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

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# Chapter 5
# Summary, discussion and perspective

## Summary

This thesis focuses on important life history traits and their trade-offs. Particularly, the trade-off between immune defense and embryonic developmental speed is a central theme of study. In short, the details of immune defenses during embryonic development of basal hexapods are studied in Chapter 2, the trade-off with developmental speed is studied in chapter 3 using selection lines of the red flour beetle *Tribolium castaneum*, while the genetics underlying developmental speed are studied in Chapter 4.

Several evolutionary innovations have made significant contributions to the extraordinary success of the insects (discussed in **Chapter 1**). However, an evolutionary novelty in insect eggs, the serosa, gets less attention from evolutionary biologists.

### The serosa and immune competence of springtail eggs

During insect embryonic development, immune protection is provided by the serosa, an extraembryonic epithelium (Jacobs et al., 2014a; Jacobs et al., 2022).

In **Chapter 2**, I expanded immune challenge to the non-insect springtail *Orchesella cincta* that does not possess a serosa, but is very close to insects in the phylogenetic tree (Figure 5-1). By injecting a mixture of Gram-positive and Gram-negative bacteria (*Micrococcus luteus* and *Escherichia coli*), I showed that immune genes in the eggs of *O. cincta* are upregulated upon infection. Thus, I concluded that the serosa is not an absolute prerequisite for an innate immune response and that other tissues produce antimicrobials in springtail eggs. Interestingly, it has been reported that eggs of the burying beetle *Nicrophorus vespilloides* lack an inducible innate immune response upon infection, despite possessing a serosa (Jacobs et al., 2014b). This further confirms that the presence or absence of a serosa in arthropod eggs does not necessarily mean presence or absence of an innate immune protection.

