Entomologia Generalis, Vol. 41 (2021), Issue 6, 591–599
Published online 28 April 2021

Article

Development of a molecular gut-content identification system to identify aphids preyed upon by the natural enemy *Coccinella septempunctata*

Da Xiao[1,*], Qingxuan Xu[1,*], Xu Chen[1], Xiaoyan Du[1], Nicolas Desneux[2], Eva Thomine[2], Hui-Jie Dai[3], James D. Harwood[1], and Su Wang[1,]**

[1] Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forest Science, Beijing, China
[2] Université Côte d'Azur, INRAE, CNRS, UMR ISA, 06000 Nice, France
[3] Weifang University of Science & Technology, Facility Horticulture Laboratory of Universities in Shandong, 262700 Weifang, China
* These authors contributed equally
** Corresponding author: wangsu@ipepbaafs.cn

With 7 figures and 1 table

Abstract: Molecular gut-content analysis has revolutionized the study of predator-prey interactions and allowed the accurate description of food webs in the field. In this study, we identified and characterized cytochrome c oxidase subunit 1 (CO1) from the aphid *Megoura japonica* (Homoptera: Aphididae) and characterized its utility for molecular gut-content research. *Coccinella septempunctata* (Coleoptera: Coccinellidae) were selected as a representative predator of two insects, *M. japonica* and *Aphis craccivora* (Homoptera: Aphididae), for analysis of predator-prey interactions using prey-specific PCR and reverse-transcription quantitative PCR (RT-qPCR). The results of prey-specific PCR showed that with increasing *M. japonica* consumption, the *MjCO1* PCR band was brightened and subsequently decreased with extended digestion time. RT-qPCR using Taqman-MGB fluorescence probe was also utilized to quantify predator-prey interaction between *C. septempunctata* and *M. japonica*. The *MjCO1* copy number in *C. septempunctata* increased with greater consumption of *M. japonica*. Moreover, *MjCO1* copy number in *C. septempunctata* decreased with increasing digestion time. Furthermore, analysis of *CO1* copy numbers in the gut of *C. septempunctata* showed no feeding preference between *M. japonica* and *A. craccivora*. Our results demonstrate the utility of molecular gut-content analysis in understanding trophic relationships between these ladybirds and aphids. In addition, RT-qPCR is shown to be an effective and accurate method that should be considered for wider application in predation studies in field settings.

Keywords: predator-prey relationship, molecular detection, gut analysis, food web, lady beetle

1 Introduction

In biological control, predator-prey relationships are characterized by prey choice and the selection of optimal predators. Several methods have been used to study predator-prey interactions, including direct observation, natural enemy manipulation, life table analysis and gut content analysis (e.g., Pfannenstiel, 2008; Harwood & Obrycki, 2005; Harwood et al., 2007; Weber & Lundgren, 2009; Moser et al., 2011; Thomine et al., 2020). The application of molecular analysis to reveal gut contents has revolutionized predator-prey relationship analysis in agro-ecosystems (e.g., Schmidt et al., 2014; Welch et al., 2014; King et al., 2015). The advantage of this approach is that it can reveal a broad range of trophic relationships with minimal disruption of natural feeding behavior and has a multitude of implications in conservation biology and management (Symondson, 2002; Sheppard & Harwood, 2005; Clare, 2014; Lundgren & Fergen, 2014). With the development of molecular gut content analysis, it has been possible to detect predation on a diverse array of pests including mosquito vectors (Zaidi et al., 1999), moths (Hoogendoorn & Heimpel, 2001; Peterson et al., 2018), whiteflies (Agustí et al., 2000; Zhang et al, 2007), aphids (Chen et al., 2000; Harwood et al., 2007) and psyllids (Agustí et al., 2003).

Polymerase chain reaction (PCR) with prey specific primers has been used to detect prey relationships and analyze trophic linkages in many organisms (Sheppard & Harwood,

DOI: 10.1127/entomologia/2021/1032

2005; Macias-Hernandez et al., 2018). This method has proven to be highly effective and versatile in laboratory trials (Symondson, 2002) and in the field (Albertini et al., 2018; Jaramillo et al., 2010; Monzó et al., 2011). However, optimization of the system is critical because PCR can provide false positive trophic connections which decrease the accuracy of reliably characterizing predator-prey interactions. Additionally, RT-qPCR can be more sensitive and accurate than general PCR and with reduced costs, it is now effective for application in predator-prey relationship studies in the field (Weber & Lundgren, 2009; Zhang et al., 2007).

In molecular gut content analysis, multi-copy genes are recommended as targets to increase detection probability (Gariepy et al. 2007; Symondson, 2002). Mitochondrial DNA (mtDNA) is present in hundreds or thousands of copies in every insect cell (Hoy, 1994; Caterino et al., 2000) and thus mitochondrial genes have been used in arthropod predator – prey research for a considerable time (Chen et al. 2000; Sheppard et al., 2004). Cytochrome *c* oxidaseI and II in mitochondria are considered ideal target genes and have been used as DNA barcodes for species-specific sequences and prey interaction analysis (Agusti et al., 2003; Simon et al., 1994; Jaramillo et al. 2010; Greenstone et al., 2014). Furthermore, both the length of prey DNA fragments and primer sensitivities have been shown to influence molecular gut analysis (Waldner et al., 2013). It is therefore important to determine target genes of prey and primer sensitivities before applying this approach to field-based studies.

The seven-spot ladybird beetle, *Coccinella septempunctata* Linnaeus 1758 (Coleoptera: Coccinellidae), has long been reported as a beneficial predator that is used as a biological control agent of aphids worldwide (Lu et al. 2012; Xiao et al., 2016; Hewlett et al. 2019; Pan et al. 2020; Rondoni et al. 2020). Both larvae and adults are voracious predators of aphids on variety of plants but the relationship and efficiency of *C. septempunctata* to aphids has been characterized in a limited and largely qualitative way. In this study, we establish a detection system for evaluating the relationship between the predator *C. septempunctata* and two important pest prey, *M. japonica* (Matsumura) and *Aphis craccivora* (Koch), using prey-specific PCR and RT-qPCR under laboratory conditions. We sought to characterize this detection system and document if it can be adapted as a valuable tool for quantitatively and qualitatively analyzing prey function of *C. septempunctata* in the field.

