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ORIGINAL ARTICLE

Analysis of digestion of rice planthopper by *Pardosa pseudoannulata* based on CO-I gene

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Real-time fluorescence PCR

Abstract In order to systematically study the predatory behavior and digestion regularity of spiders, real-time fluorescence quantification PCR technique was used to detect the number of CO-I genes in *Pardosa pseudoannulata* after it preyed on rice planthoppers in different temperatures within different periods. At 28 °C, 0, 1, 2, 4, 8, 16, and 24 h after *P. pseudoannulata* preyed on rich planthopper, DNA was extracted from cephalothorax and abdomen of *P. pseudoannulata*. Routine PCR and real-time fluorescence PCR techniques were employed for CO-I gene amplification. The results show that: The prey liquid was temporarily stored in the sucking stomach of the spider head within 2 h after prey, and gradually transferred to the midgut of the abdomen with the prolongation of time. After 4 h, CO-I gene residues of rice planthopper in the cephalothorax gradually decreased. The CO-I gene of rice planthopper was basically transferred to the abdomen after 16 h. During 0–1 h, food contained in abdominal midgut and other digestive organs was very small, CO-I gene detection was not obvious. Over time, food entered into the midgut from the sucking stomach for digestion. During 2–4 h, CO-I gene amount increased, at 2–4 h, detected CO-I gene residue reached the peak; but rapidly declined after 8, 16, and 24 h, even it is still detectable. The results at different temperatures reveal that: As the temperature increased from 26 °C to 32 °C, CO-I gene residues of rich planthopper in cephalothorax and abdomen of *P. pseudoannulata* gradually decreased, which indicated that the digestion rate increased with the increase of temperature with some range. However, when the temperature continued to increase to 34 °C, the digestion rate decreased.

1. Introduction

Spider is one of the most important predators for all kinds of agricultural pests. It is very important to know its prey species and digestion rate. The direct method of trophic analysis includes field observation (Luck et al., 1988) and digestive tract anatomy (Hengeveld, 1980). However, spider has in vitro and in vivo digestion after predation, and takes in formless prey

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tissue fluid, thus traditional morphological classification is not applicable to trophic analysis of stomach contents for spiders.

With the development of molecular biology, DNA taxonomy has been developed. DNA-based trophic analysis is to determine food organisms by identifying DNA sequences in the residual tissues of the stomach (Sheppard and Harwood, 2005). Identification of food organisms excludes degree of biological digestion, which can be used for different morphological organisms at different growth and development stages. Hoss et al. published a paper in Nature about applying DNA to identify presence of residual food organism in feces, which is the first case to adopt DNA in determination of animal diet (Hoss et al., 1992). Deagle used this technique in pyrosequencing of food organisms from arctocephalinae feces, obtaining an unbiased diet composition of arctocephalinae (Deagle et al., 2009). Reed et al. extracted DNA from feces and stomach contents of manatee, and analyzed the compositions and proportion of the food organisms with fingerprint technology, which solved the problem related to the high digestibility of prey and the difficulty in identification of traditional food identification methods (Reed and Tollit, 1995). In addition, when it comes to the spider predation effect, Weber suggested that DNA molecular marker has the advantages of rigorous reaction conditions, stable results, high sensitivity and repeatability, based on which one of the most effective methods to study the predation effect is proposed (Weber and Lundgren, 2009; Hanief, 2016). However, qualitative evaluation can only determine whether predators have the ability to prey on certain pests, while quantitative evaluation is essential to determine control capacity of the natural enemies for target pests.

Many studies have focused on the predator–prey qualitative research. On the contrary, few studies have evaluated the predatory behavior quantitatively, which is because the conventional PCR-based molecular detection technique can only qualitatively detect the predator species of the target insects, i.e. it cannot quantitatively detect capacity of predator in predation of target insects. Real-time fluorescence quantification PCR detection technique is to design a fluorescent probe based on the conventional PCR, which can detect quantitatively amount of the initial template in target DNA fragments in the system, and evaluate accurately capacity of predator in predation of target insects (Zhang et al., 2007). Therefore, it is believed to provide the possibility of precise digital quantification of ability of predators in predation of pests.

