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Identification of viruses associated with larvae of the dragonfly *Leucorrhinia dubia*, and damselfly *Coenagrion puella* from RNA sequencing data

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Dragonflies and damselflies are hosts to a variety of parasites and pathogens. However, very few studies have investigated which viruses infect dragonflies and damselflies. Here, based on next generation RNA sequencing of RNA from *Leucorrhinia dubia* (Libellulidae, Anisoptera) and *Coenagrion puella* (Coenagrionidae, Zygoptera) larvae, data on putative viruses present in odonates are reported. In both species around 20 different putative viruses, often belonging to genera known from other insect species, were found. The annotated genome structure of one novel putative iflavirus in *C. puella* and new putative iflavirus, dicistrovirus and posa-lika viruses in *L. dubia* are described. The influence of these viruses on host fitness and their modes of transmission remain to be determined.

Keywords: *Coenagrion puella;* dragonfly; *Leucorrhinia dubia;* Odonata; RNAseq; viruses

Introduction

All multicellular organisms are habitats for a plethora of parasites and pathogens. They range from highly visible ectoparasites such as ticks to intracellular parasites such as viruses. In odonates a variety of pathogens and parasites have been described including water mites, platyhelminths, single-cell eukaryotes, bacteria, and viruses (Corbet, 1999). Only gregarines, eukaryotic single-celled pathogens (Siva-Jothy & Plaistow, 1999), and water mites (Rolff, 2001) have been studied in terms of the life cycles of the pathogens and fitness effects on the hosts.

Viruses, omnipresent in all forms of life, have only been described in a handful of papers in odonates (Charpentier, 1979; Dayaram, Galatowitsch, Harding, Argüello-Astorga, & Varsani, 2014; Dayaram et al., 2013, 2015; Rosario et al., 2011, 2012, 2013) which mostly focus on circular single-stranded DNA viruses. Next-generation sequencing of genomes and transcriptomes now allows the discovery of viruses across the tree of life. Here we make use of a transcriptome assembly of the damselfly *Coenagrion puella* (Johnston & Rolff, 2013) and an unpublished transcriptome of *Leucorrhinia dubia* from two different habitats, all acquired using RNAseq technology (Haas et al., 2013), to describe putative viruses in odonates. We compare

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those findings to the literature not only in odonates but also with a focus on other groups of insects and discuss the research questions resulting from our findings.

Materials and methods

Total RNA was isolated from five *Leucorrhinia dubia* larvae from each of two populations (population 1 with fish predators, in Mjösjön; population 2 with dragonfly predators, in Grösjön; see Mikolajewski et al., 2010). Individual larvae were homogenized in cold Trizol (Sigma, Taufkirchen, Germany) with a 5-mm steel bead (Qiagen, Hilden, Germany) using a TissueLyzer (Qiagen) at 30 Hz for 30 s. Total RNA was isolated according to the manufacturers instructions after digestion with Turbo DNase (Ambion, Bremen, Germany). Equal quantities of total RNA were used to create two population pools which were then used by TGAC (Norwich, UK) to construct barcoded Truseq mRNA libraries and sequenced on a HiSeq2000 (Illumina, San Diego). Using Trinity version r2013_08_14 with a minimum kmer coverage of 2 (Haas et al., 2013), reads from both samples were combined, digitally normalized, and assembled into contiguous sequences (contigs) which were annotated using blastx versus the RefSeq protein database. Trimmed, un-normalized reads were mapped to the assembly using bowtie (Langmead, Trapnell, Pop, & Salzberg, 2009) and RSEM (Li & Dewey, 2011), and corrected for differences in library size using DESeq2 (Love, Huber, & Anders, 2014) in R. *Coenagrion puella* viral contigs were annotated as above using a previously described assembly of RNAseq data which is available from NCBI SRA under accession SRX371959 (Johnston & Rolff, 2013). Putative viral genomes were annotated using Prokka (Seemann, 2014). Maximum likelihood phylogenetic trees were constructed by aligning protein sequences using MUSCLE and Gblocks in conjunction with PhyML and TreeDyn (Dereeper et al., 2008).

