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Loss of Photoperiodic Control of Juvenile-Hormone Signaling Pathway Underlying the Evolution of Obligate Parthenogenesis in the Pea Aphid

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Phenotypic plasticity, the ability of organisms to change their phenotype depending on external stimuli, enables their survival in fluctuating environments. An extreme example is polyphenism, in which a single genotype produces discrete phenotypes in response to external cues. However, under persistent environmental conditions, natural selection would favor reduced plasticity. This study focused on the loss of reproductive polyphenism and revealed the underlying mechanism in the pea aphid *Acyrthosiphon pisum*. Although most populations exhibit reproductive polyphenism, known as cyclical parthenogenesis, with a seasonal shift between parthenogenesis and sexual reproduction, some exhibit obligate parthenogenesis. To investigate the potential role of changes in the environmental sensitivity of the juvenile hormone (JH) pathway during this evolutionary shift, we analyzed the expression of genes involved in JH synthesis and degradation. We found that five of seven JH-related genes exhibited photoperiodic responses in one cyclical-parthenogenetic strain, whereas none of them responded to photoperiod in the two obligate-parthenogenetic strains. Notably, *CYP15A* and *JHEH* genes, which are involved in the final step of JH synthesis and in the initiation of JH degradation, respectively, showed strong photoperiodic responses in the cyclical-parthenogenetic strain but showed no responses in the obligate-parthenogenetic strains. Acetone treatment induces male production in obligate-parthenogenetic strains, suggesting that the developmental pathway for male production remains functional in these strains. These results suggest that the loss of the photoperiodic response in both JH synthesis and degradation pathways is a key mechanism underlying the elimination of the sexual phase, resulting in the loss of reproductive polyphenism in aphids.

Key words: phenotypic plasticity, cyclical parthenogenesis, obligate parthenogenesis, loss of polyphenism, pea aphid

INTRODUCTION

In phenotypic plasticity, organisms can modify their phenotype by altering their developmental processes to adapt to their surrounding environment (West-Eberhard, 2003). An extreme example is polyphenism, such as seasonal color morphs in butterflies, defensive morphs in Daphnia, and caste differentiation in social insects, in which organisms produce multiple discrete phenotypes, even from a single genotype, in response to different environmental conditions. The ability to change phenotypes in response to external stimuli enables organisms to attain multiple optimal states in fluctuating environments. However, under persistent envi-

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ronmental conditions, natural selection favors genetic changes that produce a constant phenotype. Theoretical and empirical studies have suggested that both the gain and loss of phenotypic plasticity play substantial roles in phenotypic diversification and speciation (Pigliucci, 2001; West-Eberhard, 2003; Aubret and Shine, 2009; Lande, 2009; Pfennig et al., 2010; Schwander and Leimar, 2011). However, few examples have been reported regarding the proximate mechanisms underlying a reduction in plasticity, especially in natural populations.

Notably, aphids (Insecta, Hemiptera) exhibit life-cycle polyphenism, including seasonal reproductive polyphenism and wing polyphenism, although phenotypic responses vary even within a single species (Moran, 1992; Dixon, 1997; Simon et al., 2002; Ogawa and Miura, 2014; Shuo et al., 2020). Most aphid species exhibit reproductive polyphenism, also known as cyclical parthenogenesis, which involves switching between parthenogenesis and sexual reproduction depending on the season (Dixon, 1997). During the spring and summer, aphids reproduce via viviparous parthenogenesis. In the fall, parthenogenetic females produce sexual morphs, males and oviparous females, that mate and produce overwintering eggs. In most aphids, short-day photoperiods and low temperatures are the major environmental cues that trigger the production of sexual morphs (Moran, 1992; Simon et al., 2002). Because all aphid species exhibit cyclical parthenogenesis, this strategy appears to have been acquired in the early stages of aphid evolution. However, approximately 30–40% of aphid species possess populations that reproduce exclusively by obligate parthenogenesis (Fig. 1A) (Moran, 1992; Dixon, 1997; Simon et al., 2002). These populations are thought to be insensitive to photoperiodic changes, which results in the loss of the sexual phase (Lees, 1959, 1963; Simon et al., 2010).

In many insects, physiological mechanisms, particularly those of the endocrine system, control phenotypic changes in response to environmental cues (Hartfelder and Emlen, 2012). Especially, juvenile hormone (JH) plays a key role in regulating development, metamorphosis, reproduction, diapause, and polyphenism in insects (Nijhout, 1994, 1999, 2003; Zera and Denno, 1997; Tanaka, 2001; Miura, 2005; Hartfelder and Emlen, 2012). In aphid cyclical parthenogenesis, JH has been suggested to trigger the production of sexual morphs in response to short photoperiods (Hales and Mittler, 1983; Corbitt and Hardie, 1985; Ishikawa et al., 2012). Therefore, we hypothesized that modifications in the photoperiodic response of the JH signaling pathway might be a key mechanism underlying the loss of the sexual phase. The functional JH of aphids is considered to be juvenile hormone III (JHIII) (Hardie et al., 1985; Westerlund and Hoffmann,

