REVIEW

The red flour beetle *T. castaneum*: elaborate genetic toolkit and unbiased large scale RNAi screening to study insect biology and evolution

Martin Klingler[1*] and Gregor Bucher[2*]

Abstract

The red flour beetle *Tribolium castaneum* has emerged as an important insect model system for a variety of topics. With respect to studying gene function, it is second only to the vinegar fly *D. melanogaster*. The RNAi response in *T. castaneum* is exceptionally strong and systemic, and it appears to target all cell types and processes. Uniquely for emerging model organisms, *T. castaneum* offers the opportunity of performing time- and cost-efficient large-scale RNAi screening, based on commercially available dsRNAs targeting all genes, which are simply injected into the body cavity. Well established transgenic and genome editing approaches are met by ease of husbandry and a relatively short generation time. Consequently, a number of transgenic tools like *UAS/Gal4, Cre/Lox*, imaging lines and enhancer trap lines are already available. *T. castaneum* has been a genetic experimental system for decades and now has become a workhorse for molecular and reverse genetics as well as in vivo imaging. Many aspects of development and general biology are more insect-typical in this beetle compared to *D. melanogaster*. Thus, studying beetle orthologs of well-described fly genes has allowed macro-evolutionary comparisons in developmental processes such as axis formation, body segmentation, and appendage, head and brain development. Transgenic approaches have opened new ways for in vivo imaging. Moreover, this emerging model system is the first choice for research on processes that are not represented in the fly, or are difficult to study there, e.g. extraembryonic tissues, cryptonephridial organs, stink gland function, or dsRNA-based pesticides.

Keywords: Insect model system, Arthropod model system, Coleoptera, Evolution of development, Physiology, Behavior, Pest control, Systemic RNAi, Genome-wide screen, CRISPR genome editing

Natural habitat and life cycle

The kitchen is a great place to start for collecting *Tribolium castaneum*. As a stored product pest, sampling "wild" populations of *Tribolium* means collecting in the house, or in flour processing or storage industries. What was the natural habitat of these synanthropic beetles before the emergence of human agriculture and grain processing? We don't know! Some speculations about the origin of *T. castaneum* (including ancient Egyptian tales) can be found in a recent review that covers current and classical *T. castaneum* work in population genetics, ecology and evolution [1]. Here, we focus on *T. castaneum* as an experimental system for molecular and functional analysis of insect genes.

As a typical holometabolous insect, *T. castaneum* develops through several larval stages (usually 7, but 5 or 6 when starved [2, 3], and up to 11 instars based on some anecdotal accounts) followed by metamorphosis (Fig. 1). Its embryonic developmental comfort zone is between 22 and 32 °C, lasting 7 days at 25 °C and 3 days at 32 °C [3].

*Correspondence: martin.klingler@fau.de; gbucher1@uni-goettingen.de

[1] Department of Biology, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Staudtstr. 5, 91058 Erlangen, Germany
[2] Johann-Friedrich-Blumenbach-Institut, GZMB, University of Göttingen, Justus-von-Liebig-Weg 11, 37077 Göttingen, Germany
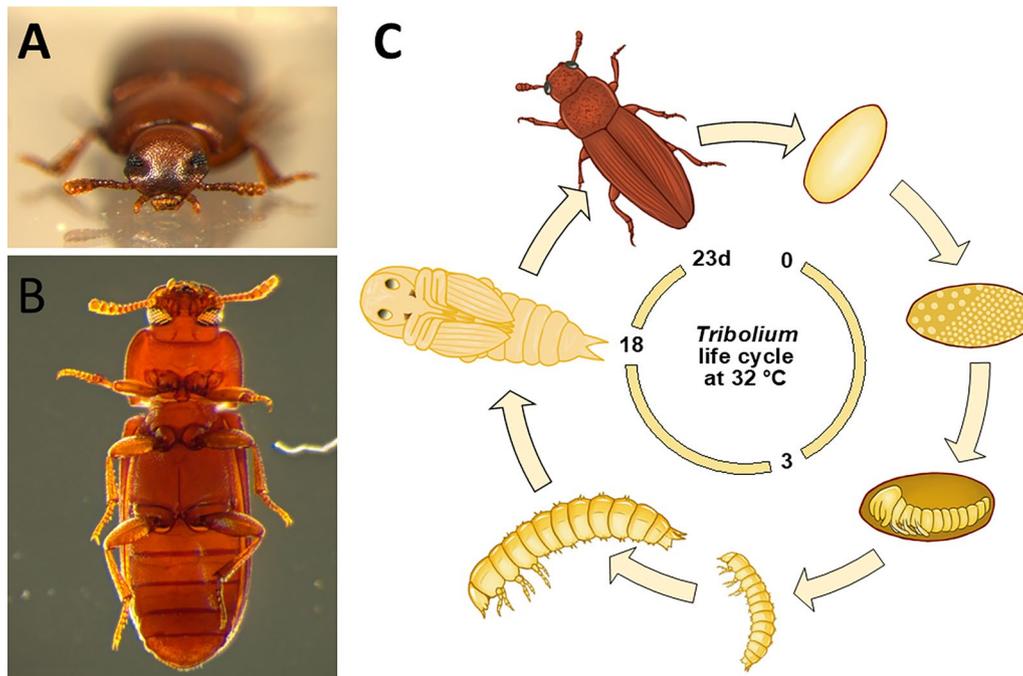
Fig. 1 The life cycle of *T. castaneum*. **A** Face-to-face with a wild type *Tribolium castaneum*. **B** Ventral view of a male under dark field illumination. White eyes (*vermilion* mutant) allow for sensitive detection of transgene eye markers. The size of an adult beetle is 3.5 mm (length). **C** Life cycle of *Tribolium:* eggs are laid into the substrate (flour) and embryonic development takes 3 days at 32 °C. Only two out of a variable number of larval stages (ca. 7) are depicted. The *pupa libera* allows visual inspection of external structures facilitating phenotypic studies of metamorphosis. Female beetles need a few days after hatching until they start laying eggs, which they continue for 3–4 months (drawings not to scale). Life cycle sketch by Benjamin Schwarz

Likewise, development from egg to adult speeds up with temperature from 74 days at 22.5 °C to about 23 days at 32 °C, which is short enough for large scale genetic experiments.

Lab culture

T. castaneum is easy to culture: it only requires organic (insecticide free) flour enriched with yeast powder (5%) for protein and vitamins. For stock keeping, whole wheat flour is recommended as it contains additional nutrients. Only for egg collection, the beetles are kept on white flour (of which "instant flour" or "doppelgriffiges Mehl" are easier to sieve). No bad odors need to be endured by *T. castaneum* workers, and unlike *Tenebrio*, the familiar "meal worm" sold in pet shops, no fresh vegetables need to be added. Flour beetles don't need to drink as they derive all their body fluids from dry matter: burning starch produces water. Beetles are best kept in incubators at a humidity between 40 and 60% and with an opening for supply of fresh air. Humidity should not drop below 30% while the upper limit is set by the emergence of mold in the flour. Beetles live up to a year but egg-laying rate is high only in the first 3–4 months.

Cultures can be protected from mites using dense foam stoppers or by covering ventilation openings with a fine gauze (100 µm mesh size). Cultures infected with microorganisms transmitted via flour can be "sterilized" by collecting eggs from the flour (using 300 µm sieves), suspending them in 3% formaldehyde for 15 min, collecting them on gauze and rinsing with water, and then placing the gauze on top of fresh flour (eggs facing down). Small stocks (50–200 animals) are kept in vials (e.g. typical fly vials with 5 cm diameter) with foam stoppers. Larger populations, e.g. for collection of eggs or animals to be injected, are kept in boxes of about 25 cm edge length with an opening for air exchange of around 5 cm diameter.

