Neocaridina denticulata: A Decapod Crustacean Model for Functional Genomics

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Synopsis

A decapod crustacean model is needed for understanding the molecular mechanisms underlying physiological processes, such as reproduction, sex determination, molting and growth, immunity, regeneration, and response to stress. Criteria for selection are: life-history traits, adult size, availability and ease of culture, and genomics and genetic manipulation. Three freshwater species are considered: cherry shrimp, Neocaridina denticulata; red swamp crayfish, Procambarus clarkii; and redclaw crayfish, Cherax quadricarinatus. All three are readily available, reproduce year round, and grow rapidly. The crayfish species require more space for culture than does N. denticulata. The transparent cuticle of cherry shrimp provides for direct assessment of reproductive status, stage of molt, and tissue-specific expression of reporter genes, and facilitates screening of mutations affecting phenotype. Moreover, a preliminary genome of N. denticulata is available and efforts toward complete genome sequencing and transcriptome sequencing have been initiated. Neocaridina denticulata possesses the best combination of traits that make it most suitable as a model for functional genomics. The next step is to obtain the complete genome sequence and to develop molecular technologies for the screening of mutants and for manipulating tissue-specific gene expression.

Introduction

A workshop was held at the 2015 annual meeting of the Society for Integrative and Comparative Biology (SICB). It involved speakers and participants in the “Pancrustacea” symposium. The purpose of the symposium was to bring together physiologists representing the breadth of research on crustaceans and insects: structure and composition, of the cuticle, transport and sensing of oxygen, osmoregulation, immune responses, regeneration of tissue, and comparative genomics. One of the ideas that emerged from the workshop was the need for a model organism, comparable to Drosophila, to transform the field and facilitate integration with insects’ systems. This policy paper is a direct outcome of the workshop. Here we define the purpose of a model species, the criteria that should be considered in selecting the species, and identify potential species as candidates. We end by making a recommendation as to how to proceed.

Purpose of the model organism

There are a number of decapods being used as model organisms. It is usually an aspect of their biology and/or their convenience of collection that makes them amenable for experimental study. We are not advocating the replacement of current model organisms by a new model organism and working exclusively on that species. However, a model species is needed for a mechanistic understanding of physiological processes, as well as for integrating the knowledge gained from variety of decapods and insects. Transcriptomics has been frequently used to identify genes involved in molting and growth, reproduction, sex determination, development, digestion, immunity, and response to stress (He et al. 2012; Harms et al. 2013; Christie 2014; Durica et al. 2014; Gao et al. 2014; Ghaffari et al. 2014; Lv et al. 2014; Shen et al. 2014; Tom et al. 2013, 2014; Ventura et al. 2013, 2014; Wei et al. 2014a, 2014b). The next
logical step is to select a genetically tractable species for genome sequencing and functional genomics. A decapod genome would facilitate the assembly and analysis of RNA-Seq data, particularly with respect to identification of alternatively spliced isoforms. Transgenic approaches can test the functionality of a gene by manipulating its expression.

**Criteria for selecting a crustacean model organism**

**Life history**

The life history of a species must be amenable for reproductive, developmental, and genetic research. Short generation time is crucial. The time from zygote to zygote must be as short as possible at moderate ambient temperatures (<6 months). Developmental and genetic studies would require a continuous supply of fertilized eggs. Adults must reproduce year-round; seasonal breeders are not suitable. Also, a species with direct development (i.e., no free-living planktonic larval stage) is preferred.

**Adult size**

The species must have an optimal adult size. Adults must be large enough for physiological research on the whole animal, as well as on individual organs and tissues. However, larger species generally have longer intergenerational times and require more physical space. For genetic studies, thousands of individuals are needed, making space for housing and raising animals a critical factor.

**Availability and ease of culture**

Animals must be readily available and be easy to rear in culture. This restricts the selection to freshwater species, as marine and estuarine species would restrict practical use to coastal locations or aquaculture facilities. Moreover, the species must be hardy and thrive over a range of ambient conditions, such as temperature, pH, and mineral composition.

