Innate immunity, developmental speed and their trade-offs in two hexapod models

Cheng, S.

Chapter 3

Correlated responses to selection for embryonic developmental time in *Tribolium castaneum*

Shixiong Cheng, Chris G.C. Jacobs, Kevin M. Bretscher, Femke Verweij, Sven Ungerer, Maurijn van der Zee

Abstract

Life-history trade-offs have played a central role in theory and interpretation of life history studies. However, understanding fitness trade-offs remains a difficult task in evolutionary biology. Here, we test if developmental time trades off with (1) pupal weight, (2) fecundity and (3) immune response in the flour beetle *Tribolium castaneum*. To this end, we artificially selected *Tribolium* eggs for fast or slow embryonic development during 21 and 17 generations, respectively. This resulted in embryonic developmental times of 125 h in the fast lines, 136 h in the non-selected lines, and 155 h in the slow line. As correlated response, larval developmental time also differentiated significantly among the selection treatments. In these selection lines, we find that (1) pupae of the slow lines are significantly heavier than pupae of the other lines. We also find that (2) 50 mothers of the fast lines lay on average 123 eggs in 4 hours, whereas 50 mothers from the non-selected lines and slow lines lay significantly more eggs, i.e. 155 and 160 respectively. Finally, using qPCR, we find significant upregulation of antimicrobial peptides (AMPs) and prophenoloxidase upon bacterial infection in all eggs, but (3) upregulation of these immune genes did not differ consistently among the selection lines. In conclusion, we found that developmental time significantly trades off with pupal weight and fecundity in *Tribolium*, but found no evidence for a trade-off with immune defense.

Introduction

The life history of an organism refers to the pattern of events that occur from birth to death related to survival and reproduction, along with the timing and occurrence of each of these events (Fox and Messina, 2013). Life-history traits (or fitness components) include egg production per brood, age-specific distribution of growth rates, size of young, the interaction of reproductive effort with adult mortality, the age distribution of reproductive effort, patterns of dormancy and dispersal, and so on (Stearns, 1976). Life-history traits are often correlated within a species. For instance, as a result of climate change, pied flycatchers optimize over a suite of different life-history traits including their laying date, clutch size and the onset of incubation. With the advance of the peak of caterpillars that act as their nestling food, the birds advanced their laying date, shortened their incubation time, and increased the clutch size to maximize their lifetime fitness (Both and Visser, 2005).

When one trait changes upon selection, correlated responses in other traits are widely observed in laboratory experiments and breeding programs as a consequence of selection (Rauw et al., 1998). For

example, a line of flies propagated by breeding old adults had longer development time from egg to adult, than fly lines in which only young flies were allowed to propagate (Partridge and Fowler, 1992). These changes in other traits than the trait under selection are called correlated responses. This change is necessarily dependent on a genetic covariance or correlation (Gromko, 1995).

Owing to finite resources in organisms, life-history traits are often negatively related to each other, resulting in trade-offs. Life-history trade-offs are associated with the strategies that organisms use for maximizing the sum of the costs and benefits (Roff, 2000). Competing requirements of growth, maintenance and reproduction prevent sole allocation of limited resources to a single trait, such as immune defense (Figure 1-8). No matter if the risk of infection is high or low, immune defense trades off with other life-history traits (Adamo et al., 2001; Schmid-Hempel, 2005). Trade-offs between immune function and growth have been demonstrated in birds and plants (Brommer, 2004; Lozano-Durán et al., 2013).

Insects are the most diverse and successful organisms and they inhabit almost all habitats and ecosystems (Grimaldi and Engel, 2005). The insect immune defense is an important component of fitness. In response to microbial infections, insects have evolved a strong and effective innate immune system consisting of interconnected cellular and humoral responses (Vilmos and Kurucz, 1998). The cellular immune response is carried out by many types of blood cells (hemocytes) which can phagocytose and kill pathogens. The humoral immune response of insects is based on the synthesis of various of antimicrobial peptides (AMPs) activated by immune signaling pathways, i.e., Toll, IMD and JAK/STAT. These AMPs are released into hemolymph to eliminate pathogens. To date, 311 AMPs have been characterized in insects, such as defensins, cecropins, attacins and moricins (Manniello et al., 2021; Tsakas and Marmaras, 2010). Despite these responses, exposure to microbial pathogens still pose serious threats to developing insects, in particular during embryonic development.

In recent years, it has clearly been demonstrated that insect eggs are able to mount immune responses to protect the embryo (Cole et al., 2020; Gorman et al., 2004; Jacobs et al., 2014a; Jacobs et al., 2022). In addition, insects such as *Tenebrio molitor* (Moret, 2006) and *Tribolium castaneum* (Roth et al., 2009) can transfer specific immune protection to the offspring. Salmela et al. reported that the transfer of immunity is mediated via the egg-yolk protein vitellogenin (Salmela et al., 2015). Furthermore, others features of the insect egg, such as the complex chorion and the cuticle, can protect the embryo from adverse conditions, such as desiccation and drowning (Zeh et al., 1989). Developing insect embryos are well protected from pathogens and adverse environmental conditions.

As the most diverse organisms on Earth, insects can provide excellent models for addressing trade-offs between immune competence and other life-history traits. Trade-offs among immune reactions and other traits, such as growth and reproduction, have been observed in a diverse range of insect species, for instance the tobacco hornworm *Manduca sexta* (Diamond and Kingsolver, 2011; Mira and Bernays, 2002; Schwenke et al., 2016). ln the host laboratory, it has been shown that eggs of the burying beetle *Nicrophorus vespilloides* lack an inducible innate immune response. At the same time, *N. vespilloides* eggs are extremely sensitive to desiccation (Jacobs et al., 2014b). Strikingly, its eggs develop extremely quickly and hatch in two days at 25°C. The model insect *D. melanogaster* eggs hatch within 24 hours at 25°C and develop extremely quickly as well. Its eggs have lost desiccation resistance and immune function too (Al-Saffar et al., 1995; Jacobs and van der Zee, 2013). Thus, it seems that egg immune competence trades off with embryonic developmental speed in insects.

In *T. castaneum*, Zou et al. reported 12 antimicrobial peptides (AMPs) involved in microbe immobilization or killing of which attacin2, cecropin3, coleoptericin1, defensin1, and defensin2 showed the most dramatic increase in transcript levels to participate in immune responses (Zou et al., 2007). As previously shown in the eggs of *T. castaneum*, all AMPs showed strong upregulation upon bacterial infection, such as attacin1, attacin2, cecropin3, coleoptericin1, defensin1 and defensin2 (Jacobs and van der Zee, 2013). Therefore, in this study, the induction profiles of mRNAs of *attacin1*, *attacin2*, *cecropin3*, *coleoptericin1* and *defensin2* will be compared in the selection lines of *Tribolium* eggs. We will also quantify the expression of *laccase2*, a prophenoloxidase involved in the melanisation reaction observed during insect immune defenses (González-Santoyo and Córdoba-Aguilar, 2012).

