Involvement of the *doublesex* gene in body color masculinization of the blue-tailed damselfly, *Ischnura senegalensis*

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Abstract

Odonata (dragonflies and damselflies) display remarkable color pattern diversity including sexual dimorphism and intrasexual polymorphism. We previously found that expression of a sex-determining transcription factor, the *doublesex* (*dsx*) gene, is associated with female color polymorphism (gynomorph for female-specific color and andromorph for male mimicking color) in the blue-tailed damselfly, *Ischnura senegalensis*. Here we investigate the function of dsx gene on thoracic coloration by electroporation-mediated RNA interference (RNAi). RNAi of the *dsx* common region changed color patterns of males and andromorphic females to patterns of gynomorphic females. Further, gynomorphic color pattern was not affected by *dsx* RNAi. The long isoform of *dsx* RNAi produced no effects, suggesting that the short isoform of *dsx* is important for body color masculinization in both males and andromorphic females. Expression pattern changes were also examined in five genes with different expression levels between sexes and female morphs. Among these genes are two melanin suppressing genes, *black* and *ebony*, that were upregulated in the *dsx*-RNAi region compared to a control region. Upregulation coincides with a gynomorphic orange color instead of the black stripe observed in males and andromorphic females. *dsx* may regulate male color differentiation by suppressing *black* and *ebony* in the thoracic region of *I. senegalensis.* Results add to the understanding of molecular mechanisms underlying the evolution of female polymorphism in Odonata.

Keywords: *doublesex*, RNA interference, regulation of gene expression, female-limited polymorphism, insects, damselfly

Introduction

Many animals and plants exhibit intraspecific color polymorphism. Color polymorphism is well studied for its ecological and evolutionary significance, including reproductive strategies, speciation, mimicry and crypsis (Gray & McKinnon, 2007). As examples, male color polymorphism of the side-blotched lizard, *Uta stansburiana,* is part of a reproductive strategy similar to the children's game, "rock-paper-scissors" (Sinervo & Lively, 1996), and female color polymorphism seen in several butterfly species is associated with Batesian mimicry (Krushnamegh Kunte, 2009; Mallet & Joron, 1999). Many Odonata (dragonflies and damselflies) species have intraspecific body or wing color variations that are sexually dimorphic and intrasexually polymorphic (Bybee et al., 2016; Corbet 1999; Futahashi, 2016, 2017; Tillyard, 1917). Most intrasexual color polymorphisms of Odonata are female-limited and have appeared independently multiple times within the order, Odonata (Fincke, Jödicke, Paulson, & Schultz, 2005). In female-limited color polymorphism, one morph, called the andromorph, typically resembles a conspecific male, while the other morph, called the gynomorph, shows female-specific color. Among Odonata, the genus, *Ischnura,* is a model group for studies of ecology and evolution of female polymorphism. Among 75 *Ischnura* species, 29 have female-limited color polymorphism, associated with mating

strategy (Rosa A. Sánchez-Guillén et al., 2018; Willink, Duryea, & Svensson, 2019). Polyandrous species often display female polymorphism, and most monandrous species are monomorphic (Robinson and Allgeyer, 1996; Sánchez-Guillén *et al*., 2018). Female-limited polymorphism in *Ischnura* is maintained by negative frequency-dependent selection and balancing selection derived from male mating harassment (Andres, Sanchez, & Cordero, 2000; Takahashi, Nagata, & Kawata, 2013; Takahashi, Kagawa, Svensson, & Kawata, 2014). In addition to body color, other phenotypic traits such as flying distance, larval duration and wing length also differ between andromorphic and gynomorphic females (van Gossum *et al.*, 2001; Abbott and Gosden, 2009). In several *Ischnura* species, female-limited color polymorphisms are controlled by an autosomal locus with two or three alleles (Cordero, 1990; Cordero & Andres, 1999; Johnson, 1964, 1966; R A Sánchez-Guillén, van Gossum, & Cordero, 2005; Takahashi et al., 2014), although little is known about the genes involved in the color polymorphisms.

