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Title: Sex differences in 20-hydroxyecdysone hormone levels control sexual dimorphism in *Bicyclus anynana* butterfly wing patterns

Short title: 20E is a sex hormone in butterflies

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Summary

In contrast to the important role of hormones in the development of sexual dimorphic traits in vertebrates [1], the differentiation of these traits in insects is attributed exclusively to variation in cell-autonomous mechanisms controlled by members of the sex determination pathway [2], such as *doublesex* (*dsx*). Although hormones can shape the development of sexual traits in insects, and interact with *dsx* to create dimorphisms, variation in hormone levels are not known to cause dimorphism in these traits [3]. Here we show that butterflies use sex-specific differences in 20-hydroxyecdysone (20E) hormone titers to create sexually dimorphic wing ornaments, without the local involvement of *dsx*. Females of the dry season (DS) form of *Bicyclus anynana* display a larger sexual ornament on their wings than males, whereas in the wet season (WS) form both sexes have similarly sized ornaments [4]. High levels of circulating 20E during larval development in DS females and WS forms cause proliferation of the cells fated to give rise to this wing ornament, and results in sexual dimorphism in the DS forms. This study advances our understanding of how the environment regulates sex-specific patterns of plasticity of sexual ornaments and conclusively shows that sex-specific variation in hormone titers can play a role in the development of secondary sexual traits in insects, just like they do in vertebrates.

Keywords: sex hormone; insect; 20E; endocrinology; sexual traits; secondary sexual traits; sexual dimorphism; butterfly; *Bicyclus anynana*

Highlights

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- Sex-specific levels of 20E, an insect molting hormone, regulate secondary sexual trait dimorphism and plasticity in butterflies.
- 20E levels above a threshold promote local patterns of cell division in one sex, but not in the other sex, to create sexually dimorphic eyespots.

eTOC

Sexual selection drives the evolution of ornaments for individuals to display to the opposite sex. Yet, the mechanisms by which sexual selection operates are still not well understood. Here Bhardwaj *et al.* provide conclusive evidence, for the first time, that male and female insects use variation in levels of hormones to create dimorphism in their sexual ornaments. Authors show that 20-hydroxyecdyone, the insect molting hormone, also functions as a sex hormone in a butterfly. They also show how the environment shapes the development of sexual ornaments at a proximate level.

Introduction

Recent studies have shown that sexual traits are neither under constant, or even similar direction of selection over time and space [5-7]. This is because organisms do not live in stable biotic and abiotic environments. One consequence of predictable and recurrent environmental changes, such as seasons, is the evolution of plasticity in sexual traits [8, 9] Understanding the mechanisms behind the development of such plastic traits can help in developing better models of phenotypic evolution by focusing research on the actual genetic loci of evolution [10].

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Bicyclus anynana butterflies evolved in a seasonal environment in Africa, experiencing predictable and recurrent dry and wet seasons (DS and WS) [11]. As a consequence of this heterogeneity this species evolved a complex pattern of plasticity in its sexual behavior as well as in the size of its sexual ornaments, the bright, UV-reflective dorsal eyespot centers (**Fig. 1**) [4]. Essentially, DS individuals display sexual dimorphism in the size of the ornaments, with the courting DS females avidly displaying their unusually large sexual ornaments to the choosy cryptic males which have overall smaller eyespots (Fig. 1) [4]. In the WS, both sexes develop large eyespots characteristic of the season and males avidly court choosy females. This leads to a pattern of sexual dimorphism in the DS and plasticity in the sexual ornament that is male-limited (Fig. 1) [4].

While the ultimate selective factors behind the patterns of sexual dimorphism and plasticity in ornament size in *B. anynana* are becoming increasingly clear [4], the proximate factors behind these patterns are not understood. Here we set out to examine the developmental mechanisms that regulate sexual ornament size dimorphism in DS individuals and male-limited plasticity in this butterfly species.

Results

Because ornament size in males is controlled by rearing temperature [4], we began by identifying the developmental window that is critical for eyespot size regulation using temperature shift experiments. Low rearing temperature typical of the DS (17^{o}C) leads to DS butterflies, whereas high temperature typical of the WS (27^{o}C) leads to WS butterflies [12]. We experimentally manipulated rearing temperature for

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91 brief windows of 48h at different stages of development by moving animals from one
92 temperature to the alternate temperature, and then returning them back to the
93 original temperature (**Fig. 2**). WS animals reared at 27°C, which were moved to 17°C
94 during the wandering (Wr) stage of larval development showed the strongest
95 decrease in eyespot size (Fig 2A). The opposite pattern, an increase in eyespot size,
96 was seen in animals reared throughout at 17°C, and moved briefly to 27°C for a 48h
97 interval during the same Wr stage (Fig 2B). These experiments show that the Wr
98 stage is critical for the determination of dorsal eyespot center size in males.
99 Therefore, we focused our subsequent investigations of eyespot center size around
100 this developmental stage.
101
102 Previous studies on the developmental basis of sexual traits in insects have pointed
103 exclusively to variation in cell-autonomous mechanisms involving the activation of
104 members of the sex-determination pathway, such as the gene *doublesex* (*dsx*), in
105 the cells that develop the trait [3, 13-15]. Therefore, we asked whether *dsx* was
106 being expressed in the eyespot centers at the wandering stage of development. *In-*
107 *situ* hybridizations with a probe generated against a common region of *dsx*, (i.e.,
108 made to identify both male and female isoforms of this gene) identified *dsx*
109 expression in the developing androconial organs, a sex-pheromone producing organ
110 [16-18] in the wings of males (**Fig. 3A**). However, no *dsx* expression could be
111 detected in the developing eyespot centers of Wr larvae (Fig. 3A, Suppl. Fig. 3).
112
113 This led us to ask whether the sexual ornaments could be under the control of sex-
114 specific hormone titers. Previous studies have implicated insect hormones in the

