cycle of freshwater jellyfish.

Ectoprocta (Bryozoans and Marine Mats)

The Bryozoa Homepage
http://bryozoa.net/
Introduction to bryozoans with an index to the described taxa and an extensive list of links to other sites devoted to bryozoans.

Bryozoa Introduction - Smithsonian Institute Marine Station at Fort Pierce
www.sms.si.edu/irlspec/IntroBryozoa.htm
Information on species diversity, habitat, reproduction, and development.

Bryozoan Home - The Field Museum of Chicago
www.bryozoan.com/index.php
Interactive global reference for the collection, preservation, and dissemination of scientific knowledge on marine, estuarine, and freshwater bryozoans.

Freshwater Bryozoans - Wright State University
www.wright.edu/~tim.wood/index.html
Information about freshwater bryozoans with links to other bryozoan websites and collections.

Platyhelminthes (Flatworms Including Turbellarians, Trematodes, and Cestodes)

Introduction to the Platyhelminthes – University of California Museum of Paleontology
www.ucmp.berkeley.edu/platyhelminthes/platyhelminthes.html
Brief overview of the diverse group of flatworms.

Marine Flatworms of the World
www.rzuser.uni-heidelberg.de/~bu6/index.html
General information, characteristics, and examples of polyclad flatworms; excellent source of quality images of the marine flatworms of the world.

Planarian Resources on the Web
www2u.biglobe.ne.jp/~gen-ju/plaweb_e.html
General resource for information on planarians.

Global Cestode Database
http://129.237.138.100/PEETII/uconnPEETII.html
Multinational collaboration for taxonomic characterization of the Cestoda.

Tapeworms.org
www.tapeworms.org
Links to workshops and databases for cestodes.

Annelids (Ragworms, Earthworms, and Leeches)

Introduction to the Annelida – University of California Museum of Paleontology
www.ucmp.berkeley.edu/annelida/annelida.html
Links to the history, evolution, systematics, and morphology of this phylum.

Annelid Research Resources
www.annelida.net/res-res.html
Links to websites on annelid taxonomy, research, and resources.

Center for Annelida Resources - Illinois Natural History Survey
www.inhs.uiuc.edu/~mjwetzel/mjw.inhsCAR.html
Links to websites on annelid taxonomy, collections, and other resources.

Earthworms – The Backyard Nature Website
www.backyardnature.net/earthworm.htm
General information on the importance, anatomy, reproduction, and behavior of earthworms.

Echinodermata (Starfish, Brittle Stars, Sea Urchins, and Sea Cucumbers)

Introduction to the Echinoderms – University of California Museum of Paleontology
www.ucmp.berkeley.edu/echinodermata/echinodermata.html
General information, characteristics, and examples of echinoderms.

The CAS Echinoderm Web Page – California Academy of Sciences
Comprehensive list of links to research, collections, and resources on echinoderms.

The Echinoid Directory – Natural History Museum of London
www.nhm.ac.uk/palaeontology/echinoids/index.html
Information on the morphology, taxonomy, and classification of echinoids.

Virtual Echinoderm Newsletter – Smithsonian National Museum of Natural History
http://invertebrates.si.edu/echinoderm/
Lists of researchers, publications, theses, and links to echinoderm information.
Sea Urchin Embryology (SUE) – Stanford University
www.stanford.edu/group/Urchin/contents.html
Comprehensive site for information on the anatomy, reproduction, development, culture, and research ideas using sea urchins in the classroom, with lesson plans, images, and supporting material.

Molluscs (Snails, Octopuses, Squid, Clams, Scallops, Oysters, and Chitons)

The Mollusca – University of California Museum of Paleontology
www.ucmp.berkeley.edu/taxa/inverts/mollusca/mollusca.php
General background information on the phylum.

Check List of European Marine Mollusca (CLEMAM)
www.somali.asso.fr/clemam/index.clemam.html
Taxonomically oriented database of the marine Mollusca of Europe and adjacent areas.

Living World of Molluscs
www.weichtiere.at/english/mollusca/index.html
General information on the major groups of molluscs, with additional information, links, and images of select species.

Snail-world
www.snail-world.com/
General information on the evolution, species, anatomy, feeding, and reproduction of snails.

Apple Snail (Ampullariidae)
http://applesnail.net/
Information (with links and images) about the anatomy, biology, care, ecology, and distribution of tropical and subtropical freshwater "apple" snails.

Freshwater Mollusk Websites – University of Minnesota
http://fwcb.cfans.umn.edu/personnel/staff/hove/Mussel.web.sites.html
Comprehensive list of links to information and research on freshwater mussels.

The MUSSEL Project Website (MUSSELp) – University of Alabama
http://mussel-project.ua.edu/
Revised classification of the Unionoida (freshwater mussels), with a database of recent unionoid species and genera described to date.

Freshwater Mussel (Unionoida) Genera of the World – Illinois Natural History Survey
www.inhs.uiuc.edu/~ksc/MusselGenera.html
Image-based website illustrating all genera of freshwater mussels (Unionoida).

Mollusk Bibliography Database – Illinois Natural History Survey
http://ellipse.inhs.uiuc.edu:591/mollusk/default.html
Searchable database of freshwater mollusc literature.

Conus Biodiversity – University of Washington
http://biology.burke.washington.edu/conus/
Information and systematics of the marine gastropod genus Conus.

Molluscs – Museum of Malacology, Cismar, Germany
www.hausdernatur.de/hncadr.htm
Extensive list of general malacological web pages, societies, journals, museums, and informal groups.

Molluscan Neuroscience Homepage – Georgia State University
www.squishybrain.org/drupal/
Links to resources related to gastropod neuroscience.

Bibliographia Nudibranchia – Nudibranch Systematic Index – University of California
http://repositories.cdlib.org/ims/Nudibranch_Systematic_Index_second_edition/
List of all names in the nudibranch bibliography.

Phylogeny of Nudibranchia – California Academy of Sciences
Descriptions of species, with natural groupings of sea slugs based on studies of anatomy and supplemented with molecular studies.

Sea Slug Forum – Australian Museum
www.seaslugforum.net/aplycali.htm
Information about the behavior, anatomy, and care of nudibranchs, bubble shells, and sea hares; with links to websites that provide taxonomic lists, information, and images of sea slugs based on geographic location.

Opisthobranchia of the World
www.medslugs.de/Opi/Opisthobranchia.htm
Links to opisthobranch images on the Web arranged alphabetically and taxonomically.

Sea Slugs of Hawaii
http://seaslugsofhawaii.com/index.html
Introductory material (with images, maps, and references for species identified from Hawaiian Islands) and links to numerous websites that provide images and information on sea slugs based on geographic location.

Arthropods (Insects, Arachnids, and Crustaceans)

Introduction to the Arthropoda – University of California Museum of Paleontology
www.ucmp.berkeley.edu/arthropoda/arthropoda.html
General overview on the largest phylum of animals, the Arthropoda.

Entomology on the Worldwide Web – Colorado State University
www.colostate.edu/Depts/Entomology/
Comprehensive list of web links to various resources in entomology including bibliographies, collections, databases, publications, and research activities.
Insects and Entomology - Iowa State University
www.ent.iastate.edu/List/
Index of web links to various resources in entomology including bibliographies, collections, databases, publications, and research institutions and societies.

Center for Insect Science Education Outreach - University of Arizona
http://insected.arizona.edu/uli.htm
Information for “Using Live Insects in Elementary Classrooms for Early Lessons in Life,” with lesson plans and fact sheets on species identification, natural history, collecting, and impacts of insects on the ecosystem.

BugGuide.Net
http://bugguide.net/node/view/15740
Online community of naturalists who share information, observations, and images of insects, spiders, and other related creatures.

The Chironomid Home Page - Museum of Zoology University of Michigan
http://insects.ummz.lsa.umich.edu/~ethanbr/chiro/index.html
Extensive list of material related to identification, checklists, culture, researchers, and references to the Chironomidae, or nonbiting midges.

Fleas of the World - Brigham Young University
http://fleasoftheworld.byu.edu/index2.htm
General information on the morphology, systematics, phylogeny, and evolution of fleas.

Fleas (Siphonaptera) - Zoological Institute, St. Petersburg, Russia
www.zin.ru/Animalia/Siphonaptera/index.htm
General information on the morphology, systematics, ecology, and fauna of fleas.

The Insect and Spider Collections of the World - Bishop Museum
http://hb.s.bishopmuseum.org/codens/codens-r-us.html
Comprehensive list and links to major insect and spider collections of the world.

The Myriapoda (millipedes and centipedes) of North America - North Carolina State Museum of Natural Sciences
http://nadiplochilo.com/index.html
General information, images, and references for millipedes and centipedes.

The Myriapoda (millipedes and centipedes) - East Carolina University
www.myriapoda.org/index.html
Introductory information on millipedes and centipedes.

Centre International de Myriapodologie (CIM) - Musée National d’Histoire Naturelle de Paris
www.mnhn.fr/assoc/myriapoda/INDEX.HTM
Comprehensive information on the Myriapoda and Onychophora worldwide.

Polydesmida
www.polydesmida.info/
General information about the millipede order Polydesmida, with links to information on the millipedes of Australia and multipedes of Tasmania.

World List of Marine, Freshwater and Terrestrial Isopod Crustaceans - Smithsonian National Museum of Natural History
http://invertebrates.si.edu/isopod/
Comprehensive list of research, collections, and bibliographic information on isopods.

Forum Flusskrebse
www.forum-flusskrebse.org/
German-based forum for general information on various crayfish species.

Garnelen
www.garnele-online.de/
German reader-based online magazine covering the taxonomy, culture, care, and breeding of invertebrates, especially freshwater shrimp.

Global Crayfish Resources - Carnegie Museum of Natural History
http://iz.carnegiemnh.org/crayfish/
Worldwide checklists, databases, identification keys, and links for freshwater crayfish.

The Crayfish by T.H. Huxley
www2.biology.ualberta.ca/palmer/thh/crayfish.htm

ZooGene: A DNA Sequence Database for Calanoid Copepods and Euphausiids
www.ZooGene.org/
International database of DNA type sequences for calanoid copepods and euphausiids.

Information Resources on the Care and Use of Insects - US Department of Agriculture
www.nal.usda.gov/awic/pubs/Labinsects/labinsects.htm
Compilation of references for the culture, rearing, and utilization of insects in the laboratory and for public display (AWIC Resource Series No. 25, 2005).

Terrestrial Invertebrate Taxon Advisory Group - American Zoo and Aquarium Association
www.titag.org/
Information about the conservation of invertebrates and the management needs of facilities that exhibit invertebrates for educational purposes, with husbandry information from various institutions for several species of invertebrates (e.g., beetles, cockroaches, tarantulas).

Caresheets - Amateur Entomologists’ Society
www.amentsoc.org/insects/caresheets/
General husbandry guidelines for a variety of insects, arachnids, and other invertebrates.
Invertebrate Husbandry Guidelines – Australian Society of Zoo Keeping
www.aszk.org.au/husbandry.invertebrate.ews
List of guidelines for invertebrates in general and specific guidelines for mantids, grasshoppers, stick insects, and several beetles and butterflies.
Invert Care
www.invertcare.com/
General husbandry guidelines for a variety of scorpions, tarantulas, centipedes, millipedes, and other invertebrates.

Invertebrate Paleontology
Invertebrate Paleontology – Cleveland Museum of Natural History
www.cmnh.org/site/researchandcollections/InvertebratePaleontology.aspx
Information about the collection, preservation, and interpretation of invertebrate fossils including sponges, bryozoans, corals, molluscs, arthropods, and brachiopods.
Invertebrate Paleontology – Harvard University (www.mc.z.harvard.edu/Departments/InvertPaleo/)
Invertebrate Paleontology – Florida Museum of Natural History (www.flmnh.ufl.edu/invertpaleo/search.asp)
Invertebrate Paleontology – Natural History Museum of Los Angeles County (http://ip.nhm.org/)
Invertebrate Paleontology – Yale University (www.yale.edu/ypmip/index.html)
Images of invertebrate fossils from various research collections.

Information about Invertebrates Commonly Used in Research, Teaching, and Display
The Nematode (Caenorhabditis elegans)
General information on the biology, ecology, and laboratory uses of C. elegans.
WormBook
www.wormbook.org/
Comprehensive collection of peer-reviewed chapters covering topics related to the biology, physiology, genetics, and development of C. elegans; plus WormMethods, a collection of protocols for maintaining C. elegans.
WormAtlas Homepage – Albert Einstein College of Medicine of Yeshiva University
www.wormatlas.org/
Database of behavioral and structural anatomy, with detailed illustrated atlas of C. elegans anatomy.
WormBase Homepage
www.wormbase.org/
Information about the genetics, genomics, and biology of C. elegans, with genetic, physical, and sequence maps.
Caenorhabditis Genetics Center – University of Minnesota
www.cbs.umn.edu/CGC/ Information about specific strains of C. elegans.
Caenorhabditis elegans Server – Southwestern Medical Center University of Texas
http://elegans.swmed.edu/
General information and links to research with C. elegans, with comprehensive list of researchers, servers, and institutional and commercial laboratories working with C. elegans.
C. elegans Project – Wellcome Trust Sanger Institute
www.sanger.ac.uk/Projects/C_elegans/
List of genome sequencing projects for C. elegans and C. briggsae.
C. elegans Gene Index – Dana Farber Cancer Institute
http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=elegans
Inventory of genes and their variants of C. elegans, with information about the functional roles of these genes and their products.
WormClassroom – University of Wisconsin-Madison
www.wormclassroom.org/
Education portal for uses of C. elegans in teaching and research; with sample curriculum, instructional materials, and multimedia resources for teachers, and sections for students.

The California Sea Hare (Aplysia californica)
California Sea Slug – Wikipedia
Foundational information on the biology and laboratory uses of A. californica.
The National Resource for Aplysia – University of Miami
http://aplysia.miami.edu/
Commercial resource and informational site for A. californica, with information about efforts to promote science education and community awareness of the relevance of research on this species.
Learning in Aplysia
http://brembs.net/learning/aplysia/
Information about the neurobiology of learning and memory in Aplysia.

The Fruit Fly (Drosophila melanogaster)
General information on the morphology, development, genetics, life cycle, and laboratory uses of the fruit fly, *D. melanogaster*.

**Drosophila - WWW Virtual Library**
www.ceolas.org/VL/fly/
List of resources, centers, laboratories, suppliers, and protocols for research on *D. melanogaster*.

**FlyBrain - University of Arizona**
http://flybrain.neurobio.arizona.edu/
Online atlas and database of the central and peripheral nervous system of *D. melanogaster*, with information on different anatomical structures, developmental stages, and imaging techniques.

**FlyBase - Consortium of four universities**
http://flybase.org/
Database with descriptions of the genes and genome of the fruit fly *D. melanogaster*.

**FlyView - Universität Münster**
http://flyview.uni-muenster.de/
Image database of *Drosophila* development and genetics; compatible with FlyBase for describing the genes and genome of the fruit fly.

**FlyMove - Universität Münster**
http://flymove.uni-muenster.de/
Online resource for university students and teachers to study the development of *D. melanogaster*, through a variety of images, movies, and interactive media.

**The Interactive Fly - Society for Developmental Biology**
www.sdbonline.org/fly/aimain/1aahome.htm

**Drosophila Information Service - University of Oklahoma**
www.ou.edu/journals/dis
Online archived journal devoted to descriptions of new mutants, techniques, research, and teaching exercises with *D. melanogaster*.

**Bloomington *Drosophila* Stock Center - Indiana University**
flystocks.bio.indiana.edu/bloomhome.htm
Commercial source for obtaining specific strains of *D. melanogaster* for research.

**Flies in Space - US National Aeronautics and Space Administration**
http://quest.nasa.gov/projects/flies/index.html#
Website designed for 5th–8th grade students with information about NASA’s life sciences research project focusing on *D. melanogaster*.

**Drosophila - University of Arizona**
http://biology.arizona.edu/sciConn/lessons2/Geiger/prelude.htm
General teaching reference for *D. melanogaster* with a guide to its care, maintenance, and genetic manipulation.

**Fruit Fly - Western Kentucky University**
bioweb.wku.edu/courses/Biol114/virgin_flies.asp
General secondary teaching reference for the development of *D. melanogaster*.

**J-fly**
http://jfly.iam.u-tokyo.ac.jp/
Japanese-based data repository for *Drosophila* researchers, with images, movies, manuals, and protocols.

**Hermit Crabs**

**Hermit crab – Wikipedia**
http://en.wikipedia.org/wiki/Hermit_crab
General information on species, anatomy, and biology of hermit crabs.

**Hermit Crabs.org** (www.hermitcrabs.org)
**Hermit-Crabs.com** (www.hermit-crabs.com)
**Hermit Crab Habitat** (www.hermitcrabhabitat.com)
Information about the care of terrestrial hermit crabs.

**The Horseshoe Crab (Limulus polyphemus)**

**Horseshoe Crabs - Wikipedia**
http://en.wikipedia.org/wiki/Horseshoe_crab
General information on the classification, anatomy, fisheries, and blood use of the four living species of horseshoe crabs.

**The Horseshoe Crab – The Ecological Research and Development Group** (http://horseshoecrab.org/)
**Horseshoe Crab – US National Oceanic and Atmospheric Administration** (www.coeo.udel.edu/horseshoecrab/)
**Horseshoe Crabs: “A Living Fossil” – Maryland Department of Natural Resources** (www.dnr.state.md.us/education/horseshoecrab/)
**Limulus polyphemus – Smithsonian Marine Station at Fort Pierce** (www.sms.si.edu/irl/spec/limulu_polyph.htm)
General information on the natural history, biology, anatomy, conservation, ecology, habitat, medical uses, research, fisheries management, and conservation of the Atlantic or American horseshoe crab, *L. polyphemus*.

**Horseshoe Crab (video) – Journal of Visualized Experiments**
www.jove.com/index/details.stp?id=958

**Horseshoe Crab**
www.fao.org/docrep/fi eld/003/AB736E/AB736E02.htm
General information on the biology, habitat, and culture of the Asian horseshoe crab, *Tachypleus tridentatus*. 
Cephalopods (Octopuses, Squid, Cuttlefish, and Nautiluses)

Cephalopod – Wikipedia
http://en.wikipedia.org/wiki/Cephalopod
General information on the classification, distribution, anatomy, life cycle, reproduction, and development of cephalopods.

Cephalopod – Tree of Life Web Project
http://tolweb.org/Cephalopoda
General information on the classification, characteristics, and references of cephalopods.

The Cephalopod Page – Waikiki Aquarium and University of Hawaii
www.thecephalopodpage.org/
General information, images, links, high school lesson plans, references, and resources for octopuses, squid, cuttlefish, and nautiluses.

CephBase – University of Texas Medical Branch and Dalhousie University
www.cephbase.utmb.edu/ or www.cephbase.dal.ca/
Database-driven interactive website with taxonomic data, life history, distribution, predator and prey data, images, videos, references, and fisheries information for all species of cephalopods (octopus, squid, cuttlefish, and nautilus).

Cephalopods – Smithsonian Institute National Museum of Natural History
http://invertebrates.si.edu/cephs/
Multimedia resource of videos that supplement traditional scientific publications on squids, octopods, and their relatives.

Cephalopods – Smithsonian Institution Research Information System (SIRIS)
http://siris-bibliographies.si.edu/ipac20/ipac.jsp?profile=biball (keyword = cephalopod)
Comprehensive research database of over 4,500 publications on cephalopods and cephalopod-related subjects.

Cephalopods of the World – Food and Agriculture Organization of the United Nations
www.fao.org/docrep/009/a0150e/a0150e00.HTM
Link to online publication, “An Annotated and Illustrated Catalogue of Cephalopod Species Known to Date,” eds. Jereb and Roper (FAO Species Catalogue for Fishery Purposes 4, vol 1, 2005).

Cephalopods of the World – Food and Agriculture Organization of the United Nations
www.fao.org/docrep/009/ac479e/ac479e00.htm

Cephalopod International Advisory Council
www.abdn.ac.uk/CIAC/
International resource for information on cephalopod biology, fisheries management, and research.

The EuroSquid World Wide Web Page – University of Aberdeen
www.abdn.ac.uk/eurosquid/
General information, research projects, analysis of European cephalopod stocks, references, abstracts, and squid-related links.

National Resource Center for Cephalopods – University of Texas Medical Branch
www.gulfbase.org/organization/view.php?oid=nrcc
Link to a program that historically provided squid, cuttlefish and other cephalopods, as well as cells and tissues to researchers in the biomedical research community.

The Octopus News Magazine Online (TONMO)
www.tonmo.com/
Online forum for issues pertaining to the care, breeding, and housing of octopuses, squids, and cuttlefish.

Squid-world – BioExpedition Publishing
www.squid-world.com/
General information on the evolution, species, anatomy, feeding, and reproduction of squids.

Amazing Cuttlefish
www.users.on.net/~jamesmosby/cuttlefish/index.html
Information about the unique features, anatomy, and life cycle of the cuttlefish.

Invertebrate Associations and Societies

American Association of Professional Apiculturists – www.masterbeekeeper.org/aapa/
American Society of Parasitologists – http://asp.usnl.edu/
Australian Coral Reef Society – www.australiancoralreefsociety.org/
Coleopterists Society – www.coleopsoc.org/default.asp
Conchologists of America – www.conchologistsofamerica.org/home/
Crustacean Society (Virginia Institute of Marine Science) – http://web.vims.edu/tcs/?srv=www
Earthworm Society of Britain – www.earthwormsoc.org.uk/
Entomological Society of America – www.entsoc.org/home
Entomological Society of Canada – www.esc-sec.org/
International Association for Aquatic Animal Medicine (including invertebrates) – http://iaaam.org/
International Association for Neuropterology – www.neuroptera.com/olinks.html
International Bee Research Association – www.masterbeekeeper.org/aapa/
International Society of Arachnology – www.arachnology.org/
International Society for Invertebrate Morphology – http://zoologi.snm.ku.dk/english/Forskning/Invertebrates/isim/
International Society for Reef Studies – www.coralreefs.org/
Lepidopterist Society – www.lepsoc.org/
Lobster Conservancy – www.lobsters.org/
National Shellfish Association – http://shellfish.org/
Royal Entomological Society – www.royensoc.co.uk/
Society for Invertebrate Pathology – www.sipweb.org/
Society of Nematologists – www.nematologists.org/
Systematic and Applied Acarology Society – www.nhm.ac.uk/hosted_sites/acarology/saas/
Unitas Malacologica (International Society for Malacology) – www.unitasmalacologica.org/index.html
Xerces Society for Invertebrate Conservation – www.xerces.org/
Abstract

All animals face hazards that cause tissue damage and most have nociceptive reflex responses that protect them from such damage. However, some taxa have also evolved the capacity for pain experience, presumably to enhance long-term protection through behavior modification based on memory of the unpleasant nature of pain. In this article I review various criteria that might distinguish nociception from pain. Because nociceptors are so taxonomically widespread, simply demonstrating their presence is not sufficient. Furthermore, investigation of the central nervous system provides limited clues about the potential to experience pain. Opioids and other analgesics might indicate a central modulation of responses but often peripheral effects could explain the analgesia; thus reduction of responses by analgesics and opioids does not allow clear discrimination between nociception and pain. Physiological changes in response to noxious stimuli or the threat of a noxious stimulus might prove useful but, to date, application to invertebrates is limited. Behavior of the organism provides the greatest insights. Rapid avoidance learning and prolonged memory indicate central processing rather than simple reflex and are consistent with the experience of pain. Complex, prolonged grooming or rubbing may demonstrate an awareness of the specific site of stimulus application. Tradeoffs with other motivational systems indicate central processing, and an ability to use complex information suggests sufficient cognitive ability for the animal to have a fitness benefit from a pain experience. Available data are consistent with the idea of pain in some invertebrates and go beyond the idea of just nociception but are not definitive. In the absence of conclusive data, more humane care for invertebrates is suggested.

