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Running title: Prey and symbiont detection by metagenomics

DEBORA P. PAULA,[*†] BENJAMIN LINARD,[†] DAVID A. ANDOW,[‡] EDISON R. SUJII,[*] CARMEN S. S. PIRES,[*] ALFRIED P. VOGLER[†$]

[*]*Embrapa Genetic Resources and Biotechnology, Parque Estação Biológica, W5 Norte, P.O. Box 02372, Brasília, DF, 70770-917, Brazil,* [†]*Department of Life Sciences, Natural History Museum, Cromwell Rd, London, SW7 5BD, UK,*[‡] *Department of Entomology, University of Minnesota, 219 Hodson Hall, 1980 Folwell Ave., St. Paul, MN 55108, USA;* [$] *Department of Life Sciences, Imperial College London, Silwood Park Campus, Ascot, SL7 5PY, UK*

Correspondence: D. P. Paula, Fax: +55 (61) 34484929;

E-mail: debora.pires@embrapa.br

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Abstract

DNA methods are useful to identify ingested prey items from the gut of predators, but reliable detection is hampered by low amounts of degraded DNA. PCR-based methods can retrieve minute amounts of starting material but suffer from amplification biases and cross-reactions with the predator and related species genomes. Here, we use PCR-free direct shotgun sequencing of total DNA isolated from the gut of the harlequin ladybird *Harmonia axyridis* at five time points after feeding on a single pea aphid *Acyrthosiphon pisum.* Sequence reads were matched to three reference databases: Insecta mitogenomes of 587 species, including *H. axyridis* sequenced here; *A. pisum* nuclear genome scaffolds; and scaffolds and complete genomes of 13 potential bacterial symbionts. Immediately after feeding, multicopy mtDNA of *A. pisum* was detected in tens of reads, while hundreds of matches to nuclear scaffolds were detected. Aphid nuclear DNA and mtDNA decayed at similar rates (0.281 and 0.11 h^{-1} respectively), and the detectability periods were 32.7 and 23.1 h. Metagenomic sequencing also revealed thousands of reads of the obligate *Buchnera aphidicola* and facultative *Regiella insecticola* aphid symbionts, which showed exponential decay rates significantly faster than aphid DNA (0.694 and 0.80 h^{-1} respectively). However, the facultative aphid symbionts *Hamiltonella defensa*, *Arsenophonus* spp. and *Serratia symbiotica* showed an unexpected temporary increase in population size by 1-2 orders of magnitude in the predator guts before declining. Metagenomics is a powerful tool that can reveal complex relationships and the dynamics of interactions among predators, prey and their symbionts.

Introduction

Molecular gut content analysis has been used to identify the prey consumed by invertebrate predators, allowing the study of specific trophic interactions that naturally occur in the field (Pompanon *et al.* 2012, Greenstone *et al*. 2014). Various approaches have been developed to assess the presence of target prey remaining in predator guts via protein-based analyses (e.g. by isoenzymes electrophoresis, ELISA, Western-Blot) or DNA-based analyses (e.g. by PCR, and qPCR) (Symondson 2002; Hardwood & Obrycki 2005; Greenstone *et al*. 2007; Weber & Lundgren 2009; Zeale *et al*. 2010). These molecular tools require the development of species-specific antibodies or DNA primers for amplification of target genes, or time-consuming cloning of PCR products and subsequent Sanger sequencing. Despite their great contribution to contemporary studies of trophic interactions due to their high specificity and sensitivity, they are limited to detect a few target prey molecules.

Since the advent of high-throughput DNA sequencing, diet analyses based on feces have been assessed in several mammals, birds and insects through barcode region sequencing, known as metabarcoding (Valentini *et al.* 2009a; 2009b; Deagle *et al.* 2010; Hereward & Walter 2012; Pompanon *et al.* 2012; Vesterinen *et al.* 2013). In these studies, DNA barcodes allow the detection of a spectrum of species against a set of DNA reference sequences, without need of cloning PCR products. However, despite being less time-consuming and very sensitive, there are still limitations, such as the need to design taxon-specific or group-specific primers that avoid predator DNA amplification (Jarman *et al.* 2004; Deagle *et al*. 2005), or to digest or block predator template DNA (Green & Minz 2005; Vestheim & Jarman 2008; Deagle *et al.* 2009, 2010; Shehzad *et al*. 2012; but see Piñol *et al.* 2014). In addition, problems with non-target template amplification (Zeale *et al*. 2010) or cross-amplification when predator and prey are phylogenetically close (Thomas *et al*. 2012) need to be addressed.

Further, the amplification of target DNA limits the study of the decay dynamics of DNA inside the predators because of the difficulties of quantifying the amount of starting material with the PCR procedure, and because of the focus on a single gene region.

Shotgun sequencing of total DNA extracted from the gut or even feces is an alternative approach that, compared to PCR-based (meta)barcoding, provides a broader taxonomic range of target organisms (Srivathsan *et al*. 2014). It could also be used to study the symbiont communities closely associated with a prey (Oliver *et al.* 2010) and the dynamics of their interactions. Conceivably, total DNA extraction from the gut content of a predator, followed by direct sequencing of any identifiable DNA fragment from the prey and from its associated symbionts could enlarge even further the spectrum of species detection. The number of genomes (nuclear or mitochondrial) elucidated and available in public databanks is increasing rapidly or can be readily generated, and these could be used as a reference to match the sequenced DNA fragments to identify prey. In that sense, three sources of DNA could possibly be used to identify the prey spectrum without need of genetic amplification: the prey nuclear and mitochondrial genomes and the genomes of its associated symbionts. As some symbionts are prey specific (Oliver *et al*. 2010), their detection could indicate or support the identification of the prey.

It is widely agreed that prey DNA susceptibility to predator digestion (Harwood & Obrycki 2005; Greenstone *et al*. 2007; Weber & Lundgren 2009) and the molecular technique (Greenstone *et al.* 2014) used for prey detection are important factors influencing the sensitivity of prey detection. Consequently, the prey detection system proposed here based on the detection of any part of the prey genomes (and on associated symbionts) and on shotgun sequencing of the DNA in the predator gut should be investigated more deeply by, for example, estimating the DNA decay rate and detectability period. These decay parameters

indicate how long prey can be detected according to the speed and DNA susceptibility to digestion, providing a basis for comparison with other molecular techniques.

This study aimed to test the detection of prey nuclear and mitochondrial genomes and bacterial symbiont genomes through a direct metagenomic approach without any amplification of genetic material, based on a feeding experiment with pea aphid *Acyrtosiphon pisum* (Hemiptera: Aphididae) in the gut of the widely invasive, aphidophagous harlequin ladybird beetle *Harmonia axyridis* (Coleoptera: Coccinellidae). We used pea aphid as a prey because its genomes have been elucidated and its associated symbionts are well characterized, which enabled the study of the fate of various bacterial symbionts after prey ingestion. These symbionts include the obligate *Buchnera aphidicola* and several facultative symbionts, such as *Regiella insecticola*, *Hamiltoniella defensa* and *Serratia symbiotica*, in addition to other known aphid symbionts, e.g., *Arsenophonus* (Oliver *et al*. 2010).

In addition, this study aimed to characterize the specificity and sensitivity of prey detection using the proposed methodology, and estimate the DNA decay rate, half-life, and detectability period. As prey items are ingested with their entire associated microbial and parasite community, the analysis of these components potentially can provide additional information on the fate of the prey and the impact of the feeding event on the predator.

