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COMMENTARY

From genes to behavior: investigations of neurochemical signaling come of age for the model crustacean *Daphnia pulex*

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Summary

The cladoceran crustacean *Daphnia pulex* has served as a standard organism for aquatic toxicity testing for decades. The model organism status of *D. pulex* rests largely on its remarkable ability to rapidly adapt morphologically, physiologically and behaviorally to a wide range of environmental challenges, as well as on its parthenogenetic reproduction and ease of laboratory culture. As in all multicellular organisms, neurochemical control systems are undoubtedly major contributors to the functional flexibility of *Daphnia*. Surprisingly, little work has focused on understanding its neurochemistry at any level. Recently, *D. pulex* has been the subject of extensive genome and transcriptome sequencing, and it is currently the only crustacean with a fully sequenced, publicly accessible genome. Although the molecular work was initiated for gene-based investigations of ecotoxicology and toxicogenomics, the data generated have allowed for investigations into numerous aspects of *Daphnia* biology, including its neurochemical signaling. This Commentary summarizes our knowledge of *D. pulex* neurochemistry obtained from recent genomic and transcriptomic studies, and places these data in context with other anatomical, biochemical and physiological experiments using *D. pulex* and its sister species *Daphnia magna*. Suggestions as to how the *Daphnia* molecular data may be useful for future investigations of crustacean neurochemical signaling are also provided.

Key words: *Daphnia pulex*, *Daphnia magna*, Cladocera, genome, transcriptome, neurohormone, neurotransmitter, peptide, amine, small molecule transmitter, gas transmitter.

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Introduction

Over its geographic range, the cladoceran crustacean *Daphnia pulex* (Fig. 1) serves as a keystone species for many freshwater ecosystems, being both the primary consumer of phytoplankton and the primary forage species for larger invertebrates and fish (Dodson et al., 2010). This crustacean is highly sensitive to changes in its environment, and possesses the ability to rapidly adapt morphologically, physiologically and behaviorally to a wide range of environmental and anthropogenic challenges (Dodson et al., 2010). *Daphnia pulex*'s remarkable functional flexibility, in combination with its parthenogenetic mode of reproduction and ease of laboratory culture, has resulted in it being used as one of the primary models for the field of environmental toxicology for over half a century (Dodson et al., 2010).

Recently, *D. pulex* has been the subject of extensive molecular investigations, including both genome and transcriptome sequencing (Colbourne et al., 2011). In fact, this species is currently the only crustacean for which a fully sequenced genome is publicly available; *D. pulex* is also one of but a few crustaceans for which a large number of transcriptome sequences have been publicly deposited. These molecular resources, in combination with the biological features described above, have resulted in the emergence of *D. pulex* as a model for the field of toxicogenomics. In addition, its phylogenetic position, presumed to be close to the point of divergence of the insects from the crustaceans (e.g. Meusemann et al., 2010), makes it a strategic target for genomic investigations of arthropod evolution.

A key factor that led to the emergence of *D. pulex* as a model organism for many biological fields is its ability to rapidly cope with environmental challenge *via* changes in its morphology, physiology and/or behavior. As in all multicellular organisms, the relative levels and/or complements of locally released paracrines and circulating hormones are undoubtedly key players in mediating this biological flexibility. Although many tissues undoubtedly participate in this chemical communication, the nervous system is a particularly rich source of both local and hormonal signaling agents. Interestingly, few studies have focused on the neurochemistry of *D. pulex* at any level. In fact, prior to approximately 2005, essentially nothing was known about neurochemical signaling in this species. One factor that has surely contributed to this lack of neurochemical knowledge is its size; adults are typically just a few millimeters in overall length. This is surely true for the identification of its neuropeptides, as the standard method for peptide discovery until ~2005 was to isolate individual peptides chromatographically and/or biochemically from large pools of starting tissue and, upon their purification, assess their structures using a combination of proteolytic cleavage, Edman analysis and mass spectrometry (Christie et al., 2010). Given its minute size and the need to manually dissect a sufficient amount of starting material, the identification of even a single *Daphnia* neuropeptide *via* this strategy would surely have been daunting, as hundreds, and in some cases thousands, of central nervous systems (CNSs) were needed for the biochemical isolation and

Fig. 1. *Daphnia pulex*. Figure used with permission from Gewin (Gewin, 2005); photograph by Paul Hebert.

characterization of individual native peptides from much larger crustaceans (e.g. Torfs et al., 2002). Similarly, the *de novo* sequencing of peptide precursor protein and receptor genes/transcripts, as well as those encoding the biosynthetic enzymes, transporters and receptors involved in amine, diffusible gas and small molecule transmitter neurotransmission, is time consuming on an individual level, and to achieve full, or at least near complete, coverage for all signaling systems, would at best have been an expensive, labor-intensive, multi-year undertaking. With the advent of new molecular technologies, the large-scale sequencing of genomes and transcriptomes is an increasingly common occurrence. When available, these resources provide alternative approaches to the identification and characterization of the predicted proteins required for the establishment of neurochemical signaling pathways, specifically genome and transcriptome mining.