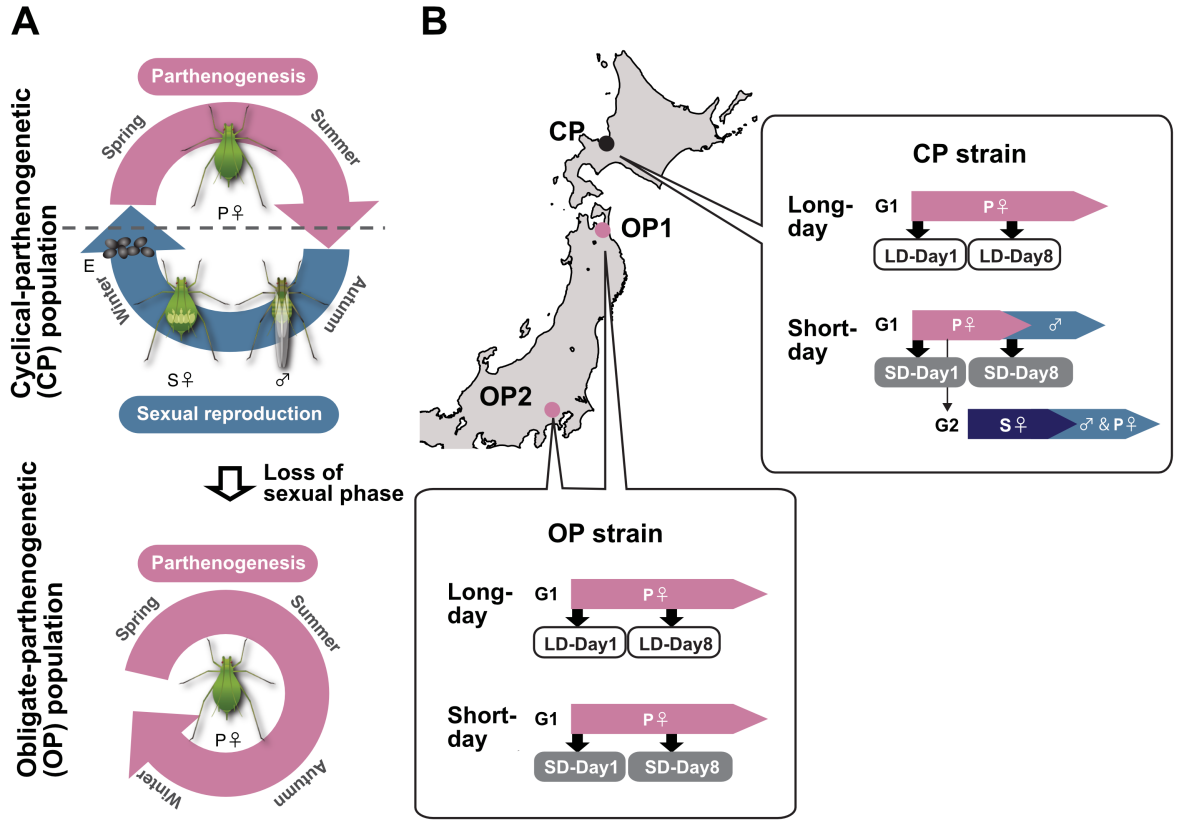

Fig. 1. The cyclical-parthenogenetic (CP) and the obligate-parthenogenetic (OP) populations of the pea aphid. P and S females indicate parthenogenetic and sexual females, respectively. **(A)** Annual life cycles of the CP and OP populations. The CP populations change their reproductive mode from parthenogenesis to sexual reproduction depending on the seasonal changes (upper). On the other hand, the OP populations have lost the sexual phase in their life cycle (lower). **(B)** The sampling points and reproductive schedules of the CP, OP1, and OP2 strains in Japan. The experimental categories are shown with the schedules. G1 and G2 indicate the first generation and second generation, respectively, after we started rearing the aphids under long-day or short-day conditions (see Fig. 3).

2004; Chen et al., 2007; Schwartzberg et al., 2008; Ishikawa et al., 2012, 2013). However, recent studies have revealed that many hemipteran species use JHIII skipped bisepoxide ($JHSB_3$) as a functional JH (Kotaki et al., 2009, 2012; Ando et al., 2020; Matsumoto et al., 2020; Villalobos-Sambucaro et al., 2020; Kodama et al., 2023). In aphids (Aphididae), both JHIII and $JHSB_3$ were detected in the hemolymph of *Myzus persicae* and *Rhopalosiphum maidis* (Yi et al., 2023), whereas only $JHSB_3$ was detected in *Lipaphis erysimi* (Yi et al., 2023). Thus, the types of chemical compounds that function as JH in aphids remain unclear. However, the synthesis pathways of JH III and $JHSB_3$ and the genes encoding the enzymes that catalyze these reactions are largely conserved (Tsang et al., 2020; Mano and Goto, 2022). Therefore, we investigated the photoperiodic responses of the JH pathway by examining the expression profiles of the genes involved in JH synthesis and degradation.

Here, we compared the photoperiodic responses of genes involved in JH synthesis and degradation between cyclical-parthenogenetic (CP) and obligate-parthenogenetic (OP) strains of the pea aphid, *Acyrthosiphon pisum* (Fig. 1A). Intraspecific variations in reproductive polyphenism have been reported in pea aphids (Kanbe and Akimoto, 2009). The OP strains are distributed in central Japan, whereas the CP strains are distributed in northern Japan. Our previous research revealed that in the CP strain, aphids reared under short-day conditions showed enhanced expression of JH-degradation genes, such as *JH esterase* (*JHE*) and *JH epoxide hydrolase* (*JHEH*). This upregulation of the JH degradation pathway likely triggers the induction of sexual morphs (Ishikawa et al., 2012). In addition to the genes related to JH degradation, we analyzed the genes involved in JH biosynthetic pathways, such as *JH acid methyltransferase* (*JHAMT*), *JH epoxidase* (*CYP15A1*), and *short-chain dehydrogenase* (*SDR*) (see Fig. 2) (Shinoda and Itoyama, 2003; Mayoral et al., 2009; Daimon and Shinoda, 2013). Furthermore, we conducted an artificial male induction in obligate-parthenogenetic strains to test whether they retained the developmental cascade for producing sexual individuals that occurs downstream of the JH signaling pathway.