Key Words: behavior; discrimination learning; invertebrate; morphology; nociception; pain; physiology; stimulus avoidance

Defining Pain versus Nociception

All species of animal are susceptible to a variety of naturally occurring hazards that can cause tissue damage. Sharp objects, such as teeth or mandibles of predators, or defensive thorns or spines in plants or animals, are common. Chemicals, blunt objects, and thermal extremes may also cause damage. Some plants (e.g., nettles) and animals (e.g., hymenoptera and coelenterates) have specialized structures that are sharp, penetrate the tissues, and transfer noxious, potentially damaging chemicals. However, animals have mechanisms that enhance their ability to maintain the integrity of their tissues through the detection of noxious stimuli and action to get away from them and/or minimize their deleterious effects.

The sensory systems that respond to noxious stimuli and mediate protective reflexes are termed nociceptors (Sherrington 1906). Nociception is defined as “the neural processes of encoding and processing noxious stimuli” (Loeser and Treede 2008, 475) or the detection and reaction “to stimuli that may compromise their integrity” (Besson and Chaouch 1987, 67). Thus nociception is the perceptual mechanism coupled with the organization of responses that typically take the animal away from the stimulus or at least are effective in terminating the perception. For example, Drosophila larvae attacked by a parasitoid wasp respond by rolling toward the stimulus, which causes the wasp’s ovipositor to pull out and the wasp to leave (Hwang et al. 2007).

By contrast, the definition of pain in humans is “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (IASP 1979, 250). Various definitions have been used with respect to animals—for example, “an aversive sensory experience caused by actual or potential injury that elicits protective motor and vegetative reactions, results in learned avoidance and may modify species-specific behaviors, including social behavior” (Zimmerman 1986, 1). A shorter definition that excludes pain assessment criteria is “an aversive sensation and feeling associated with actual or potential tissue damage” (Broom 2001, 17).

It is clear that nociception is central to the concept of pain, as without it the experience of pain is unlikely. However, simply observing a nociceptive ability does not demonstrate pain. Nociception per se is an involuntary rapid reflex response and lacks the negative emotional response or feeling associated with pain (Bateson 1991; Broom 2001). Indeed, in humans the reflex response to touching something hot precedes the experience of pain.
Although the distinction between nociception and pain is widely accepted there are semantic issues that may cloud the issue. The term “pain perception” is frequently used in pain studies (e.g., Braithwaite 2010; Sneddon 2009) and nerve fibers are said to “transmit pain” (Weary et al. 2006). Even in studies that are overtly about nociception rather than pain, nociception is described as “pain sensing” and ascending tracts in the vertebrate spinal cord are said to carry “painful sensory information” (Hwang et al. 2007) or “pain information” (Sneddon 2009). Larval *Drosophila* that lack a particular functional gene fail to roll away from thermal or mechanical stimuli and the mutation has been called “painless” even though the gene function is in the activation of transducer channels by which a neural signal results from physical stimuli (i.e., a key part of nociception) (Tracey et al. 2003). The use of these terms blurs the critical distinction between nociception and pain.

I prefer to use terms such as “pain experience” to denote an internal awareness, coupled with a negative emotional state or feeling, that results from perception of actual or potential tissue damage. It is the damage that is perceived. There is no “pain” to be perceived and the information carried to the brain in vertebrates is not in itself “painful.” The pain results from a powerful, unpleasant emotion that is part of, or coupled with, a strong motivation to terminate the experience that results from neural signals about tissue damage.

Clarity about the definitions of and distinctions between nociception and pain is essential for determining whether pain occurs in particular groups of animals.

**Function and Evolution of Pain**

As noted above, nociception provides a means of detecting and escaping from a stimulus that might continue to cause damage in the absence of action. Thus there are clear benefits in nociception and, presumably, they outweigh the costs of developing, maintaining, and using the system. What further advantage is gained by having an additional system that enables the experience of nociceptive inputs as an unpleasant emotion? It may be that the “emotional” component provides a long-lasting motivation that enables the animal to better maintain its tissue integrity (Bateson 1991).

A nociceptive response may be organized as a reflex (Sneddon et al. 2003) but may not be associated with a lasting memory and motivational change. Pain, on the other hand, might induce a long-term memory and be coupled with learning to avoid situations that gave rise to the original pain experience (Bateson 1991). The greater the tissue damage in the original experience the greater may be the unpleasant emotional response and the greater the motivation to avoid it in the future. Thus pain experience has a longer-lasting effect and protects the animal from future damage in a more effective manner than does nociception alone. Nociceptive abilities are found in most of the major animal phyla and thus are presumably a product of very early evolution. They clearly predate the Cambrian “explosion,” a period approximately 530–550 million years ago during which the major modern phyla evolved (Budd and Telford 2009). The next step in the development of pain was probably a link between nociception (with the associated reflex response) and a longer-term motivational change (with central processing and memory). But in which groups did this development take place and when? Some researchers are confident that it occurred only in vertebrates and that it is evident in fish and lamprey then the question is whether it is a novel development in the vertebrates or if the ability to experience pain predated the split of the vertebrates from the ancestors of some other phyla and could thus be present in some extant invertebrate groups? Alternatively, if there is an advantage in experiencing pain, it could be that it has evolved on more than one occasion (Elwood et al. 2009).

**How Can Pain Be Identified?**

The interest in the potential of invertebrates to experience pain lies in the quest to understand and improve welfare, as humans generally seek to avoid causing pain or suffering to animals (Broom 2007). If an animal responds to a noxious stimulus in an adaptive fashion via a nociceptive reflex and without any unpleasant experience, then welfare concerns are diminished. The key objective of this article is to examine the evidence that some invertebrates may or may not experience pain.

Inferring feelings or mental states in animals is fraught with difficulty (Dawkins 2006). A common approach is to use argument by analogy (Dawkins 1980; Sherwin 2001): if an animal responds to a potentially noxious stimulus in a manner similar to that observed to the same stimulus in humans then it is reasonable to argue that the animal has had an analogous experience (Sherwin 2001). However, Sherwin (2001) notes differences in the acceptance of this argument depending on the species rather than the behavior: observers of a dog or primate writhing in response to an electric shock accept that the animal is experiencing pain, whereas much the same response in an invertebrate is often dismissed as irrelevant to the question of pain. He suggests a more symmetrical approach when comparing vertebrates with invertebrates, with consistent acceptance or rejection of the argument by analogy (Sherwin 2001). However, empathy for invertebrates is typically low and some researchers believe that it would be “inconvenient” if these animals were believed to feel pain (Kellett 1993).

Coupled with analogy, various criteria have been proposed as collectively having the potential to demonstrate pain in mammals (Bateson 1991) and have been applied to pain in amphibians (Machin 1999; Stevens 2004), fish (Sneddon et al. 2003), and various invertebrates (Broom 2007; Elwood et al. 2009;
suitable receptors;
• a suitable central nervous system;
• responsiveness to opioids, analgesics, and anesthetics;
• physiological changes;
• avoidance learning;
• protective motor reactions;
• tradeoffs between stimulus avoidance and other activities; and
• cognitive ability and sentience.

Suitable Receptors

Sea anemones respond to mechanical stimuli and to the stings of other anemones but not to thermal stimuli (Mather 2011, in this issue). Annelids have nociceptors that respond to acid, capsaicin, and heat (although the sensitivity to acid is lower than that seen in vertebrates; Smith and Lewin 2009) and cells that respond to touch and pressure (Nicholls and Baylor 1968). Nociceptors and nociceptive behavior have been described in molluscs; for example, the snail (Cepaea nemoralis) responds to a hot plate at >40°C by lifting the anterior portion of its foot (Kavaliers et al. 1993). In Aplysia californica (Castellucci et al. 1970; Smith and Lewin 2009), once the stimulus threshold is reached, nociceptors increase firing in line with subsequent increase in stimulus strength and show maximal activity with the crushing or tearing of tissues.

Nociceptive systems have been described in particular detail in the nematode (Caenorhabditis elegans) and fruit fly (D. melanogaster) (reviewed by Smith and Lewin 2009) and molecular tools have been applied to elucidate the detailed development and functioning of their nociceptors (Goodman 2003; Tobin and Bargmann 2004). Nociceptive neurons of Drosophila larvae have multiple dendritic branches with naked endings attached to epidermal cells (Hwang et al. 2007); several classes of such multidendritic (md) neurons have been described but not all are involved in nociception. If all these md neurons are rendered inactive in particular genetic mutants, the larvae fail to respond to noxious stimuli, showing that at least one is nociceptive (Tracey et al. 2003). Selective silencing of particular classes of md neurons, however, showed that one class serves as the primary nociceptive system and that the silencing of this class of neurons eliminates the rolling response of larvae to thermal stimuli (Hwang et al. 2007). The nociceptive neurons are also responsive to attacks by parasitic wasps attempting to insert their ovipositor (as described above; Hwang et al. 2007), indicating that they are polymodal, as are those of vertebrates (Goodman 2003).

The common theme of these studies is the ability of a wide range of taxa to detect noxious stimuli and to translate them into neuronal signaling (Tobin and Bargmann 2004). The systems involved are complex but conserved across markedly different taxa, as is evident from the use of Drosophila in drug discovery for application in vertebrates, especially humans (M anev and Dimitrijevic 2005). In addition, these systems show adaptive, temporary, heightened, and reduced sensitivity and these features are also conserved across phyla (Babcock et al. 2009). One recent study, however, failed to detect nociceptors in decapod crustaceans and also noted little ability to respond to noxious stimuli (Puri and Faulkes 2010) despite organized responses to noxious chemical and electrical stimuli noted in other studies (Barr et al. 2007; Elwood et al. 2009; Elwood and Appel 2009).

Vertebrates have a variety of nociceptive fibers, some myelinated and others not. By contrast, those in invertebrate groups are only unmyelinated (Smith and Lewin 2009). However, this distinction reveals little about any taxonomic difference in pain experience because it is the unmyelinated C fibers in mammals that are most prevalent and are important in the perception of stimuli that give rise to pain (Smith and Lewin 2009). Because pain experience associated with tissue damage typically depends on nociception, a lack of nociceptors would suggest that the animal was insensitive to noxious stimuli and could not experience pain. This argument was central in recent work in fish that demonstrated nociceptors similar to those of mammals, allowing the conclusion that fish had apparatus that should be sufficient for them to experience pain (Sneddon 2003). However, that study was also right to state that the presence of nociceptors per se does not demonstrate that pain is experienced.

A Suitable Central Nervous System

Because it is clear that the human brain is suitable for pain experience there has been an assumption by some (Rose 2002) that only animals with structures very similar to those of humans have the capacity to experience pain. For example, the possibility of fish experiencing pain has been dismissed because human pain is experienced in parts of the cerebral cortex whereas fish lack this structure (Rose 2002). If one accepts this argument then the possibility of pain being experienced by any invertebrate must be dismissed because none has a central nervous system (CNS) built on the vertebrate plan. However, according to the same logic it could be suggested that because crustaceans or cephalopods lack any of the visual system found in humans they must be blind. This is not the case as both have a well-developed visual ability, each based on an entirely different CNS and receptors. Thus clearly the same function can arise in different animal taxa using different morphology, and it appears to be illogical to accept this reasoning for some experiences but to dismiss it for pain (Elwood et al. 2009).

In his review of pain criteria Bateson (1991, 834) avoided the idea that structures homologous to those of humans must be present and instead suggested that there should be “structures analogous to the human cerebral cortex.” Because
many invertebrates have a remarkably complex brain structure, albeit rather different from that of humans, some might have structures analogous to the cortex (Smith 1991). For example, it has been suggested that specific brain areas in the octopus are specialized for sensory analysis, memory, learning, and decision making and thus may be considered analogous to the human cerebral cortex (Wells 1978).

A second argument for rejecting a pain experience in invertebrates is that their brains might be too small. However, the octopus brain is larger than that of most fish and reptiles when regarded as a ratio of body weight (Smith 1991) and even the brains of many decapod crustaceans (e.g., crabs, lobsters, shrimp) are likely to be considerably larger than those of many vertebrates when regarded in an absolute comparison (Elwood et al. 2009). Broom (2007) notes that brain size does not necessarily equate to complexity of function (Broom and Zanella 2004); indeed, the brains of some invertebrates have a surprising complexity (Sandeman et al. 1992; Wells 1978), with clear functional separation of distinct areas, and thus might be sufficiently complex in function to enable pain experience (Broom 2007).

Responsiveness to Opioids, Analgesics, and Local Anesthetics

Mammals have a system for regulating pain such that the same tissue damage may result in very different responses depending on the situation. For example, humans engaged in sports often report little pain in response to tissue damage. The physiological basis of this regulation is complex but in part is dependent on endogenous opioids, release of which reduces the pain experience. Injection of the opiate morphine also reduces the pain experience and the opiate antagonist naloxone reverses this effect. For these reasons the presence of opioid receptors and responses to analgesics has been regarded as an indicator that animals experience pain (Bateson 1991; Roughan and Flecknall 2001; Sneddon et al. 2003).

A particularly persuasive approach is to offer the animal a choice between water (or food) that does or does not contain an analgesic and to observe whether a preference develops for the analgesic when noxious stimuli are applied (Colpaert et al. 1980). Danbury and colleagues (2000) found that lame chickens consumed more feed containing an analgesic than did those that were not lame. As far as I am aware this approach has not been tried with invertebrates, but various studies have applied analgesics and local anesthetics and examined their effects on responses to noxious stimuli. For example, in the crab (Chasmagnathus granulatus) electric shock elicited a defensive threat display and the percentage of animals that showed this response rose with the voltage applied. Injection of morphine hydrochloride reduced the crabs’ sensitivity to the shock in a dose-dependent manner and naloxone injection inhibited the effects of morphine (Lozada et al. 1988). Morphine also had inhibitory effects on the escape tail-flick response to electric shock in mantis shrimps (Squilla mantis) that was reversed by naloxone (Maldonado and Irilato 1982), and researchers observed a similar effect of opioids and naloxone in nematodes (Pryor et al. 2007) and snails (Kavaliunas et al. 1983).

This approach, however, is problematic because analgesics might produce a general reduction in responsiveness to all stimuli. One way around this is to create a situation in which analgesia might increase particular responses. In one such study, fruit flies placed in a tube at the darker side of a light gradient moved toward the light. If the center of the tube was heated, however, the flies were inhibited from passing through this section. The application of specific analgesics (agonists for GABAA that are effective analgesics in hot plate tests in rats; Thomas et al. 1996) reduced this inhibition and the flies passed through the heat to the lighter area (Manev and Dimitrijevic 2005).

A recent study on the glass prawn (Palaeon elegans) noted that the animal engaged in prolonged grooming of the antennae and rubbed them against the side of the tank when the antennae were treated with acetic acid or sodium hydroxide, but prior treatment with a local anesthetic (benzocaine) reduced the rubbing and grooming (Barr et al. 2008). There was no effect of benzocaine on the general locomotion of the prawn so the reduction in the two behaviors was not simply due to inactivity. However, the result with acid was not replicated in other decapod species (Puri and Faulkes 2010).

Both opioid analgesics and local anesthetics have effects that appear similar to those observed in vertebrates. But local anesthetics block sodium channels (Machin 2005) and, in crayfish (Procambarus clarkii; Leech and Rechnitz 1993), prevent the conduction of impulses from nociceptors, so it is the nociception that is reduced or eliminated. Furthermore, opioids may produce analgesia by acting on a modulatory system in the CNS (Tomsic and Maldonado 1990), but they might also have a peripheral effect (Del Seppi et al. 2007; Pryor et al. 2007). In all of these cases the nociception is or may be disrupted, so conclusions about the potential for the animal to experience pain are limited.

Physiological Changes

Noxious stimuli applied to vertebrates typically result in tachycardia, pupil dilation, and defecation. Changes in blood flow, respiratory patterns, arteriole blood gases, electrolyte imbalance, and endocrine changes are also common (Short 1998; Sneddon et al. 2003). The latter often involve corticosteroid release, which is used as a measure of stress (Stafford and Mellor 2005).

There has been limited examination of similar responses in invertebrates. Cephalopods are said to have an adrenal system that releases adrenal hormones when the animal is exposed to noxious, potentially painful stimuli, and noradrenaline and dopamine are released when the animal is disturbed (Stefano et al. 2002). Crustaceans have a stress hormone, the crustacean hypoglycemic hormone (CHH) (Chang 2005; Lorenzon et al. 2004), that functions to convert glycogen to glucose in a manner analogous to that of...
cortisol in vertebrates. Glucose rose substantially in edible crabs when a claw was removed in a manner that caused a wound but not when the crab was induced to autotomize the claw (Patterson et al. 2007). However, this might be due to the tissue damage per se rather than any negative emotional state.1

There is a clear need for more studies on physiological aspects of invertebrate responses to noxious stimuli. Of particular use would be an examination of physiological changes during avoidance learning and during presentation of just the conditioned stimuli (without the noxious event) to determine whether features akin to anxiety are present.

Avoidance Learning

I have noted that nociception allows for an immediate escape by use of a reflex response whereas pain enables motivational change and avoidance learning. The key function of pain is thus to reduce damage over a relatively long term. One would therefore expect to see evidence of rapid avoidance learning coupled with a long memory in an animal that experiences pain.

Such evidence has been reported in Drosophila that learned to associate an odor that preceded or overlapped with an electric shock: after eight (Yarali et al. 2008) or twelve trials (Tully and Quinn 1985) they avoided the odor for up to 24 hours. Researchers have used this paradigm to examine genetic influence on learning and memory and the morphology involved in terms of brain region (de Belle and Heisenberg 1994) and to create mutants for dissecting biochemical pathways involved in learning and memory (Sokolowski 2001). Curiously, Drosophila also learn to associate the odor if it occurs after the shock has ended and in this case they show a mild preference for the odor, a feature termed pain relief learning (Yarali et al. 2008).

Similarly, the crab C. granulatus associated a shock with a particular location (Denti et al. 1988) after just a single trial and retained the association for 3 (but not 24) hours. Subsequent experiments involving multtrial training, however, showed retention after a 24-hour rest interval in a different environment from that used in training (Fernandez-Duque et al. 1992).

Experiments with crayfish (P. clarkia) demonstrated an association between a light and a shock given 10 seconds later: the animals learned to respond by walking to a safe area in which the shock was not delivered (Kawai et al. 2004). But the animal did this only if it was facing the area to which it could walk to avoid the shock; if it was facing away from the safe area it exhibited a tail-flick escape response, by which it moved away tail first. Despite repeated pairings of light and shock, the animal did not learn to avoid the shock by tail flicking in response to light. However, when the animals that had experienced shocks while facing away from the safe area were subsequently tested facing toward the safe area they showed a very rapid avoidance of the shock at the onset of the light. Thus they seemed to have learned the association although they had not previously used it to avoid the shock. This finding was explained by the specific associations between cues and particular responses that are also common in vertebrates.

Hermit crabs (Pagurus bernhardus) in their shell that were shocked on the abdomen demonstrated a long-term behavioral change compared with crabs that were not shocked. The shocked crabs were more likely to approach and enter a newly offered empty shell (Elwood and Appel 2009) and, compared to those not shocked, they moved into the new shell more quickly, spent less time investigating it, and inserted their chelipeds into its aperture less often before moving in.2 This is consistent with the idea that shocked crabs assessed their original shells as being of very poor quality. Shocked crabs altered their behavior for up to a day after the initial shock (the maximum time tested), indicating a long-term shift in motivation about obtaining a new shell after the aversive experience (Appel and Elwood 2009a).

Taken together, these studies on learning and motivational change show abilities in arthropods that seem to fit this key criterion for pain experience.

Protective Motor Reactions

Protective motor reactions include reflex withdrawal from a noxious stimulus, but this is a basic feature of nociception and gives little indication of emotional state. Weary and colleagues (2006) argued that prolonged rubbing denoted an awareness of the site of the noxious stimulus and Sneddon and colleagues (2003) noted that rainbow trout (Oncorhynchus mykiss) that had noxious chemicals injected into the lip showed rubbing of the lip on the substrate, consistent with the idea of pain.

Hermit crabs induced to evacuate their shells by electric shock to the abdomen demonstrated sustained grooming by use of claws on the abdomen (Appel and Elwood 2009a,b; Elwood and Appel 2009), a response not seen when the crabs are cracked out of their shell or evicted in a shell fight. Further, when either sodium hydroxide or acetic acid solution was applied to one antenna of a glass prawn there was a significant increase in grooming of that antenna during which it was pulled repeatedly through the animal’s small pincers and mouth parts (Barr et al. 2008), and there was an increase in rubbing of that antenna against the side of the tank. The animal seemed to be aware of the specific location of the noxious stimulus and directed its attention to the treated antenna. (However, work on three species of prawn found no significant increase in directed grooming of treated antennae; Puri and Faulkes 2010.) In addition, when acetic acid was applied to an eye of a glass prawn there was a marked increase in grooming that involved both pincers moving

1Crabs lacking a claw showed a higher level of glucose when an intact crab was housed in the same tank, an effect that might be due to fear or increased alertness (Patterson et al. 2007).

2Such a minimum of investigation of the new shell is otherwise characteristic of crabs in shells of inadequate size (Elwood and Stewart 1985).
simultaneously and in very different and complex ways. The grooming was directed specifically to that eye, again demonstrating that the animal was aware of the location of the noxious event (Barr et al., unpublished data). The complexity of these prolonged responses is beyond that expected from a nociceptive reflex response and consistent with the idea of pain.

A nother protective motor response in arthropods is autotomy, in which an appendage is cast off from the body. In the spider *Argiope aurantia*, legs may be autotomized when damaged (Eisner and Camazine 1983). This was seen during attempts by these spiders to capture ambush bugs (*Phymata fasciata*), typically when the bug grasped a spider leg and probing a joint with its proboscis (the venomous saliva is painful to humans)—autotomy occurred within 5 seconds (Eisner and Camazine 1983). Simple experimental penetration of the joint with a sterile pin did not cause autotomy, indicating that the saliva had an effect. Eisner and Camazine (1983) also injected bee and wasp venom, both of which induced autotomy. They found that when individual components of the venom were injected, some, but not all, produced autotomy; effective components were histamine, serotonin, phospholipase, and melittin, all of which induce pain in humans; ineffective components were acetylcholine, bradykinin, hyaluridase, adrenaline, and dopamine. A cetylcholine and bradykinin induce pain in humans but not autotomy in spiders, and hyaluridase, adrenaline, and dopamine do not induce pain in humans, suggesting a concordance between pain effects in humans and autotomy in the spider.

Autotomy in crustaceans typically leaves a clean break at a specific joint close to the main body, which immediately seals to prevent loss of hemolymph. Cutting a membrane at a joint distal to the autotomy plane, causing hemolymph loss, elicits rapid autotomy (within a few seconds) of that appendage, preventing further loss of fluid (Patterson et al. 2007). Crabs also autotomize limbs in situations that do not involve hemolymph loss, for example if the whole animal is placed on a hot plate (Fiorito 1986) or if the leg is subject to electric shock or injected with acetic acid (Barr and Elwood, unpublished observations). The acetic acid treatment rapidly induces autotomy in a dose-dependent manner and the results are consistent with pain mediation of the autotomy response.

