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Effects of Heat Stress on the Main Organs of the Male Reproductive Tract of *Anisopteromalus calandrae* Howard (Hymenoptera: Pteromalidae)

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Abstract: ***The success of biological control against agricultural insect pests, which is one of the alternative methods to chemicals, requires bio-controlling organisms to have a good reproductive capacity. Considering insects, the reproductive capacity of the male of Anisopteromalus calandrae Howard (Hymenoptera: Pteromalidae), a cowpea bruchid parasitoid, depends essentially on the condition of development of the genital tract. In order to predict influence of current climate change on insect reproduction, the objective of this study is to determine the effects of heat stress on the dimensional and structural characteristics of the main organs of the genital tract of male of A. calandrae according to their age in lab conditions. Experimentation was carried out on nymphs and males aged 1 and 10 days and reared at 28°C and 38°C. Observations of the male genital tract structure were made with photonic microscopy. Heat stress disorganized the structure of the testis and the anterior portions of the seminal vesicles resulting to reduction of stock of spermatozoa in the lumens of the seminal vesicles. These experiments prove that a heat stress affects the structure of the reproductive system of A. calandrae which may explain the decrease of fertility of heat stressed males and then the disequilibrium of the sex ratio in this haplo-diploid insect.***

Keywords: Parasitoid wasps, heat stress, structure, testis, seminal vesicle, accessory glands.

1. Introduction

The bruchid *Callosobruchus maculatus* F. (Coleoptera: Chrysomelidae), is a major cowpea *Vigna unguiculata* (L.) Walp (Fabales: Fabaceae) pest in west Africa (Ouedraogo et al., 1996; Sanon et al., 1998) particulary in Togo (Glitho et al., 1988; Amevoin, 1998). Losses caused by this pest during the storage phase may be detrimental to food safety. This pest can cause post-harvest losses of up to 80% after 7 months (Ngamo et al., 2007). In order to reduce cowpea seed infestation rates below economic pest thresholds, control methods such as biological control are of utmost importance (Mondédji et al, 2002). Biological control involves both enriching the environment with regulatory organisms and promoting the survival and multiplication of auxiliary entomofauna such as parasitoids (Masry et al., 2018) by protecting natural reservoirs. It requires therefore a good knowledge of the reproductive capacity of bio-controllers against pests (Cameron and Walker, 2002; Khakasa et al., 2016). This is the case of *Anisopteromalus calandrae* (Hymenoptera: Pteromalidae) a nympho-larval parasitoid used against bruchids and weevils to protect crops and stocks of legumes and grains (Ji et al., 2004; Chaisaeng et al., 2010). In the case of *A. calandrae* and other Hymenoptera species, female individuals are obtained from fertilization while males are from arrhenothoic parthenogenesis (Lebreton, 2009; Lécureuil et al., 2012). It is therefore the females that ensure the survival of the species. This is why, it was important to study the reproductive capacity of male individuals in order to ensure the survival of the species by producing of spermatozoa for oocyte fertilization and then for production of females. The effectiveness of this biological method requires favorable environmental conditions to ensure the sustainability of the species, which depends among other things on the male's ability to transfer on a sufficient number of fertile spermatozoa to the female (Do Thi Khanh et al., 2005). However, any environmental factor capable of disrupting insect reproduction may also be a constraint to the effectiveness of this control method (Lacoume et al., 2006; Lacoume et al., 2009). Temperature is one of such factors with a strong impact on parasitoid wasps reproduction (El-sabrout and Bressac, 2012). In the context of global climate change, temperature variations could negatively affect parasitoid reproduction. Thus, the objective of this study is to determine in lab conditions the impacts of heat stress on the dimensional and structural characteristics of the main organs (testis, seminal vesicles and an accessory gland) of the male genital tract of *A. calandrae* used in a cowpea bruchid management program.