MATERIALS AND METHODS

Insects

To compare differences in photoperiodic responses between cyclical-parthenogenetic and obligate-parthenogenetic populations, one CP strain (ApL) and two OP strains, Hac06VsXI30 and Sait06Vs4 (hereafter called OP1 and OP2, respectively), of the pea aphid, *Acyrthosiphon pisum*, were used in this study. The CP strain was established from a cyclical-parthenogenetic population collected from Sapporo, Hokkaido, Japan (Ishikawa et al., 2012). The OP1 and OP2 strains were established from an obligate-parthenogenetic population collected from Hachinohe City, Aomori, Japan, in November 2006 and an obligate-parthenogenetic population collected from Saitama City, Saitama, Japan, in May 2006 (Fig. 1B). Based on microsatellite analysis, the OP1 and OP2 strains had MLG120 and MLG122 genotypes, respectively, which are common and widely distributed over the years in obligate-parthenogenetic populations (Kanbe and Akimoto, 2009). Thus, both strains were predicted not to produce males or sexual females under short-day conditions. Microsatellite analysis was performed as previously described (Kanbe and Akimoto, 2009).

Stock cultures of parthenogenetic aphids were maintained in tubes (diameter: 2.5 cm, height: 10 cm) in which a bean seedling (*Vicia faba*) was placed on wet vermiculite at 20°C under long-day conditions (16L8D) (Wilkinson and Ishikawa, 2000). Only wingless parthenogenetic females were used for all experiments because winged parthenogenetic females did not produce males, even under short-day conditions (8L16D, 15°C) (Ishikawa et al., 2012). Since the effect of rearing conditions lasts over two or three generations (Mackay and Wellington, 1977), aphids were kept on a 3-cm vetch seedling over three generations under long-day conditions (16L8D, 15°C) as the pre-induction to prevent to production of winged parthenogenetic females (Ishikawa et al., 2008; Ogawa and Miura, 2014).

Reproductive schedule of obligate-parthenogenetic strains under short-day conditions

To confirm the obligate parthenogenesis of the OP1 and OP2 strains, the aphids were exposed to short-day conditions (8L16D) and low temperature (15°C), in the same manner as that used to induce the production of males and sexual females in the CP strain (Ishikawa et al., 2012). Briefly, first-instar nymphs produced by single wingless mothers of the OP1 and OP2 strains were isolated and reared on seedlings under short-day conditions. When the aphids reached the adult stage and began reproducing, they were transferred daily to a new seedling in another container. The number and morphs (parthenogenetic females, males, and sexual females) of the progeny produced each day were recorded. Because the morphs could not be identified in newborn nymphs, progenies were reared until they reached fifth-instar adults for accurate identification. In the adult stage, parthenogenetic females and oviparous sexual females were distinguished by the appearance of ovaries; parthenogenetic females have parthenogenetic ovaries with many embryos, whereas sexual females possess gametic ovaries containing only haploid eggs (Blackman, 1987). Males were discriminated based on their wings, small abdomens, and male gonads. To confirm asexuality over generations, we maintained the strains under short-day conditions for several generations.

Identification of orthologs for genes involved in JH synthesis and degradation

Although the ortholog searches were conducted in our previous studies (Ishikawa et al., 2012), the genome assembly of *A. pisum* in AphidBase was updated to version 2.1. Therefore, Basic Local Alignment Search Tool (BLAST) search was conducted for *Acyrthosiphon pisum* annotation ncbi 2.1 (https://bipaa.genouest.org/is/aphidbase/) to identify the pea aphid orthologs of *SDR*, *JHAMT*, *CYP15A1*, *JHEH*, and *JHE* (Fig. 2, Table 1) (Li et al., 2019). For *SDR*, we used the protein sequence of *Aedes aegypti*, AAEL017302, as the query sequence (Mayoral et al., 2009). For JHAMT, we used the protein sequence of *Bombyx mori* (AB113578) as the query. For the *CYP15A1* gene, Daimon and Shinoda (2013) identified one ortholog, XP_001952620; therefore, we used it as a query. For the *JHEH* gene, we used the protein sequence of *Manduca sexta*, AAC47018, as a query (Wojtasek and Prestwich, 1996). For *JHE*, we use the protein sequence of *Nilaparvata lugens*, EU380769, as the query (Kamita and Hammock, 2010). Genes with more than 40% similarity were used as putative orthologs. To confirm whether the ortholog encodes a functional enzyme, we surveyed the critical residues known to characterize these enzymes in each ortholog.

Experimental designs for gene expression analysis

To test the hypothesis that the modification of the photoperiodic response of genes related to JH synthesis and degradation underlies the loss of reproductive polyphenism, we analyzed the expression of genes involved in JH synthesis and degradation in response to photoperiodic changes in the CP, OP1, and OP2

strains. As reported previously, CP strains produced sexual individuals (males and sexual females) under short-day conditions (Figs. 1B, 3A) (Ishikawa et al., 2012). After the first-instar nymphs were isolated at 15°C under short-day conditions (8L16D), the parthenogenetic females (generation 1, G1) produced only parthenogenetic progeny until 8 days after the first larviposition, and then started to produce male progeny in the late period from 10 days after the first larviposition. The parthenogenetic females (generation 2: G2) produced on the second day after the first larviposition by G1 females preferentially produced sexual females in the early period and then started to produce both males and parthenogenetic females in the late period. Under long-day conditions (16L8D), all parthenogenetic females continuously produced only parthenogenetic females (but no sexual individuals). Based on the reproductive schedule of the CP strain, gene expression was examined on the first (day 1) and eighth (day 8) day after the first larviposition under long- and short-day conditions, resulting in the following four categories: LD-Day 1, LD-Day 8, SD-Day 1, and SD-Day 8. In the obligate-parthenogenetic OP1 and OP2 strains, all parthenogenetic females on LD-Day 1, LD-Day 8, SD-Day 1, and SD-Day 8 produced future parthenogenetic female embryos (Figs. 1B; 3B, C).

