# Pardosa pseudoannulata mother adopts a compensatory strategy for reproduction deficiency resulting from the suppression of EcR/USP-1-mediated ecdysteroid signaling

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# Abstract

Spiders express specific maternal care to their offsprings. The predatory spider *Pardosa pseudoannulata* mother constructs an eggsac to protect her eggs and juvenile spiderlings. However, here we found that the female spiders ate their eggsacs when ecdysteroid signaling was suppressed. Nuclear receptors (NRs), especially ecdysone receptor (EcR) and ultraspiracle (USP), have attracted extensive attention in arthropods because of their pivotal roles in ecdysteroid signaling cascades. An *EcR* and two *USPs* were identified in *P. pseudoannulata* genome. RNAi against *EcR* and *USP-1* restrained the development of spiderlings, such as delaying moulting and decreasing moulting rate. *EcR*- and *USP-1*-silenced adult females produced more invalid eggsacs full of nonviable eggs than the control counterpart, and they ate the invalid eggsacs. *EcR* and *USP-1* responded to the changes of ecdysteroid, and *CYP307A1* knockdown led to the similar phenotypes to dsEcR and dsUSP-1 treatments. It proposed that EcR/USP-1-mediated ecdysteroid signaling regulated the development and reproduction in *P. pseudoannulata*. The period of the first reproduction cycle was 17.87 d in the ecdysteroid signaling-suppressed females, which was 7.19 d shorter than that of the control (25.06 d). The result meant that, when the female spiders detected the nonviable eggs in eggsac, they forwardly ate the invalid eggsac to terminate the useless reproduction cycle and start a new cycle by generating a new eggsac. The strategy can partially compensate for the loss of population growth due to the nonviable eggs, and eating the invalid eggsac also provide a compensation for the physiological consumption during the invalid eggsac care.


# Key Messages

1. Twenty-three *NRs* were identified in *P. pseudoannulata*, including an *EcR* and two *USPs*.
2. Two USPs had functional differentiation in the spider.
3. EcR/USP-1-mediated ecdysteroid signaling regulated the spider development and reproduction.
4. Ecdysteroid signaling-suppressed females ate the invalid eggsacs to terminate the useless reproduction cycle.

# 1 Introduction

Increased egg number is an effective strategy for arthropods to expand their population. In addition, some arthropods, such as spiders, provide extensive maternal care to protect and increase the success of their offsprings (Yip and Rayor 2014). Maternal care in spiders include eggsac construction, eggsac care, and juvenile spiderling care (Ruhland et al. 2016b). For example, the web-weaver spiders attach their eggsac to the web, while Lycosidae females carry their eggsac via spinnerets, and then carry the juveniles emerging from eggsac on notum (Yang et al. 2018; Yu et al. 2022). Egg brooding is effective for offspring protection against predators, parasites, and adverse environment (Iida and Fujisaki 2005; Ruhland et al. 2016a; Vieira and Romero 2008). *Pardosa pseudoannulata* is a predatory wolf spider against various insect pests in fields and exhibits meticulous maternal care to its offsprings. After the eggsac production, the female spiders carry their eggsacs for about 15 d until the juvenile spiderling emergence, and later

carry the new-emerged spiderlings for about 4.6 d before their dispersal (Yu et al. 2022). Although cannibalism is common in the spider, the females do not acutely prey the new-emerged spiderlings during maternal care (Yu et al. 2022). *P. pseudoannulata* females can produce five eggsacs at most in the life once mating (Yang et al. 2018), so the maternal care is alternate with the cannibalism. Ecdysteroids are characterized by their critical roles on arthropods' parental care and cannibalism (Trabalon et al. 1998; Vancassel et al. 1984).

We have identified Halloween genes involved in ecdysteroid biosynthesis and confirmed that ponasterone A (PA) was the endogenous ecdysteroid in *P. pseudoannulata* (Yang et al. 2021). In arthropods, ecdysteroids act on different nuclear receptors (NRs) to perform a variety of physiological functions. NRs, a group of ligand-activated transcription factors, are widely present in animals to regulate various biological processes (Christiaens et al. 2010). NRs are assigned to seven subfamilies (NR0-NR6) that all contain two typical domains, a highly conserved DNA-binding domain (DBD) and a less conserved ligand-binding domain (LBD), except for NR0 subfamily lacking LBD (King-Jones and Thummel 2005). NRs get a lot of attentions in insects because of their roles in embryogenesis, moulting, metamorphosis, reproduction, and homeostasis (Fahrbach et al. 2012). Since the first complete *NR* family (21 members) was reported in *Drosophila melanogaster* (Adams et al. 2000; King-Jones and Thummel 2005), *NR* family has been identified in an increasing number of insects based on whole genome sequencing, including 21 in *Anopheles gambiae* (Bertrand et al. 2004), 22 in *Apis mellifera* (Velarde et al. 2006), 21 in *Tribolium castaneum* (Bonneton et al. 2008; Tan and Palli 2008), 19 in *Bombyx mori* (Cheng et al. 2008), 20 in *Aedes aegypti* (Cruz et al. 2009), 19 in *Acyrthosiphon pisum* (Christiaens et al. 2010), and 20 in *Nilaparvata lugens* (Xu et al. 2017). Ecdysone receptor (EcR) and ultraspiracle (USP)/retinoid X receptor (RXR) are the important members of NRs and they form a heterodimer that binds to ecdysteroid to start the cascade (Christiaens et al. 2010; Thomas et al. 1993; Yao et al. 1993).

