J Comp Physiol B (2011) 181:741–750
DOI 10.1007/s00360-011-0567-1

ORIGINAL PAPER

The NF-kB like factor DIF has weaker effects on *Drosophila melanogaster* immune defenses than previously thought

Éric Le Bourg

Received: 4 September 2010 / Revised: 4 March 2011 / Accepted: 8 March 2011 / Published online: 22 March 2011

Abstract The Toll pathway of *Drosophila melanogaster*, when activated by the *Beauveria bassiana* fungus, directs the expression of the *drosomycin* and *metchnikowin* antimicrobial peptide genes by inducing the translocation into the nucleus of the DIF transcription factor. Accordingly, DIF mutants have been reported to have a lower resistance to fungi than control flies. However, as the longevity of non-infected DIF flies has not been measured in previous studies, it could be that survival times after infection are constrained by a low longevity. In the present study, DIF flies reared in conditions similar to those used in these previous studies had much lower survival time after infection than the control flies, but the longevity of non-infected DIF flies was also very low. Using rearing conditions controlling larval crowding, age of parents and mating status of experimental flies increased longevity of non-infected flies and survival time after infection in both strains. However, DIF flies had a similar survival time after infection as control ones or a slightly lower one, which shows that the effect of DIF is weaker than previously thought.

Keywords Longevity · Fungal infection · DIF · *Drosophila melanogaster* · *Beauveria bassiana*

Communicated by G. Heldmaier.

Electronic supplementary material The online version of this article (doi:10.1007/s00360-011-0567-1) contains supplementary material, which is available to authorized users.

É. Le Bourg (✉)
Centre de Recherche sur la Cognition Animale,
UMR CNRS 5169, Université Paul-Sabatier,
118 route de Narbonne, 31062 Toulouse cedex 9, France
e-mail: lebourg@cict.fr

Abbreviations

CNBW	*Cinnabar brown* control strain
DIF	Dorsal-related immunity factor
NF-kB	Nuclear factor-kappa B
ANOVA	Analysis of variance
CONT	Controlled larval rearing conditions
UNCO	Uncontrolled larval rearing conditions

Introduction

Drosophila melanogaster is widely used by biologists for more than one century. This is due to the many advantages of this fly species such as, for instance, an easy and cheap rearing or a well-known genome. This fly is also a favorite model of biogerontologists to study the longevity and traits often considered to be best studied in mammal species, such as learning, behavior, and age-linked pathologies (see respectively e.g., Le Bourg 2004; Grotewiel et al. 2005; Iijima et al. 2004). *D. melanogaster* is also used to study immunity, and there are similarities between the innate immune system of flies and that of human beings (Martinelli and Reichhart 2005). Studies of immunity in flies rely on the measurement of survival time after infection and of longevity in non-infected flies (e.g., Taylor and Kimbrell 2007).

Flies express antimicrobial peptide genes when they are infected. The Imd pathway is activated by gram-negative bacteria and the Toll pathway by fungal infection and gram-positive bacteria. When activated by fungi, the Toll pathway directs the expression of the *drosomycin* and *metchnikowin* antimicrobial peptide genes (Lemaître et al. 1997) by inducing the translocation into the nucleus of the

DIF transcription factor (reviews in Lemaître and Hoffmann 2007; Martinelli and Reichhart 2005). *Dif*^{1} mutants have been reported to be more sensitive to fungal infection than wild-type flies and do not synthesize drosomycin after infection with the fungus *Beauveria bassiana* (Rutschmann et al. 2000a). However, drosomycin and the other antimicrobial peptides are not sufficient to protect against fungal infection, because infected flies die after infection despite the synthesis of these peptides. Furthermore, the ectopic expression of drosomycin, of another antimicrobial peptide, or of a combination of two peptides in flies deficient for both the Imd and Toll pathways does not rescue the protection against *B. bassiana* (Tzou et al. 2002), which shows that a single peptide and the tested combinations of two peptides are not sufficient to protect against this fungus.

Previous studies have shown that a cold stress at young age can increase survival time of flies infected by *B. bassiana* (Le Bourg 2010; Le Bourg et al. 2009). In an attempt to know whether this better resistance could be mediated by the Toll pathway, the *Dif*^{1} mutant and its control strain were used in the hope that a cold stress would increase survival in the infected control strain, but not in the *Dif*^{1} mutant (results on the effects of cold are not reported here). In the course of this study, we have observed that, in some conditions, *Dif*^{1} mutants could resist infection (nearly) as well as control flies.

In the following experiments, the survival time to infection by *B. bassiana* was recorded either in flies reared in conditions similar to those of Rutschmann et al. (2000a) and to those of other articles of their laboratory (UNCO condition) or in flies reared as in the laboratory of the author (CONT condition). In the CONT condition, the age of the parents of experimental flies is known, there is no larval crowding, and experimental flies are virgin. In the UNCO condition, experimental flies are coming from bottles in which flies had laid for a few days. Larval crowding is thus not controlled and high, the age of parents is unknown, and experimental flies are collected lately after the beginning of emergence, which implies that they are not virgin and their age is not precisely known. However, contrary to Rutschmann et al. (2000a), who infected "males or females", but did not indicate the sex of flies in their survival results, we separated males and females in our experiments. The sex of flies infected by *B. bassiana* is also not indicated in other articles (Gobert et al. 2003; Gottar et al. 2006; Michel et al. 2001; Rutschmann et al. 2000b), but Gobert (pers. comm.) has asserted that only females were used in her experiments: it is now known that males have a better resistance to *B. bassiana* than females (Le Bourg 2010; Le Bourg et al. 2009; Le Rohellec and Le Bourg 2009; Taylor and Kimbrell 2007). Previous studies of *Dif*^{1} flies did not record the longevity of non-infected flies, as confirmed by an author of some previous articles (Ferrandon, pers. comm.), and one could wonder whether their low survival is due to a low resistance to infection or to longevity differences between *Dif*^{1} and the control strain. Therefore, the present article also studied the longevity of *Dif*^{1} flies and its control strain in UNCO and CONT conditions.

Materials and methods

Flies

The experimental flies were adult males and females of the *y w DD1*; *cn bw* and *y w DD1*; *Dif*^{1}; *cn bw* genotypes (respectively, CNBW and DIF in the following). These strains have been previously described (Rutschmann et al. 2000a). The strains are maintained by mass-mating on the standard medium (agar, sugar, corn meal and killed yeast) containing a mold inhibitor (*para*-hydroxymethyl-benzoic acid) and enriched with live yeast at the surface of the medium.