During experiments, beetles are usually kept at 30–32 °C to speed up development and for obtaining strongest RNAi phenotypes. Higher temperatures reduce embryonic viability. At 25 °C the developmental time roughly doubles compared to 32 °C. For stock maintenance the temperature can be reduced to 22–23 °C, where development from egg to adult lasts ca. 74 days. To ensure that synchronized animals of all stages are available at any time, a working stock can be kept in the following way: adult beetles are kept at 25 °C in large boxes on

white flour supplemented with yeast. Eggs are collected twice a week from this culture and transferred to another box with fresh whole wheat flour. Transfer of eggs to new flour (instead of moving the adults to a new box) minimizes the transmission of parasites via feces. To allow for synchronized development within one batch, the culture should not be too dense. Every 3–4 months the original stock is replaced by young beetles.

Stock keeping of transgenic strains requires little work. For mutants or transgenic strains kept in heterozygous condition, we select individuals carrying a visible marker once or twice a year; 20–50 beetles are transferred to new flour at least every 6 months and fresh flour is added to those cultures periodically. This way, one person with 50% working time can keep several hundreds of transgenic stocks. For stable homozygous lines, checking the eye marker may be done even less frequently.

Developmental stages can be separated using commercially available metal wire sieves of different mesh sizes: 800 μm mesh separates adults, pupae and late larvae from whole wheat flour (pre-sieved with 700 μm mesh) and from younger stages; 500 μm retains 5th instar larvae and older stages; 300 μm separates eggs from white flour (pre-sieved with 250 μm mesh). A vacuum cleaner can be used to clean the sieves and other tools, which are then dry sterilized at 70 °C to prevent contamination of stocks with extraneous eggs or larvae.

Frequent work with flour and beetles can lead to allergies. Therefore, wearing FFP1 masks, or using a chemistry hood, is highly recommended for any work involving sieving of flour.

For dsRNA injection into females or for single pair matings, sexing is required. Animals are most easily sexed at the pupal stage by external genital anlagen (larger in females). Adult males can be identified by the presence of a gland on the femur of the first pair of legs. Animals can be sorted using sprung steel tweezers. Movement of adults and larvae can be reduced by placing them in a glass petri dish on ice, or through anesthesia with carbon dioxide using standard fly equipment.

Major interests and research questions

T. castaneum has traditionally been used for developmental, ecological, behavioral and physiological studies [1, 3, 4]. In the 1960s and 70 s, a large number of mutants were recovered and studied [5]. This included for instance mutations of Hox genes, including a deficiency in the Hox cluster transforming all segments to antennal fate [6–10]. Much of the early work was summarized by Alexander Sokoloff in his three volume book [3–5]. Molecular genetics of *T. castaneum* took off in the 1990s e.g. with work in the Denell lab on the Hox complex [11, 12]. Pioneering comparisons of expression and function of orthologs of fly segmentation genes in Germany and the US [13–15] started off a major effort on macroevolutionary comparisons of patterning gene functions between fly and beetle [16–25].

In many respects the development and the biology of *T. castaneum* is more typical of insects than that of *D. melanogaster* [26, 27]. Recently, the gene sets identified to be required in the same developmental processes in *D. melanogaster* versus *T. castaneum* were systematically compared based on data from FlyBase and from the large scale RNAi screen *iBeetle* [28]. Only half of the genes involved in a given biological process were detected in both fly and beetle, while the other half were detected in only one species. This divergence probably reflects both biological divergence and technical differences in the screening approaches. These data underline the necessity of additional model systems even for well-studied processes [29]. Increasingly, *T. castaneum* is also used to study basic biological questions for which other model systems are less well suited.

Embryo polarity and patterning

The genetic underpinnings of early development are best understood in *D. melanogaster*. However, this species differs from insect-typical embryogenesis in several aspects. For instance, most cells of the blastoderm contribute to the embryo, and all segments emerge almost at the same time (long germ development) while in most other insects, a large part of the blastoderm is extraembryonic and posterior body segments are added sequentially from a posterior segment addition zone (short germ development) [26, 30]. The homologues of most developmental genes originally identified in *D. melanogaster* turned out to play similar roles in *T. castaneum*. However, quite a few of them serve different functions, demonstrating that evolution of early developmental processes is surprisingly unconstrained. For example, pair-rule genes are expressed in double-segmental stripes as in *D. melanogaster* but appear one after the other in the *T. castaneum* segment addition zone, driven by a segmentation clock [18, 31, 110] regulated by a frequency or speed gradient [32, 33]. Since primary pair rule genes are part of the segmentation clock, their knock-down leads to complete segmentation breakdown, instead of the pair rule phenotypes known from *D. melanogaster*. While later-active segmentation genes such as segment polarity and Hox genes seem to be functionally more conserved [34, 35] the functions of gap gene orthologs have diverged [17, 20, 36, 37]. Interestingly, some genes have important functions in patterning the beetle body axis, which were not know from flies [38–40]. The early gradients initiating embryonic axis formation are unexpectedly different from *D. melanogaster*, both along the dorso-ventral

[23, 24, 41], and the anterior-posterior axes. For instance, Wnt signaling is involved in early axis formation in most animals including vertebrates but not in *D. melanogaster*. In *T. castaneum*, it was found that anterior localization of the mRNA of a Wnt inhibitor led to early asymmetry of Wnt signaling involved in axis formation and proper anterior and posterior development [19, 21, 22].

Analyses of genes with conserved functions have also revealed new insights. For instance, Hox gene mutations in *T. castaneum* demonstrated a largely conserved function [8, 10] but they are located on an undivided Hox complex (similar to most animals but not *D. melanogaster*) [6, 42]. Analyses of embryonic phenotypes of HOX gene mutations and the HOX cluster deletion unequivocally demonstrated that the "ground state" of insect segment identity is antennal. This hypothesis had previously been formulated in flies based on phenotypes observed after clonal analysis in adults. However, the respective transformations had not been found in fly embryos [43]. Studying the regulation and function of the Hox cluster in *T. castaneum* may shed light on the reason for the conserved linkage of animal Hox clusters, contrasting the split observed in flies.

Morphogenetic movements and extraembryonic tissues
In some respects, the classical model system *D. melanogaster* is a poor representative of insects and *T. castaneum* is a good model system to study such processes. For instance, insects evolved extraembryonic membranes as an adaptation to life on land. In most insects (including *T. castaneum* but not *D. melanogaster*), the serosa forms a defensive layer around the embryo, protecting the growing embryo from dehydration (serosal cuticle) and invading microorganisms (serosal expression of immune genes) [44]. Formation of this extraembryonic layer necessitates additional morphogenetic movements that internalize the embryo and form the amnion, the other extraembryonic tissue found in insects. At the end of embryogenesis, this internalization (anatrepsis) becomes reversed (katatrepsis) and extraembryonic tissues are eliminated in the "dorsal organ". The mechanics of these movements are intriguing and in *T. castaneum* they are directly accessible to analysis by genetics and live imaging [45–47].

