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TECHNICAL NOTE

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A system to efficiently maintain embryonic lethal mutations in the flour beetle *Tribolium castaneum*

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Abstract Due to its small size, short life cycle, and easy maintenance, the flour beetle *Tribolium castaneum* is well suited for the genetic analysis of development. One drawback of *Tribolium* as a genetic system is, however, the difficulty of keeping embryonic lethal lines. Presently, only few lethal mutations can be kept as balanced stocks. Therefore, heterozygous carriers must be identified anew in every generation in order to maintain a recessive embryonic mutation. To alleviate this problem we have devised a block system that allows the simultaneous processing of many mutant lines or test crosses for visual inspection of larval cuticle phenotypes. Using this technique, one person can maintain about 100 embryonic lethal stocks, which makes feasible the thorough genetic analysis of embryogenesis in this species.

Key words *Tribolium castaneum* · Lethal mutants · Stockkeeping · Block system · Egg collection

Introduction

Insects constitute the largest animal taxon, and its 350 million year history has given rise to a considerable morphological diversity of embryos, larvae, and adults. Nevertheless, almost all we know about insect development is based on the study of a single species, *Drosophila melanogaster*. The sophistication of *Drosophila* genetics was instrumental for the breakthrough in molecular analysis of development in the 1980s, and still today *Drosophila* is the best understood embryological system. Based on this knowledge, the diversity in insect development can be studied by analyzing the expression of genes homologous to *Drosophila* patterning genes (see, for example, Kraft and Jäckle 1994; Patel et al. 1989, 1994; Sommer and Tautz 1993). However, while molecular-descriptive comparisons may suggest that a developmental pathway in another species deviates from its counterpart in *Drosophila*, this approach cannot unravel the mechanism of the diverged system. Therefore, functional approaches are necessary for meaningful comparisons among homologous genes and developmental mechanisms in different organisms. Since our knowledge of *Drosophila* development is based mostly on genetic data, mutational analysis of development in a few additional insect species would be most informative for such a functional comparison. One species in which such an analysis appears possible is the flour beetle *Tribolium castaneum*. *Tribolium* has a minimal generation time of about 5 weeks and can easily be bred in the laboratory in large numbers. Mutagenesis protocols have been worked out for this species, and developmental mutants have been isolated (Beeman 1987; Beeman et al. 1989; Sulston and Anderson 1996).

Many of the existing *Tribolium* mutations affect the adult morphology (Sokoloff 1974); this allows maintenance of stocks by visually selecting mutant carriers in every generation. However, most developmental mutants are lethals which only can be maintained as heterozygotes that are morphologically indistinguishable from the wildtype. In *Drosophila*, such mutations are kept in balanced stocks where it is ensured that all surviving offspring of heterozygous parents are again heterozygous carriers of the mutation, such that the lethal mutation of interest will never be overgrown by wildtype segregants. Balanced stocks rely on special chromosomes carrying defined rearrangements (Ashburner 1989). Also in *Tribolium*, embryonic lethals can be kept by this method (Beeman et al. 1989). However, the fact that there are ten rather than three major chromosomes, and that these beetles lack polytene chromosomes (which would allow

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cytological analysis of chromosomal rearrangements), makes it difficult to develop such balancer chromosomes in most regions of the *Tribolium* genome. For the time being, therefore, most embryonic lethal mutations can be maintained only by identifying individual mutant carriers in every generation via scrutinizing their offspring for embryonic phenotypes.

We have initiated a screen for early patterning mutants in *Tribolium* (Maderspacher et al. 1998) which we plan to continue until some degree of saturation is reached, i.e. until a representative collection of mutations has been obtained. To this end, we developed a technique that allows the efficient transfer of stocks and, most importantly, the rapid evaluation of large numbers of test crosses as required for stockkeeping. In this paper we describe this technique so that other *Tribolium* workers can adopt these procedures. Detailed instructions for manufacturing the tools required for this procedure are provided as an electronic supplement on the Springer server (http://link.springer.de/link/service/journals/00427/index.htm), or can be obtained directly from the authors.