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115 development and maintenance of sexual traits in insects [3], but to date no study to
116 our knowledge has ever shown sexual dimorphism in hormone titers leading to the
117 development of sexual traits in insects. Furthermore, previous research in this
118 species showed that levels of the molting hormone, 20-hydoxyecdysone (20E), were
119 involved in regulating ventral eyespot center size in females during the Wr stages of
120 development. We, therefore, asked whether levels of this hormone could be different
121 between males and females at the Wr stage.
122
123 We collected hemolymph from developing male and female larvae at finely spaced
124 intervals during the Wr stage, and observed a rise in 20E titers in all WS and DS
125 forms towards the end of this stage, just before the Wr larvae turned into prepupae.
126 Furthermore, male and female 20E titers were different within each seasonal form,
127 with females having higher titers than males ($F_{1,41}$=55.78, P<0.001) (**Fig 3B**). In
128 addition, WS titers were higher than DS titers, as previously reported for females [19,
129 20] ($F_{1,41}$=52.11, P<0.001), with no interaction between season and sex ($F_{1,41}$=0.001,
130 P=0.977).
131
132 Steroid hormones such as 20E exert effects on cells only if such cells express
133 correspondent hormone receptors [21]. We looked for the presence of the Ecdysone
134 Receptor (EcR) at two different stages during the Wr stage, an early stage (~40%
135 development) and a later stage (~90% development), flanking the period before and
136 after the rise in 20E tiers. At the early Wr stage, EcR was expressed in the dorsal
137 eyespot centers in a similar extent in each sex and seasonal form (**Fig 3C** i-iv: panel
138 1), confirming the ability of these cells to respond to the subsequent rising titers of

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20E, and the potential for this hormone to impact the developmental fate of these cells. At the later Wr stage, however, we observed a difference in the extent of EcR staining. DS males still expressed EcR in a small group of cells, whereas DS females and both WS sexes expressed EcR in a larger cluster of cells (Fig. 3C i-iv: panel 3). This suggests that the size control of the sexual ornament appears to be taking place in between these two time points, primarily via an increase in cell number.

20E levels above certain thresholds are known to promote cell division in larval wing imaginal discs [22, 23]. Therefore, to visualize whether such localized cell divisions were taking place in the region of the future sexual ornaments, we studied the localization of a mitotic marker, phospho-histone H3 (pH3) [24], using fluorescently labeled anti-pH3 antibodies in the wing discs. At 40% of the Wr stage, when the 20E titers are low, we observed no pH3 staining (green, Fig 3C i-iv: panel 2). However, at the later stage (90% Wr), when 20E titers are surging, cell division was taking place in all groups, except DS males (Fig. 3C i-iv: panel 4). We hypothesized that cell division is initiated only once a critical threshold of 20E is attained. The cells making up the sexual ornament of DS males, having the lowest 20E titers, may never reach this threshold, and hence do not experience 20E signaling at similar levels as the other groups, and do not divide.

To test this hypothesis, we manipulated 20E signaling in the four butterfly groups. We elevated 20E signaling in DS males by injecting them with 20E at approximately 60% of the Wr stage; and lowered 20E signaling in the other three groups by

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injecting individuals with a EcR antagonist, Cucurbitacin B (CucB) [25] (Fig. 4A). Injections of 20E caused an increase in eyespot center size in DS males, relative to injections with vehicle (Fig 4B i; DS M- $F_{1,37}$=18.38, P<0.01), while injections of CucB significantly reduced the eyespot center size in the other three groups relative to injections with vehicle (Fig 4B ii-iv; DS Fem: $F_{1,46}$=6.43, P=0.015, WS Mal: $F_{1,44}$=13.75, P=0.001, WS Fem: $F_{1,37}$=4.617, P=0.038), indicating a functional role of 20E signaling in dictating the size of these sexual ornaments.

Discussion

Here we have shown that sex differences in levels of a steroid hormone, during a brief period of development, controls a very localized pattern of division in cells that express the hormone receptor, which later develop into the bright UV-reflective scale cells that make up a sexual ornament in adult butterflies. Females produce more of this hormone than males, and WS forms more than DS forms. However, all groups, except DS males, produce sufficient hormone to trigger a process of local cell division at the center of the dorsal eyespots. This creates sexual dimorphism in ornament size in DS animals, and plasticity in ornament size in males.

Sexual dimorphism in some vertebrate traits, such as the length of digits in mice, is controlled by two hormones, androgen and estrogen steroids, present in different relative amounts in each sex during a small window of development [26]. Our study indicates that sexual dimorphism in insects can be achieved via the use of a single hormone, 20-hydroxyecdysone, present in each sex at different levels.

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187 It is likely that this butterfly species, which has evolved a complex mechanism for the
188 regulation of plasticity in the size of its ventral eyespots [11, 19], which function in
189 predator-prey interactions [27, 28], simply co-opted this mechanism to also regulate
190 the size of its dorsal eyespots. The selection pressures working on the dorsal
191 eyespots, however, are different from those on the ventral eyespots; so, the
192 mechanism of plasticity had to be tweaked to allow eyespots on different surfaces to
193 display different reaction norms for size in response to environmental temperature.
194 Part of the tweaking appears to have been the rise in 20E hormone titers in DS
195 females relative to DS males, allowing females to develop large dorsal eyespots in
196 the DS. Why DS females are able to maintain small ventral hindwing eyespots
197 requires further experiments. Additional work will also be necessary for a better
198 understanding of how and when the sexual dimorphism in the hormone titers actually
199 evolved.
200
201 An important advance of this work is the demonstration that different levels of a
202 steroid hormone in an insect control sexually dimorphic traits. Differentiation of male
203 and female traits in insects has, so far, been attributed exclusively to cell-
204 autonomous mechanisms involving the expression of sex-specific splice variants and
205 factors from the sex determination pathway, such as Feminizer (Fem), Transformer
206 (Tra), Fruitless (Fru), and Doublesex (Dsx), in cells that build the sexually dimorphic
207 trait [3, 29, 30]. For instance, previous work showed that the sexually dimorphic
208 mandibles of a stag beetle, *Cyclommatus metallifer*, were regulated by Juvenile
209 Hormone (JH) interacting with sex-specific isoforms of *doublesex* expressed in the
210 mandibles, but levels in JH titers across the sexes were found to be similar [31, 32].