Results

Leucorrhinia dubia sequencing yielded 36,064,334 and 32,933,205 100-bp paired-end reads from populations 1 (with fish predators) and 2 (without fish predators) respectively. Following assembly and annotation, 36 putative viral sequences (which may be endogenous or exogenous) were identified from 77,021 assembled RNA sequences (Table 1). The majority of viral contigs were short and likely represent partial transcripts originating from the most highly expressed parts of a given viral genome or partial coverage of RNA virus genomes. Three sequences with similarities to different picorna-like viruses likely represent complete genomes of approximately 9-10 kb in length (Table 1). Two of these, an *Iflavirus* and a posa-like virus, showed greater than twofold population biased coverage in population 2 (Figure 1). Comparison with RNA sequencing data from *C. puella* showed that a similar number of putative viral sequences associated with both odonate species but with different taxonomic compositions. *Iflavirus*-like sequences were abundant in assemblies from both species, as were Ambidensoviruses (Figure 2). *Coenagrion puella* viral contigs included a putative complete *Iflavirus* genome of approximately 9 kb. This iflavirus clustered well within other insect viruses and the *L. dubia* iflavirus was based in a neighboring clade (Figure 3). It is notable that both these odonate viruses display a long branch length indicative of a high substitution rate. The other novel virus described here likely belongs to the cripaviruses and is referred to as *L. dubia* dicistrovirus (Figure 4).

Both iflaviruses share identical genome organization with a single open reading frame encoding a polyprotein with capsid, RNA helicase, peptidase, and RNA-dependent RNA polymerase (RdRp) domains found in a typical *iflavirus* N- to C-terminus arrangement (Figure 5A, B).

Table 1. Summary of putative viral contigs identified from *Leucorrhinia dubia* RNAseq data.

Contig	Best RefSeq protein blastx hit	E value	Identity (%)	Family	Contig length (bp)	No. reads mapped	
						Population 1	Population 2
comp78686 c0 seq1	*Bombyx mandarina nucleopolyhedrovirus* GP64/67	5e-07	31.11	Baculoviridae	325	0	1
comp46216 c0 seq1	*Epiphyas postvittana nucleopolyhedrovirus* GP64	8e-05	31.48	Baculoviridae	741	11	11
comp17160 c1 seq1	*Uukuniemi virus* RNA polymerase	1e-06	23.09	Bunyaviridae	1765	0	111
comp6827 c0 seq1	*Triatoma virus* nonstructural protein precursor	7e-07	26.53	Dicistroviridae	1809	17	56
comp35918 c0 seq1	*Cricket paralysis virus* nonstructural polyprotein	7e-103	33.17	Dicistroviridae	9028[a]	23321	40884
comp25810 c1 seq2	*Border disease virus* nonstructural protein NS5B	5e-14	28.63	Flaviviridae	1613	51	39
comp65687 c0 seq1	*Heron hepatitis B virus* polymerase	5e-04	33.33	Hepadnaviridae	211	1	3
comp53458 c0 seq1	*Ross's goose hepatitis B virus* polymerase	2e-15	30.15	Hepadnaviridae	577	7	8
comp26355 c0 seq1	*Slow bee paralysis virus* polyprotein	2e-93	31.23	Iflaviridae	10240[a]	16	43410
comp62952 c0 seq1	*Slow bee paralysis virus* polyprotein	1e-09	47.76	Iflaviridae	201	3	1
comp58218 c0 seq1	*Kakugo virus* polyprotein	3e-07	36.62	Iflaviridae	217	3	1
comp64156 c0 seq1	*Slow bee paralysis virus* polyprotein	1e-10	37.08	Iflaviridae	266	5	1
comp52159 c0 seq1	*Brevicoryne brassicae picorna-like virus* polyprotein	4e-06	34.95	Iflaviridae	322	5	3
comp63334 c0 seq1	*Brevicoryne brassicae picorna-like virus* polyprotein	4e-31	54.17	Iflaviridae	360	4	2
comp68204 c0 seq1	*Deformed wing virus* polyprotein	8e-06	25.56	Iflaviridae	475	5	5
comp44122 c0 seq1	*Brevicoryne brassicae picorna-like virus* polyprotein	1e-11	27.53	Iflaviridae	599	11	13
comp44221 c0 seq1	*Deformed wing virus* polyprotein	3e-12	25.00	Iflaviridae	721	12	5
comp38117 c0 seq1	*Striped Jack nervous necrosis virus* protein A	3e-32	37.45	Nodaviridae	1143	0	39
comp60830 c0 seq1	*Striped Jack nervous necrosis virus* protein A	5e-14	50.67	Nodaviridae	228	0	4
comp25778 c0 seq1	*Midway virus* RNA-dependent RNA polymerase	1e-16	26.82	Nyamiviridae	3280	314	223
comp26247 c0 seq1	*Nyamanini virus* RNA-dependent RNA polymerase	3e-06	28.12	Nyamiviridae	762	18	8
comp60486 c0 seq1	*Aedes aegypti densovirus* nonstructural protein 1	5e-04	51.85	Parvoviridae	313	6	2
comp69172 c0 seq1	*Aedes aegypti densovirus* nonstructural protein 2	9e-17	48.05	Parvoviridae	322	4	2
comp12081 c0 seq1	*Aedes albopictus densovirus* AlDNVgp2	7e-08	37.33	Parvoviridae	417	10	4
comp76692 c0 seq1	*Cotesia congregata bracovirus* CcBV 30.5	2e-05	61.29	Polydnaviridae	218	1	2
comp70164 c0 seq1	*Glypta fumiferanae ichnovirus* GfV-B16-ORF1	1e-05	40.00	Polydnaviridae	220	1	4
comp22050 c0 seq1	*Cotesia congregata bracovirus* CcBV 30.5	2e-14	36.04	Polydnaviridae	340	1	4
comp9295 c0 seq1	*Glypta fumiferanae ichnovirus* GfV-B16-ORF1	2e-08	27.95	Polydnaviridae	605	15	15
comp44153 c0 seq1	*Melanoplus sanguinipes entomopoxvirus* MSV061	4e-07	41.54	Poxviridae	296	3	2
comp33248 c0 seq2	*Melanoplus sanguinipes entomopoxvirus* MSV061	4e-59	45.14	Poxviridae	815	46	56
comp6892 c0 seq1	*Eyach virus* VP2	2e-10	28.12	Reoviridae	1316	38	34
comp12483 c0 seq1	*Soil-borne cereal mosaic virus* replicase	1e-13	31.62	Rhabdoviridae	428	6	5
comp69465 c0 seq1	*Heterosigma akashiwo RNA virus* polyprotein	6e-05	48.00	Sequiviridae	241	1	2
comp57378 c0 seq1	*Hibiscus green spot virus* polyprotein	6e-04	44.44	Unassigned	266	2	1
comp30090 c0 seq1	*Posavirus non-structural* polyprotein	2e-29	26.11	Unassigned	9083[a]	25	114967
comp60375 c0 seq1	*Potato mop-top virus* polymerase	1e-04	40.74	Virgaviridae	230	4	0