Within the arthropods, the insects are the subphylum for which by far the most genomic and/or transcriptomic data are available. Over the last 10 years, this information has been used for the identification of numerous insect neurochemical pathway proteins, particularly neuropeptides and their receptors. For example, analyses of the fruit fly *Drosophila melanogaster* genome allowed for the identification of 44 G-protein-coupled receptors (GPCRs), as well as 22 peptide preprohormones, likely the sources of at least a subset of the receptors' ligands (Hewes and Taghert, 2001); the deduced complement of GPCRs was hypothesized to represent the vast majority, if not all, of the neuropeptide receptors in *Drosophila*. Similarly, 36 precursor protein genes were predicted from the honeybee *Apis mellifera* genome, resulting in the prediction of over 200 mature neuropeptides for this species (Hummon et al., 2006). The sequencing and public deposition of the *D. pulex* genome and transcriptome now allow for similar analyses of its neurochemical signaling systems. In the remainder of this Commentary, we focus on summarizing the data that have been obtained from these genomic

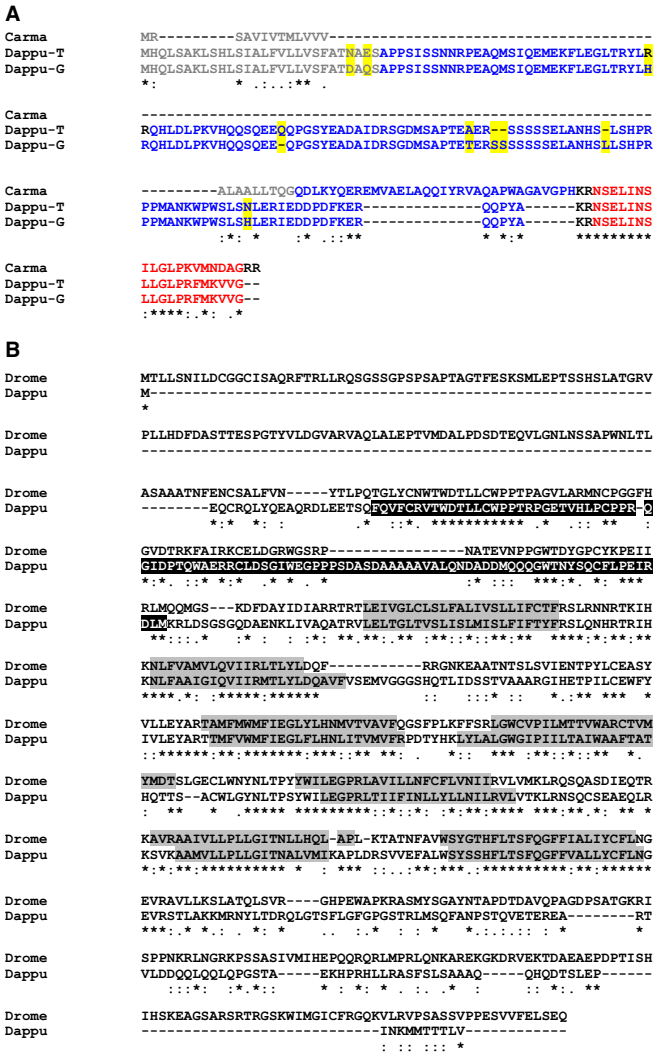

Fig. 2. Genomic and transcriptomic identification of the *Daphnia pulex* pigment dispersing hormone (PDH) precursor protein and receptor. (A) Alignment of the amino acid sequences of the *Carcinus maenas* (Carma) PDH precursor protein (Klein et al., 1992) with those deduced from the *D. pulex* transcriptome (Dappu-T) (Gard et al., 2009) and genome (Dappu-G) (Christie et al., 2011). For each protein, the signal peptide is shown in gray, with all prohormone convertase cleavage sites shown in black. Isoforms of PDH are shown in red, and all other precursor-related peptides are shown in blue. Amino acid residues that vary between the *D. pulex* transcriptome and genome predictions are highlighted in yellow. (B) Alignment of *Drosophila melanogaster* pigment dispersing factor receptor (Drome) (Adams et al., 2000) with *D. pulex* PDH receptor (Dappu) (Tilden et al., 2011). In each protein, hormone receptor and transmembrane domains are highlighted in black and gray, respectively. In both A and B, asterisks indicate amino acids that are identically conserved, whereas single and double dots denote amino acids that are similar in structure (a single dot refers to a conservative substitution and a double dot to a highly conservative substitution). Data are modified from Gard et al. (Gard et al., 2009), Christie et al. (Christie et al., 2011) and Tilden et al. (Tilden et al., 2011).

and transcriptomic investigations, placing this work in context with other data collected from anatomical, biochemical and physiological studies of neurochemical signaling in *Daphnia* species. A discussion of how this information may be useful for future investigations of neurochemical communication in this and other crustaceans is also provided.

Table 1. Peptide families and subfamilies identified in *Daphnia pulex via* genomic, transcriptomic and mass spectral analyses

		Mode of identification		
Peptide family	Subfamily	Genome	Transcriptome	Mass spectrometry
Allatostatin A		+[a,b]	+[e]	+[b]
Allatostatin B		+[a,b]	+[e]	+[b]
Allatostatin C		+[a,b]	+[e]	
Allatotropin		+[a,b]		+[b]
Bursicon		+[a,b]	+[e]	
CCHamide		+[b]		
Corazonin		+[a,b]		+[b]
Crustacean cardioactive peptide		+[a,b]	+[e]	
Crustacean hyperglycemic hormone		+[a,b]	+[e]	
DENamide		+[b]		
Diuretic hormone (calcitonin-like)		+[a,b]	+[e]	+[b]
Diuretic hormone (CRF-like)		+[b]		
Ecdysis-triggering hormone		+[a,b]	+[e]	
Eclosion hormone		+[a,b]		
FMRFamide	FIRFamide	+[b]		+[b]
	Myosuppressin	+[b]		+[b]
	Neuropeptide F	+[a,b]	+[e]	+[b]
	Short neuropeptide F	+[a,b]	+[e]	+[b]
	Sulfakinin	+[a,b]		+[b]
Intocin		+[c]		
Insulin-related peptide		+[a,b,d]		+[b]
Neuroparsin		+[b]		
Orcokinin		+[a,b]	+[e]	+[b]
Periviscerokinin		+[b]		+[b]
Pigment dispersing hormone		+[a,b]	+[e]	+[b]
Proctolin		+[a,b]		+[b]
Red pigment concentrating hormone		+[a,b]		
RYamide		+[b]		+[b]
SIFamide		+[a,b]	+[f]	+[b]
Tachykinin-related peptide		+[a,b]		+[b]

Data from: [a]Christie et al., 2011; [b]Dircksen et al., 2011; [c]Stafflinger et al., 2008; [d]Boucher et al., 2010; [e]Gard et al., 2009; [f]Verleyen et al., 2009.