In order to obtain the parents of the experimental flies of the CONT condition, flies of unknown age were allowed to lay eggs for one night in a bottle containing the medium described earlier. Flies emerging from this bottle 9–11 days after egg-laying were transferred to bottles (ca. 25 pairs in a bottle) containing the medium previously described: these flies are the parents of the experimental flies. Experimental CONT flies were obtained as follows: eggs laid by 3–5 day-old parents during 15 h period on a petri-dish containing the usual medium colored with charcoal and a drop of live yeast were transferred by batches of 25 into 80-ml glass vials containing ca. 15 ml of the medium described earlier. At emergence, virgin flies with duration of preimaginal development of 9–10 days were transferred under ether anesthesia in groups of 15 flies of the same sex to 20 ml polystyrene vials containing ca. 5 ml of the standard medium with a drop of live yeast. However, one experiment, reported in "Supplemental material", used both virgin and mated flies: for these experiments, vials contained either 16 virgin males or females or 8 pairs of flies.

In the UNCO condition, experimental flies are coming from 250-ml bottles (ca. 100 ml of medium and live yeast) in which flies of unknown age had laid for 2–4 days. Larval crowding is thus not controlled and high, which delayed development by 2 days or more when compared with the CONT condition; the age of parents is unknown; and experimental flies are harvested only once, about 2 days after the beginning of emergence. These flies are thus not virgin and their age is not precisely known. About 2 days after the beginning of emergence, experimental non-virgin

flies of the UNCO condition were thus transferred under ether anesthesia from bottles in groups of 15 flies of the same sex to 20 ml polystyrene vials containing 5 ml of the standard medium with a drop of live yeast.

Flies of UNCO and CONT conditions were transferred to new vials twice a week; they spent their life in an incubator; the rearing temperature was 25 ± 0.5°C; light was on from 0700 to 1900 hours (fluorescent lamp).

Infection procedure

The spores of the fungus *B. bassiana* kept at −80°C in 20% glycerol were incubated at 25°C in 90 mm petri dishes containing the appropriate medium (for 1 l of distilled water, the autoclaved medium contained: peptone (Sigma P463): 1 g, glucose (Fluka 49159): 20 g, malt extract (Fluka 70167): 20 g, agar: 15 g). After sporulation, which occurs ca. 4 weeks after spreading spores on the medium, flies were infected.

At the day of infection, flies were transferred to new vials before to be very slightly anaesthetized with ether and then shaken for ca. 1 min in a petri dish containing a sporulating fungal culture. After having checked under stereomicroscope that all flies were well covered with spores, flies were transferred back to their vials. The main difference between this infection procedure and that of Lemaître et al. (1997) is the fungus incubation temperature and flies rearing temperature after infection, which is 25°C here and 29°C in Lemaître et al. (1997). We reasoned that transferring flies from 25 to 29°C would be an extra-stress and that using the same temperature throughout experiment allows observing solely the effects of infection. Even at 25°C, the fungus was virulent: this procedure has been used with success in previous experiments (Le Bourg 2010; Le Bourg et al. 2009; Le Rohellec and Le Bourg 2009).

Longevity experiments in CONT and UNCO conditions

Longevity was recorded daily until the death of the last fly, either from emergence or from the day following infection, depending on experiments. However, as imagoes of the UNCO condition were harvested only once, about 2 days after the beginning of emergence (see above), emergence of these flies was arbitrarily considered to have occurred the day before harvesting flies.

Resistance to heat in CONT conditions

Resistance to heat was studied in non-infected CONT flies as an attempt to know the physiological state of CNBW and DIF flies. The survival time in a water-bath set at 37°C was visually observed at 19 days of age, i.e., at the end of the mortality plateau of both DIF and CNBW strains (see survival curves in Fig. 4). It was expected that middle-aged flies of the shorter-lived strain could be frailer and less resistant to heat than those of the most longevous strain.

Flies were transferred just before shock into empty polystyrene vials (diameter 17 mm, length 63 mm), the plug containing absorbent cotton with distilled water to prevent desiccation. Flies were observed every 5 min and those totally immobile were considered to be dead. Ten flies were used in each group of sex and genotype but, due to a technical failure, only eight DIF females were used. This procedure is routinely used in the laboratory of the author.

Statistical analyses

Longevity and survival times results were analyzed with factorial analyses of variance (ANOVA) testing, depending on the experiment, for the effect of sex, infection, genotype, age of infection, rearing condition, mating status, and all interactions. Data could be transformed to obtain a normal distribution of residuals. Percentages were analyzed with χ^2 tests.

Results

Survival to infection and longevity in UNCO conditions

In a first experiment, we aimed to verify that DIF flies have a lower survival time to infection than CNBW ones in UNCO conditions. These conditions are similar to those used by e.g., Gobert et al. (2003), except that fungus and flies were reared at 25°C and not at 29°C (see "Materials and methods"). Flies were infected at 2–4 days of age. ANOVA showed that DIF flies survived for a shorter time (Fig. 1, mean ± SEM: 2.63 ± 0.16 days) than CNBW ones (6.11 ± 0.18 days; $F(1, 205) = 210.06$, $p < 0.0001$): the survival time of DIF flies was 43% of that of CNBW ones. Sex and its interaction with genotype were not significant ($Fs < 1$). A replicate experiment with 229 flies provided similar results (statistical analysis not shown): the survival time of DIF flies was 56% of that of CNBW ones, DIF flies surviving for ca. 4 days and CNBW ones for 7 days.