Recently, a master gene that controls female-limited polymorphism was identified in several insect species (Kunte et al., 2014; Nishikawa et al., 2015; Woronik et al., 2019; Yassin, Chung, Veuille, David, & Pool, 2016; Yassin, Delaney, et al., 2016) (Kunte et al., 2014; Nishikawa et al., 2015; Yassin et al., 2016a, 2016b; Woronik et al., 2019). Among the swallowtail butterflies species in the genus, *Papilio*, a sex-determining transcription factor, the *doublesex* (*dsx)* gene, is involved in female limited Batesian mimicry (Iijima et al., 2018; Kunte et al., 2014; Nishikawa et al., 2015; Palmer

& Kronforst, 2020). Expression of the *dsx* gene is also associated with female color polymorphism in the blue-tailed damselfly, *Ischnura senegalensis* (Takahashi, Takahashi, & Kawata, 2019). Adult female *I. senegalensis* display two color morphs, gynomorphic and andromorphic. The former morph is dominant.. Males and andromorphic females display a greenish color with black mid-dorsal and humeral stripes on their thorax; gynomorphic immature females display an orange color without black humeral stripes (Fig. 2). The short isoform of the *dsx* gene is expressed predominantly in males, the long isoform of *dsx* gene is expressed predominantly in females (Takahashi et al., 2019). Notably, expression of the short *dsx* isoform is higher in andromorphic females compared with gynomorphic females (Takahashi et al., 2019), suggesting that *dsx* is involved in female color polymorphism as well as sexual color dimorphism. In this study, using electroporation-mediated RNAi techniques, the effects of the *dsx* gene on adult thoracic color patterns in the blue-tailed damselfly, *I. senegalensis,* were investigated. The *dsx* gene is essential for masculinization of body color but is not involved in expression of the gynomorphic color pattern. Also, *dsx* knockdown in males and andromorphic females promoted expression of melanin and suppressed genes, *black* and *ebony*, compared to expression in gynomorphic females. The *dsx* gene may regulate masculinization of thoracic body color through the repression of *black* and *ebony* genes in *I. senegalensis*.

Materials and Methods

Insects

Adult female *I. senegalensis* were collected at Heiwasouzou, Makabe, Kakinohana and Shikiya on Okinawa Island in May 2017, April and October 2018, and June 2019. Mature females were placed in plastic cups (diameter 11 cm, height 4 cm) with a wet paper filter at room temperature for oviposition. Newly hatched larvae were reared together for approximately 1 month, then transferred to small plastic containers (diameter 3 cm, height 5 cm) for individual rearing. Larvae were fed with *Artemia* brine shrimp and *Tubifex* worms until early final instar. Sexes of larvae were judged by the presence of an ovipositor under the abdomen. Although color morphs of larvae cannot be determined, genetically recessive andromorphic females were expected to be obtained from eggs derived from andromorphic females. It should be noted that female morph frequency differs significantly among populations (e.g., gynomorph frequency is high (>90%) at Makabe, and low (<40%) at Kakinohana), despite small genetic distances among populations at Okinawa Island (Inomata, Hironaka, Sawada, Kuriwada, & Yama, 2015).

Electroporation-mediated RNAi experiment

A small interfering RNA was designed using the siDirect program version 2.0 (http://sidirect2.rnai.jp/). For each gene of *I. senegalensis*, two siRNAs were designed and mixed (100 mM each). Target sequences for the *dsx* common region were 5′- AAC

ATG AAT TTC GTC AAA GAT GC-3′ and 5′- ACG CAA TTC TGC AAA ATG CAA GG-3′, whereas those for *dsx* long isoform-specific region were 5′- AAC GAA AAG CAG AAA AAG GAATG-3′ and 5′- AAG TGA TAA TGC GAC GTA TAT TG-3′ (Fig. 1a). siRNA for the *dsx* short isoform-specific region was not designed because only the junction site is different. Part of long isoform would include region 2 because region 2 was shown in reverse transcription PCR of gynomorphic females (data not shown). siRNAs for the *multicopper oxidase 2* (*MCO2*) gene that is essential for melanin pigmentation (Okude, Futahashi, Kawahara-Miki, et al., 2017), were used as positive controls for targeting 5′- GAGCACTTTCCGTTATCAATATA-3′ and 5′- TCCTCTTGATGCTATCTGTAATG-3′. As a negative control, siRNA for the *enhanced green fluorescent protein* (*EGFP*) gene was used for targeting 5′- CGG CAT CAA GGT GAA CTT CAA GA-3′ (Ando & Fujiwara, 2013).