[a]Putative complete genome.

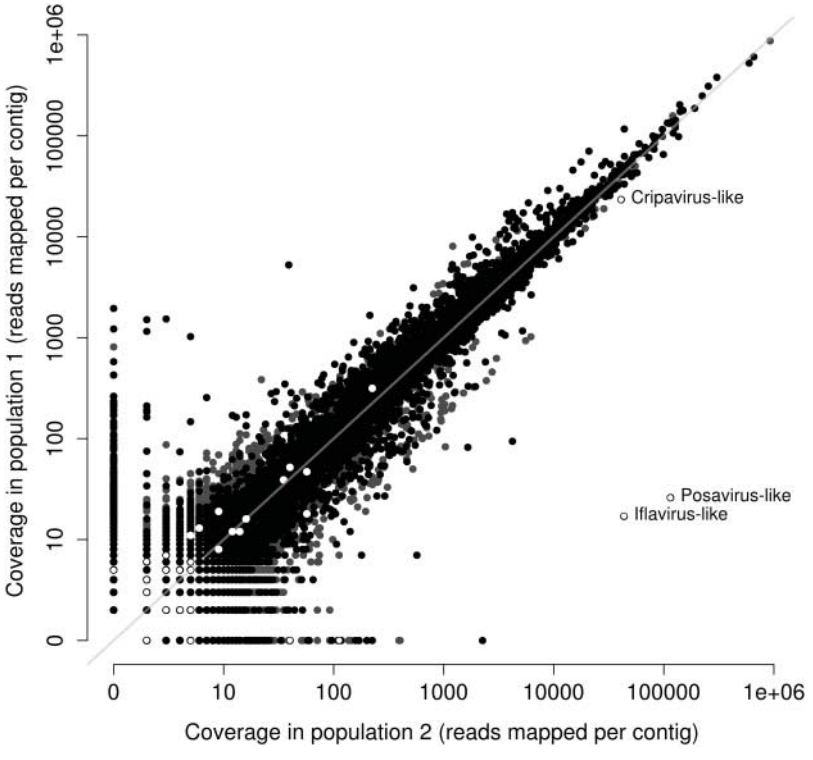

Figure 1. Viral contig coverage in two populations of *Leucorrhinia dubia*. Grey circles, unannotated contigs; black circles, annotated contigs; white circles, putative viral contigs.