In this study, replicate outbred populations of *T. castaneum* had been selected for fast or slow embryonic development for over 21 or 17 generations, respectively. Correlated responses, namely correlated post-embryonic development time, were studied in these selected populations. Furthermore, we used these selection lines to explore trade-offs with the fitness components fecundity and pupal weight. We also used this emerging insect model *T. castaneum* to study a possible trade-off between immune defense and the duration of embryonic development. We quantified the expression of immune-related genes in the eggs of the selected populations upon simultaneous infection with gram-positive and gram-negative bacteria.

Materials and methods

Artificial selection for embryonic developmental time in *Tribolium* populations

An outbred population was created by individually crossing 250 beetles which came from a bakery in The Netherlands (collected by the Dutch pest control company Rentokil) with 250 beetles from the inbred *San Bernardino* laboratory strain. This outbred population could mix and reproduce for another seven generations. From this starting population, 2h egg lays were collected. Two of these egg lays were left unselected (Non-selectedA, Na; non-selectedB, Nb). From another egg lay, we continued to select the fastest developing half for the fast line (fastA, Fa), and the slowest developing half for the slow line (slowA, Sa), and, repeated this in a biological replicate (fastB, Fb; slowB, Sb) to check for consistent differences. The selection for embryonic developmental time was carried out in a semi-automated set-up, see Figure 3-1. A brief process diagram of the artificial selection experiment is shown in Figure 3-2. All populations were kept bigger than 500 individuals in every generation. Beetle cultures were kept as in (van der Zee et al., 2005), except for the temperature which was 25 °C.

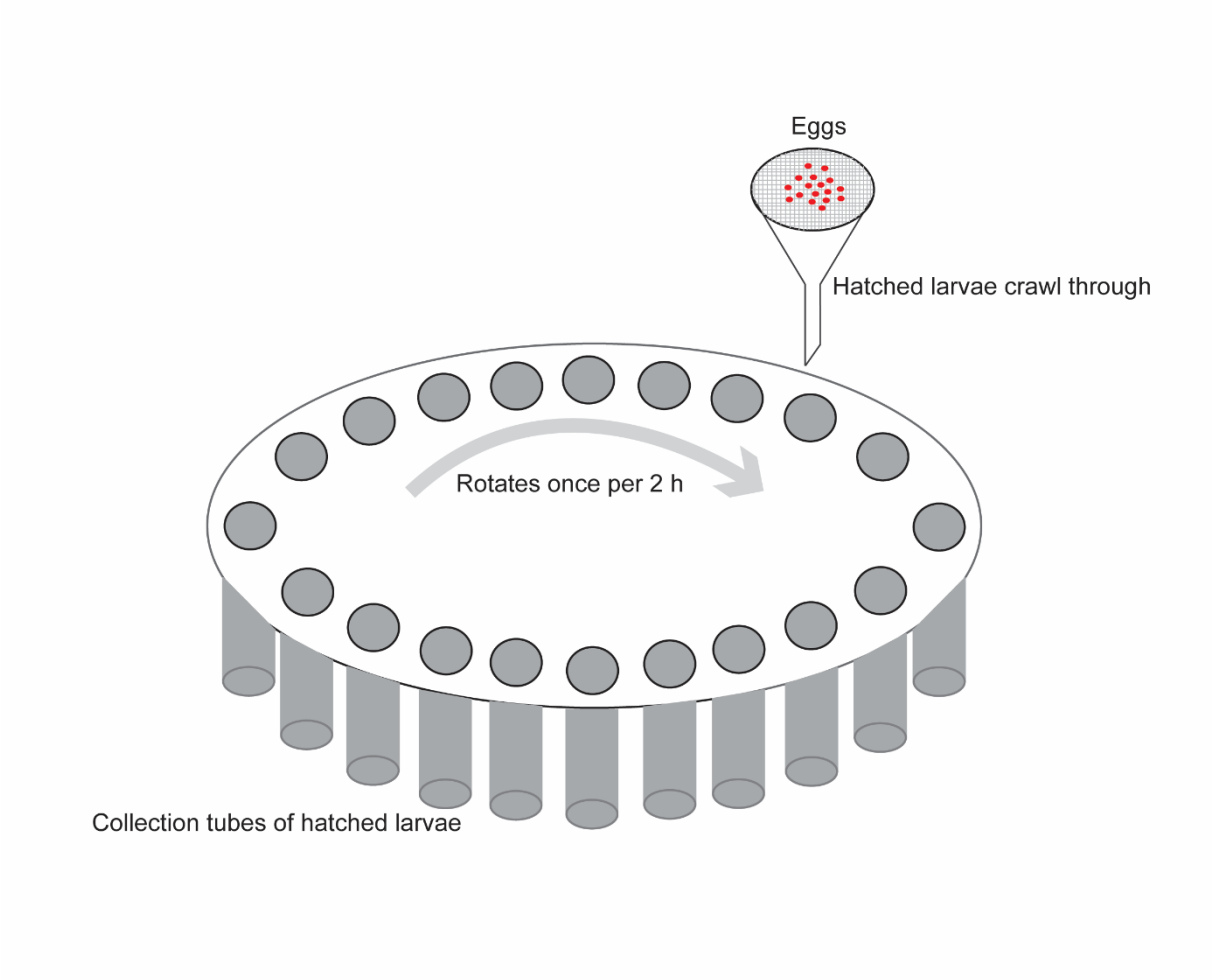

Figure 3-1. The fast or slow embryonic developmental time of *T. castaneum* were artificially selected on the rotary selection machine. The selection machine turns once per two hours in a clockwise pattern. *T. castaneum* eggs were maintained at 25°C until hatching. Once one egg hatched, it will crawl through into the below collection tube.

