Tikrit Journal for Agricultural Sciences (2024) 24 (2): 1-13
DOI: https://doi.org/10.25130/tjas.24.2.1

ISSN:1813-1646 (Print); 2664-0597 (Online)
Tikrit Journal for Agricultural Sciences
Journal Homepage: http://www.tjas.org
E-mail: tjas@tu.edu.iq

Diminish the chances of Mediterranean fruit fly *Ceratitis capitata* eggs surviving by suppressing the expression of the heat shock protein 27 gene (Hsp27)

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KEY WORDS:
Double-stranded RNA (dsRNA); biological control; insect eggs; sex ratio

Received: 30/12/2023
Revising: 15/04/2024
Accepted: 13/06/2024
Proofreading: 20/06/2024
Available online: 30/06/2024

ABSTRACT

The hypothesized role of stress-inducible heat shock proteins is to act as a buffer against environmental variations and affect fitness in suboptimal conditions. Our study examining the functions of heat shock protein 27 (Hsp27) in the eggs of the Mediterranean fruit fly (Ceratitis capitata). We utilized double-stranded RNA, specifically targeting the Hsp27 gene, to reduce its expression and assessed the consequent impact on egg viability. The investigation included the examination of early eggs (less than 6 hours post-laying) and late eggs (more than 42 hours post-laying), using varying concentrations (0.02, 0.1, and 0.2 µg/µL) of Hsp27 dsRNA. Control groups of eggs were dsRNA of ATPase, Cctra-2, or phosphate buffer solution (ph-B-S). Down-regulating Hsp27 dsRNA reduced the hatching rate of the eggs compared to the hatching rate in the control groups, especially in the ph-B-S group. The early eggs were more affected than the late eggs after soaking with Hsp27 dsRNA, where the Hsp27 dsRNA decreased the hatching rates to 12.91% early. The tested concentration of 0.2 µg/µL of Hsp27 dsRNA was achieved with the most significant reduction of 2.75 in egg viability. The sex ratio of insects hatched from treated eggs was investigated after hatching. After the down-regulation of Hsp27, there was no significant effect of the Hsp27 down-regulation on the sex ratio.

تقليل فرص بقاء بيض ذبابة فاكهة البحر الأبيض المتوسط (*Ceratitis capitata*) على قيد الحياة عن طريق قمع التعبير عن جين بروتين الصدمة الحرارية (Hsp27).27

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الخلاصة

تعمل بروتينات الصدمة الحرارية المحفزة للإجهاد كموقع عازل ضد التغيرات البيئية والتأثير على تغذية الحشرات في الظروف دون المستوى الأمثل. هدفت الدراسة الى تأثير دور بروتين الصدمة الحرارية 27 (Hsp27) في بيض ذبابة فاكهة البحر الأبيض المتوسط (Ceratitis capitata) بعد إضافة Hsp27 dsRNA. استخدم RNA عن طريق استهداف جين Hsp27، لتقليل تعبيره وتقييم التأثير على بقاء البويضة. شملت الدراسة فحص البيض الموضوع مبكرا (أقل من 6 ساعات بعد الوضع) والبيض الموضوع في مرحلة متأخرة (أكثر من 42 ساعة بعد الوضع)، باستخدام تركيزات مختلفة (0.02، 0.1، و0.2 ميكروغرام/ميكرولتر) من Hsp27 dsRNA. كانت معاملة المقارنة من البيض عبارة عن dsRNA من ATPase أو Cctra-2 ومحلول الفوسفات العازل (ph-B-S). أدى التخفيض لمعدل Hsp27 dsRNA إلى تقليل معدل فقس البيض مقارنة بمعدل الفقس في معاملة المقارنة، خاصة في مجموعة ph-B-S. كان البيض الموضوع مبكرا أكثر تأثراً من البيض الموضوع متأخرا بعد معاملته نقعا بـ Hsp27 dsRNA، حيث خفض Hsp27 dsRNA معدلات الفقس إلى 12.91%. في حين التركيز الذي تم اختباره وهو 0.2 ميكروغرام/ميكرولتر من Hsp27 dsRNA أدى الى تخفيض في نسبة فقس البيض معنويا وكان بمقدار 2.75%. كان تأثر Hsp27 غير معنوي في النسبة الجنسية للحشرة التي فقست من بيض معامل.