Material and methods

Insects and description of the study system

Harmonia axyridis (Coleoptera: Coccinellidae) was used as a model for prey detection in a common worldwide aphidophagous predator. Pupae (over 600) were collected in August 2013 (summer) in soybean fields in St. Paul, Minnesota-USA. Upon emergence adults were transferred to individual petri dishes (35x10 mm) with moistened filter paper and held under controlled conditions (25°C and 16:8 h L:D cycle) without food. After 24 h post-emergence, the individuals were used in the feeding bioassay.

Pea aphid, *Acyrthosiphon pisum* (Hemiptera: Aphididae), were used as a prey model because it has both mitogenome (GenBank gi|213948225|ref|NC_011594.1|) and nuclear genome (GenBank Assembly ID: GCA_000142985.2) elucidated (Richards *et al.* 2010). Additionally, it is the best studied aphid regarding symbionts (Oliver *et al.* 2010). Adults were obtained from a laboratory colony collected from North Dakota, USA, containing unidentified symbionts. Soybean aphids, *Aphis glycines* (Hemiptera: Aphididae), were obtained daily from the same soybean field where the *H. axyridis* pupae were collected.

The presence of symbionts was tested against the genomes of the genera *Arsenophonus, Buchnera, Hamiltonella, Regiella, Rickettsia, Rickettsiella, Serratia, Spiroplasma* and *Wolbachia*. These genera were chosen because either they are known to confer fitness advantages and costs to aphids (Wille & Hartman 2009, Oliver *et al*. 2010, Jones *et al*. 2011, Jousselin *et al*. 2012), or to coccinellids (Majerus 2006; Weinert *et al*. 2007). *Buchnera* is an obligate symbiont occurring in high numbers in specialized host organs. Except for *Arsenophonus*, all of these symbionts have been reported in *A. pisum* (Simon *et al.* 2011; Russell *et al*. 2013). In addition, *Nosema* was included, as it could be associated to *H. axyridis* (Vilcinskas *et al*. 2013), and three insect non-aphid and non-

coccinellid symbionts, *Blattabacterium, Cardinium,* and *Midichloria*, were included as false positive controls (Fein-Zchori & Bourtzis 2012).

Feeding bioassay

To estimate the decay of the prey using metagenomics in the predator gut after consumption, a feeding bioassay was conducted. Freshly emerged unfed adults were used because the gut would be totally empty, they would have the same age and physiological state, and it would avoid potential complications from secondary predation and scavenging. In addition, preliminary observations indicated that adults did not readily consume prey during the first 24 h post-eclosion. The 24-h-old beetles were individually supplied with a single *A. pisum* adult.

At six time points, immediately before feeding (negative control, denoted "Pre"), 0 h (immediately after feeding), 3, 24, 48 and 96 h after the target-species consumption, batches of 10 beetles were harvested and stored at -80°C in 100% ethanol. These time points were chosen because they contain the minimum and maximum interval time of detection currently reported in the literature for detection of a prey target molecule (protein and DNA) (Greenstone *et al*. 2014). Four hours after pea aphid consumption, *Aph. glycines* were offered once a day as a sustaining food to *H. axyridis* adults, until the last time point of the bioassay.

DNA sample preparation

The guts of the preserved predators were dissected out using clean forceps under a stereomicroscope in order to increase the chances of detecting prey DNA in the sample. Guts from the same time point were pooled into one sample. The total DNA of each sample was extracted with a DNeasy Blood and Tissue kit (Qiagen, Hilden-Germany) and quantified by fluorescence using the Qubit system (Invitrogen™) after quality checking

spectrophotometrically (ratio $A_{260/280}$ nm). The total DNA concentration of each sample was normalized to 20 ng/μL and sonicated to construct TruSeq libraries of insert size of 450 bp (250 bp paired-end, 500 cycle kit). Each library was sequenced on MiSeq-Illumina using 17% of the flowcell.

Sequence quality controls

The quality assessment of raw sequence data was made using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and PrinSEQ (Schmieder & Edwards 2011) with a minimum quality score of 20, maximum ambiguous base N of 0 and trim quality from the right (3') to minimum of 20. Overrepresented sequences (e.g. library index adapters) were trimmed with Trimmomatic (Lohse *et al.* 2012). The scripts used for the main analyses are presented in the Supporting Information (SI) section.

Predator mitogenome assembly

For the elucidation of the *H. axyridis* mitogenome, first the reads were filtered for similarity of E-value < 10^{-5} with NCBI Insecta mitogenomes that included partial and complete sequences of 587 species (taxonomic ID: 50557) using the BLASTn algorithm (Altschul *et al.* 1990). Filtering simplifies the assembly by reducing the size of the dataset and enriching it with putative mitochondrial reads. The retained reads were assembled using Celera (Myers *et al*. 2000) and IDBA-UD (Yu *et al*. 2011), and for the latter after quality control by PrinSEQ (Schmieder & Edwards 2011) with a minimum quality score and mean of 20, maximum ambiguous base N of 0 and trim quality from the right (3') to a minimum of 20. The scaffolds generated by both assemblers were concatenated in Geneious 7.0.5 (Biomatters, http://www.geneious.com/) using the parameters: no gaps allowed, minimum overlap 150,

maximum mismatches per read 0, minimum overlap identity 99%, maximum ambiguity 1. The mitogenome annotation was made by first annotating the tRNA genes using models based on the NCBI Insecta mitogenomes and the COVE software package (Eddy & Durbin 1994). The annotation process was finished manually in Geneious 7.0.5. The nearly complete mitogenome sequence of 15,322 bp includes the expected two rRNA, 22 tRNA and 13 protein coding genes arranged in the canonical gene order of Coleoptera (Timmermans & Vogler 2012). The control region was not completely sequenced. The mitogenome was deposited at GenBank under the accession code KJ778886.

Identification of aphid mtDNA

Good quality reads from all time points were matched to the NCBI Insecta mtDNA reference database of 587 species, including pea aphid and five other aphid species (November 2013), added to the sequenced mitogenome of *H. axyridis*. The matches were made by BLASTn with an E-value < 10^{-5}. Custom scripts (Supporting Information) were used to associate the GenBank general identification (gi) number and its taxonomic identification with reads obtained by Illumina sequencing requiring sequence identity >98% over a minimum hit length of 225 bp (90% of read length). A species match was retained when it equaled or exceeded the thresholds for minimum length and identity. Preliminary analyses using lower identity thresholds indicated that all false positives and ambiguous identifications were eliminated at 98% identity. Many of these false positives were repetitive DNA with high AT content. The matched pea aphid mtDNA reads were mapped onto the prey mitogenome using Geneious 7.0.5 to evaluate the overall coverage of the mtDNA. The map position of reads on the mitochondrial *A. pisum* genome was tested for a random distribution using the Poisson Dispersion test.

