Abstract Booklet

Tribolium and Friends Meeting

EURO EVO DEVO

June 25th-26th Galway 2018

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Oncopeltus, Tribolium, Drosophila – a three-taxon problem for understanding the evolution of segmentation in insects

Ariel D. Chipman, Tzach Auman and Anastasia Novikova

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Reconstructing a sequence of evolutionary events requires as a minimum a comparison among three taxa – two sister taxa and an outgroup. For understanding the evolution of segmentation in insects, we have three ideally positioned species, which form such a three-taxon group: The fruitfly *Drosophila melanogaster* and the red flour beetle *Tribolium castaneum* form a sister group relationship within Holometabola, while the milkweed bug *Oncopeltus fasciatus* forms a hemimetabolous outgroup. We have been focusing on *Oncopeltus*, dissecting its segmentation process in detail. Our results offer a comparative view and allow a reconstruction of the stages in the evolution of the different segmentation modes in insects. We have shown that simultaneous segmentation most likely appeared before the origin of Holometabola, and has been lost several times, including in *Tribolium*. We now add details about the evolution of different modes of sequential segmentation. We present the cascade involved in differentiating new segments from a posterior growth zone, and show that a hierarchy reminiscent of the classical *Drosophila* hierarchy is found in sequential segmentation as well. The cascade begins with primary pair-rule genes followed by secondary pair-rule genes, which regulate segment polarity genes. The cascade is highly redundant and RNAi phenotypes of most genes are surprisingly minor. This hierarchy was most likely ancestrally of a single-segment periodicity. However, there is evidence for a two-segment periodicity in the differentiation of the segments after their formation in *Oncopeltus*, perhaps giving a hint to the origin of the pair-rule pattern found in both *Tribolium* and *Drosophila*.

Double abdomen in a short germ insect: Zygotic control of axis formation revealed in the beetle *Tribolium castaneum*

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The distinction of anterior versus posterior is a crucial first step in animal embryogenesis. In the fly *Drosophila,* this axis is established by morphogenetic gradients contributed by the mother that regulate zygotic target genes. This principle has been considered to hold true for insects in general but is fundamentally different from vertebrates where zygotic genes and Wnt signaling are required. We investigated symmetry breaking in the beetle *Tribolium castaneum*, which among insects represents the more ancestral short germ embryogenesis. We found that maternal *Tc-germ cell-less* is required for anterior localization of maternal *Tc-axin,* which represses Wnt signaling and promotes expression of anterior zygotic genes. Both, RNAi targeting *Tc-germ cell-less* or double RNAi knocking down the zygotic genes *Tc-homeobrain* and *Tc-zen1* led to the formation of a second growth zone at the anterior, which resulted in double abdomen phenotypes. Conversely, interfering with two posterior factors, *Tc-caudal* and Wnt, caused double anterior phenotypes. These findings reveal that maternal and zygotic mechanisms including Wnt signaling are required for establishing embryo polarity and induce the segmentation clock in a short germ insect.

In addition, I plan to discuss problems arising in RNAi screens from genes that are expressed both, maternally and zygotically.

Segmentation in Arthropods: a study investigating regulatory relationship between gap genes and pair-rule genes in the beetle *Tribolium castaneum*

Rahul Sharma, Andrew Peel

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Body segmentation is a key feature found in many animal species, including insects. In the fruit fly, *Drosophila melanogaster*, segments are patterned more-or-less simultaneously via a classic genetic cascade involving the regulation of spatially periodic pair-rule gene expression by the aperiodically expressed gap genes. The beetle *Tribolium castaneum* however, exhibits the ancestral insect condition of forming its segments sequentially under the control of a segmentation oscillator/clock that is thought to comprise a negative feedback loop involving three pair-rule gene homologues (*Tc-even-skipped, Tc-odd-skipped* & *Tc-runt*). The knockdown of gap gene homologues in *Tribolium* does not result in canonical gap phenotypes, and therefore gap homologues cannot regulate periodic pair-rule gene expression as they do in *Drosophila*. We have studied gap and pair-rule gene expression and function in *Tribolium*. We find that, contrary to previous reports, a classic pair-rule phenotype is observed in *Tc-odd*$^{\text{-RNAi}}$, as well as in *Tc-odd*-CRISPR mutant, embryos, and that periodic expression of *Tc-eve* is abolished in this context. In addition, the trunk expression domains of gap genes are altered in *Tc-odd*$^{\text{-RNAi}}$ embryos. This suggests that pair-rule genes regulate gap genes in *Tribolium,* and that primary segmentation is driven by a segmentation clock involving more than just a single three-gene negative feedback loop. We continue to investigate the mechanistic details of segment formation by studying the segmentation clock genes using the CRISPR/cas9 system in *Tribolium*.