الكلمات المفتاحية: RNA, dsRNA، المكافحة الحيوية، بيض الحشرات، النسبة الجنسية

INTRODUCTION

Citrus, a genus of flowering trees in the Rutaceae family, is a crucial horticulture product with significant trade value (Ajboory & Al-Douri, 2023; Muhammad & Latif, 2022). The citrus orchards produce various fruits but face pests like leaf miners, mealybugs, swallowtail butterflies, whiteflies, scale insects, and medfly *Ceratitis capitata* (Ahmed et al., 2022; Alsabte et al., 2022; Augul & Al-Saffar, 2019). *Ceratitis capitata* (Diptera: Tephritidae) is commonly known as the Mediterranean fruit fly, abbreviated to Medfly. It is an invasive insect pest that damages horticultural produce, notably fruit (Malacrida et al., 2007). Important current challenges to managing Medfly are consumer sensitivity to pesticide residues, environmental pollution, pest resistance, climate change, global trade threats, and management of economic losses (Elfekih et al., 2014). Effective new control strategies are urgently needed to reduce reliance on environmentally damaging insecticides. Adults and their mobile stages are difficult to control because of their motility; therefore, controlling non-motile eggs is a strategy of interest. This study investigates a novel control strategy to reduce Medfly egg viability by silencing a gene.

Insects respond to stress by synthesizing heat shock proteins (Hsp) (Farahani et al., 2020). The induction of Hsp gene expression raised stress tolerance, and the level of response is related to expression levels of Hsp genes (Lü & Wan, 2011 and Muhammed and Mohamed, 2023). The expression of *Hsp* genes plays a role in the survival of organisms under stress conditions

(Hoffmann et al., 2003). Stress tolerance, including the level of *Hsp* gene expression, may differ according to gender (Dahlgaard et al., 1998), and the modalities of *Hsp* expression can differ by the type of stress exposure (Krebs, 1999).

Double-stranded RNA (dsRNA) molecules homologous to a target gene reduce or silence mRNA expression of the gene in a process known as RNA interference (RNAi) through enzyme-induced degradation of transcripts (Joga et al., 2016). The experimental introduction of RNA into cells can be used in certain biological systems to interfere with the function of an endogenous gene (Stanley et al., 2012). The application of dsRNA was used to silence insect immunity responses as a technology to improve the biological control of insects; thereby, the significance of research in this area lies in developing new pest management technologies (Stanley et al., 2012). There is potential to use RNAi as a biological control tool for managing *C. capitata*. Some features of the technology, such as lack of environmental persistence and high target gene specificity, make the RNAi technique desirable for plant protection (Zotti et al., 2018).

The methodologies of dsRNA uptake in insects include microinjection, soaking, feeding of an artificial diet, developing transgenic insects, virus-mediated uptake, and direct spray applications. Each method has advantages and disadvantages (Yang et al., 2011 and Jalal and Ahmed, 2023). For example, the disadvantage of the feeding method was highly sensitive to dietary uptake of dsRNA, while the transgenic and microinjection methods needed special conditions and professional staff and it was hard to apply in the field by farmers. The spray method is considered an unstable and uneconomic method. The soaking method is easy to apply and more economical than other ways.

The *Hsp27* gene is sensitive to different environmental stimuli, suggesting its potential as a suitable biomarker for ecotoxicological research (Martínez-Paz et al., 2014) Previous research suggests using 31 biomarkers, including Hsp27 gene, to control *C. capitata* (Anantanawat et al., 2020; Sun et al., 2016) the latter studies confirmed that *Hsp27* increase insect death rates and reduce their resistance to hard environmental conditions (Dwivedi et al., 2022; Pandey et al., 2016) Hsp27 is strongly induced during oxidative stress. In *C. capitata*, the Hsp27 gene has functions in protein folding, stress tolerance, proteasome-mediated degradation of selected proteins, cytoskeleton stabilization, actin polymerization, development and differentiation, cell death, cell cycle, and signal transduction (Economou et al., 2017). Using the combination of a transgenic GFP-CcHsp27 strain and confocal imaging, the investigation of the expression and intracellular localization of the medfly Hsp27 in mature gonads (Economou et al., 2017).

