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Full Length Research Paper

The immune enhancer, thymoquinone, and the hope of utilizing the immune system of *Aedes caspius* against disease agents

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Insects have developed an efficient defence system against microorganisms, which involves both humoral and cellular mechanisms. Recent studies on insect defence system are aimed at utilizing it in the battle against mosquito-borne diseases. However, mounting immune responses of insects has proved to impose fitness costs. The current study was conducted to test the costs of enhancing the immune responses of *Aedes caspius* by oral administration of thymoquinone, the active ingredient of the black seed oil, *Nigella sativa* L. (Ranunculaceae). The obtained results showed an effective humoral activity against *Micrococcus luteus* (NCTC 2665, Sigma-Aldrich, UK) and *Bacillus cereus* when mosquitoes were injected with lipopolysaccharide (LPS), *Bacillus thuringiensis* or *B. cereus*. However, this activity showed no effect against *B. thuringiensis*. Furthermore, oral administration of thymoquinone enhanced the humoral activity against *B. cereus* but not against *B. thuringiensis*. On the other hand, thymoquinone-enhanced immunity imposed reproductive costs in terms of higher percentages of follicular apoptosis and resorption. This observation has been confirmed by the semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis, which showed a relatively higher expression of selected caspases genes, namely: *CASP 18*, *CASP 19* and *CASP 20*, but not *CASP 21*, transcripts in immune enhanced mosquitoes compared to non-enhanced ones. Based on these results, this study suggests that enhancing the humoral activity by thymoquinone proved to be costly in terms of triggering follicular apoptosis and resorption. Thus, as part of the scenario of immunity-reproduction conflict, it was concluded that the impact of immune stimulation and/or enhancement on the vector reproduction constitutes a limiting factor to the utilization of thymoquinone in the immuno-control strategy against mosquito-borne diseases.

Key words: *Aedes caspius*, humoral immunity, thymoquinone, reproductive costs, apoptosis.

INTRODUCTION

Insect-borne diseases are major causes of death and morbidity worldwide. Mosquitoes of the genus *Aedes* are considered disease vectors as they are responsible for the transmission of a number of viral and parasitic pathogens (Christophers, 1960; Knight and Stone, 1977).

Insect haemolymph contains a number of proteins, which are involved in diverse developmental, physiological or immunological processes. Some of these proteins were found to participate in defense reactions *via* the insect defense system. This system comprised of cellular and humoral activities. Humoral immune responses are usually linked to the stimulation of cellular immune responses which includes phagocytosis by macrophage-like blood cells, activation of proteolytic cascades leading

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to localized melanization and coagulation, such as nodule formation and encapsulation (Hoffmann et al., 1996). These two activities cooperatively function in clearance of invading pathogenic microbes from the haemolymph.

The idea of targeting the mosquito immune system as a candidate in the battle against disease-causing agents (example, malaria) has recently gained the attention of the global health organizations. In the case of mosquito-malaria interaction, for instance, mosquito vectors are categorized into two groups. The first group is the refractory mosquito, which has innate immune responses that are able to block the malaria development inside its body (Collins et al., 1986; Somboon et al., 1999 and Rosenberg, 1985). The second group is the susceptible mosquito (the malaria vector), in which the malaria parasite succeeds to complete its life cycle and making it infective to a new vertebrate host. Based on this fact, the transmission blocking strategy became one of the recent rapidly evolved areas of malaria research that relies, in part, on the immuno-control strategy which takes place *via* two different hypotheses; (a) Genetically selecting malaria refractory strains that melanize ookinetes in its gut (Somboon et al., 1999) and (b) genetically modifying the vector by moving immune-genes from the refractory strain to the susceptible one (Kokoza et al., 2000) to become incompetent of malaria transmission. Furthermore, blocking malaria parasite development in the mosquito midgut is also a recent technology used as a tool to block transmission of malaria parasites in the mosquito vector. For example; using “single-chain immunotoxin construction” (Yoshida et al., 2001). This strategy relies on introducing a single-chain antibody fragment (scFv) into mosquito midgut, which works as targeting moiety to Pbs21 protein on the surface of *Plasmodium berghei* ookinetes and which eventually kills the parasite.

On the other hand, however, mounting immune responses in mosquito vector proved to be costly in terms of most fitness parameters (Yan et al., 1997; Voordouw et al., 2009). Reproductive fitness, in particular, has been significantly reduced as a result of immune induction (Ferdig et al., 1993; Moret and Schmid-Hempel, 2000; Ahmed et al., 2002; Schwartz and Koella, 2004) to the extend that refractory mosquitoes have completely lost their ability to reproduce after 25 generations of successful blocking of malaria parasite (Somboon et al., 1999). Hence, a better understanding of the costs of mounting immune responses in general, could in our opinion, help in developing a novel way of blocking the transmission of mosquito-borne diseases.

Nigella sativa L. (Ranunculaceae) seeds, commonly known as black seed, have been particularly used in the traditional Arab herbal medicine for the treatment of many diseases and ailments, that is, they are used against high blood pressure and the seed oil is used against lung diseases, arthritis and hypercholesterolemia. Therefore, *N. Sativa* has been extensively studied and many reports deal with favourable biological properties, including: anti-inflammatory, antioxidative, antitumour, antiulcerogenic and hepatoprotective (Khader et al., 2009 and references therein). In this context and as an attempt to search for a non-costly immune enhancer/inducer, we have initially tested the potential of black seed oil. Interestingly, results have indicated that black seed oil can potentially act as a promising immune-modulator with a unique dual effect on the immunity of the malaria vector *Anopheles gambiae*, acting as an immune enhancer with a normal reproductive fitness (Ahmed, 2006). Previous reports have indicated that the biological activity of *N. sativa* seeds are attributed to its essential oil components (Hajhashemi et al., 2004). The main compounds contained are thymoquinone (30 - 48%), p-cymene (7 - 15%), carvarol (6 - 12%), 4-terpineol (2 - 7%), t-anethole (1 - 4%) and the sesquiterpene longifolene (1 - 8%) (Burits and Bucar, 2000). Thus, as the main constituent of black seed oil, we have additionally investigated the immune-modulation potential of thymoquinone (Tq) in the dengue fever vector *Aedes aegypti* (Ahmed et al., 2008). Notably, Tq has been shown to exert immune-enhancive effects on both the humoral and melanization responses of *A. aegypti* against biological and non-biological agents (Ahmed et al., 2008), however, its impact on the reproductive fitness of the mosquito vector remains to be investigated.

The main objectives of the current study are two-fold: (1) To investigate whether Tq is capable of enhancing humoral antibacterial immune responses in *Aedes caspius,* a local vector for Rift Valley fever in Saudi Arabia (Al-Hazmi et al., 2005), against both *Bacillus cereus* and *Bacillus thuringiensis* bacteria. Moreover, as a control, the ability of cell-associated lipopolysaccharide (LPS) (referred to as endotoxin of *E. coli*) to enhance these immune responses against these bacterial types was also tested; (2) to investigate whether Tq-enhanced immunity exhibits negative fitness costs, on the reproductive capacities. Thus, the reproductive cost entailed on follicular development in *A. caspius* was closely investigated in terms of follicular apoptosis and resorption. The results are discussed in the light of the potential hope of implementing thymoquinone, the active ingredient of the black seed oil, as a possible tool for improving immuno-control strategies in the battle against mosquito-borne diseases in the future. The need for more efforts in the hunt for alternative non-costly and non-toxic immune-enhancers in black seed oil is also highlighted.

MATERIALS AND METHODS

Establishment of experimental mosquitoes

The target mosquito species of this study, *A. caspius*, was collected from Eastern region of the Kingdom of Saudi Arabia. Field collected mosquitoes were identified according to the classification keys of Mattingly and Knight (1956) and confirmed by the Natural History Museum (London, UK). Mosquitoes were reared in the insectary of Zoology Department, College of Science, King Saud University,

under standard conditions as previously outlined (Ahmed et al., 1999), to ensure the production of mosquitoes of similar body sizes, checked by measuring wing lengths according to Briegel (1990). Adults emerging within a 24 h period were maintained in rearing cages (30 x 30 x 30 cm each) with continuous access to a 10% glucose solution (w/v). At least 10 generations were produced prior to use for experimental purposes. After adult emergence, mosquitoes of the same age were used for this study. To maintain a stock of mosquito colony, they were routinely fed upon the blood of an anaesthetized CD mouse in order to lay eggs for new generations.