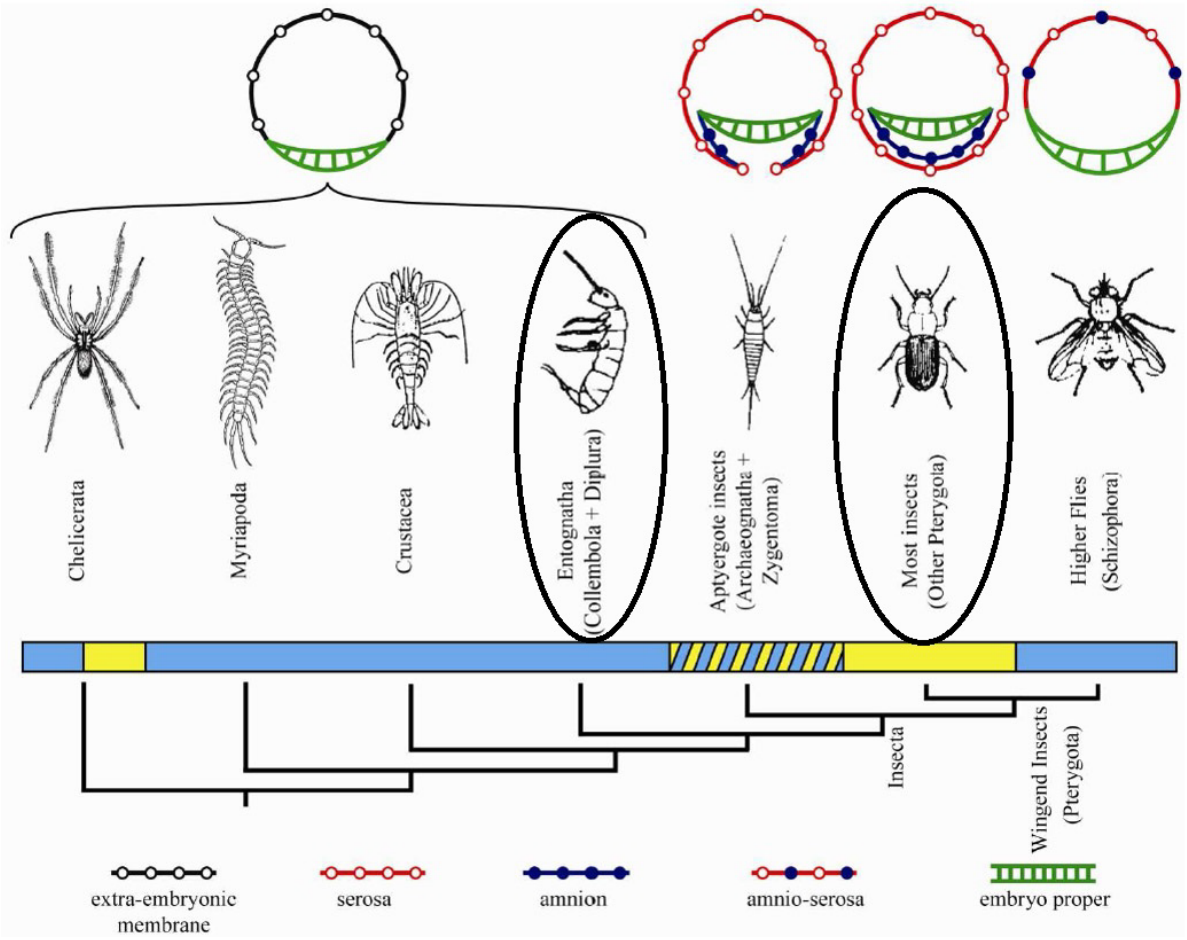


Figure 5-1. Phylogeny of all major arthropod groups. The bar under the groups shows that the eggs live in aquatic (blue), terrestrial (yellow) or humid environments (striped). The schematic drawings above illustrate the topology of embryo, amnion and serosa in these eggs. . The springtails and insects are indicated with an oval in the phylogenetic tree. Adopted from (Jacobs et al., 2013).

## Trade-offs with developmental speed

The insects *N. vespilloides* and *Drosophila melanogaster* both develop extremely quickly as embryo, and both lost desiccation resistance and immune competence of the egg (Jacobs and van der Zee, 2013; Jacobs et al., 2014b). *Drosophila* has lost the serosa altogether. Thus, egg defense seems to trade off with developmental speed in insects. Trade-offs between growth and immune defense have been shown in several organisms (Brommer, 2004; Diamond and Kingsolver, 2011; Lozano-Durán et al., 2013).

In **Chapter 3**, I selected replicate outbred populations of *Tribolium castaneum* for fast and slow embryonic development. I was thereby able to test this possible trade-off between immune defense and the duration of embryonic development. I did not find the trade-off between immune defense and embryonic developmental time in the infected selection lines, even though all genes tested showed strong upregulation upon infection. By measuring fecundity of our *Tribolium* selection lines, however, we found a strong negative correlation between developmental speed and fecundity (Figure 5-2).