2. Materials and methods

2.1. Insect rearing and production

2.1.1. Production of 4th instar larvae of *C. maculatus*

Safe cowpea seeds (black eyes variety from Madagascar) were placed with adult male and female of *C. maculatus* (Benin in West Africa strain) for 48 hours in an air-conditioned room (12: 12 h LD at 28°C continuously and between 50 and 60% RH) to allow the females to lay eggs on the seeds. The infested seeds were stored in the climatic chamber for 18 days at 30°C to obtain 4th instar larvae (L4). The seeds were kept refrigerated at 4°C to stop larval growth according to Do Thi Khanh method (2005).

2.1.2. Rearing of *A. calandrae*

A colony of *A. calandrae* (strain from Ivory Coast, West Africa) was introduced for 48 hours into a cage containing cowpea seeds infested by 4th instar larvae (L4) of *C. maculatus* for parasitoid egg laying. These seeds were then recovered and preserved until the emergence of adult of *A. calandrae* parasitoids to sustain the rearing and to conduct the various experiments.

2.1.3. Preparation of laying patches for *A. calandrae*

Individual virgin females of *A. calandrae* were laid in a petri dish containing seven (7) seeds. Each seed containing one to four larvae of the 4th instar of *C. maculatus* was attached to the bottom of the petri dish using patafix and cotton soaked in sugar water for 24 hours. After this time, the seeds brought into contact with the females of *A. calandrae* were recovered and introduced into another petri dish for 72 hours and then dissected after.

2.2. Dissection of seeds

After 72 hours, the seeds containing L4 of *C. maculatus* parasitized by *A. calandrae* were opened under a stereomicroscope to recover the second and third instar larvae (L2 and L3) of the parasitoid. Each larva was placed with its host in a development well-plate until the pupal stage was reached. The pupa were then divided into two batches. One batch was monitored until emergence (control individuals at 28°C) and the other batch was placed in an oven at 38°C for heat stress.

2.3. Heat stress and dissection of male individuals of *A. calandrae*

The pupae of *A. calandrae* were obtained approximately eight (8) days after hatching. They were incubated for 48 hours at 38°C before being returned to 28°C. The accurate stage was assessed by the color of the body (white) and the eyes (red) according to Nguyen (2013). After emergence of adults of parasitoid, control or stressed individuals were placed separately in a petri dish and fed with sugar water. Male pupae before heat stress and male adults aged 1 day and 10 days were dissected under binocular magnifying glass on a slide in Hyes solution (0.9% NaCl; 0.02% KCl; 0.02% CaCl2; 0.01% NaHCO3: pH 8.5) in order to measure the size of the genital tract and to observe the structure of the reproductive tract portions.

2.4. Genital tract organ measurements

Once photos were taken by photonic microscopy, the organs: testicles, seminal vesicle, accessory gland were measured using the "ImageJ" software version 1.4.3.67 with results in pixels. The data were later converted to µm. Then, the area of the testis and seminal vesicles and the ratio between the light and the total width of the accessory gland were calculated considering that the testis are diamonds and that the two portions of the seminal vesicle are ellipses

$$Testis\ area = \frac{\text{l test x L test}}{2}$$

$$Seminal\ vesicle\ area = \frac{\pi}{2} \text{x D x d}$$

$$Ratio = \frac{\text{L lum}}{\text{l gl}}$$

L test: testis length; l test: testis width;
D: major axis (length); d: the minor axis (width).
L lum: width of the lumen; and l g the total width of the accessory gland.
For the calculations, the anterior and posterior portions of the seminal vesicle were considered separately.

2.5. Treatment and observation of the male reproductive tract portions of *A. calandrae* under the microscope

In order to study the effects of rearing temperature on the structure of the organs of the male genital tract of *A. calandrae*, apart from measurements, semi-fine sections of these organs were performed without isolating the tract. One third (1/3) of the abdomen was put in Hyes solution. The sample was fixed at least 3 nights (4 hours at room temperature and then refrigerated) in a mixture of Paraformaldehyde and Glutaraldehyde. A series of washes (with a mixture of Cacodylate 0.4 M, sucrose 1 M and sterile water) and a post-fixing with osmium tetroxide were carried out. Then dehydration was done with ethanol at different concentrations (50°, 70°, 90° and 100°) and propylene oxide. After pre impregnation with a mixture of epon and propylene oxide and then impregnation (with pure epon), inclusion of the samples was made in the pure epon at 37°C and then at 60°C. Semi-fine cuts (thickness 0.5 µ) were made and colored with toluidine blue. The tract and semi-fine sections of the reproductive tract were observed under a photonic microscope. In order to avoid changing of the shape of the tract, lamellae were not placed before observation.

