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GENETIC VARIATION AMONG *DROSOPHILA MELANOGASTER* ISOFEMALE LINES INFLUENCES ENCAPSULATION ABILITY OF THE PARASITOID *ASOBARA TABIDA*

By

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Abstract

One of the most important factors which affect the outcome of the host-parasite interactions is the genetic variation in particular those of the host. The aim of this study was to test the effect of variation within *Drosophila melanogaster* population on the encapsulation ability against the parasitoid *Asobara tabida*. To this end, nine isofemale lines of *D. melanogaster* which represent different levels of encapsulation ability were used. The encapsulation ability and its related parameters such as infestation, avoidance, and parasitism success and super-parasitism rates were assessed following oviposition. Larval weight, protein contents and phenoloxidase in extremes and intermediate lines, were investigated. The variation among *D. melanogaster* lines influenced encapsulation, avoidance, parasitism and super-parasitism rates. Upon emergence, low success of the parasitoid associated with high mortality rates were observed in the representative lines. Although parasitized larvae may suffer from weight loss, they could survive based on mounting effective immune response. Parasitism of *A. tabida* in the *Drosophila* larvae induced high phenoloxidsae activity, particularily in the resistant ones refelecting their immune competency. In sum, the variation in encapsulation ability among isofemale lines demonstrates high underlying genetic variation.

The data might help to design successful biological control programs and to facilitate interpretation of a similar interaction of other host-parasite model systems.

Keywords: *Drosophila melanogaster,* parasitoid, encapsulation, genetic variation.

Introduction

In host-parasite interaction, genetic variability is believed to be the source used by evolutionary force to give better survival of the host or the parasite on the account of the other. Without this variation, evolution cannot take place. Hence, measurements of genetic variability is a prime task for evolutionary biologists (Jones, 1980). Hosts produced by the sexual reproduction, may have higher variability than their parasites (Stearns, 2007).

Therefore, study of genetic variation is of great importance to design better control tactics for pest populations such as whose variability is high and to choose suitable therapeutics for varia-

ble patients in medicine (Kansal *et al*, 2010; Kacsoh and Schlenke, 2012). It has been proven from the genomic analysis of human samples that more than 80% of the genetic variation lies within the same population (Owens and King, 1999). *D. melanogaster* has proved a remarkable laboratory model for studying ecological and evolutionary genetics (Schneider, 2000). Using isofemale lines (progeny of a single inseminated female) has been used as a reliable tool to investigate phenotype variability among individuals, families and populations (David *et al*, 2005). The restriction of the gene pool in isofemale line decreases the within-family genetic variance and increases the among-families variance (Moeteau *et al,* 1995).

One of the best models for the host-parasite interactions is *D. Asobara-tabida* (Kraaijeveld and Godfray, 1997; Dubuffet *et al*, 2007). *A tabida* is known to occur in Europe and North America, succeeded in parasitizing the cosmopolitan *D. melanogaster* (Carton *et al*, 1986). This type of parasitism appeared not to provoke any depressive effect on the host's immune system; the female parasitoids produce eggs with sticky chorion permitting the complete attachment into host' fat body and other visceral tissues as an avoidance response (Prevost *et al*, 2005). On the other hand, the hosts defend themselves against parasitoid's attack by formation of multi-cellular melanized capsules around the parasitoid eggs (Carton and Nappi, 1997). The encapsulation of parasitoid eggs or successful parasitism was observed to be the outcomes of multi-step process starts with infestation of the parasitoids (Eslin and Prevost, 2000). Ability of *Drosophila* to encapsulate the parasitoid eggs depends mainly on cellular response mediated by three types of haemocytes namely, plasmatocytes, lamelocytes and crystal cells (Prevost and Eslin, 1998; Moreau *et al*, 2005) cooperated with humoral response such as phenoloxidase (Rizki and Rizki, 1985; Carton and Nappi, 1997; Moreau *et al*, 2003). *Drosophila* with high haemocyte load and plenty of lamelocytes could perform better in encapsulation (Prevost and Eslin, 1998; Gerritsma *et al*, 2012). Phenoloxidase contributes to essential cellular and humoral events as a conservative or an induced factor. As such, it has been used as a measure for the status of the immune response against an invader (Adamo, 2004).