2 Materials and methods

2.1 Insects

Adult *C. septempunctata* were collected into 3.5 ml centrifuge tube from experimental wheat fields of the Beijing Academy of Agriculture and Forestry Sciences (BAAFS), Beijing, China, during May 2016. The colonies of ladybeetles were maintained under laboratory conditions and reared on the soybean aphids, *Aphis glycines* (Hemiotera: Aphididae), at the Laboratory of Natural Enemies Research, Institute of Plant and Environment Protection, BAAFS. The ambient conditions were as follows: temperature 25°C, relative humidity 65% and a 15:9 light: dark cycle (regulated by an automatic device, L-100 Suntech, Beijing, China). *C. septempunctata* were maintained in custom-built culturing cages (50 cm × 50 cm × 50 cm; 45 mesh plastic fabric on aluminum frames) at a density of 40 pairs per cage and fed daily with *A. glycines* grown on fresh seedlings of soybean.

The laboratory colony of *M. japonica* and *A. craccivora* were established from apterous individuals collected from broad bean fields since May 2012 (Yunnan province, China). The mixed colony was maintained with a continuous supply of broad bean seedlings grown on vermiculite in climatic chambers at 25 ± 2°C, 60 ± 10 % RH, 13:11 L:D. New broad bean seedlings were provided weekly to the colony and aphids were transferred by placing infested leaves on uninfected plants.

2.2 Subcloning and sequencing of *Megoura japonica*

The universal primer ((F) LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3'; (R) HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3')) (Table 1) was used to clone the cytochrome *c* oxidase subunit I of *M. japonica* (Simon et al., 1994; Folmer et al., 1994). The PCR products of the reactions were subjected to 1% agarose gel electrophoresis containing GoldenView (Coolaber, Beijing, China). The PCR bands were excised and purified using QIAEX II Agarose Gel Extraction Kit (Qiagen, Valencia, California, USA) and the purified fragment was subcloned into a pGEM – T Easy Vector (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The ligation DNA mixtures were used to transform bacterial cells using Z – Competent *E. coli* Transformation Kit and Buffer Set™ (Zymo Research Corporation, Irvine, California, USA). Plasmids were isolated from the bacterial cells and used for *MjCO1* sequencing (Invitrogen, Shanghai, China).

2.3 Feeding trials of *Coccinella septempunctata* consuming *Megoura japonica*

For the trials examining prey quantity consumed, third instar larvae of *C. septempunctata* were removed from colonies, transferred individually into petri dishes and starved for 24h. After starvation, larvae were transferred into a clean petri dish containing moistened filter paper (diameter: 60 mm) and allowed to feed on *M. japonica*. To examine the effect of prey quantity on detection, numbers of second instar nymphs of *M. japonica* (n = 5, 10, 15 and 20) were provided to *C. septempunctata*. DNA extracted from *M. japonica* were used as a positive control and dissected guts from starved third instar larvae were used as a negative control.

Table 1. Sequences and relevant parameters of the PCR primer used for molecular gut analysis.

Primer Name	Sequence (5'-3')	Temp (°C)	Product size (bp)
LCO1490 – F:	GGTCAACAAATCATAAAGATATTGG;	55.4	658
HC02198 – R:	TAAACTTCAGGGTGACCAAAAAATCA	57.3	
MjCOI – F	TGGTACAGGAACAGGATGAACTAT	57.6	323
MjCOI – R	GATCTCCTCCTCCTGCTGG	58.7	
MjCOI (Q) – F	CATTACATTTAGCAGGAATTTCATCAA	58.6	116
MjCOI (Q) – R	TGATCATGGAAAAAGTGGAATTTG	58.9	
MjCOI (Q) – Probe	TTTAGGAGCAATTAAC	67.0	16
AcCOI (Q) – F	CCCTTGATCAATCCTAATTACAGCTAT	58.3	126
AcCOI (Q) – R	CCTCCTCCTGCTGGGTCAA	59.8	
AcCOI (Q) – Probe	ATTTTATCATTACCAGTCTTAGC	68.0	23

Examination of digestion/detection time trials, third instar larvae of *C. septempunctata* were also used, removed from colonies as above and starved for 24h. Fifteen nymphs (2nd instar) of *M. japonica* were provided in each petri dish. Individuals that did not consume prey within 2h were discarded from the experiment and *C. septempunctata* were removed at 1, 2, 4, 6, 8 and 10h after feeding. The guts of *C. septempunctata* were removed by dissection with sterilized instruments and subsequently stored at – 80°C until analysis. Each experiment was repeated with three biological replicates, each biological replicate with ten technical replicates. Positive and negative controls were as described above.

2.4 Trials of *Coccinella septempunctata* feeding on *Megoura japonica* and *Aphis craccivora*

To examine food preference of *C. septempunctata, M. japonica* and *A. craccivora* were provided concurrently. Third-instar larvae, female adults and male adults of *C. septempunctata* were isolated and starved for 24 hours. Starved predators were then transferred into a small cage containing *M. japonica* and *A. craccivora* and predators were collected at 2, 4, 6 and 8 days after feeding freely on aphids. The guts of *C. septempunctata* were dissected and DNA extracted for use in subsequent quantitative experiments. DNA samples of *M. japonica* and *A. craccivora* were extracted and used as respective positive controls. DNA samples of the dissected gut from third instar, female and male adult coccinellids that were starved for 24h were used as negative controls. Each experiment was repeated with three biological replicates, each biological replicate having ten technical replicates.