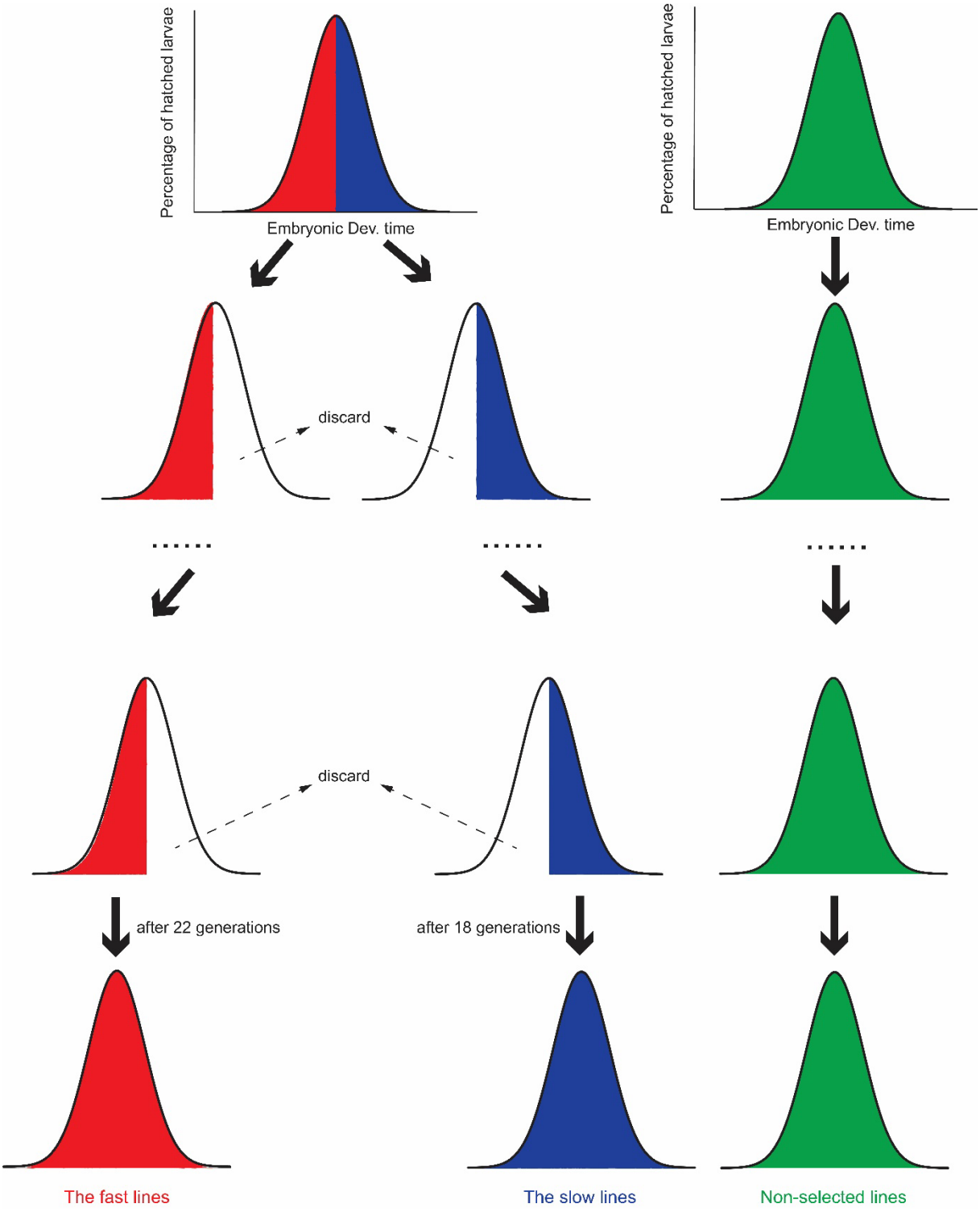

Figure 3-2. A brief process diagram of the artificial selection experiment in order to obtain our selection lines including the fast (red), non-selected (green) and slow lines (blue).

Weight

The pupae of the selection lines were weighed using a high-precision analytical balance directly after moulting from the prepupal stage (XS 105, Mettler Toledo, Germany).

Fecundity assays

To monitor fecundity among selection populations, we mixed 50 male and 50 female pupae of *T. castaneum*. After five days when they became fertile adults, we started to calculate the number of eggs laid within 4 h. Six biological replication were performed for selection lines including Fa, Fb, Na, Nb, Sa and Sb, respectively. Furthermore, we also summarize their eclosion rate when these eggs eclosed to adults.

Infection

Infection experiments were carried out as described in (Jacobs and van der Zee, 2013). All infections were performed with a tungsten needle with a 1 micron tip (Fine Science Tools). *Tribolium* eggs (24 h old) of the fast, the non-selected and the slow lines were pricked with a tungsten needle dipped in a concentrated mixed suspension of *Micrococcus luteus* and *Escherichia coli*, as standardized in our laboratory (Jacobs et al., 2013; Jacobs and van der Zee, 2013). A sterile injection served as the control. In total of 300 eggs were injected in each treatment. Six hours later, eggs were used for RNA isolation.

RNA extraction and RT-qPCR

The total RNA of 300 eggs was extracted using trizol extraction (Invitrogen) after which the RNA was purified using the RNeasy kit (Qiagen). The quality of isolated RNA was measured spectrophotometrically and on a 1.0% agarose gel. One microgram of total RNA was used for cDNA synthesis. First strand cDNA was made using the Promega Reverse Transcription system (Protocol, 2000), and 2 µL of 1:10 diluted original cDNA was used in every RT-qPCR reaction. RT-qPCR reactions were done using the SsoAdvanced Universal SYBR Green Supermix (Bio-rad, Hercules, CA, USA) on a CFX96 thermocycler (Bio-rad, Hercules, CA, USA). The RT-qPCR parameters were as follows: an initial step at 95 °C for 30s; 40 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s. This was followed by dissociation analysis of a ramp from 65 °C to 95 °C with a read every 0.5 °C. The RPL13a gene was used as internal control to calculate ΔC_T values (Lord et al., 2010). Fold upregulation was then calculated using the $2^{-\Delta\Delta C}T$ method (Livak and Schmittgen, 2001). Total RNA was isolated three times (biological replication) and each sample was measured by RT-qPCR twice (technical replication). The immune-related genes of *T. castaneum* studied here were: attacin1, attacin2, cecropin3, coleoptericin1, defensin2 and laccase 2. The primers were listed in Supplementary Table 3-1.

Statistical analysis

All data were analyzed through one-way analysis of variance (ANOVA) by SPSS software (version 27). Statistical significance was set based on Duncan's multiple range test at the 5% probability level.

Results

Developmental time of eggs of the fast lines is one day shorter than that of slow lines

During artificial selection process, a selective pressure was created for embryonic development time by using fastest or slowest 50% of the hatchlings for fast and slow lines, respectively, while two

populations were left unselected. This selection during 21 generations in the fast lines, and 17 generations in the slow lines (Figure 3-3), resulted in embryonic developmental time of on average 125 hours for the two fast lines, 137 and 136 hours for the two non-selected lines (Na and Nb, respectively), and more than 154 hours for the two slow lines (Figure 3-4).