Experimental design

The experimental design implemented in the study of humoral antibacterial activity is depicted in Figure 1. Newly emerged mosquitoes were divided into two groups, A and B, which were maintained on 10% glucose solution (w/v) or 0.3% Tq-glucose mixture (w/v), respectively, until the end of experiment. Similar body sizes were ensured by statistically comparing wing lengths (n = 20 from each group), prior to blood feeding, in order to ensure similar blood meal sizes (Briegel, 1990). As illustrated in Figure 1, six-day old mosquitoes were starved for 12 h prior to blood feeding on an anaesthetized CD mouse for 20 min. Immediately after feeding, fully engorged mosquitoes of group A or B were randomly selected and subdivided into 5 subgroups (A1 to A5) or 3 subgroups (B1 to B3) (20 mosquitoes each), respectively. Mosquitoes were, then, intra-thoracically injected with 10 ng LPS/mosquito (in 0.25 µl *Aedes* physiological saline (APS)) (Sigma, UK), *B. thuringiensis* (Bt) or *B. cereus* (Bc), as detailed in Ahmed et al. (2002). Mosquitoes left non-injected (NI) were taken as negative control, while mosquitoes sham-injected (SI) with filter sterilized APS were included as positive control. Injected mosquitoes were maintained in small cages (6 x 6 x 6 cm each) and allowed access to their relevant solutions (10% glucose or 0.3% Tq-glucose mixture), again until the end of experiment (Figure 1). At 24 h post-injection, haemolymph was collected from 5 active mosquitoes that have the ability to fly and freshly used for inhibition zone assay as described in Nimmo et al. (1997). Whole mosquitoes or ovaries, from Bc-injected group, were used for total RNA extraction or monitoring follicular apoptosis and resorption, respectively.

Oral administration of thymoquinone

Tq, the main active ingredient of the black seed oil (*N. sativa*), was used as a natural immune-enhancive agent (Sigma-Aldrich, UK). A solution of 18 mM Tq prepared in 10% glucose (equivalent to 0.3% Tq dissolved in 10% glucose solution (w/v)) was administered to mosquitoes according to Ahmed et al. (2008). Briefly, experimental mosquitoes were allowed to feed on 10% glucose solution (w/v) or Tq-glucose mixture (w/v) immediately after adult emergence and until the end of experiments. The enhancive effect of 18 mM Tq on humoral anti-bacterial activity was studied in blood fed mosquitoes (maintained on 18 mM Tq-glucose mixture or 10% glucose).

Bacterial injection, haemolymph collection and inhibition zone assays

The entomopathogenic bacteria, *B. thuringiensis* (de Maagd et al., 2001) and the non-pathogenic one, *B. cereus* (Hillyer et al., 2004), were kindly provided by the Department of Food Sciences and Nutrition, Faculty of Food Sciences and Agriculture, King Saud University and prepared for mosquito inoculation as previously described (Nimmo et al., 1997). For injection, mosquitoes were immobilized on ice and sham-injected with 0.25 µl of APS (13 mM NaCl, 0.5 mM KCl, 0.1 mM $CaCl_2$) or LPS (Sigma-Aldrich, UK) (10 ng/0.25 µl APS/mosquito) into the thoracic haemocoel of mosquito individuals using a calibrated hand-made heat-pulled micro-capillary needle as detailed in Ahmed et al. (2002), or challenged with bacteria by pricking (injection) with a fine capillary needle (dipped in a concentrated suspension of bacteria) according to Dimopoulos et al. (1997). Any mosquito that was severely bled after injection was discarded from the study. Mosquitoes were then allowed to recover and maintained in cages (16 × 16 × 16 cm each) in standard insectary conditions. No difference in the mortality of the injected or sham-injected mosquitoes was observed (data not shown). Haemolymph was collected from the thoraces of experimental mosquitoes 24 h post-treatment and subjected to an inhibition zone assay as detailed in Ahmed et al. (2002), to measure the humoral activity against each injected material. For comparison, the humoral activity against the immune-sensitive bacteria, *Micrococcus luteus* (NCTC 2665, Sigma-Aldrich, UK) was tested as a result of injection with LPS, Bt or Bc bacteria. Five replicates in each case (from five different individual mosquitoes) (n = 5) were carried out to perform statistical analysis.

Monitoring follicular apoptosis and resorption

Vitellogenic mosquitoes injected with *B. cereus* were chilled on ice for dissecting their ovaries in order to monitor either the follicular apoptosis or resorption. Follicular apoptosis was monitored in ten ovary pairs. Mosquitoes were dissected in phosphate buffered saline (PBS) 18 h post-injection/blood feeding and immediately moved into clean sterilized eppendorf tubes (one ovary pair/100 µl PBS) and used for visualizing the caspases activity in its developing follicles using CaspaTagTM Fluorescein Caspase (VAD) (Intergen Company, UK) according to the manufacturer's instructions. Briefly, FAM-Peptide-FMK was added directly to each ovary pair, mixed gently and incubated for 1 h at 37°C under 5% CO_2 and protected from light. Ovaries were then washed, mounted in Vectashield® medium and immediately examined under a fluorescence microscope using a band pass filter (excitation 490 nm, emission 520 nm). Whole follicles that contained cells displaying fluorescence and therefore exhibiting caspases-like activity were counted and the percentage (%) per ovary pair was calculated for each individual mosquito. Similarly, follicular resorption was conducted on ten ovary pairs according to Clements and Boocock (1984). Briefly, mosquitoes were dissected in APS and immediately dipped in Neutral Red (0.5% (w/v)) solution in citrate-phosphate buffer (0.1 M citric acid/0.1 M sodium citrate) at pH 6 for 1 min to assist the visualization of resorped follicles. Resorped follicles were counted under the microscope and %/ovary pair was calculated for each individual mosquito.

Histological examination of *A. caspius* larvae midgut tissues

Second-instar *A. caspius* larvae were treated with the LC_{50} of Bt-based insecticide (0.0586 mg/l) [Dipel®, Sumitomo Chemical Agro Europe (SCAE), France]. Then, larval guts were dissected 18 h post-treatment, fixed and sectioned and stained routinely as previously described (Bauer and Pankratz, 1992; Villalon et al., 2003). The midgut sections were fixed overnight in cold 2.5% glutaraldehyde in 100 mM phosphate buffer, pH 7.2 and post-fixed for 1 h at room temperature in 1% OsO_4 in the 100 mM phosphate buffer. The tissues were then dehydrated through an ethanol series, treated with propylene oxide and embedded in Poly/Bed 812 (Polysciences Inc., Warrington, PA). Thin 10 µM sections were mounted on slides, stained with haematoxylin and eosin (Sigma-