Electroporation was used to introduce siRNA into larvae (Okude, Futahashi, Kawahara-Miki, et al., 2017). Early-stage, final instar larvae (stage 1 in Okude, Futahashi, Tanahashi, & Fukatsu, 2017) were anesthetized on ice for 1 min, approximately 1 µl of 100 µM siRNA solution was injected into the left side of the thorax using a glass needle and an injector (Nanoject II; Drummond Scientific Company). Immediately after injection, droplets of ultrasound gel were placed near the injection site and on the right side of the thorax opposite the injection site. Positive and negative platinum electrodes were attached to injection and opposite sites, respectively (Fig. 1b, Okude, Futahashi, Kawahara-Miki, et al., 2017). Electroporation was

144 conducted with five or ten pulses for 25 V (each 280 ms pulses/s) using an
145 electroporator (Cure-Gene; CellProduce). Each larva was placed on a wet paper in a
146 plastic case individually for a day to confirm recovery from anesthesia. Injected larvae
147 were reared at room temperature until adult emergence. Since more than half the
148 individual larvae died before adult emergence, only adults that emerged normally were
149 used for phenotype observation. Of note, adult phenotypes were patchy around the area
150 where the positive electrode was placed (Fig. 2).

151

152 **Quantitative PCR of adult knock-down *dsx***

153 To estimate RNAi efficiency of *dsx* gene electroporation, and to investigate expression
154 of five sex and morph-associated genes (*black*, *ebony*, *chp*, *eas* and *RhoGEF*),
155 quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used. Total
156 RNA was extracted from the left side of thorax with gynomorph-like color where
157 siRNA was introduced and from the control region on the right side of the thorax of the
158 same individual (N = 4 each for male and andromorphic female adults) using
159 RNAqueous™-Micro Total RNA Isolation Kit (Thermo Fisher). cDNA was synthesized
160 using SuperScript IV Reverse Transcriptase (Thermo Fisher) and subjected to real-time
161 quantitative PCR using the StepOne Real-Time PCR System (Thermo Fisher) with
162 Power SYBR Green PCR Master Mix (Applied Biosystems). Standards for each gene
163 (*dsx* short isoform, *black*, *ebony*, *chp*, *eas* and *RhoGEF*) for qRT-PCR were generated
164 by PCR using Tks Gflex DNA Polymerase (TaKaRa) and gene-specific primers (Table

S1). The *ribosomal protein L13* (*RpL13*) gene was used as an internal standard to estimate relative mRNA expression (Table S2). The expression levels were visualized relative to maximal expression, defined as a level of 1 for each transcript. Statistically significant differences in gene expression were evaluated using the generalized linear model (GLM) assuming a gamma distribution.

Results

The *dsx* gene is involved in body color masculinization in *I. senegalensis*

We first investigated the effects of electroporation-mediated RNAi for *MCO2* (positive control) and *EGFP* (negative control) genes. Of fifteen individuals (four male, three andromorphic female, and eight gynomorphic female adults) that subjected to the *MCO2* RNAi and emerged normally, ten individuals (10/15 = 67%) exhibited patchy unpigmented regions on the left side of the thorax where the positive electrode was placed for electroporation (Table 1, Fig. 2, arrows). This observation is consistent with Okude, Futahashi, Kawahara-Miki, et al., (2017). After *EGFP* gene RNAi electroporation, 10 individuals (two male, one andromorphic female, and seven gynomorphic female adults) emerged normally, and no individuals showed an unpigmented area at the site of cathode placement (Table 2, Fig. 2).

We next performed electroporation-mediated RNAi for the *dsx* common region (both short and long isoforms) or long isoform only. Of 41 individuals (17 male, nine

185 andromorphic female, and 15 gynomorphic female adults) that subjected to RNAi of the
186 *dsx* common region and emerged normally, importantly, 14 males (14/17 = 82%) and
187 seven andromorphic females (7/9 = 78%) showed patchy orange-colored regions
188 resembling gynomorphic females (Table 3, Fig. 2, arrows). None of the 15
189 gynomorphic females exhibited this color change (Table 3, Fig. 2). RNAi of *dsx* long
190 isoform caused no noticeable effect in 31 emerged adults (nine males, four
191 andromorphic females, and 18 gynomorphic females) (Table 4, Fig. 2). The suppression
192 ratio for *dsx* expression (short isoform) was estimated and confirmed a significant
193 reduction in the left side in males ($P = 0.01963$, Fig. 3); such significant reduction was
194 not detected in andromorphic females ($P = 0.1667$, Fig. 3). Low efficiency for the
195 reduction in target gene expression is likely due to extraction of RNA from both tissues
196 affected by electroporation and from surrounding tissues.