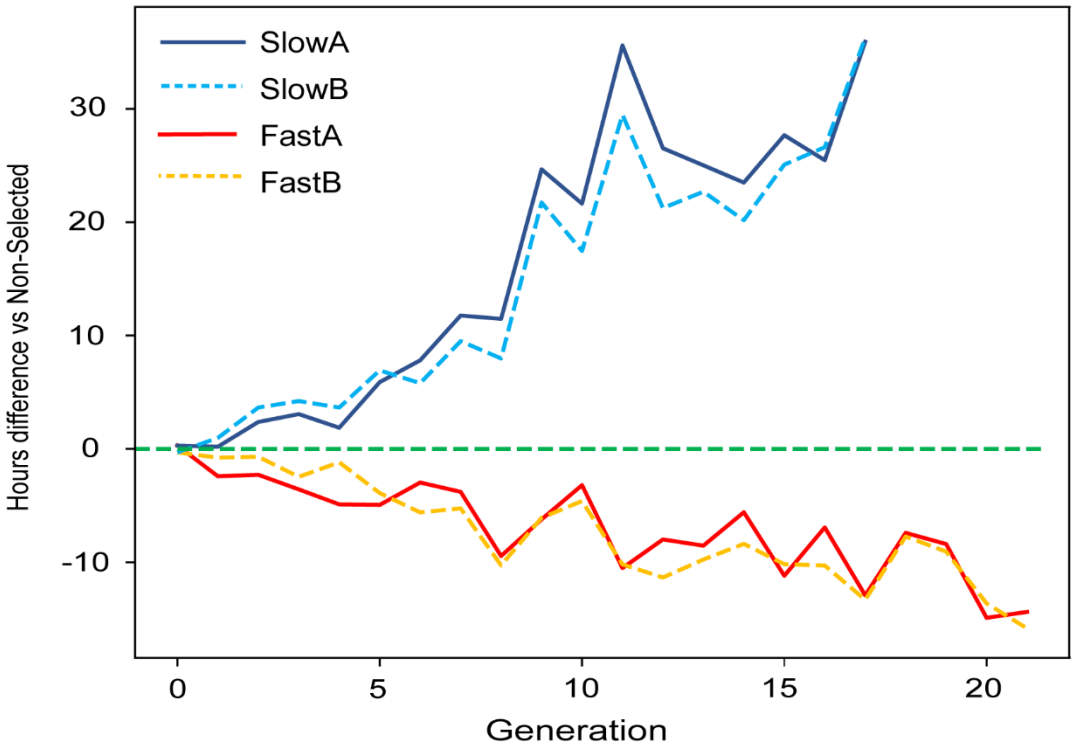

Figure 3-3. Time difference of fast and slow lines in every generation, compared to the non-selected lines. Red, orange, dark blue, and light blue lines stand for time difference of Fa, Fb, Sa and Sb respectively, compared to the non-selected lines (green).

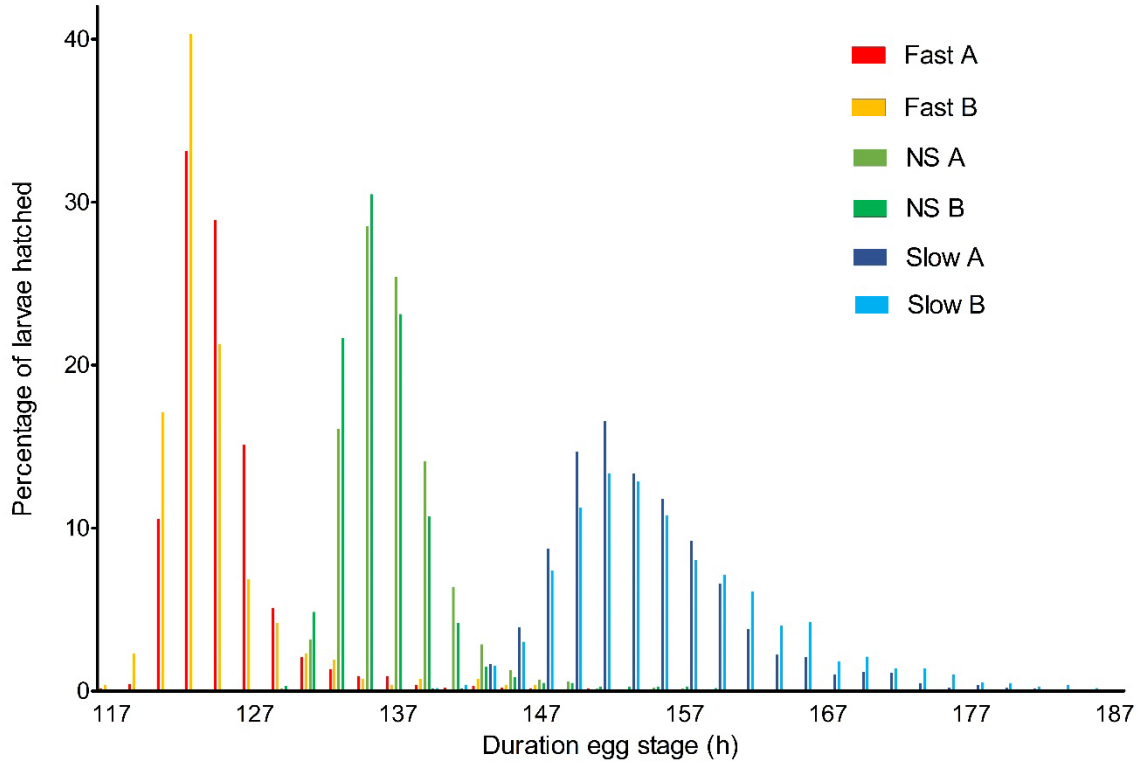

Figure 3-4. Hatching time of eggs of the selection lines. Fast, non-selected and slow lines showed the highest hatching rate at 123, 135 and 151 h post egg laying, respectively. Red, orange, green, light green, dark blue, and light blue lines stand for percentage of hatched larvae of Fa, Fb, Na, Nb, Sa, and Sb, respectively.

Correlated responses to selection in post-embryonic developmental time

To check for correlated differences in post-embryonic development, we further measured larval and pupal developmental time in the selection lines at 25 °C. We found that newly hatched larvae of the Na and Nb lines spend on average 63.5 and 63.1 days during post-embryonic development before reaching the imago stage, while the Fa and Fb lines spend 49.88 and 50.73 days, and the Sa and Sb lines spend 68 and 64.6 days, respectively (Table 3-1). In conclusion, larval developmental time of fast lines is significantly shorter than non-selected and slow lines (Figure 3-5A). Although there is a trend towards shorter pupal time in the fast lines, there is no significance of pupal developmental time among the selection lines (Figure 3-5B). At the same time, growth rate (mg/day) did not differentiate between the fast and slow lines (Supplementary Figure 3-1).

Table 3-1. Post-embryonic development time from newly hatched larvae to emergence of imago. Significance of post-embryonic development time among six selection lines was confirmed by one-way analysis of variance (ANOVA). Different superscript letters display significant difference ($P < 0.05$).