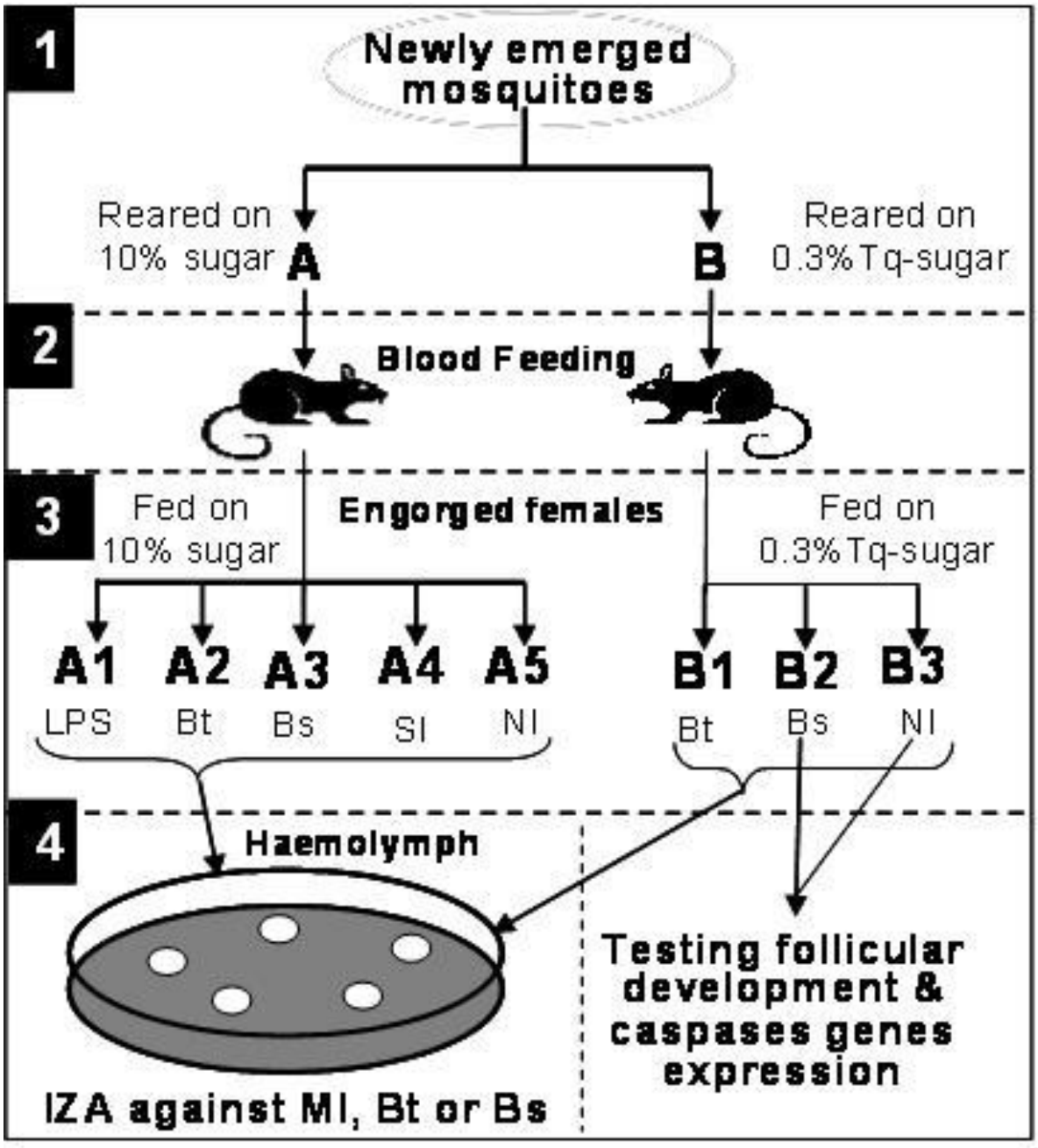

Figure 1. Schematic representation of the experimental design employed for studying the humoral anti bacterial activity and the impact of Tq-enhanced immunity on the follicular development in *A. caspius*. (1): Newly emerged mosquitoes were divided into groups A and B (≈ 300 mosquitoes each). Group A was immediately allowed to feed on 10% glucose solution and until the end of experiment, while group B was allowed to feed on 0.3% Tq-glucose mixture until the end of experiment. (2): After insuring equal body sizes, group A or B was allowed access to a blood meal from anaesthetized mouse for 20 min. (3): After blood feeding, fully engorged females from groups A or B were randomly divided into the subgroups A1 to A5 or B1 to B3, respectively. The subgroups A1 to A5 were immediately injected with LPS, *B. thuringiensis* (Bt), *B. cereus* (Bc), sham-injected (SI) with APS or left non-injected (NI), respectively. On the other hand, the subgroups B1, B2 or B3 were immediately injected with Bt, Bc or left non-injected (NI), respectively. Treated groups were then allowed access to their relevant sugar solutions again until the end of the experiment (24 h later). (4): Haemolymph from subgroups A1 to A5 or subgroups B1 to B3 was collected and used freshly for inhibition zone assays (IZA) in agar seeded with Bt, Bc or *M. luteus* (MI) for comparison. Ovaries from B2 and B3 groups were dissected for monitoring follicular resorption and apoptosis 18 h post-treatment. Total RNA was extracted from whole mosquitoes for investigating caspases genes expression via semi-quantitative RT-PCR 18 h post-treatment.

Table 1. List of primers used for RT-PCR expression analysis of caspases genes of *A. aegypti*.

Gene	Forward (F) and reverse (R) primers (5'-3')	Description	Vector base gene ID*/ GenBank accession No.**	RT-PCR product size (bp)
CASP 18	*F:* CTGTCTTGTGGTAGTTGTGATGTC *R:* CGGATGCTTGTGATTCTTCTTCTC	Caspase-1	AAEL003439	204
CASP 19	*F*: CTCGCCGTGTGACATCATAAC *R*: AAGCAAGGAAGTTCTCGTTTCTC	Caspase-1	AAEL003444	294
CASP 20	*F*:GCGGATTGCCTGATGGTATTC *R*: ATGCTTGGACTATGAACAACTTCG	Caspase-1	AAEL014658	159
CASP 21	*F*: CGATTGTAATAAAACGGTTCCTAGTCC *R*: CTATTGACATTTCTGGCATCTCTCTTAG	Caspase-1	New (not available)	115
ACTIN 6	*F*: AAGGCTAACCGTGAGAAGATGAC *R*: GATTGGGACAGTGTGGGAGAC	Housekeeping	DQ124691	159

*http://aaegypti.vectorbase.org/index.php; ** http://www.ncbi.nlm.nih.gov/Genbank/

Aldrich) and mounted with Paramount (Fisher) and examined by light microscopy using a Zeiss Axioskop 50 compound microscope (Carl Zeiss, Inc., Thornwood, NY).

Gene expression analysis by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Experimental mosquitoes were allowed to feed on 18 mM Tq-glucose mixture (Tq-fed) or 10% glucose solution (w/v) (Tq-unfed) immediately after adult emergence and until the end ofexperiments. Immediately after blood feeding, Tq-fed and Tq-unfed, were injected with *B. cereus*, as this bacterial type induced the humoral immunity measurably. At 18 h post-injection, mosquitoes were placed in sterile eppendorf tubes, snap-freezed in liquid nitrogen and stored at -70°C until use for the expression level investigations of the selected caspases genes in response to Tq treatment. Thus, gene-specific primers, designed based on *A. aegypti* gene sequences (http://aaegypti.vectorbase.org/index.php), were employed. The sequences of the specific sets of primers according to Briant et al. (2008) utilised for the amplification of each cDNA are summarized in Table 1. Thus, 10 whole immune-enhanced mosquitoes (from Tq-fed or Tq-unfed) were homogenised to fine powder (in sterile mortar and pestle) in liquid nitrogen. Then, total RNA was purified using Norgen Biotek kit, according to the manufacturer's instructions (Norgen Biotek, Canada). Purity and integrity of the isolated total RNA were verified using formaldehyde denaturing gel electrophoresis (Sambrook et al., 1989). The absence of genomic DNA in the total RNA preparations was confirmed by both agarose gel electrophoresis as well as the detection of no PCR product when specific primers for the housekeeping gene *ACTIN 6* was used. Subsequently, two-step RT-PCR protocol was carried out using GE HealthCare beads, according to the manufacturer's instructions (GE Healthcare, Sweden). When brought to a final volume of 50 µl, each reaction contains ~2.0 units of Taq DNA polymerase, 10 mM Tris-HCl, (pH9.0 at room temperature), 60 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (FPLCpure™), RNAguard™ ribonuclease inhibitor (porcine) and stabilizers, including RNase/DNase-Free BSA. A typical quantity of 2 µg total RNA was used in the RT reaction in a final volume of 50 µl. Total RNA was heated at 65°C for 5 min, snap cooled on ice for 2 min and used as a template for the first strand cDNA synthesis using pd(T)12 – 18 primer at 42°C for 45 min. Exponential phase of the PCR reaction could be empirically determined, for each apoptosis-related gene tested, by amplifying equivalent amounts of cDNA over different number of successive PCR cycle range (25 - 30) and it was found to be 27 cycles (data not shown). Hence, thermal cycling conditions that were found to be optimal for apoptosis-related genes included: 95°C, 2′ [95°C, 30″; 55°C, 30″; 72°C, 1′] x 27. Negative control reactions, where the cDNA is substituted with DNase/RNase-free water, were included in each RT-PCR run to screen for possible contamination. To compensate for variations in the input of RNA amounts/loading and an efficiency of reverse transcription, housekeeping gene *ACTIN 6* was included. PCR products were taken from PCR reactions and resolved by electrophoresis in 2.0% (w/v) agarose gel, stained with ethidium bromide and visualized under ultraviolet light by gel documentation system (Biometra Biomedizinische Analytik, GmbH).

DNA sequencing

PCR products of caspases genes were gel-purified using GE Healthcare Kit according to manufacturer's instructions (GE Healthcare, Sweden). DNA sequencing was conducted in Sequencing Core Facility at King Faisal Specialized Hospital and Research Centre (Riyadh, KSA), using Sanger Sequencing Technology on ABI Prism 3730XL (Applied Biosystems/Sanger) according to the dideoxy chain-termination method (Sanger et al., 1977). The obtained sequences were manually-cleaned and pairwise alignment against corresponding annotated caspase genes database (http:// aaegypti.vectorbase.org/index.php) was performed with BioEdit Sequence Alignment Editor (Hall, 1999).

Statistical analysis

All statistical analyses were undertaken using MINITAB software (MINITAB, Stat College, PA, v. 13.1, 2001). Data of each experiment were first tested for normality and variances homogeneity prior to further analysis with the suitable test. Because data were non-parametric, Kruskal-Wallis test was used to determine the overall effects of treatments prior to the individual comparisons between test and control mosquitoes using the non-parametric test, Mann-Whitney *U*.