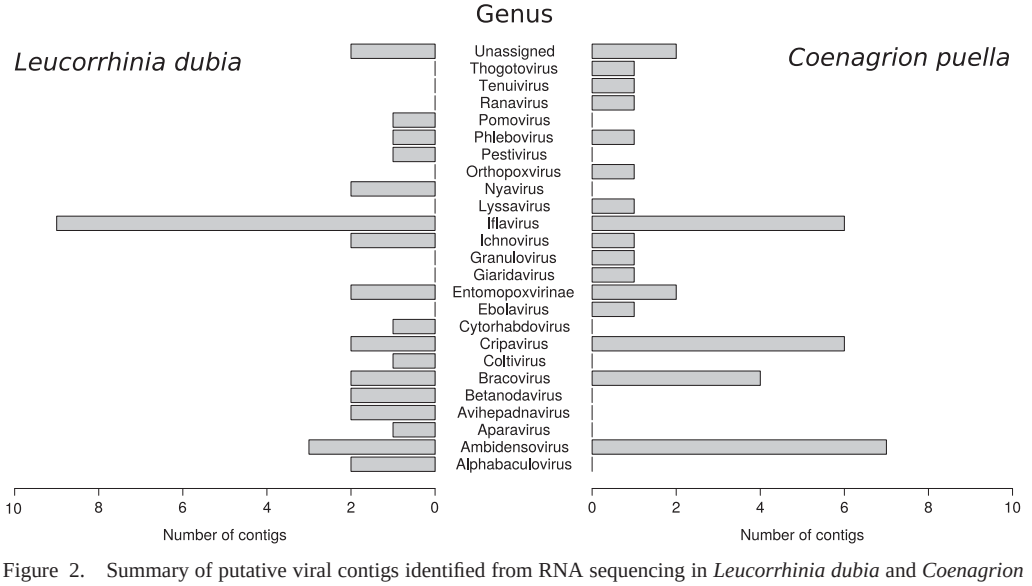

Figure 2. Summary of putative viral contigs identified from RNA sequencing in *Leucorrhinia dubia* and *Coenagrion puella* summarized by the genus of the best RefSeq protein blastx hits.

This structure was also observed in *L. dubia dicistrovirus* where a bicistronic organization is predicted to lead to the production of distinct structural and non-structural polyproteins, typical of the family *Dicistroviridae* (Figure 5C). The genome of the *L. dubia* posa-like virus showed

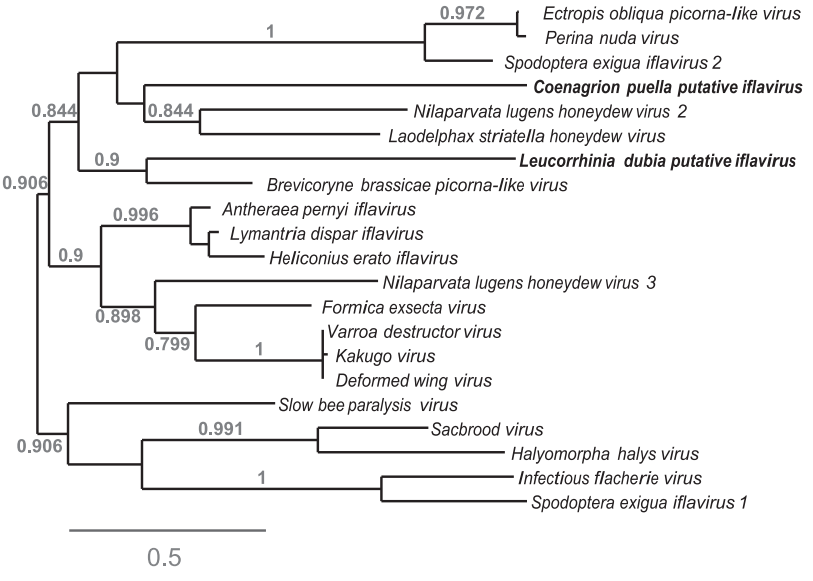

Figure 3. Maximum likelihood phylogenetic tree showing relationships among iflaviruses. Protein sequences were aligned using MUSCLE and Gblocks. Trees were constructed using PhyML and TreeDyn using phylogeny.fr webserver. Grey numbers denote confidence indices. Branch length is proportional to the number of substitutions per site.

