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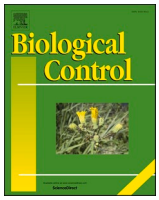

Estimating relative *per capita* predation rates from molecular gut content analysis

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HIGHLIGHTS

- Estimating predation rates is important for evaluating the effects of predators on their prey.
- We develop a broadly applicable method to estimate relative predation rates using quantitative molecular gut content data.
- This is illustrated with two examples to show how relative predation rates can be used.

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ABSTRACT

The problem of estimating predation rates from molecular gut content data has been challenging. Previous work showed how *per capita* predation rates could be estimated from quantitative molecular gut content data using the average prey quantity in the predator, the decay rate of the prey in the predator and a conversion constant to convert measured prey quantity into prey numbers or biomass. Based on this previous work, we developed and illustrated a method to estimate relative *per capita* predation rates for a single prey species consumed by one predator species. This method does not require estimation of either the decay rate of the prey in the predator or the conversion constant. We describe how gut content data from qPCR, quantitative ELISA, metabarcoding and unassembled shotgun reads (Lazaro) can be used to estimate relative *per capita* predation rates. The method was used to estimate the relative *per capita* predation rate in a laboratory feeding trial to evaluate the precision and accuracy of the method using Lazaro data. Ten independent estimates were statistically similar, but precision was related to the number of observed prey reads. We estimated the relative *per capita* predation rate by the ant *Pheidole flavens* on another ant *Pheidole tristis* in a field experiment and by the ladybeetle *Hippodamia convergens* on the aphid *Lipaphis pseudobrassicae* on organic production farms. We found that higher *P. flavens* activity-density was associated with lower relative *per capita* predation rates, therefore indicating lower predation rates on *P. tristis*. The absence of variation among farms in relative *per capita* predation by *H. convergens* suggested that the farms were biological replicates. Using relative *per capita* predation rates can provide a rapid way to assess how a predator–prey interaction changes over space and time and may help identify factors that limit or enhance biological control of pests.

1. Introduction

Molecular gut content analysis has revolutionized the detection of prey consumed by arthropod predators. The pathbreaking work of Dempster (1960) with the precipitin test, a serological test that precipitates an antigen of the prey out of solution, illuminated the potential of molecular gut content analysis. This was followed in relatively rapid succession by qualitative and quantitative ELISA (Fichter and Stephen, 1981; Miller, 1981; Ragsdale et al., 1981; Symondson et al., 1999,2000; Naranjo and Hagler, 2001; Hagler, 2006), conventional PCR (Zaidi et al., 1999; Agustí and Symondson, 2001; Foltan et al., 2005; Juen and Traugott, 2005; Lundgren et al., 2009; King et al., 2011; Davey et al., 2013), multiplex PCR (De Barba et al., 2014), quantitative PCR (qPCR, Deagle and Tollit, 2007; Lundgren et al., 2009; Weber and Lundgren, 2009), as well as other lesser used methods, such as radial immunodiffusion (McIver, 1981), quantitative electrophoresis (Lister et al., 1989)

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and species-specific alkaloids (Hautier et al., 2008). More recently, after the advent of Next Generation Sequencing (NGS) of DNA, metabarcoding (Zaidi et al., 1999; Deagle et al., 2006; Valentini et al., 2009; Pompanon et al., 2012; Taberlet et al., 2012,2018) and unassembled shotgun reads (Lazaro: Paula et al., 2015,2016,2022a,2023), which is the mapping of unassembled, unamplified shotgun reads, have expanded the reach of these molecular methods.

A longstanding goal of this work has been to estimate predation rates. Predation rates are characterized as the amount (number or biomass) of prey consumed per unit time. The challenge has been to use molecular detections to estimate a rate. Many of the historical molecular methods only generate presence or absence of prey in a predator. These include the precipitin test, qualitative ELISA and conventional and multiplex PCR. Such methods do not provide information on the number or biomass of prey consumed. Other methods do provide information about the number or biomass of prey consumed, including quantitative ELISA, quantitative PCR, quantitative electrophoresis and Lazaro. However, all of these methods lack a temporal component and the ability to distinguish the number of prey consumed from the time since consumption. Therefore, they cannot by themselves be used to estimate a predation rate. This limitation was recognized from the first published studies. Dempster (1960) converted presence-absence detections into a predation rate using independent experiments to show that predators consumed only one prey individual in a day, and consumed prey were detectable for only one day. Thus, he reasoned that the proportion of predators with detected prey could be converted to a predation rate (number consumed per day) by multiplying by one prey consumed per day and dividing by the one-day detectability period (Dempster, 1960). Rothschild (1966) generalized this idea by multiplying the proportion of positive detections by the number of prey consumed per day and dividing by the time period that consumed prey are detectable. Since those studies, six additional methods for calculating predation rates from molecular data have been proposed (Andow and Paula, 2023).

The use of quantitative DNA detection data to estimate predation rates has resulted in several different proposed methods (Andow and Paula, 2023). While the insight that predation rates required information on how much the predator consumed and how long the prey remained detectable, has been recognized by all previous methods (Dempster, 1960, Rothschild, 1966, Nakamura and Nakamura, 1977, Sunderland et al., 1987, Lister et al., 1987, Sopp et al., 1992, Andow and Paula, 2023), the ways these quantities have been measured has differed among studies. Common to all of these methods is the implicit assumption that prey decay rates in predator guts are similar enough among individuals of the same predator species and constant enough over space and time that the expected (average) decay rate of a particular prey-predator species is sufficient to accurately estimate predation rates. This assumption and its consequences have not been carefully examined, but it is likely that if the actual variation is small enough and close to normality, the expected prey decay rate will suffice. A second implicit assumption is that the prey decay rate in a predator follows a first order decay process. This means that in any individual predator the decay rate parameter is constant and does not depend on the amount of prey in the predator. This assumption also has not been extensively examined, but the existing empirical data supports it (Andow and Paula, 2023).

From these assumptions a *per capita* predation rate can be estimated using (Andow and Paula, 2023):

$$\widehat{p_{ij}} = \widehat{d_{ij}}\,\widehat{Q_{ij}} \big/ \widehat{Q_{ij0}} \tag{1}$$

where the carrot indicates an estimated value, and $\widehat{p_{ij}}$ = average *per capita* predation rate on prey i by predator j, $\widehat{d_{ij}}$ = first order rate of decay of prey i in the predator j, equal to exponential decay with units time^{-1}, $\widehat{Q_{ij}}$ = amount of detected prey (e.g., DNA, protein, antigen) i in the predator j for all individual predators, including those that did not test positive for prey i, the units of which are (detection units) × individual^{-1}, and $\widehat{Q_{ij0}}$ = amount of prey i detected in the predator j immediately after feeding on a known amount (number or biomass) of prey i with units (prey amount) × (detection unit)$^{-1}$. Thus, the right-hand-side of (1) has dimensions (detection units) × (prey amount) × (detection units)$^{-1}$ × individual^{-1} × time^{-1} = (prey amount) × individual^{-1} × time^{-1}, which is a *per capita* predation rate. The measurement of the known amount of prey consumed will depend on the predator and prey, as not all prey may be completely consumed.