Daphnia genomic resources

The genome of *D. pulex* is a unique resource for crustacean biology. The current draft genome for *D. pulex* (Colbourne et al., 2011) was assembled from over 1.5 million high-quality reads obtained from DNA collected from the naturally inbred clonal line 'The Chosen One'. The size of this genome assembly is roughly 200 megabases, which is relatively small in comparison to the genomes thus far sequenced for other species. Surprisingly, this 'small' genome is predicted to contain an unusually large number of genes, approximately 31,000, which exceeds the number found in most other species, including humans. Several factors that contribute to the ability of *D. pulex* to balance a small genome size with a large number of genes are a reduction in the length of its introns and an elevated rate of gene duplication and maintenance. Of the ~31,000 *D. pulex* genes, nearly one-third have no known homologs, and are proposed as specific to the *Daphnia* lineage. It is hypothesized that these 'unique' genes have evolved in response to *D. pulex*'s organism–environment interactions, and thus are direct contributors to this species' remarkable functional flexibility.

In addition to the sequencing of its genome, *D. pulex* has also been the subject of extensive transcriptome analysis; at present nearly 150,000 expressed sequence tags (ESTs) have been publicly deposited for this species (Colbourne et al., 2011). These ESTs were developed from 37 different cDNA libraries, which represent genes expressed under a number of distinct ecological conditions, including many commonly encountered environmental variables (e.g. differing food levels and predator kairomones) and a number of anthropogenic stressors (e.g. heavy metals and nanoparticles). These functional genomic data have validated over a third of the 31,000 predicted *Daphnia* genes, and in combination with other vetting methods (e.g. tiling microarrays), nearly 90% of the genes predicted from the *D. pulex* genome now have at least some level of additional support.

Peptidergic systems

Prior to the public deposition of its genome and transcriptome, little was known about the neurochemical signaling systems of *D. pulex*. In fact, little was known about the molecular machinery necessary for the establishment and proper functioning of neurochemical pathways in any crustacean. Although the molecules used for chemical communication are diverse in all multicellular organisms, peptides are by far the largest class used by nervous systems (Christie et al., 2010; Christie, 2011), and it is this group of compounds that was the first to be investigated *via* genome and/or transcriptome mining in *Daphnia*.

The first large-scale investigation of peptidergic signaling in *D. pulex* focused on the characterization of its peptidome using transcriptome mining (Gard et al., 2009). Here, the sequences of known insect and crustacean neuropeptide precursor proteins were used to query the *Daphnia* EST database for putative homologs; the structures of the mature neuropeptides were subsequently predicted from the deduced proteins *via* both online software programs and homology to known insect and crustacean peptide isoforms (Fig. 2A). Using this strategy, 63 peptide-encoding ESTs were identified; these encompassed 14 distinct peptide families or subfamilies (Table 1). Over 70 putative mature peptides were predicted from the proteins deduced from these ESTs. Using the

Table 2. Genomic identification of *Daphnia pulex* peptide receptors

Peptide family	Subfamily	Receptor
Allatostatin A		+*
Allatostatin B		+*
Allatostatin C		+*
Allatotropin		
Bursicon		
CHHamide		
Corazonin		
Crustacean cardioactive peptide		
Crustacean hyperglycemic hormone		
DENamide		
Diuretic hormone (calcitonin-like)		+*
Diuretic hormone (corticotropin releasing factor-like)		+*
Ecdysis-triggering hormone		
Eclosion hormone		
FMRFamide	FIRFamide	
	Myosuppressin	+*
	Neuropeptide F	+*
	Short neuropeptide F	+*
	Sulfakinin	+*
Intocin		+[a]
Insulin-related peptide		+[b]
Neuroparsin		
Orcokinin		
Periviscerokinin		
Pigment dispersing hormone		+[c]
Proctolin		+*
Red pigment concentrating hormone		+*,†
RYamide		
SIFamide		+*
Tachykinin-related peptide		+*

Data from: [a]Stafflinger et al., 2008; [b]Boucher et al., 2010; [c]Tilden et al., 2011.
*Predicted from the genome but unpublished.
†A partial protein.

transcriptome data, the genome of *D. pulex* was subsequently probed for the genes encoding these and other neuropeptide precursors (Christie et al., 2011). Genes encoding all of the transcripts identified from the EST database were found within the *Daphnia* genome, as were approximately 10 others not discovered in the initial transcriptomic study (Table 1). In total, genes encoding precursor proteins for 23 peptide families or subfamilies resulted from this work (Table 1), with the catalog of predicted mature neuropeptides expanded to over 100 isoforms using these data. For the most part, the precursor proteins deduced from the transcriptome and genome are in good agreement (Fig. 2A), providing increased confidence in both data sets, as well as in the putative mature peptides predicted from them. Several additional transcriptome and genome mining studies have also contributed to, and expanded, the predicted peptidome of *D. pulex* (Stafflinger et al., 2008; Verleyen et al., 2009; Boucher et al., 2010; Dircksen et al., 2011) (Table 1) and, taken collectively, the catalog of putative peptides predicted using *Daphnia*'s unique genomic resources represents one of the largest and most diverse sets of neuropeptides thus far known from any crustacean. Interestingly, comparisons of the predicted *D. pulex* gene structures and their mature peptide products with those of insects and decapod crustaceans show that the peptidergic signaling molecules of *Daphnia* are often more closely akin to their insect counterparts than they are to those of the decapods, further supporting the close phylogenetic relationship of the daphnid lineage to Insecta (Gard et al., 2009; Christie et al., 2011; Dircksen et al., 2011).

