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Title: A novel mechanism for left-right asymmetry establishment involving tissue remodeling and MyoID

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Abstract: How novel left-right asymmetries evolve in animals is unknown. *Drosophila pachea* males display a unique left-right asymmetry in the genital organs and mate in a right-sided position onto the female. In flies, male genitalia undergo a clockwise 360° rotation during development. To test if this tissue remodeling process is linked to asymmetry development in the male genitalia of *Drosophila pachea*, *w*e developed CRIPSR and transgenesis in this singular species. As in *D. melanogaster*, mutants of the MyoID gene homolog undergo reverse rotation of the developing genitalia. In addition, they have mirror-image asymmetric genitalia. Although their genital asymmetry is reversed mutants still adopt a right-sided copulation posture. Our results reveal a novel mechanism for establishing left-right asymmetry in animals, involving complex tissue remodeling and MyoID.

One Sentence Summary: *D. pachea* genital left-right size asymmetry is established through MyoID-mediated tissue remodelling and does not determine the direction of mating posture.

Main Text:

While the body of most bilaterian animals is superficially left-right symmetric, some internal organs display left-right asymmetries in position, shape or size (*1*). The generation of asymmetric organs during development starts with an initial symmetry-breaking step, which can involve several mechanisms: motile cilia and directional fluid flow, orientation of the cell division plane and distribution of molecules within cells (*1*). How over the course of evolution initially symmetrical organs become asymmetric remains elusive. Males of the fruitfly *Drosophila pachea* have an asymmetric phallus with a right-sided opening for sperm release (gonopore) and a pair of asymmetric external genital lobes, with the left lobe being 1.5 times longer than the right lobe (Fig. 1 A,B) (*2–4*). In addition, *D. pachea* males mate in a right-sided posture with the male being shifted about 6-8° towards the right side of the female (*3–5*). The lobes are considered an evolutionary novelty since they are not present in the three closely related sister species (*6*). The asymmetric genitalia of *D. pachea* likely evolved recently within the past 3-6

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Ma from ancestral symmetry (*6*) and this species is therefore a suitable organism to search for the evolutionary origins of genital left-right asymmetry.

During development, the male genitalia of a wide range of shizophoran flies undergoes a 360° clockwise rotation (*7*, *8*). In *D. melanogaster*, the direction of this rotation is driven by the myosin MyoID, encoded by the gene *myo31DF* (*9*), for simplicity here referred to as *myoID*. Loss of function of *myoID* in *D. melanogaster* leads to a counter-clockwise 360° genitalia rotation direction, due to the function of another counter-acting myosin, MyoIC (*7*, *9*, *10*). MyoID also controls the chirality of other organs, such as the gut and testes (*9*, *11–17*). Furthermore, MyoID appears to be a conserved key factor among different mechanisms to establish left-right asymmetry in vertebrates and invertebrates (*18*, *19*), through interaction with adherens junctions (*10*, *11*), the actin cytoskeleton (*10*, *20*, *21*), planar cell polarity pathway members (*13*, *18*) and Jun kinase pathway regulated apoptosis (*12*, *15*, *22*, *23*). Ectopically expressed, MyoID and MyoIC are sufficient to induce *de novo* directional twisting at the cell, organ, or body scale in *D. melanogaster* (*20*). However, the evolutionary transitions to *novel* left-right size asymmetries remain to be uncovered at the molecular, cellular and tissue scale.

