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ORIGINAL ARTICLE

Mosquito autogeny in *Aedes caspius* (Diptera: Culicidae): alterations of larval nourishments reservation upon bacterial infection

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Abstract The present study recorded mosquito autogeny for the first time amongst *Aedes caspius* species in the Eastern region of Saudi Arabia. Laboratory rearing showed an obligatory autogenous species of *Ae. caspius* since it foregoes blood feeding during its first ovarian cycle, even in the presence of the hosts (CD mouse), but produces its second egg batch only if ingested a blood meal. Both morphological and molecular identification confirmed that both autogenous and anautogenous strains belong to the same species of *Ae. caspius*. Data from biochemical analysis showed significant 2, 1.6, and 1.4 folds higher total carbohydrates, proteins, and lipids reserves respectively in the fourth larval instar of the autogenous strain compared to that of the anautogenous ones. In addition, exposing the fourth larval instars of autogenous strain to the infection stress by the mosquito larvicidal bacterium, *Bacillus thuringiensis* var *kurstaki* has significantly reduced total carbohydrates, proteins and lipids reserves by 29%, 35%, and 46%, respectively, at 12 h postinfection compared to those of uninfected ones. These reductions in nourishment reserves were more pronounced at 24 h postinfection in the case of proteins and lipids, but not carbohydrates. These results may indicate that bacterial infection is a health stress that significantly reduced nourishments reservation, which may interrupt the success of adult autogeny. However, the impact of infection-induced decline in larval nourishments reservation on successful adult autogeny is still to be investigated.

Key words *Aedes caspius*, anautogeny, autogeny, *Bacillus thuringiensis*, nourishments reservation

Introduction

The evolution of hematophagy in anautogenous mosquitoes has led to an essential dependence on vertebrate blood for reproduction (Briegel, 1985), and thus, mosquito-borne diseases are having a very significant impact upon human health worldwide. This refers to the fact that most mosquito species are obligatory blood feeders, termed anautogenous, and acquire a blood meal as the main protein source for egg production. Thus, many of anautogenous mosquitoes are medically important because they can feed on human blood, and therefore, have the potential to transmit human disease pathogens (Clements, 1992). Anautogenous mosquitoes can also accumulate energy reserves (lipid and glycogen) for survival and flight from a blood meal (Clements, 1992; Zhou *et al.*, 2004). Moreover, amino acids from the blood meal digestion are not only utilized for yolk protein synthesis but also have the potential to initiate and promote mosquito oogenesis (Uchida *et al.*, 2001). Sugar is an important source for building energy reserves for survival and flight in female mosquitoes (Naksathit *et al.*, 1999), and hence, can

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spare the blood meal proteins for egg production (Gary & Foster, 2001; Briegel *et al.*, 2001). The carbohydrate and lipid reserves of insects can be derived from ingested carbohydrate, and also from ingested amino acids, which constitute an important source when supplies of dietary carbohydrate are limited (Clements, 1992).

A smaller percentage of mosquito species are capable of maturing at least their first batch of eggs without a blood meal. Such females are called autogenous, a term defined by Roubaud (1929) as ''the production of eggs without ingestion of blood by the adult mosquito'' (reviewed by Attardo *et al.*, 2005). These mosquito strains utilize nourishments that are accumulated during the larval stage and carried over into the adult stage (Clements, 1994). Mosquito autogeny can be either obligatory or facultative depending upon mosquito species and the environmental conditions (reviewed by Attardo *et al.*, 2005). In fact, there are three types of autogenous mosquitoes in the extent of their reliance on larval- and adult-derived nutrients for egg production. The first type is facultative autogenous species that utilize larval stored nutrient for oogenesis if a blood meal is not available within a particular period of time after adult emergence (O'Meara, 1985). The second type is obligatory autogenous species that forego blood feeding during their first ovarian cycle even in the presence of hosts, and produce their first egg batch *via* utilizing larval reserved nutrients (van Handel, 1985). The third type is the obligatory autogenous species that forego blood feeding completely and able to lay multiple egg clutches depending solely upon larval reserved nourishments (O'Meara, 1985). For example, *Ochlerotatus atropalpus* is obligatory autogenous for its first egg cycle but may ingest blood for subsequent cycles (Telang & Wells, 2004). Moreover, larval nourishments strongly influence egg production as female larvae that were nutritionally stressed emerged as smaller adults, produced fewer eggs and emerged with less protein, lipid and glycogen stores (Telang & Wells, 2004). It has been demonstrated that number of eggs produced is significantly reduced in autogenous mosquitoes compared with anautogenous ones (O'Meara & Edman, 1975). Moreover, mosquito autogeny, whether facultative or obligatory, has been found to be genetically determined. However, its expression can be influenced by certain environmental factors during larval stage such as larval nutrition and larval density (Corbet, 1964; Russell, 1979; Wheeler & Buck, 1996), and during the adult stage such as host availability, mating, and sugar feeding (Su & Mulla, 1997; Telang & Wells, 2004).

The current study was conducted to present a first record of autogeny within mosquitoes collected from the Eastern region of Saudi Arabia. In addition, quantification of the nourishments in larval body tissues, in terms of total carbohydrates, proteins, and lipids, in an autogenous compared to anautogenous *Ae. caspius* mosquito have been investigated. Furthermore, the effect of infection with the mosquito larvicidal bacterium, *Bacillus thuringiensis kurstaki* (*Btk*), as an external health stress, on these reserved nourishments in the larvae of autogenous mosquitoes have also been investigated.