In addition to the prediction of the peptidome of *D. pulex*, several putative neuropeptide receptors have been identified *via* genome mining (Table 2). Specifically, the sequences of the receptors for insulin-like peptide (Boucher et al., 2010), oxytocin/vasopressin-like peptide (intocin) (Stafflinger et al., 2008) and pigment dispersing hormone (PDH) (Tilden et al., 2011; Fig. 2B) have been deduced. Although unpublished, genes encoding the receptors for many of the other known *Daphnia* neuropeptides also appear within the *D. pulex* draft genome assembly (Table 2).

The data obtained from genome and transcriptome analyses have allowed for additional studies of peptidergic signaling in *D. pulex*; they also complement and augment a number of earlier or contemporaneous investigations. One direct springboard from the transcriptomic and/or genomic prediction of its peptidome is a recent mass spectral analysis of the neuropeptide complement present in the *D. pulex* CNS (Dircksen et al., 2011). Here, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to probe the *D. pulex* nervous system for its native peptides using both accurate mass matching (masses calculated from the predicted mature peptide sequences; Fig. 3) and tandem MS/MS *de novo* peptide sequencing (Table 1); this study also confirmed the existence of many peptide precursor protein transcripts using reverse transcription-polymerase chain reaction (RT-PCR; the primers for PCR being derived from the predicted gene sequences; Table 1). Using this strategy, 30 neuropeptides were characterized by MS/MS fragmentation sequencing, with 40 peptides identified by accurate mass matching (Fig. 3). These data, and those obtained from the RT-PCR analyses, both vet and further expand the catalog of neuropeptides predicted for *D. pulex*.

Anatomical and physiological studies have also addressed aspects of peptidergic signaling in *Daphnia*. One recently conducted study by Strauss et al. (Strauss et al., 2011) focused on