2.5 DNA extraction and prey-specific PCR analysis

Total DNA was extracted from predator guts using DNeasy® Blood &Tissue Kits (QIAGEN Inc., Chatsworth, California, USA) following the manufacturer's instructions. Primer set *MjCOI* was used in prey-specific PCR to amplify a 323 bp fragment of *MjCOI* (Table 1). The 20μL reaction system consisted of 1× Takara buffer, 0.2 mmol/L of each dNTP, 0.2 μmol/L of forward and reverse primers, 1.25U Takara *Ex Taq*™ and 2 μL of template DNA. PCR reactions were performed in a Bio-Rad PTC –100 thermal cycler (Bio-Rad Laboratories Inc., Hercules, California, USA). The PCR cycling protocol for the *MjCOI* primer pair was 94°C for 5 min (initial denaturation), 32 cycles at 94°C for 30s (denaturation), 56°C for 30s (annealing), 72°C for 30s (elongation) and a final cycle at 72°C for 10 min. The PCR products of each reaction were subjected to electrophoresis on 1% agarose gel containing GoldenView dye (Coolaber, Beijing, China).

2.6 Specific primer for reverse-transcription quantitative PCR (RT-qPCR) and standard plasmid curve construction

The specific primers and fluorescence probe for *MjCOI* and *AcCOI* were designed and synthesized by Shanghai GeneCore Biotechnologies Company Limited (Shanghai, China) (Table 1). The standard curve of *MjCOI* plasmids were constructed before quantitative testing. Total DNA of *M. japonica* and *A. craccivora* was extracted using DNeasy® Blood & Tissue Kits following the manufacturer's instructions. Primer set *MjCOI* (forward and reverse) was used to amplify a 323 bp fragment of *COI* of *M. japonica* that included 116 bp of detected fragment and 16 bp of fluorescent probe (TTTAGGAGCAATTAAC). Primer set *AcCOI* (forward and reverse) was used to amplify a 271bp fragment of *COI* of *A. craccivora* that included a 126 bp detected fragment and 23 bp fluorescent probe (ATTTTATCATTACCAGTCTTAGC) (Table 1). The PCR products of reactions were subjected to electrophoresis on 1% agarose gel containing GoldenView. The PCR bands were excised and purified using QIAEX II Agarose Gel Extraction Kit (Qiagen, Valencia, California, USA). The purified fragment was subcloned into a pGEM-T Easy Vector (Promega) according to the manufacturer's instructions. The ligation DNA mixtures were used to transform

bacterial cells by using Z-Competent *E. coli* Transformation Kit and Buffer Set™ (Zymo Research Corporation, Irvine, California, USA). Plasmids were isolated from the bacterial cells using Zyppy plasmid Miniprep Kit (Zymo Research Corporation, Irvine, California, USA) following protocols of the manufacturer. The concentration of plasmid was measured using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The concentration of 150 ng/μL plasmid was selected as initial standard samples and diluted to six concentrations. The standard curve of these plasmids was determined by reverse transcription quantitative PCR (RT-qPCR) using Taqman – MGB fluorescence probe using a PCR detection system (Applied Biosystems QuantStudio 7 Flex, Thermo Fisher Scientific, Waltham, MA). The DNA copy number was transformed from plasmids based on the following equation: DNA (copy) = 6.02 × 10^{23} (copy/mol) × DNA amount (g)/ DNA length (dp) × 660 (g/mol/dp) (Lee et al., 2006; Whelan et al., 2003). The regression equation between Ct value and DNA copy number was obtained through linear regression analysis.

2.7 RT – qPCR analysis

The samples were amplified by RT-qPCR using Taqman – MGB probe and the ABI PCR detection system (Applied Biosystems QuantStudio 7 Flex) in 20 μL reaction mixtures containing 10 μL Probe qPCR Premix Ex Taq, 0.4 μL primer sets (forward and reverse), 0.8 μL probe and 2.0 μg DNA template. The optimized quantitative PCR program consisted of an initial denaturation at 95°C for 5min, 40 cycles at 95°C for 15s (denaturation), 61.4°C for 30s (annealing) and 70°C for 30s (elongation). Both positive and negative controls were run with each batch of RT-qPCRs. For negative controls 2 μL sterile MilliQ® H_2O was used as template and for positive controls 2.0 μg template DNA from *M. japonica* and *A. craccivora* were used to confirm reaction success.

2.8 Statistical analysis

The DNA copy number of *MjCO1* were subjected to ANOVA followed by Tukey's honestly significant difference (HSD) for multiple comparisons, to separate the means among the amount of prey and digestion time, by using ProStat software (Poly Software International, Pearl River, New York, USA). The DNA copy number of *MjCO1* and *AcCO1* were subjected to testing followed by t-test to separate the means in each detected time, using ProStat software.

3 Results

3.1 Cloning the COI gene of *Megoura japonica*

The universal primer successfully amplified *COI* in *M. japonica* and the ~700 bp specific PCR band showed clearly on the agarose gel (Fig. 1). The PCR product of *MjCOI* was purified and sequenced, then we obtained the full-length of *MjCOI* at 709 bp. This sequence showed 87.71% identity aligned with *ApCOI* (*Acyrthosiphon pisum*, KP034391), 86.74% with *SaCOI* (*Sitobion avenae*, FN868603), 85.33% with *MpCOI* (*Myzus persicae*, DQ499045) and 84.43% with *RmCOI* (*Rhopalosiphum maidis*, DQ499048).

3.2 Detection of *Megoura japonica* DNA using prey-specific PCR

The specific primers (Table 1) were used in prey-specific PCR to detect *MjCOI* in *C. semptemunctata*. The results shown on the agarose gel revealed that with increasing number of *M. japonica* consumed, the brightness of the *MjCOI* band also increased (Fig. 2). With increasing time after consumption of prey, the agarose gel showed that the brightness of the *MjCOI* band decreased (Fig. 3).