Selection lines	Days from newly hatched larvae to emergence of imago	
	Mean ± SD	N
Fa	49.88 ± 4.77[a]	33
Fb	50.73 ± 4.03[a]	33
Na	63.53 ± 6.58[b]	36
Nb	63.13 ± 5.35[b]	40
Sa	68.03 ± 5.69[c]	33
Sb	64.61 ± 5.42[b]	36

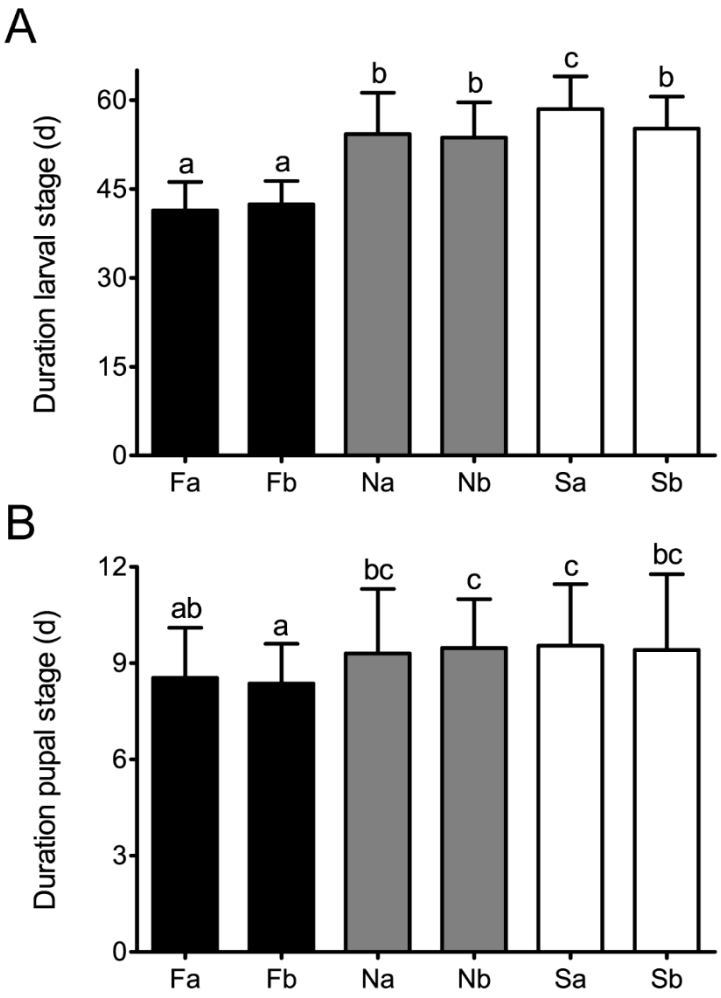

Figure 3-5. The duration of larval (A) and pupal (B) stages of the selection lines. Each vertical bar indicates Mean ± SD (n = 33 of Fa, 33 of Fb, 36 of Na, 40 of Nb, 33 of Sa, and 36 of Sb, respectively). Different letters above the vertical bar display significant difference ($P < 0.05$).

Trade-offs

Heavier individuals of the slow lines

The pupae of the slow lines were significantly heavier than the non-selected and fast lines, as shown in Figure 3-6A. The same is true for adult weight. The adults of the slow lines were significantly heavier than the non-selected and fast lines (Figure 3-6B). At the same time, the adults of the slow B line were

significantly heavier than the slow A line (Figure 3-6B). We found no significant difference in weight between female and male individuals considering all six selection lines together, as confirmed by a Welch two-samples t-test (Supplementary Table 3-2 and Supplementary Table 3-3). Within each selection line, females were significantly heavier than males only in the Sb line for both pupal and adult weight (Supplementary Table 3-2 and Supplementary Table 3-3).

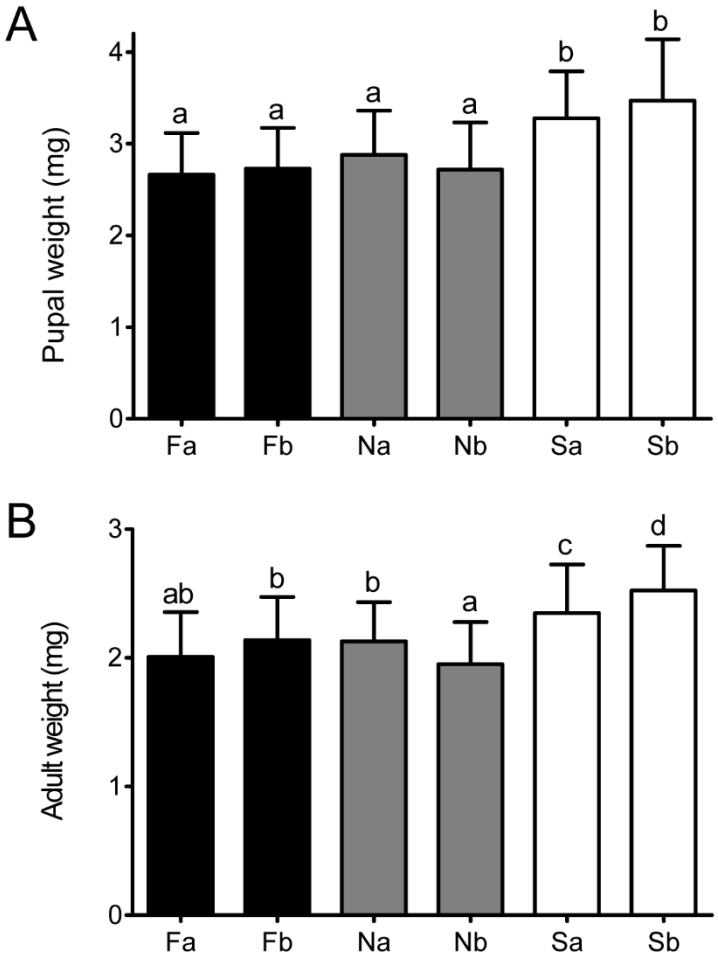

Figure 3-6. A, Weight of pupae directly after moulting from the prepupae stage. Each vertical bar indicates Mean ± SD (n = 46 of Fa, 40 of Fb, 36 of Na, 41 of Nb, 38 of Sa, and 43 of Sb, respectively). B, Weight of adults of the selection lines. Each vertical bar indicates Mean ± SD (n = 33 of Fa, 33 of Fb, 36 of Na, 40 of Nb, 33 of Sa, and 38 of Sb, respectively). Key: different letters above the vertical bar display significant difference ($P < 0.05$).

Low early fecundity of the fast lines

To compare the egg production capacity, we counted the egg number from 50 mothers of the fast (Fa and Fb), the non-selected (Na and Nb) and the slow lines (Sa and Sb) laid within 4 h. The results showed that the fast line beetles laid significantly fewer eggs than the non-selected and the slow lines (Figure 3.7A). To be precise, Fa, Fb, Na, Nb, Sa and Sb-*T. castaneum* laid on average 124, 123, 156, 155, 153 and 166 eggs from 50 adult females within 4 hour, respectively (Figure 3-7A). Interestingly, the egg number of the Sb line beetles is significantly larger than that of the others. We did not find significant differences in final successful eclosion rate of the offspring that is counted from egg to adult among the selection lines, which was showed in Figure 3-7B.