The identification and function analysis of NRs in arachnids has been slower compared to the work done in insects. Up to now, *NR* family has only been systematically identified in the genome of *Tetranychus urticae* with 30 members (Grbic et al. 2011). Scattered investigations of *NRs* have been reported in other arachnids, including *Panonychus citri* (Li et al. 2017, 2020, 2022), *Tetranychus cinnabarinus* (Shen et al. 2019), *Amblyomma americanum* (Guo et al. 1997, 1998; Palmer et al. 2002), *Ornithodoros moubata* (Horigane et al. 2007, 2008), *Liocheles australasiae* (Nakagawa et al. 2007), *Agelena sylvatica* (Honda et al. 2017), and *Parasteatoda tepidariorum* (Nicewicz et al. 2021). Most studies were focused on the functional elucidations of EcR and USP/RXR in arachnids. Although a mounting number of genomes have been sequenced, *NRs* are rarely investigated in spiders.

In this study, we completely identified *NR* family using the genome data of *P. pseudoannulata*. EcR and USP-1 are two significant members in *P. pseudoannulata* to mediate the ecdysteroid signaling. When EcR/USP-1-mediated ecdysteroid signaling was suppressed, the female spiders produced more invalid eggsacs full of nonviable eggs. To compensate for the population loss due to the nonviable eggs, female spiders initiatively ate the invalid eggsac and promoted the generating of a new eggsac in short time.

# 2 Materials And Methods

## 2.1 Identification and phylogenetic analysis of NRs in *P. pseudoannulata*

The putative *NRs* were retrieved in the chromosome-level genome (GenBank accession number: JAGEOH000000000) of *P. pesudoannulata* using the orthologs from *D. melanogaster* (King-Jones and Thummel 2005), *A. gambiae* (Bertrand et al. 2004), T. *castaneum* (Bonneton et al. 2008; Tan and Palli 2008), *B. mori* (Cheng et al. 2008), A. *aegypti* (Cruz et al. 2009), A. *pisum* (Christiaens et al. 2010), N. *lugens* (Xu et al. 2017), T. *urticae* (Grbic et al. 2011)d *citri* (Li et al. 2017) as queries via local BLAST tool (v2.7.1, downloaded from ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/). The neighbor-joining phylogenetic tree of NRs was constructed with a bootstrap of 1000 in MEGA X (v10.0.5) (Kumar et al. 2018).

## 2.2 Spiders

*P. pseudoannulata* of multiple stages were collected from paddy fields of Nanjing (Jiangsu province, China) in May 2020 and kept in plastic cups (500 mL in volume) individually at 28 ± 1°C and 16/8 h light/dark and fed with *N. lugens* until adulthood. Eleven developmental samples of egg (E), spiderling in eggsac (ES), aggregated spiderling (AS, spiderling carried by female), dispersed spiderling (DS, actively moving spiderling), virgin male (VM), virgin female (VF), mated female (MF), early-eggsac-carrying female (EESF), late-eggsac-carrying female (LESF), spiderling-carrying female (SCF), and non-spiderling-carrying female (NSCF) were harvested individually for RNA-seq at the Beijing Genomics Institute (Shenzhen, China) with 10 eggsacs or spider individuals were pooled as one sample. The newly-molted 2nd instar spiderlings (II0), the 2nd instar spiderlings 1 to 5 days post moulting (II1-II5), and the newly-molted 3rd instar spiderlings (III0) were harvested individually and 10 spiderlings each were pooled as one sample. Six tissues, brain, venom gland, intestine, fat body, ovary, and testes, were dissected from 20 adult females and males. All the above samples were prepared in three biological replicate samples.

## 2.3 RNA interference

Primers with T7 RNA polymerase promoter sequence used for dsRNA synthesis (Table S1) were designed using Beacon Designer (v7.92, PREMIER Biosoft International, CA, USA) and synthesized by Genscript (Nanjing, China). dsRNA against enhanced green fluorescent protein (*eGFP*) (GenBank accession number: KC896843) was set as negative control. Specific fragment of target gene was amplified using Phanta® Max Super-Fidelity DNA Polymerase (Vazyme, Nanjing, China) and then purified using GeneJET Gel Extraction Kit (Thermo Scientific, Carlsbad, CA, USA) according to the manufacturer's instructions. dsRNA was synthesized using T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Microinjection of *P. pseudoannulata* was as the previous description (Meng et al. 2015). (1) RNA interference in spiderlings. II1 spiderlings were kept in an agar gel plate after anesthetization with carbon dioxide. dsRNA of 50 ng in 10 nL was injected into each spiderling. The injected spiderlings were transferred into petri dishes (3.5 cm in diameter) individually and fed with the