2.6. Data Analysis

The data were analyzed using SPSS software version 16.0. The means were compared by analysis of variance (ANOVA) and were discriminated using the LSD test at the 5% threshold.

3. Results

3.1. Testis

The surface area of the testis decreases with age depending on whether the insect was stressed or not ($F_{5, 24} = 13.364$; $P = 0.000$) (Fig. 1).

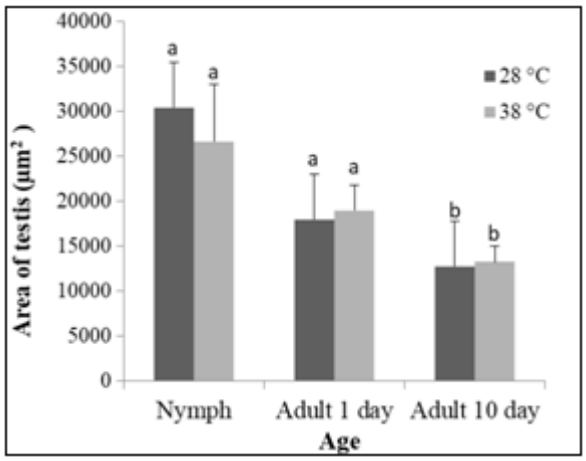
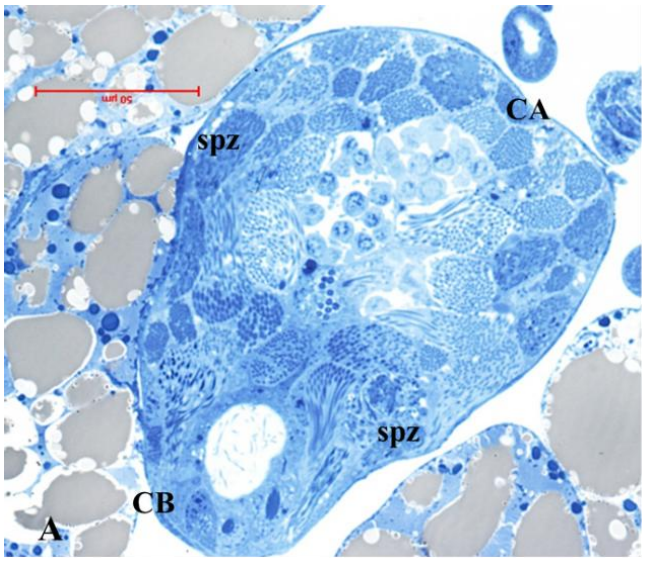


Figure 1: Testis area of *A. calandrae* males measured under microscope at pupal stage, 1 day and 10 days unmated adults. Males are either continuously maintained at 28°C or heat stressed for 48 h at 38°C at early pupal stage. Letters indicate series that are statistically different (ANOVA, P<0.05).

There is no significant difference between the testicular surface of control and stressed *A. calandrae* individuals of the same age. In terms of structure, sperm cysts occupying the apical zone of the testis and free spermatozoa in the basal zone are observed in the control for 1-day old male (Fig. 2 A). However, in the stressed 1-day-old male, in addition to sperm cysts, immature cell cysts (spermatogonia to spermatid) are observed in the basal area (Fig. 2 B). We also observe in the stressed 1-day-old male a disorganization of the disposition of the cysts: cysts with immature cells and spermatozoa in the basal zone.