A revised understanding of *Tribolium* morphogenesis further reconciles short and long germ development

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In the fly *Drosophila*, the germband forms directly on the egg surface and solely consists of embryonic tissue (termed long germ development). In contrast, most insect embryos undergo a complicated set of tissue rearrangements to generate a condensed, bi-layered germband (termed short/intermediate germ development). The ventral side of the germband is embryonic, while the dorsal side is thought to be an extraembryonic tissue called the amnion. While this tissue organisation has been accepted for around a century, and has been widely reported in different insects, its accuracy has not been directly tested in any species. I recently investigated this topic in the short germ beetle *Tribolium castaneum* using a combination of live cell tracking and differential cell labelling. Surprisingly, I found that most of the cells previously thought to be amnion actually give rise to large parts of the embryo. This process occurs via the dorsal-to-ventral flow of cells and contributes to germband extension. Through fatemapping of blastoderm cells, I found that true amnion cells originate from a relatively small region of the blastoderm. Together, my findings show that development in the short germ embryos of *Tribolium* and the long germ embryos of *Drosophila* is more similar than previously proposed. In addition, my findings show that there is no qualitative difference between the structures of the blastoderm and the short/intermediate germ germband. As such, the same tissue patterning mechanisms could function continuously throughout the cellularised blastoderm and germband stages, and easily shift between them over evolutionary time.

Spider segmentation gets its Sox on

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The Sox gene family encodes a HMG-containing domain similar to the SRY (Sex-Determining Region Y) of eutherian mammals. These genes regulate many processes during embryogenesis in metazoans. In insects, *Dichaete* is the only Sox gene known to be involved in segmentation. To determine if similar mechanisms are used in other arthropods, we investigated the role of Sox genes during segmentation in the spider *Parasteatoda tepidariorum*. While Dichaete does not appear to be involved in spider segmentation, RNAi knockdown of *Sox 21-B1* (a *Drosophila* Sox 21-B orthologue) perturbed the sequential addition of opisthosomal segments and the expression of segmentation genes. Thus, we have found that segmentation in other arthropods is also regulated by a Sox gene, but that spiders employ a different gene from insects. Our work provides new insights into the function of an important and conserved gene family across arthropods and the evolution of the development of these animals.

Using Hybridisation Chain Reaction to study Hox gene regulation in *Tribolium*

Olivia Tidswell and Michael Akam

Hox proteins are conserved transcription factors that regulate body region identity along the anterior-posterior axis of many animals. The various Hox proteins are encoded by an organised cluster of related genes - the Hox cluster - which has in some lineages become duplicated, split or dispersed.

The genes of the Hox cluster display an interesting regulatory feature – the order in which they are encoded along the chromosome is echoed by their order of expression along the anterior-posterior axis of the embryo (**spatial collinearity**) and, in some cases, in the temporal order in which they are expressed during development (**temporal collinearity**). Although spatial collinearity is an almost ubiquitous feature of Hox gene expression, temporal collinearity seems to be restricted to lineages with intact Hox clusters.

I study the regulation of Hox genes in the red flour beetle, *Tribolium castaneum*, an arthropod that has an intact colinear Hox cluster, and activates Hox genes sequentially as the germ band elongates and segments are added posteriorly.

One key question is to what extent the Hox genes of *Tribolium* are regulated like those in *Drosophila*, by localised TFs of the segmentation cascade, and to what extent their activation depends on progressive chromatin remodelling mechanisms similar to those characterised in vertebrates.

A re-inducible genetic cascade patterns the anterior-posterior axis of insects in a threshold-free fashion

Alena Boos, Jutta Distler, Martin Klingler, **Ezzat El-Sherif**

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The anterior-posterior axis of insects is divided into different fates through the action of gap genes that ultimately specify hox gene expression domains. Decades of tinkering the segmentation gene network of *Drosophila* led to the conclusion that gap genes are regulated (at least initially) through a threshold-based French Flag model, guided by both anteriorly- and posteriorly-localized morphogen gradients (namely, *bicoid* and *caudal*, respectively). Here, we show that the regulation of gap genes in the intermediate-germ insect *Tribolium castaneum* relies on a self-regulatory and threshold-free mechanism: the Speed Regulation model, mediated by a posterior gradient of the transcription factor *caudal*. We show this by re-inducing the gap gene cascade at arbitrary points in time by simply re-inducing the leading gene in the gap gene cascade (namely, *hunchback*). This demonstrates that the gap gene network is self-regulatory and is primarily under the control of a posterior morphogen in short- and intermediate-germ insects.