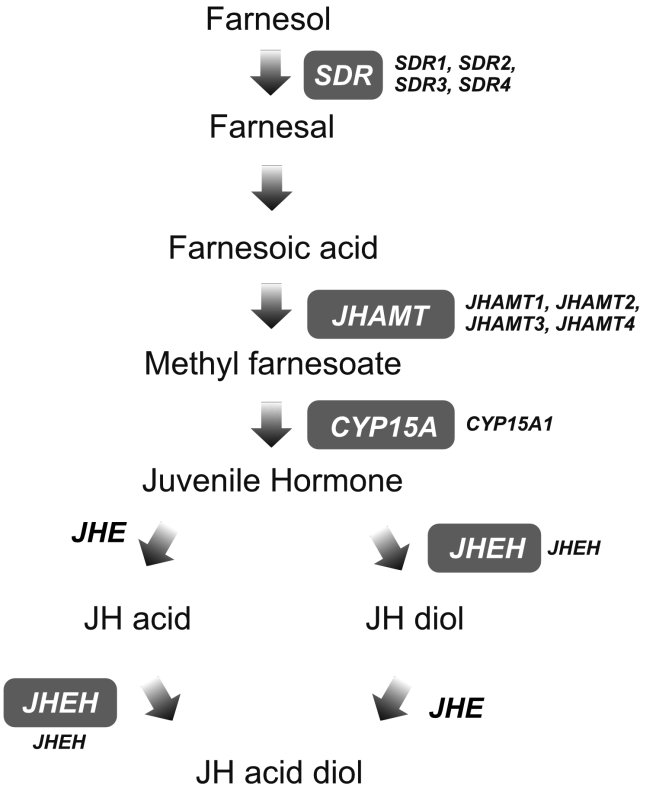

Fig. 2. Abbreviated pathways of JH biosynthesis and degradation. Genes denoted by grey boxes are analyzed in this study.

RNA extraction and quantification of gene expression involved in JH synthesis and degradation

Total RNA was extracted from whole bodies of five individuals stored at –80°C using the SV Total RNA Isolation System (Promega, Madison, WI, USA). Three biological replicates (five individuals per replicate) were prepared for each category. One microgram of total RNA from each sample was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). To compare the photoperiodic response of gene expression involved in JH synthesis and degradation pathways among the three strains, we performed real-time qPCR for *SDR1*, *SDR2*, *SDR3*, *SDR4*, *JHAMT*, *CYP15A1*, and *JHEH* genes using the SYBR Green I chemistry system and sequence detection system ABI PRISM 7500 (Applied Biosystems), as described previously (Ishikawa et al., 2012). Data acquisition and analyses were performed using ABI Prism 7500 software v2.0.1 (Applied Biosystems). The baselines and thresholds for Ct were set automatically. The relative standard curve method was used for qRT-PCR quantification, as described in User Bulletin 2 for the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Primers for both target and endogenous control transcripts were designed using Primer Express software (Applied Biosystems). *EF1a* (elongation factor 1 alpha) was used as an endogenous control, as described previously, and was determined using geNorm and NormFinder software for comparison with *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *beta-actin* genes (Ishikawa et al., 2012). For *JHAMT* and *JHEH*, we used the same primer set as in our previous study (Ishikawa et al., 2012). The primer sequences are listed in Supplementary Table S1. Two-way ANOVA was performed using R. 3.0.3 software (R Core Team, 2014).

Acetone treatment

To investigate whether the obligate-parthenogenetic strains possess a developmental cascade to produce sexual individuals, we artificially induced male production in the OP strains. Although a previous study reported that treatment with precocene dissolved in acetone induces male production in the green peach aphid *Myzus persicae* (Hales and Mittler, 1983), our preliminary experiment indicated that acetone itself induced male production. Therefore, we artificially induced males by acetone treatment in the OP1

Table 1. List of juvenile hormone-related genes analyzed in this study.

Gene symbol	Function	ACYPI ID	Reference nucleotide sequence	Reference protein sequence
SDR1	JH synthesis		NM_001160394	NP_001153866
SDR2	JH synthesis	ACYPI009545	XM_008179948, NM_001293482	XP_008178170, NP_001280411
SDR3	JH synthesis	ACYPI001301	XM_008187359	XP_008185581
SDR4	JH synthesis	ACYPI003207	NM_001162459	NP_001155931
JHAMT1	JH synthesis	ACYPI007696	NM_001162779	NP_001156251
JHAMT2	JH synthesis	ACYPI001588	XM_001943694	XP_001943729
JHAMT3	JH synthesis		XM_003244251	XP_003244299
JHAMT4	JH synthesis		XM_016802746	XP_016658235
CYP15A1	JH synthesis	ACYPI003882	XM_001952585	XP_001952620
JHEH	JH degradation		XM_003241505	XP_003241553