In depth analysis of factors affecting encapsulation of *Asobara*, using wild type and mutants of *Drosophila*, revealed that the genes responsible for encapsulation were located on chromosome number 2 (Orr and Irving, 1997). Little is known about the variation among families of the population in encapsulation ability. The preliminary results using isofemale lines of *Drosophila* and the parasitoid *Leptoplina boulardi* reported that encapsulation variation is under genetic control (Carton and Bouletreau, 1985; Wajnberg *et al*, 1985).

The aims of this study were to (i) investigate the genetic variation among the families of *Drosophila melanogaster* regarding encapsulation of the

parasitoid *A. tabida*, and (ii) determine the costs paid by *D. melanogaster* larvae following parasitism, and mount immune response in different-resistance lines.

Materials and Methods

Nine isofemale lines of *Drosophila melanogaster* were established from a population collected in Kiel, Germany during 2003 (Anagnostou *et al*, 2010). They were reared for two successive generations; thereafter the experiments were carried out. Isofemale line technique provides the most homogenous individuals within line and significant heterogeneity among lines based on genetic basis (Capy *et al,* 1994; Moreteau *et al*, 1995). *D. melanogaster* lines were regularly reared in vials supplied with 20ml sterile standard medium (cornmeal-sugar-yeast). The emerged adults of each isofemale line were diffused in cages containing 200-300 individuals. The parasitoid *Asobara tabida* was originally collected in Leiden, the Netherlands during 1998.

This parasitoid strain was routinely maintained on *D. melanogaster* population as a host. The newly emerged wasps were isolated in Nipagin-agar vials and supplied with honey and water until use for experimentation. All experiments were conducted under the laboratory conditions at 20±2°C, 60±5 RH and 16h: 8h light dark regime. Under the above mentioned conditions, variation among lines is believed to reveal genetic variability (David *et al*, 2005).

Encapsulation ability: The eggs of each flies line of *D. melanogaster* were collected overnight using hard agar medium. Next day, every 70 viable eggs were transferred to new vials containing enough rearing medium. The eggs of each line were divided onto 6 vials. After 3 days, the 2nd instar larvae in 3 vials of the six were exposed to 4 mated *A. tabida* females (not more than 15 days old) for 2 hours to oviposit their parasitoid eggs. Eight days later, the pupae were immediately freezed at -20°C until dissected in PBS (pH 7.2) to view the encapsulation under Leitz microscopy at magnification (X32). The parasitized insects were divided into those *Drosophila* having single capsule or more and those having single parasitoid larva or more. Several parameters such as the infestation, encapsulation, and the successful parasitism rates were observed as described by Moreau *et al.* (2003). The efficiency of encapsulation rate was calculated as described in Delpuech *et al.* (1996). The parasitoid avoidance and super-parasitism rates were determined according to Carton *et al.* (1987) and Prevost *et al.* (2005). The infestation rate (IR) and super-parasitism rate (SuperPR) were calculated as the number of parasitized flies (having only one parasitoid) and the number of super-parasitized flies (having more than one parasitoid), respectively in relation to the number of examined flies. The encapsulation rate (ER) and the successful parasitism rate (SPR) were calculated as number of flies having only single capsule and the number of flies having only single parasitoid larva, respectively in relation to the number parasitized flies. The

encapsulation efficiency rate (EER) and avoidance rate of parasitoids (AR) were calculated as number of parasitoid eggs encapsulated and number of parasitoid larvae, respectively in relation to the number of oviposited parasitoid eggs.

Adult emergence experiment: Only five isofemale lines represent extremes and intermediate rates of encapsulation ability were selected to carry out emergence experiment. The eggs of lines # 1, 3, 5, 7 & 9 were collected from caged flies. The eggs were treated with the same setting as described above. Thirty eggs of each line were placed onto standard rearing media in a vial and exposed to two mated female wasps to oviposit. All vials of the treated insects and control (not exposed to female parasitoids) were replicated 4 times. After successful emergence of flies and parasitoids, the IR, total ER, SPR and the mortality rate among the parasitized larvae (MR) were estimated as described by Prevost and Eslin (1998).

Based on the results extracted from this experiment, only three isofemale *D. melanogaster* lines represent both extremes and intermediate rates of the encapsulation ability were chosen for the subsequent experiments.