Materials and methods

Small and large blocks

Blocks are each composed of 25 polystyrene vials, which are glued together in a 5×5 pattern (see Figs. 1A, 2A; sb in Fig. 2) with acetone (see electronic supplements). For small blocks to be used for egg collection, vials are 27 mm wide and 64 mm high (Greiner no. 205 101). For large blocks to be used for stockkeeping, vials are 36 mm wide and 82 mm high (Greiner no. 217 101). Foam stoppers are Greiner no. 354 070 and 330 070. Small blocks usually contain 4–8 ml flour and 1–20 egg-laying females per vial (as well as one or several males), while large blocks contain up to 20 ml flour and usually one male and eight females per vial.

Small and large sanded blocks

These are similar to those above, but the top of these blocks is sanded on a level surface to make the openings of all vials perfectly level. Small (ssb) and large sanded blocks (lsb) are used in combination with beetle sieves (see below) to separate beetles from flour. For this purpose, the top of the block must adjoin the sieve precisely in order to prevent beetles from contaminating neighboring vials (see Figs. 1B, 2B). Since sanding is labor intensive, not all blocks are treated in this manner.

Small and large beetle sieves

Beetle sieves (small, sbs; large, lbs) are placed between two sanded blocks facing each other, such that beetles are retained in the upper block while the flour from each egglay is collected in a corresponding vial in the lower block (Fig. 1B). The sieve consists of a polyamide screen with 0.80 mm mesh width, which is glued onto a support that also has rails on both sides to affix the blocks in accurate position (see Fig. 2B). During sieving, the three devices are tied together with rubber bands (not shown). Beetle sieves come in two varieties, i.e., for large and small blocks, respectively.

Egg sieve and flour container

The egg sieve (es) and the flour container (fc) are tools for separating eggs from flour. The egg sieve is made from a massive block of polyacryl into which 25 holes are cut fitting the vial arrangement of small blocks (Figs. 1C, 2C,D). A stainless steel screen with 0.30 mm mesh size is affixed at the bottom (Fig. 2C). Flour that falls through the screen is collected by a flour container which fits underneath the egg sieve (Figs. 1C, 2C).

Egg funnel

The egg funnel (ef) serves to transfer eggs from the egg sieve into maturing sieves whose smaller dimension allows efficient storage during subsequent incubations, and easier handling during microscopic inspection. The egg funnel (Figs. 1D, 2E) consists of two polyacryl plates that are mill-cut to fit the egg sieve (upper panel) and the maturing sieve (lower panel), respectively. The two plates are attached with one another by four support poles, and 25 flexible tubes (polyvinyl chloride) connect corresponding borings in the two plates. Cones cut into the top plate serve as funnels to reduce the large diameter of egg sieve wells to the smaller diameter of the tubes and maturating sieve wells.

Maturing sieves

After separation from the flour, eggs are incubated in maturing sieves (ms) (Figs. 1E, 2E) until all embryos are mature. The mesh size of these sieves (0.25 mm) is selected such that unhatched eggs are retained above the screen while hatched larvae (i.e., wild-type and heterozygous larvae) tend to crawl through the screen. By placing maturating sieves onto a grid made of carton (Fig. 1E), the larvae fall into the space below the screen and are thereby removed from the egglays, while mutant embryos are retained above for further processing.

Clearing blocks

Clearing blocks (cb) are similar in format to maturing sieves, but with a glass slide instead of a screen affixed to the bottom (Figs. 1F, 2F,G). After transfer from maturing sieves into the clearing block, a 9:1 mixture of lactic acid and ethanol is added to each well. Incubation at 60°C over night results in almost complete dissolution of internal organs such that the cuticles of mature embryos can be directly analyzed using dark field optics.