Andow and Paula (2023) conducted a controlled laboratory experiment to test the accuracy of equation (1) for estimating a *per capita* predation rate. Briefly, the experiment recorded the number of aphid prey consumed by a coccinellid predator to estimate the left-hand-side of equation (1) and used quantitative prey DNA detections with Lazaro to estimate all of the terms on the right-hand side of equation (1). In addition, the data were used to compare equation (1) with previous methods proposed by Sunderland et al. (1987), Lister et al. (1987) and Sopp et al. (1992), and a generalization of the method proposed by Sopp et al. (1992). The method proposed by Lister et al. (1987) is identical to equation (1) but has been ignored since it was first proposed. The observed *per capita* predation rate (left-hand-side of equation (1)) was 3.11 aphids/h and the estimated *per capita* predation rate (right-hand-side of equation (1)) was 3.29 ± 0.27 aphids/h. The other methods estimated the *per capita* predation rate as 0.33 ± 0.02 (Sunderland et al., 1987), 1.66 ± 0.08 (Sopp et al., 1992) and 3.64 ± 0.30 (generalized Sopp et al., 1992) aphids/h, which were all significantly different from the actual value. Thus, equation (1) is supported theoretically and empirically, although additional empirical tests are needed.

In the future it is likely that increasingly more data will be collected that can use equation (1) to estimate predation rates. However, at the present time, d_i and Q_{i0} are not estimated for the vast majority of cases. In addition, while metabarcoding has become increasingly common for detecting prey in predator guts, it has been difficult to interpret the number of reads quantitatively in relation to prey quantity in the predator. Specifically, metabarcode primer preference to amplify certain taxonomic groups and variation in metabarcode primer efficiency among prey species has limited our ability to interpret metabarcoding number of reads (e.g., Kobayashi et al., 1999; Clarke et al., 2014; Deagle et al., 2014; Elbrecht and Leese, 2015,2017; Kebchull and Zador, 2015; Paula and Andow, 2023).

Here we show that, even without estimating d_i and Q_{i0}, how relative *per capita* predation rates for predation by a single predator species on a single prey species can be estimated using data from quantitative molecular gut content analysis methods. The limitation of estimating for only a single prey and predator species means that some important questions in biological control, such as determining the most significant prey species of a predator, cannot be addressed. However, many important predator–prey studies have focused on one predator species consuming one prey species (e.g., Stenseth et al., 1997, Jost et al., 2005, Hossie & Murray, 2023), so many significant questions in biological control remain and can be approached using relative *per capita* predation rates. In addition, we suggest that metabarcoding number of reads can be used to estimate relative *per capita* predation rates under some conditions. Finally, we illustrate the utility of relative *per capita* predation rates using two examples: intraguild predation on an ant species by another ant species and extraguild predation on an aphid species by a coccinellid predator.

2. Methods

Theory and assumptions. We used equation (1) to derive estimates of relative *per capita* predation rates for one prey species in a single predator. The *per capita* predation rate in equation (1) can be relativized by dividing by the maximum observed predation rate for that prey in that predator. Specifically, the *per capita* predation rate for any predator

sample, $d_{ij}Q_{ij}/Q_{ij0}$, can be relativized by dividing by the maximum per capita predation rate among predator samples, $\max(d_{ij}Q_{ij}/Q_{ij0})$. Because d_{ij} and Q_{ij0} are constants for a given prey and predator species, they cancel out when the sample *per capita* predation rates are divided by the maximum rate. Thus, relative *per capita* predation rate, r_{ij}, is simply

$$\widehat{r_{ij}} = \widehat{Q_{ij}}/max\left(\widehat{Q_{ij}}\right) \quad (2)$$

which means that estimation of relative *per capita* predation rates does not require estimation of either the decay rate, d_{ij}, or the conversion constant, Q_{ij0}. These relative *per capita* predation rates are dimensionless and should be interpreted as the proportion of the maximum rate, whatever that maximum may be. Additional research is needed to develop methods to estimate these maxima independent of the molecular data or ancillary experiments that estimate d_{ij} and Q_{ij0}, which would allow conversion of the relative rates into absolute rates.

Using the relative *per capita* predation rate, we can test general hypotheses related to temporal and/or spatial variation in predation rate by the predator on the prey. For example, we can test if there is significant variation in relative *per capita* predation rate among sites or significant variation among experimental treatments. We can test if relative *per capita* predation rates are related to predator or prey density. An important inferential limitation is that they cannot be used to estimate the effect or impact of the predator population on the prey population; the predator population size and an absolute *per capita* rate must be estimated to do this. Despite this limitation, interesting ecological variation in predation rates on a target prey can be examined as we illustrate here.

Q_{ij} can be estimated for each predator sample or for the predator population by several quantitative molecular methods, such as Lazaro, quantitative ELISA, qPCR and possibly metabarcoding. However, for various reasons elaborated in the Quantitative detection methods section below, none of these methods estimates Q_{ij} directly. Instead, Q_{ij} is estimated from the product of f_{ij} and q_{ij}, where f_{ij} is the frequency of predator j with detectable prey i and q_{ij} is the amount of prey i in a predator sample j that contains detectable prey i. Because f_{ij} is a frequency, it is estimated over all comparable samples of the same predator species and q_{ij} is estimated for each individual sample. Thus with $Q_{ij} = f_{ij}$ q_{ij}, and f_{ij} constant over all individuals, the $\max(Q_{ij}) = f_{ij}$ $\max(q_{ij})$ and therefore, the relative per capita predation rate is.

$$\widehat{r_{ij}} = \widehat{q_{ij}}/max\left(\widehat{q_{ij}}\right) \text{ for positive detections, and } \widehat{r_{ij}} = 0 \text{ otherwise.} \quad (3)$$

If the samples are a pool of individual predators instead of individual predators, p_{ij} in equation (1) is the average *per capita* predation rate of the pool, rather than the predation rate of an individual predator, as demonstrated in Andow and Paula (2023). Thus, following the foregoing logic, r_{ij} in equations (2) and (3) is the average relative *per capita* predation rate of the pool.