Materials and methods

Field collection of mosquitoes

Larval collections were made from different breeding places in AL-Ahsaa district, the Eastern region of Saudi Arabia. Collection was carried out using handled larval net consists of an iron ring (20 cm in diameter) to which a muslin sleeve (30 cm long) was attached as detailed in (Ahmed *et al.*, 2011). Collected larvae from different locations were separately labeled and moved to the rearing insectary in Zoology Department, College of Science, King Saud University for experimental purposes as detailed below. Larvae from each location were separately reared up to adult stages in separate labeled rearing cages.

Mosquito identification

Morphological identification Field collected larvae were morphologically identified according to the classification keys of Mattingly & Knight (1956) for identifying *Ae. caspius*. Morphological identification of collected samples was confirmed by the Natural History Museum (London, UK). Larvae of *Ae. caspius* collected from different locations were separately reared until adult emergence under standard insectarium conditions (26 °C, 8/12 h light/dark period) in the insectary of Zoology Department, College of Science, King Saud University, as previously outlined in Ahmed *et al.* (1999).

Determining autogeny For determining autogeny, adults emerging within a 24-h period from each original larval group were maintained in their rearing cage (30 × 30 × 30 cm) and allowed for permanent access to 10% glucose *ad libitum*. Pupae were moved into a new cage for the next 24 h adult emergence, and so on. Twenty adult groups (≈300 each in 20 different rearing cages) were obtained, and each was kept accessing glucose only until 3 weeks postemergence. Any rearing cage that showed laid eggs (raised from larvae collected from sanitation water sites) was isolated to a separate rearing room for getting more eggs. Laid eggs (≈300 eggs in total) were moved into rearing trays and mass-reared as

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Table 1 The two used universal PCR primers flanking the internal transcribed spacer 2 (ITS-2) region of the rDNA gene.

Primer name	Sequence (5′→3′)
5.8S	5′- ATC ACT CGG CTC GTG GAT CG -3′
28S	5′- ATG CTT AAA TTT AGG GGG TAG TCA C - 3′

autogenous strain of *Ae. caspius* until 20 successive generations in the laboratory.

Autogeny was confirmed by successful rearing of the resulting autogenous strain for 20 successive generations without accessing blood meals. The other mosquito groups that showed no egg production were considered as the anautogenous strain. They have been given a blood meal 3 week postemergence for laying their first egg batch. Resulting eggs were used for establishing the anautogenous colony of *Ae. caspius*. Adults of both autogenous and anautogenous strains were then morphologically identified again according to Mattingly & Knight (1956).

Molecular identification

Genomic DNA extraction To confirm the morphological identification of autogenous and anautogenous strains of *Ae. caspius*, molecular sequencing of the internal transcribed spacer 2 (ITS-2) of the ribosomal gene (rDNA) of both were compared according to Ma *et al.* (2002) and Manonmani *et al.* (2007). Genomic DNA from a single adult of a confirmed autogenous or anautogenous mosquito was extracted by illustra tissue and cells genomic Prep Mini Spin Kit (GE Healthcare, Sweden) according to the manufacturer's instructions. The quality of the purified genomic DNA was determined by agarose gel electrophoresis in TAE buffer (Sanbrook *et al.*, 1989). Purified mosquito genomic DNA was used for molecular identification as detailed below.

Conventional PCR The rDNA-ITS2 region was targeted for designing PCR primers. Conventional PCR primers were designed against the internal transcribed spacer 2 (ITS-2) regions of the ribosomal DNA (rDNA) gene. Initially, primers were designed using the Web-based software Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Subsequently, two universal primers flanking the internal transcribed spacer 2 (ITS-2) region of the rDNA gene, namely, 5.8S and 28S were used (Table 1). Designed primers were examined for hairpin, self-dimer and heterodimer formation, before they were finally approved for synthesis, using OligoAnalyzer 3.1 of Integrated DNA Technologies (IDT) (http://eu.idtdna.com/analyzer/Applications/-OligoAnalyzer).

Synthesis of approved PCR primers was carried out by Microsynth AG (Schützenstrasse 15, 9436 Balgach, Switzerland). Optimization of PCR has been carried out by utilizing GoTaq® Flexi DNA Polymerase for PCR reactions (Promega, Madison WI). Each reaction mixture was optimized to comprise 5 pmol of each primer (Microsynth AG, Switzerland), 0.4 mmol/L dNTPs (Promega, Madison WI), 5.0 μL 5× GoTaq® Flexi buffer, 2.0 mmol/L $MgCl_2$ and 1.25 units of GoTaq® Polymerase (Promega, Madison WI). To this mixture, 20–50 ng of the genomic DNA extracted from single mosquito was added and the reaction mixture was made up to 25 μL volume using sterile H_2O. These reactions were amplified in a TGradient Thermocycler machine (Biometra, Germany). Reactions without DNA served as control. The size and purity of the PCR product was determined by electrophoresing a portion of each amplification product on 1.5% agarose gel in TAE buffer along with a LowRanger 100 bp DNA molecular weight marker (Norgen, Canada).

Gel purification of PCR products

PCR reactions were loaded on 1% agarose gels and electrophoresed on low voltage (50 v) in TAE buffer to allow for efficient fractionation of PCR amplicons according to previously described methodologies (Ahmed *et al.*, 2010; Alroba *et al.*, 2011). Resulting bands of PCR amplicons were excised from gels and subjected to extraction and purification by loading onto an illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Sweden) (Ahmed *et al.*, 2010).