Microscopic inspection

The cuticle of mature embryos can be inspected using a dissecting microscope, as they are immersed in lactic acid in clearing block wells. For this purpose, the dissecting microscope must be equipped with dark field illumination. A simple but effective dark field stage can be made from an annular light pipe (for example Schott no. 138A2244, 69 mm diameter) connected to a cold light source. The annular light source is positioned ca. 30 mm below a fixed diaphragm of 15 mm inner diameter, onto which the clearing block is placed for illumination of the specimen from below. A suitable stage for such an arrangement can be obtained from Zeiss (order no. 47 52 69). For routine stockkeeping, we use a Zeiss dissection microscope Stemi SV 11 with variable magnification from 6- to 66-fold (objective 1.0×, zoom 0.6–6.6×, eye pieces 10×).

Enriched full grain and instant flour

Beetle cultures grow best on full grain wheat flour which is enriched with 5% yeast powder (Sokoloff 1974). We use organically grown flour to prevent potential problems with insecticides. In addition to yeast, we add 0.3 g Fumidil-B per kilogram flour to prevent sporozoa infections. Fumidil-B can be obtained from beekeeping supply companies in the United States (for example, Dadant & Sons, 550 E. Main, P.O. Box 385, Potterville, MI

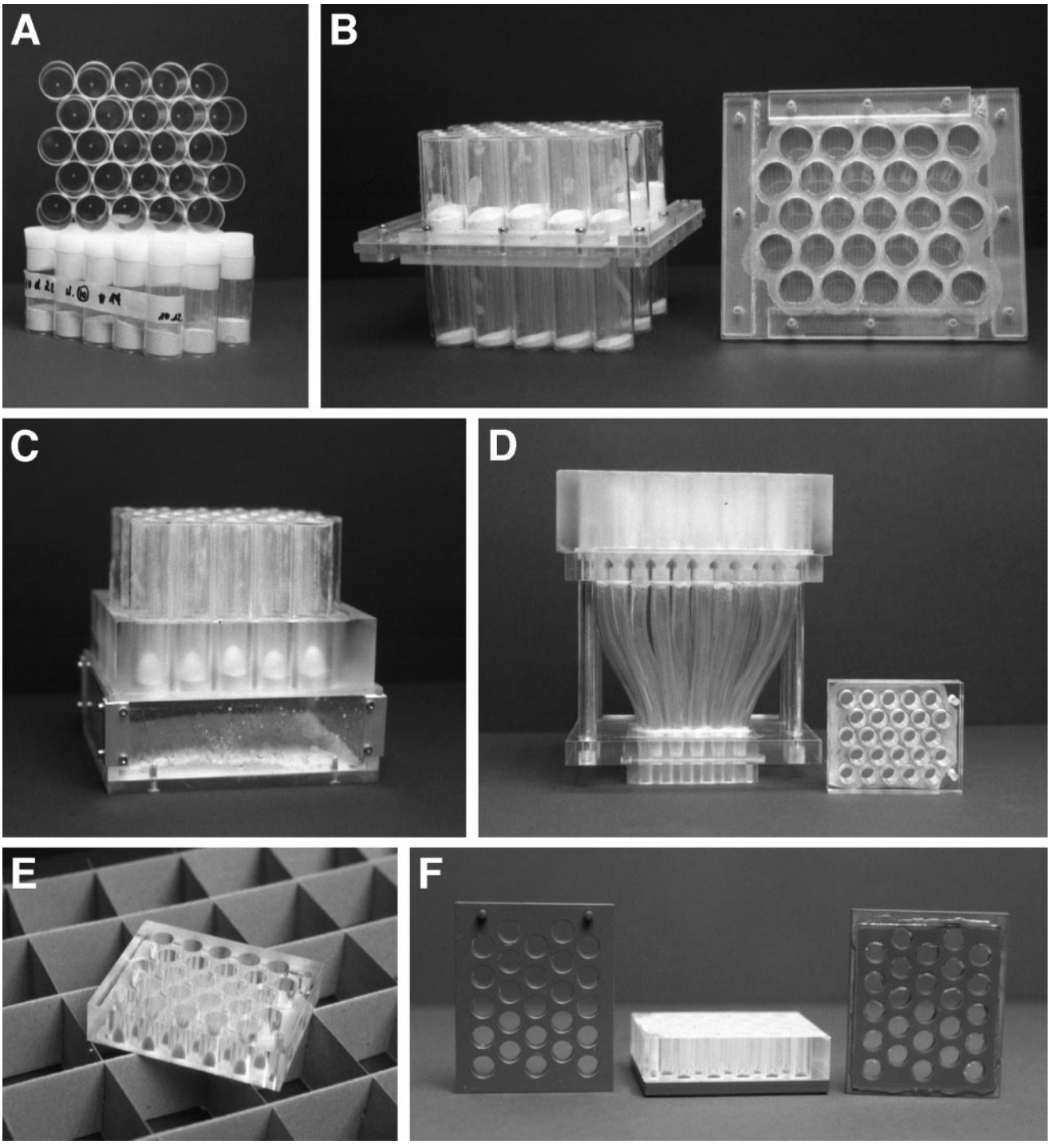