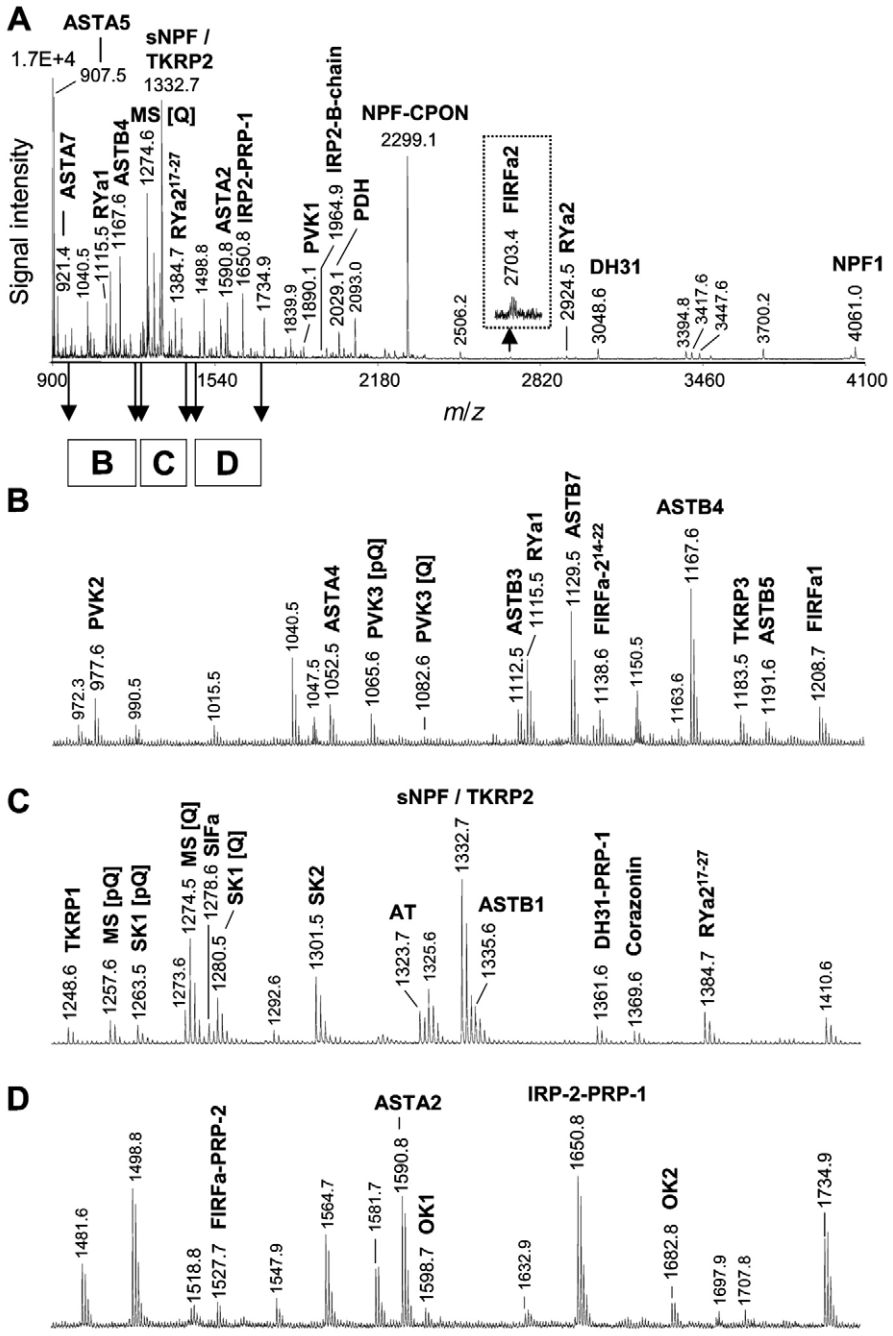

Fig. 3. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) identification of neuropeptides in the *Daphnia pulex* brain. (A) Forty-one peptides were identified by direct tissue profiling of a *D. pulex* brain *via* accurate mass matches; the inset shows an enlargement of the peak corresponding to the FIRFamide 2 peptide. (B–D) Enlargements of the boxed areas of the mass spectrum shown in A. ASTA, A-type allatostatin; ASTB, B-type allatostatin; AT, allatotropin; CPON, carboxyterminal peptide of NPY; DH31, diuretic hormone 31; IRP-2, insulin-related peptide 2; MS, myosuppressin; NPF, neuropeptide F; RYa, RYamide; SIFa, SIFamide; SK, sulfakinin; sNPF, short neuropeptide F; OK, orcokinin; TKRP, tachykinin-related peptide. Figure is reprinted with permission from Dircksen et al. (Dircksen et al., 2011).

assessing the role played by the neurons expressing PDH, a peptide initially discovered in *D. pulex via* transcriptome and genome analyses (Gard et al., 2009; Christie et al., 2011), and later confirmed *via* mass spectrometry (Dircksen et al., 2011). In this investigation, an antibody generated against PDH was used to map the distribution of this peptide in the *Daphnia* brain. Approximately 40 neurons were labeled by the PDH antibody in *D. pulex*. Based on their locations and projection patterns, 13 distinct neuron types were identified and reconstructed, including two types in the visual ganglion that resemble the medial lateral neurons of insects, neurons that produce pigment dispersing factor (the insect homolog of crustacean PDH) and that are key players in establishing circadian rhythmicity. The PDH antibody stains an identical set of neurons in *Daphnia magna*, where experiments conducted under 12 h:12 h light:dark cycles and under constant conditions (either constant light or constant dark) showed significant changes in labeling intensity of specific PDH immunopositive cells over the course of a 24 h period (Strauss et al., 2011). In addition, rhythmic changes in the activity pattern of one PDH-positive medial lateral neuron type, which showed a peak in activity in the relative ‘evening’, were seen in this species. Collectively, these data strongly suggest that PDH, and the medial lateral neurons that produce it, are key components of the circadian signaling system of *Daphnia*. Interestingly, the *Daphnia* circadian system itself has been a recent subject of genome mining (Tilden et al., 2011). Here, *D. pulex* homologs of all the major components necessary for the establishment of a *Drosophila*-like clock, e.g. period, timeless, clock and cycle proteins, as well as ones absent in *Drosophila*, but