RESULTS

Humoral anti-bacterial activity

As a control, humoral activity in the haemolymph of *A. caspius* was first tested against *M*. *luteus* at 24 h post-injection with 20 ng LPS, *B. thuringiensis* and *B. cereus*. Lysozyme equivalent humoral activity against this immune-sensitive bacterium was detected in haemolymph from mosquitoes at 24 h post-injection with LPS, *B. thuringiensis* or *B. cereus* (Figure 2A). Significantly higher (Mann-Whitney *U* test; $P < 0.05$, $n = 5$) humoral activities, expressed as lysozyme equivalent ng/µl haemolymph, were detected at 24 h post-injection in the following order: LPS (291.0 ± 18) > Bt (251.4 ± 16.7) > Bc (191.7 ±19) ng/µl, when compared to non-injected (NI, 39.85 ± 7.14) and sham-injected (SI, 67.2 ± 11.9) controls (Figure 2A). These results clearly indicate that challenging *A. caspius* with these materials has efficiently triggered a significant average increase in the anti-*M. luteus* activity (Figure 2A). Similarly, haemolymph from *A. caspius* challenged with these materials correlated with significantly higher (Mann-Whitney *U* test; $P < 0.05$, $n = 5$) anti-*B. cereus* humoral activity at 24 post-injection in the following order: LPS (169.3 ± 14.0) > Bt (154.37 ± 9.14) > Bc (124.51 ± 9.14) ng/µl, when compared to NI (49.85 ± 9.14) and SI (72.25 ± 7.47) controls (Figure 2B). These results may indicate that injecting *A. caspius* with LPS, or challenging it with *B. thuringiensis* or *B. cereus* did induce humoral activity against *B. cereus* (Figure 2B). In contrast, haemolymph from *A. caspius* challenged with these materials displayed a weak anti-*B. thuringiensis* humoral activity at 24 h post-injection in the following order at: Bc (80.0 ± 7.47) > Bt (75.0 ± 9.14) > LPS (70.38 ± 9.14) ng/µl, when compared to NI (50.38 ± 9.14) and SI (62.25 ± 7.47) controls (Figure 2C). These activities were not significantly different (Mann-Whitney *U* test; $P > 0.5$, $n = 5$) from that of the NI and SI control mosquitoes. These results may indicate that challenging of *A. caspius* with LPS, *B. thuringiensis* or *B. cereus* did not induce a significant humoral activity against *B. thuringiensis* (Figure 2C). Furthermore, injection of *A. caspius* with LPS, or challenging it with *B. thuringiensis* or *B. cereus* did induce humoral activity against *B. cereus* (Figure 2B) but not against *B*. *thuringiensis* (Figure 2C).

Histopathological effect of *B. thuringiensis* on *A. caspius* larvae midgut

Results revealed the destructive effect of *B. thuringiensis* on midgut wall of *A. caspius* larvae (Figure 3), compared to the unexposed midgut control. Midgut epithelial cells were destroyed by bacterial toxins whereas cells of the untreated control retained their structural integrity (Figure 3). Hence, being not sensitive to the humoral activity (Figure 2C) might explain the high toxicity of *B. thuringiensis* as an effective entomopathogenic biocontrol agent against larvae. The severe destruction of epithelium tissue allows the bacteria to pass to haemolymph causing cessation of feeding, septicemia and finally death (Gill et al., 1992).

Enhancive effect of Tq on humoral anti-bacterial activity

Figure 4 depicts the effect of Tq on the humoral anti-bacterial activity against both *B. cereus* and *B. thuringiensis*. Mosquitoes fed on 18 mM Tq-glucose exhibited a weak and non-enhancive effect on the humoral activity against injected *B. thuringiensis* (equivalent to 148.0 ± 15.33 ng/µl haemolymph); this weak effect was not significantly different (Mann-Whitney *U* test; $P > 0.5$, $n = 5$) from that of SI or NI-treated control mosquitoes (*v* 110.22 ± 14.46 or 88.5 ± 16.98 ng/µl haemolymph, respectively). In contrast, 18 mM Tq- glucose fed mosquitoes triggered enhancing impact on the humoral activity against *B. cereus*, equivalent to 4.5 and 5. 2 folds higher than that of SI and NI treated control mosquitoes, respectively (Figure 4). Moreover, this enhanced activity was 4.6 folds more than that of mosquitoes fed on 10% glucose only (*v* 124.5 ± 9.14 ng/µl haemolymph, Figure 2B). Taken together, these results may indicate that the observed Tq-mediated enhancive impact on the humoral activity of *A. caspius* is dependent on the type of injected bacteria.

Effect of Tq-enhanced immunity on follicular development

The impact of enhanced immunity on follicular development (in terms of triggering follicular apoptosis and resorption) as early as 18 and 20 h post-immune induction, respectively, was investigated (Figures 5A and B). Follicular apoptosis was detected as caspases-like activity which was visualized as fluorescing follicular epithelium under fluorescence microscope (Figures 6A, B and C). Data showed significantly higher percentage of follicles undergoing apoptosis in immune-enhanced mosquitoes at 18 h post-*B. cereus* injection (5.2 ± 0.37/ovary pair) compared to non-injected mosquitoes (*v* 2.0 ± 0.31/ovary pair) (Mann-Whitney *U* test; $P < 0.5$, $n = 10$) (Figure 5A). On the contrary, no significant difference in the percentage of follicles undergoing apoptosis was detected in *B. thuringiensis*-injected mosquitoes compared to non-injected mosquitoes (Mann-Whitney *U* test; $P > 0.5$, $n = 10$) (Figure 5A). Similarly, data of follicular resorption indicated significantly higher percentage of resorped follicles in *B. cereus*-injected mosquitoes at 20 h post-injection compared to the non-injected (9.8 ± 0.66 *v* 3.8 ± 0.58) (Mann-Whitney *U* test; $P < 0.5$, $n = 10$) (Figure 5B). However, no significant difference in the

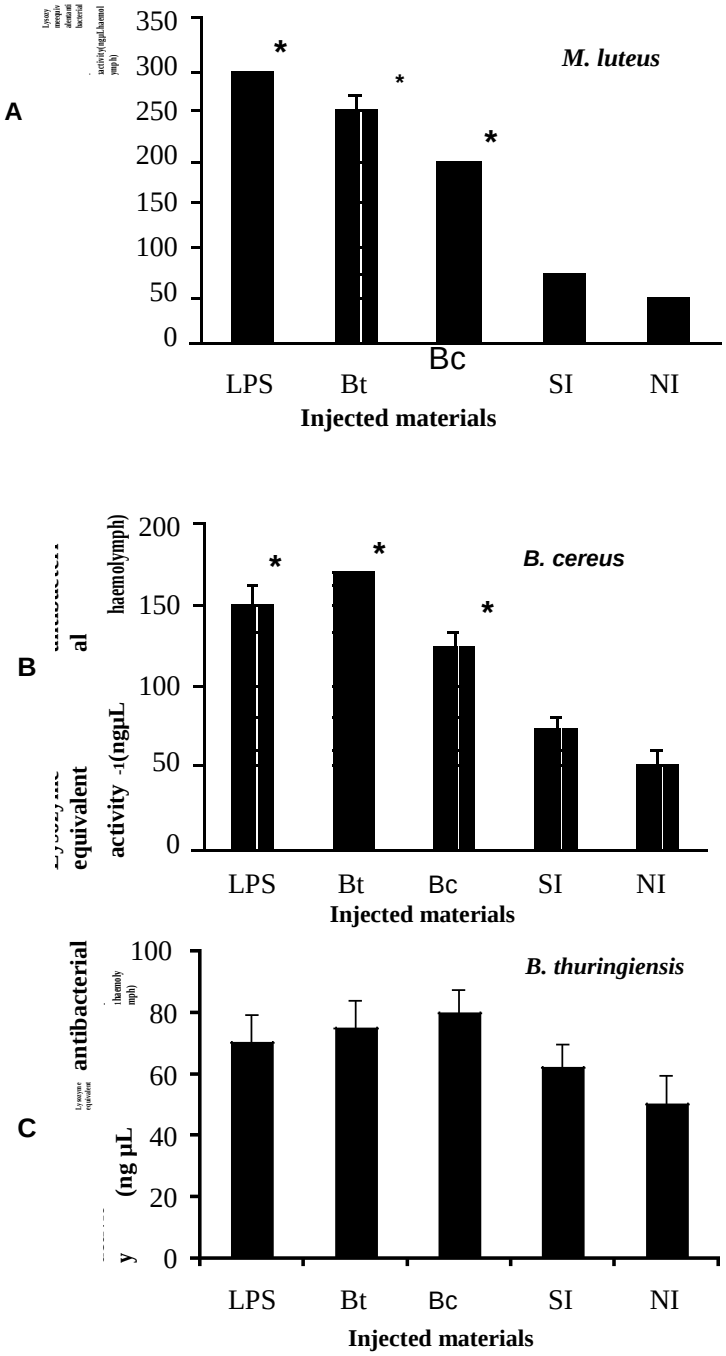

Figure 2. Humoral antibacterial activity of blood-fed *A. caspius* against *M. luteus* (A), *B. cereus* (B) and *B. thuringiensis* (C) at 24 h post-injection with LPS, *B. thuringiensis* (Bt) or *B. cereus* (Bc). Haemolymph from sham-injected (SI) or none-injected (NI) mosquitoes were used as controls. Haemolymph was subjected to an inhibition zone assay against each respective bacterial pathogen. Error bars represent standard errors on the mean (n = 5). Asterisks (*) on columns represent significantly different humoral activities as compared to that of NI or SI mosquitoes.

enhancement of humoral antibacterial activity against *B. cereus* resulted in a concomitant reproductive cost in terms of higher percentage of follicular apoptosis and resorption.