In recent years, although real-time fluorescence quantification PCR has been used for predator–prey studies (King et al., 2008), quantitative and qualitative studies of predator–prey relationships and predator control of prey by fluorescence quantification PCR are rarely reported. Specially, Harper et al. (2005) used multiple real-time fluorescence quantification PCR system to study predator's diet, finding that ground beetle can feed on aphids, weevils, earthworms and some mollusks, of which, earthworms and mollusks are the main food. Troedsson et al. (2007) studied differences in trapping and digestion efficiency of three species of algae of different size by *Oikopleura dioica* at different concentrations. Lv et al. (2005) quantitatively evaluated the control effect of field predators on *Bemisia tabaci* with real-time quantification PCR. Zhang et al. (2007) used TaqMan real-time fluorescence quantification PCR technique to quantitatively detect copy number of target DNA in *B. tabaci*, nymph, imago. Subsequently, various predators of *B. tabaci* were selected from the field. Based on the copy number of target DNA of *B. tabaci* residue in the predator, the number of *B. tabaci* larvae in the digestive tract of each predator was estimated. For pest and natural enemy communities in maize and soybean fields, using DNA molecular markers and fluorescence quantification PCR technique as tools, Song Xinyuan studied in details about the predation behavior of natural enemies for pests, fitted various influence factors to accurately and quantitatively study control effects of main natural enemies on the target pests (Song, 2008). Wang Guanghua obtained ITS gene sequences of *Sogatella furcifera*, *Nilaparvata lugens* and *Laodelphax striatellus* by PCR cloning. The primers and specific ALLGlo probes of the three planthoppers were designed and synthesized, an ALLGlo probe integrated triple real-time fluorescence quantitative detection system was established and optimized, which was further used to study capacity of common predators in rice field in predation of rice planthoppers (Wang, 2009). Using COI and SCAR molecular marker technology, Meng Xiangqin established a technique system for qualitative detection of predation of *Frankliniella occidentalis* by local natural enemies, and by TaqMan fluorescence quantification PCR, which quantitatively determined capacity of natural enemies in predation of *F. occidentalis* (Meng, 2010). Nevertheless, comprehensive research on quantitative analysis and evaluation of insect-controlling efficiency by spider has not been reported so far, demonstrating this study is quite necessary.

In this study, to understand predatory behavior and digestion regularity of spiders, real-time quantification PCR technique was used to detect the number of COI genes in *Pardosa pseudoannulata* after it preyed on rice planthoppers in different temperatures within different periods.

2. Materials and methods

2.1. Experimental materials

Mature *P. pseudoannulata* with similar size were collected in Zhuhai rice field (22° 15 min 253 s north latitude, 114° 12 min 314 s east longitude). They were put in 250 ml Erlenmeyer flask separately, and studied in experiments after hungry feeding for 7 d. Absorbent cotton was placed in the flask, only adequate water was provided to ensure that tarantula could survive under hunger.

N. lugens Seal of similar size were also collected from rice fields where *P. pseudoannulata* lived. They were placed in plastic bottle with rice, and sealed with gauze after the capture for temporary cultivation and spare applications.

2.2. Experimental design

2.2.1. Study on digestion rate of P. pseudoannulata after feeding with rice planthopper

The *P. pseudoannulata* were cultured in a bottle under starvation condition. Each of them was fed with three rice planthoppers before being placed in an incubator at 28 °C. They were killed at 0, 1, 2, 4, 8, 16, and 24 h, respectively. All walking legs

and pedipalps were removed, the cephalothorax and abdomen were cut with a scalpel. It should be noted that the abdomen is not squeezed before cutting because of the soft tissue in the abdomen. Otherwise, food juice in the midgut will flow back along the podeon to the cephalothorax, affecting the experimental results. The cephalothorax and abdomen were placed and well-marked in a 1.5 ml centrifuge tube for the subsequent processing in the next step. The negative control *P. pseudoannulata* was not fed and used directly in experiment.

2.2.2. Study on digestion rate of rich planthopper by P. pseudoannulata at different temperatures

The *P. pseudoannulata* were placed in incubators at 26 °C, 28 °C, 30 °C, 32 °C and 34 °C, respectively. Two hours after being fed with rice planthoppers, they were treated same as mentioned above in Section 2.2.1, with DNA extracted for further experiments.