Quantitative detection methods. For Lazaro ln(number of reads) has been found to be related to ln(prey quantity consumed) (Paula et al., 2023). Lazaro is a method to detect DNA of any species present and the reads of the target prey species are separated during the downstream DNA sequencing data processing when reads are filtered and assigned to the most likely prey species. The same is true for metabarcoding data. Consequently, an observation of 0 prey reads results in ln(0), which is undefined, and equation (2) cannot be used, because an observation of 0 reads should be no detection and not undefined. Instead, equation (3) must be used to estimate relative *per capita* predation rates from Lazaro number of reads, because this equation sets the lack of detection equal to 0. For qPCR, n_0, the relative initial template concentration, could be used for estimating relative *per capita* predation rates. When there is no detection in a sample, n_0 is undefined, so again, equation (3) must be used. Here, we assume that the relative initial prey template concentration, n_{0ij}, from a qPCR is proportional to the biomass of prey i preyed upon by the predator j, so that $n_{0ij}/\max(n_{0ij})$ is an estimate of r_{ij}, the relative predation rate of predator j on prey i. For similar reasons, data from quantitative ELISA must use equation (3) to estimate r_{ij}.

Metabarcoding detects the DNA of any species that is amplified by the metabarcoding primer, and similar to Lazaro, the reads of the target prey are separated by downstream processing. For metabarcoding, the number of reads has been found to be weakly related to the biomass of the species in a sample (Lamb et al., 2019). However, by restricting comparisons to a single prey species i in a single predator species j, we avoid two of the most serious problems limiting the quantitative interpretation of the number of metabarcoding reads (summarized in Paula and Andow, 2023): barcode primer selectivity to amplify only certain taxonomic groups and variation in metabarcode primer efficiency among prey species (e.g., Kobayashi et al., 1999; Clarke et al., 2014; Deagle et al., 2014; Elbrecht and Leese, 2015,2017; Kebchull and Zador, 2015; Paula and Andow, 2023). Barcode primer selectivity refers to the fact that "universal" barcode primers do not amplify all species. This selectivity will result in false negatives (prey that are present but not detected), limiting characterization of the full range of species in the diet. Variation in primer efficiency refers to the fact that detectable prey DNA will be amplified with different efficiencies. This results in noncomparable numbers of reads between different prey taxa. For example, if prey 1 is amplified with efficiency at the theoretical maximum of 2.00, meaning that the amplicon will double each amplification cycle, while prey 2 is amplified with efficiency 1.95, then starting with the same number of DNA barcode sequences, at the end of 30 amplification cycles, prey 1 will have 2.13 × more reads than prey 2. Thus, the number of metabarcoding reads cannot readily be used to compare different prey species. However, relative *per capita* predation rates do not compare among prey species and are estimated only for one prey species consumed by one predator species. Hence, the two most critical factors limiting the quantitative interpretation of metabarcoding number of reads are moot. A remaining concern with metabarcoding data is related to the competitive aspect of the PCR reaction, specifically that templates compete for primers and dNTPs. This means that the relative concentrations and diversity of DNA templates in a metabarcoding sample could influence primer efficiency. Such effects can be reduced by designing the PCR reactions to reduce template competition by having sufficient primer and dNTP concentrations.

Because metabarcoding number of reads, here symbolized by *nr*, is the result of many cycles of amplification, we assume that the logarithm of the number of reads is a better estimate of q_{ij} than raw number of reads. Assuming that $\ln(nr_{ij})$ is proportional to the biomass of prey i in predator j when prey are detected, then the absence of prey reads becomes undefined and equation (3) must be used. In some cases, metabarcoding reads for the same prey may not yield accurate estimates of r_{ij} because amplification efficiency during metabarcoding can depend on the composition of competing templates (Clarke et al., 2014; Deagle et al., 2014; Elbrecht et al., 2015, 2017), as stated above. Therefore, the interpretation of r_{ij} for metabarcoding entails the additional assumption that competing templates, i.e., those that are not the target prey i, do not cause significant enough variation in amplification efficiency for the prey i template across samples. On the other hand, if amplification efficiency is not significantly affected by the predator DNA templates, as might occur when the barcode primer anneals poorly with predator DNA templates, then it may become possible to compare relative *per capita* predation rates across predator species for the same prey.

Laboratory feeding trial to verify method. We used a previously published laboratory feeding trial (Paula et al., 2023) to evaluate the precision and accuracy of estimates of the relative *per capita* predation rate. Predaceous *Hippodamia convergens* (Guérin-Méneville, 1842) [Coccinellidae: Coleoptera] consumed 1, 3 or 6 apterous *Myzus persicae* (Sulzer) [Hemiptera: Aphididae] prey and were sacrificed 0, 3, 6 or 9 h after they had consumed the prey. For each of the three food quantities and four times of sacrifice, the guts of ten replicate predators (12 treatments, 120 individuals total) were dissected and pooled, their DNA was extracted and sequenced without barcode amplification, and the number of reads of *M. persicae* associated with each treatment was

counted using the Lazaro pipeline (Paula et al., 2022a). The samples were shipped to the Roy J. Carver Biotechnology Center (University of Illinois at Urbana-Champaign, IL, USA) and libraries were constructed with the Hyper Library construction kit (Kapa Biosystems, Wilmington, MA, USA) using unique dual indexes with 350 bp insert size and final read length 200–500 bp. Quality-checked samples were sequenced by Illumina HiSeq2500 (250 bp paired-end, HiSeq Rapid SBS sequencing kit version 2) in a single lane. The numbers of reads were normalized for all samples and treatments to equal DNA quantities (40 ng/ul) and the average sequencing depth. We eliminated the treatments that had no prey *M. persicae* reads detected, leaving 10 treatments. Additional methodological details can be found in Paula et al. (2023).

The numbers of reads for each treatment were ln transformed and divided by the ln(number of prey consumed) to standardize the results for equivalent rates of prey consumption. Previous work showed that ln (number of reads) was proportional to ln(number of prey consumed) (Paula et al., 2023). Numbers were also normalized to a 9 h decay period, as the samples were in the predator guts for different lengths of time. Read numbers from the 9 h treatments were unchanged and read numbers from the 0 h and 3 h treatments were dived by 9 and 3 respectively. This allowed us to predict that because the predator species, prey species, prey amount and decay time are controlled, the relative *per capita* predation rate would be the same for the 10 independent samples. To calculate the mean and standard deviation of the relative *per capita* predation rates, we simulated 5000 random results using the estimated ln number of reads and its variance. For each random result, the relative *per capita* predation rates were calculated, following equation (3). The mean and standard deviation for each treatment was calculated from the 5000 simulations.