Behavior has a direct impact on the space required for culture. Territorial or aggressive species must be reared under conditions that minimize agonistic interactions, such as in individual cages or compartments and/or in aquaria with suitable refuges. This is especially critical at the time of molting, when individuals are vulnerable to attack by conspecifics. Measures that reduce fighting and attacks increase the space required for maintaining the animals. Non-territorial, non-aggressive species require less space, as they can be reared in communal aquaria; more individuals of a non-aggressive species can be reared in a given space than can an aggressive species.

**Genomics and genetic manipulation**

It is a challenge to agree on what constitutes an ideal decapod genome, in which each lineage has evolved with its own distinct characteristics. A critical consideration is that a species should not have evolved faster or diverged too far from the major decapod taxa (i.e., fewer lineage-specific modifications). The more closely related the species, the more confidence one has in relating the results from studies on the model organism to other decapods. The only comprehensive crustacean genome is that of the water flea, *Daphnia pulex* (Colbourne et al. 2011). In comparison with other bilaterian genomes, this lineage has evolved rapidly and has undergone extensive duplication of genes (Colbourne et al. 2011). Certainly this model is excellent for particular applications, such as ecotoxicology (Rodriguez et al. 2007; Wang et al. 2011), but whether it can represent crustaceans more generally remains to be tested. Thus, there remains a great need for a decapod genome.

Currently, the only proven method for altering gene expression in decapod species is by RNA interference (RNAi). Injection of a dsRNA construct lowers the mRNA level of a gene and the effect can last for days (Priya et al. 2010; De Santis et al. 2011; Ventura et al. 2011; Pamuru et al. 2012; Das and Durica 2013; Chung 2014; Qian et al. 2014; Lee et al. 2015; Lezer et al. 2015). Yet, it is difficult to control for non-specific effects of RNAi. An off-target effect of the exogenous small-interfering RNA is always a concern, but can be controlled by injection of a “scrambled” dsRNA construct of a gene. However, one cannot control for systemic effects. The dsRNA does not stay localized at the site of introduction; the effects on gene expression are observed in tissues from other parts of the body (Hui et al. 2008; De Santis et al. 2011; Das and Durica 2013; Lee et al. 2015).

**Potential candidates**

There are, as yet, no decapod species that meet all the criteria, hence the question is which species satisfies most of the criteria and has the potential for meeting the rest? The list below is restricted to freshwater organisms that are widely available and grow and reproduce well at room temperature.

**Cherry shrimp, Neocaridina denticulata**

*Neocaridina denticulata* (De Haan 1884) and related species are members of the Atyidae (Suborder
Pleocyemata, Infraorder Caridea). The species is native to Asia, but is widely available as a popular aquarium organism. Adults can grow to 2.85 cm in body length (Mizue and Iwamoto 1961; Hung et al. 1993). Animals are easily acquired commercially and raised in culture, as they can tolerate a wide range in pH (pH 6.5–8.0) and temperature (up to 30°C). Optimal conditions are about room temperature (22–25°C, pH 7.0–7.5), thus saving cost on maintenance, such as operating expensive temperature-control equipment, when this eurythermal species is kept at room temperature (Mizue and Iwamoto 1961; Dudgeon 1985; Huang et al. 2006). Females of *N. denticulata* become reproductive at 6.4–7.8 mm carapace length (Oh et al. 2003). Adults molt frequently under optimal conditions, with the intermolt interval averaging 15–16 days (Niwa et al. 1998), which represents a much shorter molt cycle when compared to other decapod species. This characteristic is suitable for developmental and physiological studies, but may not be as useful for ecotoxicological studies, which may require exposures to a chemical or environmental insult longer in duration than any one molt stage. Females produce 20–30 fertilized eggs (0.57–1.08 mm diameter) (Shy et al. 1992; Hung et al. 1993), which hatch at ~30 days post-mating. Individuals attain sexual maturity at 4–6 months of age depending on water temperature (Mizue and Iwamoto 1961; Hung et al. 1993).

