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Non-canonical function of the *Sex-lethal* gene controls the protogyny phenotype in *Drosophila melanogaster*

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Running title: *Sxl* non-canonical function controls protogyny

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Abstract

Many animal species exhibit sex differences in the time period prior to reaching sexual maturity. However, the underlying mechanism for such biased maturation remains poorly understood. Females of the fruit fly *Drosophila melanogaster* eclose 4 h faster on average than males, owing to differences in the pupal period between the sexes; this characteristic is referred to as the protogyny phenotype. Here, we aimed to elucidate the mechanism underlying the protogyny phenotype in the fruit fly using our newly developed *Drosophila* Individual Activity Monitoring and Detecting System (DIAMonDS), which can continuously detect the precise timing of both pupariation and eclosion of individual flies. Via this system, following the laying of eggs, we detected the precise time points of pupariation and eclosion of a large number of individual flies simultaneously and succeeded in identifying the tiny differences in pupal duration between females and males. We first explored the role of physiological sex by establishing transgender flies via knockdown of the sex-determination gene, *transformer* (*tra*) and its co-factor *tra2*, which retained the protogyny phenotype. In addition, disruption of dosage compensation by *male-specific lethal* (*msl-2*) knockdown did not affect the protogyny phenotype. The *Drosophila* master sex switch gene—*Sxl* promotes female differentiation via *tra* and turns off male dosage compensation through the repression of *msl-2*. However, we observed that stage-specific whole-body knockdown and mutation of *Sxl* induced disturbance of the protogyny phenotype. These results suggest that an additional, non-canonical function of *Sxl* involves establishing the protogyny phenotype in *D. melanogaster*.

Author summary

A wide variety of animals show differences in time points of sexual maturation between sexes. For example, in many mammals, including human beings, females mature

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43 faster than males. This maturation often takes several months or years, and precisely
44 detecting the time point of maturation is challenging, because of the continuity of
45 growth, especially in mammals. Moreover, the reason behind the difference in sexual
46 maturation time points between sexes is not fully understood. The fruit fly *Drosophila*—
47 a model organism—also shows biased maturation between the sexes, with females
48 emerging 4 h faster than males (a characteristic known as the protogyny phenotype).
49 To understand the mechanism underlying the protogyny phenotype, we used our newly
50 developed system, *Drosophila* Individual Activity Monitoring and Detecting System
51 (DIAMonDS), to detect the precise eclosion point in individual fruit flies. Surprisingly,
52 our analysis of transgender flies obtained by knockdown and overexpression
53 techniques indicated that a physiological gender might not be necessary requirement
54 for protogyny and that a non-canonical novel function of the fruit fly master sex switch
55 gene, *Sxl*, regulates protogyny in fruit flies.

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Introduction

The time taken to reach sexual maturity is often unequal between the sexes of numerous animal species; protogyny refers to the phenotype characterized by females maturing first and protandry refers to the phenotype characterized by males maturing earlier than females [1, 2]. A search of the AnAge database (https://genomics.senescence.info/species/) revealed that approximately one-third of animals throughout the animal kingdom show sexual dimorphism in sexual maturation timing, among which poikilotherms tend to exhibit protandry, whereas homeotherms tend to exhibit protogyny (S1 Table) [3]. Although there is minimal information on arthropods in the AnAge database, several reports have indicated that male adults tend to emerge somewhat earlier than females for many insect species [2, 4-6]. Numerous hypotheses have been proposed to explain the strategy of protogyny and protandry with respect to increasing fitness; however, the detailed mechanism or benefit remains to be elucidated [1, 6-8].

To better understand the evolutionary significance of the sex bias in the sexual maturation time point, it is also important to elucidate the molecular mechanism underlying the protogyny and protandry phenotypes. However, these molecular aspects remain unclear, mainly owing to difficulties in precisely measuring the timing of maturation of individuals simultaneously and for a long period with available techniques.

In the fruit fly *Drosophila melanogaster*, adult females emerge quickly, before males (protogyny phenotype), with only a 4-h difference in eclosion timing [9]. Therefore, *D. melanogaster* offers a potentially useful model to elucidate the molecular mechanism underlying the sexual dimorphism in sexual maturation. We established a new system, *Drosophila* Individual Activity Monitoring and Detection System (DIAMonDS), which can automatically detect the phase-conversion timing of individual

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flies, such as the timing of pupariation, adult eclosion, and death, with high temporal resolution [10]. DIAMonDS enables time-lapse- and multi-scanning to simultaneously determine the time points of pupariation and eclosion in a large number of individuals under several chemical and environmental conditions and against different genetic backgrounds. Using DIAMonDS, we could precisely detect the 4-h difference of eclosion timing between sexes and further revealed that this was solely due to a difference in pupal duration [10].

In this study, we further applied DIAMonDS to evaluate the genetic regulation of the protogyny phenotype of *D. melanogaster*. As fruit flies alter their developmental rates when exposed to different environmental conditions [11-13], we first explored the effect of temperature and nutrients on the protogyny phenotype. We next manipulated the *transformer* (*tra*) gene and its co-factor *transformer-2* (*tra2*)—which play essential roles in determining the physiological sex of cells—to change the sex of the flies and evaluated the effect on the protogyny phenotype. Sex chromosome dosage compensation is also regulated differentially by sex, and the male-specific lethal complex is a key player in the dosage compensation machinery in *Drosophila* [14, 15]. Therefore, we also investigated the possibility that the dosage compensation pathway contributes to the protogyny phenotype by knocking down expression of the *male-specific lethal* (*msl*) *2* gene. Finally, we evaluated the potential role of the Sex-lethal gene (*Sxl*), which encodes an RNA splicing enzyme and acts as a master regulator of the sex-determination pathway [16-18] and also regulates the expression of its downstream genes—*tra* and *msl-2*.