Empirical data. We used Lazaro and metabarcoding data from a previously published work (Paula et al., 2022a) and qPCR data from unpublished work to illustrate the ecological utility of relative per capita predation rates. In Paula et al. (2022a), several predators were sampled from four experimental treatments during 3 seasons from plots of 100 × 200 m replicated 4 × using pitfall traps. The production systems were: 1) double-cropped annual agriculture with soybean (season 1, *safra soja* = soybean season, October-February) and maize (season 2, *safra milho* = maize season, March-July) intercropped with palisade grass (*Brachiaria brizantha*), followed by a fallow period (*entressafra* = off-season, August-September), 2) forest plantation of eucalyptus trees (hybrid of *Eucalyptus grandis* and *E. urophylla*), 3) pasture of palisade grass, and 4) integrated crop-livestock-forest (ICLF), an additive mixture of all three. Here we show data for intraguild predation by the ant *Pheidole flavens* (Roger, 1863) [Hymenoptera: Formicidae] on another ant *Pheidole tristis* (Smith, 1858) [Hymenoptera: Formicidae]. Epigeal arthropod species were sampled twice a month (Brazilian authorization SISBIO 33683–1) in 2014/2015 (August through June) in Sinop-MT/Brazil using five pitfall traps per plot, exposed for a 24-h period. Pitfall traps contained 750 mL of water and 2 drops of detergent to break surface tension and preserve the captured specimens. We counted the number of *P. flavens* and *P. tristis* in the traps and calculated the average activity-density in each replicate plot for each species for each season. Predator (*P. flavens*) and prey (*P. tristis*) activity-density were estimated from the same traps. We obtained 12 samples of the most abundant ant species, *P. flavens* (n = 200 individuals/sample). The gaster was separated and collected immediately before DNA extraction using sterilized entomological dissecting tools. Sterilization was performed by soaking the dissection tools in 0.5 % sodium hypochlorite for 10 min and autoclaving (121 °C at 1 atm for 20 min), followed by rinsing abundantly with ultrapure water (MilliQ, Burlington, MA, USA) to minimize cross contamination.

For the unpublished data, coccinellid predators associated with *Brassica oleracea* L. [Brassicaceae] crops were sampled on six organic farms in Distrito Federal, Brazil (sampling authorizations SISBIO 36950 and IBAMA 02001.008598/2012–42, sampling details are in Andow et al., 2023). Briefly, adult *H. convergens* (total n = 280) were collected twice a month for 5 months from each farm (up to 10 individuals/farm/month). Here we show results for predation of *H. convergens* on the aphid *Lipaphis pseudobrassicae* (Davis, 1914) [Hemiptera: Aphididae].

To clean external DNA from the specimens, all the specimens were soaked individually for 40 min in 2.5 % commercial bleach in 1.5 microtubes, followed by orbital rotation at 2 g at 4 °C for 40 min, discarding the washing solution and rinsing the specimens 5 × in ultrapure water (Greenstone et al., 2012). Total DNA was extracted following manufacturer protocol using either the Monarch PCR & DNA Cleanup Kit (New England Biolabs, Ipswitch, Massachusetts) for the farm samples or the DNeasy Blood & Tissue kit (Qiagen, Redwood City, California) for all others.

For Lazaro, the pertinent aliquots were normalized to 150 ng DNA/sample. For metabarcoding, a region of the 16S mitochondrial gene was amplified using the primer pair Ins16S_1short (forward 5́-TRRGACGA-GAAGACCCTATA-3́ and reverse 5́-ACGCTGTTATCCCTAAGGTA-3́) (Integrated DNA Technologies, Coralville, Iowa), which generates an amplicon of ~190 bp (Clarke et al., 2014). Primers were not tagged to eliminate bias related to the tagging process (O'Donnell et al., 2016), so an independent library was produced for each epigeal predator DNA gut sample. PCR reactions (0.2 μM primer pair) were performed in triplicate using Qiagen Multiplex PCR Master Mix (Qiagen, Redwood City, California) and adding 1.28 μg/μL of bovine serum albumin to prevent PCR inhibition (Juen & Traugott, 2006). Triplicates were pooled and purified using QIAquick PCR Purification Kit (Qiagen, Redwood City, California). Amplicons were quantified by NanoDrop (Thermo Fisher Scientific, Waltham, Massachusetts) and normalized in equimolar ratios across all the metabarcoding samples.

All Lazaro and metabarcoding samples were dried in a speed vacuum centrifuge. The dried metabarcoding and Lazaro DNA samples from the previously published field experiment were shipped to the Roy J. Carver Biotechnology Center (University of Illinois at Urbana-Champaign, IL, USA) and libraries were constructed with the Hyper Library construction kit (Kapa Biosystems, Wilmington, MA, USA) with insert size 350 bp using unique dual indexes. Quality-checked samples were sequenced by Illumina HiSeq2500 (250 bp paired-end, HiSeq Rapid SBS sequencing kit version 2) in a single lane. The Brazilian license to access the genetic heritage was provided by CGEN/SISGEN A8E3D94. Sequence data are available at Paula et al., (2022b). Fastq files were generated and demultiplexed with the bcl2fastq v2.17.1.14 Conversion Software (Illumina). The quality assessment for each dataset was done using FastQC (FastQC, RRID:SCR_014583) (v.0.11.3). Low-quality sequences (Phred < 30) and library index adaptors were trimmed by Fastqc-mcf (v.1.04.807) and Cutadapt (cutadapt, RRID:SCR_011841) (v.1.9.1). Retained high-quality Fastq reads were converted to Fasta format by SeqTK (Seqtk, RRID:SCR_018927) (v1.2).

The reference database for metabarcoding was constructed by extracting invertebrate 16S barcode regions from the European Nucleotide Sequence database (EMBL) (release 132; inv: invertebrate database/division; std: standard) using the ecoPCR version 0.2 (Ficetola et al., 2010). In addition, 16S sequences for several species that were collected in the pitfall traps were determined (Paula et al., 2022a) and added, resulting in a 16S amplicon database composed of 63,618 sequences for 39,397 species from 2,172 families. Prey detection analysis was performed using OBITools (Quéméré et al., 2013, De Barba et al., 2014, Srivanthsan et al., 2015). Identifications were made using ecotag, and only 'head' and 'singleton' sequences were used for species-level identifications with identity ≥ 98 %. We kept only the identifications with sequences having a count > 100 and removed sequences with a length shorter than 80 bp. OBITools scripts are provided in Supporting Information.

For the Lazaro reference database (Paula et al., 2015,2016), we constructed a comprehensive arthropod mitochondrial DNA database by obtaining all sequences (partial or complete, Fasta format) available from GenBank (n = 3,381, distributed in 2,779 species from 1,850 genera in 598 families). We supplemented this with 29 taxa (Paula et al. (2022a) corresponding to the main potential prey co-occurring with the

sampled epigeal predators in the experimental plots. For taxonomic prey identification, we used the Lazaro method (Paula et al., 2022a). Briefly, this method identifies and retains false mismatches, reanalyzes overlap length and percent identity, filters the best-hit matches with a threshold of 100 % identity in an overlap length of ≥ 130 bp, eliminates singleton reads, and filters the reads mapping to coding regions of the mitogenome (Paula et al., 2022a).