In this work, the effect of the down-regulation of heat shock protein on the expression profile of the *Hsp27* gene on *C. capitata* survival will be investigated by soaking the eggs of *C. capitata* in dsRNA to test if this treatment will reduce egg viability and female ratio. The finding of this work will further existing knowledge of using Hsp27 dsRNA in controlling *C. capitata.*

MATERIALS AND METHODS

Ceratitis capitata colony

The *Ceratitis capitata* colony used in this experiment was initially established in 2015 from a stock ancestry kept at the Department of Primary Industries and Regional Development (DPIRD) in Western Australia, which was periodically refreshed with the introduction of more wild flies. *C. capitata* adults were maintained in a Bugdorm-1 cage (30 × 30 × 30 cm ‘‘BioQuip products’’) with about 300 adults per cage, with access to water in a glass vial covered with a plug of cotton and also a dry 3:1 mixture of sucrose and yeast extract in a shallow glass container. Mature insects

laid eggs through the cloth sidewalls of the cages, which were collected and moved to the artificial breeding medium (carrot diet) consisting of 300 g torula yeast, 1 kg dehydrated ground carrot, 4.5 L hot tap water, 36 mL hydrochloric acid, 30 g nipagin, and 500 mL boiling water. After 13–16 days, pupae were collected and moved into the adult breeding cages. The emerged adult insects were reared on crystalline sugar, yeast hydrolysate, and water. Breeding conditions were 26.0±1.0°C, 60–70% relative humidity (RH) and darkness light (D: L) cycle of 16 dark:8 light hours. Early eggs (< 6 hours after laying and late eggs (> 42 hours after laying) of *C. capitata* were investigated in this study.

Heat shock protein 27 and control genes preparation

The dsRNA of the Hsp27 gene (Batch number: WA06887951), the dsRNA of ATPase genes (Batch number: WA06887949, which negatively affects the development of insects), and Cctra-2 (Batch number: WA06887952), which is reported to have a positive effect on insect sex ratio (increases female ratio), were used in this experiment. All molecular products were purchased from Sigma and kept at -20 °C. After bringing out Hsp27, ATPase and Cctra-2 were left to thaw for three minutes. By pipette transferred 150 microliters (µL) of each one to 200 microliter (µL) Polymerase Chain Reaction (PCR) tubes and put them into a benchtop centrifuge (Eppendorf 5415 D, United States) and centrifuged to 10,000 rpm for 4 s. Then, PCR tubes containing Hsp27, ATPase or Cctra-2 were moved to the thermocycler (Eppendorf-Mastercycler gradient- United States) and incubated at 75°C for 5 min. Three dsRNA concentrations of Hsp27, ATPase and Cctra-2 were prepared 0.02, 0.1 and 0.2 µg/µL by dilution with RNase-free water. The three concentrations of Hsp27, ATPase and Cctra-2 were stored at -20 °C for later use.

Soaking of eggs with dsRNA

A random selection of early eggs (< 6 h) was collected in a 100 mL beaker with 60 mL water. Twenty eggs were transferred by plastic dropper to eight 200 mL PCR tubes, and the water was removed by micropipette and thin strips of black filter paper. A solution of 0.02 µg/µL of Hsp27 was taken out of -20°C and kept at room temperature for 3 min before 10 µL was transferred to the 200 mL PCR tube, which included 20 eggs of *C. capitata*. Four replicate PCR tubes were prepared in this way. The other four tubes were used for controls, each tube containing 20 *C. capitata* eggs and 10 µL of phosphate buffer solution (general control group for Hsp27, ATPase and Cctra-2, as described in Table 1). The treatment tubes and the controls were left for three hours at room temperature and then the eggs were washed in RNase-free water. After washing with RNase-free water, the eggs, laid on black filter paper, were placed on the carrot diet surface into 9 cm plastic Petri dishes. The treatment dishes were labeled with egg age, Hsp27 concentration, and replicate number, while the control samples were labeled with egg age, control and replicate number. The treatment dishes and control were kept at 23±2 °C, 70-75% RH and 12:12 D: L in an incubator (model HWS, LET code 0574-88000432, Tianjin- China).

This same procedure was applied to 0.1 and 0.2 µg/µL of Hsp27 dsRNA concentrations. The procedures of the three concentrations of Hsp27 dsRNA were applied to the ATPase and Cctra-2 treatments. From day two to day six of the incubation, the number of eggs hatching was counted and recorded. After day six, the black filter papers were checked and discarded and the Petri dishes were transferred to plastic containers with a layer of sand, covered with cloth mesh, tightened with a rubber band and returned to the incubator. At the appearance of the first pupae, the sand was sieved three times weekly, and the pupae were counted and recorded, moved to sterilized Petri

dishes and incubated until adults emerged. The total number of adults, females and males, was recorded. The same protocol was also applied to late eggs (>42 h). The numbers of replicants and the total tested eggs are shown in Table 1.