Identification of aphid nuclear DNA

For each time point, nuclear reads from the guts were identified by MegaBLAST alignment to the *A. pisum* complete genome (assembly Acyr_2.0; placed and unplaced scaffolds; GenBank Assembly ID: GCA_000142985.2) (Richards *et al*. 2010). All of the reads that matched with the *A. pisum* nuclear genome with more than 245 bp of >99% sequence identity and E-value < 10^{-9} were examined. Nuclear repeat regions gave ambiguous species identifications, so the ones in *A. pisum* (including short sequence repeats-SSR), were identified and excluded with the following filters. First, we identified all rRNA reads by BLAST to the rRNA SILVA database (Quast *et al.* 2013) and discarded them. To complement the filtering of possible non-specific reads, we submitted the remaining aphid reads to the RepeatMasker pipeline (Tarailo-Graovac & Chen 2009). It first uses the TandemRepeatFinder program to detect simple tandem repeats (Benson 1999). Next, all sequences were compared to two databases of currently known structured repeats; the RepBase database specialized on repeat definitions (Jurka *et al.* 2005) and the Dfam database of repeat HMMs (Hidden Markov Models) (Wheeler *et al.* 2012). All reads containing potential non-specific SSR or microsatellites were also discarded. Finally, the filtered reads were aligned to the whole content of the NCBI Refseq Protein database with BLASTx. All translated reads matching a protein sequences associated to the pea aphid (taxonomy id:7029), with >90% sequence identity over more than 30 amino acids are considered as potential coding gene hits.

Identification of prey-associated symbionts

Thirteen bacterial genera with known insect symbiotic interactions were used to create a database of symbiont sequences. For each genus, we retrieved all available NCBI Genbank

sequences and complete genomes to build the database (Table S1, Supporting Information). DNA reads from each sample tested for the presence of these symbionts were aligned to this database with MegaBLAST and all reads aligned over > 225 bp with >95% sequence identity and E-value $<10^{-9}$ were retained. Reads similar to the conservative rRNA sequences were removed to avoid misidentification due to insufficient sequence variability between related genera. The thresholds used discarded several reads that could be associated with one of the studied genera, but the need to discriminate several genera in a bacterial family (e.g. Enterobacteriaceae) required such a measure. The number of available reference genomes used to identify symbiont reads differed for each genus, which may affect the capacity for species detection (Table S1).

Statistical analyses to estimate decay parameters

An advantage of the metagenomics method is that the number of prey reads detected in the predator guts can be used to estimate the dynamics of analyte detectability. Although considerable work has been done with detectability half-lives, little use has been made of analyte detectability parameters (Greenstone *et al*. 2014). Here we provide methods for estimating three analyte degradation parameters: analyte decay rate, analyte detectability half-life, and analyte detectability period (D_{max}). Two critical points must be kept in mind. First the detection of a prey or symbiont read is a stochastic process that combines random events associated with a) the subsample of the total DNA in the gut sample, and b) the subsample of reads sequenced from the resulting DNA library. This means that the number of reads observed at any time point is a random variable, and there is some probability that the actual number of reads was greater (or less) than the number observed. Specifically, a time point with zero observed reads must be treated as a random zero (i.e., there could have been one or

253 more reads in the original sample, but the sampling and sequencing processes did not retain
254 any of these reads), and not a true biological zero (i.e., there were no reads in the original
255 sample), and is an important and meaningful datum. [Although similar random processes
256 occur in PCR-based methods, in these methods, the sources of randomness simply add
257 variance to the estimated probability of detecting a positive individual.] Second, because the
258 bioassay used different individuals to evaluate digestion at each time point, the time points are
259 statistically independent samples of the digestion process.

260 Treating digestion as a stochastic process makes explicit the uncertainty associated
261 with the observed data. Assuming that all reads have the same probability of detection, the
262 number of observed reads at each time point will follow a Poisson process. Further, the
263 observed numbers can be used to estimate the underlying Poisson process and calculate the
264 probability distribution for possible observed read numbers. This is done using Bayesian
265 methods with a Jeffries prior. Bayesian methods were used because the observed read
266 numbers are single realizations of the underlying random process. The number of reads and
267 the Bayesian posterior distribution were normalized by the library size at each time point, and
268 multiplied by 10^6 for presentation purposes. Estimation of the detectability half-life usually
269 assumes a first-order or exponential decay in the quantity of target DNA or protein degraded
270 (Lovei *et al*. 1985; Sopp & Sunderland 1989; Weber & Lundgren 2009). Therefore, expected
271 values from the posterior distributions (not the observed numbers) were used to fit an
272 exponential decay model by non-linear regression. This initial analysis allowed the
273 identification of species and genera that did not decay exponentially in the predator guts.

274 For those that did decay exponentially, Monte Carlo simulation was used to estimate
275 the analyte decay parameters. Three parameters were estimated: a) instantaneous decay rate of
276 the DNA (analyte decay rate), b) analyte detectability half-life, and c) the maximum period

during which DNA could be detected (analyte detectability period, which is analogous to D_{max}, Sutherland *et al*. 1987). Random read numbers were drawn from the normalized posterior distributions for each time point, an exponential decay model was fit to these values by non-linear regression, and the estimated parameter values (analyte decay rate and initial number of reads) were saved. This was repeated 200,000 times to generate a joint probability distribution function (jpdf) of the two parameter values. The analyte decay rate and its 95% CI were estimated from the marginal distributions of the jpdf. The analyte detectability half-life and its 95% CI were estimated from the inverse of the decay rate. The jpdf was also used to estimate the 95% confidence region of the model parameters, and the border of this region was used to estimate the 95% confidence envelope of the non-linear regression. Analyte D_{max} and its 95% CI were estimated using the original read numbers, the analyte decay rate, and the 95% confidence envelope of the regression to calculate the time when only one read would be left. A similar method was used to estimate D_{max} from the original data published in McMillan *et al*. (2007), Kuusk *et al*. (2008) and Kerzicnik *et al*. (2012), who studied the detectability of single aphid prey using PCR. In these cases, we calculated the time when only one individual would test positive. All calculations were done in Mathematica 7.0.

Results

Library basic statistics and recovery of predator DNA

Each of the six Illumina libraries was made from the guts of 10 individuals of *H. axyridis* and corresponded to different time points after feeding on *A. pisum.* These had similar DNA concentrations and produced similar total number of reads (Table 1). Many thousands of reads in each library showed exact matches to *H. axyridis* mtDNA, and their number broadly covaried with the total number of reads in each library. Reads matching mtDNA could be assembled to recover the mitogenome of *H. axyridis,* although read coverage was not uniform and was low in some intergenic regions (Fig. S1, Supporting Information). As non-predator reads, we detected *A. pisum* and some bacterial aphid symbionts after predator feeding, detailed below, and no other species were detected.

Prey detection and decay parameters

a) mtDNA

Twenty-three reads were identified as *A. pisum* mtDNA (Table 2). As expected, there was no *A. pisum* mtDNA in the negative control, i.e. before the predator has fed. Aphid mtDNA detection occurred immediately (0 h) and 3 h after feeding, and more prey sequences were detected earlier than later. The *A. pisum* reads covered different regions of the mitogenome (Fig. 1). The majority of the genes had matches to a single read only, but some genes were repeatedly hit. The *cox1* gene was detected only once, in the sample obtained immediately after feeding.