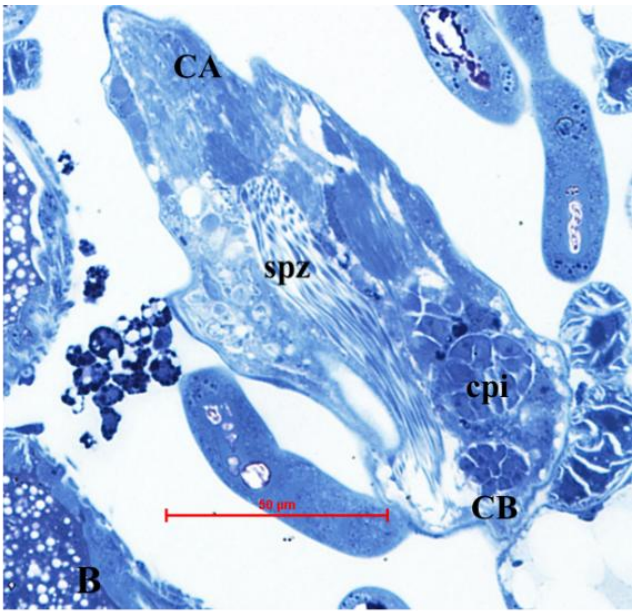


Figure 2: Testis of one-day-old control (A) and 38°C heat stressed (B) *A. calandrae* males

Basal region (CB), apical region (CA), spermatozoa (spz), cysts of immature reproductive cells (cpi).

3.2. Seminal vesicle

The surface of the anterior portion of the seminal vesicle of *A. calandrae* increases progressively with the age in control individuals (Fig. 3). From nymph to 10-day-old adult, the area of the anterior portion has almost doubled ($F_{5, 24}$ = 14.646; P = 0.000). This was not the case for stressed individuals as their surface did not change with age.

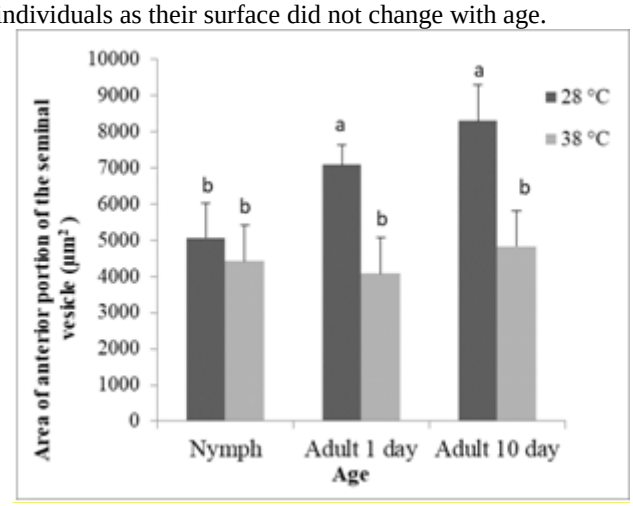


Figure 3: Area of the anterior portion of the seminal vesicle of *A. calandrae* males measured under microscope at pupal stage, 1 day and 10 days unmated adults. Males are either continuously maintained at 28°C or heat stressed for 48 h at 38°C at early pupal stage. Letters indicate series that are statistically different (ANOVA, P<0.05).

Photonic microscopic observation of the semi-fine sections of the seminal vesicle of a control and stressed 1-day-old males showed numerous spermatozoa in the lumen of the anterior portion in the control (Fig. 4 A). However, in stressed males, there were almost no spermatozoa in the lumen (Fig. 4 B).