Larval weight: To assess the costs of parasitism and mounting immune response (encapsulation), 10 larvae (4 days after parasitism) were rinsed in PBS and then dried on filter paper, before weighing in groups of ten on analytical balance. The mean weight of parasitized and control larvae was expressed in mg/10 larva. These measurements were replicated 5 times for each isofemale lines of parasitized and control insects.

Larval protein contents: Two series of insects were prepared. The first set included 8-10 larvae (4 days after parasitism) weighting about 10mg which were washed with PBS and then mashed in 100µl. The second set included ten larvae of the same age which were mashed in 200µl of the same buffer, centrifuged for 10min at 2000xg. The supernatant was transferred into new Eppendorf tubes and stored at -20°C until used. The total larval protein content was determined by the colorimetric method described by Bradford (1976). Absorbance of the samples along with BSA (standard) was measured at 595nm. Total larval protein content was expressed as µg/g for the first set and as µg/ml for the second set. This procedure was replicated 5 times for each isofemale line of parasitized and control insects.

Phenoloxidase (PO) activity: The PO activity was determined spectrophotometerically by measuring formation of dopacrome according to the method of Ashida and Soederhaell (1984). Ten ul samples of the second series in the previous experiment were added to 390µl 0.01 M ice-cold PBS and 400µl 20 mM L-DOPA (Sigma, Germany) and then incubated for 30 min at 27°C. The mixture was measured at 490 nm against blank without sample. The phenoloxidase activity is expressed as PO unit per ml of larval homogenate; one unit is the amount of enzyme required to increase the absor-

bance by 0.001 min^{-1}. This procedure was replicated five times for each isofemale line of parasitized and control insects.

Statistical analysis: Data were expressed as mean ± standard Error (SE). The effect of treatment, variation of isofemale line and their interaction were tested using PROC GENMOD (SAS, v. 9.0; SAS institute, Inc, USA). Planned comparisons were tested in case of significant difference to reveal the effect of each treatment and line alone. For encapsulation and emergence experiments, the binomial distribution and logit link-function were used, whereas, for weight, protein content, and phenoloxidase activity, normal distribution and identity link-function were used. The proportion values were normalized using Arcsine (SQRT) transformation. Correlation has been tested between relevant parameters. The genetic variation among isofemale lines was estimated using ANOVA outputs to calculate coefficient of intraclass correlation (*t*) according to formula of Hoffmann and Parsons (1988) and David *et al.* (2005).

Results

After 8 days of the parasitism of the 2nd instar larvae of different *D. melanogaster* isofemale lines by the female parasitoids of *A. tabida*, the dissection revealed significant difference in the ER and EER among lines ($F_{8,18}$ = 2.76, P = 0.035) and ($F_{8,18}$ = 7.08, P < 0.0003), respectively (Fig. 1). Encapsulated parasitoid eggs were seen in both *Drosophila* pupae and adults (Fig. 2A, B & 2D). The EER were higher than ER, which might indicate the ability of *D. melanogaster* to encapsulate parasitoid eggs even at super-parasitism conditions. IR of the parasitoid was high (51-82%) but without any significant difference among the isofemale lines. However, SPR of parasitoids in *Drosophila* showed significant variation among lines ($F_{8,18}$ = 2.78, P==0.034). SPR values showed highest value was in line #9 (90%) and lowest was in line #3 (64%).

Parasitism of the *Drosophila* by the parasitoid was successful as indicated by the presence of fully-developed *A. tabida* larvae and may be adults in the pupae (Fig. 2E & 2F). In addition, the AR of the parasitoids in response to the immune reactions of *Drosophila* and super-parasitism rates (SuperPR) were significantly different among isofemale lines ($F_{8,18}$ = 5.16, P < 0.002) and ($F_{8,18}$ = 5.21, P < 0.002), respectively. The avoidance rate showed similar order of the lines as appeared in SPR (Fig.1)

Correlation analyses of data indicated that several parameters were highly or at least moderately correlated to each others. ER showed high positive correlation to EER (r= 0.91, P < 0.0001) and at the same time there was high negative correlation to SPR (r = -0.99, P < 0.0001) and AR (r= -0.89, P <0.0001). Also, EER was inversely proportional to SPR (r= -0.90, P = <0.0001) and AR (r= -0.97, P<0.0001). Successful parasitism rate positively correlated to the AR (r = 0.89, P < 0.0001). Besides, superPR was proportional to EER (r=0.63, P=0.0004) and inversely proportional to AR (r = -0.58, P= 0.0016).