Results

Environmental stability of the protogyny phenotype in *D. melanogaster*

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The sexual difference in pupal duration was maintained under a high temperature condition of 29°C (Fig 1A) and was also not altered under several nutritional conditions, including various sugar and yeast concentrations (Fig 1B and C). These results indicated that the sexual difference of pupal duration is a very stable phenotype in *D. melanogaster.* Therefore, we further used this difference to evaluate the molecular and genetic aspects underlying protogyny.

Fig 1. Environmental stability of the protogyny phenotype. A. Effect of rearing temperature of 25°C and 29°C. B. Effect of sugar concentration in the media. High, normal, and low sugar media contain 1 M, 0.15 M, and 0.05 M sugar, respectively, in addition to the other components of the normal medium. C. Effect of yeast concentration in the media. The poor yeast medium contains one-third the yeast concentration of normal fly medium. The number of flies analyzed is indicated in parentheses on each graph. Whiskers indicate minima and maxima (***$p < 0.001$; **$p < 0.01$; Student's unpaired *t*-test).

Forced sex change does not affect the protogyny phenotype based on the genotype

A previous study [19] revealed that *tra2* knockdown or *tra* overexpression in the whole body induced a sex transformation so that the phenotypic sex was opposite to the genotypic sex, which also altered body size (Fig 2A). We confirmed that the phenotypic sex transformation of *D. melanogaster* can be controlled by genetic manipulation of *tra* or *tra2* expression independent of the sexual genotype using *UAS-tra2* RNA interference (RNAi)-mediated knockdown or *UAS-traF* overexpression with ubiquitous *GAL4* drivers (Fig 2B). Pupal durations were then compared between siblings with XX and XY genotypes, respectively (S1 Fig). The phenotypic transformation induced by

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133 *tra2* knockdown or *traF* overexpression did not alter the sexual difference of pupal
134 duration based on the chromosomal sex (Fig 2C-D). These results suggested that
135 phenotypic sex is not critical for the protogyny phenotype, which is also independent of
136 the *tra*/*tra2* pathway.
137
138 **Fig 2. Alteration of *tra2* and *tra* expression does not affect the protogyny**
139 **phenotype.** A. Schematic presentation of the sex-determination pathway and effect of
140 alteration of *tra2* or *traF* expression. B. Photographs of external morphological sexual
141 traits of wild-type, *act5c>tra2* RNAi #1, *act5c*>RNAi #2, and *da>traF* adults. C–E. Effect
142 of *act5c>tra2* RNAi #1 (C), *act5c*>RNAi #2 (D), and *da>traF* adults (E) on the
143 protogyny phenotype. The number of flies analyzed is indicated in parentheses on
144 each graph. Whiskers indicate minima and maxima (***$p < 0.001$; **$p < 0.01$; Student's
145 unpaired *t*-test).
146
147 **Disturbance of the dosage compensation pathway could not alter the protogyny**
148 **phenotype**
149 Dosage compensation machinery is not assembled in *Drosophila* females, because
150 *msl-2*, a key gene of assembly of the MSL complex is not translated. [14, 15, 20]. Thus,
151 next, we investigated the possibility of contribution of the dosage compensation
152 pathway to development of the protogyny phenotype.
153 Ubiquitous knockdown of *msl-2* (Fig 3A) successfully induced male-specific semi-
154 lethality (Fig 3B), which in turn reduced the *msl-2* expression level in males (Fig 3C).
155 However, *msl-2* knockdown did not change the sexual difference of pupal duration,
156 suggesting that the sex chromosome dosage compensation machinery does not
157 commit to the protogyny phenotype (Fig 3D and E and S2 Fig).
158

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Fig 3. Alteration of *msl-2* expression does not affect the protogyny phenotype. A. Schematic presentation of the dosage compensation pathway and effect of *msl-2* expression alteration. B. Proportion of eclosed males of *act5c>msl-2 #1* and *act5c>msl-2 #2* lines. C. Relative *msl-2* mRNA level of the *act5c>msl-2 #1 and act5c>msl-2 #2* groups. D-E. Effect of *act5c>msl-2 RNAi #1* (D) and *act5c>msl-2 RNAi #2* (E) on the protogyny phenotype. The number of flies analyzed is indicated in parentheses on each graph. Whiskers indicate minima and maxima (***$p < 0.001$; **$p < 0.01$; Student's unpaired *t*-test).

The protogyny phenotype is determined in an *Sxl*-dependent manner

Ubiquitous *Sxl* knockdown using *act5c-GAL4* was not successful owing to its lethal phenotype. Therefore, we further attempted pan-neuronal *Sxl* knockdown using *elav-GAL4*, which did not influence the sexual difference in pupal duration (S3 Fig). To avoid lethality during larval development, we used a gene-switch system, which can induce GAL4 by administrating the glucocorticoid receptor antagonist RU486 [21]. F1 larvae were derived from parents of a *UAS-Sxl* RNAi transgenic fly and an *act5c-GS-GAL4* fly reared in normal condition, and early 3rd-instar larvae were transferred to a 96-well-microplate containing media with or without the RU486. Some adult F1 females that escaped from the RU486-containing media showed partial sexual transformation morphologically, indicating that RU486-dependent transformation succeeded in this condition (S4 Fig). Moreover, only the F1 flies reared in RU486-containing media did not exhibit the sexual difference of pupal duration, suggesting that *Sxl* might regulate the protogyny phenotype (Fig 4A).