2.3. Experimental methods

2.3.1. DNA extraction: DNA fast extraction kit (animal) produced by Sangon Biotech (Shanghai) Co., Ltd was used to extract DNA

CO-I gene primers (Wang, 2009) were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Upstream primer: 5′-CAACATTTATTTTGATTTTTTGG-3′

Downstream primer: 5′-TCCAATGCACATATCTGCCATATTA-3′

2.3.2. Routine PCR

25 μL routine PCR System: 2.5 μL 10× PCR buffer, 0.5 μL 10 mmol/L dNTPs, 1 μL 10 μmmol/L upstream primer, 1 μL 10 μmmol/L downstream primer, 0.3 μL 5 U/μL Taq DNA polymerase, MLDNA template, add water to 25 μL.

PCR procedure: initial denaturation at 94 °C for 3 min; denaturation at 94 °C for 50 s; refolding for 30 s at annealing temperature 55 °C (to be lowered by 1 °C in each cycle until 50 °C); extension at 72 °C for 1 min (35 cycles); extension at 72 °C for 10 min; save at 4 °C for standby application.

After completion of the reaction, take 5 μL for electrophoresis in 1% agarose gel at 70 V for 25 min. View the results with gel imager to save resulting image.

2.3.3. Real-time fluorescence PCR

Fluorochrome SYBR Green I was used to establish a 25 μL real-time fluorescence PCR system. The liquid was homogenized and placed in a PCR 8 tube, and put in a 7500 fluorogenic quantitative PCR instrument for gene amplification. The PCR procedure was described as follows: Initial denaturation at 95 °C for 3 min, denaturation at 95 °C for 30 s; refolding for 30 s at annealing temperature 50 °C; extension at 72 °C for 1 min (a total of 40 cycles); save at 4 °C for standby application.

The expression difference was calculated with $2^{-\triangle\triangle Ct}$ method by Ct value and formula $v = \Delta C/t$, wherein, v denotes the digestion rate, ΔC denotes the total amount before the digestion- the total amount after the digestion, t denotes the digestion time. The digestion rate was calculated for each time period.

3. Results

3.1. Detection of digestion rate of P. pseudoannulata at different time after feeding by routine PCR method

According to the experimental design, gene amplification was performed by routine PCR method, and electrophoresis detection showed that no target band was found in the negative control group, which proved that the primers did not amplify the DNA of *P. pseudoannulata*, and specificity indeed exists. After gel extraction of positive results, gene sequencing proved that the amplified band represented CO-I gene of rice planthopper.

DNA was extracted from the cephalothorax and abdomen of *P. pseudoannulata* during 0–24 h after it preyed on rice planthopper, followed by conventional PCR amplification. The results are shown in Figs. 1-2.

In Fig. 1, the gel imaging results show the fact that there are obvious bright spots with length of about 900 bp in the cephalothorax at 0, 1, 2, 4 h. Among which, spots at 0, 1, 2 h are more obvious. Over time, the brightness of the electrophoretic bands decreases gradually, and becomes difficult to be observed after 16 h. This indicates that there are many residues of CO-I gene of rice planthopper in the digestive tract of cephalothorax within 2 h after predation, which gradually decrease after 4 h, and basically disappear after 16 h.

As shown in Fig. 2, after electrophoresis, there are obvious bright spots in the abdomen at 0, 1, 2, 4, 8 h, and very dark band appear after 16 and 24 h. Thus, it is believed that there were many residues of CO-I gene of rice planthopper in the abdomen within 2 h after predation, which gradually decreased after 4 h with residue remained in the abdomen after 24 h.

3.2. Detection of digestion rate of P. pseudoannulata at different time after feeding by real-time fluorescence quantification PCR technique

Reaction melting curves are of single peak, the peak value is single, and Tm value of the amplified products is uniform.