Panel Discussion: Segmentation and Evolution

Evolution of immune competence in insect eggs

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Insects comprise more than a million species and many authors have attempted to explain this success by evolutionary innovations. A much overlooked evolutionary novelty is the serosa, an extraembryonic epithelium around yolk and embryo. We have shown that this epithelium provides innate immune protection to eggs of the beetle *Tribolium castaneum.* It remained elusive, however, whether this immune competence evolved in the *Tribolium* lineage or is an ancestral function of the serosa. To test this, we expand our studies to two hemimetabolous insects with a serosa, the milkweed bug *Oncopeltus fasciatus* and the grasshopper *Locusta migratoria*. RNA sequencing reveals an extensive response, including the massive upregulation of antimicrobial peptides (AMPs). We demonstrate the antimicrobial activity of two groups of novel *Oncopeltus* peptides the Serosins and Ovicins. By qPCR, we determine that eggs become immune responsive when the serosa develops. Finally, in situ hybridizations show that transcripts of upregulated peptides are located in the serosal cells and not in the underlying embryo. This first evidence from hemimetabolous insect eggs suggests that immune competence is an ancestral property of the serosa. We also hope to present preliminary data from two Collembola just outside the insects, the springtails *Folsomia candida* and *Orchesella cincta.*

Divergent segmentation hierarchy underlies embryo patterning in ants

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The patterning cascade of gap and pair rule genes, with a few alterations, is assumed to be fundamental to all insect embryonic segmentation process, independently of the mode of their germ band development. However, we are observing an exception to this rule in the *Formicidae*. The ant family includes species with two types of embryogenesis, an intermediate germ band embryogenesis (e.g. *Lasius niger*) and a short germ band embryogenesis (e.g. *Messor pergandei*). Their difference is manifest in embryo morphology, the presence of extraembryonic cells and the different expression of some maternal mRNAs (e.g. *otd-1*) during the blastoderm stage. In the latter group, we found a number of striking modifications; during germ band extension in *M. pergandei*, we observed that runt is expressed in a gradient along the anterior/ventral to the posterior/dorsal axis, contrasting with *L. niger* embryogenesis in which *runt* expression shows the classical pair-rule stripes. Silencing of runt in *M. pergandei* resulted in axial defects, while in *L. niger*, we observed the expected segmentation defects. As these results suggest a novel regulation mechanism of early embryogenesis, we are interested in understanding how, in this group of insects, segmentation is regulated, what the role of pair-rule genes is and what the adaptive value of this modification might be.

UTR-specific knockdown of *Distal-less* and *Sp8* leads to new phenotypic variants in the flour beetle *Tribolium*

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RNA interference (RNAi) mediated knockdown serves as an effective technique for the functional analysis of developmental genes that is well established in many organisms. In the beetle *Tribolium castaneum* double-stranded RNA is applied by simple injection and distributes systemically within the tissue. Thus, systematic testing for RNAi-specificity and -efficiency is easily possible in this organism. Generally, the use of non-overlapping dsRNA fragments yielding qualitatively identical phenotypes is the method of choice to verify target-specific knockdown effects. Here we show that UTR-specific RNAi results in different effects regarding quality, severity and frequency when compared to RNAi-fragments directed at the coding-region. Furthermore, we first describe the *Distal-less*^{RNAi} antenna-to-leg transformation phenotype in the *Tribolium* larva, that has only been observed in the adult beetle and *Drosophila* so far. In addition, we unexpectedly observed sterility effects caused by 3´UTR-specific knockdown of the *Tribolium-Sp8* ortholog that is not seen when dsRNA targeted a sequence within the coding-region or the 5´UTR. We conclude that targeting UTR-sequences by region-specific RNAi can reveal unexpected new aspects of gene function applicable in basic research and crop protection.