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211 Previous reports also implicated hormones in the maintenance of sexual dimorphism
212 of adult insects [33], but no study conclusively reported sexual differences in levels
213 of insect hormones as developmental regulators of sexual traits [34]. Interestingly,
214 higher levels of 20E were found to be present in the hemolymph of females and
215 horness males *Ontophagus taurus* dung beetles, relative to horned males, during a
216 feeding stage of the last larval instar [35]. These sex-differences in titers, however,
217 were not tested for function. Here, we show conclusively, that sexual differences in
218 hormone titers can regulate dimorphism in sexual traits, without the need of cell-
219 autonomous factors also being expressed in the trait.
220
221 Sexual trait development in insects has, thus, been considered distinct from sexual
222 trait development in vertebrates, where sex-specific variation in steroid hormones,
223 such as testosterone and estrogen, are important regulators of sexual dimorphism
224 [26, 36]. Until recently, hormones were considered the exclusive means by which
225 vertebrates regulate their sexual traits [37], but the appearance of gynandromorphic
226 finches [38], displaying half male and half female plumage patterns, finally led
227 researchers to consider the presence of cell-autonomous mechanisms of sexual trait
228 development in vertebrates. The striking appearance of gynandromorphic insects
229 [39], in turn, led most biologists to assume insects used cell-autonomous processes
230 exclusively to differentiate sexual traits. Our work now conclusively shows that both
231 mechanisms are playing a role in vertebrates and insects and calls for additional
232 comparative work to understand how these two convergent mechanisms of sexual
233 trait development may have diversified and evolved.
234

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Materials and Methods

Butterfly husbandry. *B. anynana* butterflies, originally from Malawi, were reared in two climate rooms at 17°C and 27°C, at 70% relative humidity, 12:12 hours light: dark cycle, to produce the dry and wet season forms, respectively. Larvae were fed young corn, whereas adults were fed ripe mashed banana.

Eyespot and eyespot center size measurements. *B. anynana* adults from each season and sex were dissected and imaged using a Leica Stereo Microscope. Area measurements for dorsal forewings, individual posterior Cu1 eyespot, and white centers were calculated using ImageJ (NIH, v1.45s), as described previously [19].

Wandering stage sampling. Late 5th instar larvae were kept with ample food in transparent containers and imaged at 5 min intervals using the time-lapse feature of a RICOH Pentax WG-3 Camera, using method described previously [19]. Initiation of wandering stage happened when the larvae left the food and started wandering up. End of wandering stage happened when the animal begun hanging from the container, upside down.

***doublesex in-situ* hybridization.** A fragment of *doublesex* mRNA from *B. anynana* was amplified from the cDNA using the primers AM0016 (5'-GGTGTCCGTGGGCCCGTG-3'-forward) and AM0017 (5'-CCGGTCCAGCTCCAGGCG-3'-reverse) and cloned into the pGEMT-Easy vector (Promega). See **Supp. Fig. 1** for the position of the probe and primers. The insert was amplified using universal M13 primers and the amplicon was used as a

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template to synthesize DIG-labeled RNA probes. Wing discs were collected from the Wr stage larvae and used for RNA *in-situ* hybridization as described previously [40]. A Leica stereo microscope was used for imaging the stained tissues.

Semi Quantitative RT-PCR. To complement our findings from the *in-situ* hybridization, we performed semi-quantitative RT-PCR in two different sectors of the wings of Wr larvae. Late Wr stage larval wing discs were extracted and dissected into a proximal and a distal sector (**Fig. S2**). Proximal sectors contain the male androconial organ and hair pencils (only in hindwings), whereas distal sectors contain the sexually dimorphic eyespots. Wings were stored in TRIzol reagent (Life Technologies, Cat #15596-018) at -80^{o}C immediately after dissection. Extracted wing tissues were homogenized in TRIzol using a bullet blender, followed by a chloroform-isopropanol precipitation and ethanol wash. Subsequently, we treated extracted RNA with DNAse, and incubated at 37^{o}C for 15 min, followed by 3M NaoAC treatment and incubation at -80^{o}C for precipitation. Extracted RNA was followed through one round of phenol-chloroform RNA extraction. We then used 500ng of RNA from each tissue sample to do a reverse transcription by adding dNTPs, Reverse transcriptase and RNAse inhibitor at 42^{o}C for one hour to generate cDNA. A fragment of *doublesex* was amplified from this cDNA using the primers AM0462 (5'- AGTACCGCTTGTGGCCCTTC-3'-forward) and AM0463 (5'-GTCCGCGTGCGAAATACATC -3'- reverse). We used a housekeeping gene, EF-1α, as an internal control, which was amplified using primers AM0110 (5'-

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GTGGGCGTCAACAAAATGGA-3'-forward) and AM0111 (5'-GCAAAAACAACGAT-3'-reverse).

Male proximal forewing sectors, containing the androconial organ, expressed *doublesex*, whereas distal forewing sectors containing eyespots, completely lacked *doublesex* expression at this stage in development. Females, which lack the androconial organ, lacked *dsx* expression in both proximal and distal sectors. In addition, we observed similar expression patterns of *dsx* in hindwing anterior and posterior sectors. Anterior sectors, which contain androconial organs and hair pencils in males, show presence of *dsx,* which is absent in posterior sectors with eyespots. These results reinforce the idea that *doublesex* is not involved in regulating sexual dimorphism in eyespots.

Hemolymph collection. A small puncture was made to the first abdominal proleg of individual wanderers, and pre-pupae, and 20 µl of hemolymph were collected using a pipet. Hemolymph collections were taken from WS and DS male and female wonderers at five time points following the onset of wandering (20, 40, 60, 80 and 100%), and from pre-pupae (at 2 pm after the onset of pre-pupae). N = 4 per time point per seasonal form, but N≥12 for Wr 80 and Wr 100%. Sample preparation followed an established protocol [41].

Hormone extraction. We added 800 µl of HPLC grade water to the 200 µl sample of 20 µl of hemolymph + 45 µl methanol + 45 µl iso-octane and then vortexed the solution. We used a previously described protocol [19].