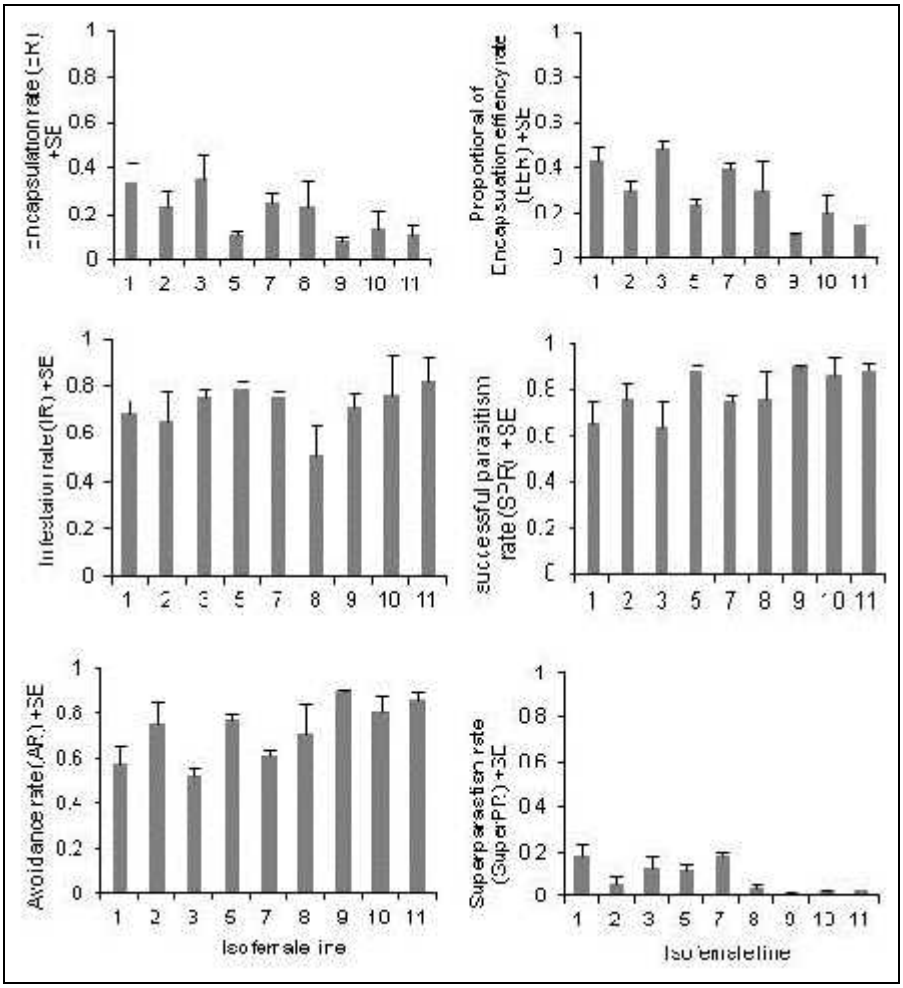

Fig. 1: Encapsulation ability, encapsulation efficiency of different isofemale lines of *D. melanogaster*, and infestation rate, successful parasitism rate, avoidance rate and super-parasitism rate of parasitoid, *A. tabida,* 8 days after parasitism of 2nd instar larvae.

After adult emergence, *Drosophila* lines were narrowed into two extremes on each side and one line with intermediate encapsulation ability. Data of emergence experiment did not show significant difference among lines in the total ER, but their values mostly were close to the values observed in encapsulation experiment. Infestation rate did showed significant difference among lines with different values than those observed in the encapsulation experiment (Fig. 3). Successful parasitism rates (SPR) of *D. melanogaster* isofemale lines were much lower than those observed in encapsulation experiment without significant difference due to line effect. There are high mortality rate among all lines without significant difference. Applying correlation proce-

dure on emergence experiment parameters, showed only negative relationship between mortality rate and total ER (r= -0.78950, P<0.0001).