Datamining BeetleAtlas to identify neuroendocrine signals that control diuresis: Introducing a novel tissue-specific expression atlas for the *Tribolium* community

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Tissue-specific expression atlases provide authoritative overviews of gene expression levels across multiple tissues, and are thus powerful tools in modern systems biology. However, the availability of such resources are highly restricted. Here, we propose to construct a new online resource, BeetleAtlas, which will provide the most detailed view yet of the spatial expression pattern of the more than 15,000 genes of the red flour beetle *Tribolium castaneum* using whole transcriptome shotgun sequencing (RNASeq). To demonstrate the utility of BeetleAtlas, we mined the transcriptomic datasets generated so far (heads, renal tubules and rectal complex of both larval and adult *Tribolium*) to identify neuroendocrine systems that control renal function in beetles. This analysis led to the discovery a gene encoding for a G-protein-coupled-receptor (GPCR), which is highly enriched in the renal (Malpighian) tubules (MTs) of *Tribolium*. Moreover, using a combination of traditional physiological and advanced genetic approaches, we characterized this neuroendocrine system, which to our knowledge is most potent regulator renal function in beetles. Although BeetleAtlas is in its infancy, we ultimately aim to generate 20 additional tissue-specific expression datasets of both larval and adult *Tribolium*, and hope to engage the community in prioritising these tissues according to end-user needs. Once completed, the BeetleAtlas dataset will be deposited unrestricted on the geo-public domain and will furthermore be integrated into existing genomic resources such as iBeetleBase and FlyAtlas to allow metatranscriptomic analyses.

Tribolium pupal pinchers support a dual origin of insect wings

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The acquisition of novel structures is a fundamental step during evolution, facilitating successful radiation and diversification of taxa. The emergence of wings in hexapods represents a profound moment in eukaryotic evolution and helped catalyze the great success of insects. Despite this, the precise tissue that gave rise to wings has been debated for over a century culminating into two prominent, yet contrasting hypotheses (the tergal origin hypothesis and the pleural origin hypothesis). New evidence is now emerging supporting a third hypothesis called the dual origin hypothesis, which can potentially unify the two competing hypotheses; however, it still requires further testing. To explore this, we investigated the genetic regulation of the tissues serially homologous to wings in the *Tribolium* pupal abdomen called gin-traps. Using an enhancer trap line of *nubbin* (a wing lineage marker) we demonstrate that formation of ectopic abdominal wings induced by homeotic transformation require contributions from two separate tissues, one from a tergal and the other from a pleural location. These results support the idea that the presence of two distinct sets of wing serial homologs per segment represents an ancestral state of the wing serial homologs. This finding further supports a dual evolutionary origin of insect wings and reveals that novelty can emerge through two previously unassociated tissues collaborating to form a new structure.

FoxB, a new and highly conserved key factor in arthropod dorsal-ventral (DV) limb patterning

Ralf Janssen

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Here I report on the discovery of a hitherto unrecognized gene that is expressed along the ventral side of all (studied) arthropods and an onychophoran, the forkhead transcription factor encoding gene *FoxB* (aka *Drosophila* paralogs *Dmfd4*/*Dmfd5* (*fd96Ca*/*fd96Cb*)). Its expression profile in *Drosophila* leg imaginal discs, the directly developing limbs of the beetle *Tribolium castaneum* the myriapod *Glomeris marginata*, the spider *Parasteatoda tepidariorum* and the onychophoran *Euperipatoides kanangrensis*, indicates a specific and conserved role in dorso-ventral (DV) limb patterning. In order to further investigate this possibility, we targeted the single spider *FoxB* gene and investigated its RNAi-induced knock-down phenotypic expression(s). We found altered leg morphology in *FoxB* RNAi-embryos that is likely correlated with disturbed DV limb-development. Expression of known (or implied by conserved expression patterns) DV limb-patterning genes such as *optomotor-blind* (*omb*), *decapentaplegic* (*dpp*), *H15*, and *wingless/Wnt1* (*wg/Wnt1*) is either missing or disturbed in *FoxB* knock-down appendages indicating a high-ranking role of *FoxB* in the gene regulatory network orchestrating DV limb patterning in spiders as well as Panarthropoda as a whole.

Your RNAi screen in *Tribolium* & Expanding the scope of iBeetle-Base

Gregor Bucher & Jürgen Dönitz

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Your RNAi screen: The iBeetle project has generated resources that allow for RNAi screens to be performed quite cost and time efficiently in *Tribolium*. I will present and discuss the design of such screens. For instance all transcription factors (700-800) could be tested in about 13 weeks at a cost of 8.000€.

iBeetle-Base: Initially, *iBeetle-Base* was developed as repository for the phenotypic data gathered in the iBeetle RNAi screen, currently covering data for 50% of the genes. In order to support the community, genome wide information on gene sequences, links to respective genomic location and homologs in other species were added. Further, it provides links to other tools and information. Since recently, *iBeetle-Base* offers the possibility to process lists of gene IDs from one species into other IDs ("query pipeline"). As a first step to allow the community to add their data we have implemented an interface to add GO terms for genes studied by the community. I will briefly review the current functionality and will discuss our plans to develop *iBeetle-Base* into an integrated database where the community can add phenotypic and other data for *Tribolium* and other species. Feedback will be highly welcome.