Table 1. The total number of *C. capitata* eggs used in four groups (Hsp27, ATPase, Cctra-2 and ph-B-S) for 3 hours exposure.

Insect stage	Group	No. of insects in each replicant	N0. of replicants	concentrations	Total No. eggs
Early eggs (<6 h)	Hsp27	20	4	3	240
	ATPase	20	4	3	240
	Cctra-2	20	4	3	240
	Control ph-B-S	20	4	3	240
Late eggs (>42 h)	Hsp27	20	4	3	240
	ATPase	20	4	3	240
	Cctra-2	20	4	3	240
	Control ph-B-S	20	4	3	240
					1,920

Statistical analysis

The egg-hatching rates and sex ratio of adults were measured as indicators of *C. capitata* response to the dsRNA effect. To determine the significant differences in *C. capitata* response to the treatment groups, a three-factor analysis of variance (ANOVA) was carried out, followed by the assessment of the significance of differences using the Least Significant Difference (LSD) test. The analysis included the data of three molecular products, three concentrations of each molecular product and the response of egg types. To indicate the significant differences between the rates, subscript letters were used. Statistical analyses were performed using SPSS software (IBM version 24 Armonk, New York, America).

RESULTS AND DISCUSSION

The egg viability experiment results indicated a significant difference between early egg hatching rates and late eggs after being treated with dsRNA of Hsp27, ATPase, and Cctra-2. The early eggs were more susceptible than the late eggs ($P<0.0005$), as shown in Figure 1 A and B and Online Resource 1. There was a significant effect between three treatments of Hsp27, ATPase and Cctra-2 ($P<0.0005$) on hatching rates in early eggs. Treatment of early eggs resulted in significant differences between Hsp27 dsRNA and ATPase dsRNA, where the rates were 19.58% and 12.91% compared to the Hsp27 in Cctra-2 and Ph-B. S, which were 86.25% and 91.25%, respectively (Figure 1 A). Comparing egg hatching rates to dsRNA concentrations revealed a substantial difference ($P<0.0005$) (Figure 1 A, Online Resource 1). The highest concentration tested (0.2 µg/µL) was more effective at reducing egg viability than the lower concentrations tested.

In the case of late egg hatching rate, Figure 1 B showed that there was not a significant effect of all interference of treatment factors on egg hatching rates ($P<0.0005$), which were 85.41, 83.33,

89.58 and 90.00% for Hsp27, ATPase, Cctra-2 and Ph-B-s, respectively. The three-factor interference of Hsp27, phosphate buffer solution Ph-B-S group, and control was the lower hatching rate of 7.00 with LSD 1.17 in the treatment of early egg and ATPase. At the same time, the lowest rate of egg hatching of bilateral interferences between Hsp27, control genes, and concentrations were 9.5 with LSD 1.65 in ATPase and (0.2 µg/µL) treatment, as shown in Online Resource 1. As a result, Hsp27 and ATPase were increased in the late egg hatching rate compared to the percentage of egg hatching rate at the early stage. The triple interferences among the four factors (egg type, Hsp27, control genes and control, and concentrations) in egg hatching were also significantly different (*P<0.0005*). Treatment of the early eggs, ATPase, and level (0.2 µg/µL) was the lowest rate of egg hatching at 12.91% with LSD 2.34 (Figure 1).