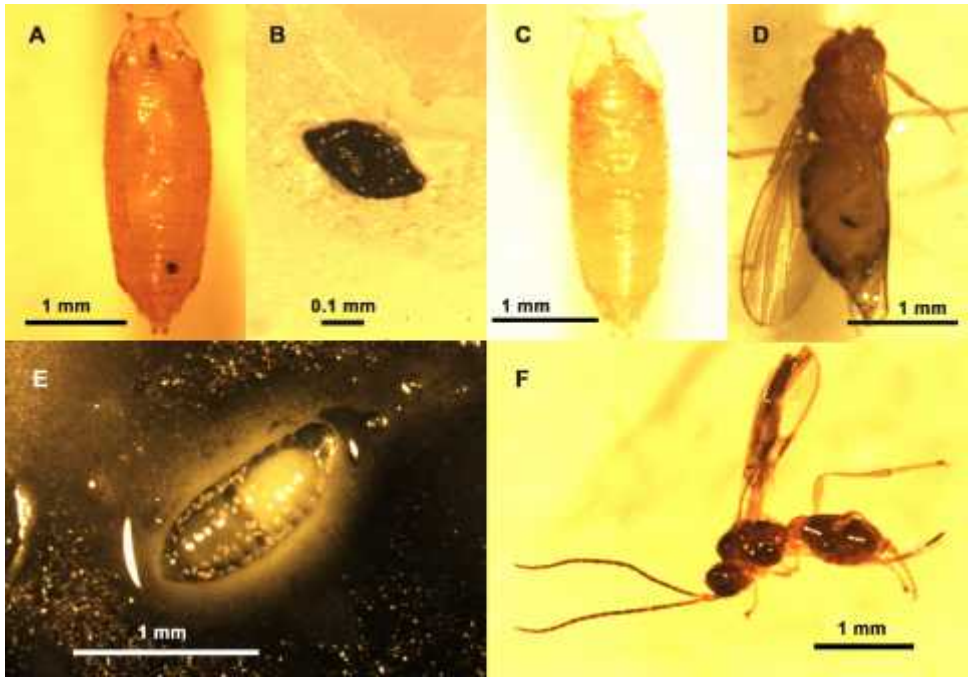

Fig. 2: Different stages extracted from *D. melanogaster* pupae parasitized by *A. tabida* and dissected 8 days later. (A) Pupa of *D. melanogaster* containing encapsulated egg of parasitoid. (B) Melanized encapsulated egg of *A. tabida*. (C) Pupa containing well-developed adult of *D. melanogaster*. (D) Adult of *D. melanogaster* containing encapsulated egg of parasitoid in abdomen. (E) Developed larva of *A. tabida*. (F) Adult female of *A. tabida*.

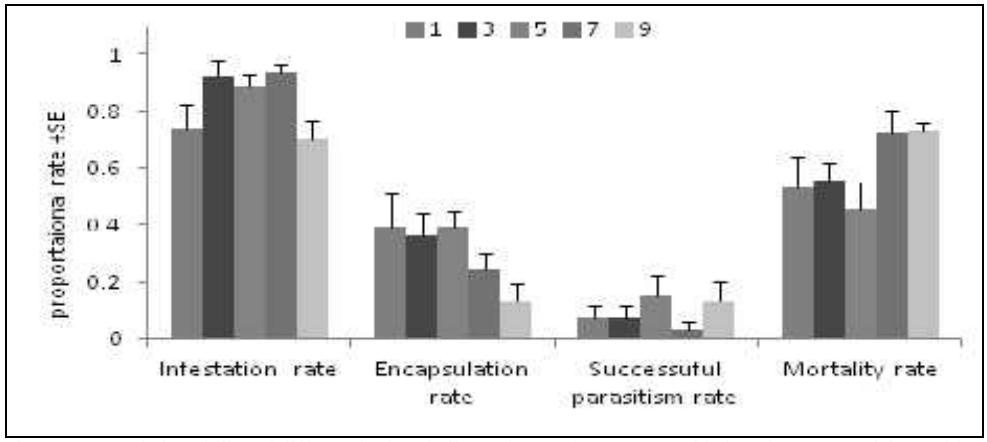

Fig. 3: Encapsulation ability of different isofemale lines of *D. melanogaster* and infestation, successful parasitism rates of parasitoid, *A. tabida* and consequently mortality rates on emergence following parasitizing 2nd instar larvae.