2nd instar *N. lugens* nymphs, and the injured spiderlings were removed within 12 h. The spiderlings were divided into two groups. Group I was used for gene quantification. Ten spiderlings were pool as one sample at 48 h and three biological replicate samples were prepared. Group II was used for phenotypic observation. The numbers of moults and mortalities were recorded at a 12 h interval until day 10. Each treatment was prepared in three biological replicates and each replicate contained 15–20 spiderlings. (2) RNA interference in females. The mated females were kept in an agar gel plate after anesthetization with carbon dioxide. dsRNA of 1 μg in 200 nL was injected into each female within 6 h after mating. The injected females were transferred into plastic cups individually and fed with *N. lugens* adults. The reproductive periods, including pre-oviposition period, eggsac-carrying period, spiderling-carrying period, and post-reproductive period, were recorded at a 12 h interval. The eggsac was weighted at 24 h after oviposition, and then returned to the female quickly. Juvenile spiderlings hatch on day 6 after oviposition and then stay in eggsacs for 9 d (Fig. S1). Therefore, an eggsac was took down from the female and opened on day 7 after oviposition to check the developmental states of eggs and count the hatched spiderlings (viable) or unhatched eggs (nonviable). Each treatment group contained at least 100 females. Random six females that had not yet oviposition were individually harvested at 72 h for gene quantification.

## 2.4 PA application

Standard substance PA (purity > 95%) was purchased from Cayman Chemical (Ann Arbor, Michigan, USA) and dissolved in absolute ethanol to prepare a stock solution of 5 mg/mL. A working solution of 2.5 mg/mL was the prepared with sterile water. Fifty percent of ethanol was set as the negative control. PA solution or ethanol was introduced by microinjection. Briefly, II1 spiderlings were kept in an agar gel plate after anesthetization with carbon dioxide. PA solution or ethanol of 10 nL was injected into each spiderling. The injected spiderlings were transferred into petri dishes individually and fed with the 2nd instar *N. lugens* nymphs. Ten spiderlings were pooled as one sample at 48 h and four biological replicate samples were prepared.

## 2.5 Real-time quantitative PCR

Total RNA was extracted using Trizol™ reagent (Invitrogen, Carlsbad, CA, USA) and then used to synthesize cDNA using PrimeScript RT Reagent Kit (TaKaRa, Kyoto, Japan) according to the manufacturer's instructions. Primers used for real-time quantitative PCR (qPCR) (Table S1) were designed using Beacon Designer and synthesized by Genscript. Elongation factor-1 alpha (*EF-1α*) and glyceraldehyde-3-phosphatedehydrogenase (*GAPDH*) (GenBank accession number: KJ888948 and KJ888949, respectively) were selected as the reference genes (Meng et al. 2015). The qPCR reaction system was composed using TB Green Premix Ex Taq II Kit (TaKaRa, Kyoto, Japan) as the manufacturer's instructions and performed on QuantStudio Real-Time PCR System (Applied Biosystems, California, USA). Each reaction was carried out with two technical replicates.

## 2.6 Data analysis

The relative expressions of target genes were related to the geometric mean of two reference genes by the $2^{-\Delta CT}$ method (Livak and Schmittgen 2001; Vandesompele et al. 2002). Gene expression, moulting rate, and mortality were presented as mean ± SEM. The FPKM (fragments per kilobase of exon model per million mapped fragments) values of each gene in the eleven developmental samples were retrieved from the normalized transcriptomes and normalized with scale function, and the heatmap was constructed using R (v4.1.1, downloaded from https://cran.r-project.org/). Significant differences were analyzed by *t*-test, one-way ANOVA with Tukey test, and Fisher's exact test at $P < 0.05$ using GraphPad Prism (v7) (Swift 1997). The spider images were taken with Leica S9i (Leica Microsystems, Wetzlar, Germany).

# 3 Results

## 3.1 Characterization of *NRs* in *P. pseudoannulata*

Twenty-three *NRs* were identified in the genome of *P. pseudoannulata*. Phylogenetic analysis showed that they were distributed in 6 subfamilies with 7 in NR1, 10 in NR2, 1 in NR3, 1 in NR4, 2 in NR5, and 2 in NR6 (Fig. 1, Table S2). *NRs* were duplicated in ecdysone-induced protein 78 (*E78*), hormone receptor-like in 46 (*HR3*), *USP*, hormone receptor-like in 51 (*HR51*), seven up (*SVP*), and hormone receptor-like in 4 (*HR4*), which had two duplicates, respectively (Fig. 1, Table S2).