Sources of nuclear and mitochondrial DNA for *N. denticulata* are available. The mitogenome and a draft nuclear genome are available at http://huilab.sls.cuhk.edu.hk/Neocaridina/ (Kenny et al. 2014; Yu et al. 2014). The Comparison of Core Eukaryotic Gene Mapping Approach (CEGMA) dataset with developmental and hormonal pathway genes indicates that the genome represents a high coverage of the expected coding sequences (Kenny et al. 2014; Sin et al. 2014). Moreover, the *N. denticulata* genome has not undergone extensive duplication and/or loss of genes compared to the highly derived *Daphnia* genome (Kenny et al. 2014; Sin et al. 2014). Analysis of the order of mitochondrial genes also suggests that it has not undergone extensive rearrangement (Kenny et al. 2014). These data suggest that the *N. denticulata* genome can represent the genomes of other decapod species.

**Red swamp crayfish, *Procambarus clarkii***

*Procambarus clarkii* (Girard 1852), native to North America, has become established in freshwater habitats in the temperate zones of Europe, South America, Africa, and Asia. It is a member of the Cambaridae (Suborder Pleocyemata). It is also an ecologically and economically important species, both as an invasive species and as a farmed species (Geiger et al. 2005; Moore et al. 2013). It is an important model for ecotoxicology and the effects of infection and stress (Barbee et al. 2010; Bonvillain et al. 2012; Lin et al. 2013; Mazurova et al. 2008; Vioque-Fernandez et al. 2007). Recently, transcriptomics has been used to study gene expression in the integument during the molt cycle (Tom et al. 2014).

Adults reach a size up to 12 cm in body length. As a generalist, it has a varied diet and can live in a variety of habitats (McClain and Romaine 2007). These traits, as well as a resistance to pathogens, make them suitable for culture (Geiger et al. 2005). A female typically produces 200–300 eggs, but up to 600 eggs per female have been reported (Geiger et al. 2005). Developmental time depends on temperature; at 23°C eggs hatch in 3 weeks and individuals reach sexual maturity in 3–5 months (McClain and Romaine 2007).

**Redclaw crayfish, *Cherax quadricarinatus***

*Cherax quadricarinatus* (von Martens 1868) is a member of the Parastacidae (Suborder Pleocyemata) and is native to Australia. The species is used widely in aquaculture, as they can be raised at relatively high densities (up to 15/m²) and a variety of feed formulations, and grow rapidly under optimal conditions (Jones et al. 2000; Saoud et al. 2012). They show non-aggressive and non-burrowing behaviors. Animals reach market size (40–200 g) and become sexually mature in 6–9 months (Ghanawi and Saoud 2012; Jones et al. 2000). They tolerate a range of temperatures, oxygen levels, mineral contents, pH, and salinities (Cimino et al. 2002; Ghanawi and Saoud 2012). Females can reproduce year-round under the proper conditions of temperature (25–26°C) and photoperiod (12L:12D) and produce between 100 and 1000 eggs per spawn; the number of eggs depends on the size of the female (Ghanawi and Saoud 2012). The duration of embryonic development is 39 days at 28°C, but the optimal temperature range is 22–25°C (Chaulet et al. 2013; Ghanawi and Saoud 2012). Considerable labor and space are required for culturing *C. quadricarinatus* (Ghanawi and Saoud 2012; Jones et al. 2000). Separate brood-stock holding tanks and spawning/nursery tanks are needed to provide an adequate supply of juveniles. As juveniles are cannibalistic and are particularly vulnerable at molting, adequate shelters or refuges must be provided to increase survival to adulthood (Ghanawi and Saoud 2012; Jones et al. 2000). To increase
profitability, aquaculture practices have placed an emphasis on selecting strains with higher growth rates and larger size at maturity (Jones et al. 2000), traits that are not suitable for a model organism. However, certain locations in northern Australia have populations in which individuals reach sexual maturity at a smaller size (Jones et al. 2000). It may be possible to use these precocious breeders as a source for stocks with a shorter generational time.