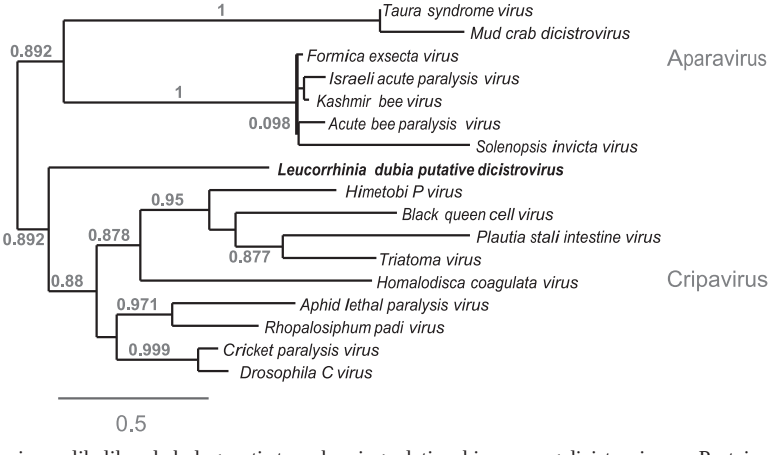

Figure 4. Maximum likelihood phylogenetic tree showing relationships among dicistroviruses. Protein sequences were aligned using MUSCLE and Gblocks. Trees were constructed using PhyML and TreeDyn using phylogeny.fr webserver. Grey numbers denote confidence indices. Branch length is proportional to the number of substitutions per site.

distinct *Posavirus* organization with capsid domains found in the C- terminus of the polyprotein (Figure 5D).

Discussion

There have been very few studies of viruses as pathogens of odonates. Following on from a microscopic study (Charpentier, 1979) only a handful of studies have demonstrated the rich diversity of viruses in dragonflies (Dayaram et al., 2013, 2014, 2015; Rosario et al., 2011, 2012, 2013). Rosario et al. (2011, 2012, 2013) describe cycloviruses in dragonflies from New Zealand,