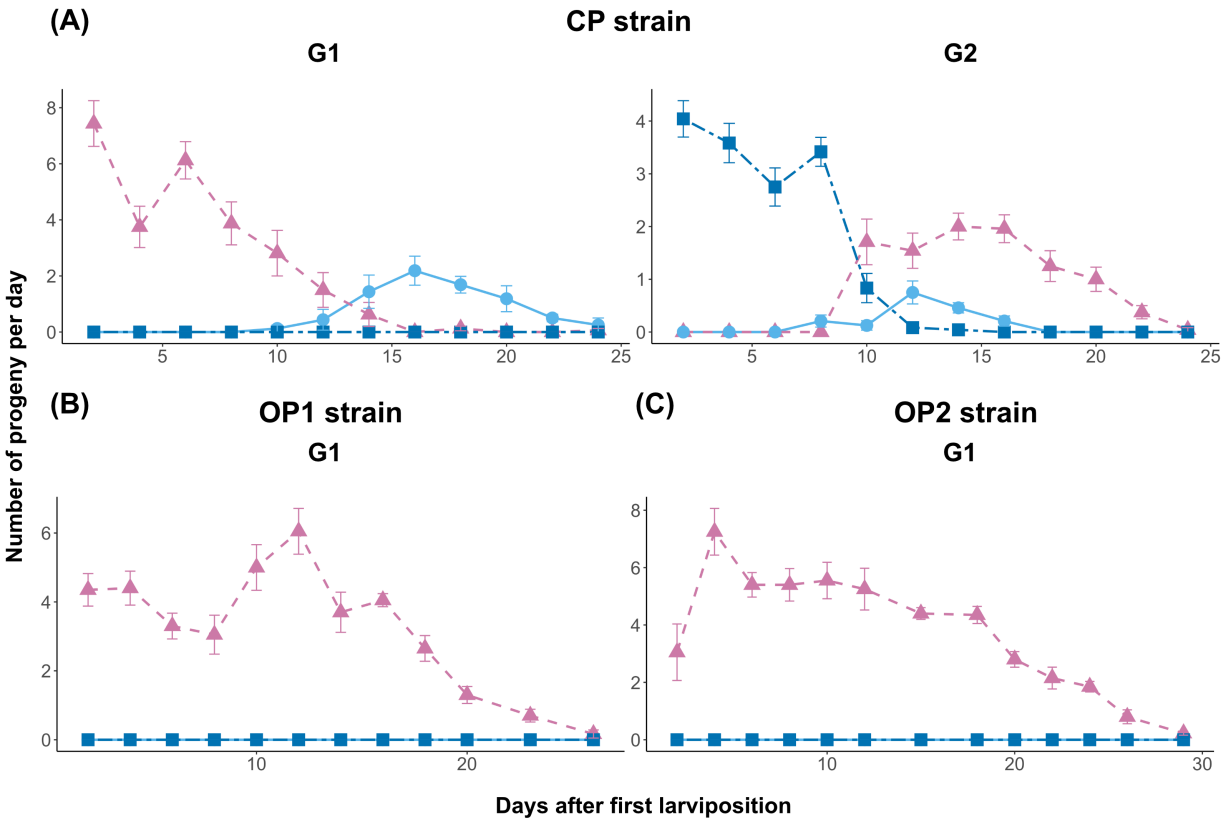

Fig. 3. Reproductive schedules of the CP **(A)**, OP1 **(B)**, and OP2 **(C)** strains under short-day conditions. Mean number and standard errors of parthenogenetic females (triangles and pink dashed line), males (circles and solid light blue line), and oviparous sexual females (squares and dash-dotted dark blue line) produced by aphids in the first generation (G1) or second generation (G2) under short-days (CP [G1]: n = 8, CP [G2]: n = 12, OP1: n = 10, OP2: n = 10). Upper panels of the CP strain are modified from Ishikawa et al. (2012).

and OP2 strains. Approximately 0.5 μL of acetone was applied to the abdomen of wingless parthenogenetic females just after the final ecdysis. As a control, we used 0.5 μL of ethanol and pentane. The aphids were then placed individually on a seedling at 20°C under long-day conditions (16L8D) and were transferred every 2–3 days to a new seedling to confirm the production of males or sexual females in the next generations. Fisher's exact test was conducted using R. 3.0.3 software (R Core Team, 2014).

RESULTS

Confirmation of obligate parthenogenesis in OP1 and OP2 strains

Under short-day conditions, all females of both the OP1 and OP2 strains produced only parthenogenetic females (Fig. 3, OP1: n = 10, OP2: n = 10). In addition, during the subsequent generations under short-day conditions, they also reproduced exclusively by parthenogenesis (data not shown).

Homologs of JH-synthesis/degradation genes in the pea aphid

After updating AphidBase, the genome database for *A. pisum*, we re-surveyed and identified the genes involved in JH synthesis and degradation by comparing amino acid sequences, before investigating the photoperiodic regulation of these genes (see Supplementary Table S2). For *SDR*, we found 16 putative orthologs in the AphidBase (ver.2.1). We excluded XP_016656002 from the NCBI database because of standard genome annotation processing. Furthermore, we narrowed these putative orthologs down to four potential orthologs using the classical SDR motif, hNhxG, on the fourth alpha helix, which was suggested to be part of the active site (see Supplementary Figure S1) (Persson et al., 2003). For *JHAMT*, we found 10 putative orthologs in the AphidBase (ver.2.1). Two orthologs, XP_016663097 and XP_008188739, were excluded from the NCBI database. Three of the orthologs, NP_001156251, XP_016663096, and XP_016663095, possessed identical protein sequences and an AphidBase ID (ACYPI007696). NP_001156251 and XP_001943729 have been identified in a previous study (Ishikawa et al., 2012). NP_001156251, XP_001943729, XP_003244299, and XP_016658235 were also identified in a recent study (Smykal and Dolezel, 2023). Because XP_016659614 and XP_001950668 showed relatively lower similarity to the query protein sequence compared to the other orthologs, we excluded them from the following analysis. For the *CYP15A1* gene, we found an ortholog, XP_016660351, in addition to XP_001952620, identified by Daimon and Shinoda (2013), which we used as a query sequence. XP_016660351 was then removed from the NCBI database. For the *JHEH* gene, we identified one JHEH ortholog, XP_003241553. For the *JHE* gene, we found no ortholog with over 40% similarity to the *JHE* gene in *Nilaparvata lugens*. Although our previous study identified two orthologs of the *JHE* gene, both were excluded from the