Tradeoffs between Stimulus Avoidance and Other Activities

Bateson (1991) suggests that one criterion for pain should be a relatively inelastic response (*sensu* Dawkins 1990), but a response that is purely mediated by nociception is an inelastic reflex— it should be the same regardless of other motivational priorities. Thus an animal that is hungry or satiated would likely exhibit the same reflex avoidance to a noxious stimulus, even if food is present. By contrast, pain is a negative emotional state, typically coupled with a very high motivation to escape that state, and thus should be given a high priority and might appear to be inelastic. Thus it seems difficult to discriminate pain from nociception by this criterion.

However, if variation in the response to noxious stimuli is dependent on other motivational requirements then there must be some higher-level interaction between competing motivational systems (McFarland and Sibly 1975). In fish, for example, those deprived of food are less likely to respond to an electric shock in a feeding area than those that are not food deprived (Millsopp and Laming 2008). A nd hens stop limping in the period leading up to egg laying but limp again after laying (Gentle 2001). Competition between different activities for expression or requirements is the essence of motivational tradeoffs. Such competition is important for pain research as it is a strong indicator that the response to the noxious stimulus is not purely reflexive; tradeoffs clearly involve some form of processing in which different needs are weighed.

Tradeoffs were the subject of two experiments in which hermit crabs were given shells with two small holes drilled and electrodes inserted so the crab could be shocked on the abdomen. When shocks of a single intensity were applied, at a level that was hoped would not cause evacuation, some crabs evacuated and were more likely to do so from a less preferred shell species (Elwood and Appel 2009). Similarly, when the shocks increased in intensity, crabs evacuated the shell at a lower shock intensity if they were in a less preferred shell species (Appel and Elwood 2009b). Thus, the animals’ response to the shock was determined in part by their normal preference for particular species of shell. Further, they were much less likely to evacuate after being shocked when the odor of a predator was present, suggesting a tradeoff between shock avoidance and predator avoidance (Wilson and Elwood, unpublished observations). These responses cannot be a reflex response as they required information from sources other than the noxious stimulus to have an effect on the response.

This approach of determining what is “traded off” against avoidance of the noxious stimulus may give insights into an animal’s priorities (Dawkins 1990)— that is, what it might “pay” to avoid the noxious stimulus in terms of lost opportunities to satisfy other motivational demands. For example, among the shocked hermit crabs that evacuated their shell some stayed near the shell and many got back into it; others, however, walked away and even attempted to climb the walls of the observation chamber (Appel and Elwood 2009a,b). This is remarkable because the shell is a vital resource and abandoning it indicates the aversive nature of the shock.

Octopuses provide another example of tradeoff in their avoidance of stinging sea anemones. Octopuses readily prey on hermit crabs but experiments have shown that when the crabs placed an anemone on their shell as protection the octopuses dramatically changed their tactics. They tried a variety of approaches such as moving below the anemone, blowing jets of water at it, and using a single outstretched arm. Thus they seemed to try to avoid the stings while attempting to maintain food intake, albeit with tactics that are less efficient for food capture (MacLean 1983; Mather 2008).
High Cognitive Ability, Consciousness, and Sentience

Several authors have considered a high cognitive ability coupled with consciousness or sentence a prerequisite for a pain experience or at least have suggested that such abilities make pain experience more likely. Bateson (1991, 832), for example, suggests that “if the animal can be shown to be conscious of what it is doing, then most people would conclude that it could experience pain.” Particular cognitive abilities are also considered important in assessing the welfare status of animals (e.g., Braithwaite 2010; Chandroo et al. 2004; Duncan 1996; Duncan and Petherick 1991). At the very least sentence probably involves awareness of internal and external stimuli (Chandroo et al. 2004; Duncan 1996), and “primary consciousness” involves the ability to generate a mental scene in which diverse information is integrated for the purpose of integrating behavior (Chandroo et al. 2004; Edelman and Tononi 2000). I consider here several examples of invertebrate abilities in integrating information from different sources to make “informed” decisions.

Spiders exhibit a variety of complex behaviors that appear to illustrate a capacity for information integration. Jumping spiders, for example, are known to adjust hunting methods depending on the type of prey and its ability to escape (Bartos 2008). The hunting spider (Portia labiata), when it hunts spitting spiders (Scytodes pallidus), which are themselves predators of spiders and thus dangerous, gathers information as to whether the spitting spider is carrying eggs in the mouth and thus less dangerous; if so, the hunting spider modifies its attack (Jackson et al. 2002). Furthermore, when hunting prey in complex environments, Portia appears to plan routes with detours that initially take it away from the prey item to avoid obstructions (Tarsitano 2006). Such behavior suggests an ability to comprehend the complex spatial relationships between itself and the prey and possible routes to a goal (Sherwin 2001).

Male giant cuttlefish (Sepia apama) exhibit a remarkable ability to change shape and color to switch between the appearance of a female and that of a male in order to foil the mate-guarding attempts of larger males. Norman and colleagues (1999) showed that small males that assumed the body shape and patterns of a female were not attacked by the larger mate-guarding male. When the larger male was distracted by another large male intruder, the small males changed body pattern and behavior to those of a male in mating display and successfully mated.

Squid use surprisingly complex color patterns for courtship and protection (Hanlon et al. 1994). A male can display a courtship coloration on one side of the body toward a female while at the same time displaying a completely different pattern on the other side to ward off an intruding male (Mather 2004, 2008). And he can switch the sides of the body showing the two displays as soon as the relative positions of the other two animals change.

Octopuses also show complex learning abilities (Edelman et al. 2004). When confronted with a maze in which the experimenter frequently changed the nature of the obstacles octopuses readily solved the maze, apparently considering the maze before proceeding (Moriyama and Gunji 1997), suggesting a level of ability that might signal consciousness.

A final example concerns hermit crabs, which when deciding to change shells systematically evaluate various components of potential new shells to determine whether they offer a gain in terms of size, shape, and weight before moving in (Elwood and Stewart 1985). The evaluation continues even after moving in and a crab may switch between the two before making a final decision (Elwood 1995). Hermit crabs that fight to take the shell from another hermit crab not only evaluate the opponent’s shell (Dowds and Elwood 1983) but also take in information about the opponent (e.g., its size and power; Briffa and Elwood 2002; Dowds and Elwood 1985). In addition, they monitor their own physiological state during the encounter in order to make effective fight decisions (Briffa and Elwood 2000, 2002, 2005)—for example, when lactate increases the attack rate slows and the attacker then stops fighting (Briffa and Elwood 2002, 2005). Hermit crabs can remember particular opponents for up to 4 days after an encounter (Gherardi and Artema 2005).

It is clear from the above examples and others (Broom 2007; Mather 2008) that some invertebrates are capable of integrating information from various sources, both internal and external, to enable complex decisions. Also apparent is a fine discrimination learning ability (Mather 2008), indicating a high cognitive ability. For example, honeybees can learn a complex learning task in which they have to select from previously unseen shapes on the basis of whether they are symmetrical or not (Benard et al. 2006; Giurfa et al. 1996) and cephalopods appear particularly adept at a range of learning tasks (Mather 2008).

Good discrimination learning may not necessarily indicate an ability to experience pain, but one might expect to see such discrimination when pain is experienced—simple nociceptive reflex avoidance results in an immediate withdrawal but does not imply any long-term motivational change. To benefit from pain experience the animal needs to be able to discriminate between the specific situation that led to the pain and other situations that did not. Animals that cannot make fine discriminations may avoid potentially harmless or even useful situations or objects. Thus it seems reasonable to speculate that the evolution of pain experience developed hand in hand with enhanced discrimination learning.

Conclusion

It is clear that the various criteria I have described differ in their usefulness in discriminating pain from nociception. Because of the wide taxonomic occurrence of functional nociceptors, the demonstration of their presence does not indicate the capacity to experience pain, and investigation of the central nervous system provides limited clues of what is or is not suitable for pain experience. The use of opioids and other.
analgesics might indicate a central modulation of responses, but potential peripheral effects may explain the analgesia. Physiological changes might prove useful but, to date, the study of their appearance in invertebrates is limited and reveals little about their pain experience.

It is thus behavior that provides the greatest insights into the likely experience of pain. Rapid avoidance learning, coupled with a prolonged memory, indicates central processing and is consistent with pain, but it is more convincing after one stimulus than after numerous repetitions. Complex, prolonged grooming or rubbing might indicate an awareness of the specific site of stimulus application and seems to be more than a reflex reaction. Tradeoffs with other motivational systems indicate central processing and may be useful to determine what an animal will “pay” to avoid the noxious stimulus. An ability to use information from various sources might indicate sufficient cognitive ability for the animal to have a fitness benefit from a pain experience.

Evidence from behavioral studies is entirely consistent with the idea that some invertebrates, particularly crustaceans and molluscs, experience pain. However, more studies must use a variety of imaginative techniques to confirm that invertebrates do indeed experience pain. Substantial research on various taxa is necessary to assess which, if any, show (1) rapid avoidance learning of noxious stimuli, (2) prolonged responses directed to the specific site on their body where the noxious stimulus was applied, and/or (3) tradeoffs between avoidance and other activities that would indicate central decision making rather than reflex reaction. Studies that demonstrate marked physiological stress responses to conditioned stimuli that herald the imminent application of a noxious stimulus would also be helpful.

Clearly, a start has been made on some of these approaches but much more is needed. Recently, Braithwaite (2010) was confident enough to state that fish feel pain but invertebrates do not. I do not share the confidence to make that discrimination. Neither do I feel confident in stating unequivocally that some of them feel pain, although it is clear that the responses described above cannot be explained just by nociceptive reflexes. While awaiting the results of further relevant studies, perhaps all who use invertebrates should consider the possibility that at least some might suffer pain and, as a precaution, ensure humane care for these animals.

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References


Abstract

Molluscs have proven to be invaluable models for basic neuroscience research, yielding fundamental insights into a range of biological processes involved in action potential generation, synaptic transmission, learning, memory, and, more recently, nociceptive biology. Evidence suggests that nociceptive processes in primary nociceptors are highly conserved across diverse taxa, making molluscs attractive models for biomedical studies of mechanisms that may contribute to pain in humans but also exposing them to procedures that might produce painlike sensations. We review the physiology of nociceptors and behavioral responses to noxious stimulation in several molluscan taxa, and discuss the possibility that nociception may result in painlike states in at least some molluscs that possess more complex nervous systems. Few studies have directly addressed possible emotion-like concomitants of nociceptive responses in molluscs. Because the definition of pain includes a subjective component that may be impossible to gauge in animals quite different from humans, firm conclusions about the possible existence of pain in molluscs may be unattainable. Evolutionary divergence and differences in lifestyle, physiology, and neuroanatomy suggest that painlike experiences in molluscs, if they exist, should differ from those in mammals. But reports indicate that some molluscs exhibit motivational states and cognitive capabilities that may be consistent with a capacity for states with functional parallels to pain. We therefore recommend that investigators attempt to minimize the potential for nociceptor activation and painlike sensations in experimental invertebrates by reducing the number of animals subjected to stressful manipulations and by administering appropriate anesthetic agents whenever practicable, welfare practices similar to those for vertebrate subjects.

Key Words: *Aplysia*; cephalopod; ethics; invertebrate; mollusc; nociception; pain; sensitization

Nociceptive Biology and Evolution

Nearly all animals that have been studied display marked behavioral responses to stimuli that cause tissue damage—the ability to sense and respond to noxious stimuli is an almost universal trait (Kavaliers 1988; Smith and Lewin 2009; Sneddon 2004; Walters 1994). Nociception, defined as the detection of stimuli that are injurious or would be if sustained or repeated, has clear adaptive advantages because it triggers withdrawal and escape during injury or in the face of impending injury.

Nociception and Nociceptive Sensitization

The first stage of nociception occurs with the activation of nociceptors, primary sensory neurons preferentially sensitive to noxious stimuli or to stimuli that would become noxious if prolonged (Sherrington 1906). Nociceptors were first demonstrated in Chordata by Burgess and Perl (1967), in Annelida by Nicholls and Baylor (1968), in Mollusca by Walters and colleagues (1983a), in Nematoda by Kaplan and Horvitz (1993), and in Arthropoda by Tracey and colleagues (2003). Preliminary studies indicate that nociception in these phyla involves many conserved sensory transduction processes (Smith and Lewin 2009; Tobin and Bargmann 2004; Tracey et al. 2003), although differences have also been found. It is not yet known whether specialized nociceptors also occur in other phyla, although this seems likely.


Nociceptive sensitization can be measured directly at the neuronal and behavioral levels and has been investigated extensively in mammals because it is thought to represent concrete, quantifiable effects that in humans are related to increased pain sensitivity. As defined by the International Association for the Study of Pain (IASP; Merskey and Bogduk 1994), increased pain sensitivity occurs in the form of hyperalgesia (greater pain in response to a normally painful stimulus) and allodynia (pain evoked by a stimulus that is not normally painful).
In a few molluscs and other invertebrates, mechanisms of nociceptive sensitization have been investigated intensively in hopes of discovering fundamental processes that contribute to hyperalgesia and allodynia in humans and other animals. Indeed, behavioral and neurophysiological alterations in nociceptors during nociceptive sensitization appear remarkably similar in snails and rats (Walters 1994, 2008; Woolf and Walters 1991), and these alterations involve many intra- and extracellular biological signaling pathways that are highly conserved (Walters and Moroz 2009).

**Interpreting Nociceptive Differences across Phyla**

There are numerous differences between the neuronal systems that mediate nociceptive sensitization in molluscs and in mammals (as well as presumably enhanced pain in the latter). At the axonal level, molluscs and other invertebrates lack myelination, so conduction of information from one part of the nervous system to another is usually much slower than in vertebrates. At the synaptic level, an evolutionary recent expansion of the synaptic proteome in vertebrates may underlie unique cognitive capabilities of this group (Ryan and Grant 2009) and in principle also contribute to a potentially unique capacity of vertebrates to experience pain.

Furthermore, at the neuroanatomical and, presumably, neural network levels, little homology exists across any of the phyla, which diverged before most of the evolution of neuroanatomical structures in contemporary animals (Farriss 2008). Thus noxious information in vertebrates is relayed from primary nociceptors via neurons in the dorsal horn of the spinal cord to brain structures including the thalamus and the somatosensory, insular, and anterior cingulate cortices (Peyron et al. 2000), but homologues to these brain structures do not exist in invertebrates. The fact that these areas are not present in the invertebrate central nervous system (CNS) does not prove that invertebrates cannot feel pain; independently derived neural structures might, in principle, have evolved the capacity to mediate the same functions. For example, some invertebrates (many cephalopods and some insects) can process highly complex visual information even though they lack a structure homologous to the mammalian visual cortex. While it is plausible that the more elaborate neural structures of mammals confer a capacity for the experience of pain, analogous processing in other phyla might mediate pain-like experiences using neural structures unrelated to and quite different from those in the mammalian brain.

Similarity across phyla of nociceptive behavior, mechanisms of nociception, and nociceptive sensitization implies either deep homology of underlying mechanisms or convergence arising from similar selection pressures among diverse and distantly related taxa. Either of these possibilities may permit insights into mechanisms important for pain and its alleviation in vertebrates from studies of animals with far simpler nervous systems. Invertebrates thus present two considerable advantages: (1) research on a complex process in a simpler system often permits a clearer picture of what is really important, and (2) the study of states bearing similarities to pain or unpleasantness in an animal that appears to have less capacity to interpret and “feel” such sensations is more palatable to scientists, to the public, and to animal welfare committees.

The presence in invertebrates of some nociceptive mechanisms that are homologous and/or convergent with those important for pain in humans presents a question of ethics: If scientists are willing to appeal to evolutionary conservatism to support the use of “lower” animals to study physiological building blocks that in humans contribute to pain and suffering (assuming that the information revealed will translate to humans and other “higher” vertebrates), is it acceptable to ignore possible implications of evolutionary conservation that similar processing of nociceptive information by higher and lower animals might in each case produce suffering? Are there reasons to think that experimental unpleasantness is less keenly felt by a snail or fly than by a mouse, monkey, or human, and if so where should one draw the line when conducting experiments that might cause suffering?

**Nociception Versus Pain**

Humans tend to experience nociception and pain as a single phenomenon, but for the study of animals it is important to draw a distinction between sensory activation and emotional perception (see Braithwaite 2010 for an excellent discussion of the separable nature of the two processes).

**Defining Nociception and Pain**

Nociception is a capacity to react to tissue damage or impending damage with activation of sensory pathways, with or without conscious sensation. Activity in nociceptive sensory pathways usually results in reflexive behavioral responses and may or may not result in other responses. Reflexive withdrawal responses tend to be mediated by very simple sensorimotor circuits optimized for speed and reliability and can occur without input from higher processing centers, although more complex escape and avoidance behaviors involve more complex neural circuits (Chase 2002; Walters 1994). Even in humans the initial reflexive response to a noxious stimulus is sometimes faster than can be consciously perceived, and nociceptors can sometimes be activated without conscious sensation (Adriaensen et al. 1980). Invertebrates that lack appropriate processing centers may be capable of only this rapid, unconscious processing.

The definition of pain widely accepted by scientific investigators is “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Merskey and Bogduk 1994). The emotional component required by this definition of pain makes its identification in other species, and especially in species quite different from humans, extremely difficult, if

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1 Abbreviation that appears ≥3x throughout this article: CNS, central nervous system.
not impossible. This is because emotion is usually defined in terms of conscious experience (e.g., Izard 2009), and while evidence of consciousness in some animals is available, proof of consciousness is not (e.g., Allen 2004).

It is therefore important to distinguish between nociception as detection of a noxious stimulus (which can be recognized scientifically by unambiguous behavioral and neural responses), and pain as the unpleasant feeling associated with that stimulus (and inferred by behavioral and neural responses of uncertain relation to consciousness).

Moreover, the emotional response during pain may be linked to cognition, “knowing” in some sense that the sensation is negative and involves a threat to the body. Whereas nociception leading to a nociceptive response can be mediated by the simplest of neural circuits (in principle just a single nociceptor connected to an effector system—e.g., a muscle), pain requires neural circuitry that incorporates additional functions, some of which might entail highly complex processing by very large numbers of neurons.

Identifying Nociception and Pain in Animals

In invertebrates, like mammals, responses to noxious stimulation can be complex. Indeed, immediate defensive responses to injury or noxious stimulation are followed by a longer-term phase (sometimes lasting weeks or months) where damaged regions are hypersensitive (e.g., Walters 1987b) and some invertebrates remember associations of the noxious event with its context (e.g., Colwill et al. 1988; Walters et al. 1981).

To date, long-term nociceptive sensitization in invertebrates has been explained by long-term alterations of primary sensory neurons (especially nociceptors) (e.g., Montarolo et al. 1986; Scholz and Byrne 1987; Walters 1987a) and motor neurons (e.g., Cleary et al. 1998; Glanzman 2008; Weragoda et al. 2004); there is little evidence for electrical activity or alterations in other types of neurons that outlast a noxious stimulus for more than tens of minutes (see Cleary et al. 1998; Marinesco et al. 2004). Interneurons and modulatory neurons have received far less experimental attention because they are much more difficult to identify and sample with the intracellular recording methods used for neurophysiological experiments on invertebrates. Truly systematic investigations of the distribution and duration of enhanced activity (possible neural correlates of pain) across an entire nervous system will benefit from the development of functional imaging methods for invertebrates (for a start, see Frost et al. 2007; Zeccevic et al. 2003) equivalent to those used to examine patterns of activity in the mammalian brain after noxious stimulation (e.g., Peyron et al. 2000; Tracey and Bushnell 2009).

Several considerations suggest that pain may be absent in at least some invertebrates. Capacities for processing all types of information, including that involved in pain or painlike phenomena, increase with the size and complexity of a nervous system, and mammals with the most complex brains have, thus far, shown the most evidence for a capacity for humanlike pain, although this might reflect a far greater experimental effort directed at vertebrates rather than genuine differences in the invertebrate pain experience.

Nociceptive reflexes and nociceptive plasticity can occur without conscious, emotional experience because these responses are expressed not only in the simplest animals but also in reduced preparations, such as spinalized animals (Clarke and Harris 2001; Egger 1978) and snail ganglia (Walters et al. 1983b). Similarly, in human patients nociceptive reflexes can occur without conscious awareness below a level of complete spinal transection (Finnerup and Jensen 2004).

But conservation or convergence of physiological and molecular mechanisms of nociception and nociceptive sensitization across distant phyla does not necessarily imply that higher-order phenomena (such as pain) that can be supported by these mechanisms are equivalent. Even if the critical mechanisms turn out not to be equivalent, it is not possible to be certain that an animal does not feel pain, and thus the ethical questions remain (Allen 2004).

How can researchers balance the opportunities some invertebrates offer for discovering mechanisms that, for example, may alleviate chronic pain or slow dementia in humans, with the possibility that the invertebrate subjects might suffer? This is a fundamental problem for all of biomedical research, but has received very little consideration in invertebrate studies. We refer readers to other articles in this issue (Elwood 2011; Mather 2011) dealing with ethical and philosophical considerations of invertebrate use.

Nociception and Painlike Phenomena in Molluscs

Molluscs have provided invaluable models for neuroscience, yielding a wealth of basic information applicable to humans and other vertebrates. A consideration of their nociceptive behavior and physiology will inform choices about their optimal use for biomedical research and improve the welfare of molluscs used in the laboratory.

Mollusca is a highly diverse and successful metazoan phylum with over 100,000 species (Ponder and Lindberg 2008) distributed among terrestrial, aquatic, and marine environments and divided into seven classes: Aplacophora, Polyplacophora, Monoplacophora, Scaphopoda, Bivalvia, Gastropoda, and Cephalopoda. Along with great diversity in body plan, lifestyle, and ecology, Mollusca encompasses enormous intraphylum variation in sensory organs and neuroanatomy (Bullock and Horridge 1965). Because the capacity for sensing and integrating information about the environment and an individual’s own body is probably important for the ability to feel pain or experience distress, this large variation in neural and sensory complexity suggests that welfare concerns may differ among groups.

The primary model species considered here are from the gastropod and cephalopod clades; members of the other classes are discussed briefly.
Aplacophora, Polyplacophora, Monoplacophora, and Scaphopoda

The classes less commonly used in neurobiological research tend to be the primitive, sedentary, or deepwater-dwelling groups.

Aplacophora contains small wormlike molluscs that burrow into the substrate (Salvini-Plawen 1981) and have paired ventral and dorsal nerve cords running along the body as well as paired cerebral and buccal ganglia in the head. Visual and vestibular organs are absent but putative mechanosensory neurons innervate the oral surface (Shigeno et al. 2007). Little is known about their behavior and nothing about the physiology of sensory neurons.

Polyplacophora contains the chitons, generally intertidal marine animals with multiple platelike shells covering the dorsal surface. They have a simple nervous system without pronounced cephalization. There are rows of primitive photoreceptors along the dorsal surface and mechanosensory organs around the mouth.

Monoplacophora is also an exclusively marine taxon of limpetlike, conically shelled molluscs. Scaphopoda have a single, tusklike shell through which water is pumped while the animal remains mostly buried in the substrate. In both of these groups the neural networks are simple and apparently unspecialized and the ganglia are small. The simplicity of their nervous systems and their behavior suggest that the possibility of these animals experiencing painlike responses to tissue insult is remote.