Fig 4. Alteration of *Sxl* expression affects the protogyny phenotype. A. Effect of *act5c-GS>Sxl* RNAi on the protogyny phenotype of flies grown in media with or without

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185 RU486. B. Effect of *Sxl* mutation on the protogyny phenotype in flies with and without
186 the *Sxl* transgene. The number of flies analyzed is indicated in parentheses on each
187 graph. Whiskers indicate minima and maxima (***$p < 0.001$; *$p < 0.05$; n.s., no
188 significant difference; Student's unpaired *t*-test).
189
190 To confirm this interpretation, we also tried to use trans-heterozygous $Sxl^{M1,\Delta 33}/Sxl^{f7,M1}$
191 masculinized females [22-24], which have low viability but exhibit the same revertant
192 eclosion to the adult stage [22]. Using our cross scheme, we were able to produce two
193 genotypes of $Sxl^{M1,\Delta 33}/Sxl^{f7,M1}$ (Sxl^{-}) flies with and without an extra *Sxl* transgene (Fig
194 4B). The Sxl^{-} flies without an extra *Sxl* transgene showed a significantly longer pupal
195 duration in comparison with that of Sxl^{+} females, reaching the same length as that of
196 the male flies. The phenotype of Sxl^{-} flies completely recovered by introduction of the
197 extra *Sxl* transgene (Fig 4B).
198
199 **Discussion**
200 In this study, we applied our recently developed DIAMonDS to explore the molecular
201 mechanism underlying the very small but consistent sex difference in eclosion timing
202 due to a difference of pupal duration.
203 Many morphological and physiological traits exhibit a sex difference, which
204 may be controlled by a canonical sex-determination pathway [25]. However, the
205 protogyny phenotype was not disturbed in genetically induced transgender flies
206 established by controlling *tra* or *tra2* gene expression or by knockdown of *msl-2*. These
207 results suggest that a morphological or physiological (dosage compensation) sex
208 difference does not play a central role in controlling the protogyny phenotype, as
209 manipulating these factors did not influence the length of male pupal duration.
210 However, further genetic manipulation experiments demonstrated that the non-

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211 canonical function of *Sxl* regulates the eclosion timing and produces the protogyny
212 phenotype in *D. melanogaster*, as females with loss-of-function mutations or
213 knockdown of *Sxl* exhibited a pupal period of the same length as that of males.
214 *Sxl* expression is activated in the presence of two X chromosomes in female
215 early embryos, and is maintained via positive auto-regulation [16, 22, 26]. Sxl also
216 regulates splicing of its downstream components, including *tra* and *msl-2*, which play
217 crucial roles in the sex-determination cascade and dosage compensation, respectively
218 [27, 28]. Therefore, our results suggest that recently identified non-canonical Sxl
219 pathways could be involved in the protogyny phenotype.
220 Indeed, Sxl protein has been suggested to interact with other targets. *Nanos*
221 (*nos*) RNA can bind directly with Sxl protein in ovarian extracts, and loss-of-function
222 studies suggested that Sxl enables the switch from germline stem cells to committed
223 daughter cells through *nos* post-transcriptional down-regulation [29]. Sxl protein can
224 also bind with *Notch* (*N*) mRNA and appears to negatively control the *N* pathway [30].
225 Genome-wide computational screening for Sxl targets also identified an ATP-
226 dependent RNA helicase, Rm62, as a novel potential target [31]. Rm62 was inferred to
227 be involved in alternative splicing regulation and is required for the RNAi machinery
228 [32, 33]. A pan-neuronal RNA- binding protein of the ELAV family, *found in neurons*
229 (*fne*), was also shown to be downregulated by *Sxl* in female heads, independent of
230 *tra*/*tra2* regulation [34]. Sxl can enhance nuclear entry of the full-length Cunitus
231 interuptus (Ci) protein, suggesting a contribution to the sex difference in growth rate,
232 although their physical interaction has not been confirmed [35]. However, there is no
233 evidence that these non-canonical targets of Sxl directly affect eclosion timing.
234 Therefore, further studies are required to demonstrate whether these Sxl targets, or
235 another novel target, could contribute to the protogyny phenotype.

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In this study, conditional knockdown of *Sxl* in the nervous system, fat body, or intestinal stem cells and enteroblasts did not disrupt the protogyny phenotype; only *Sxl* knockdown induced in the whole body could induce the delayed eclosion in females. As knockdown of *Sxl* from the early developmental stage strongly affected viability, this toxicity might be one reason for our inability to identify the responsible organ or tissue that regulates the protogyny phenotype in this study.

The independence of the protogyny phenotype from the canonical sex-determination pathway is very intriguing with respect to understanding evolution of the sex difference in sexual maturation. *Sxl* does not appear to play a role in sex determination in most insects [24, 36-38]. Several reports indicated that orthologs of *Sxl* had no sex-determinant role in non-*Drosophila* species, including in *Diptera [37, 39, 40]*. In Drosophilidae, ancestral *Sxl* was duplicated to *Sxl* and *sister of sex lethal* (*ssx*); the new *ssx* gene plays a role of ancestral *Sxl*, suggesting that *Sxl* might have evolved to function as a novel sex-determinant gene in Drosophilidae [38]. A detailed phylogenetic study revealed that a male-specific exon, and likely embryo-specific exon, originated after the divergence between the Drosophilidae and Tephritidae families but before the split of the *Drosophila* and *Scaptodrosophila* genera [41]. We presume that the implementation of *Sxl* in the sex-determination pathway may be significantly involved in acquisition of the protogyny phenotype in *Drosophila*. Therefore, we expect that identification of the target of non-canonical *Sxl* sex-specific regulation for the protogyny phenotype might help to promote understanding of the evolutionary aspects of protogyny.

Material and methods

Drosophila stocks

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All flies were maintained at 25°C on standard laboratory medium as described previously [42]. The following stocks were obtained from the Bloomington *Drosophila* stock center (BDSC): w^{1118} (wild-type; BDSC 5905), *act5c-GAL4* (BDSC 3954), *da-GAL4* (BDSC8641), *elav-GAL4* (BDSC 458), *elav-GAL4; UAS-dcr-2* (BDSC 25750), *P{CaryP} attP2* (BDSC 36303), *UAS-tra2 RNAi #1* (BDSC 56912), *UAS-tra2 RNAi #2* (BDSC 28018), *UAS-traF* (BDSC 4590), *UAS-msl-2 RNAi #1* (BDSC 31627), *UAS-msl-2 RNAi #2* (BDSC 35390), *UAS-Sxl RNAi #1* (BDSC 34393), *UAS-Sxl RNAi #2* (BDSC 38195), $Sxl^{f7,M1}$*; P{Sxl.+tCa}9A/+* (BDSC 58486), and $Sxl^{M1,f\Delta33}$*/Binsinscy* (BDSC 58487). Three gene-switch *Gal4* driver lines, *act5c-GS-GAL4, S106-GS-GAL4,* and *5961-GS-GAL4*, were a kind gift from Dr. Akagi [43].