## 3.2 Spatiotemporal expression of *EcR* and *USPs*

We studied the functions of EcR and two USPs emphatically. There were consistent expression patterns between *EcR* and *USP-2* with higher expression in spiderlings and lower expression in adults. *USP-1* showed remarkable expression in reproductive females excluding late-eggsac-carrying females, and its pattern was opposite to that of either *EcR* or *USP-2* (Fig. 2A, Table S3). In the 2nd instar spiderlings, the expression of *EcR* and two *USPs* were relatively stable in the first four days, sharply increased on day 5 and then dropped to much lower level than the beginning once moulting was completed (Fig. 2B). Spatially, *EcR* was expressed in all test tissues with higher expression in brain, venom gland, and ovary. *USP-1* was expressed in all tissues with the most abundance in brain, fat body, ovary, and testis while *USP-2* expression was mostly found in brain, venom gland, and intestine (Fig. 2C).

## 3.3 Roles of EcR and USPs on the development of *P. pseudoannulata*

*EcR* and two *USPs* were interfered in dsRNA-treated *P. pseudoannulata* spiderlings, and the *Halloween* genes involved in ecdysteroid biosynthesis were downregulated synchronously (Fig. S2A). Fifty-five percent of dsEcR-treated spiderlings died from unsuccessful moulting within 10 d (Fig. 3A, B). No significant mortalities were observed in spiderlings injected with dsRNA of two *USP* genes (Fig. 3A). Both *EcR* and *USP-1* silencing remarkably affected the spiderlings' moulting. The dsEcR-treated spiderlings were with reduced moulting rate, while the dsUSP-1-treated spiderlings exhibited the delayed moulting (Fig. 3C). However, knocking down *USP-2* did not affect the development of spiderlings (Fig. 3C).

## 3.4 Roles of EcR and USPs on the reproduction of *P. pseudoannulata*

The dsRNAs against *EcR* and two *USPs* remarkably downregulated the target genes in mated females, as well as the *Halloween* genes (Fig. S2B). Females with *EcR* or *USP-1* knockdown ate their eggsacs (Fig. 4A) that turned out to contain the ceased eggs in the dsEcR treatment, and contain both ceased and half-way developed eggs in the dsUSP-1 treatment (Fig. 4B), they were nonviable eggs. Both *EcR*- and *USP-1*-silenced females produced a significantly higher proportion of invalid eggsacs when compared to the control group (Fig. 4C), but preformed as well as the control females in terms of pre-oviposition period (Fig. 4D), egg number (Fig. 4E), and egg weight (Fig. 4F). *USP-2* knockdown remarkably decreased the egg numbers (Fig. 4E), but had no effect on the egg quality (Fig. 4B, C).

## 3.5 Transcriptional response of *EcR* and *USPs* to the changes of ecdysteroid

To verify whether *EcR* and two *USPs* could respond to ecdysteroid, we manipulated ecdysteroid level in *P. pseudoannulata* by exogenous PA application and PA biosynthesis interference via RNAi of *CYP307A1*. The results showed that the transcriptional levels of *EcR* and *USP-1* were significantly upregulated by PA application (Fig. 5A) and remarkably downregulated by *CYP307A1* knockdown (Fig. 5B). However, *USP-2* did not respond to the changes of ecdysteroid (Fig. 5).

## 3.6 Effects of ecdysteroid disruption on the development and reproduction of *P. pseudoannulata*

We also tested the effects of ecdysteroid biosynthesis suppression on the development and reproduction of *P. pseudoannulata*. The expression of *CYP307A1* and the downstream *Halloween* genes in ecdysteroid biosynthesis pathway were remarkably suppressed in the spiderlings (Fig. S3A) and females (Fig. S3B) treated with dsCYP307A1. The spiderlings moulted from 96 h on and reached a plateau after 180 h. The moulting rates of *CYP307A1*-silenced spiderlings were significantly lower than that of the control group from 96 h to 144 h (Fig. 6A). Similar to what *EcR*- and *USP-1*-silenced females did, *CYP307A1*-silenced females ate their eggsacs which were full of ceased eggs (Fig. 6B), and the proportion of invalid eggsacs in dsCYP307A1 treatment group were remarkably more than that of the control group (Fig. 6C). In addition, *CYP307A1* knockdown did not affect pre-oviposition period (Fig. 6D), egg number (Fig. 6E), and egg weight (Fig. 6F).

## 3.7 Duration of reproductive *P. pseudoannulata* females

After the first eggsac, *P. pseudoannulata* mothers prepared the next reproduction cycle by generating a new eggsac, whether the eggsac-carrying females in the control groups (dseGFP) or the eggsac-eating females in the treatment groups (dsEcR, dsUSP-1, and dsCYP307A1). In the control group, the females carried the first eggsacs for 14.72 ± 0.04 d and later the spiderlings for 4.40 ± 0.13 d. After preparation in post-reproductive stage for 5.94 ± 0.52 d, the females laid a new eggsacs (Table 1). In the treatment

group, the mothers ate their eggsacs after carrying them for 9.36 ± 0.34 d, and remained in post-reproductive stage for 8.51 ± 0.53 d before the next oviposition (Table 1). The intervals between two eggsacs were 25.06 and 17.87 d in the control and treatment groups, respectively.