Sequencing of PCR products

Sequencing of gel-purified PCR amplicons 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). Sequencing was conducted in the two directions using 5.8S and 28S primers (Table 1). The obtained sequences for autogenous and anautogenous mosquitoes were manually cleaned and pairwise alignment against *Aedes caspius* rDNA gene (NCBI Gene accession No. GU977216.1) and each other's was performed

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with BioEdit Sequence Alignment Editor (Hall, 1999), as well as publically available MultiAlign software (http://multalin.toulouse.inra.fr/multalin/multalin.html, January 2011) (Corpet, 1988)

Rearing experimental mosquitoes

Both autogenous and anautogenous *Ae. caspius* mosquito strains were reared up for the experimental purposes of the current study. Mosquitoes were reared under standard insectarium as detailed above. Anautogenous *Ae. caspius* were allowed to access both blood feeding (on anesthetized CD mouse for 30 min, 7 days postemergence) and 10% glucose solution. On the other hand, autogenous mosquitoes were forgoing blood feeding but were sugar feeding for producing their first egg batch. However, they were feeding on blood for producing their second egg batch. Blood feeding on CD mouse was performed as detailed in Ahmed *et al.* (1999). Forth instar larvae were used for experimental purposes of the current study. To maintain a stock of autogenous mosquito colony, they were kept accessing 10% glucose *ad libitum* only since blood meal is not urgently needed for egg production. Using the CD mice for mosquito feeding was ethically acceptable by the Ethics Committee at King Saud University and according to the national guidelines for animal usage in research.

Bacterial preparations and larval infection

The mosquito-larvicidal bacterium, *Bacillus thuringiensis* var *kurstaki* (*Btk*), (de Maagd *et al.*, 2001; Lee *et al.*, 2001) was used in the present study against the autogenous strain. The larvicidal form of bacteria (serotype H-3a & 3b, Biotech International Ltd, India) was obtained from the Saudi Ministry of Agriculture as a spore-crystal powder [formulation contains 5%–8% spores (w/w) and 5%–8% delta endotoxins (w/w) based on the company's instructions]. Autogenous mosquito larvae were treated with the LC_{50} of *Btk* bacteria (0.058 mg/L) according to Ahmed (2011). Total carbohydrates, proteins, and lipids have been quantified upon infection with *Btk* as detailed below.

Quantification of larval reserved nourishments

Reserved nourishments (total proteins, carbohydrates, and lipids) in body tissues of the fourth larval instar of uninfected autogenous and anautogenous *Ae. caspius* strain (for comparison), or in *Btk*-infected autogenous strain were quantified as detailed below:

Quantification of total carbohydrates In this experiment, quantifications of total carbohydrates in the fourth larval instars of autogenous and anautogenous strains were carried out according to Timmermann & Briegel (1999) and Schwartz & Koella (2004) for comparison. Briefly, 20 randomly selected autogenous or anautogenous fourth larval instars, of approximately similar body sizes, were completely homogenized in 500 μL Tris-buffer. Homogenates were then centrifuged at 3 000 r/min for 30 min at 4°C. Supernatant was collected and used for determining total carbohydrates content. An amounts of the supernatant (10 μL) was moved to a sterilized 10-mL glass vials. One mL of phenol was added and well vortexed. Then 2 mL H_2SO_4 was added and immediately vortexed then left for 20 min for cooling down. The absorbance was then read at 620 nm on an UV Visible Spectrometer (Ultrospec 2000, Pharmacia Biotech) following the manufacture's guidelines. This absorbance is compared to a standard calibration curve using glucose as a standard.

Quantification of total protein In this experiment, quantification of total protein content in fourth larval instars of autogenous and anautogenous strains was determined for comparison using the coomassie reagent (Coomassie Plus Protein, Pierce Biotechnology, Inc., Rockford, IL), based on the Bradford's assay (Bradford, 1976) and as modified by Timmermann & Briegel (1999) and Schwartz & Koella (2004). Briefly, homogenates from 20 autogenous or anautogenous larvae, of approximately equal sizes, were prepared as detailed above. Small amounts of the supernatant (10 μL) was moved to a sterilized 10 mL-glass vials, and 2 mL of Coomassie reagent was added and well-vortexed then left for 20 min. The absorbance was then read at 595 nm on an UV Visible Spectrometer (Ultrospec 2000, Pharmacia Biotech) following the manufacture's guidelines. This absorbance is compared to a standard calibration curve using bovine serum albumin as a standard.

Quantification of total lipid In this experiment, quantification of total lipids in autogenous and anautogenous larvae was determined for comparison using the vanillin assay according to Zöllner & Kirsh (1962) and Frings *et al.* (1972) since it is commonly used to estimate insect storage lipids (e.g. Schwartz & Koella, 2004; Geister *et al.*, 2008; Williams *et al.*, 2011). In this assay, lipids are hydrolysed by sulfuric acid producing fatty acids, whose hydroxyl groups react with the sulfuric acid to form the chromogen (an alkenyl cation). The alkenyl cation then

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reacts with the vanillin reagent (an aromatic hydrocarbon) to form a chromophore with maximum absorbance at 530 nm (Johnson *et al.*, 1977). Briefly, 20 randomly selected autogenous or anautogenous fourth larval instars, of approximately identical body sizes, were homogenized in 500 μL (chloroform: methanol 2 : 1 v/v). Homogenates were incubated at 100 °C until complete evaporation of solvents. Following evaporation, 30 μL sulfuric acid were added and samples were incubated at 100 °C for 10 min to produce the sulfonic acid derivatives, to which the vanillin reagent was added, immediately vortexed and the absorbance was read at 525 nm on an UV Visible Spectrometer (Ultrospec 2000, Pharmacia Biotech) following the manufacture's guidelines. This absorbance is compared to a standard calibration curve using a commercially available olive oil.