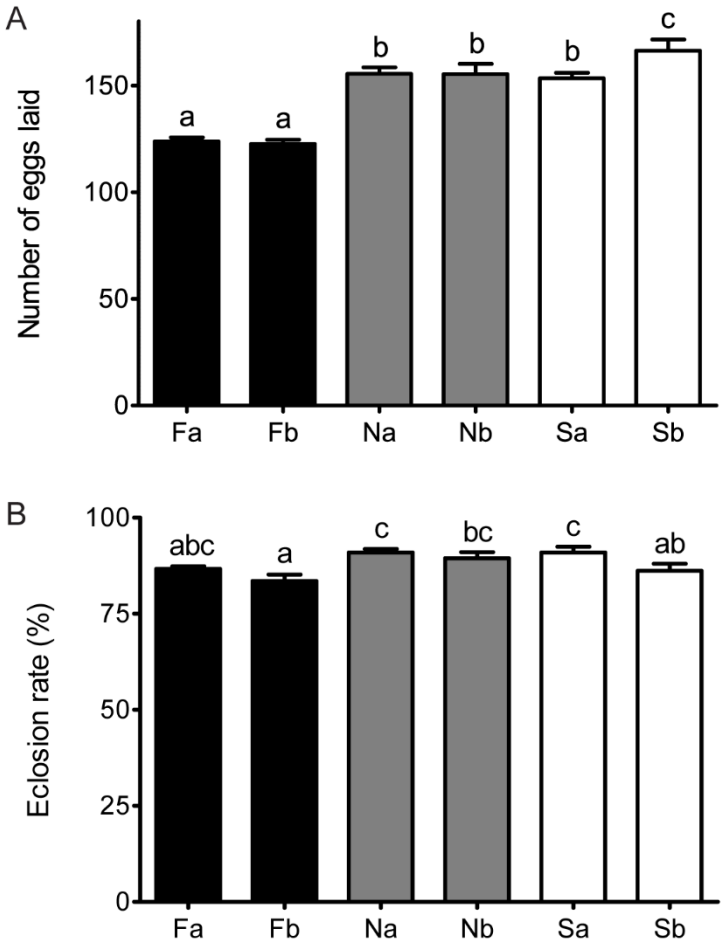

Figure 3-7. A, Fecundity of 50 *T. castaneum* females laid within 4 h among the selection lines. B, Eclosion rate (from eggs to adults) of *T. castaneum* among the selection lines. Each vertical bar indicates Mean ± SE (n = 6). Different letters above the vertical bars display significant difference ($P < 0.05$).

Induction of immune genes in eggs of the selection lines

Finally, we measured changes in the expression of several antimicrobial peptides (AMPs) and *laccase2* at 6 h post bacterial challenge in septically injured eggs compared to sterilely injured eggs. In all selection lines, the six genes showed strong upregulation upon bacterial infection (Figure 3-8). However, no consistent differences were found between the fast and slow lines. The details of the induction of immune genes were as follows:

a) Attacins

Induction profiles of mRNAs of *attacin1* and *attacin2* are shown in Figure 3-8. There are no significant differences in the up-regulation of *attacin1* in the selection lines, except that upregulation is significantly higher in Sb than in Fa, Na and Sa (Figure 3-8A). Upregulation of *attacin2* was significantly higher in the Fb and Sb lines, compared with the Fa, Na and Sa lines (Figure 3-8B).

b) Cecropins

Upon bacterial infection, we examined gene expression of *cecropin3* in *Tribolium* eggs of the selection lines. The expression change of *cecropin3* is significantly higher in the Fb line than in the Fa line, but no consistent difference between the fast and slow lines was found (Figure 3-8C).

c) Coleoptericin

In all tested genes, *coleoptericin1* showed the strongest upregulation among the selection lines in response to bacterial challenge. At the same time, upregulation of *coleoptericin1* was significantly higher in the Sb line compared to all other lines, except the Fb line. Furthermore, a significantly higher upregulation of *coleoptericin1* was found in the Fb line compared with the Fa, Na, and Sa lines, but no consistent difference between the fast and slow lines was found (Figure 3-8D).

d) Defensins

Upon bacterial infection, upregulation of *defensin2* was significantly higher in the Fb and Sb lines compared to the other selection lines, but no consistent difference between the fast and slow lines was found (Figure 3-8E).

e) Laccase

Laccase2 showed the relatively weak upregulation in response to bacterial infection compared with these AMPs. Significance in the expression changes of *laccase2* is similar to that of *cecropin3*. Upregulation in Fb is significantly higher than in Fa, but no consistent differences were found between the fast and slow lines (Figure 3-8F).

Thus, our expression data do not indicate a clear trade-off between developmental speed and immune competence.