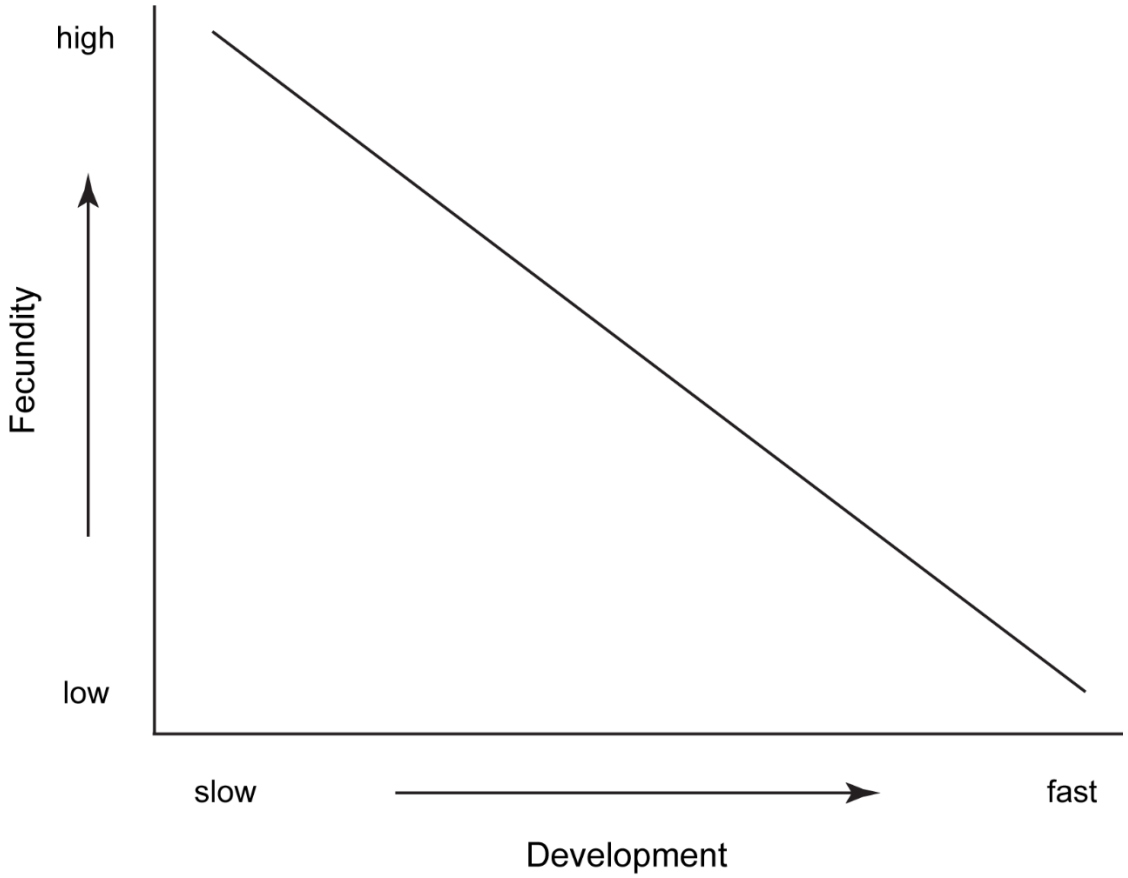


Figure 5-2. The trade-off between fast development and high fecundity found in our *Tribolium* selection lines.

## A major life history allele in the beetle *Tribolium*

Most decisions of insects on physiological, developmental, and behavioral events are mediated by hormones (Gade et al., 1997; Ketterson and Nolan, 1999; Lösel and Wehling, 2003; Stearns, 1989). The steroid hormone ecdysone (20E) is such a crucial hormone in insects (Scaraffia and Miesfeld, 2013).

In **Chapter 4**, I show a clear divergence in embryonic developmental timing of the selection lines from the start of dorsal closure onwards. I also show that a high ecdysone peak during embryonic development induces dorsal closure. Consistently, the embryonic ecdysteroid peak of the fast lines occurred earlier than in the selection lines for slow embryonic development.

Combining pooled whole genome resequencing with gene expression and a functional RNAi screen, I show that Cyp18a1, the 20E degrading enzyme was a main target of selection. Particularly, I show that a 222 bp deletion (called F allele in Chapter 4) upstream of *Cyp18a1* (Figure 5-3B), has been under positive selection in the fast lines. The deletion is located in a cis-regulatory element containing Tramtrack and Broad transcription factor binding sites, and a binding site for the Ecdysone receptor forming an ecdysone-responsive enhancer regulating *Cyp18a1* expression (Figure 5-3). The alternative allele is the S allele in which no deletion is present, but a repeated sequence (Figure 5-3A).