Fig. 1A–F Photographs of tools required for stockkeeping. **A** An empty small block placed on top of another block containing flour and beetles. Individual foam stoppers prevent beetles from escaping. **B** To separate beetles from egg-containing flour, a beetle sieve is placed between a block containing beetles and flour (*above*) and an empty block (*below*) in which the flour (and eggs) are collected. *On the right* such a beetle sieve is viewed from below to show the plastic support and the rails that ensure proper positioning of the blocks during sieving. **C** To harvest eggs from multiple egglays, the flour is poured from the bottom block in **B** (now *on top* in inverted position) into the egg sieve (*center*) which retains the eggs while the flour is disposed of into the flour container below. **D** From the egg sieve (inverted, *on top*), the eggs are transferred into the maturing sieve (*bottom*) by the egg funnel apparatus. *On the right* a maturing sieve is shown from top. **E** Eggs are incubated in the maturing sieve placed on top of a cardboard grid such that larvae crawling through the mesh fall into the space below. Mutant eggs are retained in the maturing sieve wells. **F** Finally, the eggs remaining in the maturing sieve are poured into the clearing block (a maturing sieve on top of a clearing block is depicted *in the center*). Top and bottom views of clearing blocks are shown *on left and right*. The knobs on top of the clearing sieve fit into corresponding borings in the maturing sieve to ensure proper placement during egg transfer