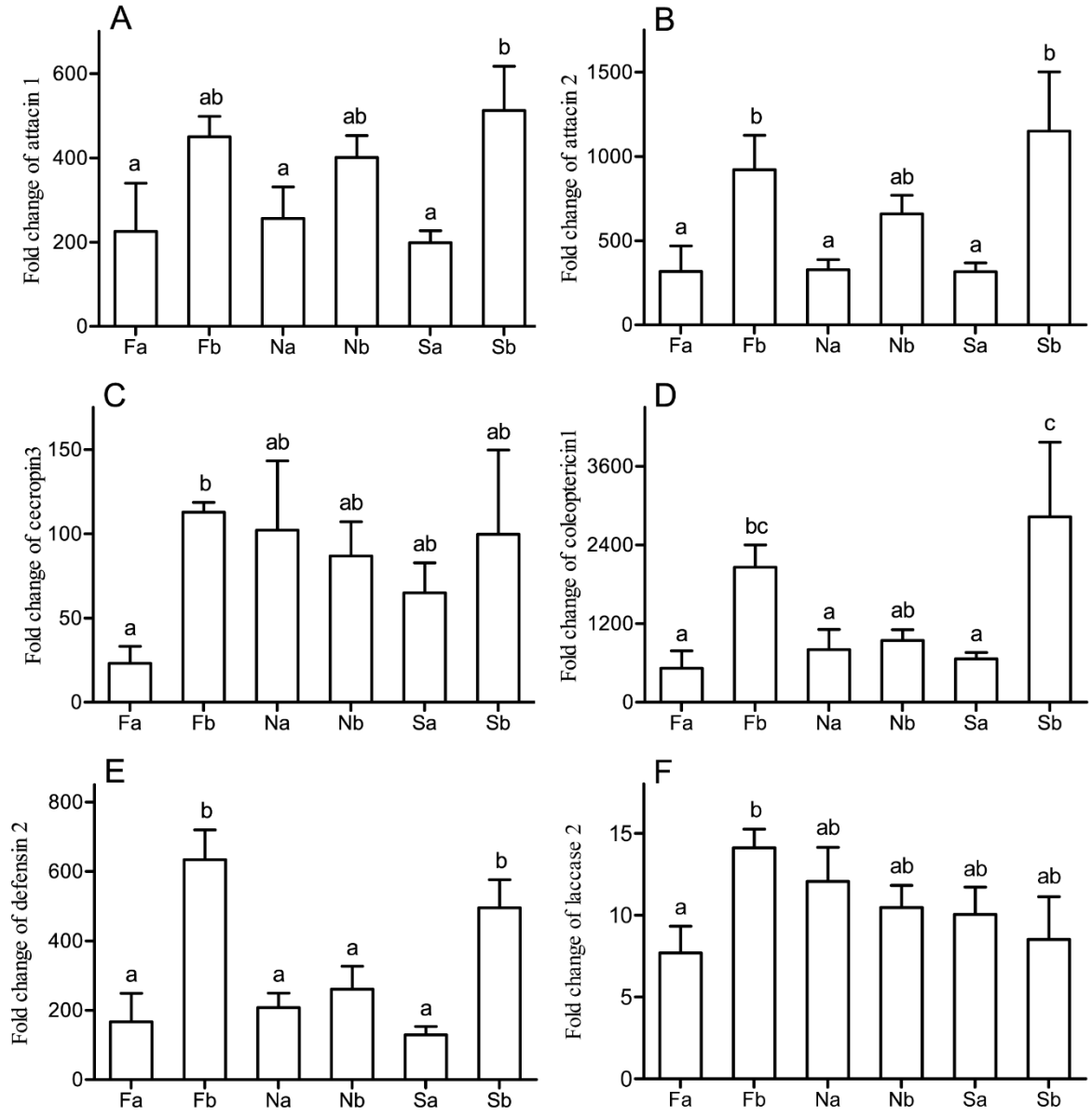

Figure 3-8. Upregulation of immune genes in the eggs of *T. castaneum* upon septic injury compared to sterile injury among the selection lines. Each vertical bar indicates mean fold change ± SE (n = 3). Different letters above the vertical bars display significant difference ($P < 0.05$).

Discussion

Artificial selection on developmental time has been carried out on *Tribolium casteneum* before (Englert and Bell, 1970; Garcia and Toro, 1990; Irwin and Carter, 2013; Roth and Kurtz, 2008; Soliman, 1982). For instance, three replicate populations of *T. castaneum* were selected for six generations, resulting in different lines with early or late pupation time (Englert and Bell, 1970). However, all these studies have been limited to larval and pupal stages. We report, for the first time, a successful response on selection for embryonic developmental time in *T. casteneum*. It is likely that such a response in life history is genetically correlated to other traits (Hill and Caballero, 1992). For instance, selection on early reproduction in *Drosophila* leads to fast larval development, whereas late

reproducing females develop slower (Zwaan et al., 1995a). We for the first time show correlated responses in larval and pupal developmental time to selection for embryonic developmental time in insects.

Trade-offs are a special category of correlated responses, as they defined as negatively correlated fitness components, for instance in size at birth, growth rate, age and size at maturity, fecundity and fertility, age- or size-specific rates of survival, and life span (Flatt, 2020). Stearns reported that there are at least 45 possible trade-offs focusing on 10 major life history traits (Stearns, 1992). Negative correlations between life-history traits are often caused by limited resources resulting in physiological trade-offs: the increment of resources allocated to one trait necessitates a decrement of resources to another trait (the traditional "Y" model of resource allocation) (Van Noordwijk and de Jong, 1986). In the red flour beetle *T. castaneum*, existence of a trade-off between life-history traits has been demonstrated before, for instance between predation avoidance and mating success in males. (Matsumura and Miyatake, 2015). Beetles of the established *Tribolium* strain with longer walking distances (L-strain) suffered higher predation risk than the strain with shorter walking distances (S-strain). At the same time, compared to S-strain males, L-strain males showed significantly increased mating success (Matsumura and Miyatake, 2015).

Artificial selection on life-history traits is regarded as one of the best empirical ways to understand the occurrence and consequences of their trade-offs (Brakefield, 2003; Hill and Caballero, 1992). Classical life-history theory predicts that developmental time trades off with weight (Flatt and Heyland, 2011; Stearns, 1992). In the model organism *D. melanogaster*, artificial selection on developmental time lead to lower adult weights in lines with fast larval development lines, and higher weights in the slow lines (Nunney, 1996; Zwaan et al., 1995a). White also found that *Drosophila* pupae of two slow developing laboratory strains (from oviposition to adult) were significantly heavier than seven field populations (White, 1984). We confirm the existence of a trade-off between developmental time and weight. Pupae and adults of the slow lines are significantly heavier than pupae and adults of the non-selected and slow lines. Given that the rate of weight increase is not different between the fast and slow lines (Supplementary Figure 3-1), the higher weight of the pupae of the slow lines is likely to be the simple consequence of the larvae having more time to accumulate biomass in the slow lines.

Trade-offs with fecundity are particularly prominent (Stearns, 1989). For instance, longevity trades off with fecundity in Drosophila (Djawdan et al., 2004; Leroi et al., 1994; Zwaan et al., 1995b). Long-lived flies, for example, start to lay only 0.24 eggs per female per 24 h and decrease quickly to 0 eggs after 7 days, whereas control females start to lay 1.8 eggs per 24h and slowly decrease to 0.32 after 20 day (Djawdan et al., 2004). Furthermore, it has been established that developmental speed trades off with fecundity. Adult females of fast developing lines show a 35% drop in fecundity after 15 generations of selection, compared to non-selected lines (Nunney, 1996). We confirm such trade-off between fast development and fecundity. Our fast developing lines lay 21% fewer eggs than the non-selected lines.

Surprisingly, we did not find evidence for a trade-off between developmental time and immune defense. Trade-offs between immune function and growth have been demonstrated in birds and plants (Brommer, 2004; Lozano-Durán et al., 2013). It could be that such trade-off between developmental time and immune defense does not exist in *Tribolium*. However, our qPCR approach on six immune genes is somewhat limited. It is also possible that some other immune genes or components associated

with the trade-off, such as the cellular response. Unexpectedly, relatively strong upregulation of all these tested genes occurred in the Fb line upon infection. This might be because the eggs of the Fb line put more resources into immune defense. Thus, the Fb beetles probably invest less resources on other life-history traits, for example, the longevity. This calls for in-depth investigation of fitness trade-offs in our selection lines, such as aging and longevity (Finch and Ruvkun, 2001; Flatt and Heyland, 2011).

It would be interesting to see if indications for a trade-off between immunity and developmental time in insect eggs can be found among insect species with different developmental time, instead of within species. The immune response in two relatively closely related insects with very different developmental times can be compared, for instance the moth midge *Clogmia albipunctata* that develops in 71 hours at 25°C (Jiménez-Guri et al., 2014), and the scuttle fly *Megaselia abdita* that develops in 27 hours at 25°C (Wotton et al., 2014). If a trade-off is present, we predict to find limited immune competence in the quickly developing *Megaselia* eggs. Furthermore, we can also compare immune competence in eggs of the mosquito pair *Culex pipiens* and *Aedes aegypti*, as *C. pipiens* develops within 46 hours at 25°C (Madder et al., 1983), while *A. aegypti* develops in 77 hours at 25°C (Vargas et al., 2014). Along the same line, a much weaker immune response in eggs of *Culex* than in *Aedes* would be indicative of a trade-off between immunity and developmental time.