A linked chain of autonomously contracting cells forms an actomyosin cable during serosa window closure in the beetle *Tribolium castaneum*

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Extra embryonic tissues in insects shows highly diverse morphogenetic strategies. Unlike *Drosophila melanogaster,* the short germ insect ***Tribolium castaneum*** undergoes dramatic epiboly like extraembryonic tissue expansion and ventral serosa window closure. This offers exceptional material to study the interplay of conserved cellular and molecular mechanisms in creating novel tissue morphologies. Therefore, we imaged live *Tribolium* embryogenesis using **multi-view fluorescence light-sheet microscopy**(**SPIM**), and characterized nuclear, membrane and actomyosin dynamics in wild type and genetically perturbed conditions. We unfold our **3D** data into **2D cartographic maps** to compare and quantify cellular events across tissues in the entire embryo. Using our 4D imaging and image analysis pipeline, we report a **contractile actomyosin cable** that forms during serosa window closure and shows unprecedented cellular dynamics. It appears as a 3D enrichment spanning the D-V axis of the embryo, initially demarcating the boundary between the embryonic and extraembryonic tissues, showing diverse shape changes over time. **Laser ablations** of the serosa tissue indicate that it expands due to a pull generated by the embryonic region, leading to an increase in membrane tensions at the cable as the window closure proceeds. Interestingly, the actomyosin cable is formed as a shifting boundary of autonomously contractile cells, which intercalate into the serosa, decreasing the cable circumference and contributing to the serosa expansion. Genetic perturbations of the serosa affect the cable and embryo morphology, indicating towards a mechanical role of serosa in embryo development. Together, our results show a novel actomyosin cable type that could show conserved cellular dynamics across insects which undergo serosa window closure.

A functional analysis reveals both shared and divergent roles for developmental patterning genes in the wing and body wall margin of *Oncopeltus fasciatus*

Cera Fisher and Elizabeth Jockusch

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The evolution of wings was arguably the key event that allowed insects to achieve enduring success and rapid expansion into every available niche. Various ancestral tissues are hypothesized for the origin of wings, including both tergal and pleural body wall outgrowths, or a combination of the two as in the dual-origin hypothesis. Recent research has identified certain characters of non-wing-bearing segments of insects that are regulated by many of the same genes involved in wing patterning, suggesting that those segments may bear wing serial homologs. In particular, overlapping, double-layered epithelium at the margins of many insect sclerites is patterned using components of the wing patterning network. Here we present results of RNA interference during metamorphosis in *Oncopeltus fasciatus*, the large milkweed bug, for genes in the canonical wing development pathway (e.g., *apterous, vestigial*) in addition to genes known to be involved in dorsal body wall patterning (e.g., *homothorax, araucan*). The resulting phenotypes show both distinct and shared functions for these genes in wing development and body wall margins. These results also bear directly on our lab's investigation of the origin of the treehopper helmet, a novel organ derived from the pronotum.

A universal vector concept for a direct genotyping of double transgenic organisms and a systematic creation of double homozygous lines

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Similarly to most eukaryotic model organisms, the red flour beetle *Tribolium castaneum* carries a diploid chromosome set. Thus, transgenic individuals are either hemi- or homozygous. Producing a line that carries a single transgene homozygously requires considerable resources. The complexity rises substantially when working with two different transgenes: typical crossing schemes, *e.g.* mating a double hemizygous individual with a genetically identical sibling, result in up to nine different genotypes. Therefore, we initially developed the *AGameOfClones* vector concept, which contains mOrange- and mCherry-based transformation markers. Lately, we evaluated our *AClashOfStrings* vector concept, which contains mCerulean- and mVenus-based transformation markers. In either concept, the markers are embedded in interweaved, but incompatible Lox site pairs. Independent Cre-mediated recombination leads to hemizygous individuals that retain only one marker, which in turn is used to produce hybrids, *i.e.* double hemizygous progeny that carry either the mOrange/mCerulean or the mCherry/mVenus marker combination. In the next generation, individuals that carry all four markers are identified as double heterozygous and give rise to double homozygous descendants that are selected by their lack of two of the markers. We prove the functionality of both vector concepts by systematically creating multiple double homozygous transgenic *Tribolium* lines suitable for long-term fluorescence live imaging. Since our approach relies on the universal Cre-Lox system, it works in most diploid model organisms, *e.g.* in rodents, zebrafish, insects and plants. It saves resources, simplifies important aspects of transgenic organism handling and, essential for ethical as well as economic reasons, it minimizes the number of wasted animals.