The decay of the mtDNA for a single *A. pisum* in *H. axyridis* fit the first order exponential decay model extremely well ($p = 1.94 \times 10^{-3}$) with an adjusted $r^2 = 0.974$ (Fig. 2A). On average, the instantaneous analyte decay rate was 0.11 reads per hour with 95% CI of

0.05 to 0.30 h^{-1}. The analyte detectability half-life was 8.9 h with 95% CI of 3.3 to 18.3 h. The analyte D_{max} to detect a single *A. pisum* read based on mtDNA, was 23.1 h with 95% CI of 9.5 to 81.4 h.

b) Nuclear genome

The number of reads with matches to the *A. pisum* nuclear genome exceeded the mtDNA reads by a factor of about 30, reaching over 500 reads at the moment of feeding (Table 2). No aphid sequences were detected in the pre-feeding negative control. Aphid nuclear DNA detection continued for all time points, including the last one at 96 h after feeding. The latter was due to the recovery of three reads, which was unexpected given the already very low counts at the two earlier time points. Over the hundreds of reads showing a nearly perfect match in the pea aphid genome at 0 h, 48 matched 29 different pea aphid protein sequences retrieved from the NCBI RefseqP database (Table S2). Similarly, at 3 h 13 reads matched 9 different aphid proteins. In many cases, both reads of the same pair matched the same aphid protein. Many aphid proteins are computational predictions based on the pea aphid genomes ("uncharacterized" and "predicted" annotations) but they were nevertheless the closest hit in the database (which includes proteins from all domains of life). Some matches seem to be linked to integrated viral genomes (XP_008184955.1, an HIV Tat-specific factor-like element), but we also uncovered genes linked to specific functions. For instance, one of the reads matched an O-linked-mannose beta-1,2-N-acetylglucosaminyltransferase (XP_001948219.2, Table S2), a protein with a domain signature (NCBI domain cd13937) conserved in animals.

The decay of the nuclear DNA for a single *A. pisum* in *H. axyridis* fit the first order exponential decay model extremely well ($p = 1.07 \times 10^{-5}$) with an adjusted $r^2 = 0.999$ (Fig.

2B). On average, the instantaneous analyte decay rate was 0.281 reads per hour with a 95% CI of 0.225 to 0.338 h^{-1}. The analyte detectability half-life was 3.6 h with 95% CI of 3.0 to 4.4 h. The analyte D_{max} was 32.7 h with 95% CI of 29.8 to 96 h. None of these values were significantly different from the corresponding parameters for *A. pisum* mtDNA, although the D_{max} was somewhat greater because many more nuclear reads were detected and reads were found at the final sampling time.

Detection characterization of prey symbionts

In addition to the detection of aphid nuclear and mitochondrial DNA, we identified reads homologous to known aphid bacterial symbionts, some of them in high numbers (Table 2). The symbionts *Buchnera aphidicola*, *Arsenophonus* spp., *Hamiltonella defensa, Regiella insecticola,* and *Serratia symbiotica* were detected only after *H. axyridis* feeding, indicating that they were exclusively associated with the ingested pea aphids. The obligate symbiont *B. aphidicola* was present in the highest numbers, with an even read sampling over its whole genome, with 1,651 reads at 0 h and 171 reads at 3 h (Fig. S2). Symbionts from the genera *Blattabacterium, Cardinium, Midichloria, Rickettsia, Rickettsiella, Spiroplasma* and *Wolbachia* were not detected at any time point, and neither was the microsporidian fungus *Nosema*. Reads matching the genomes of the genus *Serratia* (mainly *S. marcescens*) were detected in high numbers at all time points even before feeding, which indicates its association with the predator. In contrast, *S. symbiotica,* which is known to be an aphid symbiont (Lamelas *et al.* 2011), was observed only after feeding on pea aphids at all time points.

Two of the detected symbionts decayed according to the exponential decay model, and three of them did not. The first order exponential decay model fit the data for the obligatory

aphid symbiont *B. aphidicola* (p = 2.39 x 10^{-12}; adjusted r^2 = 1.000) and the facultative *R. insecticola* (p = 6.04 x 10^{-7}; adjusted r^2 = 0.998) very well. *Buchnera aphidicola* was detected in large numbers immediately after feeding and continued to be detected 24 h later (Table 2). The decay of *B. aphidicola* in *A. pisum* ingested by *H. axyridis* is presented in Fig. 2C. On average, the instantaneous analyte decay rate was 0.694 reads per hour with a 95% CI of 0.642 to 0.747 h^{-1}. The mean analyte detectability half-life was 3.4 h with 95% CI of 2.5 to 4.1 h. The analyte D_{max} was 15.4 h with 95% CI of 5.7 to 25 h. Decay of *B. aphidicola* DNA therefore was significantly faster than for either *A. pisum* mtDNA or nuclear DNA as there was no overlap in the 95% CIs.

Decay rate was similarly fast for *R. insecticola*, which however was detected in much smaller numbers than *B. aphidicola* (Table 2). On average, the instantaneous analyte decay rate was 0.80 reads per hour with a 95% CI of 0.39 to 1.34 h^{-1}. The analyte detectability half-life was 1.3 h with 95% CI of 0.7 to 2.5 h. The analyte D_{max} was only 5.1 h with 95% CI of 2.4 to 7.6 h (Fig. 2D).

The facultative symbionts *H. defensa*, *Arsenophonus* spp., and *S. symbiotica* did not fit the exponential decay model (*H. defensa*: p = 0.245, adjusted r^2 = 0.007; *Arsenophonus* spp.: p = 0.185, adjusted r^2 = 0.115; *S. symbiotica*: p = 0.072, adjusted r^2 = 0.407). All three exhibited a similar pattern, with no or almost no reads detected immediately after feeding, and a large, statistically significant increase in the number detected at 3 h after feeding, followed by a statistically significant decline in detection thereafter (Fig. 3). The rate of analyte decay with 95% CIs from 3 h onwards was 0.25 [0.13, 0.36] h^{-1} for *H. defensa*, 0.09 [0.02, 0.15] h^{-1} for *Arsenophonus* sp., and 0.04 [0.01, 0.08] h^{-1} for *S. symbiotica*. These values were significantly slower than for *B. aphidicola*, and *R. insecticola*, and equal to or slower than for *A. pisum* nuclear and mitochondrial DNA.

Discussion

Metagenomic approaches in gut analyses

This work showed that metagenomic approaches are sensitive enough to detect a single aphid prey and its associated bacterial symbionts without prior DNA amplification, based on dozens of mtDNA reads or hundreds of matches to the nuclear genome of the pea aphid. A key aspect for prey DNA recovery was the use of stringent thresholds, which not only ensured the use of high-quality reads but also limited false positives and established species identity of prey and symbionts with great precision. These parameters were clearly sufficient to discriminate the *A. pisum* mitogenome reads from *Aph. glycines*, which were provided as sustaining food later in the feeding trial. Available genome sequences serving as reference are an important resource for this approach. In the case of the pea aphid both mitochondrial and nuclear genomes had been assembled (Richards *et al*. 2010). In addition, the NCBI pea aphid scaffold archive, containing many genome segments that remained unplaced in the final genome assembly, was an important source for aphid read identifications. Thirty-four percent of complex-repeat families are in the unplaced scaffold archive, and produced a greater number of hits than the placed scaffolds. The complex-repeat families need characterization, because they can be very powerful nuclear markers for species recognition (Dodsworth *et al.* 2014). Conceivably, similar databases can be created readily for other aphid species that lack these genomic resources, e.g. by low-coverage genomic sequencing (‘genome skimming’, Straub *et al*. 2012) from which scaffolds of repetitive regions are readily assembled as a potentially large source of taxon-specific markers.