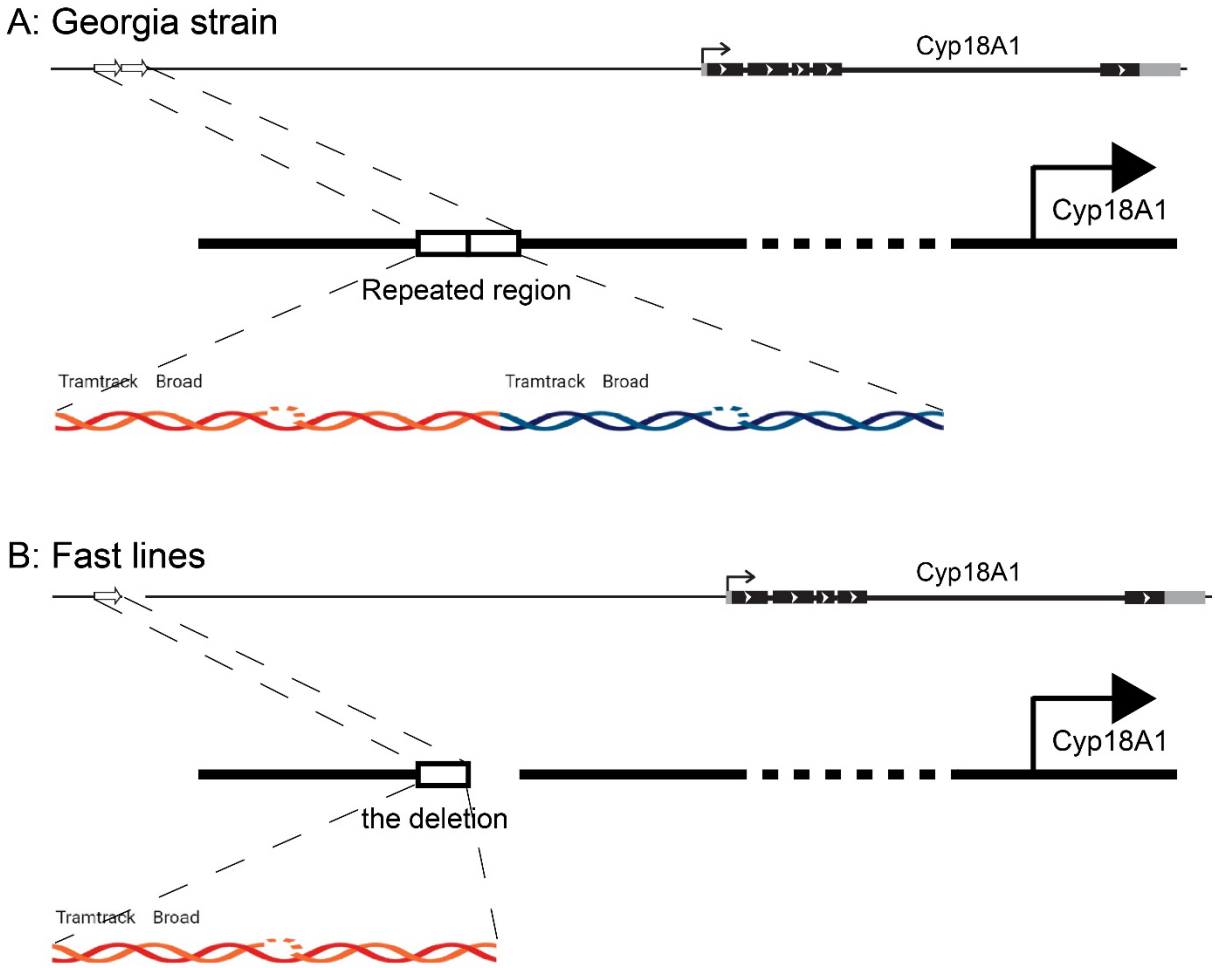


Figure 5-3. Schematic overview of the repeated region and the deletion in the upstream of *Cyp18a1*. A, The repeated region in the Georgia strain with double Tramtrack and Broad transcription factor binding sites (The S allele). B, the deleted region from the fast lines of the selection lines with single Tramtrack and Broad transcription factor binding sites (The F allele).

Interestingly, the Georgia lab strain is homozygous for the S-allele, which means that in all individuals, the222 bp sequence is duplicated (Figure 5-3A). This gave me the chance to recreate this deletion in the homogenous genetic background of the Georgia strain using CRISPR-Cas9 technology. As a result, I show that this single deletion accelerates development, delays *Cyp18a1* expression, advances the ecdysone peak, and causes a trade-off with fecundity. As the slow allele contains two Tramtrack and two Broad binding sites, instead of one, we assume that the enhancer of the slow allele more easily establishes contact with the basal promotor. A mathematical model shows that reduced sensitivity to ecdysone of the fast allele delays *Cyp18a1* expression, but advances the ecdysone peak if one assumes that ecdysone levels depend on a self-regulatory positive feedback loop.

In conclusion, I could not show that developmental speed trades off with immune defense, but I demonstrated a clear trade-off with fecundity. This life-history trade-off is genetically mediated by a simple 222bp deletion upstream of the ecdysone degrading enzyme *Cyp18a1*.

# Discussion and perspective

We found ecdysone as main target of selection for altered developmental speed. On the one hand, ecdysone is well known to control developmental time (Rewitz et al., 2013; Yamanaka et al., 2013). But on the other hand, ecdysone is also involved in starting or potentiating immunocompetence, the topic of Chapter 2 and 3. Ecdysone upregulates, for instance, the receptor of the IMD pathway PGRP-LC (Rus et al., 2013), and also directly some antimicrobial peptides (Flatt et al., 2008; Meister and Richards, 1996). However, in our study, we did not find a trade-off between developmental speed and immune defense (Chapter 3). It could be that such trade-off is not present in our selection lines, but it could also be that our qPCR approach on a limited set of immune genes was not sufficient to reveal such a trade-off.