Measurement of pupal duration

We used our recently developed DIAMonDS to measure pupa duration at the individual level. The wandering 3rd-instar larvae were collected from rearing vials, and a single larva was placed in the well of a 96-well microplate with normal medium. The plate was then placed on a flatbed CCD scanner to obtain time-lapse images until all flies were eclosed. The time-lapse image dataset was then analyzed using Sapphier software as described previously [10].

To compare the effect of *Sxl* mutation on pupal duration, $Sxl^{f7,M1}$*; P{Sxl.+tCa}9A/+* females were crossed with w^{1118}*/Y* males. The F1 progeny $Sxl^{f7,M1}$*/Y; P{Sxl.+tCa}9A/+* males were then crossed with $Sxl^{M1,f\Delta33}$*/Binsinscy* females. Each genotype of the F2 flies was then assessed for pupal duration using the DIAMonDS. To induce the gene-switch *Gal4* driver, RU486 (Mifepristone, Sigma, St. Louis, MO, USA) reagent was dissolved in ethanol and added to the medium at a final concentration of 100 μg/ml.

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To detect the sex genotype of the flies, genomic DNA was extracted from single adults by homogenization in 50 µl of squishing buffer (10 mM Tris-HCl [pH 8.2], 1 mM EDTA, 25 mM NaCl, 200 µg/ml proteinase K), and incubated at room temperature for 20 min, followed by inactivation at 95°C for 5 min. The extracted genomic DNA was subjected to polymerase chain reaction (PCR) analysis using a WD repeat-containing protein on Y chromosome (*WDY*)- and *Rp49*-specific primer mix by ampliTaq Gold 360 master mix (Applied Biosystems, Foster City, CA, USA), and then the amplified DNA fragments were separated by 2% agarose gel electrophoresis (S1 Fig).

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted from the whole adult body (for measuring *msl-2* expression knocked down by *act5c-GAL4*) and from the dissected larval central nervous system (for measuring *Sxl* expression knocked down by *elav-GAL4*) using Isogen II (Nippon Gene, Tokyo, Japan), and then RT-qPCR was performed using a One Step SYBR PrimeScript PLUS RT-PCR kit (Takara Bio, Shiga, Japan) and Applied Biosystems ABI Prism 7000 Sequence Detection System. All mRNA expression levels were normalized to the levels of *rp49* mRNA. We used the following primers for qPCR (5′-3′) for detecting *msl-2* and *Sxl* mRNA levels: *Sxl*, forward primer (5'-CCAATCTGCCGCGTACCATA-3'), reverse primer (5'-AATGGAACCGTACTTGCCGA-3'); *msl-2*, forward primer (5'-CACTGCGGTCACACTGGCTTCGCTCAG-3'), reverse primer (5'-CTCCTGGGCTAGTTACCTGCAATTCCTC-3'); and *rp49*, forward primer (5'-GATGACCATCCGCCCAGCATAC-3'), reverse primer (5'-AGTAAACGCGGTTCTGCATGAGC-3').

Statistical analysis

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All data were analyzed and graphs were plotted using Prism version 8 (GraphPad Software, San Diego, CA, USA). Data are presented as means ± standard deviation. Student's unpaired two-tailed *t*-test was performed to compare differences between two groups in each experiment, and Dunnett's one-way analysis of variance was used for multiple comparisons; $p < 0.05$ was considered to indicate a statistically significant difference.

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464

465 **Supporting information**

466 **S1 Fig. Detection of the sex genotype of single flies.** A. WDY primer sets and
467 results of single-fly PCR for females and males of w^{1118}, *act5c>tra2 RNAi #1*,

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468 *act5c>RNAi #2*, and *da>traF* fly lines. B. Results of single-fly PCR for *act5c>tra2 RNAi*
469 *#1*. C. Results of single-fly PCR for *da>traF*.
470
471 **S2 Fig. Effect of *da>msl-2 RNAi #1* and *da>msl-2 RNAi #2* on the protogyny**
472 **phenotype.** The number of flies analyzed is indicated in parentheses in each graph.
473 Whiskers indicate minima and maxima (***$p < 0.001$; Student's unpaired *t*-test).
474
475 **S3 Fig. Alteration of *Sxl* expression in the central nervous system does not affect**
476 **the protogyny phenotype.** A, B. Relative *Sxl* mRNA levels of the *elav>Sxl RNAi*
477 *#1/#2; dcr2* (A) and *elav>Sxl RNAi #1/#* (B) fly lines. C. Effect of *elav>Sxl RNAi #1* and
478 *elav>Sxl RNAi #2* on the protogyny phenotype. D. Effect of *S106-GS>Sxl RNAi #1* and
479 *5961-GS>Sxl RNAi #2* on the protogyny phenotype. The number of flies analyzed is
480 indicated in parentheses on each graph. Whiskers indicate minima and maxima (***$p <$
481 0.001; **$p < 0.01$; *$p < 0.05$; n.s., no significant difference; Student's unpaired *t*-test).
482
483 **S4 Fig.** Photographs of external morphological sexual traits of *act5c-GS>Sxl RNAi #1*
484 flies in medium with and without RU486.
485