3.3 Detection of *Megoura japonica* DNA using RT – qPCR

RT-qPCR was undertaken to detect the DNA copy number of *MjCOI* in *C. septempunctata*. The standard plasmids of *MjCOI* were constructed and used as template in RT-qPCR detection. Following this, a standard curve (Y = –3.191X + 53.028; R^2 = 0.993) of the *MjCOI* copy number and Ct value were fitted. Based on the plasmid standard curve of *MjCOI* copy number and Ct value, the regression equation (Y = 0.622X + 6.874; R^2 = 0.7548) of *MjCO1* copy number in the gut of *C. septempunctata* and *M. japonica* consumed, was fitted. Results indicated that with increasing number of *M. japonica* consumed, the detected *MjCOI* copy number in the gut of *C. septempunctata* increased (Fig. 4). We also documented that *MjCO1* copy number in *C. septempunctata* consuming 20 *M. japonica* was significantly higher than when other numbers of prey were consumed. Furthermore,

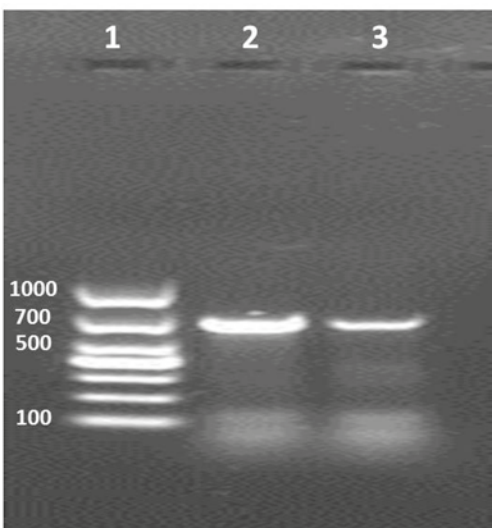

Fig. 1. Agarose gel of PCR products of COI gene using universal primer. Lane 1, 1000bp ladder; Lane 2, PCR product of *MjCOI*.

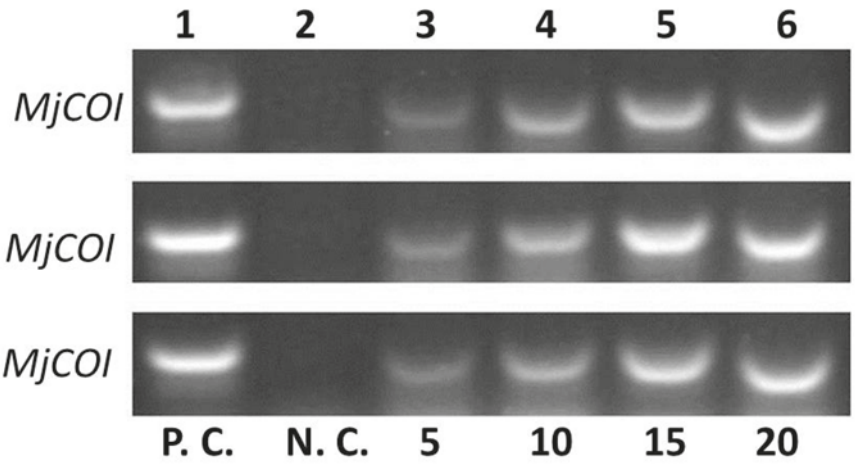

Fig. 2. Agarose gel of PCR products using *M. japonica* primer (Table 1) to determine prey number following consumption by *Coccinella septempunctata*. Lane 1, positive control *Megoura japonica*; lane 2, *C. septempunctata* fed 0 *M. japonica*; lane 3, *C. septempunctata* fed 5 *M. japonica*; lane 4, *C. septempunctata* fed 10 *M. japonica*; lane 5, *C. septempunctata* fed 15 *M. japonica*; lane 6, *C. septempunctata* fed 20 *M. japonica*.

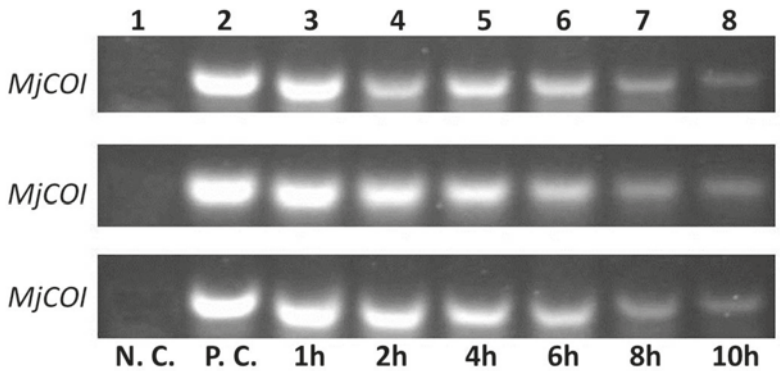

Fig. 3. Agarose gel of PCR products using *Megoura japonica* primer (Table 1) to determine digestion time following consumption by *Coccinella septempunctata*. Lane 1, negative control; lane 2, positive control; lane 3, digestion time 2h; lane 4, digestion time 4h; lane 5, digestion time 6h; lane 6, digestion time 8h; lane 7, digestion time 10h.

the regression equation (Y= –0.6296X + 10.85; R^2 = 0.9069) of *MjCO1* copy number in the gut of *C. septempunctata* and digestion time was fitted and with increasing digestion time, the *MjCOI* copy number decreased (Fig. 5).

3.4 Detection of *Megoura japonica* and *Aphis craccivora* DNA using RT – qPCR

The above results showed that both qualitative PCR and RT-qPCR are effective methods for studying the relationship between *C. septempunctata* and its prey. To detect the preference of *C. septempunctata* towards *M. japonica* or *A. craccivora*, we conducted RT-qPCR analysis as above. Firstly, the standard curve (Y = –3.164X + 52.953; R^2 = 0.998) of *AcCOI* copy number and Ct value were fitted. The results for third instar larvae showed that *MjCOI* and *AcCOI* copy number in the gut of *C. septempunctata* were approximately equal (Fig. 6). Furthermore, *MjCOI* and *AcCOI* copy number in the gut of *C. septempunctata* were also equal when female and male adults consumed these two aphids (Fig. 7).