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Hormone titer measurements using UPLC/MS. 20 µL of sample was transferred into sample vial and 5 µL of 250 µg/mL deuterated-2,2,4,4-chenodeoxycholic acid (Catalogue #DLM-6780-PK, Cambridge Isotopes Laboratories, Andover, MA, USA) (additional internal control against loss of MS sensitivity upon repeated exposure) was spiked into the sample (to make a final concentration of 50 µg/mL d4-chenodeoxycholic acid as internal standard). A series concentration of 20-hydroxyecdysone commercial hormone (Sigma-Aldrich, Catalogue#H5142, Lot#060M1390V) (1, 2, 5, 8 and 10 μg/mL) were all spiked with a constant amount of d4-chenodeoxycholic acid (50μg/mL) and analyzed via LC-MS on an Agilent 1100 LC system coupled with an ABSciex 4000 QTrap mass spectrometer. Liquid chromatography was performed on an Eclipse XDB-C18, 5μm, 4.6 × 150 mm column (Agilent Technologies Corp, Santa Clara CA). HPLC conditions: injection volume 10µL; mobile phase A and B consisted of reverse osmotic water and methanol, both containing 0.1% of formic acid; flow rate 0.5 ml/min, 30% B for 0.1min, and linearly changed to 80% B in 0.2 min; then linearly switched to100% B in 1.2 min and maintained for 1.3 min, and then linearly changed to 30% B in 2.6 min and maintained for 7.4 min. Then, the flow rate and the mobile phase were returned to the original ratio. Mass spectrometry was recorded under the positive ESI mode. A blank injection of 100% MeOH was run after each sample injection to ensure no carry over. Response factor (F) of commercial hormone to the internal standard, d4-chenodeoxycholic acid was determined. The linear range of detection for each standard was determined via the LC-MRM parameters. The result of a standard titration at 1, 2, 5, 8 and 10 μg/mL were subjected to linear regression analysis, and

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330 the correlation coefficient (R^2). Lipids of hormone samples were measured using the
331 validated LC-MRM parameters. Approximate concentration of butterfly hormone was
332 calculated using the peak area under the curve. Intensity of individual hormone
333 species was quantified by normalizing against the respective calibration curve of
334 standards and labeled steroid.
335
336 **Ecdysone Receptor and Phospho-histone H3 (pH3) immunostainings.** Wing
337 discs were dissected from wanderers at different stages. Monoclonal (mouse)
338 antibodies raised against a *Manduca sexta* EcR peptide shared across all EcR
339 isoforms (Developmental Studies Hybridoma Bank, #10F1)[42] were used at a
340 concentration of 1:5. Goat anti-mouse (Molecular Probes, #A-11001) was used as
341 secondary antibody at a concentration of 1:800. Polyclonal antibodies raised against
342 rabbit mitosis marker anti-Phospho-histone H3 (Ser 10) was used at a concentration
343 1:150 (Merck Milipore, #06-570). Goat anti-rabbit (Molecular probes, #A-11034) was
344 used as a secondary antibody at the concentration of 1:800. Wings were dissected,
345 fixed in PFA, dehydrated in MeOH at -20^{o}C, rehydrated using a gradient of MeOH
346 and water, and then treated with primary and secondary antibodies. All wings were
347 double immunostained with pH3 and EcR, and mounted with ProLong Gold
348 (Invitrogen, Carlsbad, CA, USA). Images were captured on a LSM 510 META
349 confocal microscope (Carl Zeiss, Jena, Germany). Serial Z-optic sections were done
350 in order to distinguish dorsal from ventral EcR expression. At least three biological
351 replicates were obtained for each immunostaining.
352

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353 **Hormone injections.** Male DS wanderers (60% Wr) were injected with 4 µl of 2000
354 pg/µl of 20E (8000 pg total) (Sigma-Aldrich, Catalogue#H5142, Lot#060M1390V) or
355 4 µl of vehicle (1 ethanol: 9 saline solution). Female DS and male WS wanderers
356 (60% Wr) were injected with 3 µl of 5600 pg/ul of cucurbitacin B (16,800 pg total)
357 (Sigma- Aldrich, Catalogue#C8499, Lot#035M47104V) or 3 µl of vehicle (1 ethanol:
358 9 saline solution). Female WS wanderers (60% Wr) were injected with 4 µl of 5600
359 pg/µl of cucurbitacin B (22,400 pg total) or 4 µl of vehicle (1 ethanol: 9 saline
360 solution). All solutions were stored at -20°C. The injections were done using a
361 Hamilton syringe (10 µl 700 series hand fitted microliter syringe with a 33 gauge, 0.5-
362 inch needle). The injection site was on the dorsal surface in between the integument
363 of the second and third thoracic leg after the larvae had been chilled for 30 min on
364 ice.
365
366 **Statistical analyses.** Eyespot center size was compared across seasonal forms or
367 treatments using analyses of covariance (ANCOVA), where wing area was used as a
368 covariate. Fixed factors appearing in the model were evaluated at a wing area of
369 175.265mm^2 for WS and 193.021mm^2 for DS wings. Hemolymph titers were
370 compared using 2-way ANOVAs with seasonal form and sex as fixed factors. All
371 analyses used the GLM procedure in SPSS Statistics (version 19). Data was log-
372 transformed to meet homogeneity of variance criteria (as determined by a Levene's
373 test). Pair-wise comparisons, using a Bonferroni correction for multiple comparisons,
374 were used to detect which developmental time switch points produced significant
375 differences in eyespot traits in the temperature-shift analyses. Graphs were made in

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376 Microsoft Excel (version 14.6.5 for the Mac) and Adobe Illustrator CC2015 using
377 reverse transformed data (when applicable).
378
379 **Author contributions:** Conceived and designed the experiments: SB, KLP and AM.
380 Performed the experiments: SB, KLP, AB, MDG, BRW, XT, WFC. Analyzed the data:
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Figure Legends

Fig. 1. Sexual dimorphism and phenotypic plasticity in the size of dorsal eyespot centers in *Bicyclus anynana*. A) Summary of the behavioral ecology and sexual ornament size of DS individuals and **B)** WS individuals. **C)** The eyespot centers (highlighted in yellow) are **D)** sexually dimorphic in size in DS individuals ($F_{1,37}$ = 18.215, P<0.001) and plastic in males across seasons ($F_{1,37}$ = 60.712, P<0.001) (blue symbols/outlines = males; pink = females). Sizes along the Y-axis apply to wings with an area of 208.805 mm^2. N=20 for each data point. Error bars represent 95% CI of means.