Effect of Btk *infection on nourishment reserves*

In this experiment, the impact of *Btk*-infection on larval nourishment reservation in autogenous mosquito larvae was investigated. Fourth larval instars of autogenous *Ae. caspius* were exposed to LC_{50} of *Btk* as detailed previously, or left without infection (control). Biochemical assays for quantification of total carbohydrates, proteins or lipids in *Btk*-infected or control larvae were carried out as detailed previously at 12 and 24 h postinfection.

Statistical analysis

In each of the biochemical assays experiments, data from different 5 replicates ($n = 5$) were used for statistical analysis using MINITAB software (MINITAB, State College, PA, v: 13.1, 2001) (Morrison, 2002). Briefly, data of each experiment were first tested for normality (using Anderson-Darling Normality test) prior to any further analysis. Data pertaining to all experiments were normally distributed and thus, a two-sample *t*-test (for individual comparison) was used for comparing differences in nutrient contents between autogenous and anautogenous mosquito larvae, or between *Btk*-infected and control autogenous mosquito larvae. The 5 replicates of each experiment were carried out using 5 different groups of insects.

Results

Morphological identification

Larvae of *Ae. caspius* mosquitoes were morphologically identified from the collected larvae from different breeding sites in the Eastern region of Saudi Arabia. This mosquito species was found to be the most abundant within the other collected species (Fig. 1). Most of the field-collected *Ae. caspius* from all breading sites were anautogenous. These anautogenous *Ae. caspius* mosquitoes were recorded in localities exhibited high salinity levels of around 12 800 ppm and pH levels of around 7.4 (Ahmed *et al.*, 2011). However, I noticed that this mosquito species is able to live in ddH_2O water in the lab and complete its lifecycle normally. On the other hand, the autogenous *Ae. caspius* mosquito strain was recorded within the larval samples that collected from the outmost borders of AL-Qurain village only (located North of Eastern region of Saudi Arabia). In this place, the sanitation fresh wastewater is disposed and constitutes the only mosquito breading site there. This wastewater was characterized by salinity of 2 400 ppm and pH of 8.1. Moreover, anautogenous form was found in the autogenous site but not vice versa. The selected autogenous *Ae. caspius* mosquitoes were able to lay eggs without blood for 20 successive generations before conducting the present study. Eggs production was, and still, sufficient for keeping the colony going on as well as providing enough mosquitoes for the experimental purposes of the present study.

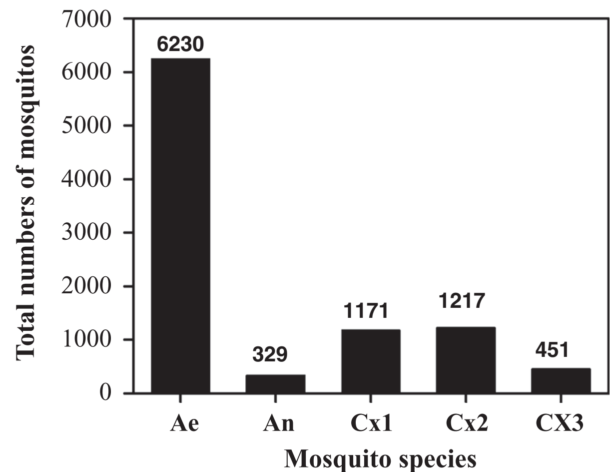

Fig. 1 A histogram showing number of collected larvae of different mosquito species from Eastern region of Saudi Arabia. Ae: *Aedes caspius*; An: *Anopheles multicolor*; Cx1: *Culex perexiguus*; Cx2: *Cx. pipiens*; Cx3: *Cx. pusillus*. Numbers on bars represent numbers of collected larvae (from Ahmed *et al.*, 2011).

Mosquito autogeny

The resulting autogenous *Ae. caspius* mosquitoes (≈300 mosquitoes) were successfully expanded *via*

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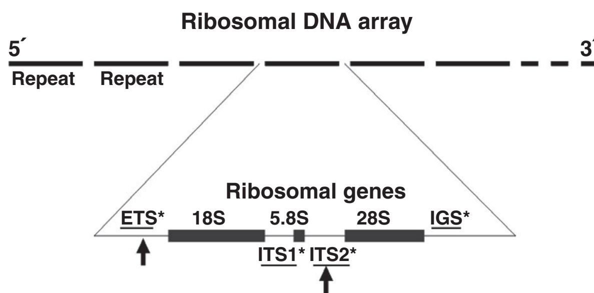

Fig. 2 Ribosomal DNA gene structure. *ETS: external transcriped spacer, ITS*: internal transcriped spacer and IGS*: intergenic spacer regions on the ribosomal DNA.

raising 20 successive generations until conducting the current study. They were kept accessing glucose solution only and producing eggs after around 7 days from emergence (for only one gonotrophic cycle in each generation). Nevertheless, they were requiring a blood meal in order to produce their second egg batch.

Molecular identification

The internal transcribed spacer 2 (ITS2) of the ribosomal rDNA gene are currently being used to detect single nucleotide polymorphisms (SNPs) for mosquito species identification (Fig. 2) (Ma *et al.*, 2002; Manonmani *et al.*, 2007) and the external transcribed spacer (ETS) regions of the intergenic spacer (IGS) (Favia *et al.*, 2001). This was done with some mosquito species from all breeding sites (Ahmed *et al.*, unpublished data). Successful PCR amplification was obtained with the primers originally designed and tested on *Aedes aegypti/albopictus* from Northern United States (Florida). These primers were originally designed from conserved and diverged regions identified in the ETS region of the IGS ribosomal DNA. As can be seen from Figure 3, differences at three nucleotide position only are observed. In fact, these differences are within the normally found variations which should not necessarily mean different species. Hence, the ITS2 region of both autogenous and anautogenous *Ae. caspius* mosquitoes are identical, which prove them as one species.