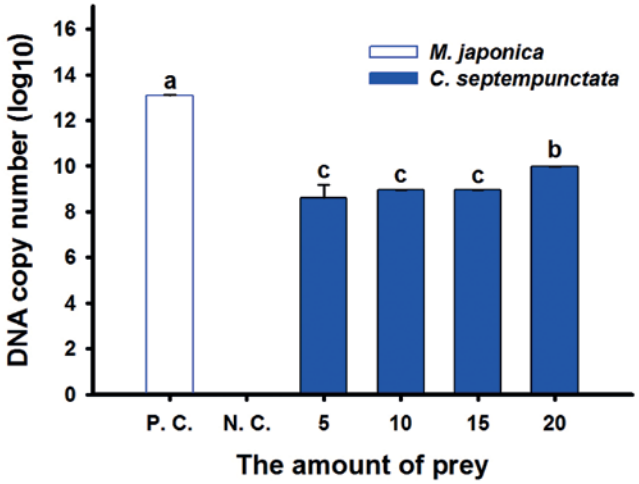

Fig. 4. The *MjCO1* copy number was determined by RT qPCR after *Coccinella septempunctata* consumed different numbers of *Megoura japonica*. DNA copy number was calculated according to the standard plasmid curve. Letters on bars indicate statistically significant difference based on ANOVA followed by Tukey's HSD multiple comparison test (P<0.05).

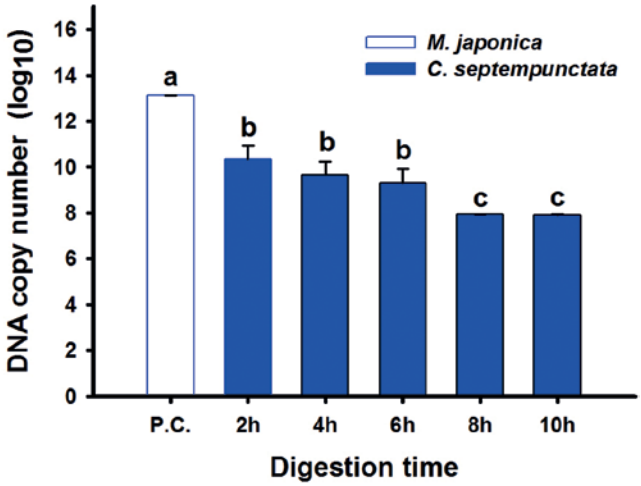

Fig. 5. The *MjCO1* copy number was determined by reverse-transcription quantitative PCR after *Coccinella septempunctata* consumed *Megoura japonica* with differing digestion time. The DNA copy number was calculated according to the standard plasmid curve. Letters on the bars indicate statistically significant difference based on ANOVA followed by Tukey's HSD multiple comparison test (P<0.05).

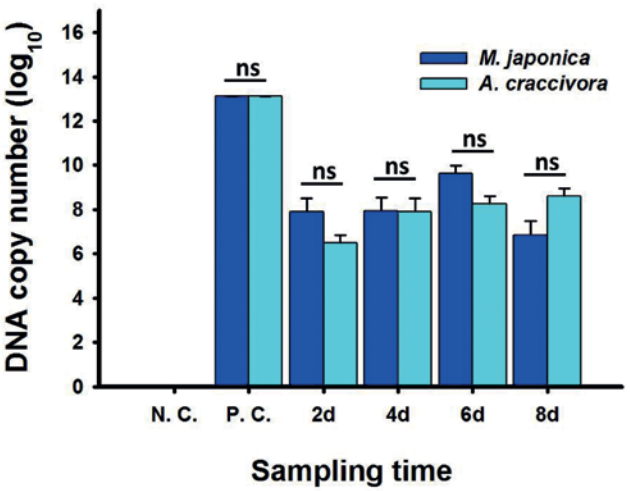

Fig. 6. The DNA copy number of *MjCO1* and *AcCO1* were determined by reverse-transcription quantitative PCR (RT-qPCR) after larval *Coccinella septempunctata* consumed *Megoura japonica* and *Aphis craccivora* with differing digestion times. The DNA copy number of *MjCO1* and *AcCO1* were calculated according to the standard plasmid curve. NS on the bars indicate no statistically significant difference based on Testing followed by t-test (P<0.05).

4 Discussion

Molecular gut-content analysis provides valuable and accurate information on prey consumption by predators and detects the presence of minute amounts of prey DNA in the digestive tract of predators. Such information greatly increases resolution in analysis of trophic relationships (Sheppard & Harwood 2005; Weber & Lundgren 2009) in the field. In the present study, we identified *COI* homologs from *M. japonica* to characterize the interactions between *C. septempunctata* and its prey, *M. japonica* and *A. craccivora,* using prey-specific PCR and RT-qPCR.

Previous research has demonstrated that several factors influence the results molecular gut content analysis including the selection of target genes, sensitivity and selectivity of the primer to prey, predator digestion time and temperature (e.g., Chen et al. 2000; Sint et al., 2012; Waldner et al., 2013; Weber & Lundgren, 2009). Thus, it is imperative to clarity the effects of prey quantity and digestion time prior to molecular gut content analysis in the field. In our study, a steady number of prey (*M. japonica*) were supplied to the predator (*C. septempunctata*) and general PCR results showed that the brightness of the *MjCOI* band increased with greater number of *M. japonica* consumed (Fig. 2). For digestion time, the general PCR results showed that the brightness of the *MjCO1* band decreased with increasing time after prey consumption (Fig. 3). Our results demonstrate that prey-specific PCR is well reflective of the predation relationship under controlled laboratory conditions. However, general PCR has some limiting factors in molecular gut content analysis; for example, if few prey are present in the predator gut this can result in low abundance of the target gene in the PCR template, resulting in ambiguity of the PCR bands in the agarose gel.