Fig. 2. Temperature-shift experiments point to wandering (Wr) stage as the most important temperature-sensitive developmental stage for eyespot center size determination. Horizontal axis labels refer to the stage of development at the start of the 48 hr shift; NS- Non-shifted Controls. L5 1-3 represent stages in larval 5th instar, Wr- Wandering stage, PP- Prepupal stage, P1-3 represent stages in pupal development. A) Animals were reared at 27°C throughout development, except for a 48hr window, where they were moved to a lower temperature of 17°C. B) Animals were reared at 17°C throughout development, except for a 48hr window, where they were moved to a higher temperature of 27°C. N=20 for each data point. Error bars represent 95% CI of means. Asterisks represent level of significance in the difference of center size observed between shifted groups and non-shifted controls (*, p<0.05; **, p<0.01; ***, p<0.001).

Fig. 3. Sex-specific differences in 20-hydroxyecdysone titers, but not *doublesex* isoforms, are associated with cell division and larger EcR expression domains in late Wr stage eyespot centres. A) (i) *dsx* mRNA is present in the pheromone producing organ of males (yellow box) but is absent from the eyespot centres (arrows).N=4 for *in-situ* stainings.**(ii)** Male forewing with male pheromone producing organ **(iii)**. **B**) 20E titers observed during fine intervals of wandering (Wr) and pre-pupal (PP) stages. Error bars represent 95% CI of means. **C)** Larval wings immunostained with EcR (Red) and pH3 (Green) antibodies at two stages of Wr stage – 40% and 90% development, zoomed in to show the developing dorsal Cu1 eyespot centres (Fig. 1C). Scale bars, 20µm.

Fig. 4. 20E signaling promotes an increase in eyespot center size. A) 20E titers in developing larvae at end of Wr stage. Dashed line represents hypothetical threshold of 20E titers required for cell division. Arrowheads next to data points represent planned manipulations to 20E signaling. **B)** 20E injections cause an increase in eyespot size in DS males (**i**), whereas reduced EcR signaling using CucB causes a decrease in eyespot size in all other groups (**ii-iv**). Error bars represent 95% CI of means. **C)** Diagram summarizing the interpretation of our results: Rearing temperature induces variation in 20E titers at the Wr stage of development. High

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560 titers result in cell division and larger eyespot centers, whereas low titers result in
561 smaller centers, as seen in DS males (blue outlines = males; pink = females). DS
562 females, despite being reared at low temperature, have sufficiently high 20E levels to
563 also undergo cell division of the wing ornament.
564

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565 **Fig. 1.** Sexual dimorphism and phenotypic plasticity in the size of dorsal eyespot
566 centers in *Bicyclus anynana*.
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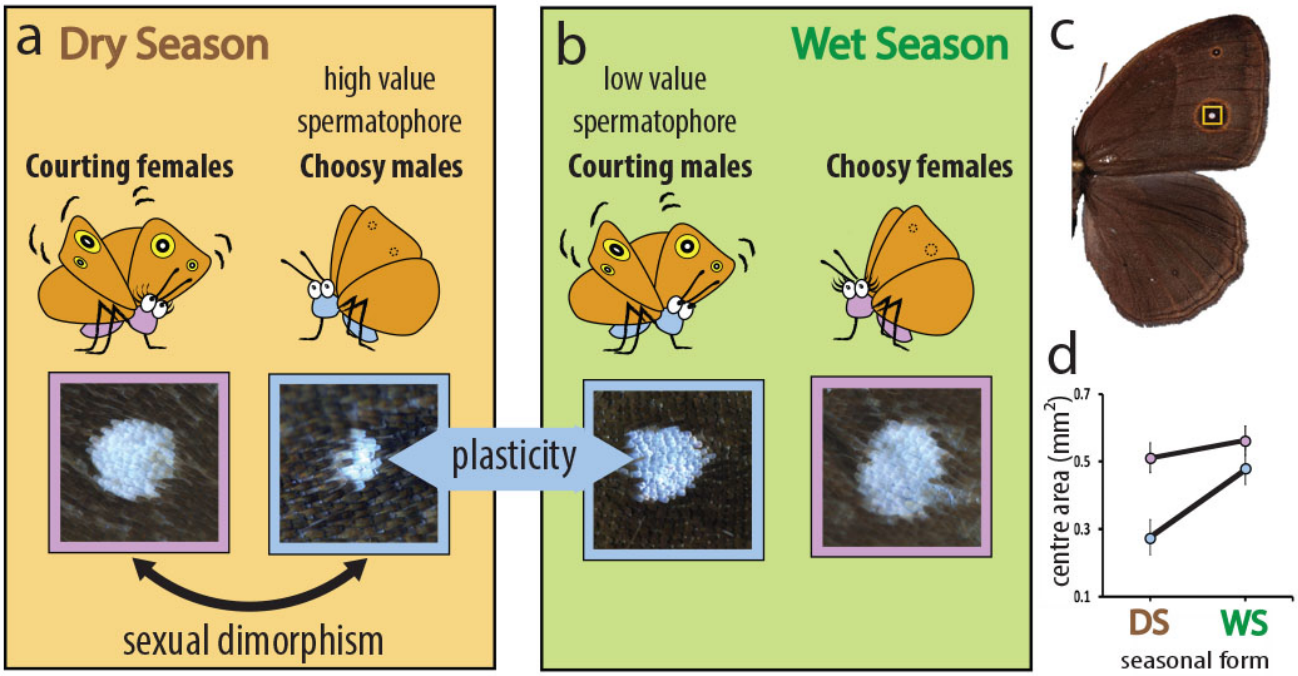
568