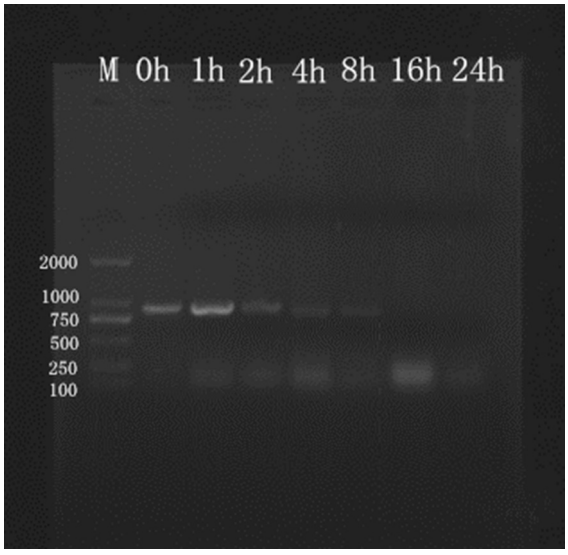

Figure 1 Results of routine PCR electrophoresis of cephalothorax of *P. pseudoannulata*.

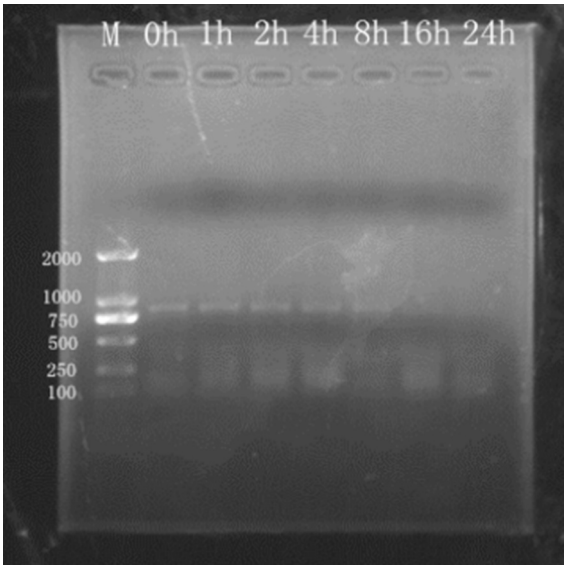

Figure 2 Results of routine PCR electrophoresis of abdomen of *P. pseudoannulata*. *Note:* M in the figure represents marker DS2000; 0 h, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h represent different time after *P. pseudoannulata* preys on rice planthopper.

The reaction specificity is good, and there is no primer dimer and nonspecific amplification. The CO-I gene of cephalothorax and abdomen of *P. pseudoannulata* after 0–24 h of predation of rice planthopper was amplified with real-time fluorescence PCR technique. The following amplification curves were obtained, as shown in Figs. 3–5.

Fig. 3 results show that CT values of amplification curve of cephalothorax at each time are concentrated between cycle numbers 14 and 16. CT value of 0, 1, 2 h is 14. Similarly, the one of the 4th hour is 15. For that of 8th and 16th hour, it is 16. CT value of the template is linear with the logarithm of the initial copy number of the template. The more the initial copy number is, the smaller the Ct value will be. It indicates that there are many residues of CO-I gene of rice planthopper in the cephalothorax of *P. pseudoannulata* within 2 h after predation, which decrease gradually with digestion time. Calculation of digestion rates of cephalothorax for each time period yielded a line graph as shown in Fig. 4.

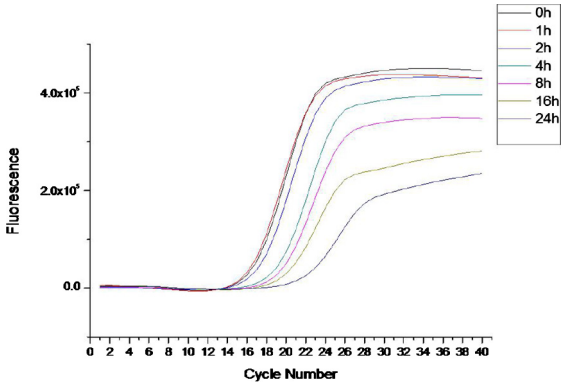

Figure 3 Amplification curve of residual CO-I gene of rice planthopper in cephalothorax.

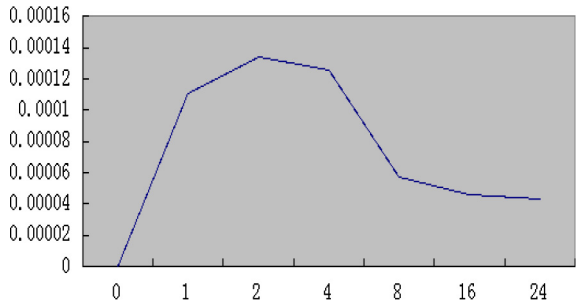

Figure 4 Digestion rate of residual CO-I gene of rice planthopper in cephalothorax.