All metabarcoding and Lazaro detections were confirmed with Melting Curve Analysis (MCA) in qPCR using taxon-specific primers. Taxon-specificity was evaluated empirically with laboratory cross-reaction tests (Paula et al., 2022a). Detection of aphid prey in the coccinellid was performed with MCA (Winder et al., 2011; Paula et al., 2022a). DNA from the prey species was used as positive controls and no template controls (NTC, negative controls) as negative controls. We designed a hypothetical aphid group-specific primer-pair, named AphGen, forward 5́-AACCACATCTTGACTTAA-3́ and reverse 5́-CTTATTAGAGGAACCTGTT-3́(amplicon size 145 bp) (Integrated DNA Technologies, Coralville, Iowa) in the mitochondrial region of the 12S gene, to screen the ladybug predator samples that could test positive for any aphid species consumption. The aphid group-specific primer-pair was designed in Primer-BLAST online tool (Ye et al., 2012). We tested its performance on the aphids [Hemiptera:Aphididae]: *Aphis citricidus* (Kirkaldy, 1907), *A. solanella* (Theobald, 1914), *Brevicoryne brassicae* (L., 1758), *Myzus persicae* (Sulzer, 1776) and *Uroleucon ambrosiae* (Thomas, 1878). MCA was performed after qPCR amplification in the Roche Applied Science LightCycler® 480 Real-Time PCR System (Roche Diagnostics Corporation, Indianapolis, Indiana) using Maxima SYBR Green/ROX qPCR Master Mix (2 ×) (Thermo Scientific™, Waltham, Massachusetts) with 1 μl of DNA in the total volume per reaction of 13 μl, in triplicate in white 384 well plates (Axygen®, corning Incorporated, Corning, New York). The optimized reaction MCA-qPCR parameters were: primer concentration at 0.2 uM; initial denaturation at 94 °C for 4 min; 45 cycles of annealing at 55 °C for 30 s and extension at 72 °C for 30 s; no final elongation. The melting temperature for the aphid group-specific primer-pair is 68.9 °C. To estimate predation on the aphid *L. pseudobrassicae* in the predator samples that tested positive for aphid consumption, a species-specific primer-pair was designed as described in Paula et al. (2022a) and verified by conducting cross-reaction tests using 1 ng of the purified DNA of each of the aphid and ladybug species known to occur in the sampled fields. Quantification of samples with positive detections was done with LinRegPCR (Ruijter et al., 2009) with manual baseline adjustment as needed.

Statistical analysis. Relative *per capita* predation rates, r_{ij}, were analyzed by glm, binomial or quasibinomial error with logit link in base R (R Core Team, 2022). The ant analysis included a blocking factor for the detection method. ANOVA tables were calculated with Anova in car (Fox and Weisberg, 2019) and least square means and standard errors were calculated with emmeans (Lenth et al., 2021). Pearson correlation was calculated in Excel and significance was tested using the Fisher transformation. York regressions were conducted to examine how prey activity-density and predator activity-density explained relative per capita predation rates for the ant data using the york function in IsoplotR (Vermeesch, 2018). York regression was used because each observation had independent measurement error in both x- and y-values. Activity-density data were log10 transformed to eliminate the variance-mean correlation in these data.

3. Results

A potential aphid group-specific primer pair. AphGen amplified all five aphid species tested (Fig. 1). According to an *in silico* analysis performed in Primer-BLAST, AphGen has the potential to amplify, without any mismatch, 170 species from the Aphidoidea superfamily, of which 118 species were Aphididae.

Laboratory feeding trial to verify method. The ten combinations of number of prey consumed and time since consumption were used to estimate a relative *per capita* predation rate for each combination. The data were normalized to equivalent prey consumption and time since consumption to calculate 10 independent estimates of relative *per capita* predation rates that were predicted to be the same. This allows verification of the accuracy of the method. There were no significant differences among the 10 estimates based on overlap of the standard deviations (Fig. 2). The estimated relative *per capita* predation rates ranged from 0.125 to 0.241, but seven of the estimates were within a narrow band between 0.150 and 0.174. The weighted mean rate was 0.150 ± 0.022 (SD). That the 10 different treatments provided a similar estimated relative *per capita* predation rate suggests that the method provided accurate estimates.

There was a significant negative correlation between the number of prey reads observed for each treatment and the standard deviation of the estimated relative per capita predation rate (Fig. 2), with the Pearson correlation, r = -0.870, z = -3.52, p = 4.28E-4. This indicates that the precision of the method is improved when more prey reads are observed. Specifically, the two largest standard deviations occurred in the treatments with 3 or 6 aphids consumed 9 h ago (3/9 and 6/9 treatments) and these two treatments had only 12 or fewer observed prey reads.

Relative* per capita *predation rates from field samples. The number of reads and relative *per capita* predation rates, *r*, of *P. flavens* on *P. tristis*

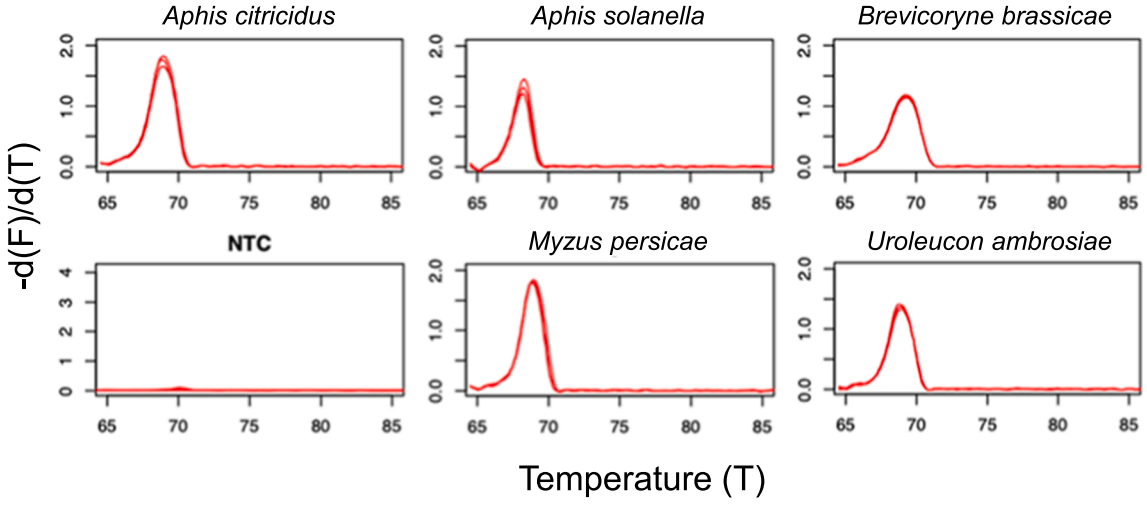

Fig. 1. Melt curves of amplicons from primer-pair AphGen for five species of aphids. NTC = no template control. Y-axis is the rate of change in fluorescence (F) per change in temperature (T).