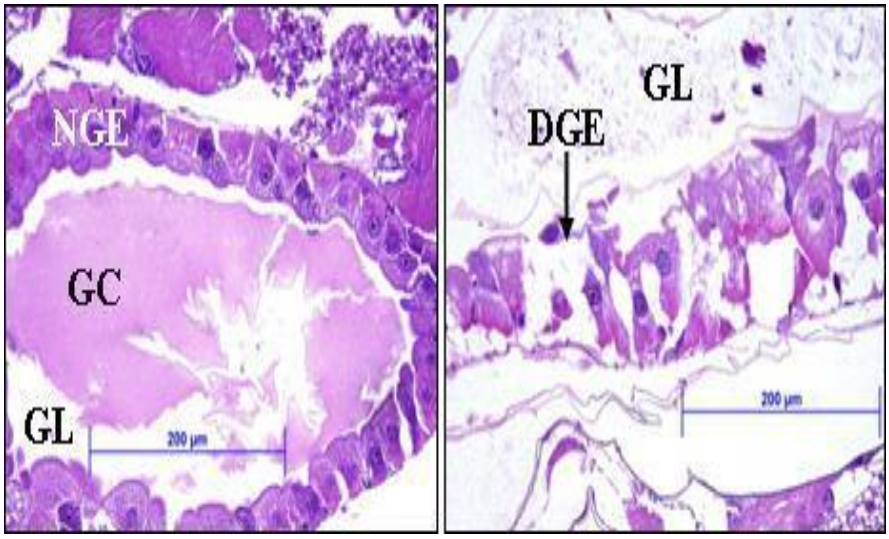

Figure 3. Histopathological examination of *B. thuringiensis*-derived toxin action in midgut epithelial tissue of *A. caspius* larvae 18 h post-treatment. Cross-section of untreated control larvae (left) shows normal gut epithelium (NGE) and nutritional gut contents (GC) filling the gut lumen (GL). Longitudinal section of treated larvae (right) depicting damaged gut epithelium (DGE) and traces of nutritional gut contents in the gut lumen. Thin 10 µM sections were examined by light microscopy using a Zeiss Axioskop 50 compound microscope (Carl Zeiss, Inc., Thornwood, NY).

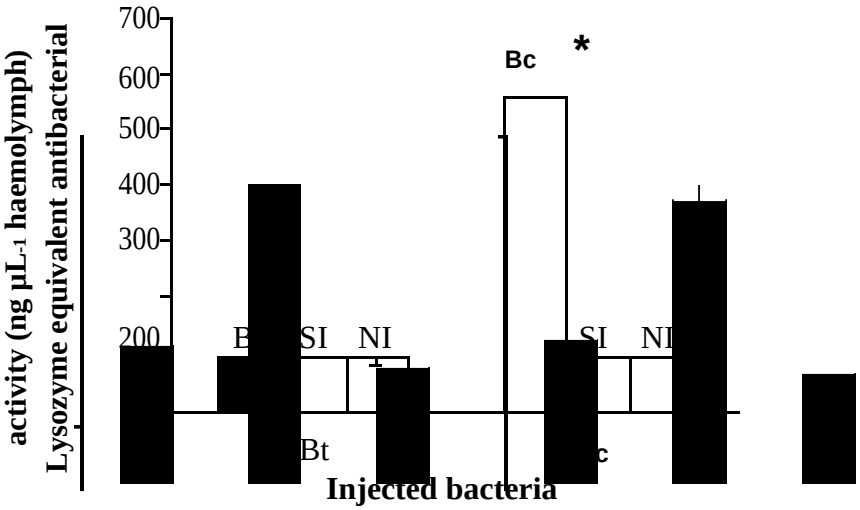

Figure 4. Enhancive effect of thymoquinone on the humoral antibacterial

Injected bacteria

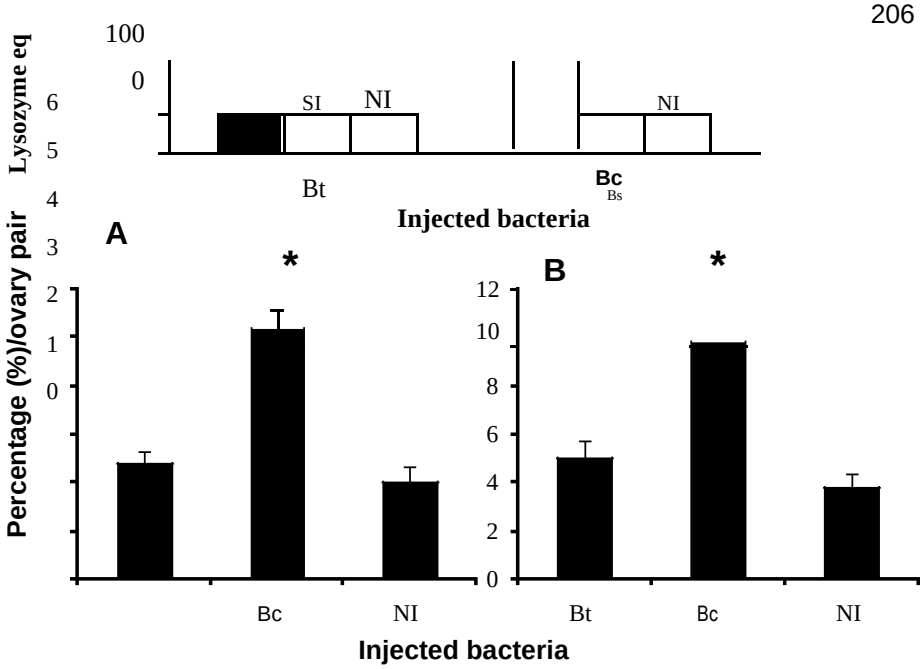

Figure 5. Enhancive effect of thymoquinone on follicular development in *A. caspius* post-challenging with *B. thuringiensis* (Bt) or *B. cereus* (Bc). Graph A shows % of apoptotic follicles at 18 h post-bacterial injection. Graph B shows % of resorping follicles at 20 h post-bacterial injection. Ovaries were dissected in APS and immediately processed for monitoring follicular caspases activity and resorption. Error bars represent standard errors on the mean (n = 5). Asterisks (*) on columns represent significantly different activities as compared to that of NI or SI mosquitoes.

Tq treatment induces the expression of a subset of selected caspases genes

The impact of Tq-mediated immune enhancement in *A. caspius* on the transcription level of selected apoptosis-related caspases genes was investigated (Figure 6D). In this context, gene specific primers of four selected *A. aegypti* caspase genes were designed and employed (Briant et al., 2008; http://aaegypti.vectorbase.org/index.php). Thus, the relative transcript levels corresponding to the four different caspases genes were analyzed by semi-quantitative RT-PCR using a combination of primer sets (Table 1). Additionally, specific primers for an actin cDNA (*ACTIN 6*) were used as an internal control of RT-PCR. After gel purification, the identity of PCR products, for each of the genes tested, was confirmed by DNA sequencing and pairwise alignment against the corresponding database gene (http://aaegypti.vectorbase.org/index.php) using BioEdit sequence alignment editor (Hall, 1999) (data not shown). When immune-enhanced mosquitoes were injected with *B. cereus* for 18 h, the level of transcripts for *CASP 18* was relatively enhanced compared with un-injected control mosquitoes (Figure 6D). Similarly, enhanced immunity exerted a similar and drastic effect on the accumulation of both *CASP 19* and *CASP 20* transcripts when compared with sugar-fed *B. cereus*-injected control, albeit to a lesser extent (Figure 6D). However, Tq-enhanced immunity did not affect the transcription level of *CASP 21* gene, which remained similar to that in the un-injected control (Figure 6D). These results may indicate that the expression of a subset of caspases genes (*CASP 18, 19 and 20*) might be associated with Tq-mediated enhanced immunity *in A. caspius* against *B. cereus.* However, a direct definitive link between Tq immune enhancement and caspase gene expression remains to be established.