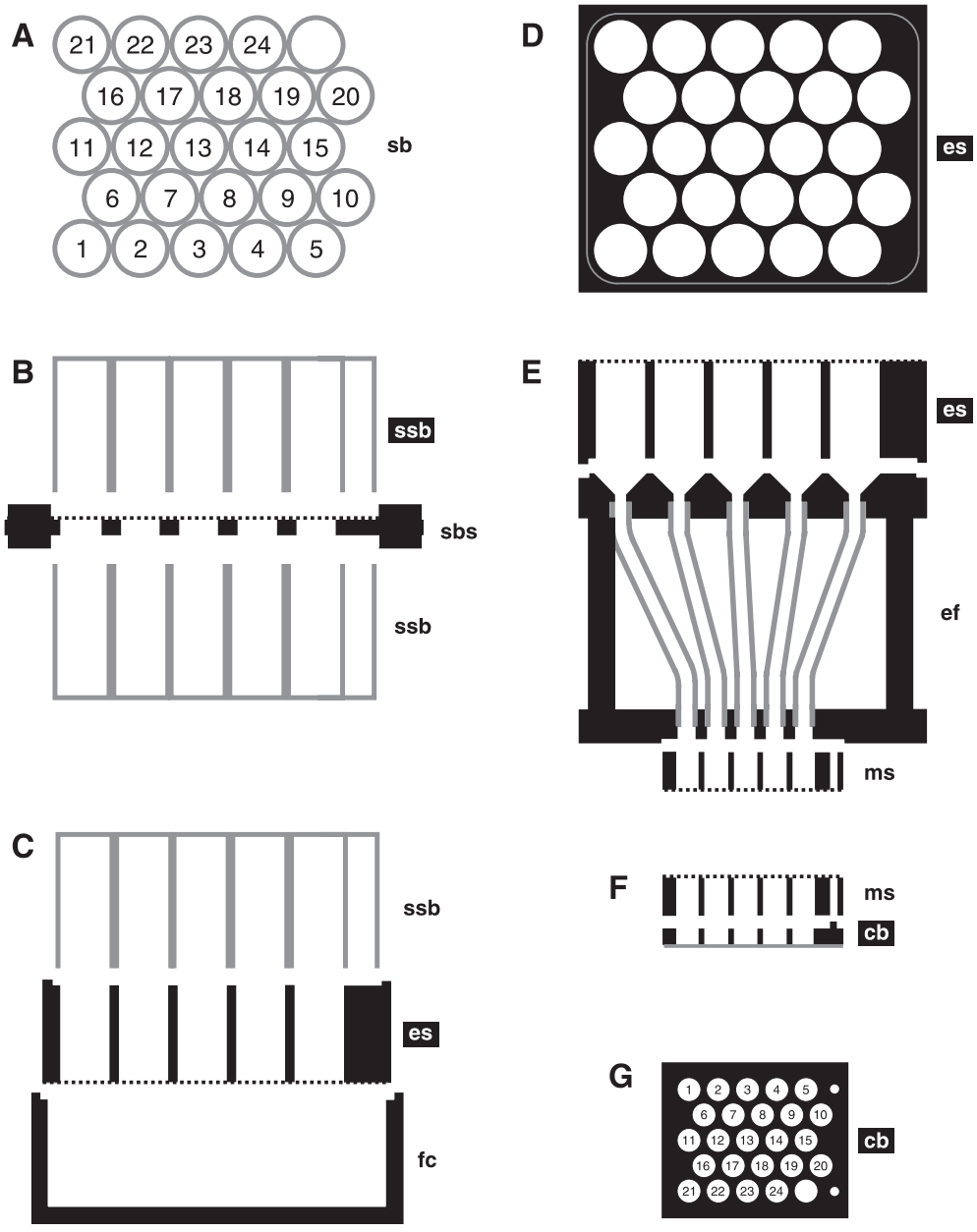

Fig. 2 Schematic description of the procedure that processes eggs from single-male egglays up to microscopic inspection. **A**,**D**,**G** Views from the top. **B**,**C**,**E**,**F** are side views. *Gray* devices are assembled from many parts; *black* devices are mill-cut from polyacryl or polyvinyl chloride. Each time flour or eggs are poured into a new block or sieve, the orientation of the egglays is flipped. For this reason, the final orientation (**G**) is inverted relative to the original orientation (**A**). *White lettering* tools with inverted orientation (i.e., *ssb*, *es*, *cb*). **A** Arrangement of vials that compose a small block (*sb*); we number the vials beginning from the lower left corner. Vial number 25 always remains unused such that the orientation of egglays is apparent during all steps of the following procedure. **B** To separate flour from the beetles the contents of the block in **A** are first poured into a small sanded block (*ssb*), which is placed above the small beetle sieve (*sbs*) which again is on top of an empty small sanded small block that collects the flour. **C** Flour (plus eggs) is then transferred from the ssb into the egg sieve (*es*), where eggs are retained while the flour is collected in the flour container (*fc*) below. **D** Top view of egg sieve. The white line demarcates the rails cut into the block that ensure precise positioning of small block and egg sieve during flour transfer. **E** Eggs are now transferred to the maturing sieve (*ms*) by means of the egg funnel (*ef*). **F** After mutant eggs have completed development (and most viable larvae have escaped through the screen) eggs are poured into the clearing block (*cb*) upon which the lactic acid/ethanol mixture is added. **G** Top view of clearing block; *numbers* indicate inverted orientation of egglays (compare to **A**). After clearing at 60°C, the resulting cuticles can be inspected under the stereomicroscope while still in the clearing block

48876). For egg collections we use instant flour (for example "Rosenmehl–Wiener Griessler–Doppelgriffiges Mehl; Type 405") which consists of normal white flour that has been processed to form larger granules. This flour does not become lumpy and block the sieves as does normal white flour. Instant flour is also supplemented with dry yeast. It is essential to presieve the instant flour through a 0.25-mm mesh sieve prior to use to remove all particles that approach or exceed the size of a *Tribolium* egg. Similarly, full-grain flour is presieved through a 0.50 mm sieve.