197

198 **_dsx_ knockdown promotes two melanin suppressing genes _black_ and _ebony_**

199 *dsx* encodes a transcription factor that regulates the expression of several downstream
200 genes (Chatterjee, Uppendahl, Chowdhury, Ip, & Siegal, 2011; Matson & Zarkower,
201 2012). In addition to the *dsx* gene, *black*, *ebony*, *chaoptin* (*chp*), *easily shocked* (*eas*),
202 and *rho guanine nucleotide exchange factor* (*RhoGEF*) genes are also differentially
203 expressed in female morphs and between sexes (Takahashi et al., 2019). These genes
204 represent candidate *dsx* target genes. Expression of these five genes between *dsx*-RNAi
205 and control regions was evaluated. Expression of two melanin suppressing genes, *black*

206 in males and *ebony* in andromorphic females, were higher on the left side (Fig. 3,
207 asterisks $P < 0.05$) and resembled expression in gynomorphic females. No significant
208 differences in expression were detected for the remaining three genes, *chp*, *eas* and
209 *RhoGEF* (Fig. 3).

210

211 # Discussion

212 *dsx* is involved in male-type color pattern formation, but not in female-specific
213 gynomorphic coloration in *I. senegalensis*. The male *dsx* isoform is essential for male
214 differentiation in most insects, while the female *dsx* isoform is involved in female
215 differentiation only in holometabolous insects (Wexler et al., 2019; Zhou, Whitworth,
216 Pozmanter, Neville, & Doren, 2018). Our results are consistent with previous work on
217 male differentiation. Like *Ischnura* damselflies, some butterflies display female color
218 polymorphism, in which one morph resembles male coloration (Kunte et al., 2014;
219 Palmer & Kronforst, 2020). Functional analysis suggests that changes in total
220 expression level and protein sequences, but not isoform patterns, are important for
221 female polymorphisms in butterflies (Nishikawa et al., 2015). These findings contrast
222 with the current results for *Ischnura* damselflies.

223 Based on qRT-PCR analyses, *black* and *ebony* were identified as *dsx* target gene
224 candidates. *black* and *ebony* encode aspartate decarboxylase and *N*-*β*-alanyldopamine
225 (NBAD) synthase, respectively. Both enzymes are essential for the synthesis of NBAD

226 that is involved in hardening of the cuticle, and for reddish and yellowish coloration
227 (Arakane, Noh, Asano, & Kramer, 2016; Futahashi & Fujiwara, 2005; Liu, Lemonds,
228 Marden, & Popadić, 2016; Wittkopp, Carroll, & Kopp, 2003). Based on phenotypes of
229 other insects, upregulation of *black* and *ebony* is consistent with a color change from
230 black humeral stripes to orange. These results strongly suggest that *dsx* suppresses *black*
231 and *ebony* in males and andromorphic females, and induces a change from a female to a
232 male color pattern. Elucidation of the regulatory mechanism of *dsx* splicing patterns
233 may clarify the evolution of female color polymorphism as well as sexual color
234 dimorphism in Odonata.

235

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241

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377

378 Data Accessibility

379 Data of RNAi adult phenotypes will be available in Dryad. The expression levels of *dsx*
380 and *dsx* target genes from real-time quantitative PCR are listed in Supplementary Table
381 S2.

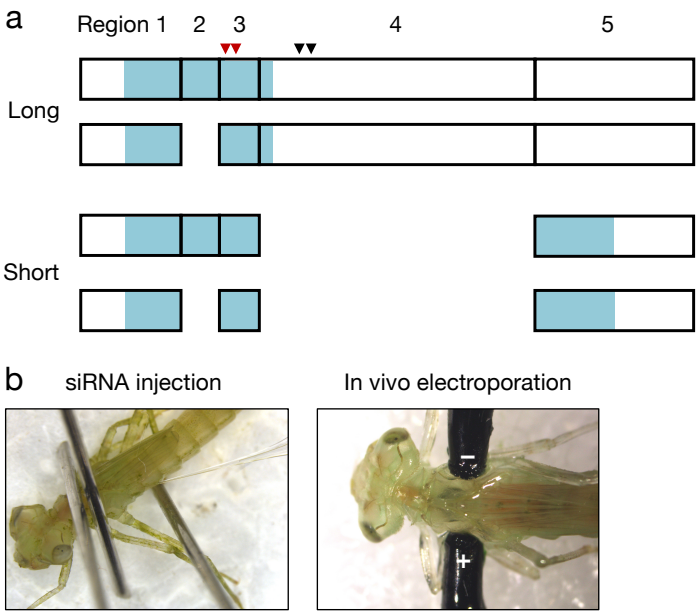
382

383 # Author Contributions

384 M. T., Y. T. and M.K. designed research. M. T., G. O. and R. F. conducted RNAi
385 experiment. M. T. conducted real-time quantitative PCR experiment and statistical
386 analysis. M. T. wrote the first draft manuscript, and all authors contributed to the
387 improvement of the manuscript.