We wondered if MyoID is involved in the establishment of left-right asymmetry in *D. pachea* male genitalia. To test this, we generated a mutation in the coding sequence of the *D. pachea myoID* using CRISPR/Cas9 mediated gene editing (*24*, *25*) (*myoID*^{mut} allele, Fig. S1). The induced mutation caused a frameshift in the seventh coding exon, leading to a premature stop codon. In homozygous *myoID*$^{mut/mut}$ males, the length ratio of left and right genital lobes was reversed with a longer right lobe and a shorter left lobe in most (81/82) dissected individuals (Data S1, Figure 1 C). Furthermore, the phallus asymmetry was reversed in *myoID*$^{mut/mut}$ males with the gonopore located at the left tip of the phallus (29/29 dissected genitalia), compared to heterozygous *myoID*$^{wt/mut}$ and *myoID*$^{wt/wt}$ males, where the gonopore was located consistently on the right side (8/8 and 13/13 dissected genitalia, respectively; Fig. S2). The locus *myoID* is autosomal (Fig. S1) and the mutation appears to be recessive since *myoID*$^{wt/mut}$ males did not have visible alterations of male genitalia morphology (Fig. 1 B). Apart from phallus and lobe morphology, homozygous mutant males revealed a genitalia rotation phenotype, with varying orientations of the male genitalia in adults, with respect to the antero-posterior midline (Fig. 1 A,D). In wild-type *D. pachea*, male claspers and genital lobes are pointing towards the ventral side (Fig. 1 A), while *myoID*$^{mut/mut}$ males revealed variable orientations of genitalia, which varied among individuals between 0 and 270° (Fig. 1 D). This latter orientation phenotype was similar to previous observations in *D. melanogaster myoID* mutants *(9)*. Our data shows that MyoID is essential to determine left-right identity in *D. pachea* male genitalia, including external genital lobes and the male phallus. This function might be mediated through its role in determining the direction of male genitalia rotation. In one *myoID*$^{mut/mut}$ males, genital lobe asymmetry was not reversed although genitalia were mis-oriented and in one individual lobes were strongly bent. However, it was unclear if their genitalia rotated clockwise or counter-clockwise during development. These mutants may have had remnant activity and expression of the *myoID*^{mut} allele, leading to a partial rotation in clockwise direction. Alternatively, other unidentified environmental or genetic factors may also contribute to the determination of genital asymmetry in *D. pachea* and were variable in our experiment.

To monitor genitalia rotation we inserted a DE-Cadherin::YFP fusion construct (DE-Cad::YFP) (Fig. S3) into the *myoID*$^{mut/mut}$ stock using piggybac transposon mediated germline transformation (*26*) (Data S2, Fig. S4). We examined male genitalia rotation in pupae that were heterozygous for DE-Cad::YFP to reduce potentially deleterious effects of the piggybac insert

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(Fig. S5). We observed that *myoID*$^{wt/wt}$ *and myoID*$^{wt/mut}$ males underwent clockwise genitalia rotation direction (n = 9/9, 25/25, respectively), as observed in most schizophoran flies (*7*, *8*) whereas in *myoID*$^{mut/mut}$ males, genitalia rotated counter-clockwise (n = 9/9) (Fig. 1E, Data S1). In 9 monitored males that had developed at least to the pharate adult stage, we were able to dissect male genitalia and to determine the orientation and the lobe length ratio of male genitalia (Fig. S2). All these *myoID*$^{mut/mut}$ males with counter-clockwise genitalia rotation direction had a reversed lobe length ratio with a longer right lobe and a shorter left lobe, although in one observation lobes were only rudimatarily developed and the measurements were not informative about the lobe length ratio (Fig. 1E). In contrast, *myoID*$^{wt/wt}$ *and myoID*$^{wt/mut}$ males had a wild-type lobe ratio (Fig. 1E). We conclude that in *D. pachea* males MyoID controls genitalia rotation direction, such as previously observed in *D. melanogaster* and that in addition, MyoID determines left-right identity of genitalia.