Good beetle laboratory practice

To reduce the risks of cross-contamination among mutant lines and disease propagation, sieves, egg funnels, and sanded blocks are cleaned of flour and eggs prior to every use with a brush attached to a vacuum cleaner. For sensitive experiments in which cross-contamination must be excluded with certainty, we sterilize all tools in a 70°C incubator to kill all eggs and small larvae that may have been overlooked. Large and small blocks are cleaned after use in a dish washer at 60°C. If electrostatics becomes a problem when using these plastic devices, this can be overcome with an anti-static gun (for example Sigma Z10,881-2).

Results and discussion

Overview of stockkeeping procedure

We keep each embryonic lethal mutation in four parallel lines, as a safeguard against loss or contamination. Therefore our present collection of 30 embryonic lethal mutants is kept as 120 lines, which occupy five "large blocks" (see above), each of which houses 24 lines. Every line consists of a single male heterozygous for the mutation in question, and eight wildtype females. These beetles are kept at 24°C and transferred to fresh food every 3 weeks. Within this time period, offspring from the cross (50% of which are also mutant carriers) are still small enough to fall through the 0.80 mm sieve used to separate beetles and flour, such that the single adult male is not replaced by a son that may not carry the mutation. The flour with eggs and young larvae is incubated until most animals are mature and is then moved to 18°C as backup. At this temperature *Tribolium* embryos fail to complete development such that no second-generation animals arise in these blocks.

After 6–9 months the lines must be reestablished since the male carriers die or their fertility declines. To again identify carriers of the mutation, single-male egglays consisting of one male and three virgin females are set up from the offspring of one of the four lines that represent a specific genotype. In most cases 24 single-male egglays (occupying one small block) are sufficient to identify at least four egglays in which the male and one (or more) of the females carry the mutation, thus producing mutant offspring. (In principle, 12 of the 24 males in one block should be heterozygous, and the probability that at least one of its three mates carries the mutation is 87.5%; therefore an average of 10 positive egglays is expected per block of 24.) Carrier males are identified by the presence of mutant phenotypes in the eggs they sired. For this purpose, cuticle preparations are generated from the egglays by parallel transfer of eggs from egg laying blocks to clearing blocks (Fig. 2F) which directly fit onto the dark field stage of a compound microscope. Four new lines for the mutation in question are then established from the male carriers, by addition of wildtype virgin females.

This method of keeping the mutant stocks as single males mated to wildtype females results in 50% carriers of the mutation in the next generation. Alternatively, one could keep the mutant stocks as populations of heterozygous males and females. However, this method would require much more effort for identifying a sufficient number of carriers in every generation, with only marginally increased percentage of heterozygotes in the offspring (66.6% vs. 50%). Furthermore, our method of continuous outcrossing with wildtype circumvents any potential inbreeding problem. It also reduces the risk of disease propagation, since our wildtype strain is kept under special scrutiny. To keep this strain disease free, we routinely isolate eggs from wildtype beetles and raise every batch on fresh food which has not been in contact with the parent generation of adult beetles. By this regimen the transmission of parasitic mites and excrement-associated micro organisms from adults to their young is dramatically reduced (it is not feasible, however, to observe this regimen with all mutant lines).

Maintenance of mutant lines

Mutant lines (each containing one mutant carrier male) are kept in large blocks composed of 25 vials. Of the 25 vials in these blocks 24 each contain 20 ml full grain flour and nine beetles; the openings of the vials are closed by foam stoppers. Each block carries a number, and a protocol sheet is kept (in duplicate) that indicates which mutant is housed in which vial. The transfer procedure requires that the content of one block is poured or sifted into other blocks. For this reason, the block design is mirror symmetric so only one type of block is required. However, the arrangement of lines within a block becomes inverted with each transfer from block to block. Therefore, to avoid numbering confusion, we leave empty one vial in each block (vial 25), such that each block contains 24 lines representing six different genotypes.