Larval total, proteins, carbohydrates, and lipids

Fourth instar larvae of the autogenous and anautogenous *Ae. caspius* mosquito strains were biochemically analyzed for their total carbohydrates, proteins, and lipids. Larvae of both strains were reared under optimal insectary conditions with food supply *ad libitum*. As shown in Figure 4, total carbohydrates, proteins and lipids reserves were significantly higher in larvae of autogenous mosquitoes compared to that of anautogenous ones (4.472 ± 0.0745 v 2.216 ± 0.0765, 40.48 ± 1.38 v 25.423 ± 0.744, and 44.12 ± 1.01 v 31.52 ± 1.15 μg/mg tissue respectively) ($P < 0.05$, $N = 5$, two-sample t-test). These data show clearly that fourth larval instars of autogenous *Ae. caspius* reserve significant more nourishments in their body tissues compared to that of anautogenous ones.

Effect of Btk*-infection on larval reserved nourishments*

Effect of *Btk* infection on total carbohydrates Fourth larval instars of the autogenous *Ae. caspius* mosquito were exposed to 0.058 mg/L (LC_{50}) of *Btk*, for 24 h or left without infection (control). Data from total carbohydrates quantification assay showed significant lower contents in the body tissues of *Btk*-infected larvae compared to control ones at 12 h postinfection (2.9443 ± 0.0882 v 4.1127 ± 0.0984 μg/mg tissue, respectively) ($P < 0.05$, $N = 5$, student t-test) (Fig. 5). This effect of *Btk*-infection has extended up to 24 h postinfection (2.9982 ± 0.0695 v 4.1127 ± 0.0984 μg/mg tissue respectively) ($P < 0.05$, $N = 5$, student t-test) (Fig. 5). However, there was no significant difference in total carbohydrate contents in the tissues of *Btk*-infected larvae at 12 and 24 h postinfection ($P > 0.05$, $N = 5$, student t-test) (Fig. 5). This may indicate that *Btk* infection has significantly decreased the amount of reserved carbohydrates to a certain level after which no further reduction occurred in *Btk*-infected larvae.

Effect of *Btk* infection on total proteins Fourth larval instars of the autogenous *Ae. caspius* mosquito were exposed to LC_{50} (0.058 mg/L) of *Btk* for 24 h or left without infection (control). Data from total protein quantification assay showed significant lower contents in the body tissues of *Btk*-infected larvae at 12h postinfection compared to that of control ones (34.406 ± 0.456 v 52.73 ± 2.4 μg/mg tissue, respectively) ($P < 0.05$, $N = 5$, student t-test) (Fig. 6). Moreover, *Btk*-infected larvae showed significant further reduction in their tissues protein contents at 24 h postinfection compared to that of control ones (26.643 ± 0.761 v 52.73 ± 2.4 μg/mg tissue respectively) ($P < 0.05$, $N = 5$, student t-test) (Fig. 6). This reduction is more pronounced at 24 h compared to that at 12 h postinfection. These data may indicate that *Btk*-infection has significantly decreased tissues protein contents in infected larvae.

Effect of *Btk* infection on total lipids Fourth larval instars of the autogenous *Ae. caspius* mosquito were exposed to LC_{50} (0.058 mg/L) of *Btk* for 24 h or left without infection (control). Data from total lipid quantification