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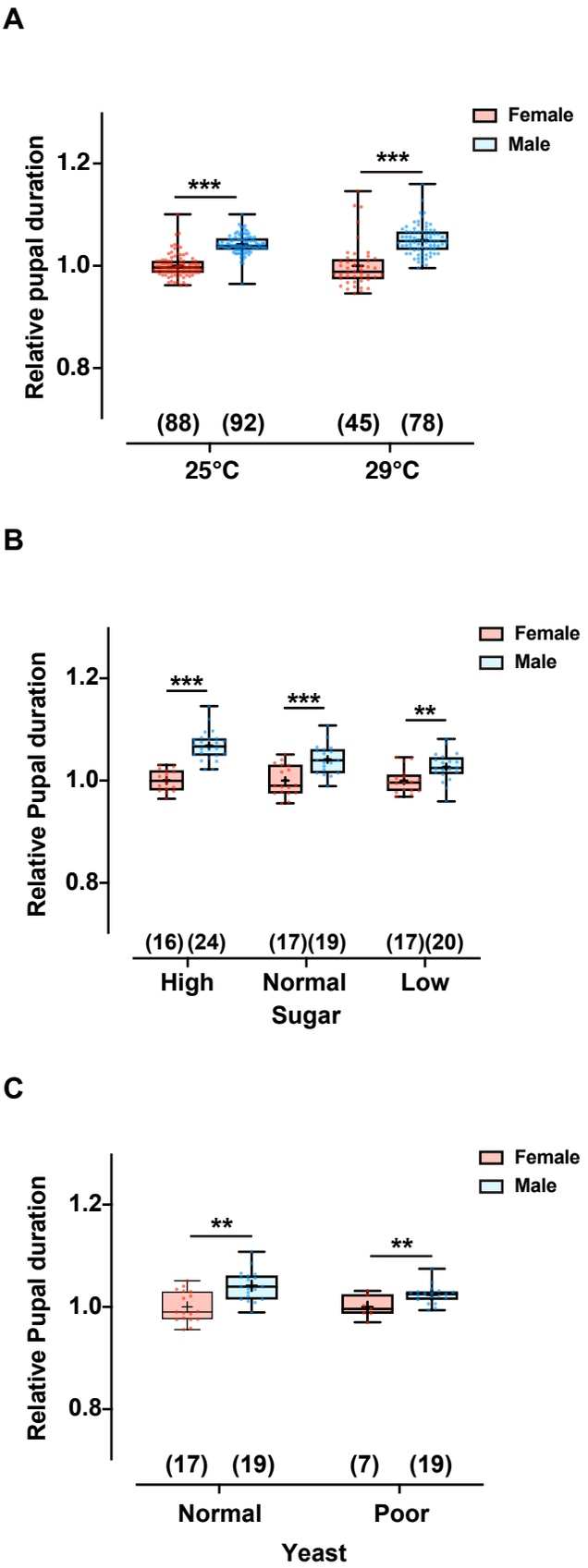

Figure 1.

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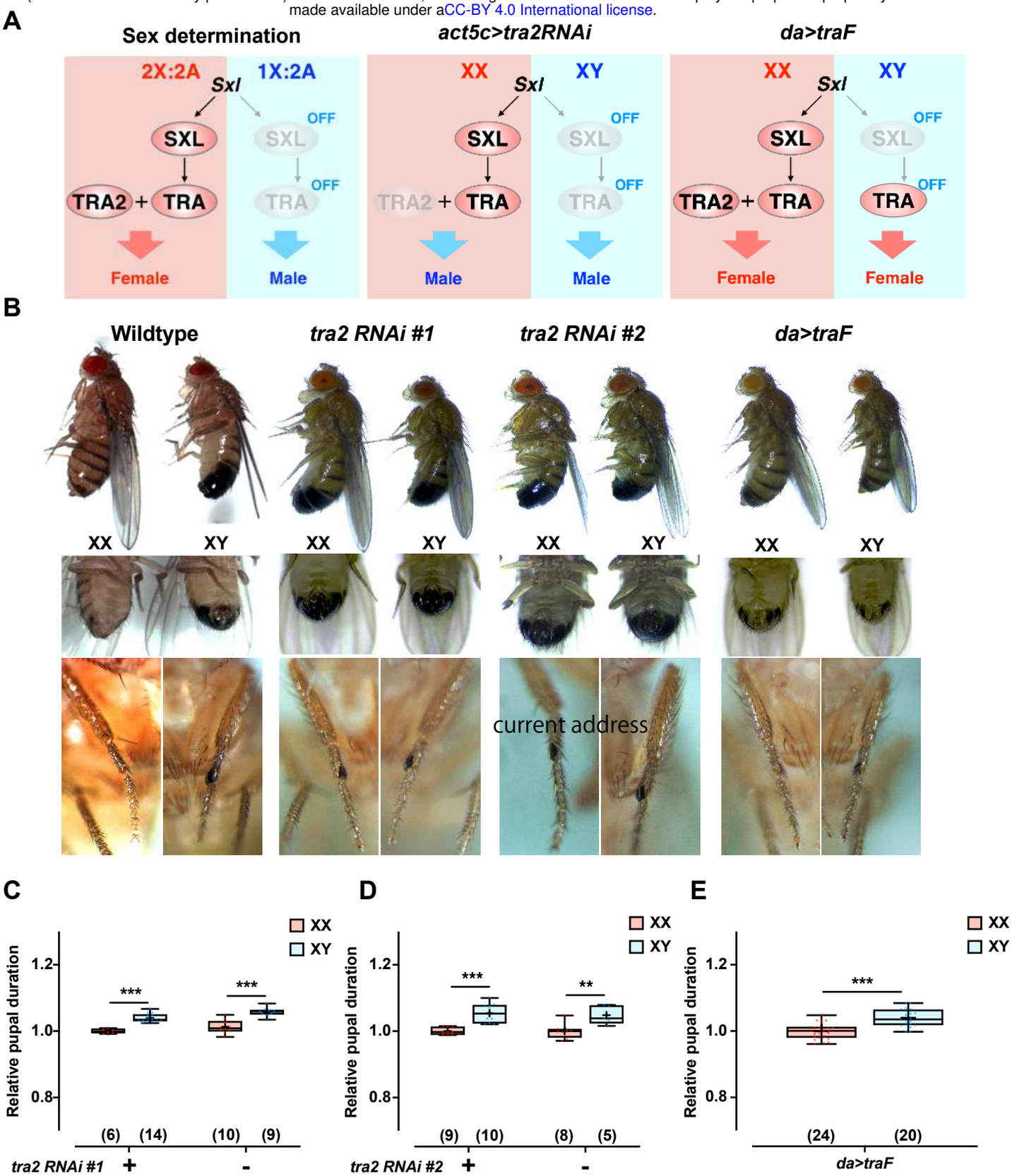

Figure 2.