Table 1
The reproductive periods of the eggsac-carrying and eggsac-eating females.

| Spider | Reproductive stage | Duration (d) | | *N* |
|---|---|---|---|---|
| | | Mean | SEM | |
| Eggsac-carrying female | Eggsac-carrying period | 14.72 | 0.04 | 138 |
| | Spiderling-carrying period | 4.40 | 0.13 | 87 |
| | Post-reproductive period | 5.94 | 0.52 | 41 |
| Eggsac-eating female | Eggsac-carrying period | 9.36 | 0.34 | 68 |
| | Post-reproductive period | 8.51 | 0.53 | 41 |

# 4 Discussion

In the present study, twenty-three *NRs* were identified in *P. pseudoannulata* and grouped into six subfamilies based on their phylogenetic analysis with no members in NR0 subfamily. It was the first complete identification and characterization of *NR* family in spiders. The number of *NRs* in *P. pseudoannulata* was close to that in insects with 19–22 members (Adams et al. 2000; Bertrand et al. 2004; Bonneton et al. 2008; Cheng et al. 2008; Christiaens et al. 2010; Cruz et al. 2009; King-Jones and Thummel 2005; Tan and Palli 2008; Velarde et al. 2006; Xu et al. 2017). Interestingly, each *NR* had single copy in all investigated insects, except for duplicates of tailless (*Tll*) in *A. gambiae* (Bertrand et al. 2004) (Table S4), whereas in *P. pseudoannulata*, six *NRs* had two duplicates each, including *E78*, *HR3*, *USP*, *HR51*, *SVP*, and *HR4*. *NR* duplication was generally occurred in arachnids (Table S4). For example, *T. urticae* had 8 hormone receptor-like in 96 (*HR96s*), 2 *USP/RXRs*, and 2 hormone receptor-like in 38 (*HR38s*) (Grbic et al. 2011). Both *A. americanum* (Guo et al. 1998)d *citri* (Li et al. 2017) had 2 *USP/RXRs*. Therefore, it raised concern about the functional differentiation between NR duplicates in arachnids, such as USP/RXRs. *A. americanum* was the first arachnid with focus on functional investigations of two USP/RXRs (Guo et al. 1998). Although the transcriptional level of *EcR* and two *USP/RXRs* were correlated with ecdysteroid titer in the developmental stages, the electrophoretic gel mobility shift assay showed that EcR/USP/RXR-1 but not EcR/USP/RXR-2 exhibited broad DNA binding specificity (Palmer et al. 2002). In *P. citri*, *EcR* had the similar temporal expression patterns to *USP/RXR-2* but different from *USP/RXR-1*, and differential expression genes in deutonymphs indicated that EcR/USP/RXR-2 and USP/RXR-1 might regulate different physiological processes to control the mite moulting (Li et al. 2022). An *EcR* and an *USP/RXR* were cloned from *L. australasiae*, and the ligand-binding assay showed that EcR had high binding ability to PA while the USP/RXR did not enhance the binding ability (Nakagawa et al. 2007). USP/RXR is required for EcR binding to ecdysteroids (Thomas et al. 1993; Yao et al. 1993), so

whether another USP/RXR in *L. australasiae* playing this role remains to be further explored. As we found here in the present study that two *P. pseudoannulata* USPs had differential functions in the development and reproduction, the duplication of *NRs* gave a more comprehensive network for the gene expression regulation in arachnids.

We thoroughly quantified the spatiotemporal expression of *EcR* and two *USPs* in *P. pseudoannulata*. *EcR* and two *USPs* were in accordance with the expression pattern of *Halloween* genes with high expression in the end of the 2nd instar spiderlings followed by a rapid dropdown once moulting was completed (Yang et al. 2021), which indicated they might involve in the moulting process. *EcR* and two *USPs* exhibited different expression patterns in eleven developmental stages, with *EcR* and *USP-2* being significantly expressed in spiderlings while *USP-1* being significantly expressed in adult females. Besides, *EcR* and two *USPs* were highly expressed in six tissues from adults, except for *USP-2* that was mostly undetectable in fat body, ovary, and testis. These results strongly indicated the functional differentiation of two USPs in *P. pseudoannulata*. Therefore, RNAi against two *USPs* showed that *USP-1* knockdown remarkably delayed spiderlings' moulting and increased the number of invalid eggsacs in females, while the development and reproduction of *P. pseudoannulata* were not affected by dsUSP-2 treatment, except for the reduced egg numbers. In addition, the phenotypes in spiderlings and females treated with dsCYP307A1 were similar to the dsEcR and dsUSP-1 treatments. Meanwhile, *EcR* and *USP-1* responded to the changes of ecdysteroid with the upregulation by PA application and downregulation by *CYP307A1* silencing. Therefore, we suggested that EcR/USP-1 mediated ecdysteroid signaling to regulate the development and reproduction in *P. pseudoannulata*.