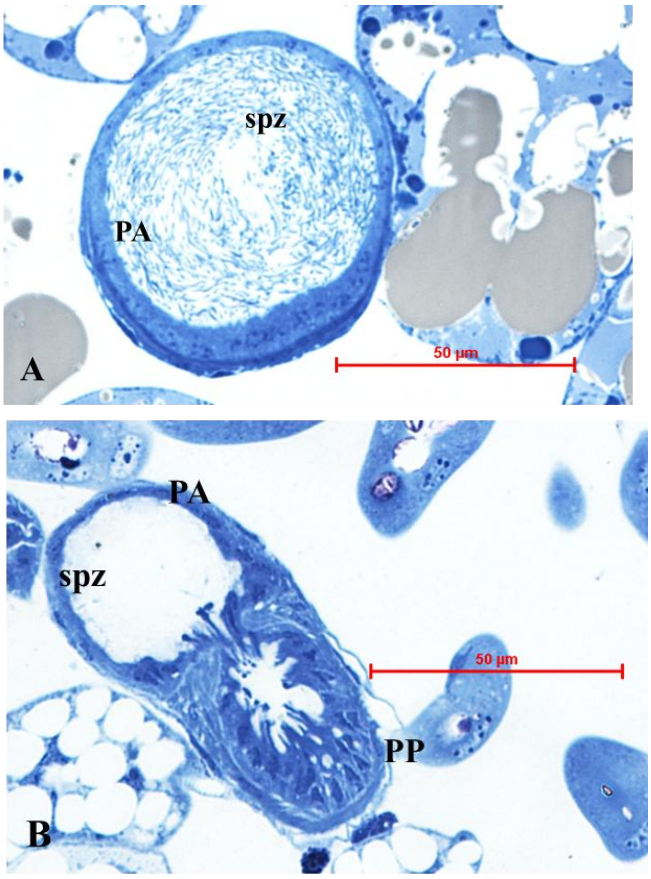


Figure 4: Structure of the anterior portion of the seminal vesicle of one-day-old control (A) and 38°C heat stressed (B) *A. calandrae* males
Anterior portion (PA), posterior portion (PP), spermatozoa (spz).

The area of the posterior portion of the seminal vesicle increases with age for both control and stressed individuals (Fig. 5) (F5, 24 = 4.394; P = 0.006).

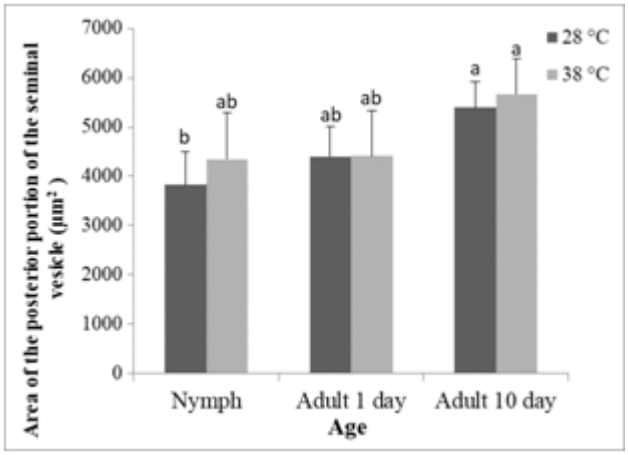


Figure 5: Area of the posterior portion of the seminal vesicle of *A. calandrae* males measured under microscope at pupal stage, 1 day and 10 days unmated adults. Males are either continuously maintained at 28°C or heat stressed for 48 h at 38°C at early pupal stage. Letters indicate series that are statistically different (ANOVA, P<0.05)

3.3. Accessory gland

The ratio between the width of lumen and the total width of the accessory gland increases with age regardless of whether individuals experienced heat stress or not (Fig. 6). The increase in this ratio was observed from the pupa to the 10th day of the life of the insect (F5, 24 = 22.853; P = 0.000).

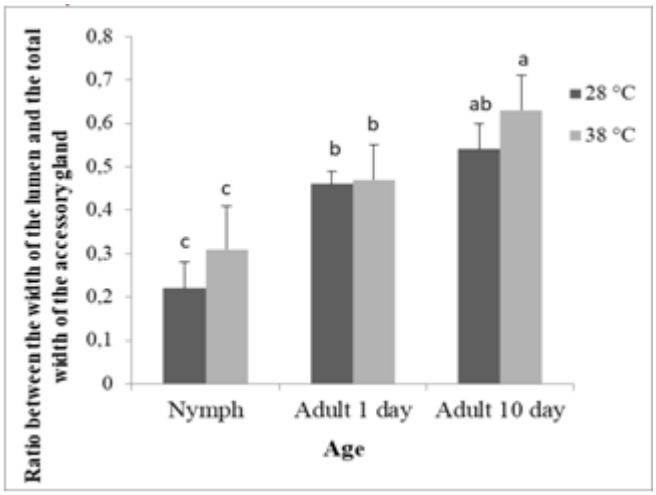


Figure 6: Ratio between the width of the lumen and the total width of the accessory gland of *A. calandrae* males measured under microscope at pupal stage, 1 day and 10 days without mating. Males are either continuously maintained at 28°C or heat stressed for 48 h at 38°C at early pupal stage. Letters indicate series that are statistically different (ANOVA, P<0.05).