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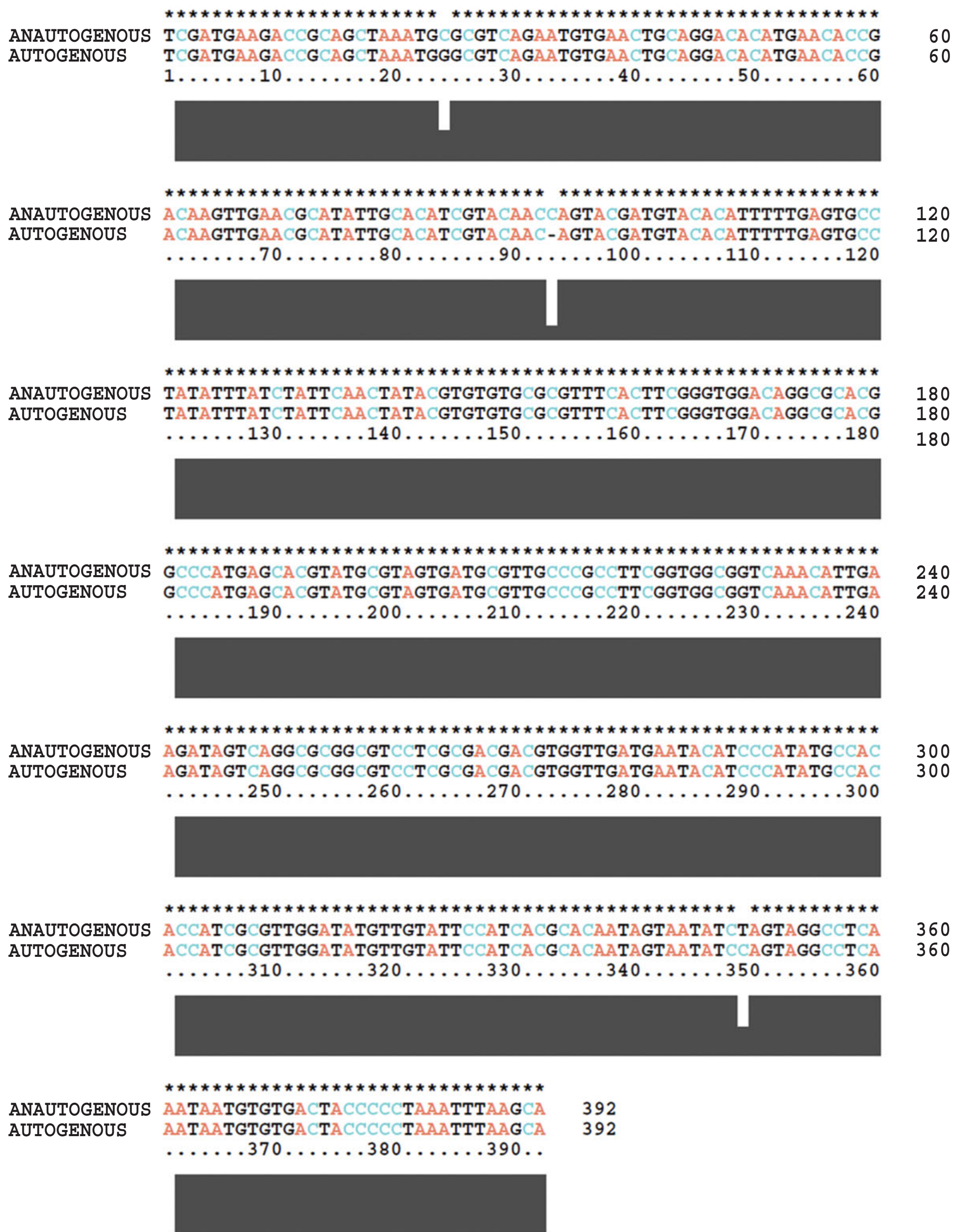

Fig. 3 Nucleotide sequence of both anautogenous and autogenous *Aedes caspius* species of mosquito from PCR-amplified 5.8S and 28S gene. The sequence alignment was generated by the ClustalW multiple alignment of BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/BioEdit/ bioedit.html). Both sequences differ only at three nucleotide position.

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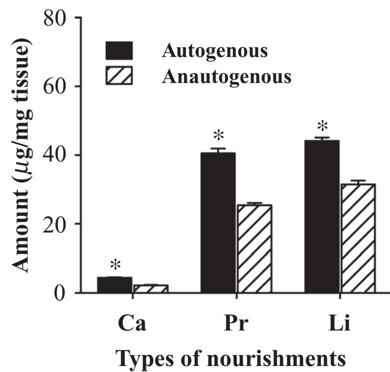

Fig. 4 Total carbohydrates (Ca), proteins (Pr) and lipids (Li) in the fourth larval instar of autogenous and anautogenous *Aedes caspius* mosquitoes (μg/mg tissue). Error bars represent standard error of means of 5 replicates ($n = 5$). Asterisks (*) represent significant higher contents in autogenous mosquito larvae comparing to that in anautogenous ones ($P < 0.05$, two-sample *t*-test).

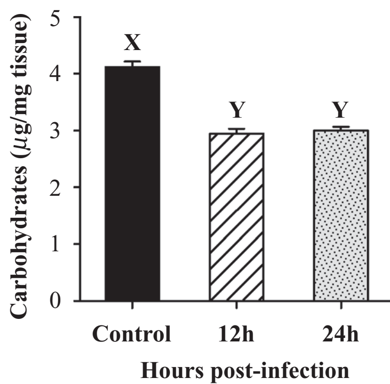

Fig. 5 Effect of *Btk* infection on total carbohydrates content (μg/mg tissue) in body tissues of the fourth larval instars of the autogenous *Aedes caspius* mosquito. Larvae were treated with LC_{50} of *Btk* spore-crystal powder or left without infection (control). Total carbohydrates titer was measured at 12 and 24 h postinfection. Error bars represent standard error of means of 5 replicates ($n = 5$). Different letters above bars represent significant differences ($P < 0.05$, two-sample *t*-test), and similar letters represent no differences ($P > 0.05$, two-sample *t*-test).

assay showed significantly lower contents in the body tissues of *Btk*-infected larvae at 12 h postinfection compared to that of control ones (24.432 ± 0.627 *v* 44.78 ± 1.1 μg/mg tissue respectively) ($P < 0.05$, $n = 5$, student *t*-test) (Fig. 7). Furthermore, *Btk*-infected larvae showed significant further reduction in their tissues lipid contents at 24 h postinfection compared to that of control ones (19.773 ± 0.833 *v* 44.78 ± 1.1 μg/mg tissue respectively) ($P < 0.05$, $n = 5$, student *t*-test) (Fig. 7). It is clear that this reduction is more pronounced at 24 h compared to that at 12 h postinfection. This may indicate that *Btk* infection has significantly decreased tissues lipid contents in infected larvae.