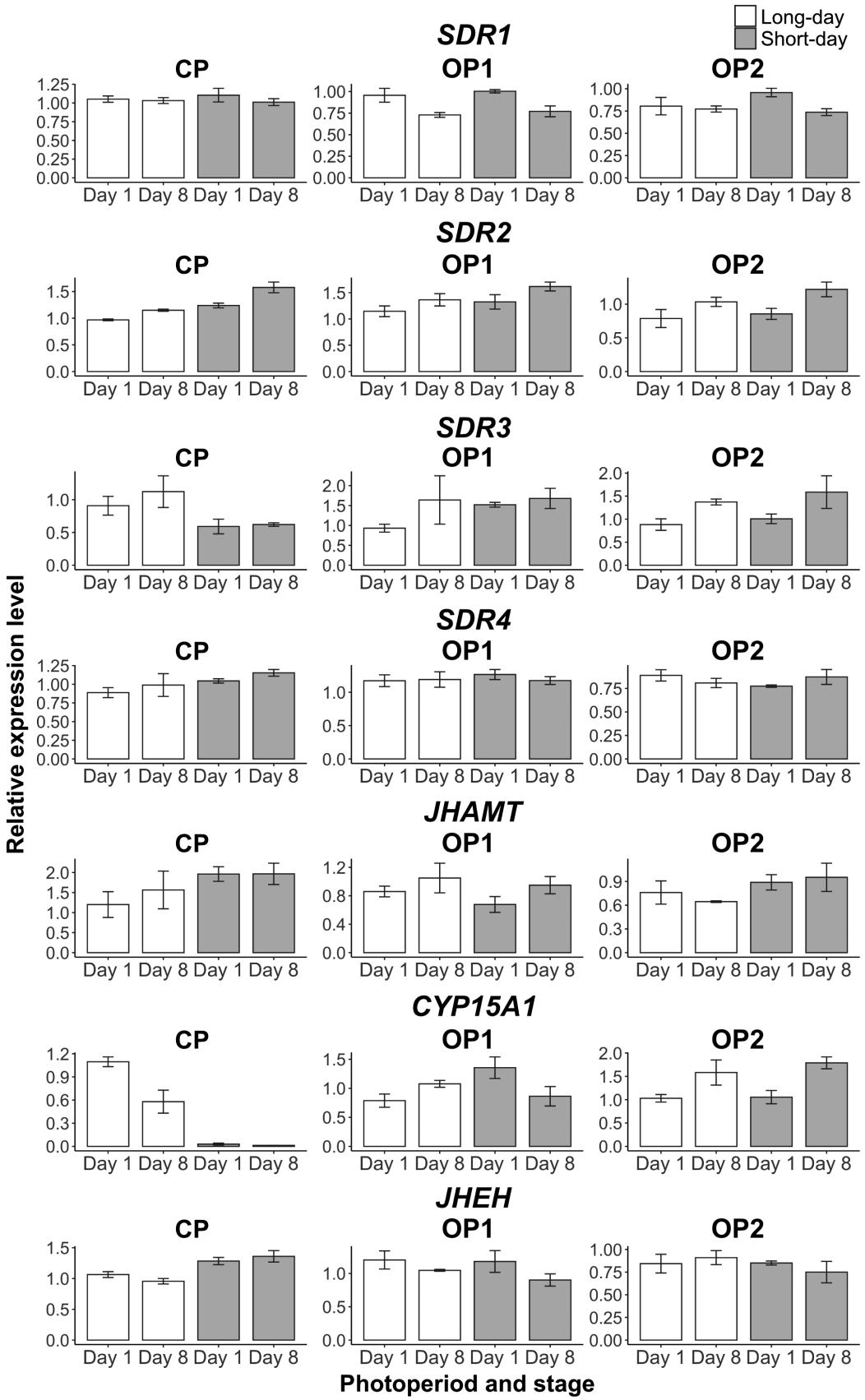

Fig. 4. Expression profiles of JH-synthesis/degradation genes under the long- and short-day conditions. Y-axes show relative expression levels, which are specific to each gene. Averages and standard errors of three biological replicates are indicated. The result of statistical analysis is shown in Table 2. The panel of *JHAMT* and *JHEH* expression levels in the CP strain are modified from Ishikawa et al. (2012).

NCBI database. Overall, we identified four orthologs of the *SDR* gene, four orthologs of *JHAMT* genes, one ortholog of the *CYP15A1* gene, and one ortholog of the *JHEH* gene (Table 1, Fig. 2).

Photoperiodic response of JH-synthesis/degradation gene expressions in cyclical-parthenogenetic and obligate-parthenogenetic strains

To investigate the mechanisms underlying the loss of reproductive polyphenism, we quantified the photoperiodic response of gene expression that acts in the direct steps of JH synthesis. Four of six genes related to JH synthesis (*CYP15A1*, *SDR2*, *SDR3*, and *SDR4*) showed significant photoperiodic responses in the CP strain (Fig. 4, Table 2). *CYP15A1* exhibited a pronounced decrease in expression under short-day conditions (two-way ANOVA, $F = 31.57$, $P < 0.0001$). In contrast, *JHAMT* did not show a photoperiodic response (two-way ANOVA, $F = 1.842$, $P > 0.05$) (Fig. 4, Table 2) (Ishikawa et al., 2012). Additionally, *CYP15A1* expression changed between days 1 and 8 after larviposition (two-way ANOVA: $F = 3.009$, $P < 0.05$), indicating an interaction between the photoperiod and age (two-way ANOVA: $F = 2.412$, $P < 0.05$). *SDR2* and *SDR4* displayed higher expression levels under short-day conditions than under long-day conditions (two-way ANOVA, $F = 1.715$, $P < 0.05$; $F = 0.549$, $P < 0.05$, respectively). Conversely, *SDR3* expression was lower under short-day conditions (two-way ANOVA, $F = 4.998$, $P < 0.05$). *SDR2* also showed increased expression on day 8 compared to that on day 1 after larviposition.