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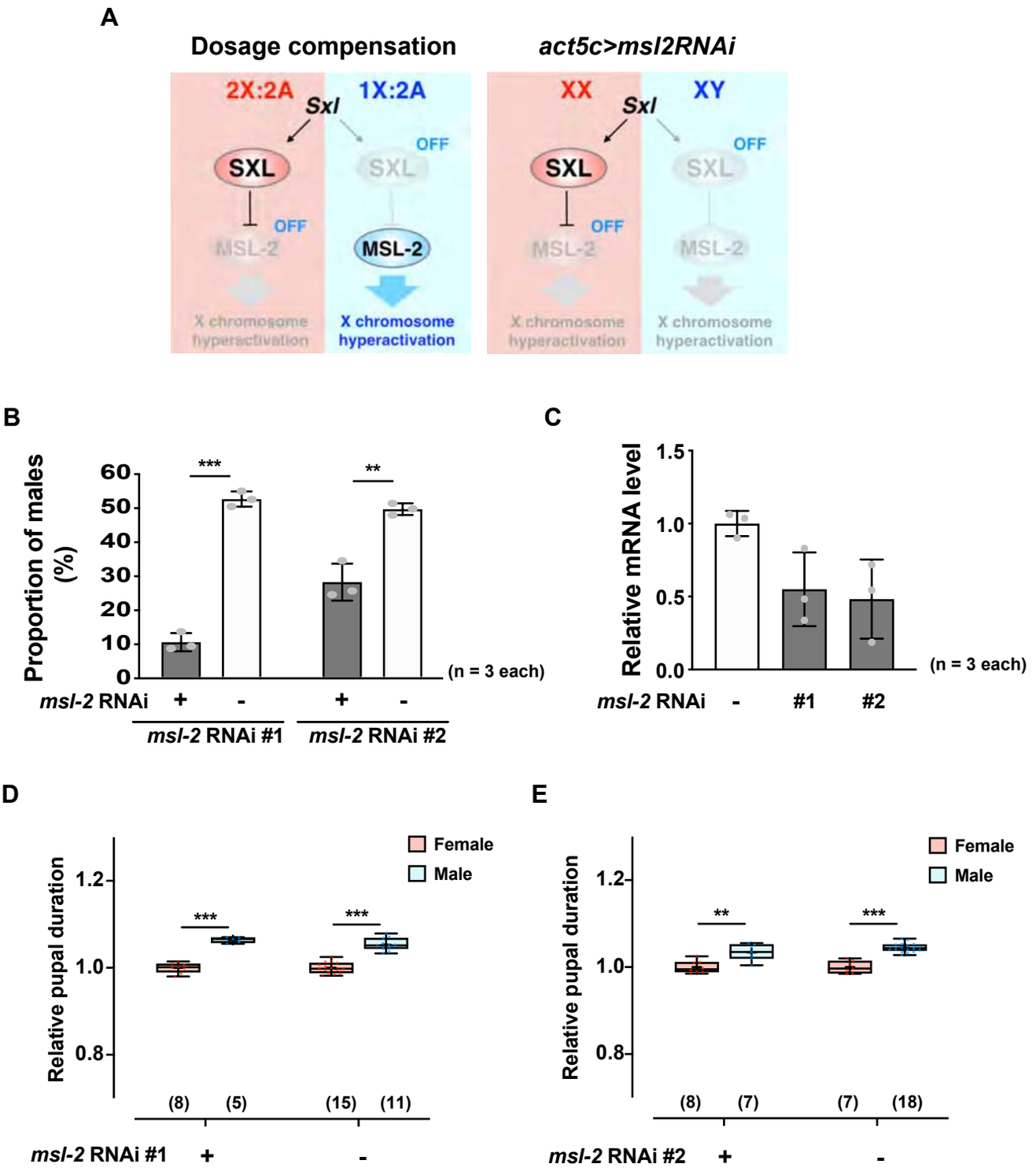