DISCUSSION

In this study, enhancement of the innate immunity of *A. caspius,* by oral administration of Tq and the concomitant impact on the reproductive fitness (in terms of follicular apoptosis and resorption) was investigated. Thus, it is important to initially clarify several key points. Firstly, mosquitoes were fed on 10% glucose or 18 mM Tq-glucose mixture immediately after adult emergence and until the end of experiment. Secondly, follicular apoptosis or resorption was monitored 18 or 20 h post-blood feeding/treatment based on previous studies (Hopwood et al., 2001). Thirdly, the size of the blood meal taken by mosquito may participate as a direct/indirect factor in most of the differences detected in treated mosquitoes of this study (that is, differences in humoral activities, follicular apoptosis and follicular resorption). Therefore, blood meal size was accounted for to be certain that these differences were not as a result of different sized blood meals. Thus, wing size (the distance from the wing tip to the distal end of the allula) was used as an estimate of body size (Table 2) and hence, blood meal size (Briegel, 1990). Fourthly, humoral activity of *A. caspius* against the immune-sensitive bacteria, *M. luteus* was first investigated as a positive control experiment for the rest of study. And finally, the reproductive cost concomitant to LPS-induced humoral activity has not been investigated here since it has previously been studied (Ahmed, 2005).

Injecting *A. caspius* with *B. thuringiensis*, *B. cereus* or LPS resulted in a strong induction of humoral activity

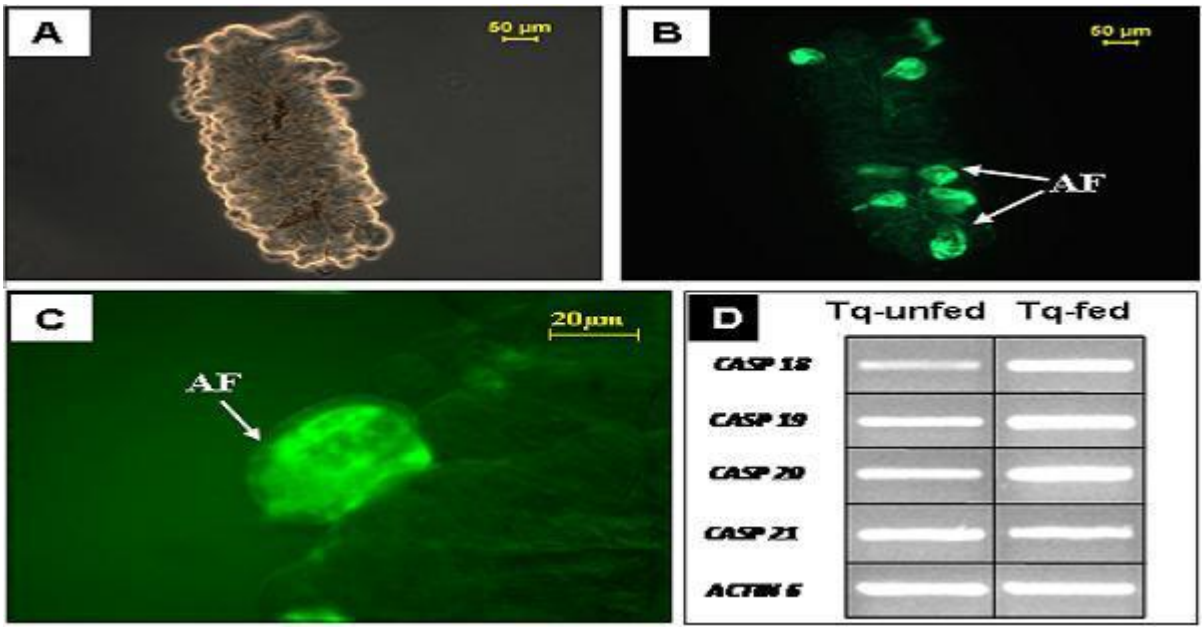

Figure 6. Molecular immune-enhancive effects of thymoquinone (Tq) on follicular development and apoptosis-related gene expression in *A. caspius*. (A) and (B) Images of a single ovary monitoring follicular apoptosis using light and fluorescence microscopy, respectively. (C) Close-up image at a higher magnification for an apoptotic follicle (AF) taken *via* fluorescence microscopy (green), using a band pass filter for excitation and emission wavelengths of 490 and 520 nm, respectively. (D) Semi-quantitative RT-PCR analysis of selected caspases gene expression 18 h post-bacterial-injection in Tq-fed compared to Tq-unfed mosquitoes as control. The housekeeping gene *ACTIN* 6 was used as reference for comparison. Specific amplification of cDNA fragments was detected after gel electrophoresis. Experiment was performed twice with similar results.

Table 2. Wing length measurements of mosquitoes used in the study of humoral anti-microbial activity.

Parameters	Experimental groups			
	Group A	Group B	Group C	Group D
Wing length	2.80 ±0.030	2.75 ±0.023	2.77 ±0.011	2.88 ±0.031
No. of mosquitoes (n)	20	20	20	20
P value	> 0.05 (Mann-Whitney *U* test)			

Groups A, B, C and D were used for injection with LPS, Bt, Bc and controls, respectively. Wing lengths were first measured and statistically compared to ensure similar body sizes and hence, blood meal sizes before any further treatments. Wing measurements were tested for normality using Anderson-Darling test prior to Mann-Whitney *U*test.

against *M. luteus* and Bc but not against Bt (Figures 2A - C). The induced activity not effective against Bt could be attributed to two reasons; First, Bt could have inhibited this immune activity. This is supported by the finding of Dongjin and Kim (2004) who reported depression of both haemocyte nodule formation and humoral activity by the entomopathogenic bacterium, *Xenorhabdus nematophila* in *Spodoptera exigua*. The second reason is that the innate immune system recognizes conserved molecular patterns that are present on the pathogen's surface, such as LPS, peptidoglycans and β-(1,3)-glucans (Medzhitov and Janeway, 2002). Upon recognition, the recognition receptors stimulate immune responses by activating humoral activities, proteolytic cascades in the haemolymph and intracellular signaling pathways in immune-responsive tissues (Kurata, 2004). Based on these mechanisms, we would suggest that either Bt has not been recognized or after being recognized, the pattern recognition receptors failed somehow to stimulate the relevant immune response in the form of humoral activity. Hence and taking into consideration the findings of Kurata (2004) who suggested that diverse peptidoglycan recognition protein members are involved in distinguishing between invading bacteria and activation of appropriate immune reactions, it could be suggested that Bc has been recognized but not Bt. This may explain the high toxicity of Bt against mosquitoes and being an effective biocontrol agent in the field of mosquito control (Knowles, 1994).

This is confirmed by the observed massive histological damage in the gut epithelium of *A. caspius* larvae (Figure 3), which may also explain the failure of *A. caspius* to develop effective gut-immunity against Bt (Lehane, 1991; Clark et al., 2005).

It has previously been reported that humoral activity is heightened in response to LPS injection which was found to be associated with a reproductive cost (Ahmed, 2005). Findings of the current study are in agreement with this observation when *A. caspius* was injected with *B. cereus* (Figure 4). In fact, mounting immune responses has proved to impose reproductive costs (Moret and Schmid-Hempel, 2000; Ahmed et al., 2002; Schwartz and Koella, 2004; Ahmed et al., 2008). Consequently, this is considered as a major limiting factor to the immuno-control strategy against mosquito-borne diseases. However, a non-costly immune induction has previously been shown to be feasible in *A. gambiae* mosquitoes, when they were allowed to feed on a mixture of 10% glucose and 0.3% crude black seed oil (Ahmed, 2006). Based on this finding, it was the primary objective of this study to test the immunization potential and the concomitant fitness cost of Tq, the principal active ingredient of the black seed oil, in *A. caspius*. Strikingly, immunization of *A. caspius* mosquitoes by Tq was found to entail a reproductive cost (Figures 5 and 6A - C). This cost was shown to take place via triggering follicular apoptosis, which lead to follicular resorption in the ovaries of Tq-immunized *A. caspius* (Figures 5 and 6A - C). These findings conform to previous studies indicating that follicular apoptosis occurs in the malaria vector *Anopheles gambiae* immunized by LPS or in response to malaria infection (Ahmed and Hurd, 2006) and hence, resulted in a reproductive cost in terms of reduced fecundity (Ahmed et al., 2002).