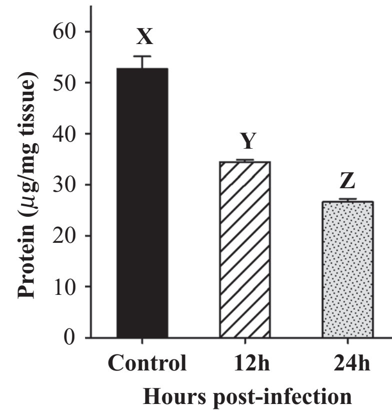

Fig. 6 Effect of *Btk* infection on total proteins content (μg/mg tissue) in body tissues of the fourth larval instars of the autogenous *Aedes caspius* mosquito. Larvae were treated with LC_{50} of *Btk* spore-crystal powder or left without infection (control). Total protein was quantified at 12 and 24 h post-infection. Error bars represent standard error of means of 5 replicates ($n = 5$). Different letters above bars represent significant differences ($P < 0.05$, two-sample *t*-test).

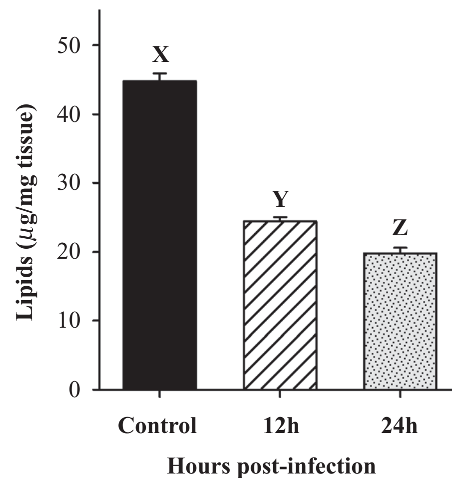

Fig. 7 Effect of *Btk* infection on total tissues lipids content (μg/mg tissue) in the fourth larval instars of the autogenous *Aedes caspius* mosquito. Larvae were treated with LC_{50} of *Btk* spore-crystal powder or left without infection (control). Total protein was quantified at 12 and 24 h post-infection. Error bars represent standard error of means of 5 replicates ($n = 5$). Different letters above bars represent significant differences ($P < 0.05$, two-sample *t*-test).

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Discussion

This study presents an autogenous species of *Ae. caspius* collected from Al-Ahsaa, the Eastern region of Saudi Arabia, as a first record. This region of the country has many mosquito genera since it has suitable mosquito breading sites (Ahmed *et al.*, 2011). Basically, females of most mosquito species are anautogenous; demand vertebrate host blood for eggs provisioning, and hence, can be medical problems because of this dependency. However, females of some mosquito species are autogenous; do not need blood for egg provisioning but, instead, acquire pre-existing nourishment reserves from larval stage. Many anautogenous mosquito species have been identified from the Eastern region of Saudi Arabia (Ahmed *et al.*, 2011). The most abundant mosquito species found in this region was *Ae. caspius* that was collected from salty water in the borders of this region but could not be found in the farms freshwater (Ahmed *et al.*, 2011). However, it was able to live normally in fresh water in laboratory (personal observation) which may indicate that it has the ability to live in both fresh and salty water. In this context, mariner-like elements are widespread in nature in a wide variety of insects (Robertson & MacLeod, 1993) including mosquitoes (Zakharkin *et al.*, 2004). Robertson and MacLeod (1993) mentioned that all mariner-like elements found in natural populations are non-functional pseudogenes, containing stop signals, deletions, frameshifts, or missense mutations that either disrupt the open reading frame or produce an inactive transposase. This may indicate that *Ae. caspius* has the ability to switch on and off the mariner-like gene element properly and appropriately based on the nature of its aquatic environment, which may explain its ability to live in tap water in the lab. Studying this particular point is now taking place.

Morphological identification (according to Mattingly & Knight, 1956) showed that both autogenous and anautogenous *Ae. caspius* strains in the current study belong to the same species. This is based on the fact that the ability of DNA barcodes to identify species reliably, quickly, and cost-effectively has shown a particular importance in medical entomology (Phuc *et al.*, 2003), where molecular approaches to species diagnosis are often of great benefit in the identification of all life stages of mosquitoes, from eggs to adults (Green *et al.*, 1985; Baimai *et al.*, 1993; Bass *et al.*, 2008). Thus morphological identification of both autogenous and anautogenous mosquito strains have been confirmed relying on the sequence of ITS2 region of the ribosomal gene (Prakash, 2006; Walton, 2007), which showed identity in both strains.

Autogenous *Ae. caspius* mosquito of the current study showed autogeny throughout 20 generations without a single blood meal until conducting the current study. This case of autogeny might be referred to the nature of its breeding site as it has been collected from sanitation water (that is rich with rotting organic materials) in the desert outside cultivated lands and human residences (i.e., it lacks blood sources). However, the anautogenous mosquitoes were collected from clear salty water of places near cultivated lands, humans and animal farms (Ahmed *et al.*, 2011) which may indicate that breeding site determines autogeny versus anautogeny. Similar observations were reported in a study done by O'Meara & Evans (1973) on different populations of *Ae. taeniorhynchus* in Florida, and referred these differences to genetic factors. They also hypothesized that host abundance at the individual mosquito breading sites has shaped these predispositions in different mosquito populations. Based on these studies, and the data of the present study, it would be assumed that autogeny is an adaptation that promotes survival and reproductive success when environmental conditions are harsh and blood resources are scarce (O'Meara & Evans, 1973; O'Meara, 1979).