388

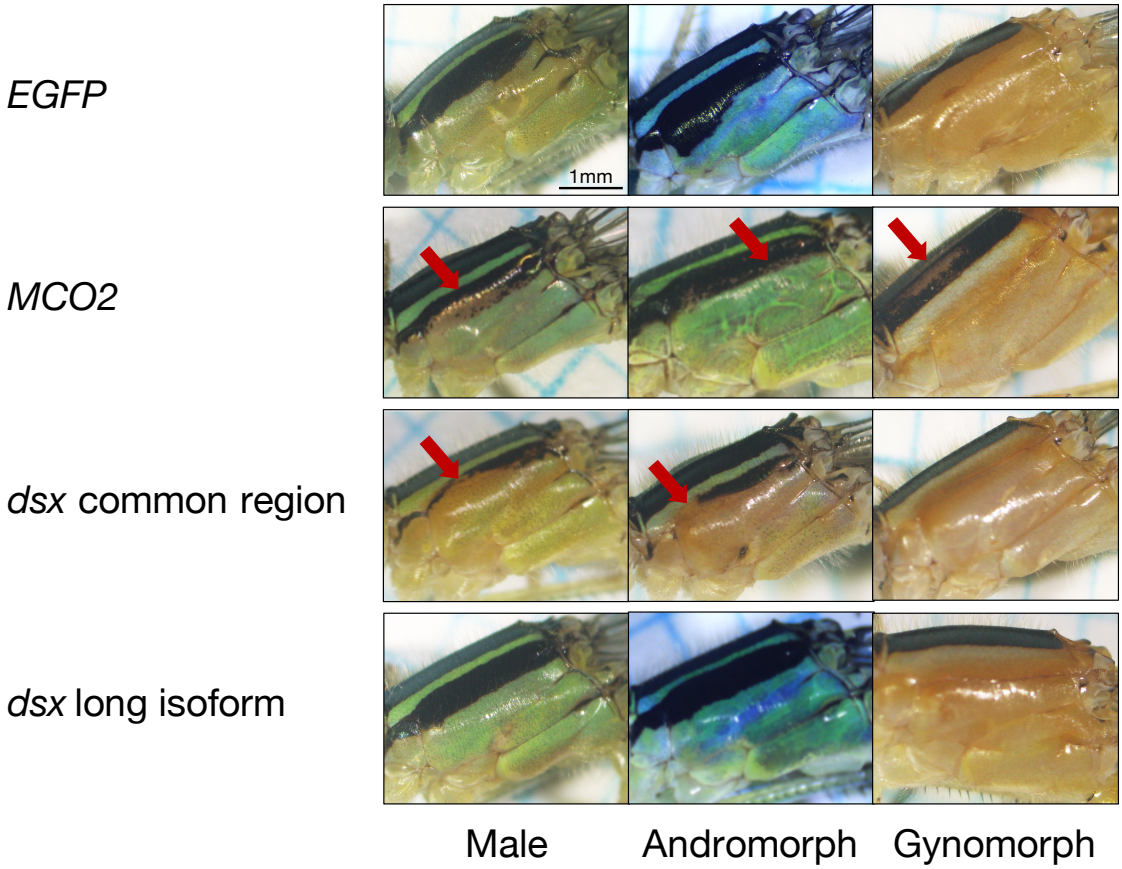
389 Figure 1.