However, the conclusion of a lower survival time to infection of DIF flies could be challenged if the longevity of non-infected flies would be very low, i.e., close to their survival time after infection. As previous authors did not measure longevity of DIF and CNBW flies in UNCO conditions, it is necessary to perform this experiment. Longevity data were log-transformed before to be analyzed with an ANOVA. DIF flies lived shorter (8.33 ± 0.81 days) than CNBW ones

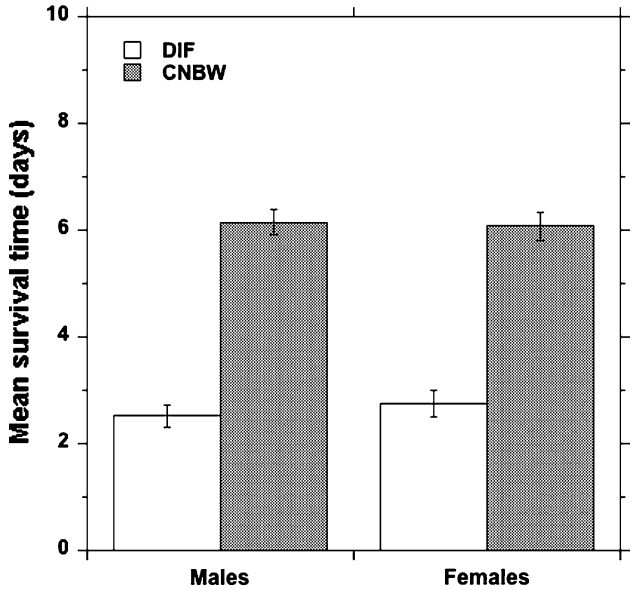

Fig. 1 Mean survival time ± SEM after infection of DIF and CNBW flies in the UNCO condition (see text). Infection was done at 2–4 days of age. Each *bar* is the mean of 49–55 flies

(13.39 ± 1.03 days; $F(1, 195) = 17.70$, $p < 0.0001$), the sex effect and its interaction with genotype being not significant ($Fs < 1$). Particularly, nearly 15% of DIF flies died the first 2 days of life, while it was the case for only ca. 5% of CNBW flies: it could be that DIF flies heavily suffered from the poor living conditions in the bottles before being transferred to vials. The longevity difference between DIF and CNBW non-infected flies was similar to that previously observed in infected flies. In addition, the mean longevities of these non-infected flies were not really different from those of infected flies (see Fig. 1), if we take into account that longevity was measured from emergence in the present experiment and from the day after infection in the previous ones.

Therefore, a new experiment measured longevity of infected and non-infected flies to confirm that infection decreases longevity in UNCO conditions. Longevity data were log-transformed before to be analyzed with an ANOVA and flies dead before 4 days of age, i.e., the day after infection, were removed from the results (10% of DIF flies and 1% of CNBW ones). CNBW flies lived longer than DIF ones ($F(1, 313) = 229.30$, $p < 0.0001$) and infection slightly decreased longevity ($F(1, 313) = 16.24$, $p < 0.0001$). The sex effect and all interactions were not significant. Particularly, the absence of a significant interaction between the infection and strain factors shows that infection decreased longevity to the same extent in CNBW and DIF flies. Results were similar when longevity was measured from emergence (Fig. 2). A replicate experiment ($n = 339$) provided very similar results (data not shown). In conclusion, CNBW and DIF flies reared in the UNCO condition have a low longevity and infection decreases it to the same extent in both strains.

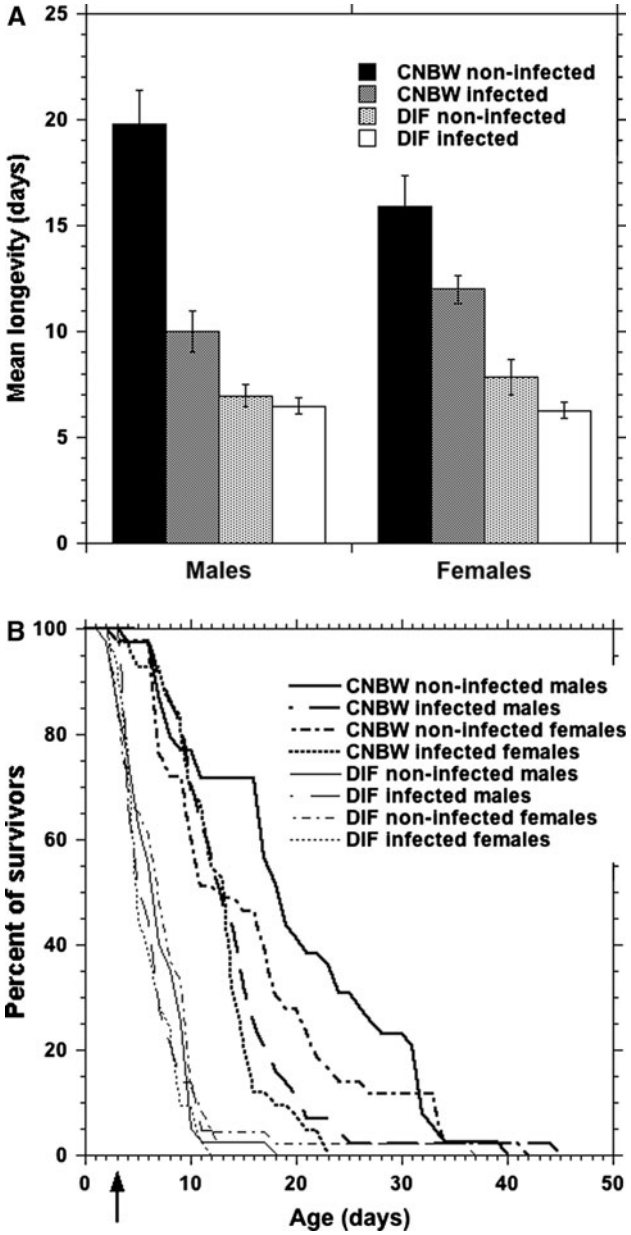

Fig. 2 **a** Mean longevity ± SEM of infected and non-infected DIF and CNBW flies in the UNCO condition. Infection was done at 3 days of age but this figure shows longevity since emergence. Each *bar* is the mean of 39–46 flies. **b** Survival curves of males and females as a function of genotype. The *arrow* under the axis of abscissas shows the day of infection. Ten percent of DIF flies were already dead at this age, but only one percent of CNBW ones

Physiological state of non-infected flies in CONT conditions

It can be suspected that UNCO conditions are not optimal for DIF and CNBW flies because longevity in UNCO

conditions is low. It could be that better conditions would increase longevity and improve physiological state. Therefore, flies reared under the CONT condition, the usual condition to measure longevity and resistance to stress in our lab, were assessed for their longevity and resistance to a lethal stress. In addition, during routine rearing of flies, it was possible to measure the viability of the eggs and the sex-ratio of emerging flies reared under the CONT conditions.

χ^2 tests showed that the viability of CNBW flies (i.e., the percentage of eggs reaching adulthood) was significantly lower than that of DIF ones in six experiments out of nine and higher in two experiments (Fig. 3). The sex-ratio was close to 50% of females in all experiments and in both genotypes, except for DIF flies of the Rep9 replicate for which it was ca. 56 ± 3.90% (other data not shown).