Bivalvia

The Bivalvia (e.g., oysters, clams, mussels, and scallops) are abundant in both marine and freshwater environments. Their nervous system includes two pairs of nerve cords and three pairs of ganglia (Brusca and Brusca 2003). There is no obvious cephalization and the nervous system appears quite simple. A population of mechanosensory neurons is activated during the foot withdrawal reflex in a razor clam, but it is not known if these are nociceptors (Olivo 1970).

Clams and scallops have simple eyes and chemosensory organs located along the periphery of the mantle and they initiate escape swimming if a threat is detected, thus some integration of information and basic decision making occurs. Escape swimming in scallops is driven by a motor pattern generator in the cerebral ganglion and usually occurs after chemosensory detection or contact with a starfish predator that would normally precede tissue destruction (Wilkins 1981), suggesting that nociception may be involved. However, to our knowledge there are no published descriptions of behavioral or neurophysiological responses to tissue injury in bivalves.

Although a number of studies have claimed that endogenous opioids (e.g., Stefano and Salzet 1999) and opioid receptors (e.g., Cadet and Stefano 1999) are expressed in the mussel Mytilus edulis, particularly in immunocytes, neither genes for proopiomelanocortin (POMC) nor opioid receptors are found in Drosophila melanogaster or Caenorhabditis elegans, and their reported existence in other invertebrates, including molluscs, is controversial (Dores et al. 2002; Li et al. 1996).

Gastropoda

This class includes terrestrial, freshwater, and marine species (e.g., snails, slugs, limpets, whelks, and many others). Typically the shell and body are coiled, although in some taxa (e.g., terrestrial slugs, sea hares, nudibranchs) the shell is absent.

Gastropods have more diverse and specialized sensory organs than the groups above and are typically motile and active foragers. A long with increased range of habitats and associated morphologies, their behavioral range is greater and this is reflected in increased neural complexity. The basic molluscan nervous system is present (Bullock and Horridge 1965) but is expanded in terms of both the number of cells and their specialization. Many gastropods have giant neuronal somata, which have been used to advantage for neuronal analyses of behavioral mechanisms (Chase 2002; Kandel 1976) and perhaps most prominently for investigations of learning and memory mechanisms (Kandel 2001). Gastropods have also provided the largest number of studies of nociceptive behavior and sensitization in invertebrates, in part because noxious mechanical and electrical stimuli have been used in many learning and memory studies. Some of these studies have used pulmonate (air-breathing) snails but most have used opisthobranch (rear-positioned gills) snails. We know of no studies of nociceptive behavior or physiology in the remaining group of gastropods—the prosobranchs.

Pulmonates

Pulmonates descended from gastropod molluscs that moved from the sea to terrestrial and freshwater habitats. The most extensively studied pulmonate is Helix (several different species on different continents), which is prey to many generalist predators such as birds and frogs. Thus the well-developed defensive and aversive behaviors elicited by noxious sensory input during failed predation attempts in this genus should be subject to continuing selection.

When presented with a potentially threatening tactile stimulus to soft tissue, Helix, like other pulmonates, withdraws reflexively, a behavior mediated by simple neural circuitry including sensory neurons that appear to be nociceptors (Balaban 2002; Ierusalimsky and Balaban 2007). Noxious stimulation can sensitize this behavior. Repeated electric shocks to the foot (which probably activate nociceptors) result in a reduced response threshold to an innocuous mechanical stimulus that persists several days after the shocks, and this long-term behavioral sensitization is associated with several alterations in the circuit that mediates the withdrawal response (Balaban 1983, 1993; Prescott and Chase 1999).
Other pulmonates used to investigate responses to noxious stimuli include *Lymnaea stagnalis* (Sakharov and Rozsa 1989) and *Megalobulimus abbreviatus* (Kalil-Gaspar et al. 2007). The latter study is of interest because it provided pharmacological evidence for nociceptive actions of an ion channel highly expressed in mammalian nociceptors (the transient receptor potential cation channel subfamily V member 1, or TRPV1, the capsaicin receptor).

As explained above, the ability to perceive noxious information as emotionally unpleasant is a delineating point between simple nociception and pain. *Helix*, with a brain containing only about 20,000 neurons in 11 ganglia, might be presumed to be incapable of higher-order processing necessary for emotional responses. Interestingly, however, some evidence suggests an emotionlike reaction in *Helix*.

Experiments using electrodes implanted into two different ganglia allowed animals to “self-stimulate” either of the two neural regions by pressing a bar (Balaban 1993; Balaban and Chase 1991). Results from this experiment suggested that different CNS regions of snails have some rudimentary “emotional coloration”: snails self-stimulated more frequently when the electrodes were placed in the mesocerebral region of the cerebral ganglion (containing some neurons involved in sexual behavior) and less frequently when they were placed in the rostral portion of the parietal ganglion, in an area where electrical stimulation of putative nociceptive sensory neurons underlying reflexive withdrawal behavior was likely (Balaban 2002). But relatively little is known about the anatomical organization and actual functions of most neurons in either of these ganglionic regions. A lack of follow-up reports of snail self-stimulation raises questions about the robustness of this finding.

An apparent ability to remember and choose to avoid a negative stimulus would suggest that a snail can selectively modify its behavior on the basis of aversive experience in ways similar to mammals. This would not show that *Helix* can experience pain but it would suggest that fundamental components of painlike information processing in vertebrates might be present in a rudimentary fashion in some molluscs.

**Opisthobranchs**

The marine opisthobranch *Aplysia californica* is the leading invertebrate model system for analyzing cellular bases of behavioral and neural plasticity in many contexts (Kandel 1976, 2001). *Aplysia* has nine central ganglia containing only about 10,000 neurons (Cash and Carew 1989), of which several hundred have been identified by soma size and location, electrophysiological properties, synaptic connections, and behavioral effects. *Aplysia* and many other molluscs also have numerous neuronal cell bodies in peripheral nerve nets (Bullock and Horridge 1965; Moroz 2006).

Most studies of learning and memory mechanisms in *Aplysia* have used known mechanosensory neurons. Initial studies used these neurons in the abdominal ganglion that innervate the siphon (Byrne et al. 1974), but later studies used homologous sensory neurons in each pleural ganglion that innervate most of the rest of the body surface (Walters et al. 1983a, 2004). Moreover, both sets of neurons function as nociceptors (Illich and Walters 1997; Walters et al. 1983a) and thus their rich plasticity is highly relevant to nociceptive functions. Both sets of nociceptors display prominent sensitizing effects, associated with short- and long-term sensitization of defensive behavior (gill and siphon withdrawal, tail withdrawal, head withdrawal) after noxious stimulation. These effects include enhancement of synaptic transmission (reviewed by Kandel 2001; Walters 1994) and hyperexcitability of the nociceptor expressed in its peripheral terminals (especially near a site of injury), neuronal cell body, axons, and near its presynaptic terminals (Billy and Walters 1989; Gasull et al. 2005; Reyes and Walters 2010; Weragoda et al. 2004). Such alterations are similar to those described in studies of nociceptive sensitization in rodents and humans, suggesting that some mechanisms that promote pain in vertebrates are also present in molluscs and, most likely, in other invertebrate taxa as well (Walters and Moroz 2009).

Interesting similarities also exist in the behavioral responses of *Aplysia* and mammals to noxious stimulation. *Aplysia* displays the nearly ubiquitous pattern of immediate withdrawal reflexes, rapid escape, and prolonged recuperative behaviors exhibited across all major phyla (reviewed by Kavaliers 1988; Walters 1994; see also Babcock et al. 2009; Walters et al. 2001).

In addition, *Aplysia* responds with a motivational state resembling conditioned fear to previously neutral chemosensory stimuli associated with noxious electric shock (Walters et al. 1981). For example, after pairing with shock, the smell of shrimp evoked a state that was not obvious unless combined with other stimuli—indeed, when only the shrimp extract was presented, the animal exhibited a response reminiscent of the freezing exhibited by rats to a conditioned fear stimulus (Walters et al. 1981). When tested in combination with weak tactile stimulation, the shrimp extract greatly facilitated head and siphon withdrawal responses, defensive inkling, and escape locomotion. Moreover, when delivered to a feeding animal, the conditioned smell inhibited the feeding.

These extensive and motivationally consistent associative alterations (see also Colwill et al. 1988) suggest that memory of a noxious event in snails can be linked to a fearlike motivational state that can dramatically alter the animal’s response to other biologically significant stimuli. Unfortunately, almost nothing is known about the neuroanatomical loci and specific neurons involved in this “higher-order” processing of nociceptive information in opisthobranch ganglia.

Nociceptive sensitization has also been reported in the marine nudibranch *Tritonia diomedea* (Frost et al. 1998), and associatively conditioned avoidance behavior after noxious conditioning stimulation has been investigated in the notaspid *Pleurobranchaea californica* (Jing and Gillette 2003; Mpitsos and Davis 1973). Both of these species offer many advantages for cellular analyses, but they are difficult to obtain commercially and have been investigated much less extensively than has *Aplysia*.
In most animals, including *Aplysia*, nociceptive pathways show a period of inhibition after strong noxious stimulation as the animal engages in escape and active defense (Mackey et al. 1987; Walters 1994). Opioids contribute substantially to nociceptive inhibition in vertebrates but, despite indirect evidence for opioid signaling—such as pharmacological actions of opioids (e.g., met-enkephalin) and opioid antagonists (e.g., naltrexone) in molluscs (Kavaliers 1988; Leung et al. 1986)—molecular evidence does not yet provide firm support for true opioid signaling in invertebrates (Dores et al. 2002). Moreover, application of enkephalins fails to produce inhibitory effects on the gill withdrawal reflex (Cooper et al. 1989) or known nociceptors (Brezina et al. 1987) in *Aplysia*. Instead another peptide, FMRFamide, may be a major transmitter that produces immediate and long-term suppressive effects on nociceptor excitability, synaptic transmission, and defensive reflexes in *Aplysia* (Belardetti et al. 1987; Mackey et al. 1987; Mottarolo et al. 1988).

Cephalopoda

Cephalopoda is an exclusively marine class that comprises squid, cuttlefish, octopus, and nautilus. The cephalopod shell is chambered and external in nautilus, internal in cuttlefish, and reduced or absent in squid and octopuses. Cephalopods are the most neurologically complex invertebrates, with centralized brains divided into specialized lobes capable of processing and integrating complex visual, chemical, and tactile sensory inputs. The octopus CNS has around 500 million cells (Young 1963), vastly more than that of gastropods. The octopus also has an enormous peripheral nervous system, separate from the CNS (Rowell 1966; Young 1963). Indeed, the number of neuronal cell bodies in the arms of the octopus is close to that of the central brain.

Cephalopods show complex behavior and are excellent learners, with reports of sensitization, habituation, associative learning, spatial learning, and even (although controversial) observational learning (see reviews by Hanlon and Messenger 1998; Hochner et al. 2006). Despite the extensive literature on cephalopod behavior, there have been no systematic behavioral or physiological investigations into nociception and nociceptive plasticity.

Cephalopods are also less commonly used for studies of neurophysiology due to the complex neuroanatomy of the cephalopod CNS, the small size of the neuronal cell bodies, and the lack of overshooting action potentials or large synaptic potentials that can be recorded from the cell bodies (Hochner et al. 2006). Nothing is known about where nociceptive information is processed in the cephalopod brain. Evidence for nociception in cephalopods is therefore exclusively behavioral.

In various learning paradigms electric shock to the arms of an octopus has been used effectively as a negative reinforcement (Boycott and Young 1955; Darmaillacq et al. 2004; Shomrat et al. 2008; Young 1961), indicating that the animal finds this stimulation aversive, but nociceptors have not yet been described in any cephalopod. Octopuses are capable of regenerating damaged or amputated arms, but there are no published studies of behavioral adaptations to damaged or healing tissue.

Although electric shock elicits defensive responses, an intriguing anecdote from Jacques Cousteau (1973, 23-24) describing an octopus’ behavior during exposure to damaging heat suggests the absence of a response to intense thermal stimulation:

> One day at Octopus City, in the Bay of Alicaster, Dumas dived with an underwater rocket and began waving it in front of an octopus’ house. Nothing happened. The animal reacted not at all. He did not try to hide, or to escape. Dumas then turned the beam directly onto the octopus, which did not even draw [sic] its arms. The game was called off, however, when Dumas saw that it was becoming cruel. The octopus showed signs of having been burned. But even then it had not tried to escape from it. This surprising insensitivity to fire has been confirmed by Guy Hilpatric, one of the pioneers of diving, who told us that he has seen an octopus, which had been brought onto shore, cross through a fire to get back into the water.

Cephalopods are chambered and external in nautilus, internal in cuttlefish, and reduced or absent in squid and octopuses. Cephalopods may be similar to those in gastropods and vertebrates (UK Animals [Scientific Procedures] Act 1986) and

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in Canada and Europe an approved welfare protocol is required for the use of cephalopod species in research (CCAC 1993; EU directive 86/609/EE.C, updated September 20103).

Cephalopods have far more complex brains and behaviors than any other invertebrate and are capable of impressive cognitive tasks (see Hochner et al. 2006, for review). For example, both the briny octopus (Boal et al. 2000) and “primitive” nautilus (Crook et al. 2009) are capable of vertebratelike spatial learning. Given these sophisticated cognitive abilities, an important question is whether nociceptive responses in cephalopods are accompanied by affective and cognitive processing functionally similar to some of the processing that is important for pain states in mammals.

Anesthesia in Molluscs

Until the early 1970s physiological and biochemical studies of molluscs dispensed with any anesthetics. This practice reflected the widespread assumption that invertebrates cannot feel pain, a view that is still common (and used to condone, for example, the preparation of living cephalopods, snails, and lobsters for human meals by methods that would cause intense, prolonged stimulation of nociceptors if applied to mammals).

Several studies of the mechanisms of action of widely used mammalian anesthetics exploited the experimental advantages of the giant axon of the squid and the giant cell bodies in gastropod molluscs such as Aplysia (e.g., Frazier et al. 1975; Winlow et al. 1992). But these anesthetics were not used by researchers during their dissections, in part because many of the mammalian anesthetics and analgesics, including opioids (Cooper et al. 1989), were not very effective in molluscs (although there were interesting exceptions, including volatile general anesthetics that potently open potassium channels that hyperpolarize and reduce excitability in Aplysia nociceptors; Winegar et al. 1996; Winegar and Yost 1998). The widespread use of anesthesia during dissection of Aplysia began when investigators interested in learning and memory realized that dissection without anesthesia could cause sensitizing effects that would interfere with the neuronal plasticity they were studying.

The anesthetic of choice for both gastropods (Pinsker et al. 1973) and cephalopods (Mesenger et al. 1985; M ooney et al. 2010) is isotonic magnesium chloride solution, typically applied by injection for gastropods and immersion for cephalopods. Magnesium ions provide an ideal anesthetic (and muscle relaxant) because they are normally present at relatively high concentrations (especially in marine molluscs) and thus are relatively nontoxic, their effects are rapidly reversible, and the agent is both inexpensive and highly effective (Walters 1987b). The effectiveness of magnesium chloride in gastropods is a result of its inhibition of neurotransmitter release at chemical synapses, its depressive effect on voltage-gated sodium channels, reducing membrane excitability (e.g., Liao and Walters 2002), and the fact that it is used at high concentrations and injection volumes (>25% of the animal volume) in combination with a balanced reduction of sodium ions (a reduction that by itself reduces excitability). Effectiveness probably also depends on leakage of the primitive blood-ganglion or blood-nerve barriers of most molluscs (Abbott 1987). A applied magnesium chloride does not penetrate these barriers in vertebrates, precluding its use as an anesthetic in mammals. Given that cephalopods also have a highly effective blood-brain barrier (ibid.) and relatively impermeable skin, the mechanisms underlying the anesthesia that occurs during the animal’s immersion in isotonic magnesium chloride are an interesting mystery.

We refer readers to Cooper (2011, in this issue) for further discussion of analgesia and anesthesia in invertebrates.

Evolutionary Selection Pressures and Painlike Phenomena in Molluscs

Insight into the extent to which nociception may lead to pain in molluscs can come from considering possible selective advantages of pain during evolution.

Nociception, like any prominent trait in animals, has been selected and refined over millions of years by evolutionary processes that act to enhance survival and reproductive success. The adaptive value of nociception is obvious and probably universal: it permits rapid avoidance of a damaging stimulus and escape from the context in which such damage occurs. Escape may be from predators, aggressive conspecifics, or threatening environmental features (e.g., rough surf).

The adaptive value of experiencing pain is more difficult to identify, although clues are available from likely consequences of nociceptive responses for survival under natural conditions. A strong negative emotion motivates rapid avoidance learning, decreasing the chances of reexposure to a noxious stimulus. This interpretation raises the question of how much neural processing power is required for emotional responses. Behaviorally expressed motivational states (defined without reference to consciousness) mediated by the actions of neuromodulators on relatively simple circuits in Aplysia have been suggested to have functional similarities to emotional states in mammals (Kupfermann 1979), and this is consistent with properties of the conditioned fearlike state in Aplysia described above. However, from a functional and evolutionary point of view, there is no need for these states to involve conscious experience by the animal.

In social animals, awareness and communication of injury can be advantageous, allowing not only the behavioral alterations that guard or rest an injured body part (at the expense of other behaviors such as foraging) but also the recruitment of caregivers to help protect and provide for an incapacitated individual during recovery. In nonsocial animals (which may include all molluscs, as even those that aggregate do so opportunistically for exploitation of a local resource, not for social interactions), obvious behavioral

3Available online (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm), accessed on April 4, 2011.
changes resulting from injury may be neutral or maladaptive (attracting the attention of predators or aggressive conspecifics). Thus it is unlikely that painlike states would have evolved in molluscs to promote communication with potential caregivers.

A mollusk with high metabolic rates, such as squid, must forage frequently to survive and thus cannot suppress active behavior for very long during recuperation (when pain is often worst in mammals), so this common behavioral correlate of pain would be maladaptive in such animals, although ongoing pain that promotes minor behavioral changes to protect an injured appendage as a tradeoff against small reductions in foraging success may be highly adaptive. Animals under predation pressure (which includes almost all molluscs, and particularly those lacking primary defenses such as a hard shell or aposematic signals) probably derive minimal value from sustained painful “feelings” if their expression renders them more vulnerable to predators. Thus, while an acute response to injury promoting escape and survival should be strongly selected, and long-term increases in sensitivity to potentially threatening stimuli are likely to be adaptive (Walters 1994), persistent painlike states that profoundly alter ongoing behavior (e.g., decreasing foraging or mating activity) may not be adaptive in molluscs.

**Conclusions**

All molluscs examined have shown a capacity for nociception as demonstrated by behavioral responses and/or by direct recording from nociceptors and other neurons. Nociception and nociceptive sensitization at the level of primary nociceptors make use of neuronal mechanisms that appear to be highly conserved and widespread throughout the animal kingdom.

But not all mechanisms related to nociceptive biology are widely shared. For example, analgesic-like effects mediated by true opioids and opioid receptors may be absent in invertebrates (Dores et al. 2002), and vertebrates may possess some synaptic mechanisms that are absent in invertebrates (Ryan and Grant 2009). Moreover, the sharing of many basic molecular building blocks does not imply sharing of higher-order processes that depend on those building blocks. For example, nearly all known brain functions in most phyla depend on action potentials generated by the operation of highly conserved sodium channels, but only a few species have brains with the capacity to learn a spoken language or do arithmetic—thus voltage-gated sodium channels are essential for learning German or solving equations, but their presence does not imply the capacity for proficiency in German or algebra. At some level this must also be true for the capacity to experience pain.

Immediate and longer-term neuronal and behavioral responses (including nociceptive memory and simple associative learning) can be mediated entirely by a single small ganglion, such as the abdominal ganglion in *Aplysia*, proving that these effects do not need complex neural structures (e.g., Antonov et al. 2003; Illich and Walters 1997). Some gastropods, which have simple nervous systems compared to cephalopods, exhibit changes in state with apparent functional similarities to emotional states, as illustrated by “conditioned fear” and “self-stimulation” in *Aplysia* and *Helix*, respectively (Balaban and Chase 1991; Walters et al. 1981).

Given the capabilities of relatively simple molluscan nervous systems, and if a key to the experience of pain is the size and complexity of the nervous system, one must seriously consider the possibility that cephalopods can experience some form of pain. The most complex and behaviorally sophisticated of molluscs, cephalopods have vastly more complex central nervous systems, with up to 500 million neurons, distinctive internal divisions of the brain, specialized integrative regions where diverse sensory inputs are processed (Boycott and Young 1955), and dense, specialized innervation of the periphery (Hochner et al. 2006). If subjects experience pain requires a minimal level of network complexity and processing power, these animals’ brains might approach that level.

Scientifically accepted definitions of pain and nociception neatly distinguish these concepts (e.g., Merskey and Bogduk 1994), but drawing a line between the two can be difficult in practice. Furthermore, no experimental observation of nonverbal animals (nonhumans) can demonstrate conclusively whether a subject experiences conscious pain (Allen 2004). Suggestive evidence for painlike experiences in some animals is available, and nociceptive responses measured at the neural and behavioral levels in molluscs have provided evidence that is both consistent and inconsistent with painlike states and functions. Unfortunately, inferences drawn from the relatively small body of relevant data in molluscs are limited and prone to anthropocentrism. Identifying signs of pain becomes increasingly difficult as the behavior and associated neural structures and physiology diverge from familiar mammalian patterns of behavior, physiology, and anatomy, making interpretation of responses in molluscs particularly difficult.

In the laboratory, molluscs are often subjected to manipulations that produce nociceptive responses, either as the aim of an experiment or as a byproduct. Profound differences between molluscs and mammals in the size, complexity, and structure of their nervous systems, as well as their lifestyles and evolutionary history, suggest that painlike phenomena, if they exist in some molluscs, are likely to be quite different from pain in mammals, although it does not follow that molluscs are incapable of experiencing pain. Gastropod and cephalopod molluscs have shown long-lasting behavioral alterations induced by noxious experience, which probably involve motivational states that can be used flexibly to alter defensive and appetitive responses. This suggests that some molluscs may be capable not only of nociception and nociceptive sensitization but also of neural states that have some functional similarities to emotional states associated with pain in humans.

While it seems improbable that any mollusc has a capacity to feel pain equivalent to that evident in social mammals,
the existence of some similarities in nociceptive physiology between molluscs and mammals, the paucity of systemic investi-
gations into painlike behavior in molluscs, and the logical impossibility of disproving the occurrence of conscious experience in other animals all suggest that it is appropriate to treat molluscs as if they are susceptible to some form of pain during experimental procedures.

In conclusion, we recommend that the design of experiments using molluscs, particularly those with larger and more complex ganglia or brains (especially cephalopods but also gastropods), take into account the possibility of a capacity for painlike experience in these animals. Effective anesthetics (e.g., magnesium chloride) should be used during dissections and, to the extent possible, during any procedure that produces tissue damage or possible stress. Investigators whose experiments unavoidably produce noxious stimulation should employ efforts similar to those required for vertebrate subjects to reduce both the number of animals and the potential for suffering to the minimum needed to test their hypotheses.

Balancing the benefits from knowledge gained in molluscan experiments with the potential for inflicting pain and distress in the experimental subjects should be an explicit consideration in molluscan studies. (For related guidance to IACUC members, we refer readers to Harvey-Clark 2011, in this issue.)