During stock transfer, beetles and flour are first poured into a sanded block which is arranged with another (empty) sanded block and a large beetle sieve (Fig. 1B) and strapped together with rubber bands (not shown). The flour (including eggs) then is sifted from one block into the other; to prevent blockage of the screen during sieving, the assembled devices are shaken by hand with the vials oriented almost horizontally such that the flour can move relative to the screen. After complete separation of beetles and flour, sieve and upper block are held together (to contain the beetles in their respective vials), separated from the lower block, and turned with the sieve towards up. Beetles still adhering to the screen are then shaken into the vials by knocking the two still assembled devices onto the table. After re-

moval of the sieve, the beetles are visually inspected and any stray larva remaining with them is removed with tweezers. Finally, fresh flour is added to the beetles and they are poured back into a fresh, empty block where the mutant lines now are arranged in the same orientation as they were in the original block.

The flour in the lower sanded block is already in the correct orientation since it has been transferred twice. To move it back in the starting block, with correct orientation, it again must be transferred twice, i.e., it is transferred into the now empty upper sanded block, and from there back into the original block.

While this procedure seems complicated when described in detail, with a little practice it becomes a quick and smooth routine which requires only simple tools that are very easy to manufacture, i.e., two sanded blocks and the large beetle sieve.

Reestablishing mutant lines from single-male crosses

To reestablish a mutant line, heterozygous animals need to be identified among the offspring of the previous male carrier. For this purpose, 24 single-male crosses are set up from sexed pupae (Sokoloff 1974) which are placed on full grain flour in small blocks (4 ml flour per vial). These blocks are kept for 2 weeks at 30°C to allow completion of pupal development and fertilization of eclosed adult females. If the male in a stock has died prematurely such that no pupae remain available, two blocks with 48 single pair matings are established from the 18°C backup stocks, i.e., using nonvirgin females. Also these nonvirgin crosses are left for 2 weeks at 30°C to allow for sperm replacement such that their offspring will mostly represent the genotype of the male in question rather than that of previous mates.

After 2 weeks, the full grain flour is removed from the beetles (using the beetle sieve) and replaced by 4 ml fresh instant flour per vial. Instant flour is much easier to sieve than generic white flour, which greatly reduces the labor required for egg harvesting. Beetles are allowed to lay eggs on instant flour for 3 days at 30°C. Then the flour is separated from the beetles using the small beetle sieve (mesh size 0.80 mm), and the eggs are again separated from the flour using the egg sieve (mesh size 0.30 mm). With help of the egg funnel apparatus (Figs. 1D, 2E) eggs are now transferred into the smaller egg maturing sieves. To transfer the eggs, the three devices are first assembled in inverse orientation (egg sieve on bottom, maturing sieve on top, and egg funnel between them) and then turned around such that the eggs fall through the funnels and tubes into the corresponding wells of the maturing sieve. The maturing sieve then is incubated at 30°C for another 3 days to allow all eggs to complete development. Freshly hatched larvae display geotropism and tend to crawl through the sieve (mesh size 0.25 mm) and fall into the space below, such that the eggs retained above the screen are enriched for mutant embryos. More importantly, this procedure also prevents the larvae that hatch first from devouring their sibling eggs during this incubation period.