The longevity of non-infected flies of the 46/2008 group was measured. CNBW flies had a lower longevity than DIF ones (Fig. 4, $F(1, 207) = 62.52$, $p < 0.0001$). Females lived slightly longer than males ($F(1, 207) = 9.25$, $p = 0.0027$), and the sex by genotype interaction was not significant. CNBW flies lived for ca. 25 days and DIF ones for ca. 36 days. This lower longevity of CNBW flies was confirmed in other experiments where DIF males and females had, respectively, a mean lifespan of 34 and 40 days ($n = 55$ in each sex), whereas these values were 23 and 27 days in CNBW flies ($n = 55$ or 60 in each sex). Therefore, both DIF and CNBW flies lived much longer in CONT conditions than in UNCO ones and the direction of the strain-linked longevity difference was reversed: CNBW flies outlived DIF ones in UNCO conditions, but the contrary was observed in CONT conditions.

Regarding resistance to lethal heat, CNBW flies were, however, not inferior to DIF ones, as shown by the ANOVA of survival time at 37°C ($F < 1$). Females survived for a longer time than males ($F(1, 34) = 13.16$, $p = 0.0009$, means of males and females, respectively: 114.25 ± 6.63 and 181.67 ± 7.79 min). However, the sex by genotype interaction was not significant.

This set of results shows that CNBW and DIF flies differ by their physiological state: CNBW flies have lower longevity and viability than DIF ones in CONT conditions, but they have a similar resistance to lethal heat. However, it happened that the viability of CNBW flies was higher in two experiments out of nine.

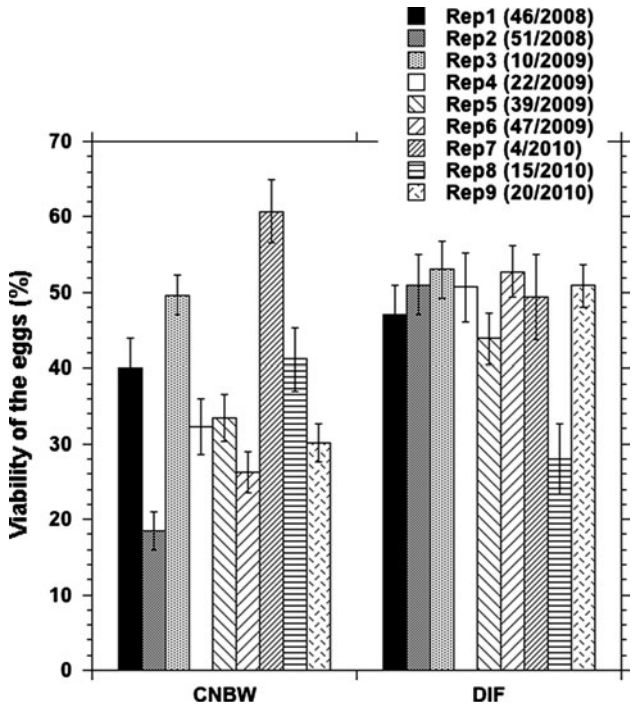

Fig. 3 Mean viability of the eggs ± confidence interval (at $p = 0.05$) of DIF and CNBW flies. The number of flies emerging from the CONT condition was counted in several replicates (Rep1 to Rep9) during the course of experiments. For each replicate and genotype, the number of harvested eggs was in the range 300–1,400 and there was 25 eggs/vial. The figure only shows data for which the two strains were observed at the same time (in parentheses, the order of the week in the year). The only non significant difference between CNBW and DIF flies was observed for the week 10 of 2009 (Rep3). CNBW flies had a lower viability than DIF ones in the other replicates, except in the replicates 7 and 8 for which CNBW flies had a higher viability

Survival to infection in CONT conditions

When DIF and CNBW flies are reared in UNCO conditions, their survival time after infection is dependent on

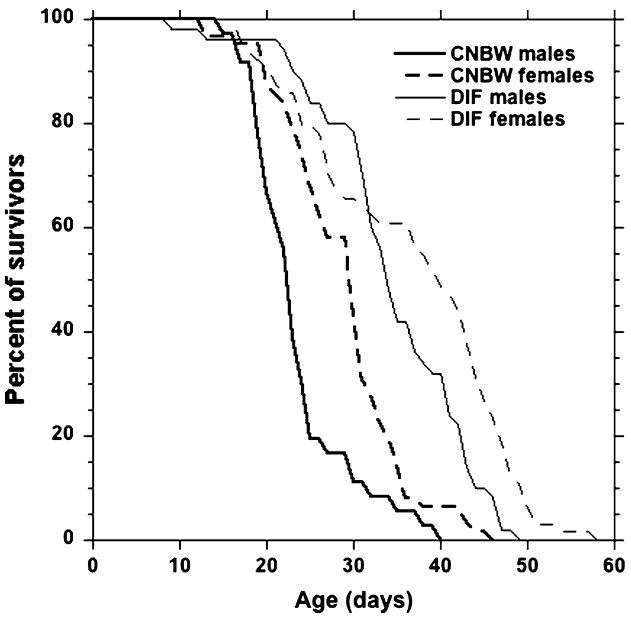

Fig. 4 Longevity curves of CNBW and DIF flies. The number of flies is 36 for CNBW males (mean longevity ± SEM: 23.67 ± 0.94 days), 61 for CNBW females (28.69 ± 0.85 days), 50 for DIF males (34.58 ± 1.21 days), and 64 for DIF females (36.92 ± 1.40 days)

their longevity (see Fig. 2). To verify it was also the case in CONT conditions, we measured longevity of infected and non-infected flies of the 10/2009 group in CONT conditions, applying infection at an age close to the mean longevity of the CNBW strain, i.e., 19 days of age. Since this age is close to the mean longevity of CNBW flies (see Fig. 4), we could expect to observe that infected DIF flies outlive CNBW ones, if the DIF transcription factor is of no importance to resist infection.