Head development, brain evolution and behavior
The head of adult flies is highly derived, and even more so is the involuted head of fly larvae. Beetle larvae, in contrast, have an insect-typical head capsule with well-formed head appendages. How the insect head morphology comes about during development was first understood in *T. castaneum* [48, 49]. While many head (and brain) genes are widely conserved [48], there is

much variation in head development between beetle and fly, some of which may be causally related to changes in head morphology [49, 50]. The brain of fly larvae is also strongly reduced, lacking a *central complex* until metamorphosis, while many insects including beetles show a reduced larval form of that structure [51]. Studies of embryonic patterning of the central complex in *T. castaneum* provide the basis for understanding evolutionary shifts of developmental timing in the brain [52–54]. Likewise, *T. castaneum* shows a more ancestral state with respect to the neuroendocrine system and the subesophageal ganglion has been studied with respect to adult neurogenesis [55–57]. Since the *T. castaneum* CNS is also amenable to systemic RNAi [58, 59], there should be a future for behavioral genetics via RNAi in the beetle, for example to study circadian activity or in death feigning behavior [60, 61].

Appendage development and regeneration
Segmented appendages are the defining character of arthropods, and it is unfortunate that formation of these important structures is still incompletely understood. *D. melanogaster* research has identified the early acting appendage "gap genes" *Distal-less, dachshund* and *homothorax*, whose role is conserved in *T. castaneum* [62–64]. However, how the size and number of podomeres, and the position of joints are specified by the early acting appendage genes is not known. The fact that legs form during embryogenesis in *T. castaneum* allows studying the process with simple standard tools while studying leg formation during fly metamorphosis requires clonal analysis or transgenic RNAi. Given the genome-wide RNAi screening opportunities in *T. castaneum,* this model is predestined to study appendage segmentation in arthropods.

While legs arise in most insects as cylindrical outgrowths of the embryonic epidermis, in *D. melanogaster* limb formation is delayed and occurs within invaginated and flattened imaginal discs. Hence, the comparison of the genetic networks between fly and beetle should reveal how the derived state of fly limb development evolved [63]. Another process that is amenable to genetic analysis in *T. castaneum* is the ability of larval appendages to regenerate between larval molts [65].

Metamorphosis and development of adult shape and organs
Many genes essential for postembryonic development of pupae and adults also function during embryogenesis. Systemic RNAi makes is easy to knock down an essential gene during metamorphosis without killing the embryo as would happen with a null mutation. Applying dsRNA

to late larvae or pupae has revealed many late gene functions in hormonal regulation, the formation of adult limbs and wings, or other organs including hard adult cuticle, adult flight musculature, development and operation of defensive glands, or the development of gonads and gametes [2, 66–71].

Population studies and physiology

Microevolutionary studies are actively pursued by a substantial part of the *Tribolium* research community (for review see [1]). In addition, several questions concerning physiological adaptations can be genetically analyzed in *T. castaneum*, including the use of defensive odoriferous glands producing toxins without poisoning the carrier [70]. Moreover, the species' impressive adaptation to drought make it an ideal model system to study this phenomenon e.g. by studying the cryptonephridial organ [72]. Further, the life-span is considerably longer than that of *D. melanogaster* providing the opportunity to find additional anti-aging related processes.

Pest management, systemic RNAi and selfish genetic elements

As a major pest of stored products, *T. castaneum* has long been used for investigating pest management and insecticide resistance [81]. While earlier studies focused on mechanisms and spread of pesticide resistance, recent interest has been building for using *Tribolium* as a model system for testing dsRNA-based pesticides in beetles and other insects. In RNAi mediated pest control, dsRNAs that target essential genes of pest species are sprayed onto plants, or produced by transgenic crops [82, 83]. Upon feeding on the crop, the dsRNA induces RNAi in the pest leading to its death. So far, *T. castaneum* has served as a screening tool for identifying the most efficient target genes [84, 85]. In the future, it might be useful for optimization of target sequences and to study RNAi dynamics. Since the capability to induce RNAi systemically varies widely among beetles and other pest species, the mechanism of dsRNA uptake and spread has also come into focus, which can be thoroughly studied in *T. castaneum* [86, 87].

A most intriguing example of selfish DNA was discovered by RW Beeman in *T. castaneum*, causing skewed segregation ratios in genetic crosses, named MEDEA (Maternal-Effect Dominant Embryonic Arrest, [88]). This element can spread in beetle populations through vertical transmission, by killing all offspring that do not inherit the MEDEA element. The principle underpinning MEDEA, consisting of a maternally expressed toxin and a zygotically expressed antidote, has inspired artificial

selfish constructs that should similarly spread in natural populations and could be used for species-specific suppression of pest and vector species [89].

Experimental approaches

The workhorse technique: robust and systemic RNA interference

Gene knock-down via RNAi is an exceptionally robust and efficient technique in *T. castaneum* [90, 91]. The penetrance of phenotypes is usually close to 100% (i.e. all injected animals and all their offspring usually show a phenotype), and phenotypic strength comparable to the Null-phenotypes of genetic mutations are observed in most cases, e.g. for the Hox gene *Tc-sex combs reduced* [9], the patterning genes *Tc-Krüppel* and *Tc-knirps* [37, 92] and *Tc-Distal-less* [62, 91], as well as the eye color enzyme *Tc-vermillion* [93]. All the tissues and processes tested so far are amenable to RNAi, including physiological processes like cuticle [94] and pigment formation [95], immunity [96, 97] and neuroendocrine processing [56]. Development has been studied with respect to embryonic [17, 22] and postembryonic patterning of the epidermis [98], extraembryonic tissues [99], brain [53] and muscle formation [100].

Importantly, the RNAi effect is environmental, i.e. extracellular dsRNA is taken up by cells and triggers silencing. Hence, after injection into the body cavity, dsRNA is distributed via the hemolymph and appears to be taken up by all cells in the entire organism. This was convincingly shown when EGFP expressed from a ubiquitous promoter was silenced in all tissues [58]. We are not aware of tissues or stage that would not be amenable to RNAi. Hence, injection into the body cavity of any life stage leads to gene knock-down in most if not all cells. Embryos can be injected at late stages, leaving earlier gene functions unaffected, and injection into L5 or L6 larvae is often used to obtain phenotypes affecting metamorphosis. The RNAi effect is even transmitted from mother to offspring, such that injection into female pupae or adults leads to knock-down in the offspring as well [91]. The strength of silencing can be adjusted either by titrating the concentration of the dsRNA injected, or by collecting embryos at different time points after injecting their mothers [91]. dsRNAs are also taken up by cells in culture [101]. The molecular basis of dsRNA uptake remains enigmatic in *T. castaneum* but it seems to involve Clathrin-dependent endocytosis [86, 102]. Trials with oral uptake of dsRNA have met divergent success in different labs [103]. Whether RNAi silencing can spread from cell to cell (i.e. systemic RNAi sensu stricto) remains to be tested. In summary, virtually every gene in every tissue can be expected to be strongly silenced upon injection of dsRNA into any stage of *T. castaneum*.

Cost- and time-efficient RNAi screening for unbiased detection of gene function

An outstanding strength of *T. castaneum* is the ease of large- or mid-scale RNAi screens. As part of the *iBeetle* screen [28, 29], a genome-wide collection of templates was produced, based on which dsRNAs can be commercially ordered, obviating the need for cloning (Eupheria Biotech, Dresden). This resource, together with the ease of microinjection (students learn the procedure within a day), enables mid or large scale RNAi screens to search for novel gene functions or to test gene lists derived from omics analyses. In our experience, the upper limit of the throughput is about 100 genes per week per screener (including injection, processing and screening for a simple phenotype like lethality). The *iBeetle* screen was a particularly complex screen because many different types of phenotypes were scored in parallel, resulting in relatively low throughput of 21 genes per week per screener. Based on these experiences, testing for example all transcription factors (around 800 genes) would take between 8 and 13 weeks depending on the complexity of the readout. Thus, *T. castaneum* is an excellent system for quickly testing the function of many genes, e.g. based on omics candidate gene lists.