As can be seen from Fig. 4, digestion rate of cephalothorax accelerates within 2 h after predation, which slows down during 2–24 h. The results of routine PCR and real-time fluorescence PCR show that residue of CO-I gene of rice planthopper in cephalothorax decreases after 2 h. It is because after capture of prey, *P. pseudoannulata* will inject venom and primary digestive juice secreted from the midgut, so that soft part of the prey is decomposed into liquid as a result of the in vitro primary digestion. The digestive juice mainly contains amylase, protease which only initially digests carbohydrates, proteins, etc., while DNA is not digested as sucking stomach located in cephalothorax does not digest it after sucking prey liquid, but temporarily stores it. Then, the liquid maintains the original state and constantly enters the abdomen after 2 h, so CO-I gene volume in the cephalothorax decreases, demonstrating a downward trend.

Fig. 5 shows CO-I gene amplification of rice planthopper in the abdomen of *P. pseudoannulata*. As can be seen, CT values of the abdomen at various times are concentrated between 15 and 19. Specially, CT value of the fourth and eighth hours is 15; and that of 0, 1, 2, and 16 h is 16, which significantly increases to 19 after 24 h. Digestion rate of residual CO-I gene of rice planthopper in abdomen is shown in Fig. 6.

As can be seen from Fig. 6, less CO-I gene residue of rice planthopper can be detected at the 0th and 1st hour. The detected CO-I gene residue of rice planthopper reaches the maximum at 2–4 h, which decreases at 8, 16, 24 h, but the difference is not obvious. The results show that there are less CO-I gene residue of the planthopper in the abdomen within 2 h after predation, and the food is digested in the abdomen during 2–24 h.

Considering the characteristics of the digestive system of *P. pseudoannulata*, the reason for this phenomenon is probably that just after the predation, a lot of food exists in the esophagus and sucking stomach of the cephalothorax, while there is very little food contained in midgut and other digestive organs of the abdomen, so CO-I gene detection volume is not obvious during 0–1 h. Over time, food successively enters into the midgut from the sucking stomach for massive digestion. During 2–4 h, CO-I gene volume increases, the midgut in the abdomen and developed digestive gland can secrete a variety of digestive enzymes including nuclease, and most macromolecules including CO-I gene are degraded, digested and absorbed here, showing accelerated digestion rate. After massive digestion of CO-I gene, there is not much residue in the abdomen, thus CO-I gene digestion rate remains almost the same during 8–24 h.

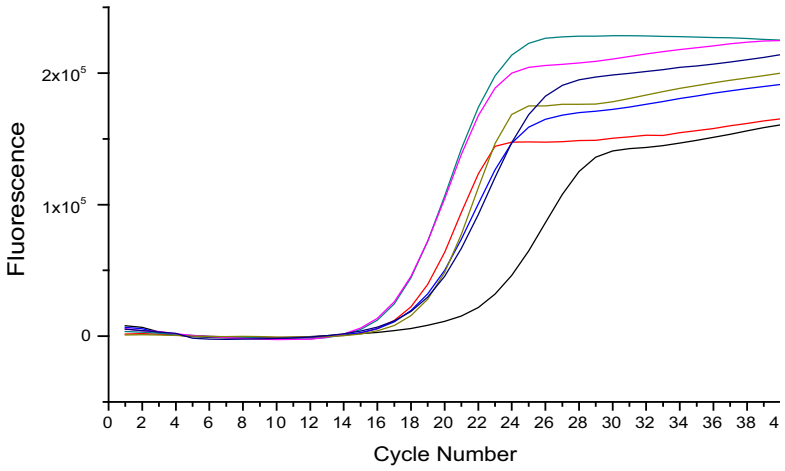

Figure 5 Amplification curve of residual CO-I gene of rice planthopper in abdomen.