Figure 5 reports longevity after the day of infection. DIF flies outlived CNBW ones ($F(1, 350) = 24.31, p < 0.0001$) and females lived longer than males ($F(1, 350) = 10.00$, $p = 0.0017$). The significant sex by strain interaction showed that the sex effect was mainly observed in CNBW flies ($F(1, 350) = 5.38$, $p = 0.0209$). Infection decreased longevity ($F(1, 350) = 139.80, p < 0.0001$) and the infection by strain interaction ($F(1, 350) = 22.30$, $p < 0.0001$) showed that infected flies of both strains had the same longevity after infection, ca. 7 days, whereas non-infected DIF flies (ca. 17 days) outlived CNBW ones (ca. 12 days), as previously observed (see Fig. 4). The other interactions were not significant (*Fs* close to 1).

Therefore, in CONT conditions, non-infected flies outlive infected ones by 5 and 10 days, respectively, in CNBW flies and DIF ones, whereas infected flies of both strains survived for the same time. Survival time after infection applied at middle age is thus not correlated to the longevity of DIF and CNBW strains. Another experiment using a lower number of only infected flies (51/2008 group) confirmed that, when infection was done at 19 days of age, CNBW flies survived for the same time as DIF ones (ca. 6 days, data not shown).

These experiments show that it is possible to disconnect survival time after infection from longevity of non-infected flies, but they do not show that, in absolute terms, DIF flies have a lower survival than CNBW ones. This could be explained by the absence of effect of the DIF transcription factor. It could also be that the DIF factor is required to survive infection at young age, but not at middle age. Finally, CNBW flies could survive as long as DIF ones simply because the age at infection, 19 days, is close to the mean longevity of CNBW flies. If the latter explanation were correct, CNBW flies would survive longer if infected at younger ages. Studying survival to infection at younger ages is thus necessary to clarify the issue.

Virgin DIF and CNBW flies of the 39/2009 group were subjected to infection at 4, 12, or 18 days of age, i.e., 1, 2 or 3 weeks of age: this experiment did not use non-infected flies. Some CNBW flies were observed to be moribund just after infection and we decided to discard all flies dying the first 2 days after infection, i.e., six CNBW and one DIF 4-day-old males, and three 12-day-old CNBW females were discarded. As most discarded flies were of the CNBW

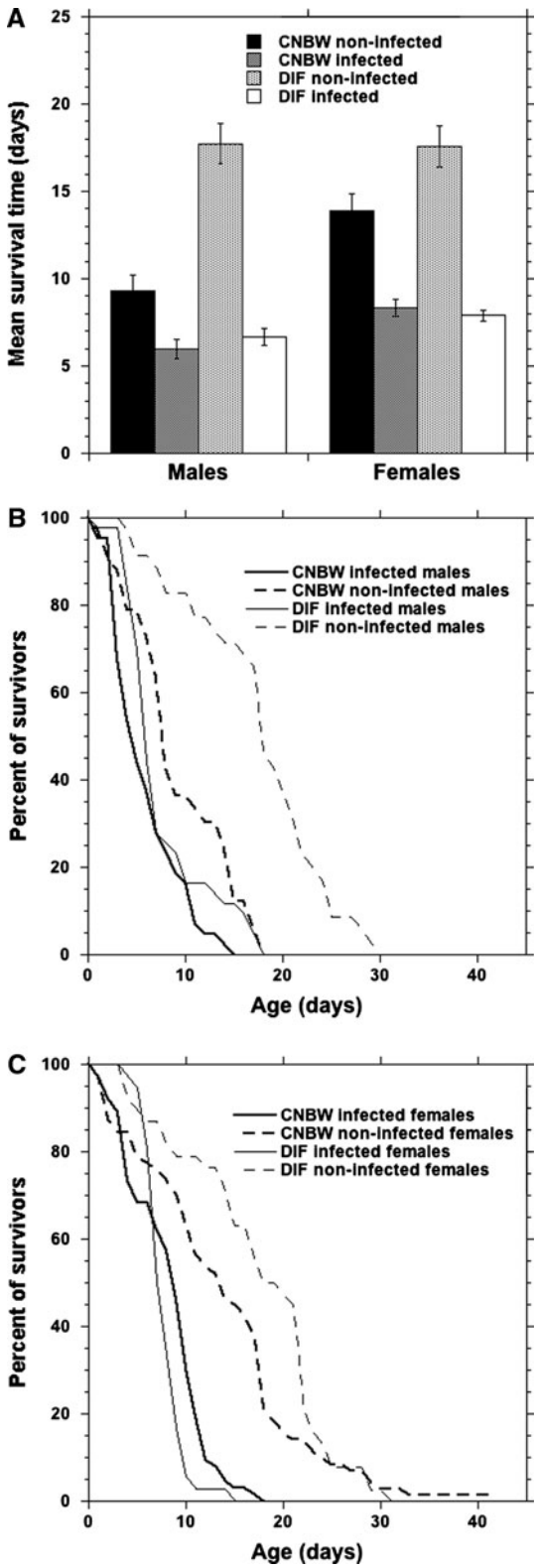

Fig. 5 **a** Mean survival time ± SEM of infected and non-infected DIF and CNBW flies from the CONT condition. Infection was done at 19 days of age and day 0 is the day of infection. Each *bar* is the mean of 36–63 infected flies or 33–71 non-infected ones. **b** and **c** Survival curves of males and females, respectively, as a function of genotype, infection status, and sex. Day 0 is the day of infection

group, this obviously increased the lifespan of CNBW flies. The survival times of the remaining 504 flies were log-transformed before to be analyzed with ANOVA.

DIF flies survived for a shorter time after infection (ca. 11 days) than CNBW ones (ca. 13 days, $F(1, 492) = 13.74$, $p = 0.0002$, Fig. 6a). The sex effect was nearly significant ($F(1, 492) = 3.58$, $p = 0.059$), males tending to survive 1 day longer than females. The significant sex by genotype interaction ($F(1, 492) = 9.65$, $p = 0.0020$) showed that DIF females survived for a shorter time than males, whereas no sex-related difference was observed in CNBW flies. Age of infection had a significant effect ($F(2, 492) = 11.07$, $p < 0.0001$), the lowest survival time being observed when infection was done at 12 days of age. The significant sex by age interaction showed that this effect of age was mainly due to females ($F(2, 492) = 6.71$, $p = 0.0013$) and the significant age by genotype interaction ($F(2, 492) = 3.96$, $p = 0.0196$) showed that DIF flies had a low survival time when infection was done at 12 days of age. The second-order interaction was not significant. When the results of each age were analyzed separately, the genotype effect was significant only in 12-day-old flies, the mean differences between strains being ca. 2, 3, and 0 day when infection were done, respectively at 4, 12, or 18 days of age.