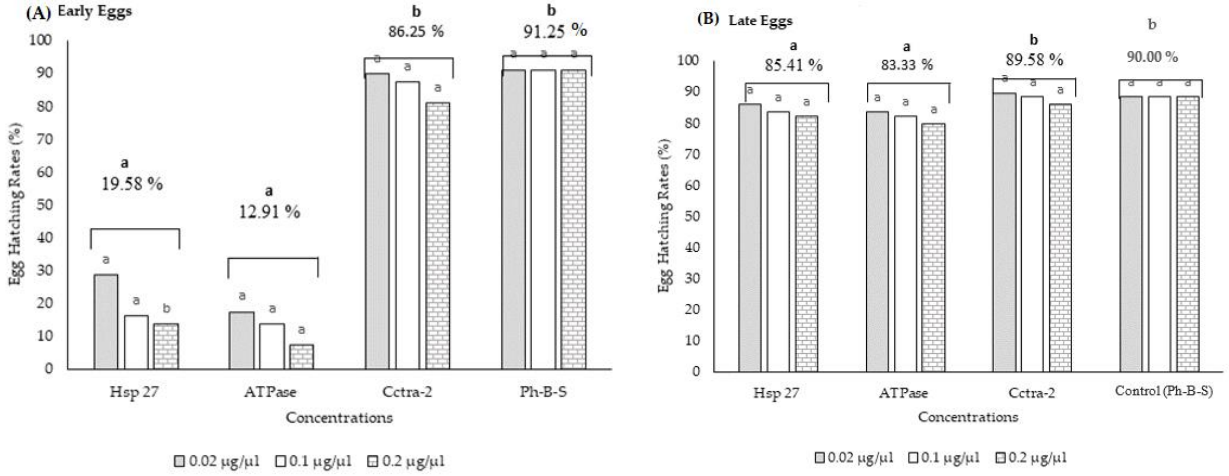

Figure 1. Hatching rates of *C. capitata* after being separately treated with Hsp27, ATPase, and Cctra-2 in three concentrations compared to the control. **(A)** Early egg treatment, **(B)** Late egg treatment. The lowercase letters a and b indicate the significant differences between egg-hatching rates.

In the sex ratio experiment, the proportion of female adults grew there was no significant difference in the sex ratio between treatment groups and control groups. The results of the female percent of statistical analysis (Online Resource 2) indicated that there were no significant differences in female percentages of early eggs and late eggs ($p>0.05$). There were no significant effects of Hsp27, ATPase and Cctra-2 on the female ratio (Figure 2). Online Resource 2 of the statistical analysis indicated that there were no significant differences in the female ratio of Hsp27, ATPase and Cctra-2 (*p>0.05*). The results (Figure 2) confirmed that the female ratio was nearly 50%, where *p>0.05* (Online Resource 2). The bilateral and triple interferences of experimental factors did not influence the female ratio, where the value of *P* was 0.93 (Online Resource 2). Overall, the Hsp27 dsRNA and genes of the control group treatments on *C. capitata* did not affect the sex ratio.

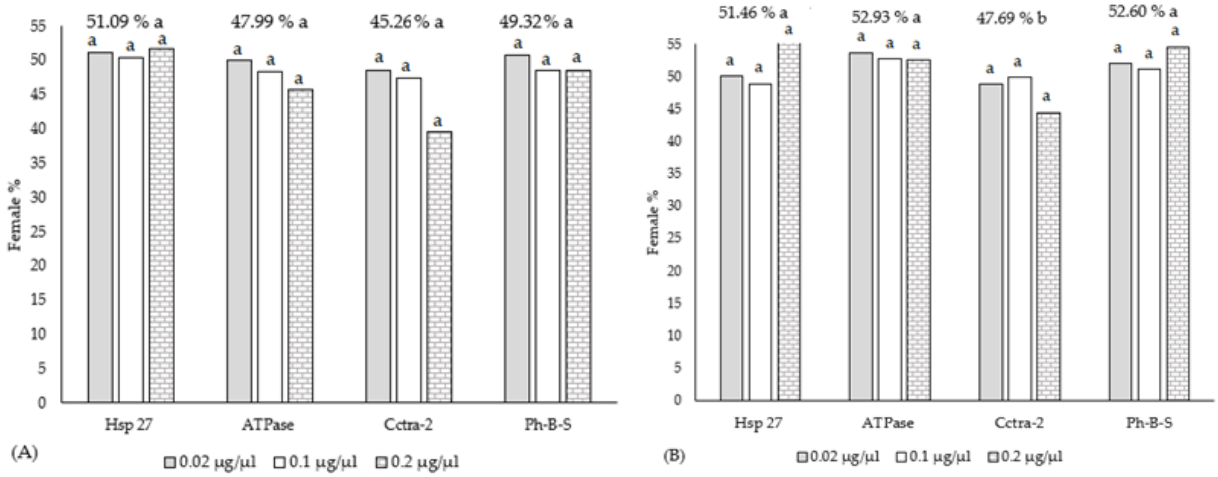

Figure 2. The percentage of *C. capitata* females after treated with three levels of Hsp27, ATPase and Cctra-2 comparing to the control group (Ph-B-S), (A) The percentage of female after-treated early eggs compared to the control group, (B) The percentage of female after-treated late eggs compared to the control group. The Lowercase letters a and b indicate the significant differences between egg-hatching rates.