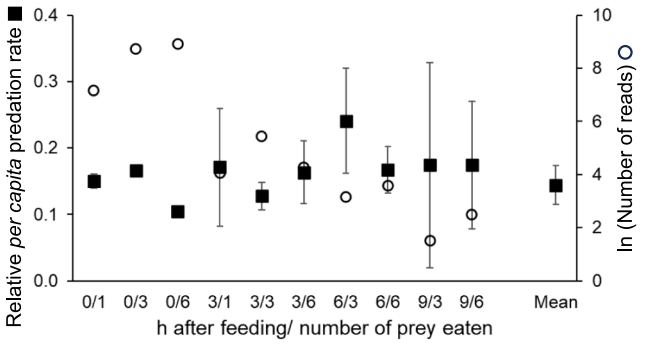

Fig. 2. Relative *per capita* predation rates (mean and standard deviation) and number of Lazaro prey reads estimated from a laboratory feeding trial allowing *Harmonia axyridis* to feed on 1, 3, or 6 *Myzus persicae* apterae. Predators were sacrificed 0, 3, 6 or 9 h after feeding. Mean is the inverse variance weighted mean of the 10 values with the weighted standard deviation.

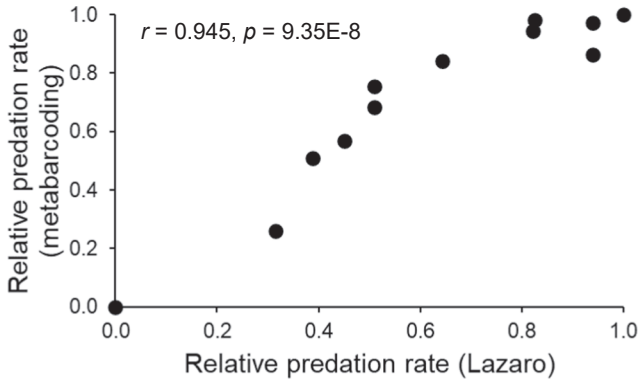

Fig. 3. Pearson correlation and *p*-value between relative *per capita* predation rates estimated by metabarcoding and Lazaro for 12 samples of *Pheidole flavens* preying on *Pheidole tristis*.

for 12 samples of *P. flavens* are provided in Table 1. Each sample was analyzed independently by metabarcoding and Lazaro. In previously published work (Paula et al., 2022a), we showed that log number of reads of detected *P. tristis* was significantly correlated with independent quantification by qPCR. Here we found that the relative *per capita* predation rates determined by metabarcoding and Lazaro were also highly correlated (Fig. 3, Pearson correlation = 0.945, $z = 5.339$, $p = 9.35E\text{-}8$). This suggests that, in this case, metabarcoding probably provided accurate estimates of the relative per capita predation rate.

The relative *per capita* predation rate of *P. flavens* on *P. tristis* was analyzed with quasibinomial error because there was significant overdispersion (dispersion parameter = 128.5). The treatment × season effect was significant ($p = 0.0456$), but the main effects of treatment and season were not. Relative *per capita* predation was higher in the crop treatment than the forest and integrated treatments, and lower in the forest treatment than the crop and integrated treatments for all of the seasons (Fig. 4). For these three treatments, it was higher during season 1 (*safra soja*) than the off-season or season 2 (*safra milho*). The pasture

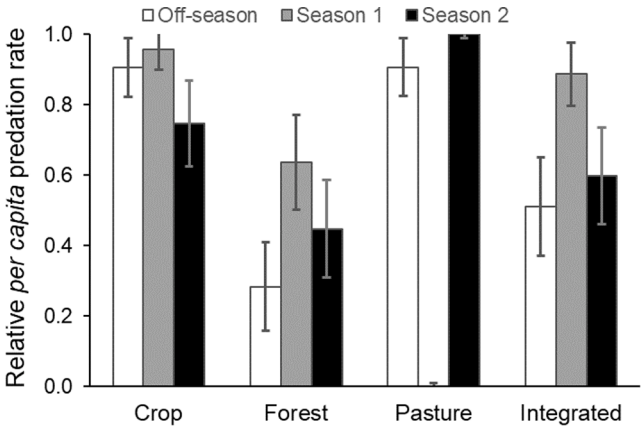

Fig. 4. Relative *per capita* predation rate of *Pheidole flavens* on *Pheidole tristis* in four experimental treatments during three cropping seasons. Bars are standard errors.

Table 1
Number of reads detected for the prey *Pheidole tristis* consumed by the predator *Pheidole flavens* and relative predation rates, *r*. Treatments and seasons are described in the main text; Off = off-season; S1 = season 1; S2 = season 2. Each sample was 200 pooled predator workers.

Treatment	Season	Method	*Pheidole tristis*	
			Number of reads	*r*
Crop	Off	Lazaro	2666	0.940075
Forest	Off	Lazaro	14	0.314504
Integrated	Off	Lazaro	44	0.450972
Pasture	Off	Lazaro	1014	0.824873
Crop	S1	Lazaro	2654	0.939537
Forest	S1	Lazaro	72	0.509662
Integrated	S1	Lazaro	986	0.821536
Pasture	S1	Lazaro	0	0
Crop	S2	Lazaro	222	0.643852
Forest	S2	Lazaro	26	0.388276
Integrated	S2	Lazaro	72	0.509662
Pasture	S2	Lazaro	4408	1
Crop	Off	Metabarcoding	130,941	0.864248
Forest	Off	Metabarcoding	35	0.260785
Integrated	Off	Metabarcoding	2313	0.568192
Pasture	Off	Metabarcoding	647,683	0.981509
Crop	S1	Metabarcoding	566,549	0.971692
Forest	S1	Metabarcoding	29,809	0.755694
Integrated	S1	Metabarcoding	392,573	0.944784
Pasture	S1	Metabarcoding	0	0
Crop	S2	Metabarcoding	96,634	0.841963
Forest	S2	Metabarcoding	1027	0.508639
Integrated	S2	Metabarcoding	11,120	0.683366
Pasture	S2	Metabarcoding	833,380	1

treatment was completely different. It had the lowest relative *per capita* predation rate in the first season and had the highest relative rate in the other two seasons of all of the treatments (Fig. 4). York regressions showed that higher predator activity-density was associated with lower relative *per capita* predation rate (slope = -0.501 ± 0.084 (SE), 10 *df*, $p = 1.44E\text{-}4$, Fig. 5A), while there was no relationship associated with prey activity-density (slope = 0.106 ± 0.065 (SE), 10 *df*, $p = 0.136$, Fig. 5B).