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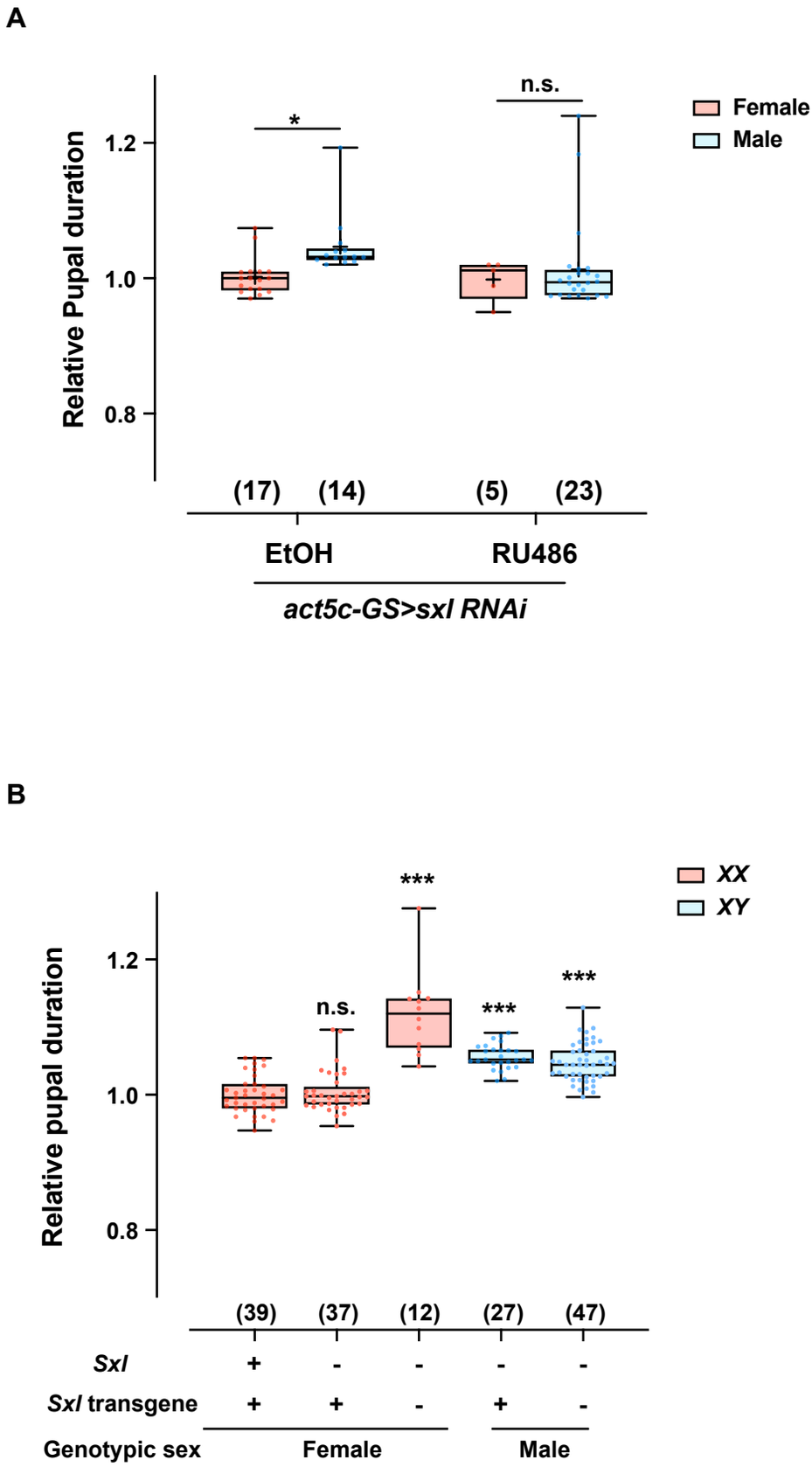

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S1 Table. Abundance of Protandry and Protogyny in vertebrate species

Class	Protandry	Protogyny	No bias	Total (number of species)
Poikilotherm				
Chondrichthyes	43	8	10	61
Teleostei	58	27	150	235
Amphibia	20	2	56	78
Reptilia	14	1	55	70
Homeotherm				
Aves	12	21	645	678
Mammalia	77	233	264	574
Total	224	292	1180	1696

Calculated from dataset of AnAge Database (https://genomics.senescence.info/species/)

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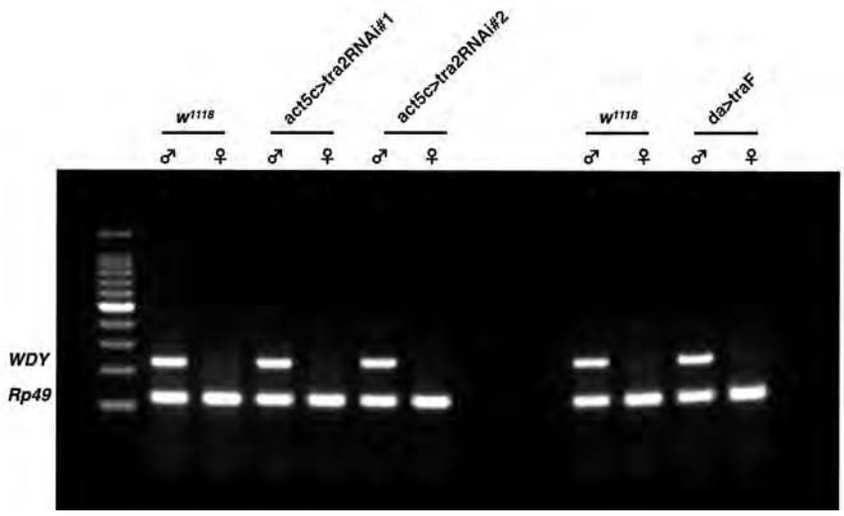
A

Y* chromosome specific gene: *WDY

Forward primer: **5'-TGGACGACGATGACTCCTCT-3'**
Reverse primer: **5'-TTGAACCTGCCCTGGTTGTT-3'**



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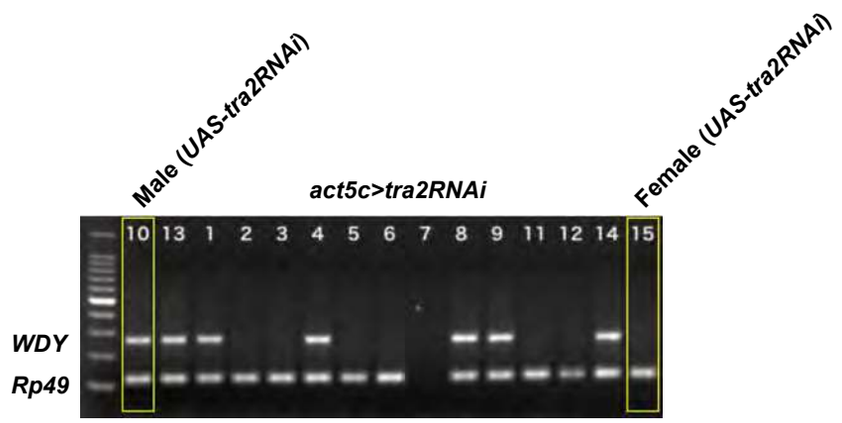