Apart from its establishment during development, the role of genital asymmetry with respect to reproduction is not well understood (*27*). Genital asymmetry has been hypothesized to co-evolve or to evolve in response to changes in mating position (*27*). Alternatively, asymmetric genitalia may cause lateralized mating behavior since the asymmetric parts mediate the coupling of the female and male abdomen and therefore potentially direct the relative orientation of the female and male bodies. We investigated if males with reversed genital asymmetry would adopt a reverted copulation posture by monitoring copulation behavior (Fig. S6, Data S3) of *myoID*$^{wt/wt}$, *myoID*$^{wt/mut}$, *or myoID*$^{mut/mut}$ males with wildtype *myoID*$^{wt/wt}$ females. We annotated mating behavior (Data S4) and copulation postures (Data S5, Fig. S7). A copulation was considered to occur when the male mounted the female and achieved to settle into an invariant copulation position for at least 30 seconds. Alternatively, males were sometimes observed to mount the female, but leg and abdomen movements did not settle and eventually the male separated from the female within a few minutes, which we considered to be a failed mounting attempt (106/140 total attempts, Fig. S6). Only 26% (10/38) of the couples with *myoID*$^{mut/mut}$ males that mounted the female also adopted a stable copulation position, compared to 11/11 and 13/13 for *myoID*$^{wt/wt}$ and *myoID*$^{wt/mut}$ males, respectively (Fig. S6). Genitalia orientation angles of *myoID*$^{mut/mut}$ males that managed to adopt a stable copulation posture were found to deviate at most by 16.7° from wild-type orientations, while it was higher in the majority of *myoID*$^{mut/mut}$ males (18/29) that failed to copulate (Fig. 1D, Fig. S6). Such defects in genitalia orientation were previously reported to be detrimental for copulation and efficient genitalia coupling for *D. melanogaster myoID* mutants (*28*). Regardless of the genotype and direction of genital asymmetry, *myoID*$^{wt/wt}$, *myoID*$^{wt/mut}$ and *myoID*$^{mut/mut}$ males consistently adopted a right-sided average copulation posture (Fig. 2). We conclude that the right-sided mating posture in *D. pachea* does not depend on the direction of male genital asymmetry.

Overall, our study reveals functional conservation of the unconventional myosin MyoID to control clockwise genitalia rotation in developing *D. pachea* male genitalia. In addition, the same protein got co-opted during evolution of *D. pachea* to contribute to determination of the direction of a novel male genital asymmetry. The latter function may be mediated through the genitalia rotation process itself since rotation direction and genital asymmetry direction are perfectly associated in our live-imaging trials (Fig. 1E). Mechanical forces between rotating genitalia and non-rotating outer tissue may act in opposite direction at the left and the right side and in function of the sense of rotation. Male genital asymmetry in *D. pachea* does not determine the posture itself since wildtype males and also mutant males with reversed genital asymmetry consistently mate in a right-sided copulation posture. Right-sided mating is therefore female-

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controlled or may be a hard-wired behavior that could have possibly favored the evolution of morphological asymmetry as an instructive cue to optimize genital contacts (*27*). This study provides the first experimental insights into *de novo* Evolution of left-right asymmetry. It involves recruitment of a conserved left-right regulator and is associated with a pre-existing tissue remodeling process.

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Supplementary Materials:

Materials and Methods

Figures S1-S7

Table S1

Movies S1-S2

External Databases S1-S5

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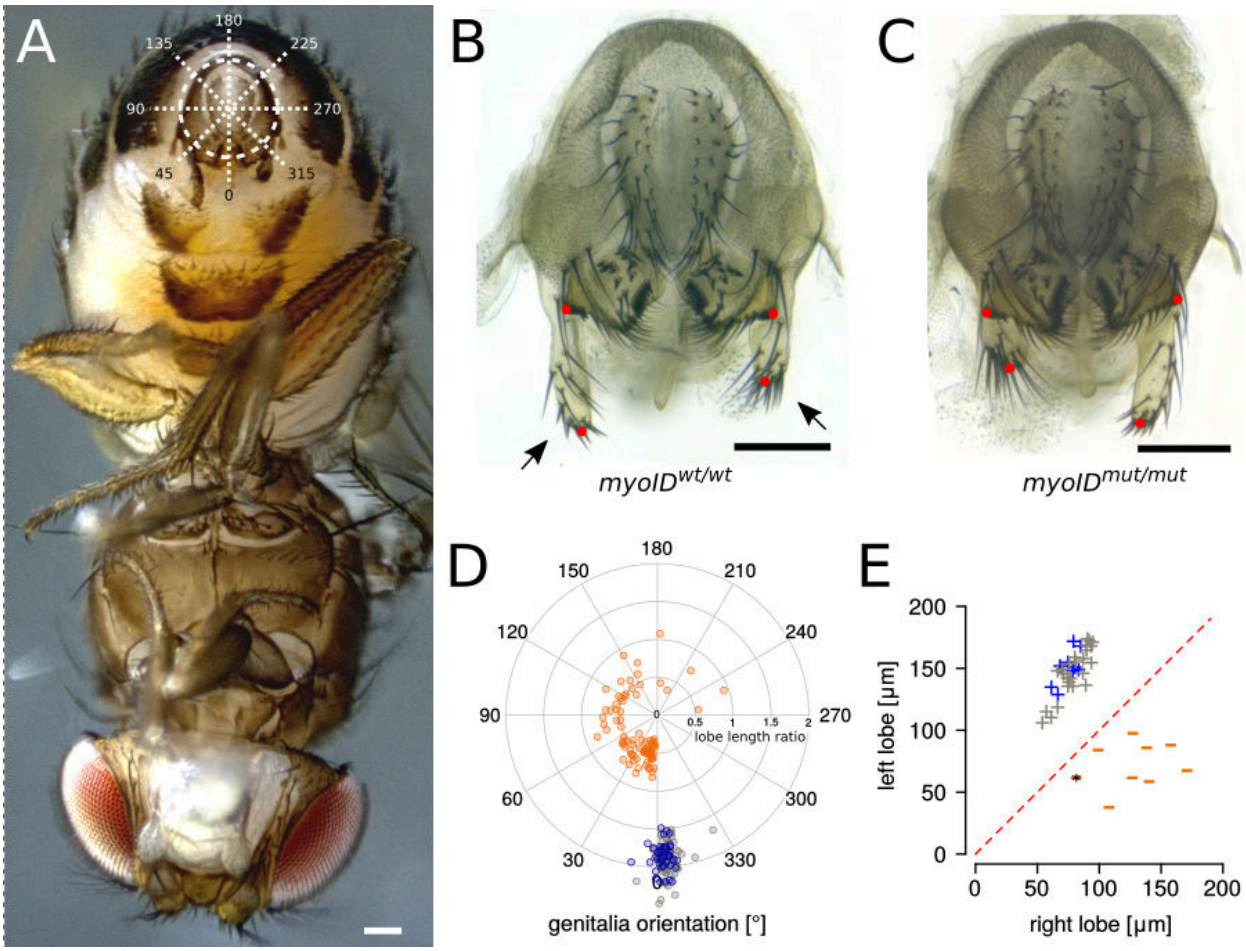

Fig. 1. M*yoID* mutants display reversed genital asymmetry and genitalia rotation defects. **(A)** Wild-type *D. pachea* male in ventral view. The orientation of genitalia is measured as an angle between the midpoint of male genital claspers relative to the male midline, as depicted by the dashed polar diagram. The scale is 100 µm. **(B)** External adult male genitalia of **(B)** a wild-type male *myoID*$^{wt/wt}$ with asymmetric genital lobes at the ventral side (arrows). **(C)** External adult male genitalia of a *myoID*$^{mut/mut}$ mutant male with reversed genital asymmetry. **(B-C)** Lobes are indicated by arrows and lobe lengths measurement landmark are shown as red dots. The scale is 100 µm. **(D-E)** Male genitalia orientation and lobe length asymmetry ratio in wild-type *myoID*$^{wt/wt}$ males (blue), heterozygous *myoID*$^{wt/mut}$ mutant males (grey) and homozygous *myoID*$^{mut/mut}$ mutant males (orange). **(D)** The polar diagram illustrates genitalia orientation angles. **(E)** The direction of genital lobe asymmetry is associated with the direction of male genitalia rotation. Left and right lobe lengths are plotted for individual males monitored by time-lapse microscopy. Genitalia rotation direction is indicated as +: clockwise, and as -: counter-clockwise. The diagonal red dashed line indicates the 1:1 lobe length ratio. The star indicates an observation with rudimentarily developed lobes in form of buds on both sides.