An unusual case that the female ate her invalid eggsac occurred in dsEcR, dsUSP-1, and dsCYP307A1 treatments, in which EcR/USP-1-mediaed ecdysteroid signaling was suppressed, to stop the useless maternal care. The wolf spiders, including *P. pseudoannulata*, express parental care to their offsprings by carrying their eggsacs and juvenile spiderlings (Ruhland et al. 2016b). Spider mothers termly detected their juveniles' statuses and opened the eggsac at a certain time to release the juveniles, the exact timing of eggsac opening partially depends on stimuli from juvenile movements in eggsac (Ruhland et al. 2019; Viera et al. 2007). In this study, the ecdysteroid signaling-suppressed *P. pseudoannulata* mothers might not receive the signals from the eggsac full of nonviable eggs. Carrying an invalid eggsac did not give any benefit for population growth of *P. pseudoannulata*. Alternately, they selected to eat the invalid eggsacs to terminate the useless reproduction cycle and provide chances for the generating of a new eggsac in advance. This selection of eating invalid eggsac shortened the useless reproduction cycle, which partially compensated for the reproduction loss due to the suppression of EcR/USP-1-mediaed ecdysteroid signaling from the point of view of time. Eating invalid eggsac also partially recovered the energy costs because maternal care was costly for females, even to invalid eggsacs (Ruhland et al. 2016b). In fields, some pesticides may disrupt the EcR/USP-mediaed ecdysteroid signaling and lead to the generating of the nonviable eggs, such as ecdysteroid analogues (Borchert et al. 2005; Zhang et al. 2021).

In summary, the suppression of ecdysteroid signaling severely constrained the population growth of *P. pseudoannulata* by generating the invalid eggsacs. The wolf spider forwardly selected to eat the nonviable eggs as a compensatory strategy. The spider adopted this compensatory strategy to reduce costs in population growth and consumption from carrying invalid eggsacs.

# Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Authors' contributions

ZMY and ZWL conceived and designed research; ZMY, YYY, and YW conducted experiments; ZMY analyzed data; ZMY wrote the original draft; NY and ZWL reviewed and edited the manuscript; ZWL provided fund resources. All authors read and approved the final manuscript.

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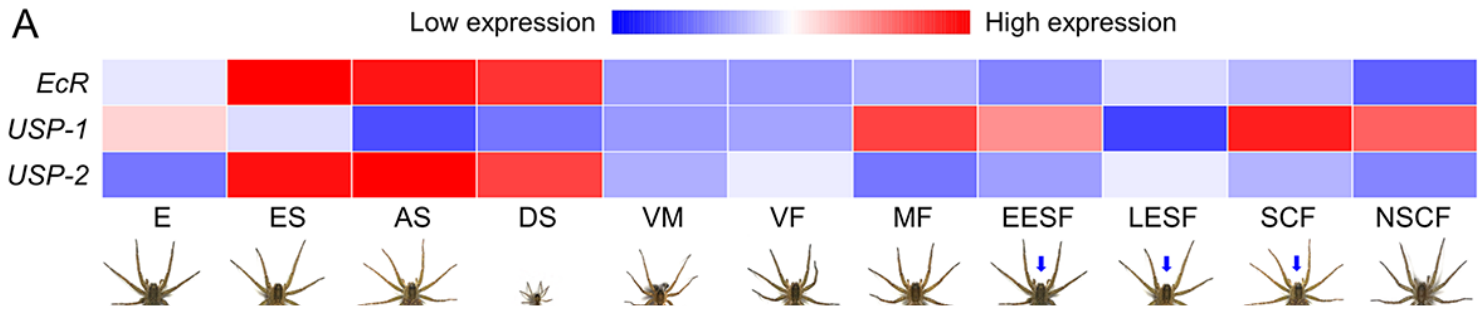
# Figures

### Figure 1

**Phylogenetic tree of NRs.** Drome, *Drosophila melanogaster* (King-Jones and Thummel 2005); Anoga, *Anopheles gambiae* (Bertrand et al. 2004); Trica, *Tribolium castaneum* (Bonneton et al. 2008; Tan and Palli 2008); Bommo, *Bombyx mori* (Cheng et al. 2008); Aedae, *Aedes aegypti* (Cruz et al. 2009); Acypi, *Acyrthosiphon pisum* (Christiaens et al. 2010); Nillu, *Nilaparvata lugens* (Xu et al. 2017); Tetur, *Tetranychus urticae* (Grbic et al. 2011); Panci, *Panonychus citri* (Li et al. 2017); Parps, *Pardosa pseudoannulata*. NRs with the same color form one subfamily.




## Figure 2

**Spatiotemporal expression of *EcR* and *USPs* in *P. pseudoannulata*.** (A) Spiders of eleven developmental stages. (B) The whole 2nd and newly-molted 3rd instar spiderlings. (C) Six tissues from adult spiders. E, egg (early-eggsac); ES, spiderling in eggsac (late-eggsac); AS, aggregated spiderling (spiderling carried by female); DS, dispersed spiderling (actively moving spiderling); VM, virgin male; VF, virgin female; MF, mated female; EESF, early-eggsac-carrying female; LESF, late-eggsac-carrying female; SCF, spiderling-carrying female; NSCF, non-spiderling-carrying female. The transcriptional level (i.e., FPKM value) of *EcR* and two *USPs* in (A) were derived from the average of three biological replicate samples (Table S3). Red arrows in (A) indicate eggsac or juvenile spiderling samples. Blue arrows in (A) indicate female samples. II0 and III0 represent the newly-molted 2nd and 3rd instar spiderlings, respectively. II1- II5 represent the 2nd instar spiderlings on day 1-5, respectively. Asterisk indicates the significant difference of the expression between two *USPs* analyzed by *t*-test at $P < 0.05$.