C

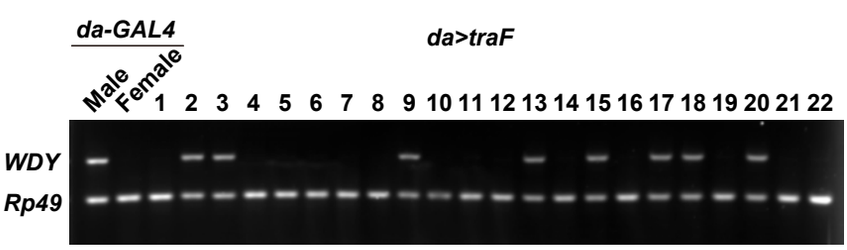

Figure 2-Supplementary figure 1.

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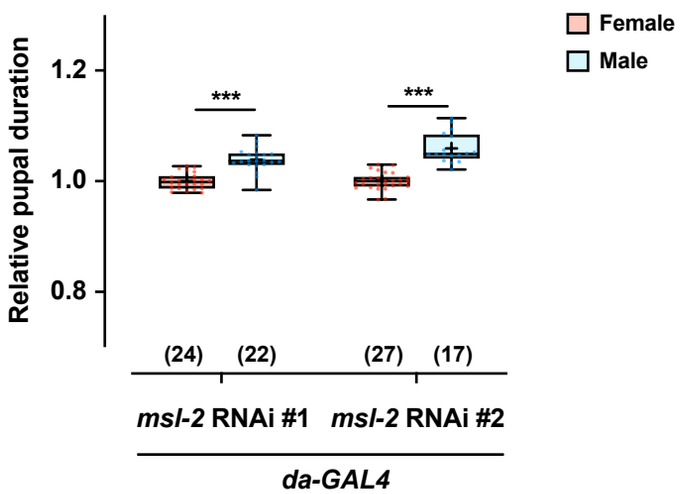

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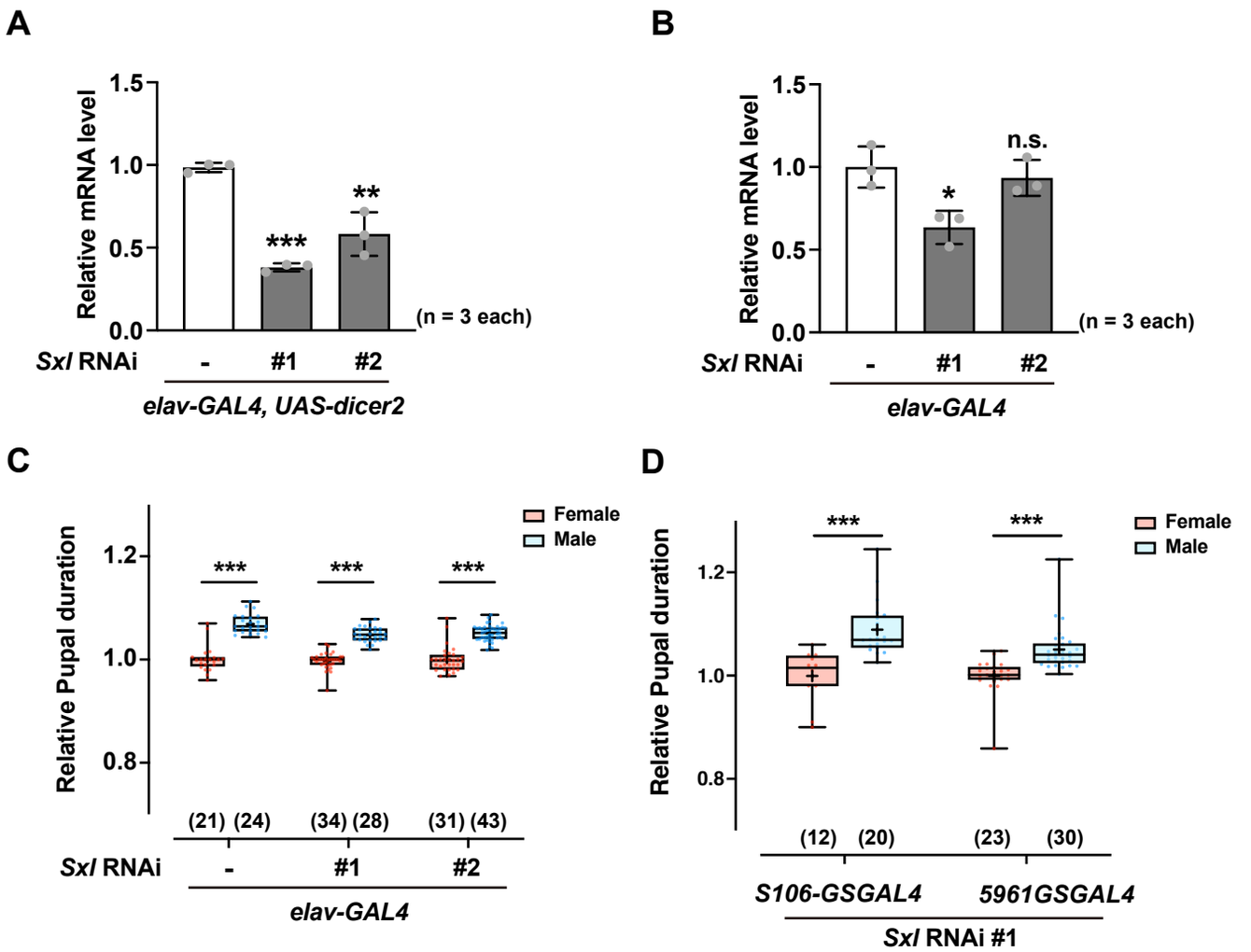

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