With reduced costs of RT-qPCR, this technique is more frequently being used for molecular gut content analysis. RT-qPCR contrasts to qualitative PCR in that is relies on

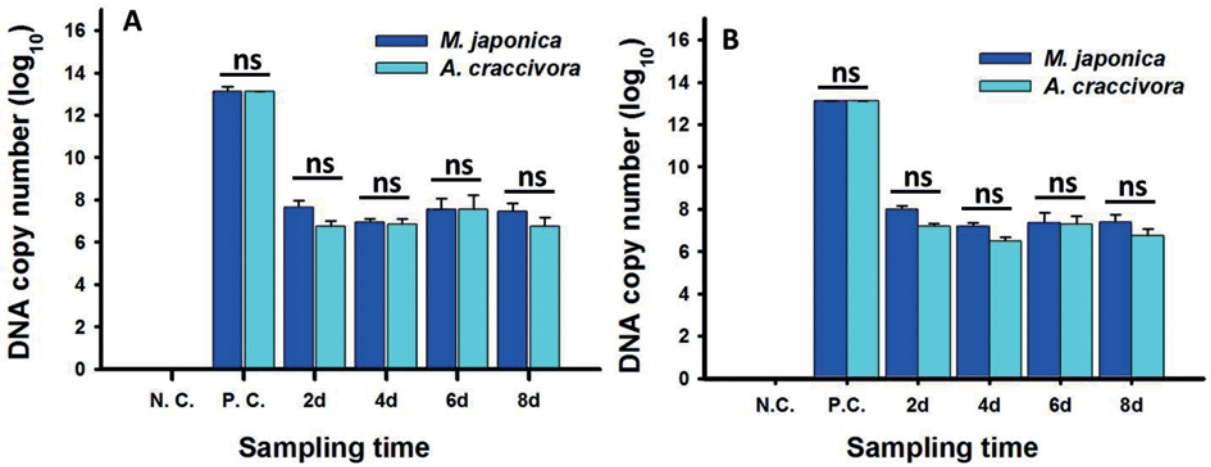

Fig. 7. The DNA copy number of *MjCO1* and *AcCO1* were determined by RT-qPCR after adult *Coccinella septempunctata* consumed *Megoura japonica* and *Aphis craccivora* with differing digestion times (A: Female; B: Male). The DNA copy number of *MjCO1* and *AcCO1* were calculated according to the standard plasmid curve. NS on the bars indicate no statistically significant difference based on Testing followed by t-test (P<0.05).

flourometric quantitation rather than visual band detection on an agarose gel, thus reducing analysis time and the subjectivity of the results (Weber & Lundgren, 2009). Previous studies demonstrated the utility of this technique in analysis of the relationship between predators and their prey (King et al., 2008; Redd et al., 2014; Zhang et al., 2007). In our study, RT-qPCR with Taqman-MGB probes were used to analyse the predatory relationship between *C. septempunctata* and *M. japonica*. The standard curve of the *MjCOI* plasmid and Ct value was fitted and DNA copy number of *MjCOI* transformed based on a general equation. Our results showed that with an increasing number of *M. japonica* consumed, the DNA copy number of *MjCOI* also increased in the gut of *C. septempunctata* (Fig. 4). The results of digestion time analysis showed that with an increase in digestion time of *C. septempunctata* there was a decrease in DNA copy number of *MjCOI* (Fig. 5). Compared with general PCR, the advantage of RT-qPCR in molecular content analysis is that DNA copy number can be subjected to statistical analysis (Lundgren & Fergen, 2014; Redd et al., 2014) and can therefore evaluate the predation relationship more accurately than prey-specific PCR. For example, the brightness of the PCR band in prey-specific PCR in which *C. septempunctata* consumed 15 or 20 *M. japonica* were similar (Fig. 2) whereas the consumption of 20 *M. japonica* provided a significantly higher DNA copy number of *MjCOI* in the gut compared 15 *M. japonica* consumed (Fig. 4).

DNA-based gut-content analysis can allow rapid screening of multiple prey for a given predator (Juen & Traugoot, 2007), enabling the assessment of prey preference. In our study, *A. craccivora* was selected as the second prey option in food preference tests because this aphid co-infests broad beans with *M. japonica* in the field. Third instar larvae, female adults and male adults of *C. septempunctata* were selected to test these food preferences. Our results demonstrated that both larvae and adults of *C. septempunctata* routinely preyed upon *M. japonica* and *A. craccivora* at the same time. Additionally, there were no significant differences in DNA copy number between *MjCOI* and *AcCOI* in the gut of *C. septempunctata* (Figs. 5, 6). Thus, we can conclude that *C. septempunctata* had no food preference in the laboratory but field-based variation could be observed and needs careful examination.

In summary, our results have provided clear evidence that RT-qPCR is sufficiently accurate and sensitive for its subsequent application in studying trophic interactions in the field. A remaining challenge for molecular gut-content analysis is to transform qualitative assay results into quantitative data that can be used to infer the impact of predation on prey population dynamics. Thus, further research on how to quantify prey number using a probe method would help to strengthen predator-prey studies in the future.

Acknowledgement: This research was supported by the Youth Scientific Research Fund of Beijing Academy of Agricultural and Forestry Science (QNJJ201725; QNJJ201806), Sciences and Technology Innovation Ability Construction of BAAFS (KJCX20200203), National Key Research and Development Program (SQ2017ZY060059) and EUCLID (EU-CHINA Lever for IPM Demonstration, H2020-SFS-2014, grant number: 633999).