A fraction of the selected aphid reads corresponded to potential non-species-specific reads, i.e. highly conserved regions such as rRNAs or Simple Sequence Repeats (SSRs). A small proportion (3.5%) of SSRs was present in the NCBI pea aphid scaffold archive, but they

generally did not produce matches to our read-to-genome BLAST-based mapping. Nevertheless, any detected rRNAs and SSRs matches were excluded, and therefore species misidentifications based on these sequences are unlikely.

While the number of mtDNA reads detected for the predator was the overwhelming fraction of the reads and was always at least 400 times higher than for the prey, by dissecting the guts we recovered sufficient genetic signal for the detection of prey DNA and for analysing decay rates. The metagenomic approach provided a refined estimate of abundance and ultimately the decay rate because detection is less limited by amplification efficiency of one or a few target genes, but is related to the degree of preservation of a broader portion of the prey genome. By avoiding the amplification step of prey DNA, the number of detected reads is more directly correlated to the amount of prey material, which was neatly confirmed by the decay of read numbers over time after feeding.

In addition to improved DNA abundance measures, the metagenomic approach is powerful due to its holistic analysis of the gut content. This includes the recovery of the obligate *B. aphidicola* genome that produced a roughly uniform distribution of matching reads over its genome of 643.5 kb, nearly all of which were exact (100%) matches (Fig. S2). With the read mapping approach used here, the recovery relies on the completeness of the reference databases used to match the sequenced DNA community. Additional reference databases can be constructed to search for other associated organisms, such as pathogens, parasitoids, and possible food plants. The metagenomics approach holds the advantage that the number of reads can be interpreted quantitatively for the entire system simultaneously without the vagaries of PCR reactions on multiple targets.

Prey decay in the predator gut

The use of time-points separated by 24 h intervals, which bracketed the known D_{max} periods for PCR-based methods, seemed to be too long for mtDNA detection of only a single aphid prey item without amplification of a target prey mtDNA gene. More prey mtDNA might have been detected using a shorter evaluation interval of perhaps up to 12 h after prey ingestion. This might improve the precision of the decay parameters for mtDNA and reduce the large confidence region around the regression (Fig. 2A), but the values for the analyte decay rate and analyte D_{max} would not change much. On the other hand, the use of a library with an insert of 450 bp might have precluded the detection of prey mtDNA reads for periods longer than 3 h after prey ingestion, as most of the prey mtDNA in the predators' guts content could have already been digested to smaller lengths (Chen *et al*. 2000). By increasing the number of reads detected after 3 h, the analyte decay rate would be reduced, and analyte D_{max} would be longer.

The analyte detectability half-life of *A. pisum* genetic materials was 3.6 to 8.9 h, which is similar to the 2.0-4.9 h detectability half-life for PCR-based detection of a single aphid consumed by different predators (Greenstone *et al*. 2014). However, analyte D_{max}, determined here from the metagenomic data, was 2-11 times longer than PCR-based D_{max}. We estimated the D_{max} for a single aphid prey using PCR to be 4.0 h for *Pardosa sternalis* (Aranae: Lycosidae) and 9.8 h for *Tetragnatha laboriosa* (Aranae: Tetragnathidae) (data from Kerzicnik e*t al*. 2012), 12.9 h for *Pardosa* spp. (data from Kuusk *et al*. 2008), and 16.1 h at 14ºC and 14.5 h at 21ºC for *Adalia bipunctata* (Coleoptera: Coccinellidae) (data from McMillan *et al.* 2007). When considering the decay of similar prey items, metagenomic sequencing appears to enable prey detection for a longer period of time than PCR-based methods.

Symbiont detection and population dynamics in the predator gut

The secondary detection of several genera and species of prey symbionts in this work was possible because we could construct a symbiont reference database from GenBank. Secondary detection is defined here as the detection of exogenous DNA that was inside the first source of exogenous DNA (prey, in this case). Usually the detection of insect symbionts has been done with PCR based-methods, including metabarcoding through 16S rRNA (Jones *et al*. 2011, Hirsch *et al*. 2012). As found here, metagenomics can be used to monitor symbiont population fluctuations after prey ingestion.

The detected *B. aphidicola*, *R. insecticola*, *H. defensa*, *Arsenophonus* spp., and *S. symbiotica* are all known to be aphid symbionts, and none have been reported from coccinellids, and they were not found in the negative control. In contrast, the genus *Serratia*, which includes the widespread, non-symbiotic *S. marcescens* and other free-living species, was detected in large numbers in all of the bioassay treatments, including the never-fed, negative control.

Two kinds of decay patterns of prey symbionts were detected. One kind was for *B. aphidicola* and *R. insecticola*, which decayed according to the first order exponential decay model similar to prey DNA. This result suggests that the population dynamics of *B. aphidicola* and *R. insecticola* in the gut of *H. axyridis* could be characterized as a pure death process, where they are introduced into the predator gut via their aphid host and then die and are digested at a fixed rate. *Buchnera aphidicola* was detected in large numbers immediately after feeding, and up to 24 h after feeding, but not thereafter. A similar dynamic was found for *R. insecticola*, which is only known from aphids (Oliver *et al.* 2010). Interestingly, both decayed at a faster rate than *A. pisum* nuclear or mitochondrial DNA. Although *Aph. glycines* aphids were provided once a day, starting four hours after *A. pisum* aphid feeding, no *B.*

485 *aphidicola* or *R. insecticola* were found at 48 and 96 h after feeding on *A. pisum*. Their decay
486 rates may have been so fast that any *B. aphidicola* or *R. insecticola* DNA introduced via *Aph.*
487 *glycines* aphids was already degraded by the time the predators were collected at 48 and 96 h
488 in the bioassay.

489 The second kind of decay pattern was observed for *H. defensa, S. symbiotica* and
490 *Arsenophonus* spp. *Hamiltonella defensa* and *S. symbiotica* are associated with *A. pisum*
491 where they coexist with *Buchnera* in bacteriocytes and also occur in sheath cells around
492 bacteriocytes and in the hemolymph (Oliver *et al*. 2010). *Arsenophonus* is widespread in
493 related Aphidinae, but not in pea aphid (Jousselin *et al*. 2012), and never has been reported
494 from any beetle species. Because the only food consumed by the 3 h post-feeding *H. axyridis*
495 was *A. pisum*, *Arsenophonus* was most likely present in the North Dakota *A. pisum* population
496 used in this study.

497 One possible explanation for the unusual decay pattern is that it was generated due to
498 a random association of infected aphid hosts with beetles at the different time points, because
499 the facultative symbionts do not infect all of their aphid hosts (Russell *et al*. 2013). We
500 rejected this possible explanation, by calculating the probability that this could have happened
501 just by chance. An upper bound on this probability is 0.33% (see Supporting Information), so
502 the observed patterns probably reflect changes in the relative population size of these three
503 symbionts in the predator gut. In addition, the large number of reads at 3 h could not have
504 come from *Aph. glycines* aphids, as none of the predators had access to this food until 4 h
505 after consumption of *A. pisum*.