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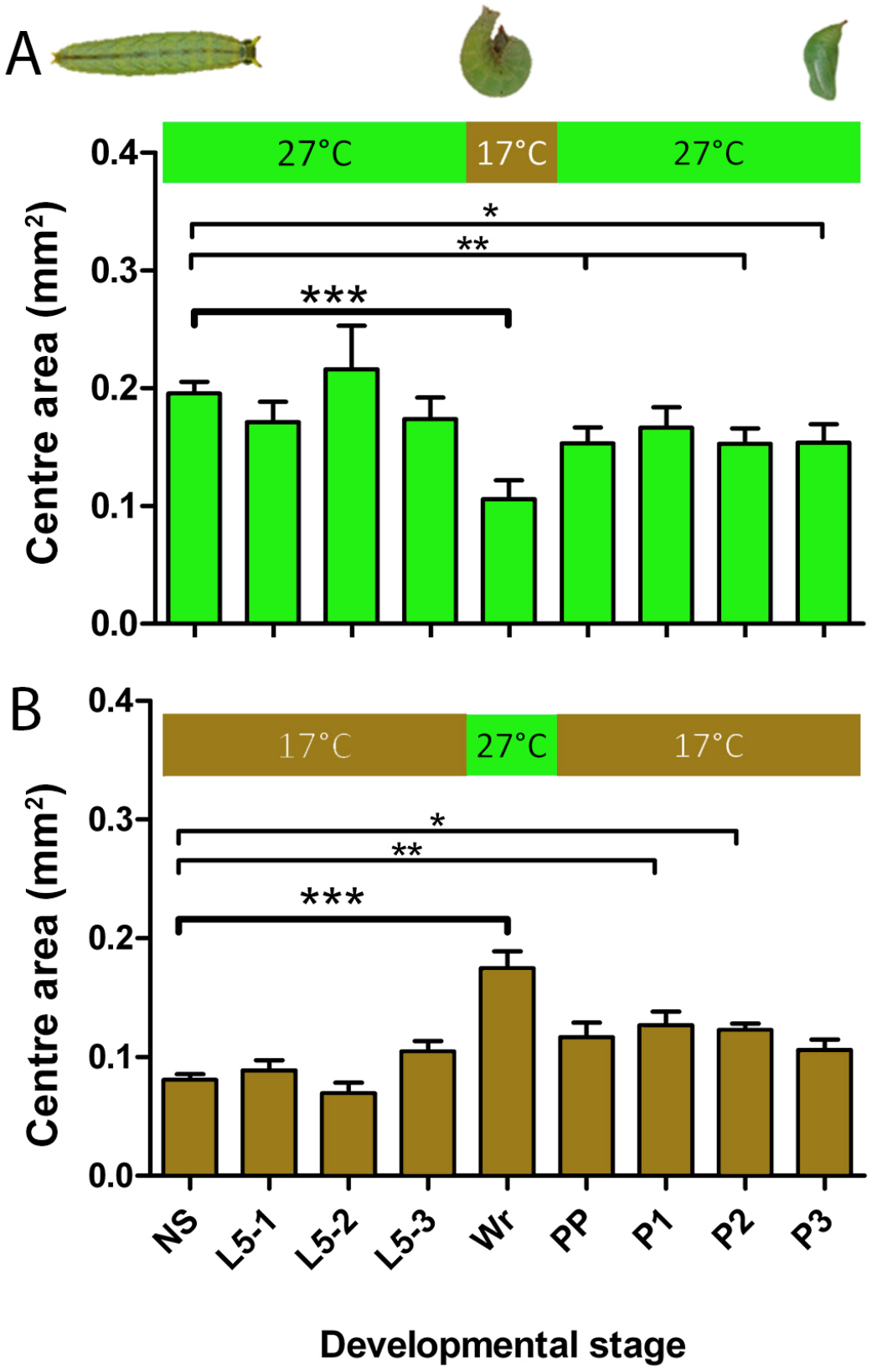
572 **Fig. 2.** Temperature-shift experiments point to wandering (Wr) stage as the most
573 important temperature-sensitive developmental stage for eyespot center size
574 determination
575



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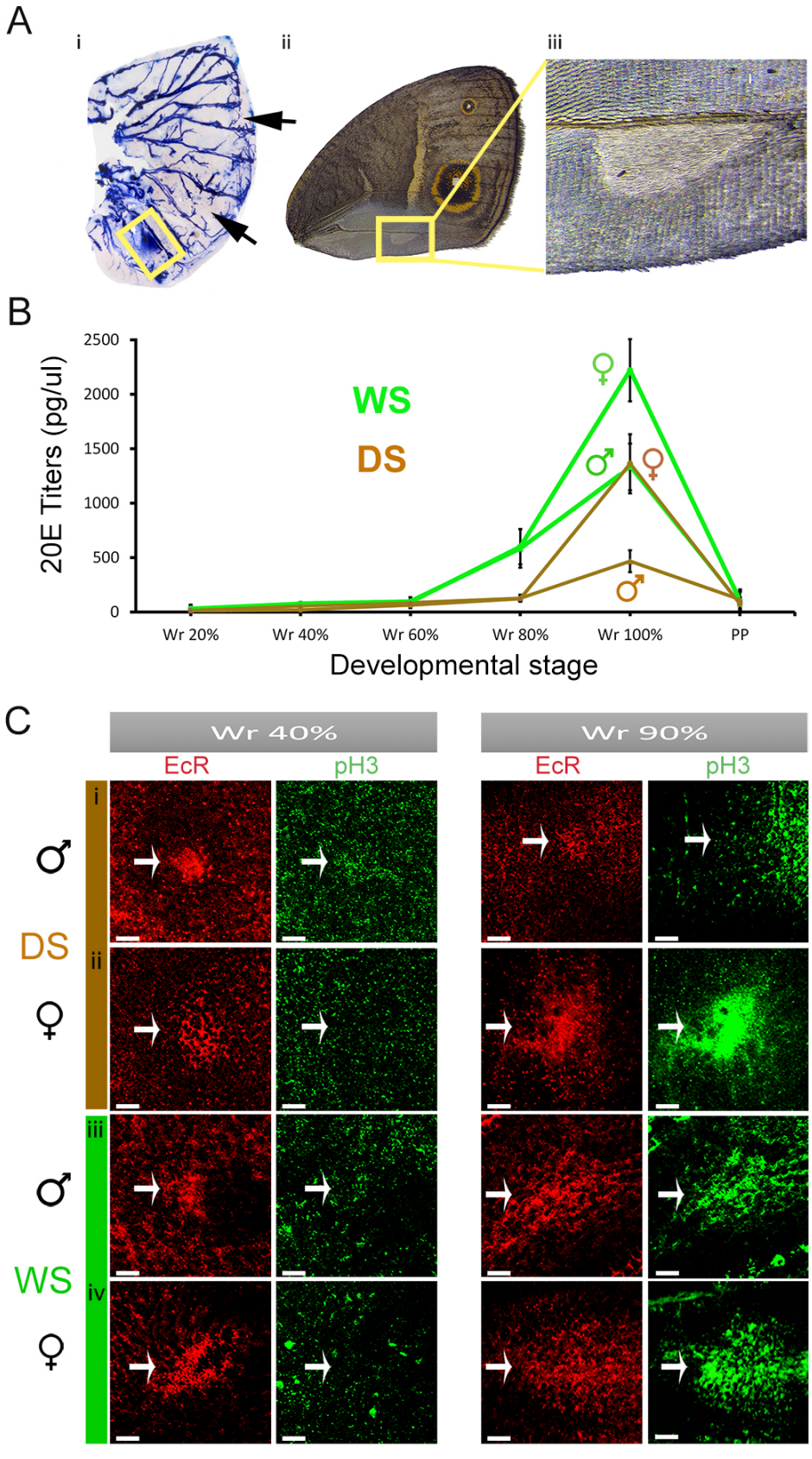
578 **Fig. 3.** Sexually dimorphic 20-hydroxyecdysone titers, but not *doublesex* isoforms,
579 are associated with cell division and larger EcR expression domains in late Wr stage
580 eyespot centres.
581