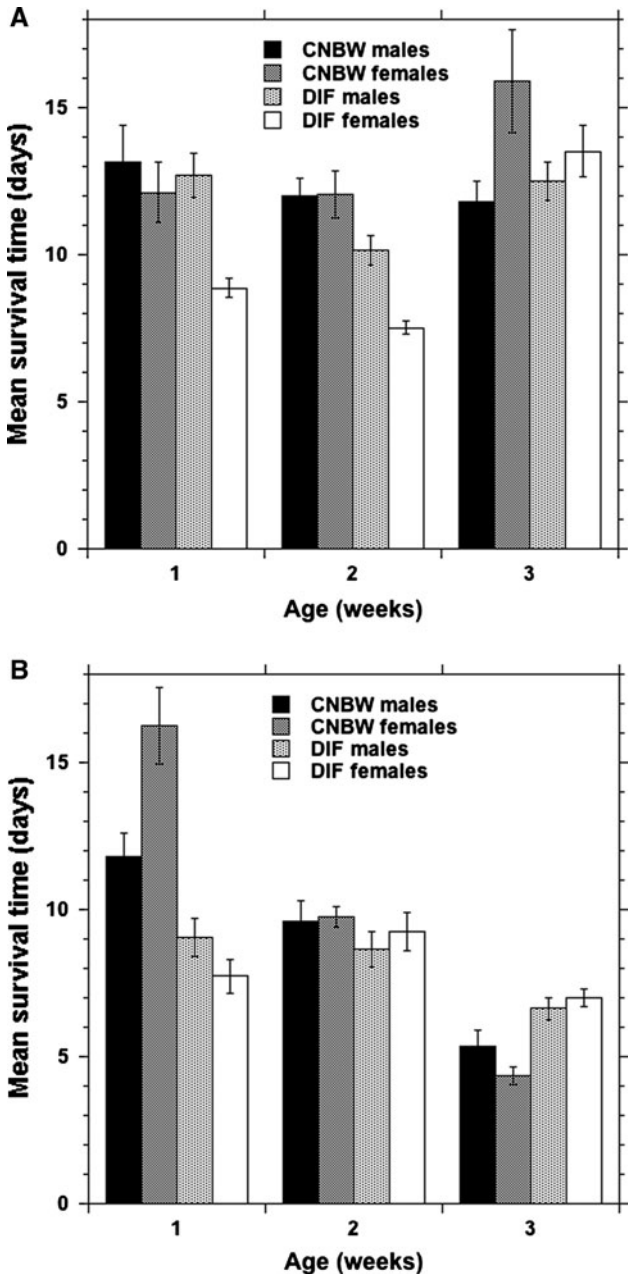

Fig. 6 **a** Mean survival time ± SEM after infection of DIF and CNBW flies from the CONT condition. Infection was done at 4, 12, or 18 days of age (week 1, 2, or 3 on the figures). Each *bar* is the mean of 19–63 flies. **b** Mean survival time ± SEM after infection of DIF and CNBW flies from the CONT condition in the replicate experiment. Infection was done at 5, 12, or 19 days of age (week 1, 2, or 3 on the figures). Each *bar* is the mean of 33–60 flies

This experiment was replicated. Virgin DIF and CNBW flies of the 20/2010 group were subjected to infection at 5, 12, or 19 days of age, i.e., 1, 2 or 3 weeks of age. This experiment also used a group of non-infected flies and ANOVA of longevity of these non-infected flies showed that females lived longer than males ($F(1, 211) = 6.66$, $p = 0.0105$), but DIF flies only marginally lived longer than CNBW ones ($p = 0.0841$). The sex by genotype interaction was not significant. Mean longevities (±SEM) of male and female CNBW flies were 22.32 ± 1.20 and 24.11 ± 1.58 days, respectively; those of DIF flies being 22.61 ± 1.60 and 30.00 ± 1.95 days. Thus, in this experiment, flies, particularly DIF ones, lived for a shorter time than in the previous ones (see Fig. 4). Survival times after infection were square-root-transformed before to be analyzed with ANOVAs. Infected DIF flies survived for a shorter time (ca. 8 days) than CNBW ones (ca. 9 days, $F(1, 532) = 10.09$, $p = 0.0016$, Fig. 6b). The sex effect and its interaction with age of infection were not significant ($Fs < 1$). Age of infection had a significant effect ($F(2, 532) = 63.28$, $p < 0.0001$), survival time decreasing with age at infection. The significant age by genotype interaction ($F(2, 532) = 35.08$, $p < 0.0001$) showed that CNBW flies survived longer than DIF ones if infection was done at 1 week of age, had a similar survival time when infection was done at 2 weeks of age, and a lower one when infection was done at 3 weeks of age. The significant second-order interaction was mainly due to the high survival time of CNBW females infected at 1 week of age ($F(2, 532) = 6.83$, $p = 0.0012$). When the results of each age were analyzed separately, the genotype effect was highly significant in 1-week-old flies ($F(1, 196) = 47.90$, $p < 0.0001$), CNBW flies surviving 5 days longer than DIF ones. The genotype effect was not significant at 2 weeks of age ($F(1, 182) = 2.89$, $p = 0.0905$), and it was significant

at 3 weeks of age ($F(1, 154) = 32.72$, $p < 0.0001$), CNBW flies surviving 2 days shorter than DIF ones.

Therefore, these two experiments show that infected DIF flies could survive for a shorter time than CNBW ones, but this effect was significant only at 2 weeks of age in the first experiment and at 1 week of age in the replicate experiment. In the replicate experiment, DIF flies survived longer than CNBW ones when infection was done at 3 weeks of age, and this result can probably be explained by the low longevity of CNBW flies.

As flies are mated and subjected to larval crowding in UNCO conditions, but not in CONT ones, other experiments were done to know whether mating status or larval crowding could explain why DIF flies have not a very shorter survival time after infection than CNBW ones in CONT conditions. However, mating status and larval crowding had no effect on survival to infection (see Online Resource).

Discussion

These experiments have shown that a large effect of the DIF transcription factor on immune defenses is observed when flies are reared in UNCO conditions (larval crowding, mated flies, age of parents and duration of development unknown), as reported in previous studies of this mutant (e.g., Rutschmann et al. 2000a): young DIF flies, which do not synthesize drosomycin after infection with *B. bassiana*, survive for ca. 3 days to fungal infection and CNBW ones for ca. 6 days (Fig. 1). Therefore, in UNCO conditions, DIF flies had a lower survival time to infection (ca. 50%) than CNBW flies. This result is similar to those of e.g., Gobert et al. (2003) or Gottar et al. (2006), even if it is somewhat difficult to compare the present results with previous ones, because these authors did not report mean survival times but only survival curves.