506 All three symbionts (*H. defensa*, *Arsenophonus* spp., and *S. symbiotica*) started with
507 small or undetectable numbers immediately after *H. axyridis* fed on *A. pisum*, and by 3 h
508 later, their populations grew in the predator guts by 1-2 orders of magnitude. Subsequently

509 they declined at different rates, with *H. defensa* declining fastest and *S. symbiotica* declining
510 slowest. The predator gut appears to be suitable for initial high rates of reproduction of these
511 symbionts, suggestive of an infection attempt during the 3 h after *A. pisum* ingestion. Indeed,
512 Degnan *et al.* (2009) found that *H. defensa* had abundant putative pathogenicity loci and
513 regulatory genes that may be important for infecting new hosts. In addition, Costopoulos *et al*.
514 (2014) fed the coccinellid *Hippodamia convergens* with aphids containing either *H. defensa*
515 or *S. symbiotica* which, compared to a control diet, reduced coccinellid survival and increased
516 adult size. The transient increase in symbiont populations reported here could account for how
517 a prey symbiont could affect the predator. The observed decline in symbiont populations later
518 in the bioassay indicates deterioration of the predator gut environment, possibly caused by the
519 predator immunity defense and increased competition from other gut bacteria.
520 Although infective horizontal transmission of prey symbionts to predators has not
521 been reported, it eventually could happen through repeated transient infections by prey
522 symbionts after prey ingestion, especially if the symbiont conferred advantageous ecological
523 effects. From our results, we can hypothesize that only the less specialized symbionts can
524 survive such transmission. *Hamiltonella* and *Regiella* species are generally distinguished from
525 their “free-living” Enterobacteriaceae relatives by their reduced genomes and the loss of some
526 essential pathways (Moran *et al.* 2005; Degnan & Moran 2008; Rao *et al.* 2012). On the other
527 hand, *Arsenophonus* species possess larger genomes and are morphologically and functionally
528 very diverse in different aphid lineages, while *Serratia* species are widespread in many insects
529 (Nováková *et al.* 2009; Russell *et al.* 2013). The fastest decay rates observed for *Hamiltonella*
530 and *Regiella* species and the slowest decay rates observed for *Arsenophonus* and *S.*
531 *symbiotica* seem to be directly correlated to these different levels of symbiosis.
532

Broader implications

The use of metagenomics in predator gut content analysis is a powerful tool that can reveal complex relationships among predators, prey, and their symbionts. Because the copy number of the genetic materials does not change during sample processing, the dynamics of these relationships can be studied quantitatively. Although it does not require development of specific PCR primers or antibodies, it requires reference DNA databases to make possible species identification. These databases could focus on either prey nuclear or mitochondrial DNA or symbiont genomes, and can be acquired from GenBank, or provided by the investigator. The prey DNA databases allow definitive identification of prey species, while the symbiont database may reinforce the prey identifications and reveal prey symbiont population dynamics in the predator gut. Finally, because of its high analyte D_{max} and specificity, metagenomics can be especially useful for trophic interaction studies with a high number of prey species to be detected at the same time, identifying unknown prey and revealing species not previously known to be preyed upon by a predator.

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Data Accessibility

The metagenomic data have been deposited in MG-RAST in project ??? (or Dryad ?). The *Harmonia axyridis* mitogenome sequence was deposited at GenBank under the accession code KJ778886. The Bioinformatics scripts are provided in the online Supporting Information.

Author Contributions

DPP and APV designed the research. DAA conducted the field sampling, the feeding bioassay and the decay statistical data analyses. DPP performed the molecular biology experiments. DPP and BL analyzed the bioinformatics data. DPP, DAA, BL and APV wrote the paper. DPP, DAA, BL, APV, CSSP and ERS revised it.

Figure legends

Fig. 1 Coverage of *A. pisum* mtDNA for a single aphid in the prey feeding bioassay. The tRNA genes are represented by amino acid single letter codes. The rRNA genes are represented by "16S" and "12S". The non-coding region (D-loop and AT rich) is represented by "Misc". Protein-coding genes are represented by their standard abbreviations.

Fig. 2 Decay (analyte decay rate, analyte half-life and analyte D_{max}) of the genetic material of a single prey as a function of time after predation detected through metagenomics. A) the mtDNA of *A. pisum*; B) nuclear genome of *A. pisum*; C) genome of the obligatory symbiont *B. aphidicola*; D) genome of the facultative symbiont *R. insectiola*. The number of reads was normalized by the library size. Heavy solid lines: expected decay process; Light solid lines: 95% confidence envelop for decay process; Solid circles: expected observed reads with 95% credibility intervals based on posterior Bayesian distribution.

Fig. 3 Mean number of reads of the prey bacterial symbionts found in the gut content of *H. axyridis* as a function of time after aphid predation with 95% credibility intervals (from posterior Bayesian distribution). The number of reads was normalized by the library size.

727 **Table 1** Number of reads obtained in the TruSeq libraries and MiSeq-Illumina sequencing for the
728 feeding bioassay after quality control. The total number of reads in each library was used to normalize
729 the data among the treatments for estimating the decay parameters

Reads number	**Elapsed time after feeding**					
	Pre	**0 h**	**3 h**	**24 h**	**48 h**	**96 h**
DNA (μg/mL)	24.39	28.73	24.81	28.01	20.70	22.70
Forward (R1)	1,751,599	1,967,870	1,664,734	2,072,981	2,115,223	1,602,152
Reverse (R2)	1,750,653	2,022,493	1,652,913	2,083,512	2,119,968	1,598,851
Predator mtDNA	7,427	10,849	9,165	13,442	10,963	7,191

730

Table 2 Number of reads obtained for the mtDNA and nuclear genome for the prey, *A. pisum*, and for the complete genomes of the bacterial symbiont genera and species detected for the each time point in the feeding bioassay. The high *Serratia* spp. read numbers included an abundant species associated with the predator and the prey

	Elapsed time after feeding					
	Pre	**0 h**	**3 h**	**24 h**	**48 h**	**96 h**
Acyrthosiphon. pisum mtDNA	0	13	10	0	0	0
Acyrthosiphon pisum nuclear DNA	0	518	185	10	6	3
Buchnera aphidicola	0	1,651	171	2	0	0
Arsenophonus spp.	0	0	76	12	11	13
Hamiltonella defensa	0	0	577	0	0	4
Regiella insecticola	0	27	2	0	0	0
Serratia spp.	12,450	18,939	10,761	21,270	16,680	12,220
Serratia symbiotica	0	1	9	2	3	2