Elaborate and expanding transgenic toolkit

T. castaneum was the first species where the 3xP3 eye marker, based on an artificial Pax-responsive enhancer, was successfully applied [104]; 3xP3 has become a standard element of transgenic constructs in many insects. Besides the *piggyBac* transposon, other transposable element systems like *Minos* [105] or maize *Ac/Ds* (Distler & Klingler unpublished) work as transgenesis vectors as well. Transgenesis is performed as in *D. melanogaster,* by injecting early blastoderm stages with a plasmid containing transposon sequences flanking the cargo, mixed with a helper plasmid or mRNA that serve as a source of the transposase. The efficiency of transgenesis is similar to that using the P-element in *D. melanogaster*, and the genomic distribution of insertions is quite random [106]. Heat-shock and UAS/Gal4 mediated gene misexpression have been established [107, 108], the activity of several enhancers has been tested [109] and dedicated in vivo imaging lines have been established [110, 111]. Of note, enhancers and core promoters taken from *D. melanogaster* show poor activity in *T. castaneum,* such that the use of endogenous elements is recommended. Core promoters that have proved effective in enhancer trapping and in genetic constructs include the *Tc-hsp68* and the super core promoter 1 (SCP1)-the latter being active in both *D. melanogaster* and *T. castaneum* [109]. Cre-Lox mediated recombination is working efficiently [111]

opening the way for flip-out technologies like brainbow [112].

The identification and characterization of enhancers driving specific patterns is the limiting step in many applications. Several enhancers expected to drive ubiquitous expression such as *Tc-alpha-tubulin*, *Tc-EF1-alpha* and *Tc-poly-ubiquitin* appear not to be active in all cells at all stages and often show patchy or mosaic expression (Averof and Bucher labs, unpublished observation). A number of efforts testing putative developmental enhancers by placing them in front of reporter genes have failed. The likelihood of identifying active enhancers is improved by taking into account chromatin accessibility and using additional bioinformatics predictions [109]. The *cis*-regulatory elements of ubiquitously expressed genes are often located within few kilobases upstream of the transcription start site.

Forward genetic screens-from irradiation to transposon mutagenesis

The relatively short generation time of *T. castaneum* allows performing classic forward mutagenesis screens. Irradiation and chemically induced mutagenesis were used to generate homeotic mutations that allowed to identify the single HOX complex [42]. A screen based on chemical mutagenesis led to the identification of pair-rule and gap-gene mutants [34, 37, 92, 113] and tools for parallel processing of up to 25 single pair matings have been developed [114]. The mobilization of piggyBac transposons has been applied for large-scale insertional mutagenesis and enhancer trapping in the *GEKU screen* [106] (Fig. 2). Based on mutants and molecular markers, a genetic map was generated, which was crucial for guiding a chromosome level assembly of the genomic sequence in times prior to long read sequencing [76, 78, 79].

Given the challenge of characterizing the often complex mutations induced by irradiation, the difficulty of mapping mutations and the effort of keeping such lines, we think that the use of transposon-mediated screening remains the most convenient forward genetics option, since the transposon marks the mutated locus and facilitates stock keeping based on a visible eye marker. However, given the effort it takes to reach saturation in genetic screens and the subsequent manpower needed for stock keeping, forward genetic screens seem less attractive. Hence, instead of performing forward genetic screens, approaches using large scale RNAi screening and genome editing to generate specific mutants may be more convenient for asking most questions.
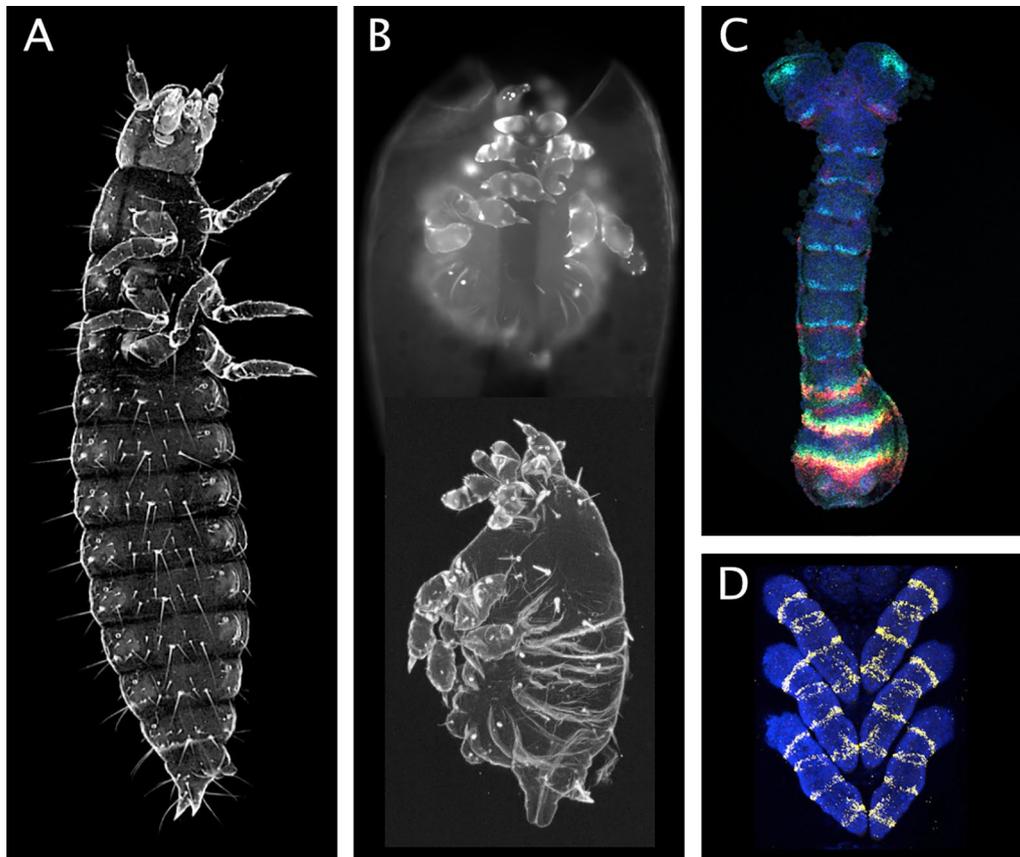
Fig. 2 Cuticle phenotypes and in situ stainings. **A**, **B** Cuticle autofluorescence images of a wildtype 1st instar larva (**A**) and two larvae homozygous for a transposon insertion into the first exon of the *Tc-cycD* gene. Reduced cell proliferation results in smaller embryos with shortened thoracic appendages (**B**). (**C**, **D**) Hybridization chain reaction staining of the pair rule genes *Tc-runt* and *Tc-odd-skipped* together with *Tc-wingless* as segmental marker in an elongating germ band (**C**) and of *Tc-odd-skipped* in the embryonic legs (**D**). Anterior is up in all panels. The larva in (**A**) is 1.4 mm in length, the embryo in (**C**) is 0.87 mm long. Image (**C**) by Felix Kaufholz, (**D**) by Christine Zellner

Genome editing for transgene insertion, gene modification and clonal cell marking

Genome editing based on CRISPR/Cas9 is highly efficient in *T. castaneum*, with knock-out efficiencies including mosaicism in > 50% of the injected animals and a high rate of germline transmission [115]. Knock-in of constructs using non-homologous end joining works reasonably well, with 0.1 to 10% of injected animals having at least one transgenic offspring. Constructs for generating targeted enhancer trap lines using this technique are available [53, 116]. Homology directed repair is efficient when replacing the EGFP of a transgene with another fluorescent protein [115], but less efficient when replacing larger stretches of DNA of essential genes (Bucher, unpublished results). Part of the problem may be the lethal effects of somatic mutations, which could be overcome with a transgenic line restricting expression of Cas9 to the germline. Efforts to generate such lines are underway (Gilles, Schinko, Bucher, personal communication)

[117]. A system to enable stochastic clonal cell marking and clonal analysis of gene functions (Valcyrie) based on CRISPR/Cas9 driven excision has been established, opening interesting applications [118].