However, the conclusion that the DIF transcription factor is important to resist fungi is challenged because in UNCO conditions non-infected DIF flies live for a very few days (see e.g., Fig. 2). Therefore, survival times after infection of DIF flies are constrained by this very low longevity and infected CNBW flies thus survive longer than DIF ones. In conclusion, experiments on flies reared in UNCO conditions do not allow to conclude that the DIF transcription factor is of any use to resist fungal infection because the low survival after infection of DIF flies appears to reflect their low longevity in UNCO conditions.

It could be expected that the use of controlled rearing conditions (CONT) would increase longevity and reveal an effect of the DIF transcription factor on resistance to fungi. Indeed, CONT conditions increased mean longevity in CNBW flies (by ca. 1 week) and more importantly in DIF ones (up to 4 weeks, compare e.g., Figs. 2 and 4). CNBW flies could survive longer than DIF ones when infected, but this effect was not always significant when infection was done at 1 or 2 weeks of age. When flies were infected at 3 weeks of age, DIF and CNBW flies had a similar survival time and DIF flies could even survive longer than CNBW ones, probably because this age was close to the mean longevity of CNBW flies. Therefore, the DIF transcription factor can be of some help to resist fungal infection, at least at young age, but this effect can be low or even absent. Figure 7 summarizes the relative survival of DIF flies infected at less than 1 week of age in UNCO conditions and at 1, 2 or 3 weeks of age in CONT conditions. This figure clearly shows that the relative survival of DIF flies is higher in CONT conditions than in UNCO ones.

Studying resistance to infection in CONT conditions promoting higher longevity of non-infected flies leads to somewhat different conclusions to those which are deduced from experiments in UNCO conditions. UNCO conditions decrease longevity to the extent that studying resistance to infection is meaningless, as the survival time to infection of DIF flies is only a mere consequence of their low longevity. The only means to really observe an effect of the DIF transcription factor is to use rearing conditions promoting high longevity. In such conditions, infected DIF flies can survive up to 4 days shorter than CNBW ones, but this effect is often weaker and even absent. These experiments thus do not show that DIF has no effect on resistance to infection. Rather, they show that this effect is weaker than previously thought, not always observed, and dependent on the age of flies, as it is not observed at 3 weeks of age, probably because the longevity of CNBW flies is close to this age.

These results showing a weak role for DIF are maybe not so surprising because double mutants deficient for the Toll and Imd pathways, i.e., flies unable to resist both bacterial and fungal infections, are not rescued against *B. bassiana* infection by ectopic expression of antimicrobial peptides, and particularly drosomycin (Tzou et al. 2002). Thus, even if *B. bassiana* induces the expression of drosomycin (see e.g., Fig. 5 in Taylor and Kimbrell 2007), this peptide is probably of a secondary importance to resist infection. As concluded by Lemaître and Hoffmann (2007) in a review article, "the mechanisms mediating Toll resistance against *B. bassiana* have yet to be established".

It seems that no longevity study of DIF flies was done before the present article (Ferrandon, pers. comm.). Concerning CNBW flies, i.e., *cinnabar brown* mutants, results are scarce. Narise (1974) reported that the mean longevity of mated females was 42 days at 25°C and that the percentage of eggs reaching adulthood (viability) was 62%. Hiraizumi (1985) reported that mean longevity of mated females was around 19 days at 23–24°C and Civetta and

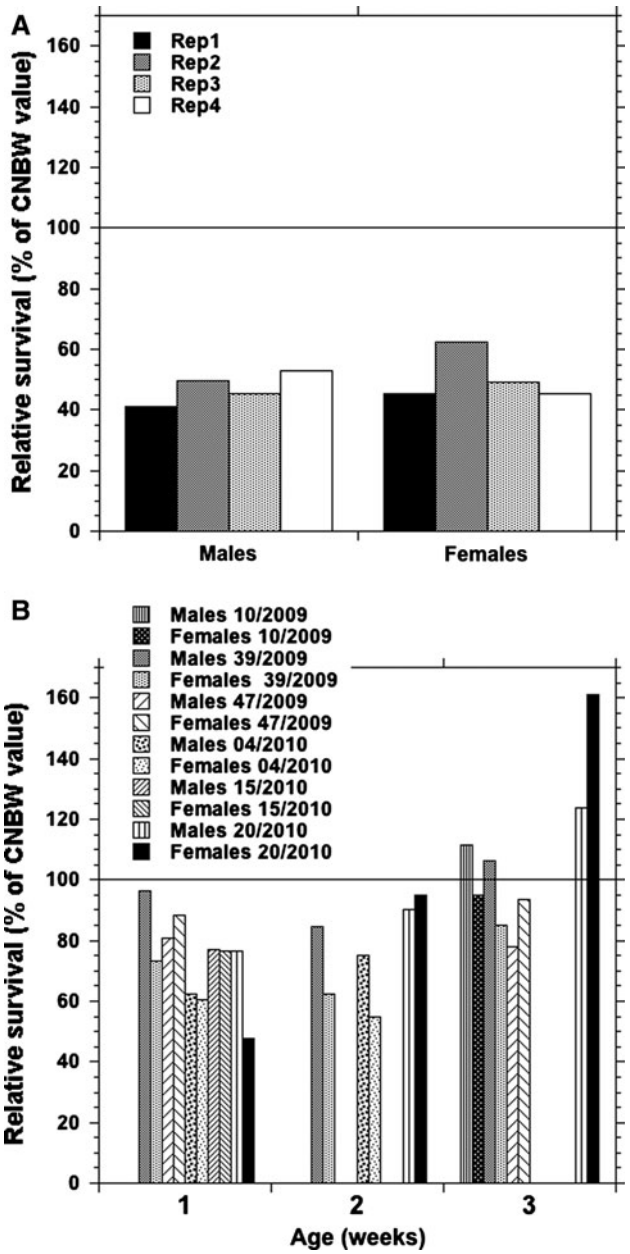

Fig. 7 Relative survival time after infection of DIF flies compared to that of CNBW flies of the same sex observed in the same experiments. A *line* shows the 100% level, i.e., when DIF and CNBW flies have the same survival time. **a** Experiments in UNCO conditions at young age. The order of the replicates follows the order of results presentation in the text and includes the replicates for which no detailed results were described. **b** Experiments in CONT conditions. As in Fig. 3, the numbers indicate the order of the week in the year. Virgin flies were reared under the CONT condition and infection was applied at 1, 2 or 3 weeks of age. Two experiments (39/2009 and 20/2010) were done at each of these three ages, and the others were done at one or two ages

Clark (2000) that it was 43 days (at 25°C) in virgin females and between 37 and 53 days in mated ones. In the present CONT conditions, virgin *cn bw* flies lived for ca. 25 days and viability was in the 20–60% range. By contrast, DIF flies could live for ca. 35 days and their viability was rather stable and around 50% (Fig. 3). Paradoxically, DIF flies appear to be healthier than CNBW ones when reared in CONT conditions, even if no strain difference was observed concerning survival time at 37°C.