4. Discussion

The male genital tract organs did not evolve in the same way with age. Study of the effects of the rearing temperature (38°C) on the dimensional and structural characteristics of the main organs of the male genital tract of *A. calandrae*, showed that this factor did not have effect on the surface of the testicles and on the ratio between the width of the lumen and the total width of the accessory gland. However, it had an effect on the structure of the testis and therefore on the spermatogenesis process resulting in a delay in cyst maturation. The results showed that in non-stressed males, a clear arrangement of cysts is observed: immatures at the apical part and mature ones at the basal level of testis. This type of arrangement was also observed in *Schistocerca gregaria* Forsk. (Orthoptera: Acrididae) under normal conditions by Ouali-N'Goran (2013). However, when *A. calandrae* males were under heat stress, there was disorganization of the cysts arrangement in the testis. This result is in accordance with that of Chirault et al (2015) who reported that heat stress affects the induction of the spermatogenesis process in *Nasonia vitripennis* Walker (Hymenoptera: Pteromalidae). Similarly, temperature influenced the activity of the seminal vesicle which surface area did not increase. This led to a reduction of the sperm stock in the lumen of the anterior portion of the seminal vesicle. By counting the number of spermatozoa stored in the seminal vesicle, Nguyen (2013) proved that in males of *A. calandrae*, the quantity of spermatozoa stored in this organ decreased when individuals are stressed at 38°C. This confirms our results based on observation of testicular

structure. El-Sabrout & Bressac (2012) stated that the rate of spermatozoa stored in the spermatheca of a female mated with a male of A. calandrae stressed at 38 °C is much lower than that stored after mating with a control male (30°C). This result confirms the negative effect of heat stress on spermatogenesis process of *A. calandrae*. Similarly in *N. vitripennis*, heat stress leads to a reduction in the quantity of spermatozoa stored in the seminal vesicle (Chirault et al., 2015). This could be as a result of spermatozoa failing to move from the testis to the seminal vesicle. Furthermore, Porcelli et al., 2017 reported that heat stress temperature during juvenile and adult stages influences egg-to-adult viability, adult sperm motility and fertility of *Drosophila subobscura*. The found temperature effects on sperm motility. Thus, in the case of *A. calandrae*, climate change could have negative effects on the success of biological control. Thus for better performance of *A. calandrae* which product males by parthenogenesis as a control agent, sperm must be well formed under ideal conditions (at an appropriate temperature) giving strength and vigour to males to fertilize eggs. Fertilized eggs giving females assume the sustainability of the species and therefore advantageous ecological distributions in terms of the distribution of a good biological control agent.

5. Conclusion

Study of the effects of heat stress on the main organs of the male genital tract of *A. calandrae* per age showed that a heat period at 38°C during early pupal stage did not affect the surface of the testis, the posterior parts of the seminal vesicles and the ratio between the width of the lumen and the total width of the accessory glands. However, it had a negative impact on the structure of the testis that could influence normal course of spermatogenesis. Heat stress also impacts the activity of the seminal vesicle which did not increase in surface area regardless of age but led to a reduction of the sperm stock in the lumens of the anterior portions of the seminal vesicles. Heat stress has also disorganized the cysts arrangement in the testis. Thus heat stress delayed spermatogenesis and reduced the number of spermatozoa that moved from the testis to the seminal vesicles.

The results of our experiment suggest the study of the effects of heat stress on the structures of the male genitals of *A. calandrae* according to age for more information on the fertility of the species in possible global warming situations. It is obvious that heat stress has consequences on the structures of the males genitals of *A. calandrae* thus on the fertility of males of such beneficial insects, and then on the sex ratio of natural and controlled populations of parasitoid wasps.

Thus, the rearing of *A. calandrae* is recommended to be carried out at a temperature suitable for its use in a biological control programme.

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