In vivo *imaging and biophysics*

Markers for live imaging based on injected mRNA or transgenic lines (reporter constructs and enhancer trap lines generated by random insertion or CRISPR approaches) are available, providing a global view of embryonic development, including patterns of cell proliferation, morphogenetic movements, cell migration and innervation, enabling the analysis of the underlying biophysical mechanisms [45, 110, 116, 119–121].

Genetic mapping

Surprisingly few QTL and GWAS studies have been conducted in *T. castaneum* so far [73–75], even though SNPs

are frequent, and other mapping markers have been available for some time [76–78]. Frequently used strains are the San Bernardino (SB, mainly in the European evo-devo community-originally obtained from A. Sokoloff by K. Sander), Georgia (GA) and cSM strains (in the USA). An inbred line derived from GA was used for genome sequencing (GA2) [79, 80]. An India-derived strain (Tiw-1) has been used for genetic mapping experiments in combination with SB and other American strains, as its genetic distance from most other strain offers plenty of molecular markers (about one SNP per 200 bp when compared to SB) [37, 76, 77].

Research community and resources

The *iBeetle*-Base (http://ibeetle-base.uni-goettingen.de/) provides gene sequence information, links to respective *Drosophila* homologs, and phenotypic information from the *iBeetle* RNAi-screen [28, 122, 123]. Further, it allows users to download the sequences of the entire genome, individual genes or coding sequences, and includes a genome browser. The *T. castaneum* morphological ontology *TrOn* describes a machine-readable semantic representation of morphological terms and their definitions [124]. An extensive transcriptomics database similar to the FlyAtlas [125] is being established (BeetleAtlas; https://motif.mvls.gla.ac.uk/BeetleAtlas/; K.V. Halberg, personal communication).

The first genome sequence included genetic mapping data [79] and was enhanced by re-sequencing and by adding extensive RNA-seq data [80]. An assembly based on long read technology is in the making.

dsRNAs targeting any set of *T. castaneum* genes can be ordered from Eupheria Biotech GmbH, Dresden.

A community mailing list is open for discussion of technical questions and is also used for announcing meetings and courses (subscribe at https://listserv.gwdg.de/mailman/listinfo/T. castaneum-community). Community meetings, which are often attached to the Euro-EvoDevo (EED) meeting, are announced via this list.

References
1. Pointer MD, Gage MJG, Spurgin LG. Tribolium beetles as a model system in evolution and ecology. Heredity. 2021. https://doi.org/10.1038/s41437-021-00420-1.
2. Chafino S, Ureña E, Casanova J, Casacuberta E, Franch-Marro X, Martín D. Upregulation of E93 gene expression acts as the trigger for metamorphosis independently of the threshold Size in the Beetle *Tribolium castaneum*. Cell Rep. 2019;27:1039-1049.e2.
3. Sokoloff A. The biology of Tribolium: with special emphasis on genetic aspects. Oxford: Clarendon Press; 1974.
4. Sokoloff A. The biology of Tribolium: with special emphasis on genetic aspects. Oxford: Clarendon Press; 1972. p. 1972.
5. Sokoloff A. The biology of Tribolium: with special emphasis on genetic aspects. Oxford: Clarendon Press; 1977.
6. Stuart JJ, Brown SJ, Beeman RW, Denell RE. A deficiency of the home-otic complex of the beetle Tribolium. Nature. 1991;350:72–4.
7. Shippy TD, Guo J, Brown SJ, Beeman RW, Denell RE. Analysis of maxillopedia expression pattern and larval cuticular phenotype in wild-type and mutant tribolium. Genetics. 2000;155:721–31.
8. Brown S, DeCamillis M, Gonzalez-Charneco K, Denell M, Beeman R, Nie W, et al. Implications of the Tribolium deformed mutant phenotype for the evolution of Hox gene function. Proc Natl Acad Sci U S A. 2000;97:4510–4.
9. Curtis CD, Brisson JA, DeCamillis MA, Shippy TD, Brown SJ, Denell RE. Molecular characterization of Cephalothorax, the Tribolium ortholog of sex combs reduced. Genesis. 2001;30:12–20.
10. Shippy TD, Ronshaugen M, Cande J, He J, Beeman RW, Levine M, et al. Analysis of the Tribolium homeotic complex: insights into mechanisms constraining insect Hox clusters. Dev Genes Evol. 2008;218:127–39.
11. Beeman RW, Stuart JJ, Haas MS, Denell RE. Genetic analysis of the homeotic gene complex (HOM-C) in the beetle *Tribolium castaneum*. Dev Biol. 1989;133:196–209.
12. Beeman RW, Stuart JJ, Brown SJ, Denell RE. Structure and function of the homeotic gene complex (HOM-C) in the beetle *Tribolium castaneum*. BioEssays. 1993;15:439–44.
13. Sommer RJ, Tautz D. Involvement of an orthologue of the Drosophila pair-rule gene hairy in segment formation of the short germ-band embryo of Tribolium (Coleoptera). Nature. 1993;361:448–50.

14. Brown SJ, Patel NH, Denell RE. Embryonic expression of the single Tribolium engrailed homolog. Dev Genet. 1994;15:7–18.

15. Nagy LM, Carroll S. Conservation of wingless patterning functions in the short-germ embryos of *Tribolium castaneum*. Nature. 1994;367:460–3.

16. Brown S, Fellers J, Shippy T, Denell R, Stauber M, Schmidt-Ott U. A strategy for mapping bicoid on the phylogenetic tree. Curr Biol. 2001;11:R43–4.

17. Bucher G, Klingler M. Divergent segmentation mechanism in the short germ insect Tribolium revealed by giant expression and function. Development. 2004;131:1729–40.

18. Choe CP, Miller SC, Brown SJ. A pair-rule gene circuit defines segments sequentially in the short-germ insect *Tribolium castaneum*. Proc Natl Acad Sci U S A. 2006;103:6560–4.

19. Bolognesi R, Farzana L, Fischer TD, Brown SJ. Multiple Wnt genes are required for segmentation in the short-germ embryo of *Tribolium castaneum*. Curr Biol. 2008;18:1624–9.

20. Marques-Souza H, Aranda M, Tautz D. Delimiting the conserved features of hunchback function for the trunk organization of insects. Development. 2008;135:881–8.

21. Fu J, Posnien N, Bolognesi R, Fischer TD, Rayl P, Oberhofer G, et al. Asymmetrically expressed axin required for anterior development in Tribolium. Proc Natl Acad Sci U S A. 2012;109:7782–6.

22. Ansari S, Troelenberg N, Dao VA, Richter T, Bucher G, Klingler M. Double abdomen in a short-germ insect: Zygotic control of axis formation revealed in the beetle *Tribolium castaneum*. Proc Natl Acad Sci. 2018. https://doi.org/10.1073/pnas.1716512115.

23. van der Zee M, Stockhammer O, von Levetzow C, Nunes da Fonseca R, Roth S. Sog/Chordin is required for ventral-to-dorsal Dpp/BMP transport and head formation in a short germ insect. Proc Natl Acad Sci U S A. 2006;103:16307–12.

24. Nunes da Fonseca R, von Levetzow C, Kalscheuer P, Basal A, van der Zee M, Roth S. Self-regulatory circuits in dorsoventral axis formation of the short-germ beetle *Tribolium castaneum*. Dev Cell. 2008;14:605–15.