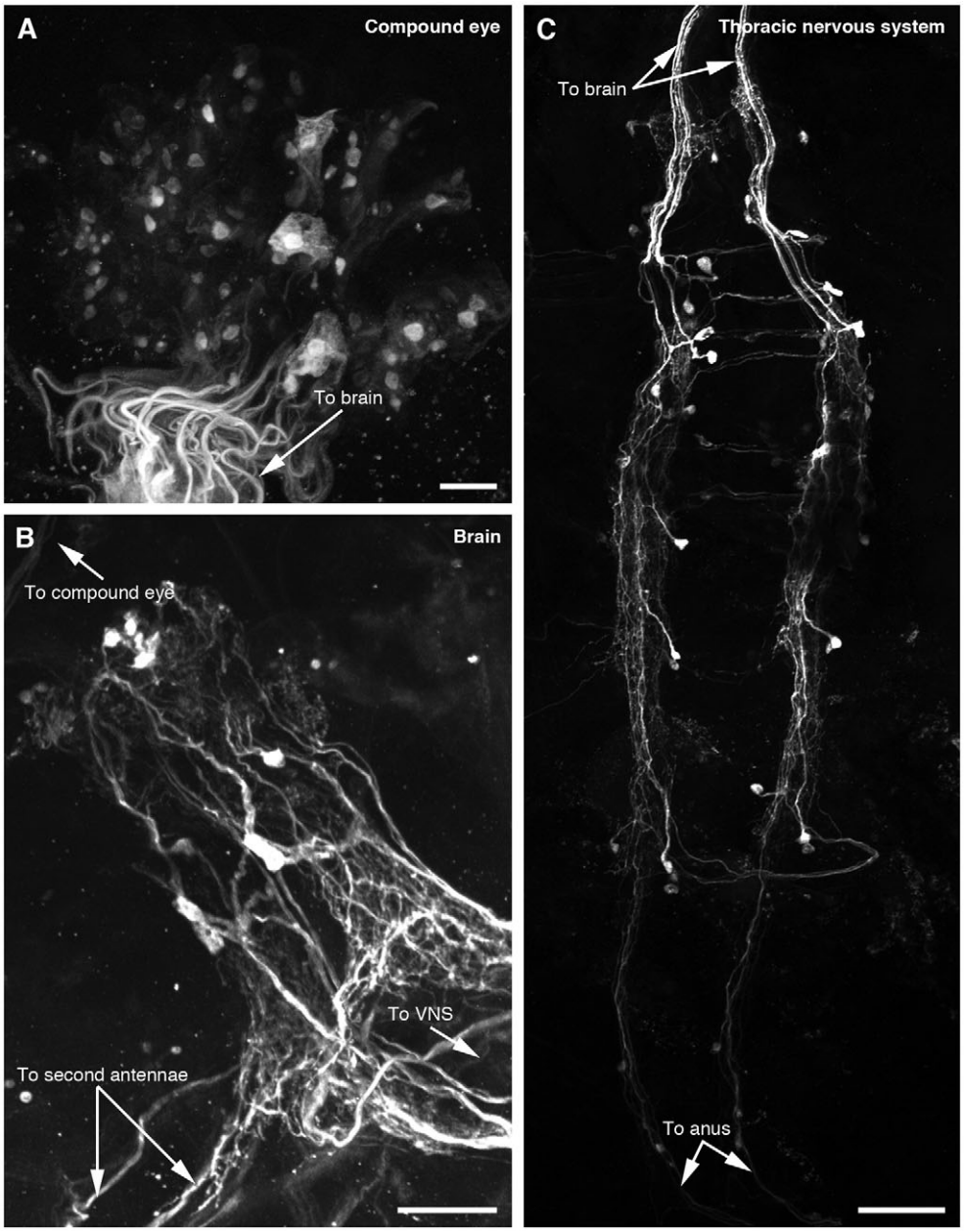

Fig. 4. Confocal micrographs showing histamine-like immunoreactivity in the central nervous systems of *Daphnia pulex* and *Daphnia magna*. (A) Histamine-like labeling in the *D. pulex* compound eye. (B) Histamine-like labeling in the *D. magna* supraoesophageal ganglion (brain). (C) Histamine-like labeling in the *D. magna* thoracic nervous system. Scale bars, 50 μm. VNS, ventral nervous system. Figure is reprinted from McCoole et al. (McCoole et al., 2011).

present in other more ancestral insect circadian systems, e.g. cryptochrome 2, were identified.

Aminergic systems

A second class of commonly used neuronal signaling molecules is amines. In crustaceans, these include dopamine, histamine, octopamine and serotonin (Christie, 2011). As is the case for the peptides, multiple proteins involved in each aminergic signaling pathway have been mined from the *D. pulex* genome using known *Drosophila* proteins as references (McCoole et al., 2011; McCoole et al., 2012a). Specifically, genes encoding the biosynthetic enzymes for each amine were identified, as were genes encoding putative receptors for each of these molecules (Table 3). In addition, genes encoding dopamine and serotonin transporters were found (Table 3). Taken collectively, this suite of data represents the largest number of aminergic genes and/or proteins thus far identified from any crustacean.

Included among the amine pathway proteins identified from the *D. pulex* genome were histidine decarboxylase, the rate-limiting biosynthetic enzyme for histamine, and two histamine-gated chloride channels, hclA and hclB (McCoole et al., 2011). These data strongly support the production of histamine in this species, and were the impetus for both immunohistochemical and biochemical analyses of the roles played by this amine in *Daphnia*, particularly its participation in their phototactic response to ultraviolet (UV) light (McCoole et al., 2011). Using immunohistochemistry, an extensive network of histaminergic somata, axons and neuropil was identified within the *Daphnia* CNS, including labeling of photoreceptor cells in the compound eye and projections from them to the brain, data supporting a role for histamine in the visual system (Fig. 4). To assess its actions on phototaxis directly, a behavioral assay was developed in which animals (here *D. magna*, whose complement of histaminergic neurons is identical to that of *D. pulex*; Fig. 4) were exposed to

Table 3. *Daphnia pulex* aminergic pathway proteins identified *via* genome mining

Amine	Protein	Putative function
Dopamine	Tryptophan-phenylalanine hydroxylase[a]	Biosynthesis
	Tyrosine hydroxylase[a]	Biosynthesis
	DOPA decarboxylase[a]	Biosynthesis
	Type 1-like dopamine receptor[a]	Receptor
	Type 2-like dopamine receptor[a]	Receptor
	Dopamine transporter[a]	Transporter
Histamine	Histidine decarboxylase[b]	Biosynthesis
	Histamine-gated chloride channel A[b]	Receptor
	Histamine-gated chloride channel B[b]	Receptor
Octopamine	Tryptophan-phenylalanine hydroxylase[a]	Biosynthesis
	Tyrosine decarboxylase[a]	Biosynthesis
	Tyramine-β-hydroxylase[a]	Biosynthesis
	α-Adrenergic-like octopamine receptor[a]	Receptor
	β-Adrenergic-like octopamine receptor[a]	Receptor
Serotonin	Tryptophan hydroxylase[a]	Biosynthesis
	Tryptophan-phenylalanine hydroxylase[a]	Biosynthesis
	DOPA decarboxylase[a]	Biosynthesis
	Type 1-like serotonin receptor[a]	Receptor
	Type 7-like serotonin receptor[a]	Receptor
	Serotonin transporter[a]	Transporter

Data from: [a]McCoole et al., 2012a; [b]McCoole et al., 2011.

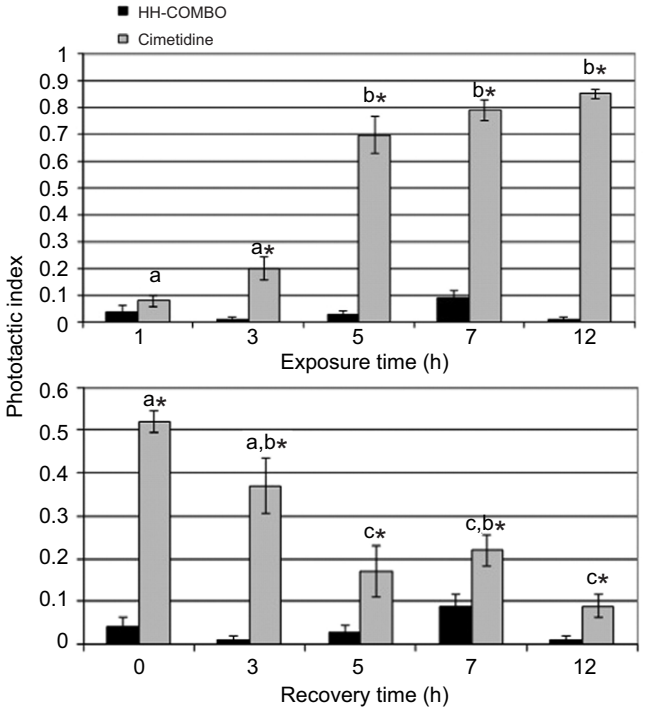

Fig. 5. Effects of cimetidine (2×10^{-3} mol l^{-1}) on the phototactic response of *Daphnia magna* to UV light. (A) Cimetidine-induced inhibition of the phototactic response to UV. (B) Recovery of the phototactic response following washout of a 5 h exposure to cimetidine. In both A and B, a phototactic index of 0 represents the normal negative phototactic response seen upon UV light exposure. *Statistically significantly different from respective control (P<0.05). Lowercase letters in the two panels denote treatments that are not significantly different from one another in their respective panel. HH-COMBO, high-hardness COMBO medium (a defined freshwater culture medium). Figure modified from McCoole et al. (McCoole et al., 2011).