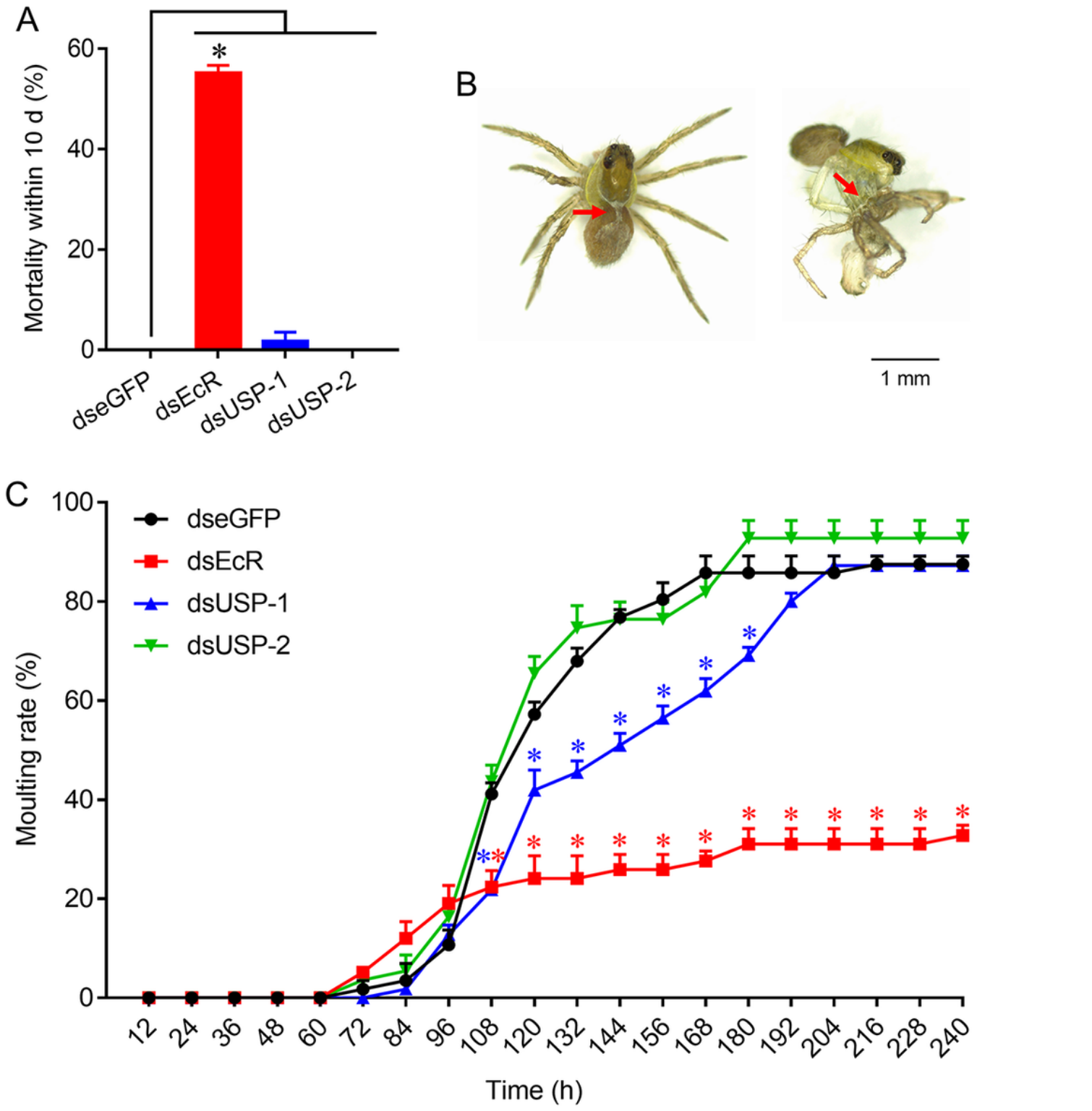



**Figure 3**

**Effects of knocking down *EcR* and *USPs* on the development of *P. pseudoannulata*.** (A) Mortality. (B) The dead spiderlings by *EcR* silencing. (C) Moulting rate. Red arrows in (B) indicate the abnormal moults. Asterisk indicates the significant difference of mortality and moulting rate between the dsEcR and dsUSP treatment and the control groups analyzed by one-way ANOVA with Tukey test at $P < 0.05$.