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References

Abstract

Invertebrate animals have long played an important role in biomedical research in such fields as genetics, physiology, and development. However, with few exceptions, scientists, veterinarians, and technicians have paid little attention to the anesthesia, analgesia, and euthanasia of these diverse creatures. Indeed, some standard research procedures are routinely performed without anesthesia. Yet various chemical agents are available for the immobilization or anesthesia of invertebrates, ranging from gases or volatile liquids that can be pumped into either an anesthetic chamber (for terrestrial species) or a container of water (aquatic species), to benzocaine and other substances for fish. Many invertebrates are not difficult to immobilize or anesthetize and the procedures recommended in this article appear to be safe; however, none should be considered totally risk-free. Analgesia of invertebrates is as yet a largely unexplored field; until scientific data are available, other measures can promote the well-being of these animals in the laboratory. For euthanasia, various methods (physical or chemical or a combination of both) have been recommended for different taxa of invertebrates, but most have not been properly studied under laboratory conditions and some can be problematic in the context of research procedures and tissue harvesting. Furthermore, relevant data are scattered, sometimes available only in languages other than English, and there is no international approach for seeking and collating such information. In this article I review various methods of anesthesia, analgesia, and euthanasia for terrestrial and aquatic invertebrates, as well as areas requiring further research.

Key Words: analgesia; anesthesia; euthanasia; humane care; invertebrate; laboratory animal; welfare

Introduction

Invertebrates: An Overview

Most living animals belong to the large group commonly termed “invertebrates.” There are at least 1 million species of invertebrate compared with 4,000 mammals, nearly 10,000 birds, 6,000 reptiles, 6,000 amphibians, and over 25,000 fish. The vertebrates all belong to one phylum (Chordata), whereas there are over 30 phyla of invertebrate animals. They range from the single-celled protozoan to the more complex and larger metazoan (multi-cellular animals) such as arthropods and molluscs. The emphasis in this article is on the metazoan species, with particular reference to those commonly used in research.

Most invertebrates are ectothermic (“cold-blooded”), although a few species (mainly moths [Lepidoptera]) that live in cold areas or are active in the winter can generate metabolic heat to raise their body temperature sufficiently to counter freezing. A similar adaptation is evident in bumble-bees (Hymenoptera; Bombidae), which can raise their body temperature by contraction of flight muscles.

Emerging Interest in Invertebrate Welfare

Although some species of invertebrates have been domesticated for hundreds or even thousands of years (Beavis 1988), relatively little is known about the physiology and responses of most (with the notable exception of the work by Young and others on cephalopods; e.g., Young 1963), and therefore only limited scientific evidence is available to guide research workers.

Belief in Europe that some insects could be sentient goes back at least to the Middle Ages. In Measure for Measure William Shakespeare wrote that “the poor beetle that we tread upon, in corp’ral sufferance finds a pang as great as when a giant dies.” In the early 19th century, enlightened naturalists began to express concern about the treatment of animals. Some entomologists and collectors introduced methods of killing insects that were thought to be more humane and in keeping with the rapidly developing philosophy of “kindness to animals” (see, e.g., Stephens 1834). This concept of giving invertebrates the benefit of the doubt was remarkably far-sighted.

In the past 50 years, researchers recognized the need to manage and work with invertebrates in the laboratory in a...
humane way. Early editions of the UFAW *Handbook on the Care and Management of Laboratory Animals* (e.g., UFAW 1967) included chapters on the fruit fly, house fly, and blow fly, the American cockroach, locusts, crickets, beetles, ticks, and molluscs.

Until relatively recently, however, little attention was paid to the health and welfare of invertebrates in captivity (Cooper 1980, 1987a,b; Cooper and Zwart 2005, 225-245; Frye 1986, 1990; Williams 2002). The situation is changing largely because of the importance and value of these animals when they are kept or managed in zoos, in private collections, and in conservation projects and because invertebrates play an increasingly significant part in research (see below). Wilson-Sanders (2011, in this issue) illustrates the range of research, testing, and educational procedures in which invertebrates are used.

Nonetheless there is much debate as to whether invertebrates can feel pain, although most species show responses to adverse stimuli (Cooper 2006). Many (e.g., the cephalopods) have a well-developed nervous system, and in some species researchers have identified opioid systems similar to those associated with pain sensation in mammals (Kavaliers et al. 1983). Elwood (2011, in this issue) explores in detail the hypothesis that invertebrates might experience pain, carefully distinguishing such experience from pain reception (nociception).

The focus of this article is the use of anesthesia, analgesia, and euthanasia in invertebrates that are kept for research. Much of the text is also pertinent to the care of these animals when they are kept in captivity for other purposes such as display, exhibition, or pleasure (Cooper 1987a, 1998; Cooper and Zwart 2005).

**Anesthesia**

Anesthesia of invertebrates is necessary either (1) to immobilize an animal to facilitate examination or sampling or (2) to permit the performance of procedures (e.g., magnetic resonance imaging; Figure 1) that may be stressful or painful (in such instances it may be used in conjunction with analgesia).

In the past, research scientists and others working with invertebrates tended to perform procedures without anesthesia or use only hypothermia, sometimes with little regard for the animals’ well-being. This approach is probably not conducive to good experimental technique or accurate scientific results. It is increasingly unacceptable to conduct invasive procedures on invertebrates without some form of chemical restraint, preferably through the use of an agent with known anesthetic properties (Cooper 2001). There may also be legal arguments for anesthetizing invertebrates when they are used in research; animal welfare legislation in some countries covers some or all invertebrates (ME Cooper 1987).

It is important for researchers and technicians who deal with invertebrates to be familiar with and able to use appropriate anesthetic techniques for these species. The “higher” (i.e., more complex) invertebrates are generally not difficult to anesthetize, and most of the procedures recommended in the sections below seem to be relatively safe; indeed, some taxa, such as cockroaches, are remarkably resistant to hypoxia. However, as with other species, no method is totally risk-free and the old British maxim that “there are no safe anesthetics, only safe anesthetists” remains true, even when one is dealing with invertebrates. Particularly in this context, the scientist, technologist, or veterinarian who is familiar with biology and natural history and who understands the needs of the species has an advantage over the person with no such knowledge.

The excellent book on invertebrate medicine edited by Lewbart (2006; the second edition is in press, Lewbart 2011) presents methods for the anesthesia of over a dozen different taxa of invertebrates. For some that have been widely used in research, such as the Nematoda (Bodri 2006), a whole range of techniques is available and there is extensive supporting literature. However, there is still, it appears, a dearth of information about the use of anesthetic agents in many groups. For example, Foster (2006, 242) stated that “there are currently no known reports of anesthesia use in chaetognaths” but, as with other such claims, it is not clear whether the statement follows a full literature search in languages other than English. Similarly, Smolowitz (2006, 74) claimed that “little work has been done to identify appropriate anesthetic agents” in gastropods and Lewbart (2006, 129) observed that “there is little in the literature on anesthesia of leeches.” Clearly, a more international approach is necessary for seeking and collating relevant information on anesthesia and other topics relating to invertebrates.

The following methods of chemical immobilization for terrestrial and aquatic invertebrates are recommended based largely on my experience working with various species in...
the laboratory, in zoological collections, and in veterinary practice. I also draw on the observations of Zwart (both cited in Fehr et al. 2005 and from personal communications). Although it is not clear whether some of the agents documented produce a true state of anesthesia or merely immobilize the animal, they usually prove to be safe (in terms of patient recovery), are generally well tolerated, and greatly facilitate the handling and manipulation of individual animals and groups.

Terrestrial Species: Inhalation Anesthesia

Isoflurane (5–10%), sevoflurane, halothane (5–10%), or carbon dioxide (CO₂; 10–20%) are now the most commonly used agents for the anesthesia of terrestrial invertebrates. They are administered either by a jar or, more appropriately, an anesthetic chamber (Figure 2). A chamber for use with insects, arachnids, and crustaceans is available (Applebee and Cooper 1989), but such a sophisticated and relatively expensive item is not essential. Suitable chambers can be improvised from a variety of items, notably from “recycled” plastic bottles that can be disinfected or discarded after use; Pizzi (2006) describes the use of this method with spiders. It is important to place gauze or something similar over the end of the tubing in such systems because certain invertebrates may attempt to escape (Chitty 2006).

Induction with an inhalation agent usually takes several minutes but can vary according to the species and the ambient temperature. Recovery can be prolonged (2–5 hours) but is usually uneventful.

Many arthropod species can be anesthetized safely with inhalation agents (Figure 3) and some are remarkably tolerant to hypoxia. In one unpublished trial I exposed American cockroaches (Periplaneta americana) to 100% CO₂ for 2 hours; recovery took several hours but there were no fatalities. Cockroaches may be unusual in this respect, but I have also found the routine use of 20% CO₂ or 10% isoflurane, halothane, or sevoflurane to be successful and safe in several other species of arthropod. If a procedure is considered to be potentially painful, there may be merit in using isoflurane, halothane, or sevoflurane rather than CO₂, because the extent to which the latter induces analgesia in invertebrates is not known, and its use in vertebrate animals is controversial because of concerns about its effects on the animals’ health and welfare.


Terrestrial or amphibious molluscs are often best anesthetized using water-soluble agents (as discussed below). Snails and slugs can be placed foot down in a glass receptacle with 0.5 to 5 cm of water (depending on the animal’s size) containing the chemical.

Aquatic Species

Noga and colleagues (2006) provided an excellent table of agents that can be effective for immobilizing/anesthetizing crustaceans and many of the agents and techniques they advocated are likely applicable to other aquatic taxa. Based on my experience, the following information applies to freshwater and marine species as well as temperate and tropical aquatic species (Figure 4). At high (tropical) temperatures, induction of and recovery from anesthesia usually occur more rapidly, in keeping with the ectothermic nature of invertebrates.

Aesthetic agents are best administered to invertebrates in a specific container. I use a clean glass container that, depending on the size of the animal, holds between 0.5 and 5.0 liters of fluid (Figure 5). The water in the container can either be from the animal’s own tank or, preferably, made up fresh. The latter must be carefully prepared, especially for

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2In this article the term “anesthesia” implies that an animal is rendered insensitive to noxious stimuli; “immobilization” means that the animal cannot move but may or may not be aware of such stimuli.
marine species, for which salt concentration can be crucial. The water in the container should be at the same temperature as that from which the animal is transferred. If necessary, anesthesia can be induced in the animal’s home tank, although complications can arise with this approach if several invertebrates are present, not all of which require anesthesia, or if there is a filtration and water circulation system that can dilute or remove the anesthetic agent.

Absorption Anesthesia Using Tricaine Methanesulfonate (MS-222) or Benzocaine

Tricaine is soluble in water, but benzocaine must first be dissolved in acetone; in each case 100 mg of the anesthetic agent is generally used per liter of water. Various species (e.g., coelenterates) can be anesthetized safely using tricaine (Stoskopf 2006).

Once immobile, the animal is removed and can usually be kept out of the water for 10 to 15 minutes, during which it must remain wet; in addition to being placed on a damp surface (Figure 6), it can be sprayed periodically either with the water that contains the anesthetic agent (to maintain anesthesia) or with freshly prepared oxygenated water (to hasten recovery). Oxygenated water is produced by pumping pure oxygen from a cylinder for 5 minutes through chlorine-free water. I have never, knowingly, encountered “gas bubble disease” in my invertebrate patients but the possibility must be considered.

Recovery from absorption anesthesia generally takes place in 30 minutes or more, after which the animal can be returned to its own tank.

Carbon Dioxide

Carbon dioxide offers a reliable and relatively inexpensive technique for the “anesthesia” of aquatic invertebrates. Although doubt exists about the degree of humaneness and analgesia induced by CO₂, in practice the technique is well tolerated in invertebrates and appears not to compromise their welfare; it has, for example, been used on many occasions to immobilize, examine, and swab medicinal leeches (Hirudo medicinalis) kept for medical research (Cooper et al. 1986; Cooper 2001). However, if CO₂ is used in water, the resulting changes in pH can lead to an increase in acidity that may be injurious to a sensitive skin (or gill) and may influence research results.

Carbon dioxide can be bubbled from a cylinder through water in a suitable container (usually a glass or plastic jar) and this has the advantage of some control over concentration. Alternatively, the water can be diluted 50:50 with commercial soda water. In a real emergency, a product such as Alka-Seltzer that liberates CO₂ gas can be used but this should not be considered good practice.

Other Agents

Various other agents have been tried in different invertebrates (see Lewbart 2006). For example, chloretone, ethanol,
and chloroform can be used in water to immobilize various taxa of invertebrates (Fehr et al. 2005). Scimeca (2006) provided valuable information on the anesthesia of cephalopods and recommended magnesium chloride and ethanol, either separate or combined, for this purpose. Magnesium chloride is also effective for marine polychaetes (Lewbart 2006), isobutanol and lidocaine can be given by injection to crustaceans (Noga et al. 2006), and saturated mephenesin has been used to anesthetize leeches (Tettamanti et al. 2003).

**Limited or Discontinued Methods**

Hypothermia can be used in many species to slow the animal’s metabolic rate and to facilitate handling and noninvasive techniques, such as surface sampling. Thirty minutes in a refrigerator (+4ºC) is usually sufficient for this purpose but other techniques can be effective; for example, nematodes can be chilled using a stream of carbon dioxide (Bodri 2006). However, hypothermia should not be used for surgical or other invasive procedures. Some invertebrates (e.g., certain Solifugae, or camel spiders) do not appear to tolerate chilling very well and can die as a result (Pizzi 2006).

The concept of using chemical agents, especially inhalable substances, to immobilize invertebrates is not new—for hundreds of years apiarists have used smoke to calm honeybees (*Apis mellifera*) (Figure 7). As mentioned earlier, scientists studying fruit flies (*Drosophila melanogaster*) have long used anesthetic ether to immobilize their subjects, to the extent that strains of *Drosophila* were categorized on the basis of whether or not they were “ether sensitive.” But despite its long history and many good features, the use of ether is no longer advisable for any living organisms.

Traditionally in work with *Drosophila*, “etherizers” were developed to anesthetize the flies for experimental purposes (Demerec and Kaufmann 1962). These sometimes allowed precise administration of ether through the use of a rubber bulb attached to a chamber containing a wad of cotton or gauze soaked in the agent; a squeeze of the bulb forced relatively uniform volumes of ether-air mixture through rubber tubing into the chamber holding the flies. For larvae, nitrogen gas was often used. But Zwart (cited in Fehr et al. 2005) warned that ether, on account of its irritancy, should not come into contact with the head of the animal and cited chloroform as another possible agent. Snails (*Helix aspersa*) exposed to ether vapor demonstrate an adverse response in the form of marked production of mucus (Peer Zwart, University of Utrecht, personal communication, 2005). Another alternative, methoxyflurane, proved useful in various species in the past (Cooper 2001; Cooper and Knowler 1991) of an anesthetic agent (Figure 8). An unhealthy animal, or one that is in poor body condition or dehydrated, is less likely to tolerate the procedure.

A assessment of depth of anesthesia in invertebrates is problematic and few guidelines exist. Immobility and the loss of a righting reflex are usually indicators of full anesthesia. Some species, such as certain Lepidoptera, can “sham death” or go into a state of immobility that does not necessarily indicate that they are anesthetized. Twitching of limbs, antennae, and palps and the presence of muscle tone are features of both induction and recovery in many terrestrial species but are unreliable signs (or perhaps have not been properly studied). A quatic invertebrates show similar changes but with marked variation. In my work on the anesthesia of leeches, I assessed responses on the basis of (1) whether the leeches remained attached to the side or bottom of the holding jar, (2) the speed and extent of the animal’s swimming movements, (3) muscle tone (i.e., the extent to which the leeches, when removed, were turgid or flaccid), (4) whether the caudal sucker was functioning, and (5) response to stimulation (touching and handling). In gastropod molluscs, a response to pricking of the foot can be used as an indicator (Cooper 2001; Cooper and Knowler 1991).
Anesthetic Emergencies

Occasionally problems (e.g., accidental overdose by inexperienced personnel; Cooper 1998) arise during anesthetic procedures and prompt action is necessary to revive the animal and save the experiment (thus preventing the need to repeat it and to use additional animals).

If a terrestrial invertebrate takes a long time to recover, or appears to be dead, it should be returned to the anesthetic chamber and exposed to pure oxygen for 10 to 30 minutes. Failure to show any signs of recovery 12 hours later—and even then, only with signs suggestive of death such as rigor mortis or a fetid odor—is a clear indication that the organism is dead.

As indicated earlier, some species of invertebrate can be very refractory to hypoxia. Zwart (personal communication, 2005) has questioned whether artificial respiration, by exerting pressure on the body, might help in eliminating inhaled anesthetic agents, especially in insects where respiration is maintained by altering the shape of the thorax at each action of the flight muscles.

Similar emergency measures may be effective with aquatic species, but the O₂ should be bubbled through the water, either continuously for 10 to 20 minutes or intermittently for 1 to 2 hours.

Recovery

Animals should receive supportive care during and after anesthesia, but as yet little is known about the needs of different invertebrates. As in all such work, an understanding of the physiology of the species is helpful.

Maintenance of fluid balance is particularly important, as it is in all animal species. Most invertebrates are small, with a large surface area in proportion to body mass, and are therefore prone to desiccation. Spiders and some other species will imbibe water or saline given orally with a pipette or syringe (Figure 9). In describing the use of isoflurane in myriapods, Chitty (2006) advocated humidification of the vapor because centipedes are very susceptible to dehydration; similar precautions could be adopted with other sensitive species.

Terrestrial gastropods require special consideration as they are especially susceptible to desiccation. Indeed, they have developed various strategies to conserve water. One such strategy is “feces-sitting” (Bleakney 1991; the snail rests with its muscular foot on a pile of feces; fresh feces are 80% water and the snail may limit fluid loss by remaining in contact with them.

Fluid balance is obviously less of a consideration in aquatic species. However, it can be an important factor if, for example, surgery or a wound in the animal’s integument permits the ingress or egress of fluids and electrolytes.

Analgesia and Other Methods to Promote Invertebrate Welfare

For decades scientists and welfarists have debated whether invertebrates can experience pain or discomfort (Alumets et al. 1979; Cobby 1988; Cooper 2006; Fiorito 1986; Wigglesworth 1980), and the debate is likely to continue (see Elwood 2011). Experiencing pain and “suffering” from it may not be the same. Until more is known, there would appear to be merit, on both scientific and humanitarian grounds, for minimizing the extent to which laboratory invertebrates are exposed to adverse stimuli that may prove “stressful” or painful. As stated earlier, whenever possible the animals should be afforded the benefit of the doubt.

Judging from the available literature in English, French, and German (e.g., Gabrisch and Zwart 2005), the use of analgesics in invertebrates does not yet appear to be feasible. One alternative is to use anesthesia for any procedures that might possibly be painful or that may necessitate either prolonged restraint or disruption of the animal’s normal behavior.

Whenever invertebrates are kept for research or study, it is helpful to draw up codes of practice—quite apart
from any legal requirements—to help promote high standards of care and welfare (CCAC 1982; Collins 1990; Invertebrate Working Group 1990). There are also practical ways to maintain the well-being of invertebrates in the laboratory:

- Provision of an environment and social groupings that match, as closely as possible, those that the species favors in the wild. If in doubt, a choice of environmental parameters and habitat should be offered.
- Avoidance of unnecessary or insensitive handling or restraint.
- Maintenance of high standards of management, preferably by personnel with a genuine interest in and knowledge of invertebrates and their care.

Data on behavioral needs and the effect of stressors on survival are available for some taxa and should be used in efforts to enhance the well-being of invertebrates in the laboratory. Such information is available for millipedes (Bailey and Kovaliski 1993; Dangerfield and Chipfunde 1995) and molluscs (Cowie 1985), and Smith and colleagues (2011, in this issue) provide detailed information about laboratory-reared cephalopods. For the latter, the provision of a correct environment is crucial and, no less than the type of research procedures, may determine whether the welfare needs of such species are met in the laboratory.

Indeed, an emerging approach to humane care as a means to not only enhance welfare but also reduce pain—in both human and veterinary medicine—involves physical therapy and environmental changes as well as the administration of analgesics, anesthetics, and other drugs.

Mather (2011, in this issue) discusses moral and ethical approaches to invertebrate use in detail. She emphasizes the importance of knowing about the biology of the invertebrates with which one works in order to recognize “suffering.” She further stresses the need for personnel to be properly educated in this respect so that they can participate knowingly in decisions about the management and use of invertebrates in laboratories.

### Euthanasia

Official texts on the euthanasia of experimental animals have generally made little or no mention of invertebrates (e.g., Commission of the European Communities 1993; Working Party 1996, 1997). In contrast, for over 60 years the UFAW Handbook series, to its credit, has not only covered laboratory invertebrates but also incorporated information on anesthesia and euthanasia for many species; for example, the section on Birds, Poikilotherms, and Invertebrates (UFAW 1967) included methods for “tranquillizing and killing insects and ticks” as well as more specific notes on the “narcotisation and anaesthesia” of molluscs.

A nimal welfare publications, on the other hand, increasingly include discussion of the euthanasia of invertebrates. For example, some years ago the World Society for the Protection of Animals (WSPA 1994) cited various methods for killing crustaceans, ranging from pithing (of crabs) and electrosurgery (crabs and lobsters) to chemical euthanasia (all species), and criticized the use of hot or boiling water to prepare crustaceans for cooking. The killing of surplus or diseased invertebrates in zoological collections has also attracted attention; among others, the Invertebrate Working Group (1990) provided guidance on humane methods to kill terrestrial species (e.g., in butterfly houses) and more recently Hackendahl and Mashima (2002) considered the euthanasia of aquatic invertebrates.

In the research literature, various methods of euthanasia have been recommended for different taxa of invertebrates (Lewbart 2006), but most have not been properly studied. However, information is available about some of the idiosyncrasies of ectothermic animals, specifically reptiles and amphibians; for example, methods that involve severing the nervous tissue (i.e., the spinal cord in vertebrates) may not be relevant to “higher” invertebrate taxa (Cooper et al. 1989). Zwart (cited in Fehr et al. 2005) provided a table of both physical methods (e.g., decapitation of species with a well-defined head) and chemical methods (e.g., the injection of snails with pentobarbitone). Zwart (personal communication, 2005) has also found that the pH of commercially available pentobarbitone can vary between 9.5 and 11.0 and that, when injected into the hemocele of snails, these unbuffered substances produce a rubbery ball of coagulated protein that, inter alia, can make the tissue unsuitable for histological examination. It seems likely that such an effect is injurious, and possibly painful, in the live snail.

For the euthanasia of spiders, Pizzi (2006) recommended immersion in 70% ethanol. He warned against rapid freezing of these (and other) invertebrates because the resulting tissue damage compromises histological examination, an important consideration in invertebrates kept for research purposes.

In summary, methods of euthanasia for invertebrates are inadequately researched and warrant more attention (Cooper 2006; Murray 2006).