25. van der Zee M, Berns N, Roth S. Distinct functions of the Tribolium zerknullt genes in serosa specification and dorsal closure. Curr Biol. 2005;15:624–36.

26. Tautz D, Friedrich M, Schröder R. Insect embryogenesis - what is ancestral and what is derived? Development. 1994;1994:193–9.

27. Nagy LM. Insect segmentation A glance posterior. Curr Biol. 1994;4:811–4.

28. Schmitt-Engel C, Schultheis D, Schwirz J, Ströhlein N, Troelenberg N, Majumdar U, et al. The iBeetle large-scale RNAi screen reveals gene functions for insect development and physiology. Nat Commun. 2015;6:7822.

29. Hakeemi MS, Ansari S, Teuscher M, Weißkopf M, Großmann D, Kessel T, et al. Screens in fly and beetle reveal vastly divergent gene sets required for developmental processes. BMC Biol. 2022;20:38.

30. Davis GK, Patel NH. Short, long, and beyond: molecular and embryological approaches to insect segmentation. Annu Rev Entomol. 2002;47:669–99.

31. El-Sherif E, Averof M, Brown SJ. A segmentation clock operating in blastoderm and germband stages of Tribolium development. Dev Camb Engl. 2012;139:4341–6.

32. Zhu X, Rudolf H, Healey L, François P, Brown SJ, Klingler M, et al. Speed regulation of genetic cascades allows for evolvability in the body plan specification of insects. Proc Natl Acad Sci U S A. 2017;114:E8646–55.

33. Boos A, Distler J, Rudolf H, Klingler M, El-Sherif E. A re-inducible gap gene cascade patterns the anterior-posterior axis of insects in a threshold-free fashion. Elife. 2018;7:e41208.

34. Maderspacher F, Bucher G, Klingler M. Pair-rule and gap gene mutants in the flour beetle Tribolium castaneum. Dev Genes Evol. 1998;208:558–68.

35. Peel AD, Telford MJ, Akam M. The evolution of hexapod engrailed-family genes: evidence for conservation and concerted evolution. Proc R Soc Lond B Biol Sci. 2006;273:1733–42.

36. Cerny AC, Grossmann D, Bucher G, Klingler M. The Tribolium ortholog of knirps and knirps-related is crucial for head segmentation but plays a minor role during abdominal patterning. Dev Biol. 2008;321:284–94.

37. Cerny AC, Bucher G, Schroder R, Klingler M. Breakdown of abdominal patterning in the Tribolium Kruppel mutant jaws. Development. 2005;132:5353–63.

38. Tidswell ORA, Benton MA, Akam M. The neuroblast timer gene nubbin exhibits functional redundancy with gap genes to regulate segment identity in Tribolium. Dev Camb Engl. 2021. https://doi.org/10.1242/dev.199719.

39. Savard J, Marques-Souza H, Aranda M, Tautz D. A segmentation gene in tribolium produces a polycistronic mRNA that codes for multiple conserved peptides. Cell. 2006;126:559–69.

40. Schoppmeier M, Fischer S, Schmitt-Engel C, Löhr U, Klingler M. An ancient anterior patterning system promotes caudal repression and head formation in Ecdysozoa. Curr Biol CB. 2009;19:1811–5.

41. Rousso T, Lynch J, Yogev S, Roth S, Schejter ED, Shilo B-Z. Generation of distinct signaling modes via diversification of the Egfr ligand-processing cassette. Dev Camb Engl. 2010;137:3427–37.

42. Beeman RW. A homoeotic gene cluster in the red flour beetle. Nature. 1987;327:247–9.

43. Brown SJ, Shippy TD, Beeman RW, Denell RE. Tribolium Hox genes repress antennal development in the gnathos and trunk. Mol Phylogenet Evol. 2002;24:384–7.

44. Jacobs CGC, Spaink HP, van der Zee M. The extraembryonic serosa is a frontier epithelium providing the insect egg with a full-range innate immune response. Elife. 2014. https://doi.org/10.7554/eLife.04111.

45. Hilbrant M, Horn T, Koelzer S, Panfilio KA. The beetle amnion and serosa functionally interact as apposed epithelia. Elife. 2016. https://doi.org/10.7554/eLife.13834.

46. Jain A, Ulman V, Mukherjee A, Prakash M, Cuenca MB, Pimpale LG, et al. Regionalized tissue fluidization is required for epithelial gap closure during insect gastrulation. Nat Commun. 2020;11:5604.

47. Panfilio KA. Extraembryonic development in insects and the acrobatics of blastokinesis. Dev Biol. 2008;313:471–91.

48. Posnien N, Koniszewski NDB, Hein HJ, Bucher G. Candidate gene screen in the red flour beetle tribolium reveals Six3 as ancient regulator of anterior median head and central complex development. PLoS Genet. 2011;7: e1002418.

49. Kittelmann S, Ulrich J, Posnien N, Bucher G. Changes in anterior head patterning underlie the evolution of long germ embryogenesis. Dev Biol. 2013;374:174–84.

50. Schinko JB, Kreuzer N, Offen N, Posnien N, Wimmer EA, Bucher G. Divergent functions of orthodenticle, empty spiracles and buttonhead in early head patterning of the beetle *Tribolium castaneum* (Coleoptera). Dev Biol. 2008;317:600–13.

51. Dreyer D, Vitt H, Dippel S, Goetz B, El Jundi B, Kollmann M, et al. 3D standard brain of the red flour beetle *Tribolium Castaneum*: a tool to study metamorphic development and adult plasticity. Front Syst Neurosci. 2010;4:3.

52. Farnworth MS, Eckermann KN, Bucher G. Sequence heterochrony led to a gain of functionality in an immature stage of the central complex: a fly-beetle insight. PLoS Biol. 2020;18: e3000881.

53. He B, Buescher M, Farnworth MS, Strobl F, Stelzer EH, Koniszewski ND, et al. An ancestral apical brain region contributes to the central complex under the control of foxQ2 in the beetle Tribolium. Elife. 2019;8: e49065.

54. Koniszewski NDB, Kollmann M, Bigham M, Farnworth M, He B, Büscher M, et al. The insect central complex as model for heterochronic brain development-background, concepts, and tools. Dev Genes Evol. 2016;226:209–19.

55. Dippel S, Kollmann M, Oberhofer G, Montino A, Knoll C, Krala M, et al. Morphological and transcriptomic analysis of a beetle chemosensory system reveals a gnathal olfactory center. BMC Biol. 2016. https://doi.org/10.1186/s12915-016-0304-z.

56. Fritzsche S, Hunnekuhl VS. Cell-specific expression and individual function of prohormone convertase PC1/3 in Tribolium larval growth highlights major evolutionary changes between beetle and fly neuroendocrine systems. EvoDevo. 2021;12:9.

57. Trebels B, Dippel S, Schaaf M, Balakrishnan K, Wimmer EA, Schachtner J. Adult neurogenesis in the mushroom bodies of red flour beetles (*Tribolium castaneum*, HERBST) is influenced by the olfactory environment. Sci Rep. 2020;10:1090.

58. Miller SC, Brown SJ, Tomoyasu Y. Larval RNAi in drosophila? Dev Genes Evol. 2008;218:505–10.

59. Miller SC, Miyata K, Brown SJ, Tomoyasu Y. Dissecting systemic RNA interference in the red flour beetle *Tribolium castaneum*: parameters affecting the efficiency of RNAi. PLoS ONE. 2012;7: e47431.