The estimated relative initial template concentration (n_0) calculated from the qPCR data and the relative per capita predation rates, *r*, of *H. convergens* on *L. pseudobrassicae* are provided in Table 2. The relative predation rate did not vary among the six organic farms (Fig. 6A, $p = 0.7111$) or among the five months studied (Fig. 6B, $p = 0.6252$).

4. Discussion

Theoretical considerations. In this paper, we have elaborated a method to estimate relative *per capita* predation rates from quantitative gut content data and made explicit the assumptions that underlie the method. Quantitative gut content data are generated by methods that allow measurement of relative prey quantity in a predator sample, which includes methods such as quantitative ELISA, quantitative electrophoresis, qPCR and Lazaro (Paula et al., 2022a). Methods that provide only presence-absence of prey in a sample, such as conventional PCR and qualitative ELISA, cannot be used with this method. We show that the relative *per capita* predation rates can be derived from an equation that estimates absolute *per capita* predation rates from

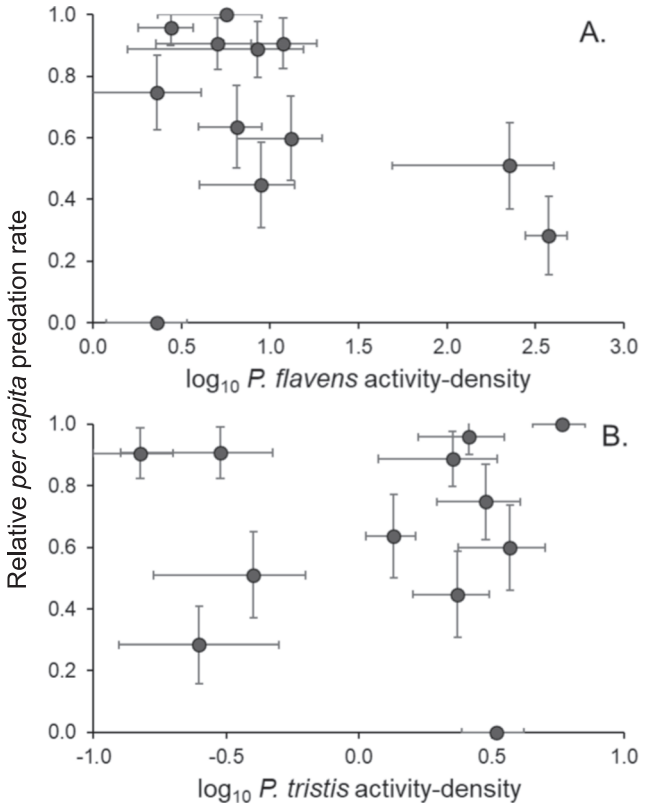

Fig. 5. Relation between (A) predator activity-density (*Pheidole flavens*) or (B) prey activity-density (*Pheidole tristis*) and relative *per capita* predation rate in four experimental treatments during three cropping seasons. Bars are standard errors.

Table 2
Estimated relative initial template concentration from qPCR, n_0, and relative *per capita* predation rates, *r*, for predation by *Hippodamia convergens* on *Lipaphis pseudobrassicae*. *n* = number of predator beetles in a sample; NA = missing values.

n	Farm	Month	*Lipaphis pseudobrassicae*	
			n_0	*r*
10	1	May	2.40246E-10	0.204481
7	2	May	1.10293E-10	0.093874
10	3	May	6.67307E-10	0.567966
8	4	May	4.08672E-10	0.347833
0	5	May	NA	NA
7	6	May	3.33995E-10	0.284274
10	1	June	3.16251E-10	0.269172
10	2	June	3.40445E-10	0.289763
10	3	June	3.22444E-10	0.274442
10	4	June	1.70841E-10	0.145408
8	5	June	3.46085E-10	0.294563
10	6	June	1.17491E-09	1
10	1	July	3.29235E-10	0.280222
10	2	July	2.46794E-10	0.210054
10	3	July	5.58228E-10	0.475125
10	4	July	9.48184E-11	0.080703
10	5	July	2.01368E-11	0.017139
10	6	July	2.60957E-10	0.222109
10	1	Aug	2.39223E-10	0.20361
10	2	Aug	5.09636E-10	0.433768
10	3	Aug	5.21434E-10	0.443809
10	4	Aug	4.04077E-10	0.343923
10	5	Aug	5.08299E-10	0.432629
10	6	Aug	2.70683E-10	0.230387
10	1	Sept	4.37485E-10	0.372357
10	2	Sept	3.67981E-10	0.313201
10	3	Sept	2.78144E-10	0.236737
10	4	Sept	4.40389E-10	0.374829
10	5	Sept	6.14571E-10	0.523081
10	6	Sept	3.39314E-10	0.288801

quantitative gut content data (Andow & Paula, 2023). The equation was verified empirically to provide an accurate estimate of absolute per capita predation rates using a controlled experiment (Andow & Paula, 2023). We relativized these absolute rates by dividing all observations with the highest observed rate. In doing so, several terms cancel, and the relative *per capita* predation rate is proportional to the quantified prey content in a sample. The original, absolute method (Andow & Paula, 2023) assumed that the decay rate of the prey in the predator follows a first order decay process and that the decay rate is similar among the predator samples. While additional data are needed, the available data supports these assumptions (Andow & Paula, 2023). The relative *per capita* predation rate developed here requires these, but no additional assumptions.

Application of the relative *per capita* predation rate is presently restricted to comparison of relative predation rates of one predator species on a single prey species, because decay rates are known to vary among predator and prey species (Greenstone et al., 2010, Andow & Paula, 2023). While this does not allow a comparison of predation on different prey species by a predator or comparison of predators on a prey species, it does allow comparison of predation by a predator on a prey across time, space or individuals.

The use of the number of metabarcoding reads to quantify the prey in a predator sample remains controversial. A few studies have successfully interpreted metabarcoding reads quantitatively (Willerslev et al., 2014, Kartzinel et al., 2015, Krehenwinkel et al., 2017), and a *meta*-analysis has shown a small, but significant relation between the number of reads and species abundance (Lamb et al., 2019). As metabarcoding relies on PCR, read abundance has quantification biases related to variation in species-specific primer efficiency, amplicon length (shorter amplicons may artificially increase species richness and evenness), primer tag jumps, and barcode primer preference to amplify certain taxonomic groups (Amend et al., 2010; Engelbrektson et al., 2010; Berry et al., 2011; Ihrmark et al., 2012; Pinto & Raskin, 2012; Deagle et al., 2013,2014; Clarke et al., 2014; Elbrecht and Leese, 2015; Alberdi et al., 2018), which leads to lack of or misrepresentation of taxa and over- or under-estimation of abundance (Yu et al., 2012; Leray et al., 2013; Deagle et al., 2014; Elbrecht and Leese, 2015; Pinol et al., 2015; Bista et al., 2018; Lamb et al., 2019; Pinol et al., 2018). Several studies have examined the use of spike-in standards (e.g., Thomas et al., 2014,2016) as a correction factor for different prey species, but these methods were neither sufficiently accurate nor general.