### Conclusions

As the Council of Europe’s Charter on Invertebrates (Pavan 1986) pointed out, invertebrates are the most important component of wild fauna—providing food and contributing to agriculture and forestry (e.g., providing useful models for mathematical and other studies on genetics and biodiversity), medicine, and industry and crafts (e.g., as the basis of designs for carvings, bee hives, furniture, carpets, curtains). Moreover, they are increasingly attracting interest among researchers. As I have expressed in the context of the care of invertebrates in zoos and in private hands (Cooper 2006), I believe that the care of these animals in the laboratory should be of as high a quality as that for vertebrates and that this care should be reflected in the research in which these animals are used.
It is worth noting that over 100 years ago (January 5, 1901), an editorial in the Veterinary Record declared that, for anesthesia, “The use of chloroform by practitioners is now common, and the amount of pain and suffering prevented is enormous. By its aid we are enabled to perform operations with success which without it were seldom satisfactory.” A century later, sophisticated methods of anesthesia are used routinely in both veterinary medicine and laboratory animal science and those words seem quaint. Yet the ability to use such methods on invertebrates remains very much at a comparable embryonic stage, as is widespread knowledge of appropriate methods of euthanasia. Research in these areas is needed to rectify this situation and to ensure both invertebrate welfare and quality research. Furthermore, with the increasing globalization of research, methods should be established for the collation and translation of information available only in languages other than English.

Acknowledgments and Dedication

I am grateful to Sally Dowsett for typing this article and to my wife, Margaret Cooper, for providing most of the photographs and for commenting on the legal section. My friend and former colleague Ken A pplebee produced images of the anesthesia chamber and agreed to their use. Peer Zwart, a pioneer of invertebrate medicine and pathology, has been an ongoing source of inspiration and encouragement. Cameron H. Fletcher, Manager Editor of the ILAR Journal, has been most helpful and considerate during the metamorphosis of this contribution: merci beaucoup!

This paper is dedicated to all veterinarians, research workers, and animal technologists whose interest in invertebrates has led them to promote a better understanding of the health, welfare, and conservation of these oft-neglected creatures.

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Abstract

People who interact with or make decisions about invertebrate animals have an attitude toward them, although they may not have consciously worked it out. Three philosophical approaches underlie this attitude. The first is the contractarian, which basically contends that animals are only automata and that we humans need not concern ourselves with their welfare except for our own good, because cruelty and neglect demean us. A second approach is the utilitarian, which focuses on gains versus losses in interactions between animals, including humans. Given the sheer numbers of invertebrates—they constitute 99% of the animals on the planet—this attitude implicitly requires concern for them and consideration in particular of whether they can feel pain. Third is the rights-based approach, which focuses on humans’ treatment of animals by calling for an assessment of their quality of life in each human-animal interaction. Here scholars debate to what extent different animals have self-awareness or even consciousness, which may dictate our treatment of them. Regardless of the philosophical approach to invertebrates, information and education about their lives are critical to an understanding of how humans ought to treat them.

Key Words: cephalopod; consciousness; contractarian; ethics; invertebrate; nociception; pain; rights; utilitarian

It is probably not surprising that people’s attitudes toward invertebrates, and indeed all animals, are human-centered. After all, as infants we are first aware only of ourselves, truly egocentric (Berk 2000). Gradually we learn that there is a world separate from us, and by age 3 or 4 a child is busy exploring and cataloguing it. Later, in adolescence we go through another stage of egocentrism where we focus tightly on our place in this burgeoning universe. So anthropocentrism, the attitude that we are the measure of everything and the universe revolves around us, is predominant in human thinking.

This attitude is fostered by Western science (see Balcombe 2010). Western society is individual rather than communally focused, emphasizing competition instead of cooperation and thus reinforcing an individual focus and increasing the egocentrism. Furthermore, there is a strong belief in the objectivity of this view, so that it is not critically evaluated but assumed to be correct. This is supported by the Judeo-Christian view of humans as having dominion over the earth and all things in it. Some have pointed out that this dominion should mean protection and care, but in practice the industrial complex has used it as an excuse for exploitation.

This attitude goes hand in hand with the arrangement of animals on a scala naturae, a sort of tree of life with “lower” organisms at the base, rising through simpler vertebrates to the peak—primates and, of course, humans. This hierarchical approach originated with Aristotle as a way of logically organizing all life, and was taken up by the Christian church. God was at the top of the chain of life, indicating perfection, and humans were the next step down, working toward it (Balcombe 2010).

Surprisingly, there are modern versions, now with three peaks, showing insects, molluscs, and vertebrates increasing in neural complexity toward the apex of intelligence. Even now, people talk of “highly evolved animals” at these peaks. Of course this is untrue—simpler animals like nematode parasites and blind cave fishes are very highly evolved for their demanding environment. And horseshoe crabs, whose fossils can be found in rocks from the late Ordovician, are an enduring model that may survive when recent “generalists” like octopuses and humans have passed on.

No animal is better than any other, yet studies (Bekoff 1994; Eddy et al. 1993) show that humans value intelligent animals and those similar to us over all others. For example, although invertebrates comprise 99% of the animals on the planet, Eddy and colleagues (1993) proved the vertebrate-centered view by choosing only 3 invertebrates for their sample of 30 animals.

Whatever the particular bias, attitudes toward animals have a philosophical and ethical basis, even though those who hold them may not realize or explore the meaning of their attitudes toward animals in general and invertebrates in particular. There is a lot of variation, but the attitudes can be roughly categorized as contractarian, utilitarian, and rights based (Nussbaum 2001).

The Contractarian Approach

This philosophy presumes the complete separation of humans and nonhuman animals. Writing long ago, Descartes...
proclaimed that humans merited consideration in terms of
pain and suffering because we have souls; all other animals
did not have souls and thus were no better than automata
(Balcombe 2010). He was supported in this by the Christian
church, which sanctioned much cruelty to animals.

Disconcerting as it is to those who care for animals, this
approach has one advantage: it asks us to evaluate the effects
of our interactions with animals for our own good. Rollin
(1985), talking about treatment of animals for research, places
the responsibility for and benefit of ethical behavior on our
shoulders and argues that we must act fairly toward animals.
As people who control animals’ lives, we must treat them well
not because they “deserve” it but because it demeans us not to.
For example, he asks undergraduate students using shock as a
deterrent in their animal studies to first try the shocks on them-
selves, so that they are aware of what they are doing. He calls
on all humans to be moral actors.

Even industries that inflict suffering on animals can min-
imize it in the name of moral action. A vertebrate-centered
example of this approach is that of Temple Grandin (1995),
who has described her understanding of the worldview of
cattle and has developed a career in designing holding facili-
ties and slaughterhouses for them. She has designed places
that minimize their daily stress and the trauma of death and
notes her satisfaction in knowing that they are as well cared
for as possible.

Grandin also discusses the effect of working in such
facilities on people. The Jewish man who conducts ritual
slaughtering of kosher beef considers this a calling and an act of
religious piety; and Grandin (1995) sees his attitude as ref-
lecting a need for ritual and respect at the death of animals,
a need that is present in many cultures. Even so, routine kill-
ing can’t help but affect those who do it, and may engender
internalized pain or repudiation as a means of emotional or
psychological protection from the reality of large-scale ani-
mal slaughter. Grandin advises slaughterhouse managers to
rotate the actual job of killing to enable workers to retain a
moral stance—so that the act of killing never becomes com-
monplace and no one gets callous about life because of it.

A further challenge is that the modern, urbanized, tech-
nical world removes humans farther and farther from organisms
of all kinds. In addition, Balcombe (2010) suggests that media
coverage deliberately distances us from animals and distorts
the understanding of their lives by focusing on the “excite-
ment” of the chase and the drama of death. But octopuses, for
example, are predators that actually spend three-quarters of
their daytime lives resting or sleeping, which doesn’t make
“good press” (Mather 1988). Balcombe (2010) also points out
that, in order to make ourselves seem more important, we use
demeaning and uncritical words not only to describe animals but also to insult humans—think of “beastly,” “brut-
ish,” “savage,” or even simply calling someone “an animal.”

How much more true this uncaring attitude is toward inver-
terbrates! It is not just that their importance is seen as much less
than their numbers indicate, but also either that they are simply
not considered (Bekoff 1995) or, in the case of insects, that we
are uneasy and even fearful around them (Hardy 1988).

The most positive of human attitudes toward animals is
Wilson’s (1984) “biophilia.” He writes that humans should
retain an “inherent human affi nity for life and lifelike pro-
cesses” and that we will act ethically if we appreciate the
living inhabitants of the planet in all their diversity. Again,
the point is not the type of animal but that all animals benefi t
when we care. Wilson (1984) has a long way to go to per-
suade us to love all animals.

The Utilitarian Approach

A Gain-Loss Approach to Animal Ecology

The utilitarian approach to the importance of animals is ob-
jective: humans should assess gains and losses when making
any decisions and judgments about animals. According to
this practical view, the dominance of invertebrates in terms
of number of animals means that they are critical to the sur-
vival of life on the planet and should therefore be respected
and protected (New 1993).

Kellert (1993) makes the point by emphasizing the value
of invertebrates in waste decomposition, as food for humans
and for the organisms that humans eat, as sources of chemi-
cals and drugs of immense benefi t to humans, and as indi-
cators of the health of ecosystems. Recent prominent
developments illustrate this close-knit relationship between
invertebrate and human welfare. Observing the decimation
of bee colonies by disease, experts pointed out that bees’
pollination enables the production of many human food
produced. And coral reef animals, which form the backbone
of one of the most productive marine ecosystems (Ponder et al.
2002), are threatened by a number of human actions.

Assessing Pain as a Cost

The gain-loss equation applies to evaluation of the impact of
human actions in particular situations and on specifi c ani-
mals or populations. For example, what are the impacts of
fishing, keeping animals in aquariums, and using them as
experimental subjects? Unfortunately, it is often the human
gains that are the major consideration and the losses to ani-
mals secondary.

Debate has focused especially on the possibility that hu-
mans inflict pain and suffering on animals and on how these
conditions can be assessed in nonhuman animals. Evaluation
of emotions in animals is fraught with subjectivity; indeed,
many Western scientists refused until quite recently to even
speculate that animals had emotions at all because it was not
possible to properly prove their existence. Griffin’s (2001)
efforts over many years have helped but the problem of eval-
uating animal emotions is a huge one still.

The Signifi cance of Pain versus Nociception

Pain is valuable from an ecological perspective as it allows
an organism to be aware of danger and to avoid situations in
which damage might occur or recur. Yet it is not a simple sensory modality. Even in humans, who can describe their physical sensations, it is not easy to understand (see Matlin and Foley 1997 for a textbook description). Merskey’s (1986) definition of pain as “information about actual or potential tissue damage, or interpreted in terms of such damage” conveys the variability of the sensation.

The experience of humans shows that receptor signals of damage are not automatically processed and passed undistorted to the central nervous system and brain. There are many examples of rituals, sports, and wartime experiences in which humans are not aware of painful major tissue damage for minutes or even hours (Matlin and Foley 1997); clinically diagnosed pain disorder (American Psychiatric Association 2000) is not tightly associated with actual damage, only triggered or even unaccompanied by actual damage; and phantom pain, the sensation from a lost limb, is the result of a central representation that endures after the periphery is no longer sending signals. Because of all these variations in pain, it is obvious that it has sensory, emotional, and cognitive aspects in humans, and these make it difficult to identify in nonhuman animals, whose ways of communicating discomfort are often less well understood by humans.

One way to separate these different aspects of pain experience is to define and evaluate nociception, the purely sensory experience of the damage signal (Kavaliers 1988; also see Elwood 2011, in this issue). It is too simple to suggest that nonhuman animals have only nociception, especially if they exhibit learning and expectation of future stimuli—and many invertebrates do demonstrate at least simple learning.

**Evidence of Pain in Vertebrates (Fish)**

Braithwaite’s (2010) study of whether fish feel pain shows how such an investigation could be carried out for invertebrates. She notes that fish, as vertebrates, should have structural and brain similarities with mammals that experience pain, and her assessment of the anatomy of receptor systems similar to those of other vertebrates does show a clear parallel. She looks at data about brain regions and finds that, although the specific brain arrangement is different among the vertebrate classes, similarly functioning regions are present (this is harder to show in invertebrates). She then measures increases in response to a chemical cue in the effluent of their prey and octopuses, which learn to avoid the crabs as prey (Maclean 1983). One study showed that chemical stimuli in water that contained an octopus stimulate hermit crabs to put anemones on their shells (Ross and von Boletzky 1979), presumably to repel predators. However, there is no evidence that the hermit crab learned this behavior.

The physiology of stress responses is clearly similar across many phyla. Stefano and colleagues (2002) point out that the immediate rise in immunocytes and a later increase in opiates in mussels and leeches subject to cold water shock are not only normal stress responses but the same as found in humans after coronary artery bypass surgery. These animals also show adrenocorticotropic hormone (ACTH) downregulation of immunocyte activation, again similar to that of mammals. Even more interesting is the heart rate increase of juvenile queen scallops (*Aequipecten opercularis*) under predation threat when on a substrate that offered no refuge (Kamenos et al. 2006). And the heart rate of mussels increases in response to a chemical cue in the effluent of their predator, the dog whelk (*Nucella lapillus*; Rovero et al. 1999). While these observations are scattered, they make it clear that the physiological systems are very similar across widely diverse vertebrate and invertebrate animals.

Elwood (2011) and his associates are the only researchers that have explicitly studied whether the nociception that an invertebrate shows to noxious stimuli could be extended to pain. The first study (Barr et al. 2007) was on prawns’ antennal grooming. Prawn antennae are crowded with tactile and chemical receptors and are a major area for evaluation of waterborne sensory stimuli. Application of chemicals or gentle pinching caused grooming of the specific antenna and

Given the positive answers for these studies, Braithwaite concludes that yes, fish do feel something like pain.

**Assessing Pain versus Nociception in Invertebrates**

This structural parallel between fish and other vertebrates makes it easier to prove pain in those species than in invertebrates, but an examination of nociception across different invertebrate phyla is a good starting point.

Cnidarian sea anemones, for example, have stinging cells that they use on each other and that are also painful to humans and repellent to animals of many species (Braithwaite 2010; Mather 2001). The anemones live in clones (groups of identical individuals), holding fast to the rocks of the seashore and catching drifting small animals and detritus. Clones that encounter each other have “wars,” stinging each other and inflicting considerable damage. They flinch from these attacks and eventually one clone retreats from the other and is the loser in the encounter. These animals have no centralized brain and only a nerve net, yet they clearly exhibit nociception in this situation.

The anemones’ weapons are also used by other animals, in situations that seem to demonstrate learning. Hermit crabs pull anemones off the substrate and place them on their borrowed gastropod shells where they repel both crabs and octopuses, which learn to avoid the crabs as prey (Maclean 1983). One study showed that chemical stimuli in water that contained an octopus stimulate hermit crabs to put anemones on their shells (Ross and von Boletzky 1979), presumably to repel predators. However, there is no evidence that the hermit crab learned this behavior.

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Elwood (2011) and his associates are the only researchers that have explicitly studied whether the nociception that an invertebrate shows to noxious stimuli could be extended to pain. The first study (Barr et al. 2007) was on prawns’ antennal grooming. Prawn antennae are crowded with tactile and chemical receptors and are a major area for evaluation of waterborne sensory stimuli. Application of chemicals or gentle pinching caused grooming of the specific antenna and
rubbing against the substrate, which was reduced by application of the local anesthetic benzocaine (which did not reduce general arousal, only antennal grooming). Because the grooming is a targeted and not a generalized response, the authors suggested that it parallels the responses of fish and other vertebrates in similar situations and can be considered evidence of pain.

E. wood (2011) argues that separating immediate nociceptive responses from longer-term cognitively guided pain responses will always be difficult because pain is such an imperative system that response is usually immediate. According to the utilitarian approach, the solution is simply to accept the importance of minimizing the effect (i.e., “costs”) of these stimuli on animals, whatever their perception of the stimulus might be.

The Rights-Based Approach

According to the rights-based approach both the agent and the receiver in any interaction have value and thus deserve consideration and respectful treatment (Regan 2003). This is the only one of the three viewpoints that focuses on the individual and accords animals the right to bodily integrity and physical liberty. The spotlight is thus on the experience of the animals themselves.

Nussbaum (2001) suggests taking into account an animal’s life, health, physical integrity, and emotional well-being. Such a perspective raises some difficult questions, such as whether it is defensible to exhibit animals (including invertebrates) in aquariums and zoos, thus depriving them of liberty. And one of my colleagues asked whether this approach requires consideration of the potentially conflicting welfare of a parasite or its host.

Furthermore, it is necessary to understand the basic physiology of the animal itself, whereas such knowledge is missing for most invertebrates. How can humans protect the right of the clam, the luna moth (Actias luna), or the neid worm without understanding how the animal lives? Davis and colleagues (1999) make this point about the ascidians, a primitive chordate that is common but whose ecology is very poorly known: we might like or need to protect them if we knew how.

The rights-based approach demands close study of the experiences and awareness of animals and evaluation of the situations to which humans subject them. Does the animal have the learning capacity to recognize and respond to a stimulus that signals an event? Does it have the self-awareness to know how trouble will affect it and the mobility to avoid trouble? The animal’s capacity to learn is important; Bekoff (1994) points out that suffering might be less bearable without cognition, which remembers the past and plans for the future, only dealing more effectively with an unpleasant present. Many people say that invertebrates do not have consciousness or self-awareness, so it is important to examine their capacity in this area to evaluate what humans’ treatment of them might mean.

According to Broom (2007, 99), a sentient animal is “one that has some ability to evaluate the actions of others in relation to itself and third parties, to remember some of its own actions and their consequences, to assess risk, to have some feelings, and to have some degree of awareness.” This account of cognition and awareness certainly applies to cephalopod molluscs, which studies have shown are heavily dependent on learning (Alves et al. 2008; Wells 1978) and may have consciousness (Mather 2008). The following sections therefore focus largely, but not exclusively, on these invertebrate species.

Self-Referencing, Self-Awareness, and Self-Consciousness

Bekoff and Sherman (2004) propose three levels of understanding of self: self-referencing, self-awareness, and self-consciousness. They believe that an animal’s fit in these categories is dictated more by its behavioral ecology (e.g., whether it is social) than its brain size (within limits) or phylogenetic derivation. And presumably the animal’s place in these categories should dictate the treatment it receives from humans. For instance, a Korean-style restaurant in New York City cooks mixed shellfish and octopuses alive in a frying pan at the table. Do some of these animals or none of them deserve this treatment?

Self-Referencing

Self-referencing is the matching of a target individual to oneself and does not usually involve learning or cognition. Courtship of hetero- or homosexual individuals entails the identification of species, sex, and readiness to reproduce before mating actually takes place. Dual-sexed hermaphrodites may compete with one another to see which will be which sex in the reproductive act (Anthes 2010). Simple awareness of self and of the identity of the target animal is necessary.

Self-Awareness

Self-awareness involves recognition that one is a self and that conspecifics are others, as well as a sense of one’s place in the world or possessions (e.g., shelter, territory). The animal may demonstrate learning and cognition, and the factors that influence its “decisions” may be simple or complex. Hermit crabs, for example, fight one another over the possession of shells in which to hide their vulnerable abdomen; they evaluate new shells for different durations and fight others with different intensities in different circumstances, including whether the contended shell is of a more desirable species (E1wood 1995). There may be evidence that they

1Although some marine animals do not even do this—sessile species such as coral and bivalves use broadcast fertilization, simply releasing gametes into the water.
assess both their ability in relation to a rival and the “value” of the shell possession to calculate whether a fight should proceed (Elwood 2011).

Further demonstrating self-awareness, cephalopods are excellent navigators for short distances (Alves et al. 2008), and some of their ability to locate themselves in the environment is learned. Field studies of octopuses (Mather 1991a) showed they forage freely across the ocean bottom, returning to a sheltering “home” from a distance and over a time that necessitates spatial memory. They can return home by detours even after they have been displaced from their foraging path (Mather 1991b). They also remember which areas they have been foraging in over the last few days and do not repeat searches in these locations where no prey is likely to be found.

Interestingly, cephalopods’ “decision” of whether to consume a prey species in hiding near the capture location or whether to take it home to consume it is based on the distance to the home (Mather 1991a), clearly an indication of the awareness of oneself and one’s relative location. This ability has also been proven in the laboratory for octopuses and cuttlefish, and is an interesting parallel with the spatial ability of bees and mammals (Shettleworth 2010), suggesting parallel competence across several phyla.

**Self-Consciousness**

What of self-consciousness, which likely involves cognition? The mirror test has become the critical evaluation of this capacity in primates (Gallup et al. 2002) and lately has been used on other vertebrates, although not without controversy (Moses 1994). A simple version of the test is to expose the animal to its image in a mirror and evaluate reaction. A more stringent test is to make an innocuous mark on a non-visible area of the animal’s body (e.g., its head), then to expose the animal to a mirror image of the area and see if it touches the mark.

Cephalopods have the best potential of self-consciousness of all the invertebrates, as they exhibit exploration and play, personalities and problem solving (Mather 2008). Octopuses exposed to mirrors show alerting and approach behavior, no different from their reactions to a view of another octopus (Mather and Anderson, submitted).

Two problems have been cited concerning the exposure of animals such as octopuses and cuttlefish to mirrors, assuming their failure at the task (Bekoff and Sherman 2004; Mather and Anderson, in press). First, although octopuses have excellent visual acuity, they may not depend on that sense in the same way that mammals do. Second, they are generally asocial and so may not have complex behavioral responses to either the image or the actual presence of another octopus.

**Deception**

A nother way to evaluate invertebrate animals for self-awareness is to look at whether they show deception. A gain the ability comes in three levels, with the simplest and most automatic being permanent deception (e.g., visual or other sensory camouflage); many invertebrates have a deceptive appearance—for example, the lemon yellow dorid nudibranch gastropod (*Tochuna tetraqueta*) is a perfect match for the yellow sponges on which it lives and feeds. Similarly, kelp crabs (*Puguitia producta*) are excellent matches for the algae on which they live, and several species of shrimp mimic their gorgonian resting places exactly. Some insects have exquisite matching to aspects of the environment such as leaves and sticks, and moths mimic the bark of trees on which they hide. But these deceptive appearances are permanent and selection is the machinery that determines them; no learning or cognition is present.

A second level is deception based on time and place. Cephalopods with their changeable skin show such ability with ease and often with a wide repertoire of displays for use in different situations. One example of such deception is the deimatic dots on the dorsal surface of cuttlefish (Langridge et al. 2007) and squid (Mather 2010). These dots appear on the skin surface in the presence of a low-level threat, as when a potential but not imperatively dangerous predator approaches. The cuttlefish shows two dots and the squid two of four on their large dorsal surfaces, presumably mimicking the eyes of a larger animal, as the dorsal surface is often turned toward the approaching fish. Indeed, there is a significant correlation of appearance of lateral dots with the direction of the approaching fish, as the animals exhibit dots on the part of their body that can be most easily seen by the potential predator. Squid do not direct such warning displays toward conspecifics but toward a chosen target (Mather 2010), thus they seem not to be automatic but chosen.

True deception occurs when animals give misinformation about resource-holding power or their ability to win contests. Caribbean reef squid (*Sepioteuthis sepioidea*) have “honest” formalized zebra display contests, in which the intensity of the display is greater on the squid taking a position above, and that individual is the one that claims resources in terms of consortships with a nearby females (Mather 2004). There is no deception, as the animals trade places if the display of the lower animal is more intense. Juvenile squid, however, respond to the mating displays of an adult pair by engaging the male with a high-intensity zebra that does not accurately represent its ability to win a fight. Such a deceptive display intensity might mean that the juvenile later was able to mate with the female.