The efficiency of this approach has relied upon the development of accessible and affordable ways to introduce dsRNA within insects (Yang et al., 2011 and Al-Obaidy et al., 2023). The soaking method is easy for certain insect cells and does not require complicated application techniques (Jang, 1992). Previous studies did not apply Hsp27 to *C. capitata* via the soaking method (Whyard et al., 2009). The ATPase gene is known for its effects upon less hatching of fruit fly eggs; therefore, it was used as an extra comparing group to ensure the results. The Cctra-2 has an impact on sex transformation, changes the sex ratio, and produces pseudo-males in *C. capitata* as well it is yet another indication that *Ceratitis* has a functionally conserved tra-2 homolog that contributes to the maintenance of transformer-positive autoregulation. Cctra functions as a crucial master switch and an epigenetic memory device for determining the gender of females (Roux et al., 2010). The current study on two types of *C. capitata* eggs explores the role of heat shock protein 27. *C. capitata* early eggs and late eggs were soaked in solutions of Hsp27, ATPase and Cctra-2 within three concentrations treatments for three hours. Egg viability (the ability of viable larvae to hatch), pupation, emerged adults and the female-to-male ratio were recorded and were used to elucidate Hsp27 function.

The use of dsRNA techniques in insect post-embryonic development is crucial for crop protection (Dias et al., 2019). This study examined the efficacy of soaking delivery of Hsp27 dsRNA to investigate egg viability in *C. capitata* early and late eggs with the aim of evaluating this technology's potential and Hsp27's effectiveness in decreasing the viability of the eggs of this pest. With further improvements, a method to reduce egg viability may provide an effective alternative to some control methods such as pesticides, radiation, and modified atmosphere in *C. capitata* management (Kalosaka et al., 2009).

Experimental results confirmed that Hsp27 dsRNA reduced egg viability compared to the control groups via soaking delivery. The findings are consistent with the data collected by (Kokolakis et al., 2008), who found that significant levels of Cchsp27 mRNA in the freshly

deposited eggs support the existence of CcHsp27 in the oocyte (Kokolakis et al., 2008). They applied five dsRNA in four concentrations to eggs of the fruit fly *C. capitata*. They found that only three dsRNA were effective in reducing egg hatching by more than 85% (Ali et al., 2017). In addition, our findings agreed with another study that used different temperatures pretreatment enhanced thermotolerance by increasing Hsp70 in medfly (Kalosaka et al., 2009).

The current study's findings disagreed with the findings of Salvemini et al., 2009 (Roux et al., 2010). They injected *C. capitata* early eggs (<6h) with 2.7 µM of cctra-2 dsRNA. They found that cctra-2 dsRNA reduced early egg development in adults by about 49%. The critical function of this gene in developing female-specific traits is demonstrated by Cctra-2 dsRNA embryonic injection. We can conclude that Cctra-2 is necessary for Ceratitis capitata female development (Roux et al., 2010). The use of the injection method of dsRNA delivery and the concentration of dsRNA may be the reason for the disagreement between our results and their results. In our experiment, eggs of *C. capitata* responded differently to each Hsp27 dsRNA and the gene controls. Early egg results indicated that Hsp27 dsRNA treatments were most effective in comparison to late eggs. We showed that the early eggs were more susceptible to Hsp27 dsRNA than late eggs, which is consistent with those of other researchers who studied the analysis of the structural characterization, developmental expression, heat shock, and ecdysone control of the Drosophila Hsp27 homolog in medflies, revealing significant differences in gene regulation across different developmental stages (Kokolakis et al., 2008).

Due to there being no previous studies about using low concentrations of soaking dsRNA to establish the sensitivity to Hsp27 in the control of *C. capitata*, in this study, a range of 0.02, 0.1 and 0.2 µg/µL dsRNA was used. Lower concentrations of Hsp27 dsRNA could be effective, which is useful since it is predicted to cause fewer off-target effects. Furthermore, by determining the dose-response relationship, a concentration could be chosen at which efficiency is maintained but at minimal risk of non-specific effects because a stage- and cell-specific pattern of CcHsp27 expression occurs during oocyte development (Fire et al., 1998; Pujol-Lereis et al., 2014).