An early crossbreeding study by Spielman (1957) showed that there are multiple genes responsible for conferring a tendency toward mosquito autogeny or anautogeny which appears to be gene dosage dependant in mosquitoes. For instance, *O. atropalpus*, has both autogenous and anautogenous strains, showed significant different genetics of autogeny, as the trait of autogeny is conferred by a single dominant autosomal gene (O'Meara & Craig, 1969). Furthermore, O'Meara (1972) explained that while the gene conferring autogeny is dominant in this species, there are other modifier/enhancer genes that optimize the level of fecundity in mosquitoes carrying the autogeny gene. Thus, these genes could be responsible for conferring or supporting autogeny or anautogeny status. Thus, it would be suggest that both autogenous and anautogenous *Ae. caspius* of the current study may have the same autogeny gene but there may be different modifier/enhancer. Moreover, differences have also been noted in the expression of genes coding for larval storage proteins between autogenous and anautogenous mosquitoes (Gordadze *et al.*, 1999; Zakharkin *et al.*, 2001). This may interpret the autogeny of the target mosquito in this study as they were collected from a desert sites of sanitation wastewater with scarce blood source, as factors that may triggered this kind of genes. Based on the autogeny categorization by Attardo *et al.* (2005), I would categorize *Ae. caspius* autogeny of the current study as obligatory since it foregoes blood feeding during their first ovarian cycle even in the presence of hosts (personal observation), and produce their first egg batch *via* utilizing larval

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reserved nutrients as previous recorded by van Handel (1985).

Nutrition, in terms of both the quality and the quantity of food of the larval and adult stages, is probably the most important factor that affects reproductive fitness of most of insect species (reviewed by Englmann, 1970). There are other factors in addition to feeding (e.g., mating, environmental factors and larval population density) which may also affect insect reproduction (Englmann, 1970). In either autogenous or anautogenous insects, there is no controversy about the universal requirement of proteins and lipids for their reproduction and optimal egg production (reviewed by Yuval *et al.*, 2007). In this context, an inverse relationship between protein availability and mortality, and a positive relationship between protein availability and fecundity, longevity and reproductive patterns in different insect models have been reported (Carey *et al.*, 2008; Zur *et al.*, 2009). And hence, a peak survival and reproduction rates in the tephritid fruit fly, *Anastrepha ludens*, for instance, were obtained at different proportions of protein and carbohydrates (Carey *et al.*, 2008). However, overconsumption of protein was found to account for reduction of longevity in fruit fly *Anastrepha fraterculus* (Oviedo *et al.*, 2011), thus, this fly regulates ingestion of carbohydrates and proteins to optimize survival and reproduction. However, in mosquitoes, proteins are essential of reproduction, and a significant positive correlation between blood meal size (amount of proteins and lipids) and fecundity has been approved (Briegel, 1990).

Biochemical assays of the current study showed 2, 1.6, and 1.4 folds higher carbohydrates, proteins and lipids contents respectively in the fourth larval instar tissues of the autogenous *Ae. caspius* compared to that of the anautogenous ones. These reserved nourishments are essential for egg production in the adult autogenous stage (Clements, 1992) since blood meal is not essential at least for the production of the first egg batch. Moreover, the observed declines in these nourishments upon *Btk* infection could be attributed to the cellular damage in the infected gut (Tanada & Kaya, 1993; Ahmed *et al.*, 2010) and cessation of feeding by larvae (Goldberg & Margalit, 1977; Feitelson *et al.*, 1992). However, the decline in carbohydrate content in *Btk*-infected larvae seems to be *Btk*-infection independent. Therefore, infected larvae may have the ability to compensate carbohydrate nourishments on the cost of protein contents to maintain the energy reserves to meet the need for physiological processes, including survival (Day *et al.*, 1994), and hence, maintain activities if become adult (Briegel, 1985). Thus, autogenous-adult producing larvae of the current study may have ensured *Btk*-infection unaffected carbohydrate reserves for securing sugar source as one of the main important factors determining the fate of blood meal protein amino acids on the utilization of reserved protein by adult female mosquitoes for vitellogenesis (Foster, 1995; Naksathit *et al.*, 1999). Evidence for this is the domestic mosquito, *Ae. aegypti*, which lives in domestic environment where low sugar is available, while blood sources are rich, and thus, females seldom feed on sugar and tend to take supplementary blood meals to improve their energy reserves in the field (Foster, 1995; Zhou *et al.*, 2004). This may further explain the significant higher protein content observed in the autogenous mosquito larvae compared to that of the anautogenous ones in the current study, as they forego blood meal during production of the first egg batch.

Under nutritive stress (i.e. starvation), female *Ae. aegypti* mobilized 4%–84% of lipids, almost all total carbohydrates and 14%–31% of proteins (Briegel, 1990). These findings, as well as the data of the current study, may further explain the higher nutrient reserves in the larvae of autogenous mosquitoes compared to that in anautogenous ones, which act as nutrient reserves needed for the autogenous adult mosquito to live and reproduce without the blood meal. It is important also to point out that, in mosquitoes, sugar is not only directly contributes to egg proteins and lipids, but also may play an extremely important role in energy production and survival postegg laying. Furthermore, adult female mosquitoes maintain a substantial amount of larval stage derived lipid reserves, which also contribute to egg lipid and energy production (Zhou *et al.*, 2004). It is also expect that the amount of reserved nourishments in larval tissues play an important role in the immune and antioxidant responses in the adult autogenous *Ae. caspius* mosquitoes reported in a previous study (Ahmed, 2011). Finally, assessing the type of this autogeny, the possibility of interbreeding between the anautogenous and autogenous forms, a quantitative analysis on the autogenous versus anautogenous composition of each breading site in the target area of study, and the effect of bacterial infection of larvae on the fecundity, fertility and life span of the adult stages are all now under investigation.

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Disclosure

Author confirms that there is no conflict of interests. This article reports the results of research only. Mention of a commercial or proprietary product does not constitute an endorsement of the product by the United States Department of Agriculture.

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