Similarly, male cuttlefish have alternate morphs (skin displays) when they are courting a female (Hall and Hanlon 2002). One of these displays signals dominance, and the male sets up a consortship and later mates with the female. A n alternate deceptive strategy is for a male to adopt the same display as female cuttlefish and become a “sneaker,” changing its body pattern to then court and mate with a female when her consort is temporarily away. Unfortunately, Hall and Hanlon (2002) did not continue to watch sneakers over time, so they did not observe whether tactics differed from one individual to the next or changed with maturity (for
a discussion of alternate reproductive strategies, see Taborsky and Brockmann 2010). In reef squid, males adopt peripheral positions and sneaking strategies as subadults, then join visual display contests and hold consortships as they grow to maturity (Mather, unpublished observations).

A striking example of deception over time is the mantis shrimp fighting technique (Caldwell 1986). Individuals occupy and often successfully defend their burrows in the substrate. The meral claw strike is dangerous, so shrimp seldom actually fight but favor visual displays to indicate their size and ability to hold the burrow. When females lay eggs or when any shrimp molts, their resource holding potential is considerably reduced and fights often result in displacement from a burrow. Weakened individuals instead use a meral claw spread to indicate size and resource holding potential. They also initiate fights and are more aggressive in other ways just before they molt, enhancing their “reputation” just before it could most easily be challenged.

Scientific Evidence in Support of the Rights-Based Approach

Given this evidence that many invertebrates may have simple sentience sensu Broom (2007), and that the rights theorists believe that these animals have the right to a full, rich life, how should humans behave morally when interacting with them? It is helpful to look beyond simple evaluations and tap into the life history of the animal to see to what extent its natural behavior and needs are fulfilled in captivity.

Moltschaniwskyj and colleagues (2007) offer wide-ranging guidance for appropriate care of a variety of cephalopod species. They also urge use of the 3Rs (reduce numbers of animals, refine procedures, and replace animals with alternatives) for invertebrate subjects in experimentation. This advice is important because there is a trend to replace vertebrate animals in experimental work with invertebrates; but this reduces animal welfare concerns only if people believe invertebrates are not aware of the consequences of many human actions.

For example, crabs caught in commercial pot traps are declawed and then returned to the ocean (Patterson et al. 2007, 2009), a practice that seems justified by the fact that crabs sometimes autotomize a claw when threatened by a predator; declawing is thus seen as “natural” and not detrimental to the crab. The opposite turns out to be true: the effects are extensive and change several important aspects of the crabs’ lives. Studies show that claw removal is a significant stressor (Patterson et al. 2007), raising the level of glucose, lactate, and glycogen in the hemolymph much higher than baseline both in handled crabs and in crabs induced to autotomize. Furthermore, glucose and lactate were higher and glycogen lower when crabs were placed with conspecifics after claw removal. Crabs are aggressive with one another and the crab with one or more claws missing, whether by autotomy or removal, is at a significant disadvantage to compete for and hold high-quality territories.

Not only do crabs without a chela move down in the hierarchy, they are at a disadvantage in feeding. Crabs with only one claw had significant difficulty consuming mussels, their common prey (normally the crab holds the mussel with one chela and crushes it with the other). In captivity they consumed a lot of alternative food (pieces of fish), but this option is likely not available in the wild. Some of the declawed animals even died from hemolymph loss and others would have starved or lost significant amounts of weight.

Thus an intervention that looks like a harmless mimic of a natural situation is instead devastating to all aspects of the crab’s life. Patterson and colleagues (2009) therefore strongly recommend that this method be stopped.

The Importance of Supporting Species-Specific Normal Behavior

For rights theorists, even benign captivity can deprive an animal of its rights, and acting morally means ensuring all animals a full and complete life, including the rights to appropriate housing, stimulation, reproduction, and feeding opportunities (see Broom 2001 for an extensive discussion of these topics mostly in mammals).

The need to carefully choose appropriate species for captivity was recently highlighted by Mason (2010; although she ignored the invertebrates, as pointed out by Carere et al., in press). A gain a good example is the cephalopods, for which Moltschaniwskyj and colleagues (2007) note clear limits in knowledge of their biology. They point out that ommastrephid squid suffer 100% mortality in rearing and are therefore not suitable for captivity.

Octopuses, for example, explore and learn well, play, have personalities, and solve problems (Mather 2008). Is it right to keep these intelligent animals in a barren, restrictive aquarium tank? Studies clearly show that enrichment makes a difference to their biology: captive young cuttlefish in an enriched environment grew faster and ended up larger than those in a barren one (Dickel et al. 2000).2 Enrichment for these (and many other) animals helps to maintain healthy activity levels, alleviate the effects of confinement, and enable animals to pursue species-specific normal activities.

A particular risk for intelligent and social animals is boredom (Wemelsfelder 1993). Common symptoms of boredom are repetitive route retracing, constant attempts to break out of confinement, and abnormal sleep and resting patterns. Octopuses in captivity are well known for their ability to escape from the confinement of their tank, taking advantage of the manipulative ability of their arms. A aquarium owners use a wide variety of techniques to confine them, not always successfully. The first recorded event was in the Brighton Aquarium over 100 years ago, when the captive octopus visited a neighboring tank and dined on a lumpfish each night for several nights before returning to its home. But bored octopuses

2Wells (1978), however, has argued that octopuses are used to hiding in a protective home and moving out for food, and that their solitary lifestyle makes them preadapted to thrive in captivity.
can also be destructive; Anderson (2005) describes an octopus that attacked her tank by repeatedly moving rocks around and scratching the glass, blowing gravel up from the bottom, and finally biting through the ties holding the undergravel filter in place, pulling it up, and tearing it into pieces, which were found floating at the water surface the next morning.

One way to provide enrichment for octopuses in an aquarium is to provide substrate that is comparable to the animal’s natural environment, with rocks to make a sheltering home and gravel (cuttlefish bury in gravel and should never be deprived of it; Mather 1986). Enrichment for octopuses also includes the provision of novel objects for manipulation and play (Anderson and Wood 2001). Octopuses will take apart any complex many-pieces object, and the children’s Mr. Potato Head toy is a favorite at several aquaria.

If appropriate and at all possible, enrichment should also provide the animal for release into its natural environment; for instance, hatchery-reared salmon are notoriously unaware of predators and are easily picked off (Brown and Day 2002). Cephalopods should receive live prey so that they can exercise their natural predatory response.

Anderson (2000), for example, describes the release of the giant Pacific octopus (Enteroctopus dofleini) Ursula, who had come to the Seattle Aquarium by donation when she was very small. She was growing too large for her aquarium tank, so her keepers fed her live crabs for several days before her release to give her the opportunity to catch appropriate prey. When she was released (to a huge blast of publicity), divers followed her progress down to the seafloor below the waterfront aquarium. The divers checked on her for the next 40 days and noted the remains of three species of local crabs piling up in front of her den, so she was hunting successfully. When she was last seen, three males were also observed near her, so it is likely that she also fulfilled the rights criterion of being able to reproduce.

Thus it is clearly possible with captive octopuses to accommodate the special needs of an intelligent invertebrate.

Conclusion

Although theorists have not necessarily thought specifically of invertebrates in postulating humans’ attitudes toward animals, human attitudes are important to the welfare of these (and all) animals. Contractarian theorists value our human-ness in caring about animals, utilitarians consider the importance of the 99% of animals that invertebrates represent, and rights theorists’ concentration on animals’ essential needs is useful for enriching the everyday lives of invertebrates.

Education is key (Meheen 1995): as invertebrates are better understood, people—whatever their value system—will come to appreciate and take better care of them.

Acknowledgments

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Abstract

With billions of individuals and possibly hundreds of thousands of genera, invertebrates represent the largest number and greatest diversity of all animals used in research. Although the capacity for nociception is recognized in many invertebrate taxa, researchers and IACUC members are challenged by a lack of clear understanding of invertebrate welfare and by differing standards of moral concern for these taxa. In practice this has led IACUCs to consider invertebrates in two major groups: species worthy of increased moral concern approximating that shown to vertebrate species (this group includes cephalopods and to some extent decapod crustaceans) and all others. This dichotomy has led to differences in how invertebrate research is regulated and documented. This article presents two case studies illustrating specific concerns in invertebrate research protocols and then provides relevant information to address practical IACUC matters related to regulatory and ethical issues, sourcing and record keeping, risk management, assessment of pain and nociception in invertebrates, housing and husbandry, invasive procedures, veterinary care, and humane endpoints.

Introduction

A fundamental concern of institutional animal care and use committee (IACUC) members is the welfare of all animals used in research regardless of their phylogenetic position. The increase in knowledge of functional and comparative genomics has revealed extensive genetic homology between humans and other species and underscores the fact that although there are great differences, there are also fundamental similarities in all eukaryotes. Yet with invertebrates humans seem to be more aware of the differences than the similarities, notwithstanding the vast numbers of organisms and variety of species that populate every corner of the planet, accounting for over 90% of animal biodiversity.

A Passion for Invertebrates?

The Creator would appear as endowed with a passion for stars, on the one hand, and for beetles on the other.

- JBS Haldane (1949)

The sheer number of invertebrate species is what impressed the biologist Haldane. Within one order (Coleoptera) of the phylum Arthropoda there are over 300,000 species, and the total numbers of arthropod taxa—in excess of 750,000 species—outnumber all other animals on the planet threefold.

Invertebrate species range from comparatively simple single-cell life forms (e.g., protozoa) to colonial aggregations of cell types (e.g., sponges and coelenterates) to complex animals that share morphological and physiological convergence with vertebrates (e.g., cephalopods and crustaceans).

Some of the longest-lived colonial and noncolonial animals on the planet are invertebrates: the common red sea urchin (Strongylocentrotus franciscanus) may live 200 years (Ebert and Southton 2003); it has been estimated that vestimentiferan worms found near deep-sea hydrothermal vents live up to 250 years, and some corals and sponges may be thousands of years old (Bergquist et al. 2000).

Invertebrates in Research

Invertebrates are used both in a wide range of field research on biodiversity and conservation and in the laboratory as animal models for a variety of science questions. Uses range from acute toxicity assays in aquatic invertebrates such as Hydra and Daphnia to invasive neurophysiology in the sea hare Aplysia. Wilson-Sanders (2011) describes a large number of well-defined invertebrate models using fruit flies and the nematode C. elegans in diverse research areas such as drug screening, cell death, aging, retrovirus biology, memory, muscular dystrophy, Parkinson’s disease, wound healing, aging, amyloidosis, programmed cell death, diabetes, and immunology.

The diversity of invertebrates requires particular IACUC care in dealing with research ethics issues. A central challenge is the lack of scientific consensus on what constitutes pain and suffering in these species and whether they are applicable to even "advanced" invertebrate species. Elwood (2011) discusses efforts and methods to distinguish between pain and nociception in invertebrates, and Crook and Walters (2011) elaborate on the nociceptive behavior and physiology of molluscs; but
scientific evidence of pain and suffering in invertebrates remains poorly researched and controversial. This subject is discussed in greater detail in the section below on the Challenge of Assessing Pain and Nociception in Invertebrates.

IACUC Invertebrate Protocol Case Studies

The following case studies illustrate the challenges that IACUC members confront in their review of protocols for research involving invertebrates. These cases raised questions about proper scientific procedure, animal welfare, containment, handling and human safety, and attitudes toward invertebrates. Both cases underscore the importance of thoughtful and informed IACUC review.

Case Study 1

The Protocol

An IACUC received a protocol from a newly recruited principal investigator (PI) working with orb-weaving spiders. The PI proposed to conduct single-cell electrode recordings from the giant ganglia in the spider’s leg, which he proposed to pull off without the use of anesthesia. One or two legs would be sufficient for each day’s recording sessions, and each spider would yield a total of eight legs over the course of 1 to 2 weeks. The PI asserted that this was an accepted and widespread practice among neurophysiologists working with this model. He also indicated that stepping on the spiders was the preferred method of physical euthanasia.

IACUC Concerns and Resolution

The IACUC was sufficiently concerned about issues of both human safety and animal welfare that they requested a meeting with the investigator, a neurophysiologist, to discuss the protocol.

A s a justification for the technique proposed the PI made the point that this species of spider has a natural detachment line at the base of each leg for separation of limbs by autotomy and does so to avoid predation in the wild. He also stated that a number of investigators worldwide use this technique and that it is accepted practice in his discipline. When asked how many legs could be harvested, he answered “all the legs.” When asked if the spider would be able to self-feed with fewer than three or four legs, he was unsure. When asked if he did indeed step on the spiders to euthanize them he indicated that he had been facetious and that they were generally killed with ether in a bell jar.

The committee consulted with the institutional veterinarian who cited evidence in the veterinary literature that arachnids could be anesthetized with veterinary gas. The PI, while concerned that this could result in the accidental death of valuable animals before their full use for research purposes, agreed to work with the veterinarian to refine the model, use gas anesthesia for the removal of legs, and restrict the number of legs removed after recovery to four, after which the last leg removal would be a terminal procedure. The PI agreed to use gas anesthetic overdose for euthanasia (the use of ether was prohibited because of the risk of explosion).

The IACUC included a member from the department of occupational health and safety who asked the PI about safety measures for the handling and use of venomous spiders. The PI indicated that there was no specific antidote for the venom of the spiders and that their venom was not fatal in humans but analogous to a wasp sting. The room the animals were held in had not only an insect escape-proof anteroom and doorway for secondary containment but also a primary screen-sealed holding container for each spider. The room was locked and secure at all times and labeled that it contained venomous animals. The animals were physically handled by the PI wearing gloves and using a set of long forceps.

Finally, the PI was informed that the protocol form was a legal record and that facetiousness was inappropriate, and he agreed to modify the form before approval to reflect best practices as discussed.

Case Study 2

The Protocol

The IACUC was approached by a PI who had been contracted by a company involved in the transportation of live lobsters. The purpose of the protocol was to determine the critical maximum and minimum temperatures that caused mortality in lobsters during transport. The proposal was to raise or lower the water temperature by 5°C per hour until the lobsters stopped moving, which was the proposed endpoint.

IACUC Concerns and Resolution

The committee was concerned about the project, which was industrially driven and lacked any form of academic review (although it was fully funded), and requested a peer review of the protocol. The IACUC members met with the PI and discussed the endpoints. It was agreed that a more precise endpoint would be the point at which the lobster stopped responding to a gentle pinch to the antennae and/or failed to right itself when inverted. The temperature change regime would be fixed at 2°C per hour. The IACUC included a member from the department of occupational health and safety who asked the PI about safety measures. The PI proposed to conduct single-cell electrode recordings from the giant ganglia in the spider’s leg, which he proposed to pull off without the use of anesthesia. One or two legs would be sufficient for each day’s recording sessions, and each spider would yield a total of eight legs over the course of 1 to 2 weeks. The PI asserted that this was an accepted and widespread practice among neurophysiologists working with this model. He also indicated that stepping on the spiders was the preferred method of physical euthanasia.

Attitudes and Their Impact on Regulations and Review

As Mather (2011) eloquently explains, how animals are used in research depends not only on the regulatory and ethical
environment but also on attitudes about which species are considered worthy of moral concern. It is clear from a survey of the literature that in practice invertebrates tend to be ranked at the lowest levels of moral concern for living animals; indeed, some institutions require no formal review of their use for research, although funding approval generally entails at least a scientific merit review and institutional approval (primarily vested in the IACUC) is usually linked to the release of research funds.

 Among IACUCs, many members adopt a binary approach to invertebrate welfare, classifying the animals as "advanced" invertebrates (i.e., cephalopods and occasionally decapod crustaceans) and all other species. Boyle (1991, 7) explained the basis for the special status conferred on some cephalopod species, in particular octopuses:

In the laboratory, numerous experimental studies have described remarkable capabilities of sensory discrimination, especially of visual stimuli; they have demonstrated that true learning occurs and is likely to be an integral part of the normal life of an octopus. These qualities of behavioral complexity, sensory discrimination and learning in cephalopods bear comparison with those of many lower vertebrates and provide ample cause for considering their welfare in the laboratory for humane and scientific reasons.

However, in the absence of evidence to the contrary, such a distinction is not appropriate and all invertebrates should receive care and treatment that ensure their welfare.

International Regulations and Guidelines

At the regulatory level, fish and invertebrates are excluded from the US Animal Welfare Act, for example, although Public Health Service (PHS)-approved research protocols require ethical justification for all animal use (PHS 2000). Thus whether invertebrates meet the legal definition of an animal under this policy is unclear. In Canada, Canadian Council on Animal Care guidelines for the use of invertebrates are more specific in articulating ethical concern for, and IACUC oversight of, research using advanced invertebrates such as cephalopods (CCAC 1991):

> Protocols must be submitted to an appropriate review committee for all studies and courses which involve the use of vertebrates and some invertebrates in Categories B through E. Cephalopods and some other higher invertebrates have nervous systems as well developed as in some vertebrates, and may therefore warrant inclusion in Category B, C, D, or E.

The CCAC guidelines, which have been translated and used as the basis for animal care practices in a number of countries, do not specify which "other higher invertebrates" are indicated based on nervous system complexity, but in practice some IACUCs extend this guideline to species of arthropods, notably decapod crustaceans. Otherwise, the CCAC categorizes experiments using "most invertebrates" at the lowest invasiveness level (Category A), similar to studies that involve the use of tissues collected after slaughter of domestic animals.

In the United Kingdom, the Cruelty to Animals Act 1876 specifically excludes invertebrates. On the other hand, the Council of Europe issued a "Charter on Invertebrates" that recognizes the "compelling positive values of invertebrates including their use in science and medicine" (New 1995, 16).

A further example of the relatively limited moral concern for invertebrate use at the undergraduate level, Youth Science Fair Canada (YSF 2010) regulations governing students' use of animals in science fairs permit the unrestricted use of bacteria, fungi, protozoa, insects, plants, and invertebrate animals, but are far more restrictive where vertebrate animals are concerned, prohibiting their use in any fashion that may be "deleterious to the animals."

IACUC Review

Many institutions have developed a policy of reviewing all protocols involving invertebrate use, whereas other IACUCs may refuse to review invertebrate protocols in jurisdictions where invertebrates do not meet the legal definition of "animal." When IACUC review of invertebrate research is not strictly required legally, some investigators object to it as an unnecessary regulatory burden. Review is nonetheless appropriate as part of the due diligence of research oversight and principles of parsimonious, ethical animal use. One institution, San Jose State University, has a voluntary invertebrate protocol review policy that embraces this concept:

For the use of invertebrate species, the IACUC requires review and approval of projects that entail permission from a government agency to access, collect, or deploy the species being studied.... The provision for IACUC approval of invertebrate studies also extends to work involving animal species considered venomous or a threat to public health, endangered, threatened or of special concern [Endangered Species Act, 1972] and for projects involving invertebrate species in which the pain and distress category is considered a category level V (as defined by the IACUC for vertebrate species...). All other invertebrate studies do not require IACUC review and approval. However, it is highly recommended... that investigators pursue committee approval for graduate or research projects to be kept on file with the University Animal Care office.

2Category A covers "experiments on most invertebrates or on live isolates," including, for example, "the use of tissue culture and tissues obtained at necropsy or from the slaughterhouse; the use of eggs, protozoa or other single-celled organisms; experiments involving containment, incision or other invasive procedures on metazoa" (from the CCAC website, http://ccac.ca/en_/standards/policies/policy-categories_of_invasiveness, accessed on April 4, 2011).

2Available online (www.sjsu.edu/gradstudies/iacuc), accessed on April 4, 2011.
The value of a conscientious IACUC in oversight of the use of invertebrates in research cannot be overstated, reflecting the leadership and overall institutional conscience concerning the use of all animals.

Special Considerations for the IACUC

Permits, Collection, and Transfers

Many species of invertebrates used in research may be exotic to the region where they are being used, where they may represent a threat to biodiversity or a direct disease or pest threat not only to other laboratory animals but also to agriculture and aquaculture, so their housing and containment may be subject to local, regional, and federal regulation.

In the United States fish and wildlife regulatory authorities in each state typically enforce requirements for collection permits and annual reports of animals collected from the wild. In coastal regions of Canada, the use of laboratory-reared cuttlefish other than in complete containment systems is subject to licensing by the federal Department of Fisheries and Oceans (DFO) and permits are issued under the local DFO Introductions and Transfers Committee. This committee delegates the responsibility to local DFO aquaculture authorities and veterinarians to inspect and regulate the proposed holding facility for the nonendemic species; such inspection may entail visits, effluent water containment, continuous effluent water decontamination and testing, and testing of the introduced species for the presence of unwanted or reportable disease entities.

For at-risk, threatened, or endangered invertebrates or those listed by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) there may be additional layers of federal or state/provincial permitting required.

Sourcing Invertebrates

The sources, transportation methods, procedures for quarantine, and disease monitoring methods for invertebrates are as diverse as the animals themselves. Some species are available from mainstream laboratory or biological supply sources, together with turnkey life support holding systems, special diets, and good information sources for standards maintenance.

For some species, laboratory-reared pathogen-free specimens are available from a well-defined source (e.g., the National Resource Center for Aplysia; http://aplysia.miami.edu/) analogous to the commercial suppliers of research rodents in terms of microbial and genetic integrity. Other species may be available only from the wild, pet stores, or specialized hobbyists, or they may be obtained in ways that are on the margins of the comfort zone for the average IACUC member. In these instances communication between the PI, the IACUC members, and the animal care and veterinary personnel is particularly important.

Record Keeping

Many institutions require little record keeping for the research use of invertebrates other than cephalopods. IACUC forms for invertebrate use are often abbreviated and contain minimal information, limited to the name of the PI, the species used, the end use of the animals in teaching or research, brief descriptions of the techniques used in the study, disposition of the animals at the end of the study, and safety issues if isotopes or hazardous chemicals are involved.

In Canada, Invertebrate Protocol Forms for the majority of invertebrate species (other than cephalopods) may simply report the species used, numbers of animals used, and the grant number of the PI. Furthermore, institutions are not obligated to keep records of the numbers of invertebrates used, although they are not discouraged from developing animal use practices and culture for the use of invertebrates, such as record keeping, standard operating procedures (SOPs) for the care and maintenance of the animals, and IACUC review of animal care protocols. For cephalopods, the categorization of protocols and their ethical review parallel those for the use of vertebrates, with requirements for anesthesia, reasonable aseptic conditions for surgery, postoperative monitoring, and endpoint definition.

Numbers of Invertebrates Used

A part from cephalopods, the numbers of invertebrates used for research are seldom tracked, at least in part because many of the species studied (Drosophila, C. elegans, Artemia) may teem in uncountable millions in a single research laboratory, let alone in an institution, region, or country, and accurate accounting for such numbers is not feasible. When the research involves advanced invertebrates such as cephalopods or rare and endangered invertebrates of greater economic or ecological value, there may be more proactive efforts to track the numbers of animals used for purposes of accounting to regulatory agencies.³

Risk Management: Toxic and Injurious Species, Field Work

Invertebrates can be a hazard to people and the environment, capable of escape, harmful environmental impacts if introduced as pest species, and injury to humans through physical or chemical means (e.g., the venomous bite of the blue-ringed octopus, the sting of a lion’s mane jellyfish, the venom of certain spiders used in neurophysiology). The IACUC needs to be satisfied that the PI and holding facility can maintain hazardous invertebrates in safe conditions for animals and humans alike (more on this under Housing and Husbandry below).

