

DIVISION OF EVOLUTIONARY DEVELOPMENTAL BIOLOGY



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The Division of Evolutionary Developmental Biology was started in June 2015. We focus on the evolutionary novelties acquired by insects through evolution, in order to elucidate the molecular and evolutionary mechanisms leading to the large variety of traits that they display. From this wealth of exciting traits, our lab currently focuses on promoting research into (1) the origin and diversification of insect wings, (2) wing color patterns and mimicry of ladybird beetles, and (3) acquisition and diversification of beetle horns.

I. Origin and diversification of insect wings

Of the various flying animals on the earth, insects have evolved a unique flight organ. Despite over two centuries of debate, the evolutionary origin of the insect wing is still an enigma. We try to approach this issue using evo-devo methods. In *Drosophila melanogaster*, the wing master gene *vestigial (vg)* and its interaction partner *scalloped (sd)* play pivotal roles in the formation of wing field identity. For this reason, these genes are ideal candidates for investigating wing origin and evolution.

One way to identify the structure from which insect wings first evolved is to explore the function of “wing” genes in ancestral wingless (apterygote) species. We chose the firebrat, *Thermobia domestica*, as a model (Figure 1A). *T. domestica* belongs to Thysanura, phylogenetically the closest extant relative of winged (pterygote) insects, making it ideal for elucidating wing origin. We cloned *vg* and *sd* orthologs from *T. domestica* (*Td-vg* and *Td-sd*). To examine the functions of these genes, we developed RNA interference (RNAi) based methods for *T. domestica*. We are currently testing for



Figure 1. The firebrat, *Thermobia domestica* (A), the two-spotted cricket, *Gryllus bimaculatus* (B).

functional effects of altered transcription of each of these wing genes in the ancestrally wingless firebrats. In addition, we are performing comparative analyses of the function of these same genes in “primitively winged” (hemimetabolous) insects, to obtain additional clues relevant to understanding the origin and evolution of insect wings.

Interestingly, our previous work showed that *vg* expressing epidermal tissue forms lateral outgrowths in non-winged segments in the mealworm beetle (Ohde *et al.*, 2013). From these facts, we hypothesize that ancestral lateral body wall outgrowths evolved into functional wings. To test this hypothesis, we are now comparing the role of *vg* and other “wing genes” between wings and lateral outgrowths in a basal winged insect, *Gryllus bimaculatus*, and non-winged insect, *Thermobia domestica* (Figure 1).

II. Wing color patterns and mimicry of ladybird beetles

Insect wing color patterns demonstrate a tremendous range of diversity and have evolved to fulfill various ecologically important functions such as intraspecific sexual signaling, mimesis, mimicry, and warning against predators. The molecular mechanisms responsible for generating such patterns, however, remain unknown for most species. To investigate the developmental mechanisms of color pattern formation, we chose the multicolored Asian ladybird beetle, *Harmonia axyridis*, which has conspicuous and variable wing color patterns consisting of black and red pigments (Figure 2A). Vivid wing color patterns of ladybirds function as a warning signal to predators that they are distasteful, and ladybird beetles are mimicked by various insect species. Mimicry provides an exciting opportunity to study how independent lineages of insects converge on similar color patterns. For exploring color pattern formation in a mimic, we use the leaf beetle, *Argopistes coccinelliformis*, which has color patterns similar to *Harmonia*, and which is thought to be a Batesian mimic of ladybird beetles (Figure 2B). To elucidate the molecular mechanisms underlying these wing color patterns, we have established a technique for germline transformation using a *piggyBac* vector and RNAi in the ladybirds.

Recently, genome-editing technologies using TALEN and CRISPR/Cas9 provide rapid and potent genetic analysis systems including not only gene knockout but also gene knock-in. To establish TALEN-mediated genome editing in *Harmonia*, we used a transgenic ladybird strain that has single copies of exogenous *EGFP* and *N-acetyl transferase* (NAT, anti-melanin synthesis enzyme) genes. Messenger

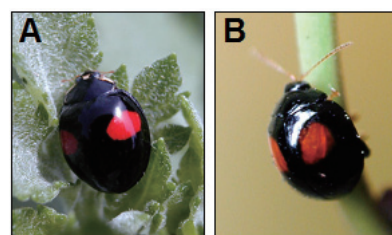


Figure 2. The ladybird beetle, *Harmonia axyridis* (A) and the leaf beetle, *Argopistes coccinelliformis* (B).

RNAs of TALENs targeting only the EGFP transgene were microinjected into the fertilized eggs. As a result, 20.8% of G0 founders produced EGFP-negative and NAT-positive knockout progenies. This efficiency was higher than that of germline transformation by *piggyBac* vectors (about 5%). We are continuing to develop both CRISPR/Cas9 and *piggyBac* methods in our lab and will use both approaches to perturb expression of candidate genes putatively involved in wing color pattern formation.

Based on the knowledge obtained from *H. axyridis*, we are trying to understand how the similar wing-color patterns of model and mimic are generated – for example, do they use conserved or divergent mechanisms?