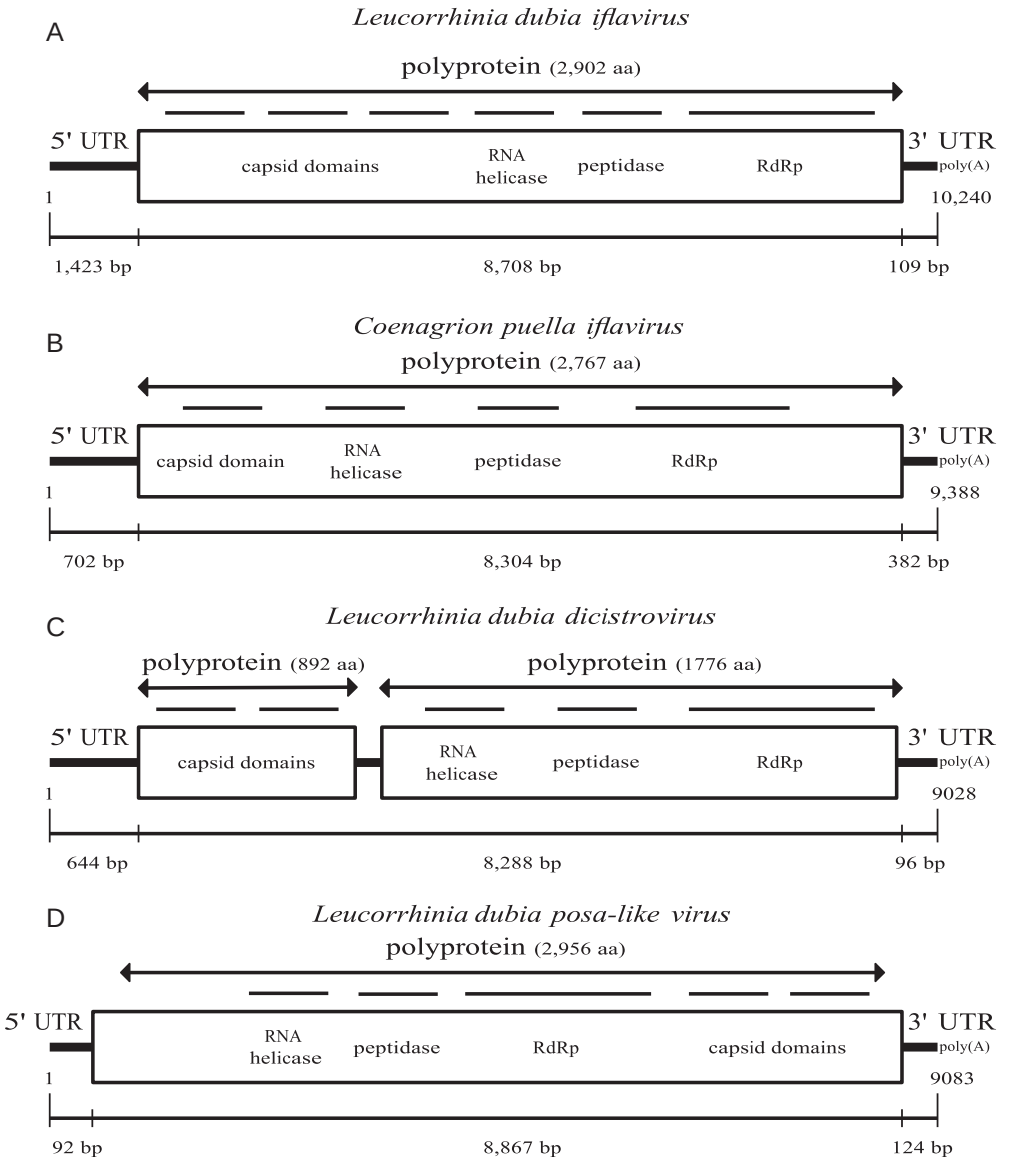

Figure 5. Genome organization of putative RNA viruses of odonates. The order of the conserved protein domains as well as the predicted sizes of untranslated regions, open reading frames, and polypeptides are given for A, *Leucorrhinia dubia iflavirus*; B, *Coenagrion puella iflavirus*; C, *Leucorrhinia dubia dicistrovirus*; D, *Leucorrhinia dubia* posa-like virus. UTR, untranslated region; bp, base pair; aa, amino acid; RdRp, RNA-dependent RNA polymerase.

Australia and North America. Cycloviruses are closely related to circoviruses, which are usually found in vertebrates including humans. Yet we did not find any evidence for cycloviruses in our *L. dubia* samples from Sweden, possibly due to a methodological bias of RNAseq towards RNA viruses.

Charpentier's (1979) morphological description of a densovirus from *L. dubia* seems to be the earliest description of any virus in odonates (Corbet, 1999). Here we found evidence of densoviruses in *L. dubia* from two populations. Overall, the two populations harbored a very similar virome (Table 1, Figure 1) with two viruses showing notably elevated coverage. Exposure

to non-pathogenic bacteria is known to reinforce the negative effects of predation risk in *C. puella* larvae (Janssens & Stoks, 2014). The differential threat of fish predation experienced in these two populations raises the possibility of a link between predation and viral susceptibility, as has been investigated in amphibian larvae (Haislip, Hoverman, Miller, & Gray, 2012).

We believe that we present here a first description of the viral communities of zygopterans. We recovered a similar set of putative (insect) viruses from *C. puella* as we recovered from *L. dubia* (Figure 2). Whether this reflects a wide spread of particular viruses or is caused by lack of power to distinguish between certain viruses remains to be tested. While the majority of viruses in *L. dubia* and in *C. puella* were found in all pooled samples, the *C. puella* iflavirus was only present in one of the sequenced pools, which is an indication for variation of infection rates in within the population.

The role of viruses in the biology of odonates is unknown. All we can state at this time is that viruses are abundant and diverse in the two odonate species we studied, similar to other insect species. Many of the viruses we found, such as iflaviruses, dicistroviruses and densoviruses, are known to reduce fitness in other insect species by either impacting on survival or reproduction (Mondet, de Miranda, Kretzschmar, Le Conte, & Mercer, 2014; Szelei et al., 2011).

Moreover, nothing is known about the transmission of these viruses. We extracted RNA from larval *L. dubia* and larval *C. puella*, indicating that they were infected in the aquatic environment. A recent study on the fecal virome of wild rodents (Phan et al., 2011) noted an abundance of insect-related viruses including members of the Dicistroviridae, Iridoviridae, Polydnaviridae and the subfamily Densovirinae, indicating the potential for transmission via predation. Another noteworthy speculation is based on the abundance and prevalence of water mites in a wide variety of odonate species (Rolff, 2001): it is plausible that parasitic water mites may vector viruses within and between life stages of their odonate hosts as occurs in other insects such as honey bees (Bowen-Walker, Martin, & Gunn, 1999).

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Supplemental data

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