³However, a review of the CCAC figures for animal use in 2008 showed that, of 2,272,815 animals used, just 7 were cephalopods; it is likely that this figure represents underreporting.
Risks are inherent in some types of field and laboratory work, for example in scuba diving for specimen collection or census purposes and in the handling of venomous or injurious species. Such work requires primary and secondary containment, escape mitigation plans, emergency supplies and training, and special training for first responders and other individuals not directly involved with the project who may need to access the holding area in an emergency. In all cases it is important to enumerate such risks and to mitigate them by taking precautions at the institutional and local levels to ensure that (1) SOPs, equipment, and animal holding/housing are in place and approved for the safe handling of the species in question, (2) antivenin and other emergency treatment supplies are on hand, (3) medical personnel are identified and familiar with the risks and treatment, and (4) records are maintained through the institutional occupational health and safety program.

The Challenge of Assessing Pain and Nociception in Invertebrates

Because IACUCs represent the ethical conscience of their institutions and on a wider scale reflect the morality of the culture and community where they exist, it is essential that their members understand and address issues of pain and suffering in invertebrates when making decisions about their research use (see Cooper 2011; Elwood 2011). Such understanding begins with a grasp of the distinction between pain and nociception in animals.

The International Association for the Study of Pain defines pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage” (Merskey and Bogduk 1994). The cognitive-emotional component of pain, which requires higher brain center function through the limbic system in vertebrates, is considered its important aspect, not the activation of pain sensors (nociceptors). “A cility induced in the nociceptive pathways by a noxious stimulus is not pain, which is always a psychological state, even though... pain most often has a proximate physical cause” (Merskey and Bogduk 1994). Under this definition, species capable of feeling pain are those that have not only the nociceptors but also the brain neuroanatomical and neurophysiological features and behaviors that allow the expression of fear, anxiety, distress, and terror, akin to human and other advanced vertebrate responses to noxious stimuli.

Many species of invertebrates, including annelids, nematodes, molluscs, and insects, have the capacity for nociception and withdraw from damaging stimuli (St. John Smith and Lewin 2009), and in some species there are putative connections between nociceptors and brain learning centers (Puri and Faulkes 2010; Sandeman et al. 1992). In molluscs the central nervous system is not required for pain withdrawal reflexes, which can be mediated by the peripheral nervous system (Crook and Walters 2011). In leeches, specialized sensory nociception cells induce bending away from damaging stimuli, and the bending reflex can function with as few as 10 abdominal ganglial cells (Sahley et al. 1994). In arthropods, recent work suggests that nociceptive fibers, like all other sensory neurons, can be inhibited by a variety of neuromodulators (Pfeiffer and French 2009). And studies in Aplysia provide evidence that nociception and sensitization can lead to long-term influences on memory and behavior in invertebrates (Woolf and Walters 1991).

However, the presence or modulation of nociception does not conclusively demonstrate that invertebrates are capable of experiencing the mental/emotional state of pain that is acknowledged in many vertebrate species. Imaging studies in mammals expressing painlike behaviors in response to noxious stimuli have demonstrated activation of brain areas (e.g., the limbic system) associated with emotion (Maliszewski et al. 2003). This type of cause and effect evidence is lacking in invertebrates, and the poorly understood nature of invertebrate nervous systems makes proof by analogy difficult.

Some insect neurobiologists have argued that the distributed nature of the invertebrate nervous system (except in cephalopods) precludes higher-order information processing, including the existence of any kind of mental state (Eisenmann et al. 1984). Invertebrates other than cephalopods also have much lower (by two to three orders of magnitude) numbers of neurons compared to vertebrates, a difference that could preclude the ability to experience mental states such as emotion and pain, and there is not much evidence in the scientific literature of an emotional state akin to “distress” in invertebrates such as insects.

Importantly, none of the evidence cited is conclusive in either confirming or refuting the possibility of pain and distress homologues in invertebrate species. Clearly this is fertile ground for study. In the absence of conclusive results, however, there is no evidence that the scientific community should ignore the welfare of invertebrates used as research animals.

Standards of Care

Given the exceptional diversity of invertebrates, housing and husbandry needs can range from accommodation for the very large (e.g., for Enteroctopus dofleinii at 40 kg) to the very small and even microscopic (e.g., for Drosophila or C. elegans). The IACUC, which in most institutions likely deals only occasionally with invertebrate protocols, needs to concern itself with the details of species-specific requirements and of the life support systems, containment measures for hazardous species, and experience and training of the personnel who will maintain the animals.

The myriad details of invertebrates’ basic care requirements, food, a healthy and enriched environment, and safe handling for both operators and animals is beyond the scope of this paper. Smith and colleagues (2011, in this issue) provide an overview of the maintenance of invertebrates; there is an abundance of literature on the care, maintenance, and housing of various invertebrate species, from the laboratory...
animal, zoo/aquarium, wildlife rehabilitation, and pet and hobbyist literature; and the Association of Zoos and Aquariaums (www.aza.org) has guidelines for the maintenance of many species of invertebrates in captivity.

In addition, both the IACUC members and the scientists using invertebrates usually benefit from mutual education, the former through laboratory visits and other methods to become familiar with the particular invertebrate models in use, and the PI through protocol writing workshops and other opportunities to interact with the IACUC and educate its members.

Housing and Husbandry

Housing and Containment

Housing design is important for both the provision of life support and, particularly in the case of insect pest and toxic/injurious species, containment to prevent escape (together with clear procedures for recapture in the event of an escape). In general species should be housed separately to prevent aggression or predation but there are polyculture and experimental exceptions to this rule.

Rooms may require higher than normal temperature and humidity, and lighting systems should be controllable for photoperiod. Many invertebrate species benefit from some form of environmental complexity such as branches or hollow pipes for hiding spaces. All surfaces (including those of items provided for environmental enrichment) should be sanitizable.

It may be necessary to ensure the availability of features to contain escaped individuals, such as an “air knife” at the door level to keep flying insects inside, or a bath or moat system to keep crawling insects inside containers. In some cases electrified “bug zappers” or nonlethal electric barriers are used to enhance containment and prevent escaped specimens from spreading through a facility. There should also be screens or other means to isolate the area and the HVAC systems from surrounding areas to prevent the appearance of unwanted guests in other areas of the building.

It is highly advisable to hold escape-prone and destructive pest species (e.g., dermestid beetles, which damage biological and book collections) and species that generate strong odors (e.g., Phanea carrion flies) in isolated buildings away from other activities, valuable collections, and personnel.

Water

Water sources for invertebrates, whether for drinking or life support, require careful attention as the addition of certain chemicals in city water supplies may be acutely or chronically toxic to some species. Marine species require high-quality seawater that has undergone the removal of particulates, metabolites, and pathogens (e.g., through UV or biofiltration) and other treatments such as ozonification. For all marine invertebrate species there should be an emergency backup for life support systems.

The importance of measures to maintain water quality cannot be overstated; in one case in the author’s experience zoonotic {\textit{Vibrio vulnificus}} was isolated in pure culture from the anterior chamber of cuttlefish {\textit{(Sepia officinalis)}} exhibiting bilateral hypopyon as a primary symptom. The source of the {\textit{Vibrio}} was intake seawater from a sewage-polluted source in a marine flow-through system.

The CCAC has extensive guidelines for maintaining marine species on its website (www.ccac.ca).

Food

Provision of foodstuffs for invertebrates could be the subject of an entire paper. General principles are similar to those for other species: food must be nutritionally balanced and fresh, and food that is contaminated (e.g., with mold) or that may have been sprayed with insecticides or other toxic materials should be strictly avoided. Food should not be permitted to undergo decomposition, although even here there are exceptions (for instance, autoclaved cow’s liver provided to blowflies for the production of fly larvae may be in the process of autolysis).

Contamination of invertebrate food may have unforeseen consequences. In one case contamination of fruit flies used as a protein source for captive rufous hummingbirds ({\textit{Selasphorus rufus}}) proved to be the means of transmission of epi- ornithic {\textit{Aspergillus}} infection in the birds (Harvey-Clark 1993).

Invasive Procedures, Veterinary Care, and Treatment

Surgery, anesthesia, and veterinary care for invertebrates are a relatively new subspecialty of zoo and exotic animal veterinary medicine, and may therefore need some translation both by PIs and by veterinarians serving on IACUCs to be fully understood and effectively evaluated. Collegial communication and consultation with persons versed in methods used in invertebrate research, and especially in invasive procedures, are important. Experience with the species in question often enables recognition of subtle signs of health status changes and earlier interventions, supporting better outcomes for both the research and the animal care and welfare.

Resources are available to IACUC members for information on the health management and veterinary care of invertebrates (Cooper 1980, 2004, 2011; Lewbart 2006; Smith et al. 2011). However, due to the lack of comprehensive information on many of these species, clinical assessment and treatment options may be limited and this may mean that euthanasia is exercised earlier and/or more frequently than in comparable studies with research animal species that are well characterized.
Humane Endpoints and Euthanasia

The American Veterinary Medical Association Guidelines for Euthanasia (AVMA 2007) fail to include techniques and ethics for invertebrate species. For invertebrates that show clear signs of morbidity, standardized guidelines and methods are needed to ensure timely intervention by skilled personnel. In many cases, euthanasia may be the first and most compassionate option.

In practice, as for mammals, when feasible the use of physical euthanasia methods should be preceded by a chemical method that obtunds the animal, unless the PI can provide compelling scientific arguments to the contrary. Various chemical and physical means are available to achieve rapid, humane euthanasia for invertebrate species and are reviewed elsewhere (Cooper 2004, 2011; Lewbart 2006).

After euthanasia the IACUC needs to ensure that animal remains are disposed of in an appropriate fashion (e.g., biowaste incineration), as components of some species may remain venomous after death, some invertebrates are vectors for infectious or zoonotic diseases, and others may harbor viable eggs or propagules that can cause invasive species infestations, damage to agriculture, or other unintended effects.

Conclusion

The wise man regulates his conduct by the theories both of religion and science. But he regards these theories not as statements of ultimate fact but as art-forms.

- JBS Haldane (1927)

The art of working with the immense diversity of invertebrate species—for which investigators and IACUC members alike do not always fully apprehend all aspects of care and biology—lies in the principles of humane parsimony.

In the absence of a definitive understanding of welfare implications in these species, scientists and IACUC members should strive to respect life, follow 3Rs principles (reduce, refine, replace), and minimize the trauma and severity of procedures when possible. Investigators can bring much to the table by educating the members of the IACUC in the biological and scientific aspects of invertebrate models, and the IACUC needs to work collegially with the PI to ensure the fulfillment of its role in ethical oversight and the thoughtful and responsible use of research animals.

Work with species that are not fully characterized as animal models will continue to be iterative and requires a flexible institutional culture that incorporates good scholarship, best practices, and use of the most current information. In all cases, invertebrates should not be regarded as “second-class citizens” by either the IACUC or investigator but should, as with all research animals, be subject to rigorous professional standards of animal care oversight.

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Chimpanzees in biomedical and behavioral research constitute a national resource that has been valuable in addressing national health needs. However, the expected level of use of the chimpanzee model in biomedical research did not materialize, creating a complex problem that threatens both the availability of chimpanzees and the infrastructure required to ensure their well-being. This report examines the issues and makes recommendations. ISBN 0-309-05891-0; 1997, 108 pages, 6 × 9, paperbound

Definition of Pain and Distress and Reporting Requirements for Laboratory Animals: Proceedings of the Workshop Held June 22, 2000
The goal of this ILAR/NIH joint workshop was to provide feedback from the scientific community to the USDA regarding the lack of a functional definition of “distress” as well as the efficacy of continuing to use current categories to report pain and distress. Speakers’ areas of expertise and perspectives ranged from scientific research to animal welfare policy, protocol review, and relevant organizations or institutions. ISBN 0-309-0698-6; 2000, 132 pages, 6 × 9, paperbound

The Development of Science-based Guidelines for Laboratory Animal Care: Proceedings of the November 2003 International Workshop
The purpose of this workshop was to bring together experts from around the world to assess the available scientific knowledge that can affect the current and pending guidelines for laboratory animal care. Workshop presentations and discussions focused on identifying gaps in the current knowledge to encourage future research endeavors; assessing potential financial and outcome costs of nonscientifically based regulations, facilities, and research; and determining possible negative impacts of arbitrary regulations on animal welfare. ISBN 0-309-09302-3; 2004, 264 pages, 6 × 9, paperbound

Education and Training in the Care and Use of Laboratory Animals
Federal law requires that institutions provide training for anyone caring for or using laboratory animals. This volume provides the guidelines and resources needed to coordinate a quality training program, as well as to meet all legal requirements. ISBN 0-309-08691-4; 1991, 152 pages, 8.5 × 11, paperbound

Guide for the Care and Use of Laboratory Animals, 8th ed.
The Guide for the Care and Use of Laboratory Animals has been updated by a committee of experts to incorporate new scientific information on commonly used laboratory animals, including aquatic species, and provides extensive references. It is organized around major components of animal use: key concepts of animal care and use; animal care and use program (including the roles and responsibilities of the institutional official, attending veterinarian, and the institutional animal care and use committee; regulatory considerations; program and personnel management; and program oversight); animal environment, husbandry, and management; veterinary care (including animal procurement and transportation, preventive medicine, and clinical care and management); and physical plant design considerations. ISBN 0-309-15400-6; 2010, 248 pages, 6 × 9, paperbound

Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research
Expanding on the Guide for the Care and Use of Laboratory Animals, this report provides current best practices for animal care and use and discusses how the regulations and guidelines provided by the Guide, the Animal Welfare Act, the Animal Welfare Act Regulations, and PHS Policy can be applied to neuroscience and behavioral research. The report treats the development, evaluation, and implementation of animal-use protocols as a decision-making process, not just a decision. It encourages the use of professional judgment and careful interpretation of regulations and guidelines to develop performance standards that ensure animal well-being and high-quality research. This report is an indispensable resource for researchers, veterinarians, and institutional animal care and use committees. ISBN 0-309-08903-4; 2003, 224 pages, 6 × 9, paperbound
Guidelines for the Humane Transportation of Research Animals
Transporting research animals is a necessary part of the biomedical enterprise that can have substantial effects on the physiological and psychological condition of the animals. Individuals at research facilities often find arranging transportation of animals a challenge. In order to address a plethora of sometimes confusing and burdensome regulations pertaining to transportation of research animals, this report recommends that an interagency working group be established to coordinate federal inspections and permitting activities. It further recommends that steps be taken to ensure the availability of safe, reliable air and ground transportation for research animals. The report also establishes science-based good practices for transporting research animals and advises that research institutions designate a single individual to be responsible for ensuring safe shipment and receipt of animals. ISBN 0-309-10122-0; 2006, 160 pages, 6 × 9, paperbound

Immunodeficient Rodents: A Guide to Their Immunobiology, Husbandry, and Use
This volume is an indispensable reference on the nature of immune defects in rodents and the special techniques necessary to maintain and breed them. The authors describe 64 inbred, hybrid, and mutant strains of rodents, each with some immune defect; explain mechanisms for ensuring genetic purity; and provide a standardized nomenclature for different varieties. ISBN 0-309-03796-4; 1989, 260 pages, 6 × 9, clothbound

Infectious Diseases of Mice and Rats
This edition—a must for all researchers who use these animals—provides practical suggestions for breeding, keeping, and identifying pathogen-free laboratory rodents. ISBN 0-309-06332-9; 1991, 415 pages, 6 × 9, paperbound

Companion Guide to Infectious Diseases of Mice and Rats
This companion to Infectious Diseases of Mice and Rats makes practical information on rodent diseases readily accessible to researchers. ISBN 0-309-04283-6; 1991, 108 pages, 6 × 9, paperbound

Nonhuman primates (NHP) continue to play an important role in the research of many human diseases such as malaria and AIDS. Changes in the need for different species of NHP, the adequacy of the current supply of NHP, and projections of future needs for NHP are issues that concern scientists, veterinarians, and funding authorities from countries that are major users of NHP, as well as countries that produce and supply these animals. In this volume, workshop participant discussions relate to current shortfalls and excesses in NHP breeding and exportation programs, the status of breeding and conservation programs internationally, the development of specific pathogen-free colonies, difficulties in transporting NHP, and challenges in the management of NHP colonies. ISBN 0-309-08945-X; 2003, 262 pages, 6 × 9 paperbound

Microbial and Phenotypic Definition of Rats and Mice: Proceedings of the 1998 US/Japan Conference
This workshop is part of a long-term program to nurture international collaborative and information-exchange activities. As genetics and genomics affect the study of biology and medicine, the role of comparative medicine cannot be understated. Workshop contributors seek to enhance the genetic and microbiologic integrity of laboratory rat and mouse colonies worldwide. The mouse has been a critical model for the discovery of genes responsible for several cancers and many other diseases. The rat model “functionally” characterizes mammalian model systems. This meeting sought to help global scientific enterprise harmonize the mouse and rat models and to meet the research challenges of the 21st century. ISBN 0-309-07389-8; 1999, 110 pages, 6 × 9, paperbound

Monoclonal Antibody Production
Monoclonal antibodies are important reagents used in research, diagnosis, and treatment of diseases. They are produced by injection into the abdominal cavity of a suitably prepared mouse or by tissue culturing cells in plastic flasks. This report weighs the costs and benefits of each method and makes recommendations for their uses. ISBN 0-309-07511-4; 1999, 74 pages, 6 × 9, paperbound

National Need and Priorities for Veterinarians in Biomedical Research
This report identifies various factors that contributed to creating an unfulfilled need for veterinarians in the biomedical research workforce, including an increase in the number of NIH grants utilizing animals and the burgeoning use of transgenic rodents, without a comparable change in the supply of appropriately trained veterinarians. The committee developed strategies for recruiting more veterinarians into careers in biomedical research. ISBN 0-309-09083-0; 2004, 102 pages, 6 × 9, paperbound

Nutrient Requirements of Laboratory Animals, 4th ed.
In the years since the third edition of this indispensable reference was published, a great deal has been learned about the nutritional requirements of common laboratory species: rat, mouse, guinea pig, hamster, gerbil, and vole. The fourth edition presents the current expert understanding of the lipid, carbohydrate, protein, mineral, vitamin, and other nutritional needs of these animals. The extensive use of tables provides easy access to a wealth of comprehensive data and resource information. ISBN 0-309-05126-6; 1995, 192 pages, 8.5 × 11, paperbound

Occupational Health and Safety in the Care and Use of Nonhuman Primates
The field of occupational health and safety constantly changes, especially as it pertains to biomedical research.
New infectious hazards are of particular importance at non-human-primate facilities. For example, the discovery that B virus can be transmitted via a splash on a mucous membrane raises new concerns that must be addressed, as does the discovery of the Reston strain of Ebola virus in import quarantine facilities in the U.S. The risk of such infectious hazards is best managed through a flexible and comprehensive occupational health and safety program (OHSP) that can identify and mitigate potential hazards. This report is intended as a reference for vivarium managers, veterinarians, researchers, safety professionals, and any other persons who are involved in developing or implementing an OHSP dealing with nonhuman primates. This report attempts to list the important features of an OHSP and provide the tools necessary for informed decision-making in developing an optimal program that meets all particular institutional needs. ISBN 0-309-08914-X; 2003, 184 pages, 6 × 9, paperbound

**Occupational Health and Safety in the Care and Use of Research Animals**

Much has been written about the care of research animals, yet little guidance has appeared on protecting the health and safety of the people who care for or use these animals. This report, an implementation handbook and companion to the Guide, identifies principles for building a program and discusses the accountability of institutional leaders, managers, and employees for a program’s success. ISBN 0-309-05299-8; 1997, 168 pages, 6 × 9, paperbound

**The Psychological Well-Being of Nonhuman Primates**

A 1985 amendment to the Animal Welfare Act requires those who keep nonhuman primates to develop and follow appropriate plans for promoting the animals’ psychological well-being. The amendment, however, provides few specifics. *The Psychological Well-Being of Nonhuman Primates* recommends practical approaches to meeting those requirements. ISBN 0-309-10359-2; 1998, 184 pages, 6 × 9, paperbound

**Recognition and Alleviation of Distress in Laboratory Animals**

The use of animals in research adheres to scientific and ethical principles that promote humane care and practice, and these principles and standards of practice must be updated based on scientific advances in our understanding of animal physiology and behavior. This report focuses on the stress and distress experienced by animals used in research. It aims to educate laboratory animal veterinarians; students, researchers, and investigators; institutional animal care and use committee members; animal care staff; and animal welfare officers about current scientific and ethical issues associated with stress and distress in laboratory animals. The report evaluates pertinent scientific literature to generate practical guidelines, focusing on the following areas: scientific understanding of causes and functions of stress and distress; the transformation of stress to distress; and the identification of principles for the recognition and alleviation of distress. The report also discusses the role of humane endpoints in situations of distress and principles of minimization of distress in laboratory animals. Finally, the report identifies areas in which further scientific investigation is needed to improve laboratory animal welfare. ISBN 0-309-10817-9; 2008, 118 pages, 6 × 9, paperbound

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This report, the second of two revising the 1992 publication *Recognition and Alleviation of Pain and Distress in Laboratory Animals*, focuses on pain experienced by animals used in research. This book aims to educate laboratory animal veterinarians; students, researchers, and investigators; institutional animal care and use committee members; and animal care staff and animal welfare officers about the current scientific and ethical issues associated with pain in laboratory animals. The committee evaluated pertinent scientific literature to generate practical and pragmatic guidelines for recognizing and alleviating pain in laboratory animals, focusing on the following areas: physiology of pain in commonly used laboratory species; pharmacologic and nonpharmacologic principles to control pain; identification of humane endpoints; and principles for minimizing pain associated with experimental procedures. Finally, the report cites areas in which further scientific investigation is needed to improve laboratory animal welfare. ISBN 978-0-309-12834-6; 2009, 270 pages, 6 × 9, paperbound

**Recognition and Alleviation of Pain and Distress in Laboratory Animals**

Clear guidelines on the proper care and use of laboratory animals are being sought by researchers and members of the many committees formed to oversee animal care at universities as well as the general public. This report provides comprehensive information about behavior, pain, and distress in laboratory animals. ISBN 0-309-07525-4; 1992, 160 pages, 6 × 9, paperbound

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**Science, Medicine, and Animals Teacher’s Guide**

*Science, Medicine, and Animals* explains the role that animals play in biomedical research and the ways in which scientists,
governments, and citizens have tried to balance the experimental use of animals with a concern for all living creatures. A n accompanying Teacher’s Guide is available to help teachers of middle and high school students use Science, Medicine, and Animals in the classroom. A s students examine the issues in Science, Medicine, and Animals, they will gain a greater understanding of the goals of biomedical research and the real-world practice of the scientific method in general. The Teacher’s Guide was reviewed by members of the National Academies’ Teacher Associates Network and is recommended by the National Science Teacher’s Association. ISBN 0-309-10117-4; 2005, 24 pages, 8.5 × 11, paperbound

**Scientific and Humane Issues in the Use of Random Source Dogs and Cats**

This report examines the value of random source animals in biomedical research funded by the National Institutes of Health (NIH) and the role of Class B dealers who acquire and resell live dogs and cats to research institutions. The report addresses (1) the important biomedical research questions and common research topics in contemporary NIH-funded research where Class B dogs and cats are desirable/necessary as well as the frequency of these research topics (i.e., number of grants where the potential exists or the source of the animal is identified as a Class B dealer); (2) the specific characteristics (e.g., physiological, anatomical, or genetic) of the animals that make them particularly well suited for certain types of research; and (3) recommendations for the use of Class B dogs and cats. ISBN 978-0-309-13807-9; 2009, 136 pages, 6 × 9, paperbound

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