Statistical analysis showed that *D. melanogaster* larvae weight was influenced by parasitism ($F_{1,24}$ =10.86, P = 0.003) and isofemale line variation ($F_{2,24}$=5.69, P=0.009). Parasitism effect on the weight of larvae can be attributed to the significant decrease in weight of parasitized *Drosophila* larvae of the line #3 (resistant) (Fig. 4). Variation among isofemale lines was due to significant differences observed among parasitized isofemale lines ($F_{2,12}$ = 4.33, P = 0.041).

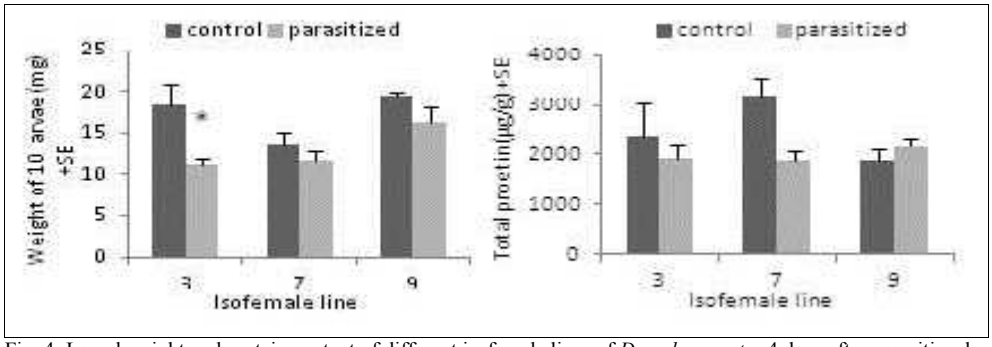

Fig. 4: Larval weight and protein content of different isofemale lines of *D. melanogaster* 4 days after parasitism by the parasitoid *A. tabida*. * indicates significant difference compared to control when $P < 0.05$.

Neither parasitism by *A. tabida* nor variation of the isofemale lines was able to affect the protein content of *D. melanogaster* larvae ($F_{1,24}$ = 2.97, P = 0.0851) and ($F_{2,24}$ = 1.2, P = 0.3019), respectively (Fig. 4). PO activity was affected by parasitism ($F_{1,24}$ = 10.56, P = 0.0034) and variation among isofemale lines of *Drosophila* ($F_{1,24}$ = 40.58, P < 0.0001). Variation among isofemale lines are attributed to the significant differences observed in the control ($F_{1,12}$ = 14.6, P = 0.0006) and parasitized larvae ($F_{1,12}$ = 26.93, P < 0.0001). The parasitism variation was due to significant increase of PO activity in resistant and susceptible lines # 3 & 9, respectively. PO specific activity was only affected by parasitism ($F_{1,24}$ = 13.45, P = 0.0012) as evidenced by significant increase seen in the line #3 (resistant) (Fig. 5).

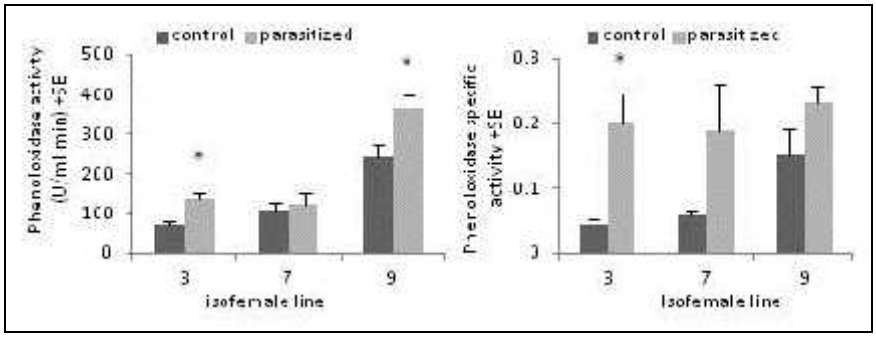

Fig. 5: Phenoloxidase activity phenoloxidase specific activity of *D. melanogaster* larvae of different isofemale lines 4 days after parasitism by parasitoid *A. tabida*. * indicates significant difference compared to control when $P < 0.05$.