Overall, we have artificially selected replicate outbred populations of *Tribolium* for fast and slow embryonic development, alongside a control. This resulted in a spectacular response, and lead to correlated responses in larval and pupal developmental time. We did not find evidence that developmental time trades off with immune defense. However, we did demonstrate that developmental time trades off with pupal and adult weight, and with fecundity. It would be interesting to know what the mechanistic and genetic mediators of these trade-offs would be. Likely, hormones such as juvenile hormone (JH), 20-hydroxyecdysone (20E), and insulin/insulin-like growth factor-like signaling (IIS) play a role as key switches for regulating life-history trade-offs (Flatt et al., 2005; Ketterson and Nolan, 1999; Stearns, 1989). These hormones regulate growth in Drosophila melanogaster (Nijhout et al., 2014). They could mediate the trade-off with fecundity as JH and insulin directly influence oogenesis (Abu-Hakima and Davey, 1975; LaFever and Drummond-Barbosa, 2005; Parthasarathy and Palli, 2011). In addition, such hormones could regulate potential trade-offs with immunity too, as 20E is a known potentiator of the immune response and required for embryonic immunity (Schwenke et al., 2016; Tan et al., 2014). Thus, we may expect alleles in genes involved in these pleiotropic hormone signalling pathways to underlie the observed trade-offs in our study. Our selection lines offer a great opportunity to start unravelling the genetic underpinnings of life-history trade-offs.

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Supplementary information

Supplementary Table 3-1. Primers for immune sequences of *Tribolium castaneum*.

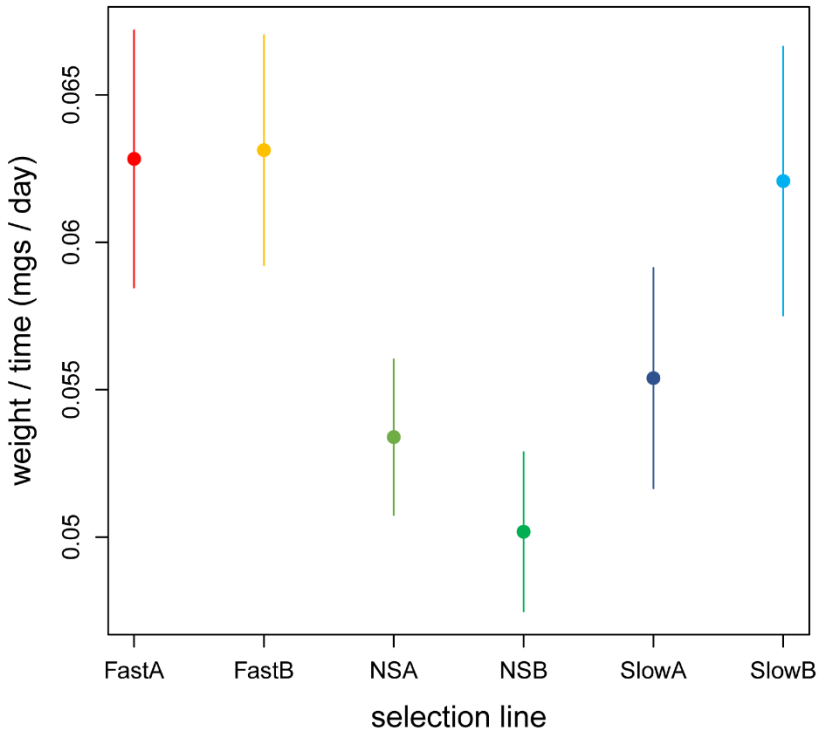
Gene name	TC number	Forward primer (5'-3')	Reverse primer (5'-3')
Tribolium castaneum			
Attacin1	TC007737	TTTTGCCTCCAAACAATTCC	CACCGACGTTTAGGTTCGAT
Attacin2	TC007738	CCCGGAATCCTCAAACTACA	GGGGCATCTTTATTGACGAA
Cecropin3	TC000500	GCTGTTCCCGTGGTTAAAAA	ACTGGAGGCGCATACTGAAT
Coleoptericin1	TC005093	TTTGGCACTTTTTGCACTTG	GGGATGTCCTGTTCTACGGA
Defensin2	TC010517	TCACTTGTGACGTCCTCAGC	CGCGTTTCTTCAAAAAGAGG
Laccase 2	TC010489	TACAACAGACATTTAGTTGCACCA	AGGTGGGGCCATGTAGGAAA

Supplementary Table 3-2. Pupal weight of female and male of the selection lines.

Selection line	Female weight (mg)		Male weight (mg)		Welch two-samples t-test	
	Mean ± SD	N	Mean ± SD	N	T value	P value
Fa	2.64 ± 0.39	27	2.69 ± 0.55	19	-0.3134	0.7562
Fb	2.82 ± 0.53	20	2.64 ± 0.33	20	1.2529	0.2195
Na	2.90 ± 0.44	22	2.84 ± 0.56	14	0.3582	0.7234
Nb	2.75 ± 0.59	21	2.68 ± 0.43	20	0.4251	0.6733
Sa	3.34 ± 0.64	16	3.23 ± 0.41	22	0.6120	0.5464
Sb	3.64 ± 0.79	24	3.26 ± 0.41	19	2.052	0.0475
All lines	3.00 ± 0.67	130	2.90 ± 0.51	114	1.3700	0.1720

Supplementary Table 3-3. Adult weight of female and male of the selection lines.

Selection line	Female weight (mg)		Male weight (mg)		Welch two-samples t-test	
	Mean ± SD	N	Mean ± SD	N	T value	P value
Fa	2.05 ± 0.32	19	1.95 ± 0.39	14	0.7759	0.4451
Fb	2.17 ± 0.39	17	2.10 ± 0.27	16	0.6099	0.5468
Na	2.17 ± 0.31	22	2.07 ± 0.31	14	0.9363	0.3572
Nb	2.00 ± 0.33	20	1.90 ± 0.32	20	0.8894	0.3794
Sa	2.35 ± 0.39	14	2.35 ± 0.38	19	0.0102	0.9919
Sb	2.63 ± 0.36	22	2.37 ± 0.27	16	2.5621	0.0148
All lines	2.23 ± 0.41	114	2.13 ± 0.37	99	1.9609	0.0512



Supplementary Figure 3-1. Growth rate defined as adult weight divided by total postembryonic time (larval development time and pupal development time) of an individual (mg/day; means ± 2 x s.e.m.). Selection regime had an effect ($\chi 2$=11.099; df=2; p=0.0039), but the fast lines are not significantly different from the slow lines ($\chi 2$= 2.34; df=1; p= 0.127).