Apoptosis has been established as a component of the innate immune response in baculovirus infections of lepidopteran insects. In addition, cross-talk exists between innate immunity pathways and apoptosis pathways in insects (Clem, 2005). For example, in mosquitoes, apoptosis occurs during *Plasmodium* and arbovirus infection in the midgut, suggesting that apoptosis plays a role in mosquito innate immunity (Clem, 2005). The process of apoptosis is largely carried out by a family of cysteine proteases called caspases. These enzymes are expressed as zymogens and are activated by multiple stimuli. Therefore, we examined the impact of Tq-immunized mosquitoes on the transcription level of selected caspases genes (Figure 6D). Our results clearly indicated that *CASP 18*, *19* and *20*, but not *CASP 21*, were highly expressed in Tq-immune-enhanced *A. caspius* mosquitoes as compared to non-immune enhanced control (Figure 6D).

In this context, the expression level of potential apoptosis regulators, at various developmental stages (that is, early and late larvae, pupae, adults, females, midgut and adult body minus the midgut), throughout the life cycle of *A. aegypti*, has previously been examined, via quantitative reverse transcriptase PCR (qRT-PCR) (Briant et al., 2008). Interestingly, caspases were expressed at the highest levels in the midgut of *A. aegypti* as compared to the other tissues or stages (Briant et al., 2008). Moreover, expression level of selected caspases genes, examined via qRT-PCR, has been reported to be heightened in response to the treatment of *A. caspius* larvae with Bt-derived toxins, 24 h post-treatment (Aboul-Soud et al., unpublished data). Taken together, it was suggested that Tq-immunization in *A. caspius* against *B. cereus* results in the elevation of a subset of caspases genes expression (Namely: CASP 18, 19 and 20), mimicking the induction of apoptosis-related genes during pathogen invasion, adding up additional fitness costs on the immunized mosquitoes. However, a direct link between Tq-mediated immune enhancement in mosquitoes and caspase gene expression remains to be investigated. Numerous recent reports clearly indicate that Tq exerts several medicinal effects including: anticancer and anti-inflammatory properties. Its anticarcinogenic activity has recently been shown to be exerted via stimulating suicidal death of tumor cells (Qadri et al., 2009). However, the molecular pathways of Tq action are not clear. Nevertheless, Tq is known to induce apoptosis by p53-dependent and p53-independent pathways in cancer cell lines. Moreover, Tq has been shown to act on the immune system by modulating the levels of inflammatory mediators (Gali-Muhtasib et al., 2006). A recent study evaluated the antiproliferative and pro-apoptotic effects of Tq in two human osteosarcoma cell lines with different p53 mutation status (Roepke et al., 2007). Tq was confirmed to result in a significantly greater extent of apoptosis in a p53-independent fashion through the activation of caspase cascade enzymes including caspases-9 and -3 (Roepke et al., 2007). Therefore, the link between Tq and apoptosis signal transduction pathway has previously been established in cancer cell lines. However, to the best of our knowledge, the detailed Tq-mediated apoptosis in mosquitoes is seriously lacking.

In the course of the experiment, feeding mosquitoes on 18 mM Tq [0.3% (w/v), 10% glucose] resulted in no apparent mortality toxicity signs, up to 10 day-post-treatment, as compared to the sugar-fed control (personal observation). Hence, the observed effect of Tq on the induction of apoptosis-related genes and exerting a fitness cost entailed on ovary development in terms of follicular apoptosis and resorption may not simply be directly related to mild toxicity of the Tq concentration implemented in this study. Moreover, this same concentration has previously been used with no apparent toxicity observed (Ahmed, 2008). Recent reports, however, have indicated that Tq exhibits *in vitro* cyto- and genotoxic effects in a concentration-dependent manner: it induced significant anti-proliferative effects at 20 µM and acute cytotoxicity at higher concentrations. Thymoquinone significantly increased the rates of necrotic cells at concentrations between 2.5

and 20 µM. Furthermore, it induced significant genotoxicity at concentrations ≥ 1.25 µM (Khader et al., 2009). Hence, it appears that the concentration employed in this current study of 18 mM is much higher than the cytotoxic Tq concentrations reported in Khader et al. (2009). However, since the solubility of pure thymoquinone is relatively low in water according to the supplier's description, substantially lower concentration is expected. Nonetheless, this observation does not rule out that the observed Tq-mediated induction of caspase genes expression in *A. caspius* may be due to mild toxic effects in mosquitoes. Taken together, these results indicate that Tq is not suitable as a candidate for immune-control strategies due to the concomitant fitness cost observed in ovaries development, namely: follicular apoptosis and resorption and the probable toxic effect that might lead to the induction of apoptosis-related gene expression.

Thus, stimulation of mosquito immunity resulted in the induction of apoptotic pathways in the developing ovarian follicles leading to follicular resorption is likely to be the major mechanism behind fecundity reduction that might be due to the allocation of some resources (in the fat body) towards production of immune peptides into the haemolymph against injected bacteria. We would suggest that this is more likely to occur on the cost of vitellogenin production and hence, egg formation (Ahmed et al., 2002). This kind of resources re-allocation is more likely to be the direct result of follicular apoptosis and hence, resorption. In support of this suggestion, the crud black seed oil has been shown to induce a non-costly immunity since follicular apoptosis has not been significantly triggered (Ahmed, 2006). This may occur via three possible mechanisms; the first is a tight control of caspases gene transcription by black seed oil and thus, proper follicular development was maintained. Evidence for this is that, the black seed oil have been shown to prevent the progressive apoptosis in Hep-2, laryngeal carcinoma cells, which were pre-treated with apoptosis-inducing factors (Corder et al., 2003). The second, chromosomal fragmentation has been reported as a sign of follicular apoptosis in mosquitoes (Hopwood et al., 2001), it would be assumed that black seed oil may have prevented chromosomal fragmentation in mosquitoes, thus, maintaining normal follicular development. This is shown by the fact that black seed oil has been proved to be a protective agent against the chromosomal fragmentation induced in mouse cells (Aboul-Ela, 2002). The third, based on previous findings (Ahmed, 2006), suggested that black seed oil may have significantly increased immune responses in blood fed mosquitoes via providing adequate nutrition for immunity as well as for compensating the concomitant shrinkage of nutrition allocated for vitellogenesis from the blood meal. And hence, both immunity and vitellogenesis (in terms of normal follicular apoptosis and resorption processes) occurred efficiently in parallel. This has been supported by reports indicating that adequate nutritional resources resulted in complete melanization of the sephadex beads (C-25) by *A. stephensi*, compared to inadequately-fed ones (Koella and Sørensen, 2002). Tq might have induced a costly immune activity, more likely as the immune elicitor LPS (Ahmed and Hurd, 2006).

In conclusion, black seed oil induced a non-costly immunity (Ahmed et al., 2008) but not its active ingredient, the thymoquinone, alone as shown in the current study. Hence, it is envisaged that further detailed investigations, focus on the potential of other constituents of black seed oil, namely: p-cymene, carvarol, 4-terpineol, t-anethole and the sesquiterpene longifolene, individually and in combination, in inducing a non-costly immunity and the possibility of its utilization in support to the immuno-control strategies, are still needed to be carried out.

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Abbreviations

LPS, Lipopolysaccharide; **RT-PCR,** reverse transcriptase polymerase chain reaction; **Tq,** thymoquinone; **NI,** non-injected; **SI,** sham-injected; **APS,** *Aedes* physiological saline; **qRT-PCR,** quantitative reverse transcriptase polymerase chain reaction; **Bt,** *Bacillus thuringiensis*; **Bc,** *Bacillus cereus*.

REFERENCES

Aboul-Ela IE (2002). Cytogenetic studies on Nigella sativa seeds extract and thymoquinone on mouse cells infected with *Schistosomiasis* using karyotyping. Mutat. Res. 516: 11-17.

Ahmed AM, Taylor P, Maingon R, Hurd H (1999). The effect of *Plasmodium yoelii nigeriensis* on the reproductive fitness of *Anopheles gambiae*. Invertebr. Reprod. Dev. 36(1-3): 217-222.

Ahmed AM, Baggott S, Maingon RL, Hurd H (2002). The costs of mounting an immune response are reflected in the reproductive fitness of the mosquito *Anopheles gambiae. Oikos*, 97: 371-377.

Ahmed AM (2005). The humoral anti-bacterial response of *Anopheles gambiae* and the immunity-reproduction trade-off conflict: between the hope and limitation of the malaria immuno-control strategy. Proceedings of the 3rd International Conference of Applied Entomology, Cairo University, 23rd-24th of March (2005), pp. 351-374.

Ahmed AM (2006). A Dual Effect for the Black Seed Oil on the Malaria Vector A*nopheles gambiae*: Enhances Immunity and Reduces the Concomitant Reproductive Cost. J. Entomol. 4(1): 1-19.

Ahmed AM, Hurd H (2006). Immune stimulation and malaria infection impose reproductive costs in *Anopheles gambiae* via follicular apoptosis. Microb. Infect. 8: 308-315.