References

Agusti, N., De Vicente, M. C., & Gabarra, R. (2000). Developing SCAR markers to study predation on *Trialeurodes vaporariorum*. *Insect Molecular Biology, 9*(3), 263–268. https://doi.org/10.1046/j.1365-2583.2000.00185.x

Agusti, N., Unruh, T. R., & Welter, S. C. (2003). Detecting *Cacopsylla pyricola* (Hemiptera: Psyllidae) in predator guts using COI mitochondrial markers. *Bulletin of Entomological Research, 93*(3), 179–185. https://doi.org/10.1079/BER2003236

Albertini, A., Marchi, S., Ratti, C., Burgio, G., Petacchi, R., & Magagnoli, S. (2018). *Bactrocera oleae* pupae predation by *Ocypus olens* detected by molecular gut content analysis. *BioControl, 63*(2), 227–239. https://doi.org/10.1007/s10526-017-9860-6

Caterino, M. S., Cho, S., & Sperling, F. A. (2000). The current state of insect molecular systematics: A thriving tower of Babel. *Annual Review of Entomology, 45*(1), 1–54. https://doi.org/10.1146/annurev.ento.45.1.1

Chen, Y., Giles, K. L., Payton, M. E., & Greenstone, M. H. (2000). Identifying key cereal aphid predators by molecular gut analysis. *Molecular Ecology, 9*(11), 1887–1898. https://doi.org/10.1046/j.1365-294x.2000.01100.x

Clare, E. L. (2014). Molecular detection of trophic interactions: Emerging trends, distinct advantages, significant considerations and conservation applications. *Evolutionary Applications, 7*(9), 1144–1157. https://doi.org/10.1111/eva.12225

Dodd, L. E., Chapman, E. G., Harwood, J. D., Lacki, M. J., Rieske, L. K. (2012). Identification of prey of *Myotis septentrionalis* using DNA-based techniques *93*(4), 1119–1128. https://doi.org/10.1644/11-MAMM-A-218.1

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology, 3*(5), 294–299.

Gariepy, T. D., Kuhlmann, U., Gillott, C., & Erlandson, M. (2007). Parasitoids, predators and PCR: The use of diagnostic molecular markers in biological control of Arthropods. *Journal of Applied Entomology, 131*(4), 225–240. https://doi.org/10.1111/j.1439-0418.2007.01145.x

Greenstone, M. H., Payton, M. E., Weber, D. C., & Simmons, A. M. (2014). The detectability half-life in arthropod predator-prey research: What it is, why we need it, how to measure it, and how to use it. *Molecular Ecology, 23*(15), 3799–3813. https://doi.org/10.1111/mec.12552

Harwood, J. D., Bostrom, M. R., Hladilek, E. E., Wise, D. H., & Obrycki, J. J. (2007). An order-specific monoclonal antibody to Diptera reveals the impact of alternative prey on spider feeding behavior in a complex food web. *Biological Control, 41*(3), 397–407. https://doi.org/10.1016/j.biocontrol.2007.02.008

Harwood, J. D., Desneux, N., Yoo, H. J. S., Rowley, D. L., Greenstone, M. H., Obrycki, J. J., & O'Neil, R. J. (2007). Tracking the role of alternative prey in soybean aphid predation by *Orius insidiosus*: A molecular approach. *Molecular Ecology, 16*(20), 4390–4400. https://doi.org/10.1111/j.1365-294X.2007.03482.x

Harwood, J. D., & Obrycki, J. J. (2005). Quantifying aphid predation rates of generalist predators in the field. *European Journal of Entomology, 102*(3), 335–350. https://doi.org/10.14411/eje.2005.051

Hewlett, J. A., Szczepaniec, A., & Eubanks, M. D. (2019). The effects of sugarcane aphid density in sorghum on predation by lady beetles and lacewings. *Biological Control, 129*, 171–177. https://doi.org/10.1016/j.biocontrol.2018.10.015

Hoogendoorn, M., & Heimpel, G. E. (2001). PCR-based gut content analysis of insect predators: Using ribosomal ITS-1 fragments from prey to estimate predation frequency. *Molecular Ecology, 10*(8), 2059–2067. https://doi.org/10.1046/j.1365-294X.2001.01316.x

Hoy, M. A. (1994). *Insect Molecular Genetics, An Introduction to Principles and Applications*. San Diego, California: Academic Press.

Jaramillo, J., Chapman, E. G., Vega, F. E., & Harwood, J. D. (2010). Molecular diagnosis of a previously unreported predator-prey association in coffee: *Karnyothrips flavipes* Jones (Thysanoptera: Phlaeothripidae) predation on the coffee berry borer. *Naturwissenschaften, 97*(3), 291–298. https://doi.org/10.1007/s00114-009-0641-7

Juen, A., & Traugott, M. (2007). Revealing species-specific trophic links in soil food webs: Molecular identification of scarab predators. *Molecular Ecology, 16*(7), 1545–1557. https://doi.org/10.1111/j.1365-294X.2007.03238.x

King, R. A., Symondson, W. O., & Thomas, R. J. (2015). Molecular analysis of faecal samples from birds to identify potential crop pests and useful biocontrol agents in natural areas. *Bulletin of Entomological Research, 105*(3), 261–272. https://doi.org/10.1017/S0007485314000935

King, R. A., Read, D. S., Traugott, M., & Symondson, W. O. C. (2008). Molecular analysis of predation: A review of best practice for DNA-based approaches. *Molecular Ecology, 17*(4), 947–963. https://doi.org/10.1111/j.1365-294X.2007.03613.x

Lee C, Kim J, Shin SG, Hwang S. (2006). Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli. Journal of Biotechnology 123*(3), 273–280. http://doi.org/10.1016/j.jbiotec.2005.11.014

Lu, Y., Wu, K., Jiang, Y., Guo, Y., & Desneux, N. (2012). Widespread adoption of Bt cotton and insecticide decrease promotes biocontrol services. *Nature, 487*(7407), 362–365. https://doi.org/10.1038/nature11153

Lundgren, J. G., & Fergen, J. K. (2014). Predator community structure and trophic linkage strength to a focal prey. *Molecular Ecology, 23*(15), 3790–3798. https://doi.org/10.1111/mec.12700

Macias-Hernandez, N., Athey, K., Tonzo, V., Wangensteen, O. S., Arnedo, M., & Harwood, J. D. (2018). Molecular gut content analysis of different spider body parts. *PLoS One, 13*(5), e0196589. https://doi.org/10.1371/journal.pone.0196589