Ecdysone is a typical developmental timer. Indeed, growth rate (expressed in mg/days) is not significantly different between the fast and slow lines (Chapter 3); only developmental time is. However, among the targets of selection is also *melted*, a protein that interacts with Tsc1 and FOXO to enhance insulin/TOR activity (Teleman et al., 2005). Insulin signalling is a pivotal pathway enhancing growth rate (Baker and Thummel, 2007; Nijhout et al., 2014). Another potential target identified is *CAT1*. CAT is orthologous to slimfast, an amino acid sensor in the fat body that can overrule peripheral insulin/TOR activity by PI3K modulation affecting developmental time (Baker and Thummel, 2007; Colombani et al., 2003). Both *Melted* and *CAT1* RNAi lead to developmental delay (Figure 4-6).

These two major regulatory systems, ecdysone and insulin/TOR signalling could mutually regulate developmental speed. Upon stimulation by insulin and insulin-like growth factor (IGF) or amino acids, insulin and TOR pathways are activated. The second major target in our selection lines, *Melted*, balances these two pathways (Baker and Thummel, 2007; Teleman et al., 2005), which in the end also has an output in ecdysone biosynthesis (Kannangara et al., 2021; Niwa and Niwa, 2016; Yamanaka et al., 2013) (Figure 5-4). With the activation of ecdysone signaling, developmental timing is also affected.