60. Li C-J, Yun X-P, Yu X-J, Li B. Functional analysis of the circadian clock gene timeless in *Tribolium castaneum*: functions of timeless in *Tribolium castaneum*. Insect Sci. 2018;25:418–28.

61. Matsumura K, Sasaki K, Miyatake T. Responses to artificial selection for locomotor activity: a focus on death feigning in red flour beetle. J Evol Biol. 2022. https://doi.org/10.1111/jeb.14012.

62. Beermann A, Jay DG, Beeman RW, Hulskamp M, Tautz D, Jürgens G. The Short antennae gene of Tribolium is required for limb development and encodes the orthologue of the Drosophila Distal-less protein. Development. 2001;128:287–97.

63. Prpic N-M, Wigand B, Damen WG, Klingler M. Expression of dachshund in wild-type and distal-less mutant Tribolium corroborates serial homologies in insect appendages. Dev Genes Evol. 2001;211:467–77.

64. Angelini DR, Smith FW, Jockusch EL. Extent with modification: leg patterning in the beetle *Tribolium castaneum* and the evolution of serial homologs. G3 Bethesda. 2012;2:235–48.

65. Chou J, Ferris AC, Chen T, Seok R, Yoon D, Suzuki Y. Roles of Polycomb group proteins enhancer of zeste (E(z)) and polycomb (Pc) during metamorphosis and larval leg regeneration in the flour beetle *Tribolium castaneum*. Dev Biol. 2019;450:34–46.

66. Konopova B, Jindra M. Juvenile hormone resistance gene Methoprene-tolerant controls entry into metamorphosis in the beetle *Tribolium castaneum*. Proc Natl Acad Sci U S A. 2007;104:10488–93.

67. Suzuki Y, Squires DC, Riddiford LM. Larval leg integrity is maintained by distal-less and is required for proper timing of metamorphosis in the flour beetle *Tribolium castaneum*. Dev Biol. 2009;326:60–7.

68. Schönbauer C, Distler J, Jährling N, Radolf M, Dodt H-U, Frasch M, et al. Spalt mediates an evolutionarily conserved switch to fibrillar muscle fate in insects. Nature. 2011;479:406–9.

69. Clark-Hachtel CM, Linz DM, Tomoyasu Y. Insights into insect wing origin provided by functional analysis of vestigial in the red flour beetle *Tribolium castaneum*. Proc Natl Acad Sci U S A. 2013;110:16951–6.

70. Li J, Lehmann S, Weißbecker B, Ojeda Naharros I, Schütz S, Joop G, et al. Odoriferous defensive stink gland transcriptome to identify novel genes necessary for Quinone synthesis in the red flour beetle. Tribolium castaneum PLoS Genet. 2013;9: e1003596.

71. Teuscher M, Ströhlein N, Birkenbach M, Schultheis D, Schoppmeier M. TC003132 is essential for the follicle stem cell lineage in telotrophic Tribolium oogenesis. Front Zool. 2017;14:26.

72. Koyama T, Naseem MT, Kolosov D, Vo CT, Mahon D, Jakobsen ASS, et al. A unique Malpighian tubule architecture in *Tribolium castaneum* informs the evolutionary origins of systemic osmoregulation in beetles. Proc Natl Acad Sci U S A. 2021;118: e2023314118.

73. Zhong D, Pai A, Wang M-H, Keech N, Yan G. Fine-scale analysis of parasite resistance genes in the red flour beetle *Tribolium castaneum*. Genetics. 2013;195:253–61.

74. Drury DW, Jideonwo VN, Ehmke RC, Wade MJ. An unusual barrier to gene flow: perpetually immature larvae from inter-population crosses in the flour beetle, *Tribolium castaneum*: perpetual immaturity via hybridization. J Evol Biol. 2011;24:2678–86.

75. Jagadeesan R, Fotheringham A, Ebert PR, Schlipalius DI. Rapid genome wide mapping of phosphine resistance loci by a simple regional averaging analysis in the red flour beetle *Tribolium castaneum*. BMC Genomics. 2013;14:650.

76. Beeman RW, Brown SJ. RAPD-based genetic linkage maps of *Tribolium castaneum*. Genetics. 1999;153:333–8.

77. Zhong D. AFLP-based genetic linkage map for the red flour beetle (*Tribolium castaneum*). J Hered. 2004;95:53–61.

78. Lorenzen MD. Genetic linkage maps of the red flour beetle, *Tribolium castaneum*, based on bacterial artificial chromosomes and expressed sequence tags. Genetics. 2005;170:741–7.

79. Richards S, Gibbs RA, Weinstock GM, Brown SJ, Denell R, Beeman RW, et al. The genome of the model beetle and pest *Tribolium castaneum*. Nature. 2008;452:949–55.

80. Herndon N, Shelton J, Gerischer L, Ioannidis P, Ninova M, Dönitz J, et al. Enhanced genome assembly and a new official gene set for *Tribolium castaneum*. BMC Genomics. 2020;21:47.

81. Campbell JF, Athanassiou CG, Hagstrum DW, Zhu KY. *Tribolium castaneum* : a model insect for fundamental and applied research. Annu Rev Entomol. 2022;67:080921–75157.

82. Baum JA, Bogaert T, Clinton W, Heck GR, Feldmann P, Ilagan O, et al. Control of coleopteran insect pests through RNA interference. Nat Biotechnol. 2007;25:1322–6.

83. Mao Y-B, Cai W-J, Wang J-W, Hong G-J, Tao X-Y, Wang L-J, et al. Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. Nat Biotechnol. 2007;25:1307–13.

84. Ulrich J, Dao VA, Majumdar U, Schmitt-Engel C, Schwirz J, Schultheis D, et al. Large scale RNAi screen in Tribolium reveals novel target genes for pest control and the proteasome as prime target. BMC Genomics. 2015. https://doi.org/10.1186/s12864-015-1880-y.

85. Knorr E, Fishilevich E, Tenbusch L, Frey MLF, Rangasamy M, Billion A, et al. Gene silencing in *Tribolium castaneum* as a tool for the targeted identification of candidate RNAi targets in crop pests. Sci Rep. 2018;8:2061.

86. Tomoyasu Y, Miller SC, Tomita S, Schoppmeier M, Grossmann D, Bucher G. Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in Tribolium. Genome Biol. 2008;9:R10.

87. Wynant N, Santos D, Vanden BJ. Biological mechanisms determining the success of RNA interference in insects. In: International review of cell and molecular biology. Amsterdam: Elsevier; 2014. p. 139–67.

88. Lorenzen MD, Gnirke A, Margolis J, Garnes J, Campbell M, Stuart JJ, et al. The maternal-effect, selfish genetic element Medea is associated with a composite Tc1 transposon. Proc Natl Acad Sci U S A. 2008;105:10085–9.

89. Chen C-H, Huang H, Ward CM, Su JT, Schaeffer LV, Guo M, et al. A synthetic maternal-effect selfish genetic element drives population replacement in drosophila. Science. 2007;316:597–600.

90. Brown SJ, Mahaffey JP, Lorenzen MD, Denell RE, Mahaffey JW. Using RNAi to investigate orthologous homeotic gene function during development of distantly related insects. Evol Dev. 1999;1:11–5.

91. Bucher G, Scholten J, Klingler M. Parental RNAi in Tribolium (Coleoptera). Curr Biol. 2002;12:R85–6.

92. Peel AD, Schanda J, Grossmann D, Ruge F, Oberhofer G, Gilles AF, et al. Tc-knirps plays different roles in the specification of antennal and mandibular parasegment boundaries and is regulated by a pair-rule gene in the beetle *Tribolium castaneum*. BMC Dev Biol. 2013;13:25.