DIF flies cannot live long if reared in the poorly controlled UNCO conditions and longevity is not rescued by providing after emergence the same conditions as those used in CONT conditions (vials renewed twice a week containing 15 flies of the same sex: see "Materials and methods"). The effect of UNCO conditions is not mediated solely by larval crowding or mating activity at a very early age (see Supplemental material). Rearing in bottles is a very harsh condition and it is probable that only robust flies can survive this challenge. DIF flies thus appear to be fragile and unable to live long if subjected during their development to these conditions. Is it due to a direct effect of the mutation they bear? For instance, it could be hypothesized that bottles are replete of fungi which kill DIF flies soon after emergence. If so, one could expect that routine rearing of DIF flies in bottles would be impossible, but they were observed to thrive in bottles like other strains. The reason for the low longevity of DIF flies in UNCO conditions thus remains unknown, but one can conclude that providing flies with rearing conditions promoting normal longevity should be a prerequisite before studying their survival time to a stress, particularly, if this time is rather long as it is the case for resistance to fungi.

However, a curious result of this study is that a mutant suffering from a lower resistance to infection, at least if infection occurs at 1 or 2 weeks of age (see Fig. 7b), can outlive its short-lived control strain (ca. 35 vs. 25 days) and has often a higher viability in CONT conditions (Fig. 3). A new, and unexpected, effect of DIF is thus a longevity and viability increase if flies are reared in CONT conditions. This is a clear genotype-environment interaction: the effect of DIF is detrimental in UNCO conditions, but it can be beneficial in CONT ones, at least as far as longevity and viability are concerned.

Acknowledgments Many thanks are due to Isabelle Massou for preparing the sporulating cultures of *Beauveria bassiana* and to Vanessa Gobert for providing the strains *y w DD1*; *cn bw* and *y w DD1*; *Dif*^{1}; *cn bw*, her helpful advices and stimulating discussions. Thanks are also due to the anonymous referees for their helpful comments.

References

Civetta A, Clark AG (2000) Correlated effects of sperm competition and postmating female mortality. Proc Natl Acad Sci USA 97:13162–13165

Gobert V, Gottar M, Matskevich AA, Rutschmann S, Royet J, Belvin M, Hoffmann JA, Ferrandon D (2003) Dual activation of the *Drosophila* Toll pathway by two pattern recognition receptors. Science 302:2126–2130

Gottar M, Gobert V, Matskevich AA, Reichhart JM, Wang C, Butt TM, Belvin M, Hoffmann JA, Ferrandon D (2006) Dual detection of fungal infections in *Drosophila* via recognition of glucans and sensing of virulence factors. Cell 127:1425–1437

Grotewiel MS, Martin I, Bhandari P, Cook-Wiens E (2005) Functional senescence in *Drosophila melanogaster*. Ageing Res Rev 4:372–397

Hiraizumi Y (1985) Genetics of factors affecting the life history of *Drosophila melanogaster*. I. Female productivity. Genetics 110:452–464

Iijima K, Liu HP, Chiang AS, Hearn SA, Konsolaki M, Zhong Y (2004) Dissecting the pathological effects of human aβ40 and aβ42 in *Drosophila*: a potential model for Alzheimer's disease. Proc Natl Acad Sci USA 101:6623–6628

Le Bourg E (2004) Effects of aging on learned suppression of photopositive tendencies in *Drosophila melanogaster*. Neurobiol Aging 25:1241–1252

Le Bourg E (2010) Combined effects of suppressing live yeast and of a cold pretreatment on longevity, aging and resistance to several stresses in *Drosophila melanogaster*. Biogerontol 11:245–254

Le Bourg E, Massou I, Gobert V (2009) Cold stress increases resistance to fungal infection throughout life in *Drosophila melanogaster*. Biogerontol 10:613–625

Le Rohellec M, Le Bourg E (2009) Contrasted effects of suppressing live yeast from food on longevity, aging and resistance to several stresses in *Drosophila melanogaster*. Exp Gerontol 44:695–707

Lemaître B, Hoffmann J (2007) The host defense of *Drosophila melanogaster*. Annu Rev Immunol 25:697–743

Lemaître B, Reichhart JM, Hoffmann JA (1997) *Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proc Natl Acad Sci USA 94:14614–14619

Martinelli C, Reichhart JM (2005) Evolution and integration of innate immune systems from fruit flies to man: lessons and questions. J Endotoxin Res 11:243–248

Michel T, Reichhart JM, Hoffmann JA, Royet J (2001) *Drosophila* Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. Nature 414:756–759

Narise T (1974) Relation between dispersive behavior and fitness. Jpn J Genet 49:131–138

Rutschmann S, Jung AC, Hetru C, Reichhart JM, Hoffmann JA, Ferrandon D (2000a) The Rel protein DIF mediates the antifungal but not the antibacterial host defense in *Drosophila*. Immunity 12:569–580

Rutschmann S, Jung AC, Zhou R, Silverman N, Hoffmann JA, Ferrandon D (2000b) Role of *Drosophila* IKKg in a Toll-independent antibacterial immune response. Nat Immunol 1:342–347

Taylor K, Kimbrell DA (2007) Host immune response and differential survival of the sexes in *Drosophila*. Fly 1:197–204

Tzou P, Reichhart JM, Lemaître B (2002) Constitutive expression of a single antimicrobial peptide can restore wild-type resistance to infection in immunodeficient *Drosophila* mutants. Proc Natl Acad Sci USA 99:2152–2157