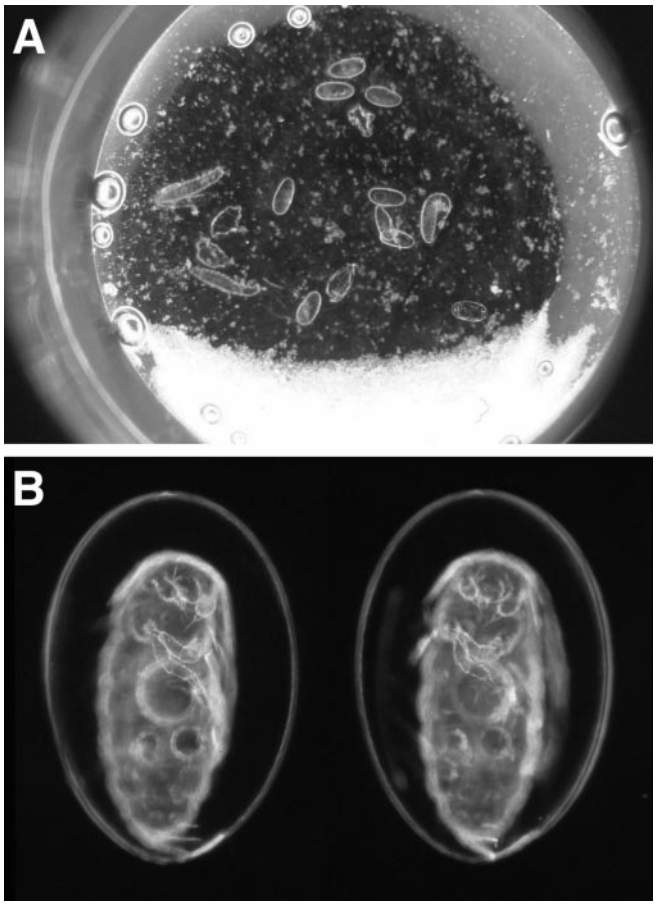

Fig. 3 Cuticle preparations as seen through the dissecting microscope. **A** Low magnification of an egglay in one well of a clearing block (the well is 10 mm wide). Tilting of blocks during clearing ensures that most of the flour accumulates in one corner of the well (*below*). **B** Stereo view of an *itchy* mutant larva (Maderspacher et al. 1998), as visualized by an Leica MZ12 dissecting microscope (objective Planapo 1.6×, zoom 5×, eye piece 10×). Labrum, antennae, labium, second thoracic legs and four abdominal segments can be recognized; the remaining segments are deleted in this pair-rule mutant. Remnants of the serosal cuticle can be seen behind the embryo

To process the remaining eggs for microscopic inspection, they are again poured from the maturing sieves into clearing blocks, upon which 0.2 ml of a lactic acid/ethanol mixture (9:1) is added to each well. Clearing blocks are then incubated at 60°C overnight to dissolve all internal organs of the embryos and larvae. *Tribolium* eggs are inevitably covered by a layer of flour that sticks to the vitellin membrane. Since instant flour consists of relatively large particles, a considerable amount of flour ends up in the clearing block wells together with the eggs. While lactic acid clears embryos fairly well, it does not dissolve the flour; however, the flour dissociates from the vitellin membrane during this incubation and sinks to the bottom while the eggs float near the surface. By arranging the clearing blocks in a tilted position dur-

ing the incubation, it is ensured that the flour collects in one corner of the wells (Fig. 3A) such that it does not obstruct the microscopic image later.

Mutant embryos are identified by inspection of clearing blocks under a dissecting microscope using dark field optics. To allow visualization of morphological details, embryos can be appropriately positioned in the lactic acid solution by means of a bent tungsten needle. While this inspection method does not allow visualization of embryonic features in as much detail as upon embedding in Hoyer's lactic acid and inspection in a high-resolution microscope, with a little practice it is possible to recognize almost all embryonic phenotypes (an example is shown in Fig. 3B). Once mutant embryos are detected, the position of the respective well in the clearing block can be correlated with the corresponding vial in the small block containing the parent beetles (note that the orientation of wells in the clearing sieve is inverted relative to the parent block, see Fig. 2A,G). The parent beetles now can be supplemented by additional wildtype females and set up as a fresh mutant line.

Efficiency of the procedure

Currently, our stock collection of 30 embryonic lethal lines occupies five blocks (i.e., 120 vials). To distribute the labor intensive reestablishing of mutant lines, we keep the collection in a rolling system, i.e., if the collection at a given time occupies blocks numbered from 11 to 15, mutant lines in block 11 are the next to be reestablished (as block 16), then those in block 12 (which become block 17), and so on. Maintenance of the collection currently requires less than one-third of a technician's working time, i.e., one full time person would be able to keep at least 100 lethal mutants (as 400 lines) using this procedure.

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