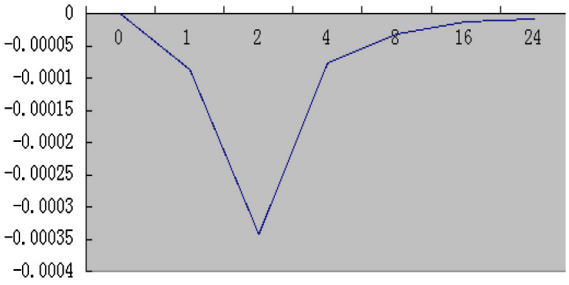

Figure 6 Digestion rate of residual CO-I gene of rice planthopper in abdomen.

3.3. Detection of impact of temperature on digestion rate of P. pseudoannulata by routine PCR method

As can be seen from Figs. 7 and 8, the amplified band of CO-I gene of rich planthopper in the cephalothorax of *P. pseudoannulata* is significantly brighter than that in the abdomen within 2 h of feeding under the same temperature, which is in agreement with the digestive results of *P. pseudoannulata* at different times in earlier stage after feeding. It is clearly seen from the results of cephalothorax electrophoresis, electrophoretic band brightness gradually become darken from 26 °C to 32 °C, which becomes the darkest at 32 °C.

3.4. Detection of impact of temperature on digestion rate of P. pseudoannulata by real-time fluorescence quantification PCR technique

As can be seen from Figs. 9 and 10, the total CT value of CO-I residual gene amplification of rich planthopper in cephalothorax of *P. pseudoannulata* is in the range of 14–17. Specially, CT value is the smallest at 26 °C, which is only 14. On the contrary, CT value is 15 at 28 °C, 16 at 30 °C, and becomes the largest (reaching 17 in this case) at 32 °C. A similar trend is observed in real-time fluorescence PCR amplification curve of the abdomen. The larger the CT value is, the less the residual CO-I gene of rice planthopper in *P. pseudoannulata* is, and the faster the digestion rate is, vice versa. The results show that temperature can affect digestion rate of rice planthopper by *P. pseudoannulata* to a certain extent.

4. Discussion

4.1. The relationship between the CO-I gene residue of rice planthopper and the digestion time

Experiments were carried out in an incubator at 28 °C, which effectively reduced the effect of temperature on the digestion

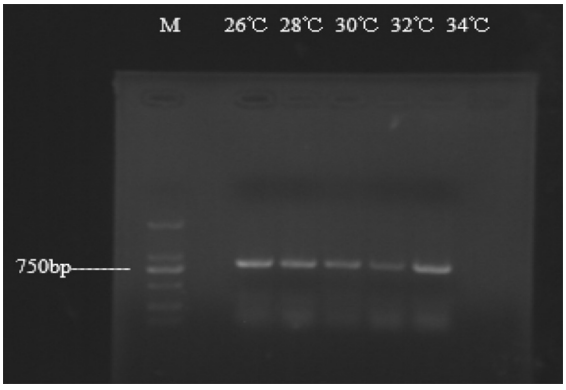

Figure 7 Routine PCR electrophoresis of cephalothorax of *P. pseudoannulata* at different temperatures.

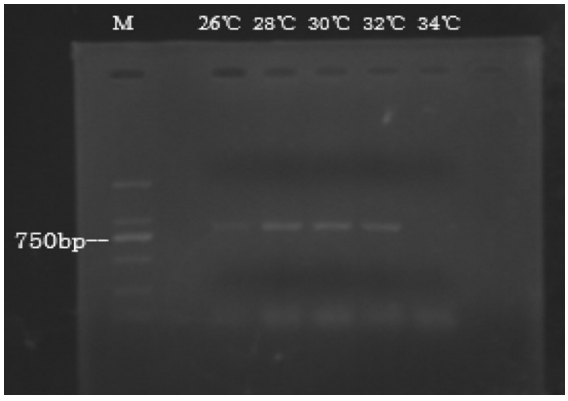

Figure 8 Routine PCR electrophoresis of abdomen of *P. pseudoannulata* at different temperatures.