In contrast to the CP strain, neither OP1 nor OP2 displayed significant photoperiodic responses in any of the JH synthesis genes (Fig. 4, Table 2). However, in both strains, some genes showed different expression levels between days 1 and 8 after larviposition. In the OP1 strain, the *SDR1* gene showed higher expression on day 1 (two-way ANOVA: $F = 1.217$, $P < 0.05$). In the OP2 strain, *SDR2*, *SDR3*, and *CYP15A1* showed higher expression levels on day 8 (two-way ANOVA: $F = 0.287$, $P < 0.05$; $F = 0.455$, $P < 0.05$; and $F = 0.101$, $P < 0.05$, respectively).

We also investigated the response of the JH degradation gene *JHEH* to photoperiodic changes in the OP1 and OP2 strains. Our previous study on the CP strain showed that *JHEH* gene expression significantly increased under short-day conditions (two-way ANOVA: $F = 1.339$, $P < 0.05$) (Fig. 4, Table 2) (Ishikawa et al., 2012). However, in the OP1 and OP2 strains, *JHEH* expression did not differ significantly between day-length conditions (two-way ANOVA: $F = 0.074$, $P > 0.05$; $F = 0.166$, $P > 0.05$, respectively) (Fig. 4, Table 2). Our gene expression analysis revealed that obligate-parthenogenetic strains did not show photoperiodic responses in JH-related genes, suggesting a potential link to the loss of the sexual phase.

Male production from obligate-parthenogenetic strains induced by acetone treatment

To investigate whether obligate-parthenogenetic strains retain the downstream developmental pathway for male production, we artificially induced male production by acetone treatment in the OP1 strain. After the application of

Table 2. Two-way ANOVA results to detect the photoperiodic and age effects on genes involved in JH synthesis and degradation.

Gene	Strain	Sexual reproduction	*F-value*			*P-value*		
			Photoperiod	Age	Photoperiod x age	Photoperiod	Age	Photoperiod x age
SDR1	CP	+	0.019	0.234	0.161	0.5035	0.0902	0.1496
	OP1	–	0.038	1.217	0.002	0.43634	**0.00266	0.96325
	OP2	–	0.071	0.473	0.197	0.3606	0.0696	0.1573
SDR2	CP	+	1.715	1.004	0.174	***0.000115	***0.000654	0.060744
	OP1	–	0.441	0.592	0.008	0.0896	0.0517	0.7491
	OP2	–	0.287	1.651	0.089	0.2459	*0.0167	0.5807
SDR3	CP	+	4.998	0.313	0.393	*0.0242	0.4848	0.5137
	OP1	–	0.482	1.875	0.683	0.376	0.231	0.434
	OP2	–	0.455	3.449	0.077	0.4172	*0.0269	0.8273
SDR4	CP	+	0.549	0.414	0.025	*0.0468	0.1248	0.5954
	OP1	–	0.011	0.015	0.044	0.668	0.688	0.548
	OP2	–	0.019	0.006	0.244	0.665	0.884	0.149
JHAMT	CP	+	1.842	0.349	0.128	0.0956	0.5179	0.6727
	OP1	–	0.483	1.364	0.014	0.335	0.133	0.775
	OP2	–	1.403	0.027	0.349	0.12	0.843	0.498
CYP15A1	CP	+	31.57	3.022	2.412	***7.73e-06	*0.0102	*0.0162
	OP1	–	0.647	0.199	2.426	0.2428	0.49	*0.0238
	OP2	–	0.101	3.993	0.039	0.51476	**0.00533	0.60484
JHEH	CP	+	1.339	0	0.138	**0.00104	0.91741	0.15437
	OP1	–	0.074	0.866	0.044	0.4913	0.0985	0.6136
	OP2	–	0.166	0.002	0.116	0.412	0.852	0.372

*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$

acetone, 16.7% of the aphids (n = 18) began to produce males (Fig. 5). Male producers produced one to three males (mean ± standard error: 11.1 ± 3.8%) at 9–10 days after the treatment. No males were observed in the control groups treated with ethanol (n = 21) or pentane (n = 27) (Fisher's exact test: $P < 0.05$). Acetone treatment also induced male production in 3% of the aphids in the OP2 strain (n = 33).

DISCUSSION

In the present study, we investigated the endocrine mechanisms underlying the loss of reproductive polyphenism in natural aphid populations. We found that five out of seven genes involved in the biosynthesis and degradation of JH showed photoperiodic responses in the CP strain. In contrast, none of these genes exhibited photoperiodic responses in the OP1 and OP2 strains. Notably, *CYP15A*, which encodes an enzyme for the final step of JH synthesis, showed a distinct response, with high expression under long-day conditions and decreased expression under short days in the CP strain. However, this response was completely absent in the OP1 and OP2 strains. Similarly, *JHEH*, which is responsible for initiating JH degradation, exhibited higher expression under short-day conditions in the CP strain; however, this response was not observed in the OP1 and OP2 strains. These results strongly suggest that the loss of the photoperiodic response in both the JH synthesis and degradation pathways may be a key mechanism underlying the elimination of the sexual phase in the aphid life cycle (Fig. 6). Aphid reproductive strategies are heavily influenced by the winter climate (Simon et al., 2010). Cyclical-parthenogenetic populations pay a two-fold cost of sex and require longer developmental arrest (Maynard-Smith, 1978), whereas they can produce cold-resistant overwintering eggs (Simon et al., 2010). In contrast, obligate-parthenogenetic populations can potentially reproduce year-round through parthenogenesis and exhibit shorter developmental periods. However, parthenogenetic females lack resistance to low temperatures (Simon et al., 2010). Therefore, reproductive polyphenism, which switches between parthenogenesis and sexual reproduction, is advantageous in areas with harsh seasonal variations. However, obligate-parthenogenetic populations potentially driven by the loss of the photoperiodic response in JH signaling may be favored in environments with milder winter temperatures (Dedryver et al., 2008).