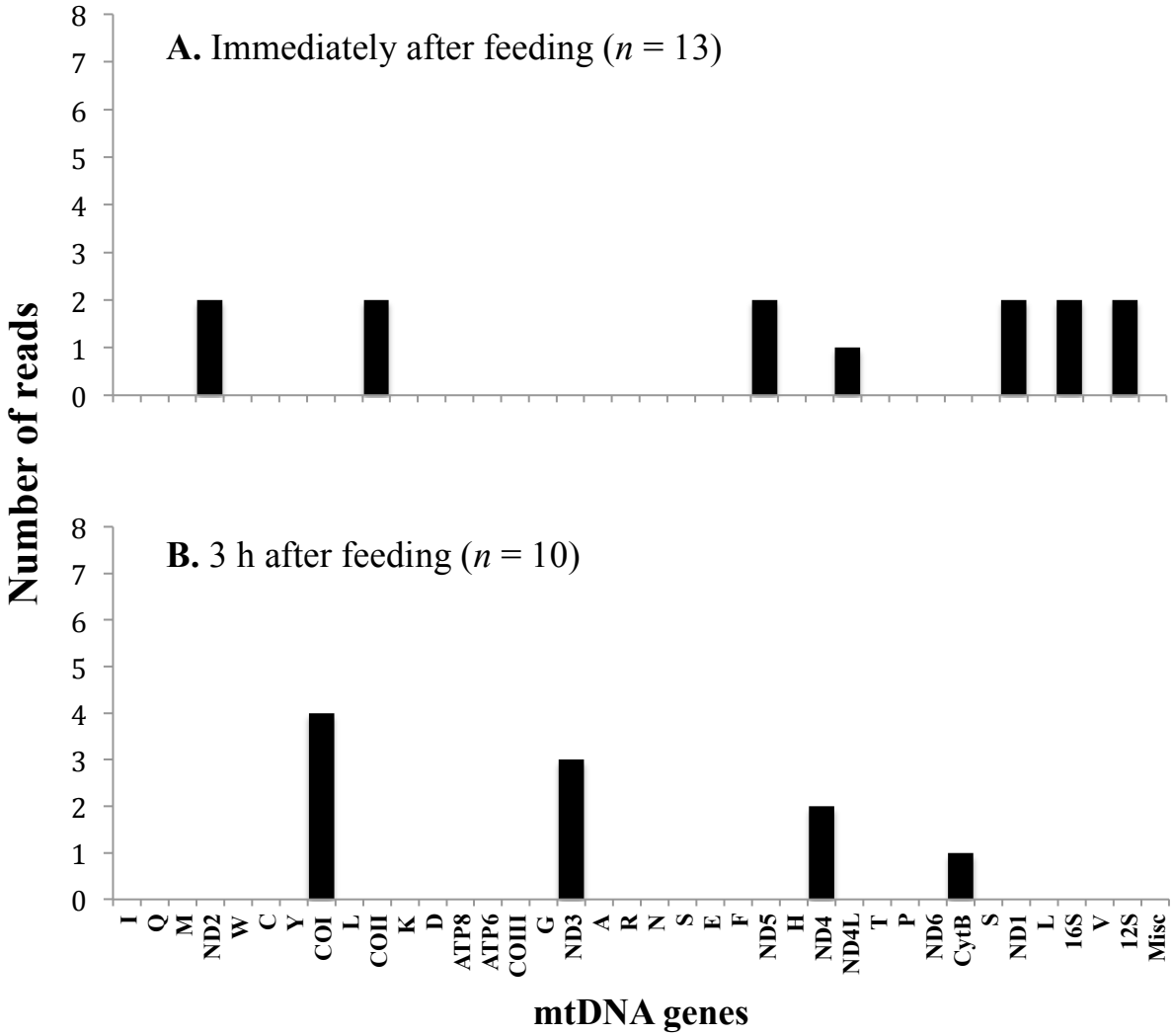
A. Immediately after feeding (n = 13)
B. 3 h after feeding (n = 10)
Number of reads
0
1
2
3
4
5
6
7
8
I
Q
M
ND2
W
C
Y
COI
L
COII
K
D
ATP8
ATP6
COIII
G
ND3
A
R
N
S
E
F
ND5
H
ND4
ND4L
T
P
ND6
CytB
S
ND1
L
16S
V
12S
Misc
mtDNA genes

Fig. 1

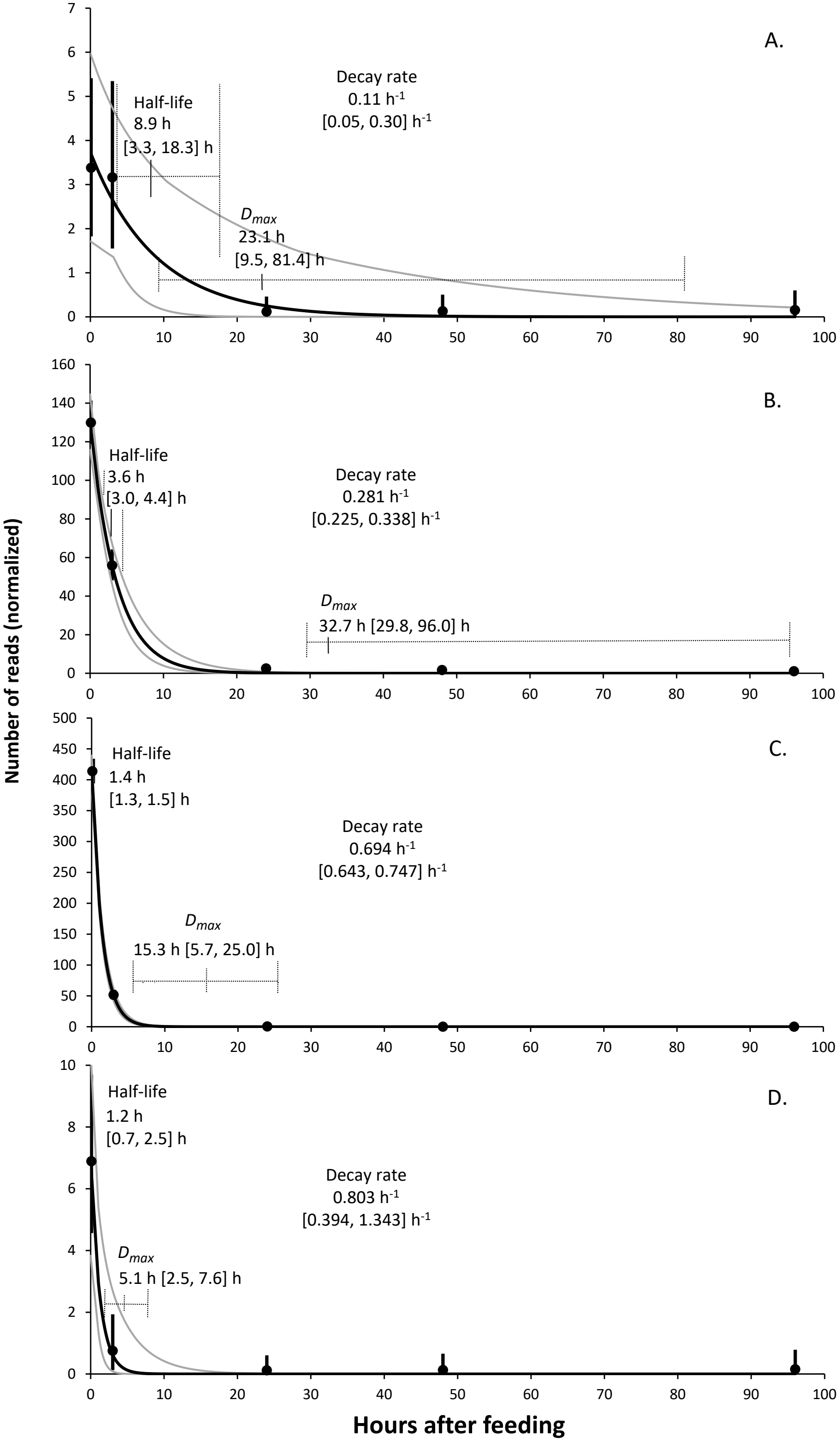
A.
Half-life
8.9 h
[3.3, 18.3] h
Decay rate
0.11 h-1
[0.05, 0.30] h-1
Dmax
23.1 h
[9.5, 81.4] h
B.
Half-life
3.6 h
[3.0, 4.4] h
Decay rate
0.281 h-1
[0.225, 0.338] h-1
Dmax
32.7 h [29.8, 96.0] h
C.
Half-life
1.4 h
[1.3, 1.5] h
Decay rate
0.694 h-1
[0.643, 0.747] h-1
Dmax
15.3 h [5.7, 25.0] h
D.
Half-life
1.2 h
[0.7, 2.5] h
Decay rate
0.803 h-1
[0.394, 1.343] h-1
Dmax
5.1 h [2.5, 7.6] h
Number of reads (normalized)
Hours after feeding