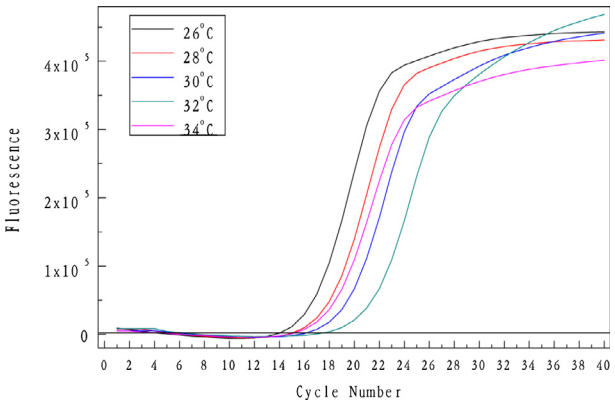

Figure 9 Real-time fluorescence PCR amplification curve of residual CO-I gene of rice planthopper in cephalothorax of *P. pseudoannulata*.

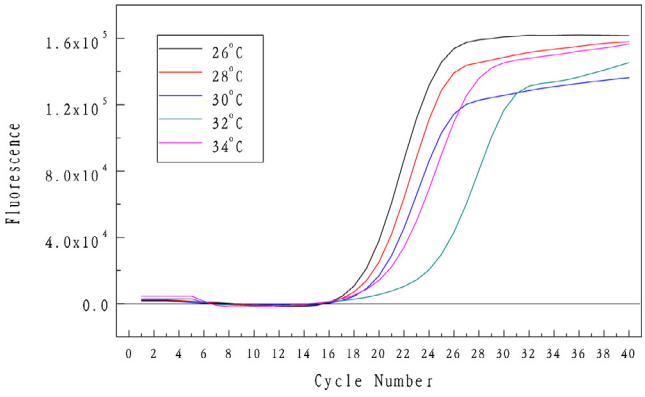

Figure 10 Real-time fluorescence PCR amplification curve of residual CO-I gene of rice planthopper in abdomen of *P. pseudoannulata*.

rate (Hoogendoorn and Heimpel, 2001). At the same time, starvation for 7 days (De León et al., 2006; Harper et al., 2006) before the experiment could ensure that predator had consumed residue food before feeding, which increased its predation probability, with relatively accurate predatory capacity detected. The presence of other prey increased the detection rate of prey DNA (Dodd, 2004), so this experiment only fed single prey, rich planthoppers, to the *P. pseudoannulata*. Within 2 h after predation, the sucked prey liquid was not completely digested, but temporarily stored in the sucking stomach of spider cephalothorax, which gradually transferred to the midgut of the abdomen with the time. After 4 h, CO-I gene residues of rich planthopper in cephalothorax decreased gradually, which was totally transferred to the abdomen at 16 h. Therefore, during 0–1 h, food amount in the midgut and other digestive organs of the abdomen was very low, so CO-I gene detection volume was not obvious. Over time, food successively entered into the midgut from the sucking stomach for massive digestion. During 2–4 h, CO-I gene volume increased, the midgut in the abdomen and developed digestive gland could secrete a variety of digestive enzymes including nuclease, and most macromolecules including CO-I gene were degraded, digested and absorbed during this range, showing accelerated digestion rate. Detected CO-I gene residue of rice planthopper reached the peak during 2–4 h, which dropped rapidly at 8, 16 and 24 h, but still detectable. Studies pointed out that time of detectability of number and type of prey in indigestive tract of predators depends on the predator itself, while food quality can also affect the spider's metabolic rate (Anderson, 1974). Large prey increases time of detectability of prey, with a longer detection period even in the absence of feeding of alternate preys (Sheppard et al., 2005). The longest time of detectability can range from a few hours to five days (Chen et al., 2000; Ma et al., 2005).

4.2. Effect of temperature on the digestion rate of P. pseudoannulata

Temperature can significantly affect the digestion rate of predators (Zhao, 2001; Liu, 2014). As the temperature increased from 26 °C to 32 °C, CO-I gene residues of rich planthopper were decreased gradually in cephalothorax and abdomen of *P. pseudoannulata*, indicating that digestion rate increased with increasing temperature within a certain range. However, when the temperature continued to increase to 34 °C, the digestion rate decreased. It is possible that enzymatic activities of the spider such as digestive enzymes are affected by different temperatures. As a result, digestive capacity, mobility of *Oxyopes sertatus* maintain at a better state under 28–32 °C (Wang and Yan, 2006), while excessive temperature will cause a negative effect on physiological activity of spiders (Xu et al., 1995).

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