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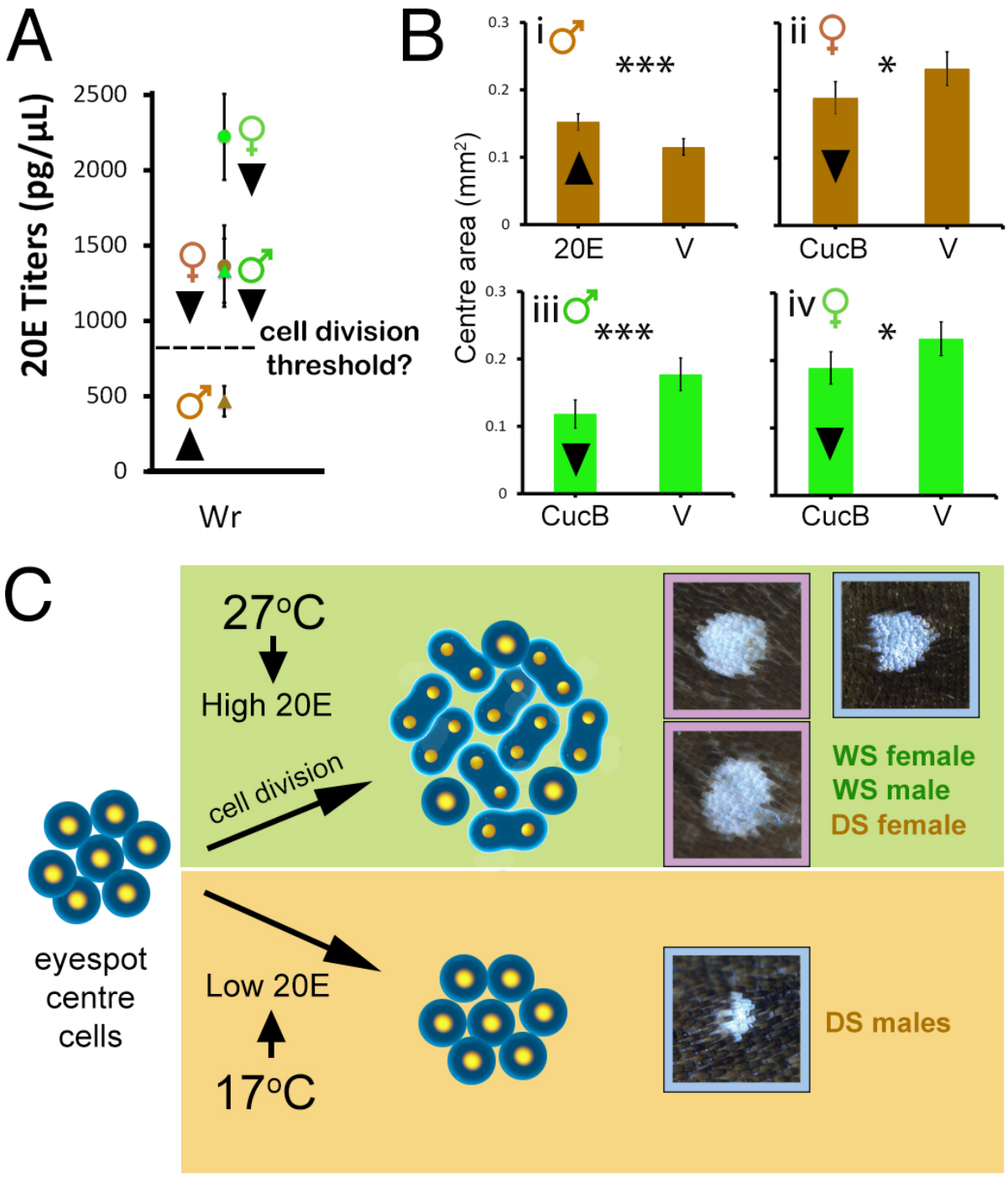
583 **Fig. 4.** 20E signaling promotes an increase in eyespot center size.
584
585