Figure 2

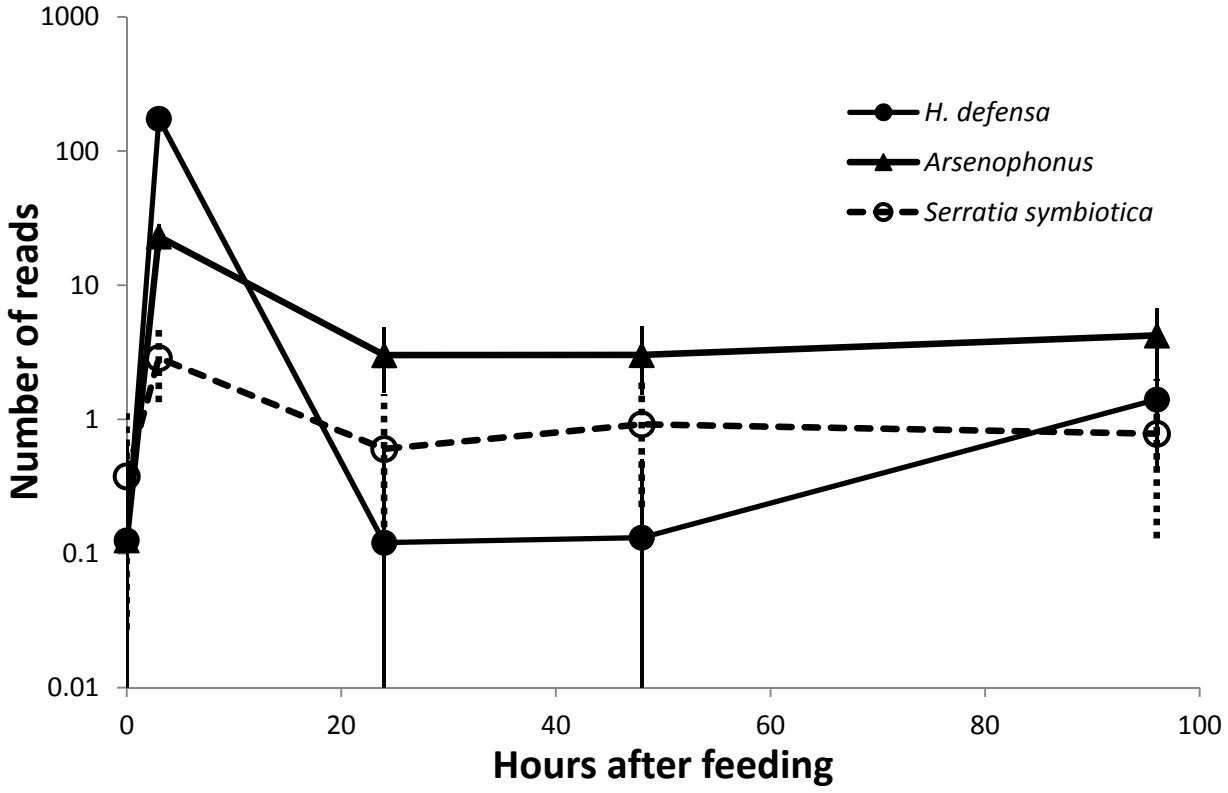
1000
100
10
1
0.1
0.01
Number of reads
0
20
40
60
80
100
Hours after feeding
H. defensa
Arsenophonus
Serratia symbiotica

Figure 3

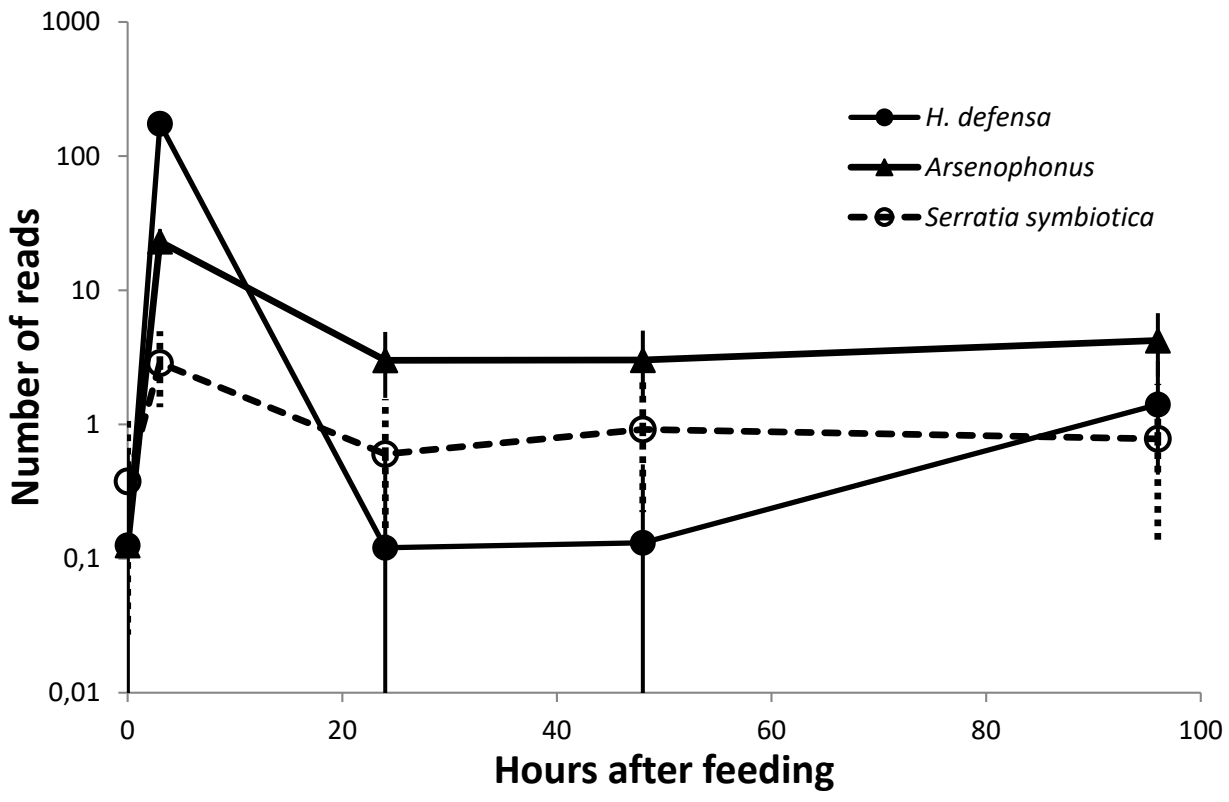
1000
100
10
1
0,1
0,01
Number of reads
0
20
40
60
80
100
Hours after feeding
H. defensa
Arsenophonus
Serratia symbiotica

Figure 3