cimetidine, a type-2 histamine receptor antagonist, which is known to block both the hclA and hclB channels in *D. melanogaster* (the species whose proteins were used as queries for the discovery of the *D. pulex* hclA and hclB genes). In the presence of this channel blocker, *D. magna*'s negative phototactic response to UV exposure was inhibited in a reversible, time-dependent manner (Fig. 5). Taken collectively, these genomic, anatomical and behavioral data demonstrate the role of histamine in mediating the negative phototaxis seen in response to UV light exposure in *Daphnia*, one of the behaviors commonly employed as an assay for determining the effects of environmental and anthropogenic stressors on these animals.

Diffusible gas and small molecule transmitter systems

The genome of *D. pulex* has also been mined for putative proteins involved in diffusible gas and small molecule transmitter signaling (McTaggart et al., 2009; McCoole et al., 2012b). For the former class of molecules, the nitric oxide and carbon monoxide pathways were investigated, whereas for the latter group, the acetylcholine, glutamate and γ-aminobutyric acid (GABA) systems were targeted. Genes encoding proteins required for the establishment of each gas and small molecule transmitter signaling system, including biosynthetic enzymes, receptors and transporters, were identified (Tables 4, 5). As for the peptides and amines, these data represent the largest number of gas and small molecule transmitter pathway genes and/or proteins thus far identified from any single crustacean.

Although no studies have focused on the physiological roles of neurally released gas transmitters, there is evidence that small molecule transmitters play roles in the expression of predator-induced morphological changes in *Daphnia*, specifically, in the induction of the neck teeth, structures that are postulated to reduce the animals' susceptibility to predation. Early studies noted that pesticides that target the acetylcholine and GABA signaling systems modulate the kairomone-induced expression of these structures in *Daphnia* (Hanazato and Dodson, 1993; Barry, 1998). To more directly link this modulation to the cholinergic and GABAergic systems, Barry (Barry, 2002) investigated the actions of stimulators and inhibitors of both acetylcholine and GABA neurotransmission on the *Chaoborus* (a dipteran insect with an aquatic larval stage)-induced expression of neck teeth in *D. pulex* neonates. Enhancement in the development of these structures was seen in the presence of both stimulators of cholinergic transmission and inhibitors of GABA; inhibitors of acetylcholine suppressed the expression of this inducible defense mechanism. The author proposed that acetylcholine or GABA released from secretory neurons were the targets of the compounds used, and that modulation of the cholinergic and/or GABAergic systems of *D. pulex* by substances affecting these systems could lead to inappropriate expression of neck teeth in environments with low predations rates or to their suppression in environments where there is a high risk of predation, effects with significant consequences for

Table 4. *Daphnia pulex* diffusible gas transmitter pathway proteins identified *via* genome mining

Gas	Protein	Putative function
Carbon monoxide	Heme oxygenase 2	Biosynthesis
	Soluble guanylyl cyclase[a]	Receptor
Nitric oxide	Nitric oxide synthase	Biosynthesis
	Soluble guanylyl cyclase[a]	Receptor

All data are from McCoole et al., 2012b.
[a]Both α- and β-subunit proteins identified.

Table 5. *Daphnia pulex* small molecule transmitter pathway proteins identified *via* genome mining

Small molecule transmitter	Protein	Putative function
Acetylcholine	Choline acetyltransferase	Biosynthesis
	Vesicular acetylcholine transporter	Packaging
	Nicotinic acetylcholine receptor[a]	Receptor
	Muscarinic acetylcholine receptor	Receptor
	Acetylcholine esterase	Inactivation
Glutamate	Glutaminase	Biosynthesis
	Vesicular glutamate transporter	Packaging
	Type-I ionotropic glutamate receptor	Receptor
	Type-II ionotropic glutamate receptor[b]	Receptor
	NMDA-type ionotropic glutamate receptor	Receptor
	Metabotropic glutamate receptor	Receptor
	Excitatory amino acid transporter	Reuptake
	Glutamate dehydrogenase	Recycling
	Glutamine synthetase	Recycling
GABA	Glutamic acid decarboxylase	Biosynthesis
	Vesicular GABA transporter	Packaging
	$GABA_A$ receptor	Receptor
	$GABA_B$ receptor[c]	Receptor

All data are from McCoole et al., 2012b.
[a]α1-, α2-, α3-, α4-, α5-, α6-, β1- and β2-subunit proteins identified.
[b]IID- and IIE-subtype receptors identified.
[c]Subtype 1, 2 and 3 receptors identified.

individual fitness and, as a keystone species, for the fitness of ecosystems as a whole.

Future directions

As outlined in earlier parts of this Commentary, much recent work has focused on elucidating the neurochemical signaling systems of *D. pulex* using its publicly accessible genomic resources. This included studies of its peptidergic, aminergic, gas and small molecule transmitter signaling systems, which encompass the vast majority of pathways used by the nervous system for chemical communication. Taken collectively, the knowledge amassed through these investigations makes the molecular neurochemistry of *D. pulex* the most completely cataloged of any crustacean; it now rivals that of the best-studied insects.