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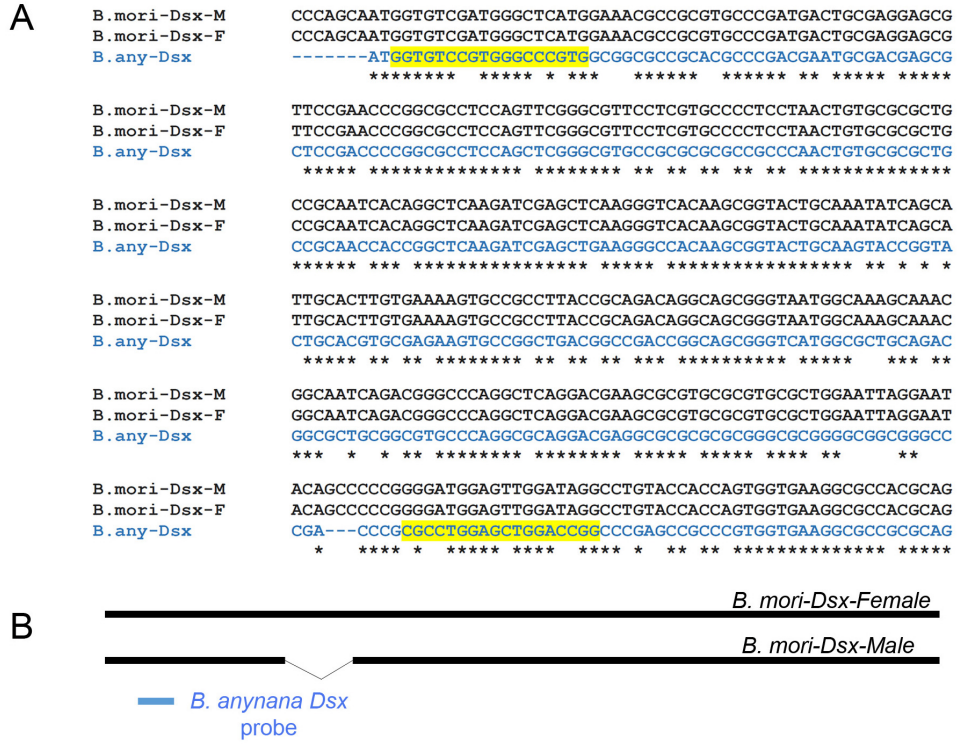
589 **Supplementary information**
590



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592 **Supplemental Fig S1. Partial *dsx* sequence alignment in *B. anynana* and *in situ***
593 **probe location**. (A) Partial alignment of the male and the female isoforms of *B.*
594 *anynana dsx* coding sequence. The forward and the reverse primers used to amplify
595 a region of the *dsx* sequence for RNA *in-situ* hybridization are highlighted in yellow.
596 Note that the amplified fragment is common to both the female and the male
597 isoforms. (B) Schematics showing the position of *B. anynana dsx* probe relative to
598 the male and female isoforms of *dsx* from *B. mori*.
599

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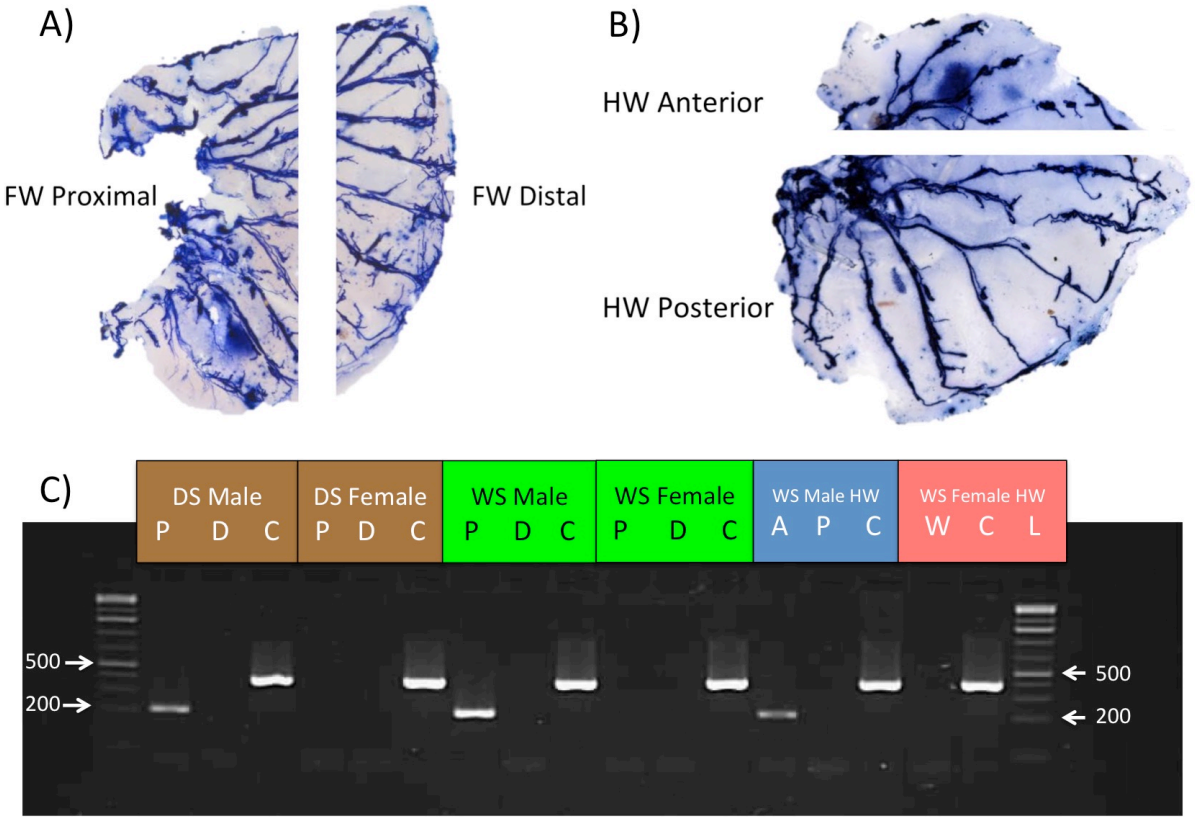

Supplemental Fig S2 (To fig. S3A). *Dsx* is not expressed in the developing eyespot centers in *B. anynana*, but is present in male androconial organs. (A) Proximal and distal forewing (FW) sectors in B. anynana (B) Anterior and posterior hindwing (HW) sectors. FW proximal and HW anterior sectors in males have androconial organs, which are absent in females. (C) Proximal sectors in FW in DS males and WS males express dsx. Similar expression in observed in WS male HW anterior sectors, which also contain androconial organs. dsx is absent in wing regions with eyespots in both males and females. EF-1α is present as a control in all treatments. (P- Proximal, D- Distal, C- Control, A-Anterior, P-Posterior, W-entire wing, L-1Kb plus ladder). We used three biological pools (Number of wings in each pool = 5) of males and females of each seasonal form for these experiments and the results were identical across all biological replicates.