Monzo, C., Sabater-Munoz, B., Urbaneja, A., & Castanera, P. (2011). The ground beetle *Pseudophonus rufipes* revealed as predator of *Ceratitis capitata* in citrus orchards. *Biological Control, 56*(1), 17–21. https://doi.org/10.1016/j.biocontrol.2010.09.004

Moser, S. E., Kajita, Y., Harwood, J. D., & Obrycki, J. J. (2011). Evidence for utilization of Diptera in the diet of field-collected coccinellid larvae from an antibody-based detection system. *Biological Control, 58*(3), 248–254. https://doi.org/10.1016/j.biocontrol.2011.05.014

Pan, H. S., Liu, B., Jaworski, C. C., Yang, L., Liu, Y. Q., Desneux, N., … Lu, Y. H. (2020). Effects of aphid density and plant taxa on predatory ladybeetle abundance at field and landscape scales. *Insects, 11*(10), 695. https://doi.org/10.3390/insects11100695

Peterson, J.A., Burkness, E.C., Harwood, J.D., Hutchison, W.D. (2018). Molceular gut-content analysis reveals high frequency of *Helicoverpa zea* (Lepidoptera: Noctuidae) consumption by *Orius insidiosus* (Hemiptera: Anthocoridae) in sweet corn. *Biological Control* 121, 1–7. https://doi.org/10.1016/j.biocontrol.2018.02.006

Pfannenstiel, R. S. (2008). Spider predators of lepidopteran eggs in south Texas field crops. *Biological Control, 46*(2), 202–208. https://doi.org/10.1016/j.biocontrol.2008.03.011

Redd, K. S., Ling, S. D., Frusher, S. D., Jarman, S., & Johnson, C. R. (2014). Using molecular prey detection to quantify rock lobster predation on barrens-forming sea urchins. *Molecular Ecology, 23*(15), 3849–3869. https://doi.org/10.1111/mec.12795

Rondoni, G., Borges, I., Collatz, J., Conti, E., Costamagna, A. C., Dumont, F., … Cock, M. J. W. (2020). Exotic ladybirds for biological control of herbivorous insects – a review. *Entomologia Experimentalis et Applicata*. https://doi.org/10.1111/eea.12963

Schmidt, J. M., Barney, S. K., Williams, M. A., Bessin, R. T., Coolong, T. W., & Harwood, J. D. (2014). Predator-prey trophic relationships in response to organic management practices. *Molecular Ecology, 23*(15), 3777–3789. https://doi.org/10.1111/mec.12734

Sheppard, S. K., & Harwood, J. D. (2005). Advances in molecular ecology: Tracking trophic links through predator-prey foodwebs. *Functional Ecology, 19*(5), 751–762. https://doi.org/10.1111/j.1365-2435.2005.01041.x

Sheppard, S.K., Henneman, M.L., Memmott, J., Symondson, W.O.C. (2004). Infiltration by alien predators into invertebrate food webs in Hawaii: a molecular approach. *Molecular Ecology 13*, 2077–2088.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., & Flook, P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America, 87*(6), 651–701. https://doi.org/10.1093/aesa/87.6.651

Symondson, W. O. C. (2002). Molecular identification of prey in predator diets. *Molecular Ecology, 11*(4), 627–641. https://doi.org/10.1046/j.1365-294X.2002.01471.x

Thomine, E., Rusch, A., Supplisson, C., Monticelli, L. S., Amiens-Desneux, E., Lavoir, A. V., & Desneux, N. (2020). Highly diversified crop systems can promote the dispersal and foraging activity of the generalist predator *Harmonia axyridis*. *Entomologia Generalis, 40*(2), 133–145. https://doi.org/10.1127/entomologia/2020/0894

Waldner, T., Sint, D., Juen, A., & Traugott, M. (2012). The effect of predator identity on post-feeding prey DNA detection success in soil-dwelling macro-invertebrates. *Soil Biology & Biochemistry, 63*, 116–123. https://doi.org/10.1016/j.soilbio.2013.03.030

Whelan, J. A., Russell, N. B., & Whelan, M. A. (2003). A method for the absolute quantification of cDNA using real time PCR. *Journal of Immunological Methods, 278*(1–2), 261–269. https://doi.org/10.1016/S0022-1759(03)00223-0

Welch, K. D., Schofield, M. R., Chapman, E. G., & Harwood, J. D. (2014). Comparing rates of springtail predation by web-building spiders using Bayesian inference. *Molecular Ecology, 23*(15), 3814–3825. https://doi.org/10.1111/mec.12721

Weber, D. C., & Lundgren, J. G. (2009). Assessing the tropic ecology of the Coccinellidae: Their roles as predators and as prey. *Biological Control, 51*(2), 199–214. https://doi.org/10.1016/j.biocontrol.2009.05.013

Xiao, D., Zhao, J., Guo, X., Chen, H., Qu, M., Zhai, W., … Wang, S. (2016). Sublethal effects of imidacloprid on the predatory seven-spot ladybird beetle *Coccinella septempunctata*. *Ecotoxicology (London, England), 25*(10), 1782–1793. https://doi.org/10.1007/s10646-016-1721-z

Zaidi, R. H., Jaal, Z., Hawkes, N. J., Hemingway, J., & Symondson, W. O. C. (1999). Can multiple-copy sequences of prey DNA be detected amongst the gut contents of invertebrate predators? *Molecular Ecology, 8*(12), 2081–2087. https://doi.org/10.1046/j.1365-294x.1999.00823.x

Zhang, G.-F., Lu, Z.-C., Wan, F.-H., & Lovei, G. L. (2007). Real-time PCR quantification of *Bemisia tabaci* (Homoptera: Aleyrodidae) B-biotype remains in predator guts. *Molecular Ecology Notes, 7*(6), 947–954. https://doi.org/10.1111/j.1471-8286.2007.01819.x

Manuscript received: 16 February 2020
Revisions requested: 22 July 2020
Modified version received: 8 August 2020
Accepted: 28 December 2020