Despite all the concerns regarding metabarcoding data for quantitative purposes, by focusing on one prey in one predator species, relative *per capita* predation rates avoid the problems of species-specific primer efficiency and primer preference for certain taxa. Further, because tag-jumps are random and amplicon length will be the same for all the species amplified in a sample, then virtually all of the major limitations to quantitatively interpreting the number of metabarcoding reads are eliminated. The remaining potential complication is if amplification efficiency for the one prey species is strongly dependent on the mixture of templates in the sample, which can occur via template competition for the barcode primers. This possibility can be mitigated by adjusting the relative concentration of primer and dNTPs to template. Thus, it is possible that in many cases, the number of metabarcoding reads can be used to calculate relative per capita predation rates.

Laboratory feeding trial. To verify that the theory could be used to generate accurate and precise estimates of relative per capita predation rates, we conducted a controlled laboratory feeding trial. The trial used a ladybeetle feeding on variable numbers of aphids with the gut contents analyzed at different periods post ingestion using Lazaro (Paula et al., 2022a), which detects prey in the unamplified gut contents. We hypothesized that when the data were normalized to comparable numbers of prey and comparable post-ingestion periods, estimates of the relative

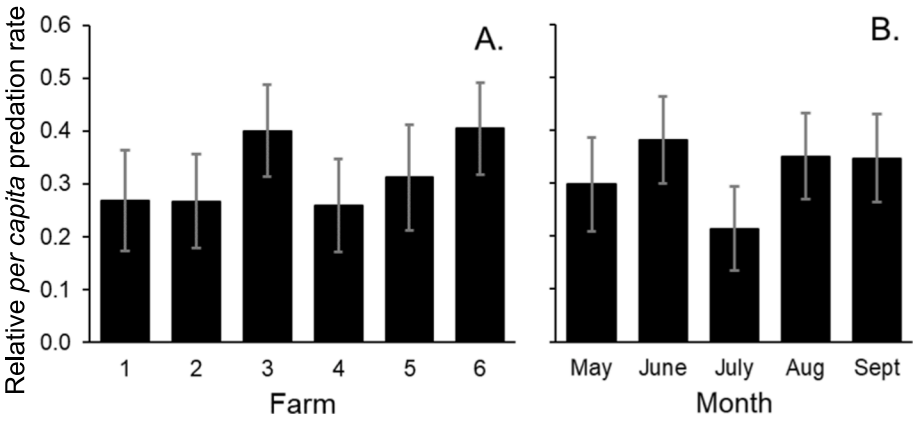

Fig. 6. Relative per capita predation rate of *Hippodamia convergens* on *Lipaphis pseudobrassicae* (A) on six organic farms and (B) during five months. Bars are standard errors.

per capita predation rate would be similar. The results from the ten independent estimates gave statistically similar estimates of a relative *per capita* predation rate. This similarity indicated that the theory and estimating equations can provide accurate estimates of the relative *per capita* predation rate. Precision was worse when few prey reads were observed, suggesting that when using Lazaro, additional samples may be needed to generate precise, accurate estimates of the relative *per capita* predation rate when few prey reads are observed. It would be useful to repeat such laboratory feeding trials on diverse predator species feeding on different prey to test the generality of our findings.

Additional trials are also needed to investigate the factors affecting the precision and accuracy of estimates of relative *per capita* predation rates using data from other quantitative methods, including qPCR, quantitative ELISA and metabarcoding. It might be predicted that metabarcoding data would generate less precise and possibly less accurate estimates of the relative *per capita* predation rate than the other quantitative methods. Because metabarcoding relies on DNA amplification, the effect of stochastic events on the number of reads would be multiplied exponentially, which would reduce precision and possibly reduce accuracy.

Empirical examples. Here we have illustrated how relative *per capita* predation rates can be used to compare predation during three seasons in a large-scale field experiment, and during five months of observations in six organic farms. The large-scale field experiment used data generated from Lazaro and metabarcoding and the farm observations used data generated from qPCR.

In the field experiment, we found that the relative *per capita* predation rate by *P. flavens* on *P. tristis* was higher in the crop treatment than the forest and integrated treatments, and lower in the forest treatment than the crop and integrated treatments. Predation in the pasture treatment varied with the season and was highest during the off-season and season 2 and lowest during season 1. The estimates from Lazaro and metabarcoding gave the same statistical inferences. This predator is geographically widespread throughout tropical and subtropical North, Central and South America and prefers to nest in rotting wood, beneath the bark of trees, in dead knots on tree trunks, in sod on rocks, in the soil beneath stones, and in epiphyte masses (Wilson, 2003). Based on its nesting biology, the highest predation rate might have been predicted to occur in the forest treatment and the lowest in the crop treatment, which was not supported by our results. However, we also found that the relative *per capita* predation rate was negatively associated with predator activity-density possibly implying that prey were limiting. This was supported by the absence of an association of predation with prey activity-density, probably because prey were not as abundant as the predator and present at similar activity-densities in all of the treatments. Thus, the lower/higher relative *per capita* predation rate on *P. tristis* in the forest/crop system by *P. flavens* was not inconsistent with a prediction based on its nesting biology. Its nesting biology would predict higher predator activity-density in the forest treatment and lower predator activity-density in the crop treatment, resulting in lower predation in the forest treatment and higher relative *per capita* predation rate in the crop treatment.

In the observational study using data from qPCR, we found no difference in relative *per capita* predation rate by *H. convergens* on *L. pseudobrassicae* across six farms and five months of observations. Potential differences may have been obscured by the large standard errors in the estimated relative *per capita* predation rates within farms and months. In any event, this result suggests that the farms may be considered biological replicates for understanding predation in future and previous analyses (Andow et al., 2023a,2023b) associated with this study.

Estimating predation rates is an important step toward understanding the spatio-temporal dynamics of a predator–prey interaction. As described here, the use of quantitative molecular gut content analysis to calculate relative *per capita* predation rates can provide a rapid way to assess how this interaction changes over space and time. Ultimately, this may help identify factors that limit or enhance biological control of pests.

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CRediT authorship contribution statement

David A. Andow: Writing – review & editing, Writing – original draft, Visualization, Software, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Débora Pires Paula:** Writing – review & editing, Project administration, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2024.105499.

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