Ahmed AM, Al-Olayan EM, Amoudy MA (2008). Enhancing the humoral and melanization responses of *Aedes aegypti* mosquito: a step towards the utilization of immune system against dengue fever. J. Entomol. 5: 305-321.

Al-Hazmi A, Al-Rajhi AA, Abboud EB, Ayoola EA, Al-Hazmi M, Saadi R, Ahmed N (2005). Complications of Rift Valley Fever Outbreak in Saudi Arabia. Ophthalmol. 112: 313-318.

Bauer LS, Pankratz HS (1992). Ultrastructural effects of *Bacillus thuringiensis* var. san diego on midgut cells of the cottonwood leaf beetle. J. Invertebr. Pathol. 60: 15-25.

Briegel H (1990): Fecundity, metabolism, and body size in *Anopheles* (Diptera: Culicidae), vectors of malaria, J. Med. Entomol. 27: 839-850.

Briant B, Blair CD, Olson KE, Clem RJ (2008). Annotation and expression profiling of apoptosis-related genes in the yellow fever mosquito, Aedes aegypti. Insect Biochem. Mol. Biol. 38: 331-345.

Burits M, Bucar F (2000). Antioxidant activity of Nigella sativa essential oil. Phytother. Res. 14: 323-328.

Clark TM, Hutchinson MJ, Huegel KL, Moffett SB, Moffett DF (2005). Additional morphological and physiological heterogeneity within the midgut of larval *Aedes aegypti* (Diptera: Culicidae) revealed by histology, electrophysiology, and effects of *Bacillus thuringiensis* endotoxin. Tissue Cell, 37: 457-468.

Clem RJ (2005). The role of apoptosis in defense against baculovirus infection in insects. Curr. Trop. Microbiol. Immunol. 289: 113-130.

Clements AN, Boocock MR (1984). Ovarian development in mosquitoes. Stages of growth and arrest, and follicular resorption. Physiol. Entomol. 9: 1-8.

Collins FH, Sakai RK, Vernick KD, Paskewitz S, Seeley DC, Miller LH, Collins WE, Campbell CC, Gwadz RW (1986). Genetic selection of a *Plasmodium*-refractory strain of the malaria vector *Anopheles gambiae.* Science, 234: 607-610.

Corder C, Benghuzzi H, Tucci M, Cason Z (2003). Delayed apoptosis upon the treatment of Hep-2 cells with black seed. Biomed. Sci. Instrum. 39: 365-70.

Christophers SR (1960). The Yellow Fever Mosquito: Its life history, bionomics and structure. Cambridge University Press, Cambridge.

De Maagd RA, Bravo A, Crickmore N (2001). How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. Trends Genet. 17: 193-199.

Dimopoulos G, Richman A, Muller HM, Kafatos FC (1997). Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. Proc. Nat. Acad. Sci. USA. 94: 11508-11513.

Dongjin JI, Kim Y (2004). An entomopathogenic bacterium, *Xenorhabdus nematophila*, inhibits the expression of an antibacterial peptide, cecropin, of the beet armyworm, *Spodoptera exigua*. J. Insect Physiol. 50: 489-496.

Ferdig MT, Beemtsen BT, Spray FJ, Jianyong L, Christensen BM (1993). Reproductive costs associated with resistance in a mosquito-filarial worm system. Am. J. Trop. Med. Hyg. 49: 756-762.

Gali-Muhtasib H, Roessner A, Schneider-Stock R (2006). Thymoquinone: A promising anti-cancer drug from natural sources. The Int. J. Biochem, Cell Biol. 38: 1249-1253.

Gill SS, Cowles EA, Pietrantonio PV (1992). The mode of action of *Bacillus thuringiensis* δ-endotoxins. Annu. Rev. Entomol. 37: 615-636.

Hajhashemi V, Ghannadi A, Jafarabadi H (2004). Black cumin seed essential oil, as a potent analgesic and antiinflammatory drug. Phytother. Res. 18: 195-199.

Hall TA (1999). Bio Edit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids. Symp. Ser. 41: 95-98.

Hillyer JF, Schmidt SL, Christensen BM (2004). The antibacterial innate immune response by the mosquito *Aedes aegypti* is mediated by hemocytes and independent of Gram type and pathogenicity. Microb. Infect. 6: 448-459.

Hoffmann JA, Reichhart JM, Hetru C (1996). Innate immunity in higher insects. Curr. Opin. Immunol. 8: 8-13.

Hopwood JA, Ahmed AM, Polwart A, Williams GT, Hurd H (2001). Malaria-induced apoptosis in mosquito ovaries: a mechanism to control vector egg production. J. Exp. Biol. 204: 2773-2780.

Khader M, Bresgen N, Eckl PM (2009). *In vitro* toxicological properties of thymoquinone. Food Chem. Toxicol. 47: 129-133.

Knight KL, Stone A (1977). A catalogue of the mosquitoes of the world. Geo. W. King Co., Baltimore, MD.

Knowles BH (1994). Mechanism of action of *Bacillus thuringiensis* insecticidal delta-endotoxins. (ed. Evans PD), Academic Press, London. Adv. Insect Physiol. 24: 275-308.

Koella JC, Sørensen FL (2002). Effect of adult nutrition on the melanization immune response of the malaria vector *Anopheles stephensi*. Med. Vet. Entomol. 16: 316-320.

Kokoza V, Ahmed A, Cho WL, Jasinskiene N, James AA, Raikhel A (2000). Engineering blood meal-activated systemic immunity in the yellow fever mosquito, *Aedes aegypti*. Proc. Nat. Acad. Sci. USA, 97: 9144-9149.

Kurata S (2004). Recognition of infectious non-self and activation of immune responses by peptidoglycan recognition protein (PGRP)-family members in *Drosophila*. Dev. Comp. Immunol. 28: 89-95.

Lehane MJ (1991). Biology of blood sucking insects. Harper Collins Academic, University Press, Cambridge.

Mattingly PF, Knight KL (1956). The mosquito of Arabia. I. Bull Br. Mus. Nat. Hist. (Ent.) 4(3): 89-141.

Medzhitov R, Janeway Jr. CA (2002). Decoding the patterns of self and non-self by the innate immune system. Science, 296: 298-300.

Moret Y, Schmid-Hempel P (2000). Survival for immunity: the price of immune system activation for bumblebee workers. Science, 290: 1166-1168.

Nimmo DD, Ham PJ, Ward RD, Maingon R (1997). The sand fly *Lutzomyia longipalpis* shows specific humoral responses to bacterial challenge. Med. Vet. Entomol. 11: 324-328.

Qadri SM, Mahmud H, Föller M, Lang F (2009). Thymoquinone-induced suicidal erythrocyte death. Food Chem. Toxicol. 47: 1545-1549.

Roepke M, Diestel A, Bajbouj K, Walluscheck D, Schonfeld P, Roessner A, Schneider-Stock R, Gali-Muhtasib H (2007). Lack of p53 augments thymoquinone-induced apoptosis and caspase activation in human osteosarcoma Cells. Cancer Biol. Ther. 6: 151-160.

Rosenberg R (1985). Inability of *Plasmodium knowlesi* sporozites to invade *Anopheles freeborni* salivary glands. Am. J. Trop. Med. Hyg. 34: 687-691.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sanger F, Nicklem S, Coulson AR (1977). DNA sequencing using chain termination inhibitors. Proc. Nat. Acad. Sci. USA, 74: 5463-5467.

Schwartz A, Koella JC (2004). The cost of immunity in the yellow fever mosquito, *Aedes aegypti* depends on immune activation, J. Evol. Biol. 17: 834-840.

Somboon P, Prapanthadara L, Suwonkerd W (1999). Selection of *Anopheles dirus* for refractoriness and susceptibility to *Plasmodium yoelii nigeriensis.* Med. Vet. Entomol. 13: 355-361.

Villalon JM, Ghosh A, Jacobs-Lorena M (2003). The peritrophic matrix limits the rate of digestion in adult *Anopheles stephensi* and *Aedes aegypti* mosquitoes. J. Insect. Physiol. 49: 891-895.

Voordouw MJ, Anholt BR, Taylor PJ, Hurd H (2009). Rodent malaria-resistant strains of the mosquito, *Anopheles gambiae*, have slower population growth than susceptible strains. BMC Evol. Biol. 9: 76.

Yan G, Severson DW, Christensen BM (1997). Costs and benefits of mosquito refractoriness to malaria parasites: implications for genetic variability of mosquitoes and genetic control of malaria. Evolution, 51: 441-450.

Yoshida S, Ioka D, Matsuoka H, Endo H, Ishii A (2001). Bacteria expressing single-chain immunotoxin inhibit malaria parasite development in mosquitoes. Mol. Biochem. Parasitol. 113: 89-96.