An understanding of the sex determination mechanisms in insects of agricultural or public health may help develop improved methods for their control using the sterile insect technique or dsRNA-produced pseudo males (Ali et al., 2017; Fire et al., 1998).

Statistically, there were no significant effects of the Hsp27 dsRNA and other genes on the female-to-male ratio, although observations showed that there was a difference in Cctra-2 effect on the female ratio only. Observations were consistent with the data collected by (Roux et al., 2010), Where the cctra-2 achieved the lowest female percentage, 47.78%, while (Roux et al., 2010 and Sarhan, 2023) reported that the cctra-2 achieved 3.22% of *C. capitata* female. The findings confirmed that Hsp27 dsRNA was effective in reducing rates of egg development. The lowest concentrations of Hsp27, ATPase and Cctra-2 tested did not yield significant results. Results are consistent with (Roux et al., 2010) about the cctra-2 effect of reducing female percent, but the percent difference may be because of using the interference method and different concentrations, as previously discussed.

CONCLUSIONS

These findings reveal the different responses of *C. capitata* eggs to dsRNA and the delivery method and contribute to the current understanding of insect dsRNA mechanisms and provide important information for the further application of dsRNA as a genetic tool and pest control strategy. We found that early eggs were more susceptible, and this should be considered in future

experiments related to the use of dsRNA and *C. capitata* eggs. This study confirmed that Hsp27 dsRNA was effective in reducing rates of egg development. The lowest concentrations of Hsp27, ATPase and Cctra-2 tested did not yield significant results. Therefore, we recommend that urging more research on targeting eggs inside the fruit.

CONFLICT OF INTEREST

The authors declare no conflict of interest associated with this manuscript.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the staff of the Department of Plant Protection, College of Agricultural Engineering Sciences, University of Baghdad and Murdoch University, Australia for their technical and general support.

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Supplementary Data

Table S1. The egg-hatching rates of *C. capitata* with LSD values after being treated with three levels of Hsp27 heat shock protein compared to the three control groups.

Eggs ages	Hsp27, ATPase and Ccra-2	Concentrations				Interferences of eggs, Hsp27, ATPase and Ccra-2
		(0.02 µg/µl)	(0.1 µg/µl)	(0.2 µg/µl)	(Control)	
Early (<6h)	ATPase	3.50	3.50	2.75	18.25	7.00
	Hsp27	5.75	3.25	2.75	18.25	7.50
	Cctra-2	18.00	17.50	16.25	18.25	17.50
Late (>42h)	ATPase	17.00	16.75	16.25	18.00	17.00
	Hsp27	17.50	17.00	16.75	18.00	17.31
	Cctra-2	18.25	18.00	17.50	18.00	17.50
LSD	**2.34**					**1.17**
Eggs averages						
Interferences of eggs and concentrations	Early eggs	9.08	8.08	7.25	18.25	10.67^{b}
	Late eggs	17.58	17.25	16.83	18.00	17.42^{a}
LSD	**1.35**					**0.67**
Hsp 27+ATPase+Cctra-2 averages						
Interferences of (Hsp27+ATPase+Cctra-2) and concentrations	ATPase	10.25	10.12	9.50	18.12	12.00^{b}
	Hsp27	11.62	10.12	9.75	18.12	12.41^{b}
	Cctra-2	18.12	17.75	16.87	18.12	17.72^{a}
LSD	**1.65**					**0.89**
Concentration average		13.33^{b}	12.67^{b}	12.04^{bc}	18.12^{a}	
LSD	**0.95**					

Note: The small letters refer to significant differences; similar letters are not significant different
LSD = least significant difference

Table S2. The female emerging ratio of *C. capitata* eggs, which were treated with three concentrations of three Hsp27, compared with ATPase, Ccta-2, and Ph-B-S

Source	Type III Sum of Squares	Degree of freedom (df)	Mean Square	F- teste	significant.
Corrected Model	9665.613[a]	23	163.824	.670	.962
Intercept	590862.572	1	590862.572	2418.017	.000
Eggs	563.138	1	140.785	.576	.680
Proteins	411.116	2	205.558	.841	.433
Concentrations	164.885	2	54.962	.225	.879
Interferences of eggs, Proteins and Concentrations	8526.475	17	170.529	.698	.932
Error	43984.503	180	244.358		
Total	644512.688	240			
Corrected Total	53650.116	239			

a. R Squared = .180 (Adjusted R Squared = -.089)