390

391

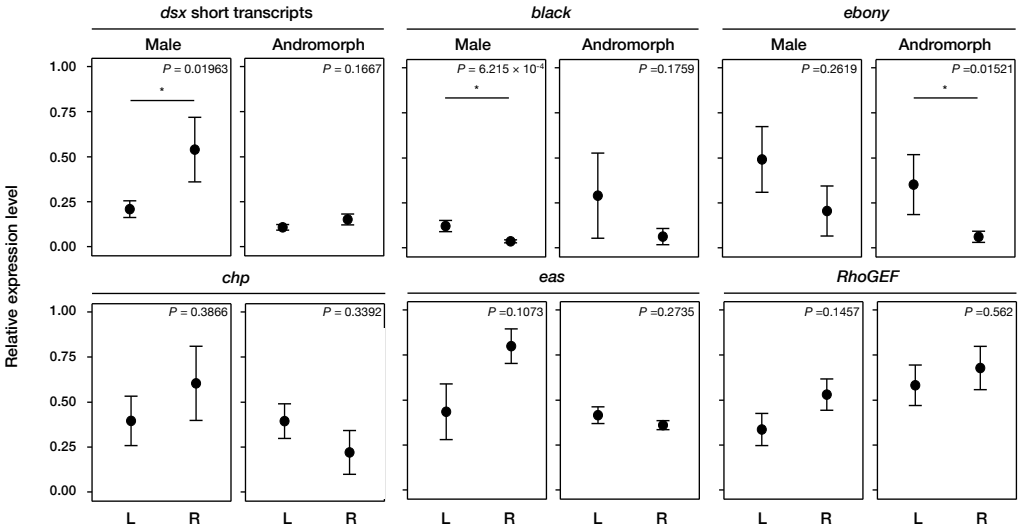
392 Figure 2.



393

394

395 Figure 3.



396

397

398 **Titles and legends to figures**

399

400 **Fig. 1** The positions of *dsx* small interfering RNAs (siRNAs)(**a**) and
401 electroporation-mediated RNA interference (RNAi) process (**b**). **a** Summary of
402 *dsx* isoform (modified from Takahashi et al., 2019). The red and black
403 arrowheads indicate siRNA regions for the common region and long isoform,
404 respectively. The blue boxes indicate open reading frames. Numbers above boxes
405 are region identifiers. Based on RNAseq and qRT-PCR analyses, region 2 was
406 sometimes skipped for both long and short isoforms. **b** After siRNA injection,
407 electroporation is performed with positive and negative electrodes placed on left
408 and right side of the thoraxes, respectively, with droplets of ultrasound gel.

409

410 **Fig. 2** RNAi knockdown phenotypes observed in the thorax of *I. senegalensis*.
411 Vertical axis is target genes of RNAi. No phenotypic effects in the body color of
412 *EGFP* RNAi individuals were observed. *MCO2* RNAi individuals of all the three
413 morphs mostly exhibited patchy unpigmented regions on the left side of the
414 thorax (arrows) where the positive electrode was placed for electroporation. The
415 thorax colors of males and andromorphs were changed into orange by *dsx*
416 common region RNAi (arrows). RNAi of *dsx* long isoform did not cause any
417 color changes in the thorax of all the three morphs.

418

419 **Fig. 3** qRT-PCR analyses of *dsx* and candidates for *dsx* target genes between *dsx*
420 (common region) RNAi region (L; left side of the thorax) and control region (R;
421 right side of the thorax) ($N = 4$ for each male and andromorph). (a) *dsx* (short
422 isoform), (b) *black*, (c) *ebony*, (d) *chaoptin* (*chp*), (e) *easily shocked* (*eas*) and (f)
423 *rho guanine nucleotide exchange factor* (*RhoGEF*). Data represent mean ± S.E.
424 Asterisks in the figure indicate significant differences ($P < 0.05$) evaluated using
425 the Generalized Linear Model (GLM) assuming a gamma distribution.

426 Table 1. Summary of RNAi results for *MCO2* gene

Morph	Emerged adults	Normal pigmentation	Partial changes of pigmentation
Males	4	1 (25%)	3 (75%)
Andromorphs	3	1 (33%)	2 (67%)
Gynomorphs	8	3 (37.5%)	5 (62.5%)

427

428 Table 2. Summary of RNAi results for *EGFP* gene (negative control)

Morph	Emerged adults	Normal pigmentation	Partial changes of pigmentation
Males	2	2 (100%)	None
Andromorphs	1	1 (100%)	None
Gynomorphs	7	7 (100%)	None

429

430 Table 3. Summary of RNAi results for *dsx* gene (common region)

Morph	Emerged adults	Normal pigmentation	Partial changes of pigmentation
Males	17	3 (18%)	14 (82%)
Andromorphs	9	2 (22%)	7 (78%)
Gynomorphs	15	15 (100%)	None

431

432

433 Table 4. Summary of RNAi results for *dsx* gene (long isoform only)

Morph	Emerged adults	Normal pigmentation	Partial changes of pigmentation
Males	9	9 (100%)	None
Andromorphs	4	4 (100%)	None
Gynomorphs	18	18 (100%)	None

434