**Figure 4**

**Effects of knocking down *EcR* and *USPs* on the reproduction of *P. pseudoannulata*.** (A) Females injected with different dsRNAs. (B) The development of eggs. (C) The number of valid/invalid eggsacs. (D) Pre-oviposition period. (E) Egg number. (F) Egg weight. Black arrows in (A) indicate that a dseGFP- or dsUSP-2-treated female carrying her eggsac (left) and a dsEcR- or dsUSP-1-treated female eating her eggsac (right). Blue arrows in (B) indicate the developed appendages. Significant difference of the number of valid/invalid eggsacs between the dsEcR and dsUSP treatment and the control groups were analyzed by Fisher's exact test. Asterisk indicates the significant difference of pre-oviposition period, egg number, and egg weight between the dsEcR and dsUSP treatment and the control groups analyzed by one-way ANOVA with Tukey test at $P < 0.05$.

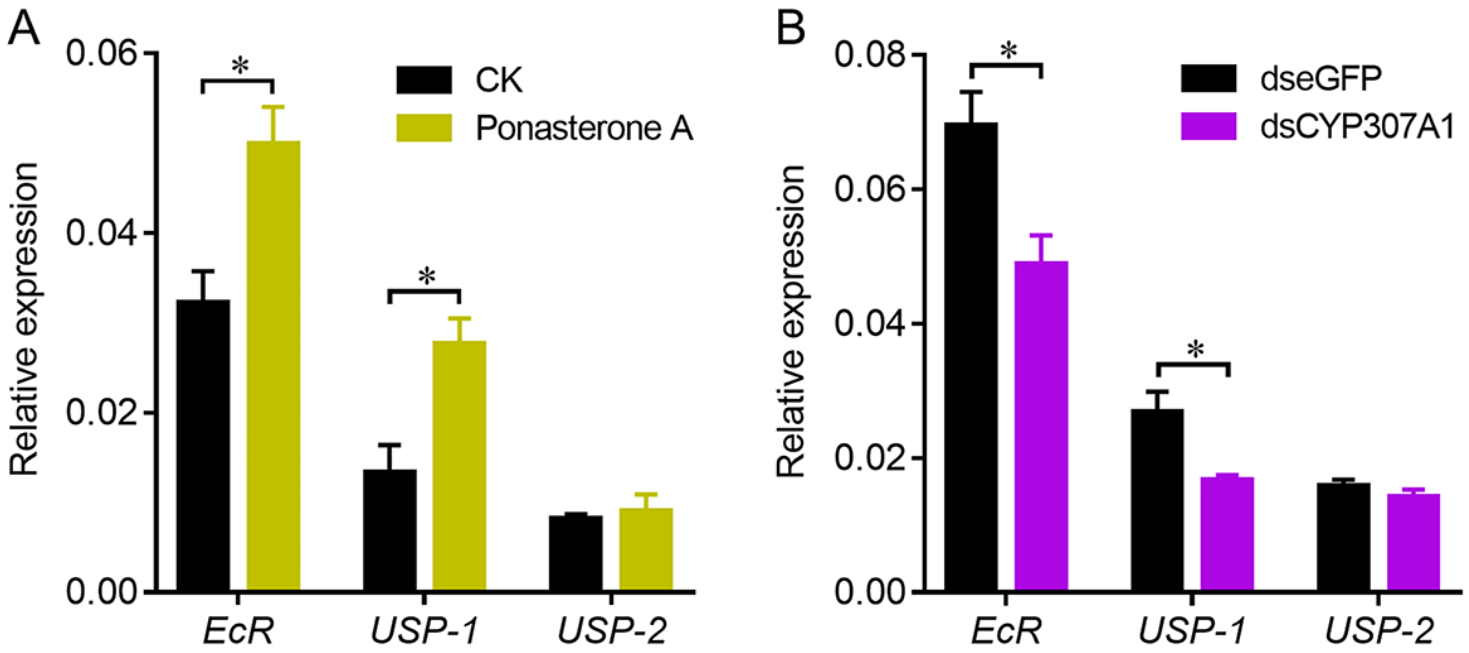



**Figure 5**

**The transcriptional level of *EcR* and *USPs* in *P. pseudoannulata* treated with ponasterone A (A) and dsCYP307A1 (B).** CK, 50% ethanol. Asterisk indicates the significant difference of the expression of *EcR* and two *USPs* between the treatment and the control groups analyzed by *t*-test at $P < 0.05$.

**Figure 6**

**Effects of *CYP307A1* knockdown on the development and reproduction of *P. pseudoannulata*.** (A) Moulting rate. (B) The development of eggs. (C) The number of valid/invalid eggsacs. (D) Pre-oviposition period. (E) Egg number. (F) Egg weight. Asterisk indicates the significant difference of moulting rate, pre-

oviposition period, egg number, and egg weight between the dsCYP307A1 treatment and the control groups analyzed by *t*-test at $P < 0.05$. Significant difference of the number of valid/invalid eggsacs between the dsCYP307A1 treatment and the control groups was analyzed by Fisher's exact test.

## Supplementary Files

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