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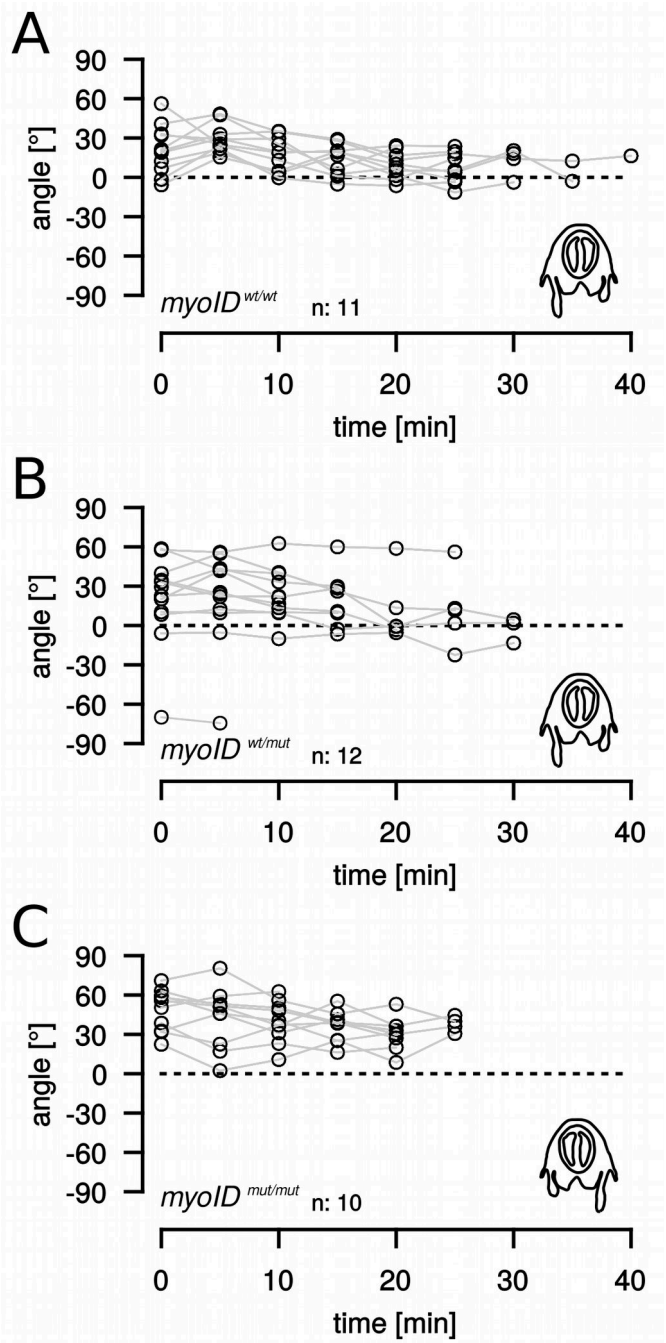

Fig. 2. The right-sided *D. pachea* mating posture does not depend on the direction of male genital asymmetry. Mating angles (α) (Supplementary Materials) of *D. pachea* copulating with a male being either **(A)** *myoID*$^{wt/wt}$ with the wild-typle lobe length ratio, **(B)** *myoID*$^{wt/mut}$ heterozygous males with wildtype lobe length ratio, **(C)** *myoID*$^{mut/mut}$ males with reverted lobe asymmetry ratio. Females had a *myoID*$^{wt/wt}$ genotype in all conditions. The hypothesis angle = 0 was rejected for each male genotype (GLM fit angle ~ genotype, all $p < 1 \times 10^{-10}$). Data acquisition and processing is illustrated in Figs. S6, S7. Positive angle values correspond to right-sided orientations of the male head relative to the female midline. Data points that correspond to the same trial are connected by grey lines. The number of trials are indicated by n, a drawing of the external male genitalia of each genotype is shown on the right.