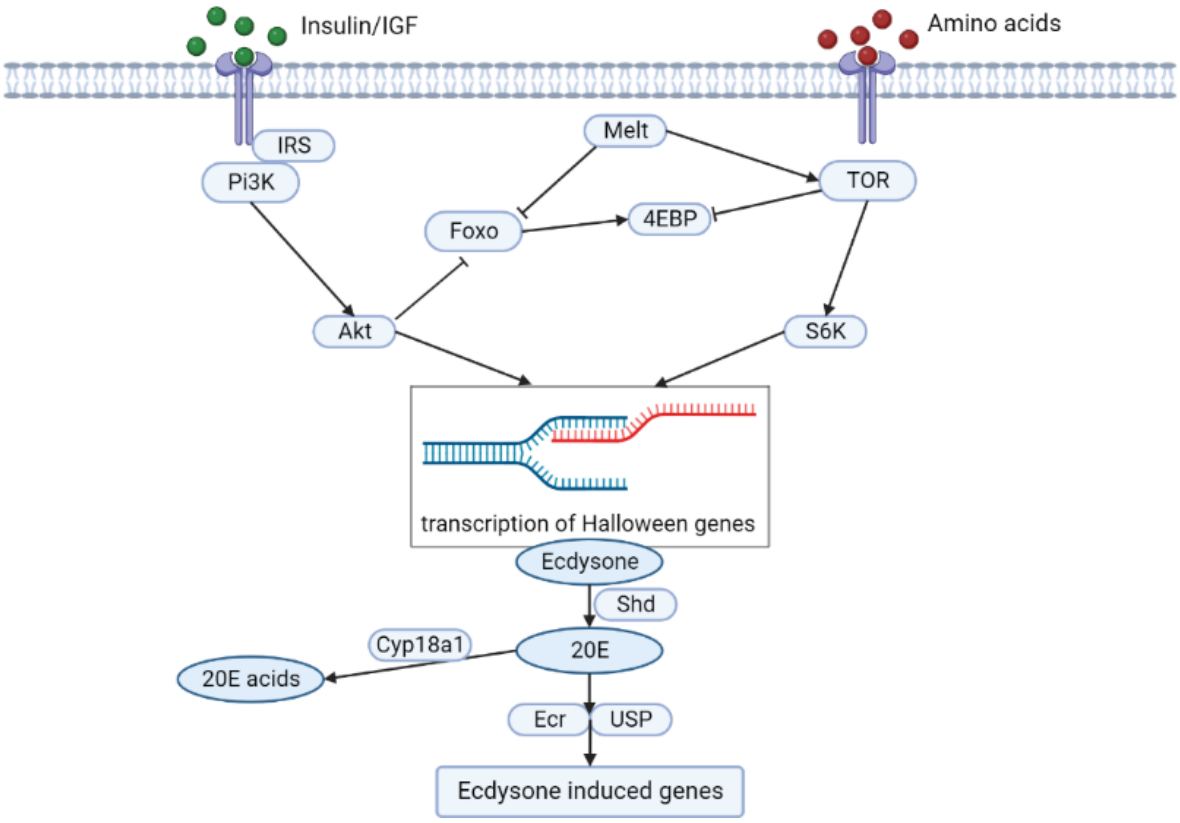


Figure 5-4. Cross talk between ecdysone signaling, insulin and target of rapamycin (TOR) pathways. Key: green balls, insulin and insulin-like growth factors; red balls, amino acids. These balls are transported through phospholipid bilayer into the cell as stimulation. After binding to the IRS or TOR, they will activate insulin and TOR pathways in the IRS/PI3K/Akt/Foxo and TOR/S6K signaling branches, respectively. Meanwhile, Melted attends to balance these two pathways by regulating Foxo and TOR. As a result, Akt and S6K turn on the transcription of Halloween genes, synthesizing ecdysone and activating 20E signaling pathway. IRS, insulin receptors; Pi3K, phosphoinositide 3-kinase; Akt, protein Kinase B; FoxO, forkhead box O; Melt, protein melted; 4EBP, eukaryotic translation initiation factor 4E-binding protein; S6K, S6 Kinase; Ecr, ecdysone receptor; USP, Ultraspiracle.

So far, I did not have time to functionally study the role of insulin pathway in development in *Tribolium*. In future experiments, injections with a synthetic insulin peptide will be informative, as recently reported method to study roles of insulin in long-lived reproductive caste of ants (Yan et al., 2022). Finally, I did not find any evidence for involvement of another important hormone, juvenile hormone, which is responsible for growth, development and reproduction of insects as well (Jindra et al., 2013; Williams, 1956). In general, juvenile hormone and ecdysone have opposite roles on growth, immunity and reproduction in insects (Schwenke et al., 2016; Spindler et al., 2009). Thus, I speculate that juvenile hormone may be associated with slow embryonic development.

## Ecological importance

Current global warming is ~1.1°C above preindustrial, which has raised concerns among scientists and the public. It is estimated that the warming will reach ~1.5°C by 2030, and even ~2 to 3°C expected under current policies by 2100. Strikingly, global warming of 1.5 to 2°C triggers multiple climate tipping points according to the Paris climate agreement. When reaching one of the tipping points, global warming may result in substantial Earth system impacts (Armstrong McKay et al., 2022).

When species respond differently to global warming, ecological mismatches in seasonal timing ("phenological mismatches") occur. Such mismatches have also been reported for plants and their pollinators, leading to global threats for pollinators that are crucial to humans (Freimuth et al., 2022; Gérard et al., 2020). Phenological mismatches must exert strong selection on insect developmental

time (Renner and Zohner, 2018; Singer and Parmesan, 2010). My study has shown that polymorphisms affecting developmental speed are present in natural insect populations. This means that developmental timing may be much more evolvable than expected for such a supposedly highly optimized life history trait (Stearns, 2000). Maintenance of the polymorphism may be explained by balancing selection requiring rapid adult dispersal when food sources are temporary, or allowing slow development and higher fecundity in more stable ecological conditions (Charlesworth, 2015). Classically, quantitative traits are thought to be determined by many alleles of small effect (Charlesworth and Edwards, 2018). My work, however, has demonstrated that polymorphisms can be alleles of very large effect. In conclusion, insect populations may be able to adapt their developmental time and life histories rapidly to global warming.

However, I have also demonstrated that changes in key life history traits such as developmental timing are highly correlated to other traits. Although changes in developmental timing may not immediately affect immune defenses of insects, my work has clearly shown that climate-induced changes in developmental time will have consequences for weight and fecundity of insects. Such changes could still disrupt ecological networks. Nevertheless, my thesis has revealed substantial evolvability of insect developmental speed.

## Conclusions

In summary, it has been well established that insect eggs are immune-protected by the serosa. I have shown, however, that the serosa is not a prerequisite for an immune reaction in eggs, as infection upregulates immune genes in springtail eggs that do not possess a serosa. I could not find a trade-off between immune protection and developmental speed in insect eggs. I did, however, find a clear trade-off between developmental speed on the one hand and weight and fecundity on the other hand in selection lines of the beetle *Tribolium castaneum*. Strikingly, a single natural allele mediates this trade-off to a large extent, as demonstrated by CRISPR/Cas9 reconstruction. Such alleles may play a crucial role when insects have to adapt their developmental speed to current climate change.

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