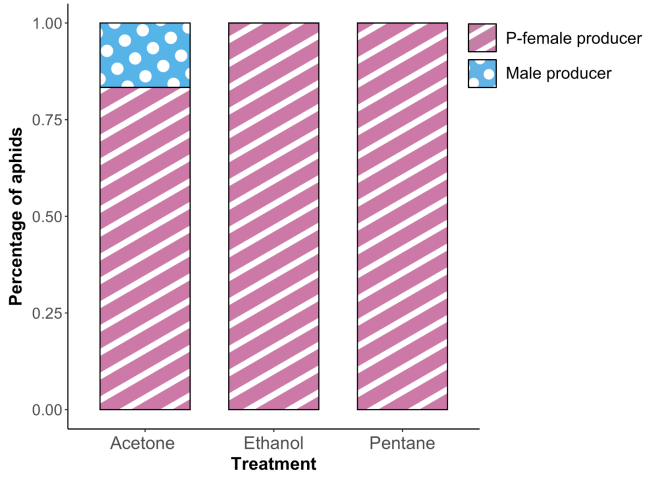

Fig. 5. Percentage of parthenogenetic female (P-female) producers (stripes with pink) and male producers (circles with light blue) induced by acetone, ethanol, and pentane treatments in the OP1 strain.

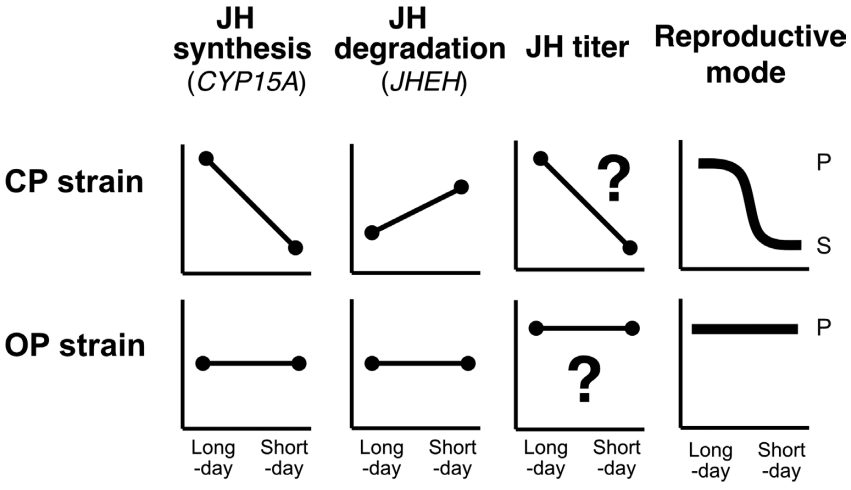

Fig. 6. Photoperiodic response of JH pathway in the CP and OP strains. The CP strain exhibits upregulation of the JH-synthesis pathway (*CYP15A* expression) and downregulation of the JH-degradation pathway (*JHEH* expression) in response to short-day length, which may be responsible for the decrease of JH titer, leading to the change of reproductive mode from parthenogenesis (P) to sexual reproduction (S). However, the OP strains do not show photoperiodic responses of the JH-synthesis/degradation gene expressions, resulting in obligate parthenogenesis. This suggests that modification of the photoperiodic sensitivity of the JH signaling pathway would underlie the multiple loss of reproductive polyphenism in the aphid life cycle.

Interestingly, we also demonstrated that topical application of acetone can induce the production of sexual males in OP strains. This result indicates that the OP1 and OP2 strains retained the developmental cascade to produce sexual males. Hormones act as molecular switches that alter gene expression patterns and initiate developmental events (Nijhout, 1999). In aphid reproductive polyphenism, JH has been suggested to integrate information on photoperiod and temperature, leading to the production of sexual morphs (Ishikawa et al., 2012). Thus, modifying the JH signaling pathway might be an effective way to eliminate the entire developmental process of the sexual phase from the aphid's annual life cycle. A previous study on *Manduca sexta* also showed that artificial selection to decrease larval color polyphenism resulted in the loss of sensitivity of JH titers to heat shock (Suzuki and Nijhout, 2006). Those previous findings taken together with the results of the present study indicate that changes in environmental sensitivity of hormone biosynthesis and degradation may play an important role in the evolutionary reduc-

tion of plasticity in both natural and artificially selected populations.

However, the specific mutations underlying the loss of photoperiodic responses in these genes remain unknown. Recent genomic studies have revealed that a single genomic region on the X chromosome controls the loss of ability to produce sexual females in response to a short photoperiod in pea aphids (Nouhaud et al., 2014; Huguet et al., 2024). In addition, several photoreceptors, circadian clock genes, and insulin-like peptides, such as ILP4, have been suggested to control the reproductive mode change from parthenogenesis to sexual reproduction (Goto, 2022; Colizzi et al., 2024). These genomic regions and genes are strong candidates for upstream factors that cause the loss of photoperiodic response in JH-related genes. Recently developed molecular tools, such as genome editing tools like CRISPR/cas9, can potentially enable functional assays for candidate genes or sequences that may underlie the modification of the photoperiodic control of the JH pathway. These techniques will also help to reveal the synthetic and degradation pathways of JH and identify functional JH in pea aphids. Further studies are required to fully understand the evolutionary processes that lead to the loss of reproductive polyphenism.

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COMPETING INTERESTS

We declare we have no competing interests.

AUTHOR CONTRIBUTIONS

AI designed the study, and performed experiments and data analysis. TK provided the aphid strains. AI wrote the first draft of the manuscript with HG, KO, TK, SA, and TM making significant contributions to editing. All authors agree to be held accountable for the content therein and approve the final version of the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online (URL: https://doi.org/10.2108/zs240075)

Supplementary Figure S1. Alignment of putative orthologs of SDR.

Supplementary Table S1. List of primer sequences for qRT-PCR.

Supplementary Table S2. Putative orthologs of JH synthesis and degradation genes.

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