III. Acquisition and diversification of beetle horns

Insects show a tremendous range of diversity in “horns”, rigid body outgrowths that function as weapons. Horns are exciting for evo-devo studies because they have arisen multiple times *de novo*, as evolutionary “novelties”. However, the molecular mechanisms involved in sexually dimorphic horn formation are still poorly understood. To investigate the developmental mechanisms of horn formation, we focus on the Japanese rhinoceros beetle, *Trypoxylus dichotomus* (Coleoptera), which exhibits remarkable sexual dimorphisms in head and thoracic horns (Figure 3A). The male-specific horns of *T. dichotomus* are one of the best models to study how an extreme, sex-specific morphology is formed. We have developed a technique for larval RNAi in *T. dichotomus*, permitting us to rigorously and systematically test the functional roles of a large suite of candidate developmental genes, revealing for the first time the molecular mechanisms responsible for growth of male rhino beetle horns. In addition, we are employing a high throughput approach. To identify novel genes involved in the sexually dimorphic horn development in *T. dichotomus*, mRNA of the developing horn discs has been assessed by deep-sequencing transcriptome analysis.

To understand how, molecularly, beetle horns have diversified, we are extending our analyses to include additional

beetle species with different types of exaggerated horns, including rhinoceros beetles with diverse horn structures as well as horned beetles in other phylogenetic groups (Figure 3).

Previously, we have shown that the sex-determination gene *doublesex* (*dsx*) plays crucial roles regulating horn development in *T. dichotomus* (Scarabaeoidea, Scarabaeidae) (Ito *et al.*, 2013). The function of *dsx* on weapon traits in Scarabaeoidea is conserved among beetles tested so far. Although sex-specific weapon traits have evolved independently in various Coleopteran groups, developmental mechanisms of sex-specific expression have not been studied outside of the Scarabaeoidea. Therefore, we focused on the sexually dimorphic broad-horned flour beetle *Gnatocerus cornutus* (Tenebrionidae, Tenebrionidae). In this beetle, only males have well-developed mandibles and genae, and a pair of short horns on the head (Figure 3C). We investigated the function of *dsx* in this beetle using larval RNAi. Our results show that sex-specific *G. cornutus dsx* isoforms have an antagonistic function for all sexually dimorphic head structures. Combining these results with previous studies in other sexually-dimorphic weaponed beetles in the Scarabaeoidea, indicated that sex-specific regulation of weapon development via differential *Dsx* isoforms is a common mechanism shared by sexually-dimorphic beetles, even though their specific weapon traits have evolved independently.

Publication List:

[Original papers]

- Gotoh, H., Ishiguro, M., Nishikawa, H., Morita, S., Okada, K., Miyatake, T., Yaginuma T., and Niimi, T. (2016). Molecular cloning and functional characterization of the sex-determination gene *doublesex* in the sexually dimorphic broad-horned beetle *Gnatocerus cornutus* (Coleoptera, Tenebrionidae). *Sci. Rep.* 6, 29337.
- Gotoh, H., Zinna, R.A., Warren, I., DeNieu, M., Niimi, T., Dworkin, I., Emlen, D.J., Miura, T., and Lavine, L.C. (2016). Identification and functional analyses of sex determination genes in the sexually dimorphic stag beetle *Cyclommatus metallifer*. *BMC Genom.* 17, 250.
- Ozawa, T., Mizuhara, T., Arata, M., Shimada, M., Niimi, T., Okada, K., Okada, Y., and Ohta, K. (2016). Histone deacetylases control module-specific phenotypic plasticity in beetle weapons. *Proc. Natl. Acad. Sci. USA* 113, 15042-15047.
- Zinna, R., Gotoh, H., Brent, C.S., Dolezal, A., Kraus, A., Niimi, T., Emlen, D., and Lavine, L.C. (2016). Endocrine control of exaggerated trait growth in rhinoceros beetles. *Integr. Comp. Biol.* 56, 247-259.

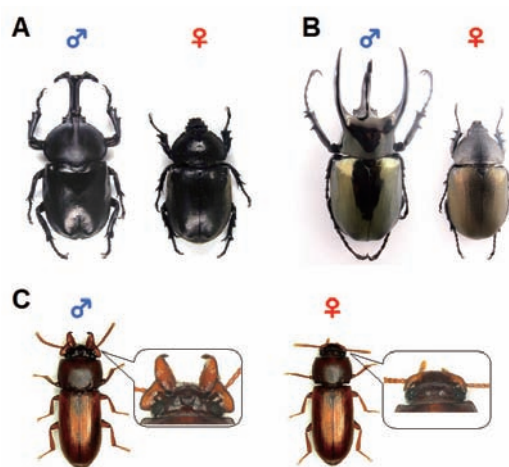


Figure 3. The Japanese rhinoceros beetle, *Trypoxylus dichotomus* (A), the Atlas beetle, *Chalcosoma atlas* (B) and the broad-horned flour beetle, *Gnatocerus cornutus* (C). Adult male (Left) and female (Right).