Although only a handful of anatomical, biochemical and physiological studies of neurochemical signaling have been conducted on *Daphnia*, the molecular data generated from genome and transcriptome mining should allow for rapid expansion in these areas. For example, the sequences of the neurochemical pathway genes and transcripts described in these studies can be used to generate primers for PCR-based investigations of gene expression (e.g. Strauss et al., 2011). These sequences can also be used to design gene-specific probes for *in situ* hybridization analyses of the cellular distribution of the expressed genes (e.g. Hsu et al., 2008a; Hsu et al., 2008b), both spatially and temporally. Similarly, the protein sequences deduced from the gene and/or transcript sequence data will allow for the production of synthetic proteins and/or peptides, which can be used to generate antibodies for both spatial and temporal immunohistochemical mapping studies of gene products (e.g. Gard et al., 2009; McCoole et al., 2011; Strauss et al., 2011). Lastly, the synthesis of putative mature neuropeptides will allow for direct physiological investigations of their bioactivities both in *Daphnia* and in heterologous crustacean and insect models (e.g. Marco and Gäde, 2010).

One area in which the molecular data amassed from *Daphnia* will surely be of use is as an intra-subphylum reference for future crustacean genome or transcriptome assembly and annotation. There is a general consensus that one of the major difficulties in undertaking any large-scale genome or transcriptome project is the identification and use of a proper reference set for assembling and annotating the sequence data from the species being investigated. Although numerous insect genomes have been assembled and annotated, for example those of the fruit fly *D. melanogaster* (Adams et al., 2000) and the honeybee *A. mellifera* (Weinstock et al., 2006), the genome of *D. pulex* is the only crustacean resource of this type currently extant. As genomic information has been of significant use in elucidating neurochemical signaling system in many insects (Hewes and Taghert, 2001; Riehle et al., 2002; Hauser et al., 2006; Hummon et al., 2006; Liu et al., 2006; Predel and Neupert, 2007; Hauser et al., 2008; Li et al., 2007; Hauser et al., 2010), it is likely that the neurochemical data set amassed for *D. pulex* will provide a critical, and thus far unique, intra-subphylum reference for assembling and annotating future crustacean genomes and transcriptomes.

Another novel and exciting contribution that the *Daphnia* genomic and transcriptomic data may provide to the field of crustacean neurochemistry is its use in the development of RNA interference methods for studying the role(s) played by specific signaling systems in the control of physiology and behavior. As outlined earlier, a large number of proteins involved in peptidergic, aminergic, gas and small molecule transmitter signaling have been identified in *D. pulex*. Although this catalog is in and of itself an important contribution to the field, it also generates the problem of how to begin to understand the individual functions of the newly discovered genes and proteins. One method that has been used for such analyses in non-transformable animals is double-stranded RNA (dsRNA)-mediated RNA interference, which allows for specific gene silencing. Kato et al. (Kato et al., 2011) have recently established such a method using ovulated *D. magna* eggs. Specifically, freshly ovulated eggs were injected with dsRNA for the distal-less gene, a gene controlling appendage development. These injections appeared to successfully silence distal-less mRNAs, resulting in a dose-dependent truncation of the second antenna. In support of this silencing being gene-specific were demonstrations that non-overlapping dsRNAs generated for the

distal-less gene produced the same alteration in phenotype, and that injections of unrelated dsRNA induced no changes in morphology. Given the extensive characterization of *Daphnia*'s behavioral repertoire, similar studies targeting neurochemical pathway genes are likely to provide major insights into the functional roles served by their encoded proteins in these animals and, using *D. pulex* as a proxy, in crustaceans more broadly.

Concluding remarks

Prior to 2009, little was known about neurochemical signaling in *D. pulex* on any level, a surprising fact given the model organism status of *Daphnia* for many biological fields and its long history of behavioral study, the modulation of which is undoubtedly rooted in its neurochemistry. The sequencing and public deposition of the *D. pulex* genome and transcriptome have dramatically changed this situation over the past several years, with genome and transcriptome mining allowing for the identification of large numbers of peptidergic, aminergic, gas and small molecule transmitter pathway proteins from this species. Taken collectively, these data now make *D. pulex* perhaps the best characterized crustacean in terms of its molecular neurochemistry, and our understanding of the putative proteins involved in its neurochemical signaling systems now rivals or surpasses those of most insects. The molecular data that have been obtained for *Daphnia*, in combination with its well-documented behavioral repertoire, now positions the field of crustacean neurochemistry to make significant strides in understanding how specific neurochemical pathway proteins manifest themselves in the overt expression of a myriad of behavioral and physiological outputs. Clearly this information will be of great benefit to our understanding of *Daphnia* biology (e.g. phenotypic plasticity, circadian rhythmicity, escape behavior, etc.) and, for that matter, of neurochemical signaling in a general sense.

Glossary

Ecotoxicology
: The study of the effects of toxic compounds on organisms, typically at the level of populations and/or ecosystems.

Genome
: The full complement of genetic material for an individual, generally encoded in DNA.

Hormone
: A signaling molecule whose target cells, tissues, etc. are located at a distance from the release site of the molecule in question, typically transported to a target *via* the circulatory system.

Paracrine
: A signaling molecule whose target cells, tissues, etc. are located in close proximity to the release site of the molecule in question.

Peptidome
: The full complement of peptides present in a given cell, tissue, organism or population of organisms.

Toxicogenomics
: The study of the structure and function of genomes in response to adverse xenobiotic exposure.

Transcriptome
: The full complement of RNA transcripts produced by a given cell, tissue, organism or population of organisms at the particular moment in time at which the sample in question was collected.

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