Coefficient of intraclass correlation, *t* of encapsulation ability was calculated. The values of *t* indicated high genetic variation of most traits among lines. These values increased in the IR and ER in the emergence experiment and in the weight and PO after parasitism, whereas they decreased in the SPR on emergence and in protein contents and specific PO after parasitism as shown in table (1). The phenotypic variability was significantly ($P < 0.05$) observed in EER, AR and superPR in dissecting experiment. The weight was variable only in the parasitized larvae, while the specific PO was only variable in the unparasitized ones. PO was variable in both states (parasitized & unparasitized). Genetic variation among lines was underlying significant phenotypic variability recorded by ANOVA (not shown). Homogeneity of variances in lines was compared using Bartlett' test, but not shown. Significant heterogeneity was only observed among lines for the EER, AR, control weight and parasitized specific PO.

Table 1: Values of intraclass correlations of encapsulation ability to the parasitoid *A. tabida* and related traits in different isofemale lines of *D. melanogaster*

Trait	From dissection experiment[1] or control[2]	From emergence experiment[3] or treated[4]
IR	0.40[1]	0.72[3]
ER	0.62[1]	0.70[3]
EER	0.80*[1]	
AR	0.75*[1]	
SPR	0.61[1]	0.38[3]
superPR	0.82*[1]	
MR	0.67[3]	
Weight	0.74[2]	0.80*[4]
Protein content	0.68[2]	0.31[4]
PO	0.94*[2]	0.96*[4]
Specific PO	0.85*[2]	0.39[4]

* Significant variation among lines when $P < 0.05$.

Discussion

This study was conducted to shed light on a top priority point in genetic variation which is the difference among families in a population of *D. melanogaster* as a model for host-parasite interaction. Specially, outcomes of the interactions between *Asobara* virulence and *Drosophila* resistance may depend critically on genetic structure (Dubuffet *et al*, 2007).

Data obtained from the dissecting experiment demonstrated that the infestation rates of this strain of *A. tabida* were high (51-82%), but with no significant difference among isofemale lines. This might open the door for another hypothesis that infestation rates may be affected by the variation of the parasitoid itself. The evolutionary relationships between the hosts and parasites sharing the same environment or nearby geographical regions may render them most suitable to each other than those living in different environments as reported on insect-parasitoid (Prevost *et al*, 2005) and on insect-

fungus interactions (Meshrif, unpublished data). Following oviposition of one parasitoid's egg into the 2nd instar of *D. melanogaster*, it was observed that other females of *A. tabida* tended to lay their eggs too in already parasitized larvae in a process called super-parasitism. The variation among isofemale lines contributed significantly to the rate of super-parasitism. This implies that the increase of eggs number within insect body may increase the chance to be recognized by the immune system due to increase of antigenecity. Otherwise, if they succeed to develop into larvae, they often act as competitors to each other seeking the limited food source available. Consequently, this may lead to incomplete development or increase mortality rate of pupae as reported by Prevost and Eslin (1998).

Upon reaching the oviposited eggs to the target location within larval body, there were 2 scenarios depending on the position and number of the parasitoid eggs in addition to the immune response of the host. The first scenario is that a part of the larvae succeeded in mounting the effective cellular encapsulation against single egg as reported in the current study. Moreover, the results indicated that the encapsulation rate and its efficiency were influenced by the variation of the *Drosophila* isofemale lines. The results can be supported by other observation in the current study on genetic variation among lines illustrated by the coefficient of intraclass correlation. The latter results is in consistent with the finding reported by Hoffmann *et al.* (2001) that for starvation and desiccation resistance, variation were highest among *Drosophila* isofemale line from the same population. Isofemale lines can provide an indication on the comparative genetic architectures among populations (Parsons, 1983). The line # 3 was designed as resistant one and the line # 9 was the susceptible one based on the results of encapsulation rate and efficiency. The results of emergence experiment confirmed previous ones supplied by dissecting experiment. Another part of the larvae, which represent most of the parasitized ones, showed inability to recognize and encapsulate the parasitoid eggs. In the second scenario, the dissecting results showed great success of the eggs to hatch and complete development within the *D. melanogaster* larvae (64.3-90.5%) and develop as feeding parasitoid larvae. These results were supported by high values of avoidance rate of the parasitoids (51.6-89.6%), indicating that most of the eggs laid by the parasitoid females had good chance to develop within the *D. melanogaster* rather than being encapsulated. By narrowing the isofemale lines into three, representing the extremes and the intermediate one, the results of emergence experiment showed that the success of parasitism was very lower than that observed in the dissecting experiment. This low success of the parasitoid in the larvae of *D. melanogaster* can be interpreted by the high mortality (55-73%). Surprisingly, it was found that the total encapsulation rate is inversely proportional to the mortality rate. This may