**Recommendation: *N. denticulata***

It is our recommendation that the crustacean research community unites behind a coordinated effort to develop *N. denticulata* as a decapod model for functional genomics. *Neocaridina* sp. have been used for reproductive and developmental studies (Dudgeon 1985; 1987; Jirikowski et al. 2013; Oh et al. 2003) and ecotoxicological studies (Huang and Chen 2004; Huang et al. 2006; Huang et al. 2004; Liu and Sung 2011; Sung et al. 2011; Wang et al. 2010). *N. denticulata* is superior to other potential freshwater species in terms of life history, size, availability, and ease of culture. *Neocaridina denticulata* is resistant to bacterial infection (Sung et al. 2011) and a related species, *N. davidi*, is resistant to a crayfish pathogen (Svoboda et al. 2014). Moreover, a preliminary genome database is available (Kenny et al. 2014).

An important advantage that *N. denticulata* has over *C. quadricarinatus* and *P. clarkii* is that the exoskeleton of *N. denticulata* is relatively transparent due to its low degree of sclerotization and calcification as well as its variation in the distribution and pigmentation of chromatophores (Flores and Chien 2011; Sin et al. 2014). This transparency allows non-invasive monitoring of internal organs and tissues for the study of reproduction, development, and molting. For example, the stage of molt is assessed by examination of setogenesis in the exopodites of the uropods (Flores and Chien 2011). More importantly, this also provides for large-scale mutant selection when techniques for genetic manipulation become available. This is an advantage for monitoring staining of tissues with vital dyes (Niwa et al. 1998) and for the *in vivo* expression of a reporter gene, such as green fluorescent protein, in transgenic animals.

In summary, *N. denticulata* has the same attributes as other successful model species, such as fruit flies, nematodes, and zebrafish. It has a relatively short life cycle, it is easy to rear in large quantities, and it is easily obtained and distributed to researchers. *Cherax quadricarinatus* and *P. clarkii* grow quickly and are easy to culture in large numbers, but they require much more space. As a benthic species, crayfish are restricted to a two-dimensional space. *Neocaridina denticulata* live on plants hence they can occupy a three-dimensional space. Moreover, the calcified cuticle of the crayfish species prevents monitoring of molt stage and reproductive condition and the screening of transgenic individuals. Taken together, the cherry shrimp best meets the criteria for a decapod model.

**Next steps**

1. Obtain a complete genome sequence: A genome sequence is necessary for understanding gene regulation and protein isoforms generated by alternative splicing. Preliminary draft genome analysis suggests that *N. denticulata* could potentially represent a prototypical decapod crustacean genome, or at least, it has not undergone extensive lineage-specific modifications, such as duplications or losses of genes. Deep genome sequencing and transcriptome sequencing are now underway.

2. Establish transgenic methods for controlling gene expression: To determine gene function, it is critical that a gene can be over- or under-expressed in specific tissues at specific times. RNAi down regulates gene expression, but there are no established methods to up regulate specific gene expression. Developing molecular techniques for disrupting the expression of a targeted gene in a specific tissue is a better approach. For example, *Daphnia* embryos expressing an EcRE/reporter gene construct were used to monitor ecdysteroid activity (Asada et al. 2014). The use of Transcription activator-like effector nuclease (TALEN) in *Daphnia* (Hiruta et al. 2014) indicates that techniques for editing genomes can be applied to crustaceans to establish stable lines. Furthermore, genetic transformation has also been established for the amphipod *Parhyale hawaiensis* (Pavlopoulos and Averof 2005). All these require the introduction of gene constructs in the early embryo. The external development of the fertilized eggs (~30 per animal) allows access for injection and manipulation (Kenny et al. 2014), which provide opportunities for the knockdown of individual genes (e.g., morpholino or siRNA/dsRNA on target gene) (Pickart and Klee 2014; Raouane et al. 2012), genome editing (e.g., zinc finger nuclease, TALEN, or CRISPR/Cas9) (Gaj et al. 2013), manipulating gene
expression (e.g., GAL4/UAS)(Duffy 2002), and large-scale creation of random mutations (e.g., PiggyBac transposon)(Kim and Pyykko 2011).

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