93. Lorenzen MD, Brown SJ, Denell RE, Beeman RW. Cloning and characterization of the *Tribolium castaneum* eye-color genes encoding tryptophan oxygenase and kynurenine 3-monooxygenase. Genetics. 2002;160:225–34.

94. Arakane Y, Dixit R, Begum K, Park Y, Specht CA, Merzendorfer H, et al. Analysis of functions of the chitin deacetylase gene family in *Tribolium castaneum*. Insect Biochem Mol Biol. 2009;39:355–65.

95. Arakane Y, Muthukrishnan S, Beeman RW, Kanost MR, Kramer KJ. Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning. Proc Natl Acad Sci U S A. 2005;102:11337–42.

96. Knorr E, Schmidtberg H, Vilcinskas A, Altincicek B. MMPs regulate both development and immunity in the Tribolium model insect. PLoS ONE. 2009;4: e4751.

97. Yokoi K, Hayakawa Y, Kato D, Minakuchi C, Tanaka T, Ochiai M, et al. Prophenoloxidase genes and antimicrobial host defense of the model beetle *Tribolium castaneum*. J Invertebr Pathol. 2015;132:190–200.

98. Tomoyasu Y, Wheeler SR, Denell RE. Ultrabithorax is required for membranous wing identity in the beetle *Tribolium castaneum*. Nature. 2005;433:643–7.

99. Horn T, Panfilio KA. Novel functions for Dorsocross in epithelial morphogenesis in the beetle *Tribolium castaneum*. Dev Camb Engl. 2016;143:3002–11.

100. Schultheis D, Weißkopf M, Schaub C, Ansari S, Dao VA, Grossmann D, et al. A large scale systemic RNAi screen in the red flour beetle *Tribolium castaneum* identifies novel genes involved in insect muscle development. G3 Bethesda Md. 2019;9:1009–26.

101. Hahn N, Knorr DY, Liebig J, Wüstefeld L, Peters K, Büscher M, et al. The insect ortholog of the human orphan cytokine receptor CRLF3 is a neuroprotective erythropoietin receptor. Front Mol Neurosci. 2017. https://doi.org/10.3389/fnmol.2017.00223.

102. Xiao D, Gao X, Xu J, Liang X, Li Q, Yao J, et al. Clathrin-dependent endo-cytosis plays a predominant role in cellular uptake of double-stranded RNA in the red flour beetle. Insect Biochem Mol Biol. 2015;60:68–77.
103. Mehlhorn S, Hunnekuhl VS, Geibel S, Nauen R, Bucher G. Establishing RNAi for basic research and pest control and identification of the most efficient target genes for pest control: a brief guide. Front Zool. 2021;18:60.
104. Berghammer AJ, Klingler M, Wimmer EA. A universal marker for trans-genic insects. Nature. 1999;402:370–1.
105. Pavlopoulos A, Berghammer AJ, Averof M, Klingler M. Efficient transfor-mation of the beetle *Tribolium castaneum* using the Minos transposable element: quantitative and qualitative analysis of genomic integration events. Genetics. 2004;167:737–46.
106. Trauner J, Schinko J, Lorenzen MD, Shippy TD, Wimmer EA, Beeman RW, et al. Large-scale insertional mutagenesis of a coleopteran stored grain pest, the red flour beetle *Tribolium castaneum*, identifies embryonic lethal mutations and enhancer traps. BMC Biol. 2009;7:73.
107. Schinko JB, Hillebrand K, Bucher G. Heat shock-mediated misexpres-sion of genes in the beetle *Tribolium castaneum*. Dev Genes Evol. 2012;222:287–98.
108. Schinko JB, Weber M, Viktorinova I, Kiupakis A, Averof M, Klingler M, et al. Functionality of the GAL4/UAS system in Tribolium requires the use of endogenous core promoters. BMC Dev Biol. 2010;10:53.
109. Lai Y-T, Deem KD, Borràs-Castells F, Sambrani N, Rudolf H, Suryamohan K, et al. Enhancer identification and activity evaluation in the red flour beetle, *Tribolium castaneum*. Dev Camb Engl. 2018. https://doi.org/10.1242/dev.160663.
110. Sarrazin AF, Peel AD, Averof M. A Segmentation clock with two-seg-ment periodicity in insects. Science. 2012. https://doi.org/10.1126/science.1218256.
111. Strobl F, Anderl A, Stelzer EH. A universal vector concept for a direct genotyping of transgenic organisms and a systematic creation of homozygous lines. Elife. 2018;7: e31677.
112. Livet J, Weissman TA, Kang H, Draft RW, Lu J, Bennis RA, et al. Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. Nature. 2007;450:56–62.
113. Sulston IA, Anderson KV. Embryonic patterning mutants in *Tribolium castaneum*. Development. 1996;122:805–14.
114. Berghammer A, Bucher G, Maderspacher F, Klingler M. A system to efficiently maintain embryonic lethal mutations in the flour beetle *Tribolium castaneum*. Dev Genes Evol. 1999;209:382–9.
115. Gilles AF, Schinko JB, Averof M. Efficient CRISPR-mediated gene target-ing and transgene replacement in the beetle *Tribolium castaneum*. Dev Camb Engl. 2015;142:2832–9.
116. Farnworth SM, Eckermann KN, Ahmed HMM, Mühlen DS, He B, Bucher G. The red flour beetle as model for comparative neural development: genome editing to mark neural cells in Tribolium brain development. In: Sprecher SG, editor. Methods in brain development. 2nd ed. New York: Humana; 2019.
117. Rylee JC, Nin-Velez A, Mahato S, Helms KJ, Wade MJ, Zentner GE, et al. Generating and testing the efficacy of transgenic Cas9 in *Tribolium castaneum*. Insect Mol Biol. 2022. https://doi.org/10.1111/imb.12778.
118. Gilles AF, Schinko JB, Schacht MI, Enjolras C, Averof M. Clonal analysis by tunable CRISPR-mediated excision. Development 2019;146(1):dev170969. https://doi.org/10.1242/dev.170969.
119. Benton MA, Akam M, Pavlopoulos A. Cell and tissue dynamics during Tribolium embryogenesis revealed by versatile fluorescence labeling approaches. Dev Camb Engl. 2013;140:3210–20.
120. Strobl F, Stelzer EH. Long-term fluorescence live imaging of Tribolium castaneum embryos: principles, resources, scientific challenges and the comparative approach. Curr Opin Insect Sci. 2016;18:17–26.
121. Münster S, Jain A, Mietke A, Pavlopoulos A, Grill SW, Tomancak P. Attach-ment of the blastoderm to the vitelline envelope affects gastrulation of insects. Nature. 2019;568:395–9.
122. Dönitz J, Gerischer L, Hahnke S, Pfeiffer S, Bucher G. Expanded and updated data and a query pipeline for iBeetle-Base. Nucleic Acids Res. 2018;46:D831–5.
123. Dönitz J, Schmitt-Engel C, Grossmann D, Gerischer L, Tech M, Schopp-meier M, et al. iBeetle-Base: a database for RNAi phenotypes in the red flour beetle *Tribolium castaneum*. Nucleic Acids Res. 2015;43:D720–5.
124. Dönitz J, Grossmann D, Schild I, Schmitt-Engel C, Bradler S, Prpic N-M, et al. TrOn: an anatomical ontology for the beetle *Tribolium castaneum*. PLoS ONE. 2013;8: e70695.
125. Chintapalli VR, Wang J, Dow JAT. Using FlyAtlas to identify better Drosophila melanogaster models of human disease. Nat Genet. 2007;39:715–20.

Publisher's Note