indicate that mounting an effective immune reaction against the parasitoid eggs does not affect the *Drosophila* physiology, but the death of parasitized *Drosophila* could be due to the inability of the parasitoid to regulate the host physiology as reported by Vinson and Iwantsch (1980).

In order to find out the relationships between the parameters following the dissecting experiment, correlation coefficient indicated that encapsulation rate of the *Drosophila* was inversely proportional to avoidance rate and successful parasitism rate, while the encapsulation efficiency rate was also proportional to super-parasitism rate. This means that the increase of eggs of parasitoids laid in individual *Drosophila* mostly induced higher percentage of recognition and encapsulation. Otherwise, the avoidance rate of the parasitoid was proportional to successful parasitism rate and the inversely proportional to super-parasitism rate.

Trade-off between the parasitoid resistance and other life history traits of *Drosophila* such as larval competitive ability and larval weight have been reported due to the parasitism by Kraaijeveld and Godfray (1997) and Moreau *et al.* (2002). In the current study, larval weight of the *Drosophila* larvae was not only influenced by the parasitoid infestation, but also was influenced by the isofemale line variation. The effect of the parasitism was only significant in resistant line (i.e. less resistant lines did not significantly lost weights compared with the controls). This information may indicate that the lost weights are not only due to parasitism, but could also be due to the encapsulation ability that requires substantial energy to maintain optimal functioning (Siva-Jothy *et al*, 1998). These costs paid either by susceptible or resistant strains of *D. Melanogaster* may interpret partly the high rates of mortality observed in the emergence experiment. So, the balance of resource allocation by the parasitized fly between defense and fitness traits is critical for life continuation as suggested by Fellowes *et al.* (1999).

The results of larval protein did not show any significant change due to parasitism nor isofemale line, but it is desirable to measure other energy reserves such as the lipids and carbohydrates. Parasitism and variation among isofemale line affected the phenoloxidase activity especially in the resistant line. This indicates that despite both susceptible and resistant lines had significantly higher phenoloxidase activity, phenoloxidase could contribute effectively to encapsulation in the resistant line. This observation could be supported by genomic study of *Drosophila-Asobara* interaction, that revealed up-regulation of JAK/STAT, Toll pathways and genes functioning with melanin deposition (Wertheim *et al*, 2005).

The coefficient of intraclass correlations of the studied traits showed high genetic variation among the isofemale lines based on the significant variability of the phenotypes, and heterogeneity of variances observed. Of course, the optimal is to find this variation among the lines under control

and parasitism conditions as observed in the phenoloxidase activity.

However, under quantitative traits such as what is measured herein; the phenotypic-based genetic variability may be only detected under one condition normal or stressed as what observed in the weight, and specific phenoloxidase activity, presumably because the within-line variance is more than the among–lines variance.

This can be interpreted with the findings of Pal (1998) who reported that genetic variation was suppressed in benign environment. Other traits were reported to have genetic variation under stress (Rohlfs, 2006). The traits can be more affected by within-line variance that may harbour significant genetic components (Petavy *et al*, 2004).

Conclusion

The variation of encapsulation ability of *A. tabida* and related traits in *D. melanogaster* could be due to different genetic makeup among isofemale lines. Although parasitized may suffer from weight loss, they could survive based on mounting effective immune response.

As future perspectives, *Drosophila* population may be considered promising to study evolution of host resistance under different settings such as geographical variation, different nutrients and parasites.

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