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EcR/USP-1-mediated ecdysteroid signaling regulates wolf spider (*Pardosa pseudoannulata*) development and reproduction

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ABSTRACT

Lycosidae females demonstrate meticulous maternal care of offspring by carrying egg sacs and juvenile spiderlings during the reproductive stage. Nuclear receptors (NRs), especially the ecdysone receptor (EcR) and ultraspiracle (USP), have attracted considerable attention in the regulation of arthropod development and reproduction due to their pivotal roles in ecdysteroid signaling cascades. In the present study, 23 *NRs*, including one *EcR* and two *USPs*, were identified in the genome of the predatory wolf spider *Pardosa pseudoannulata*. RNA interference (RNAi) targeting *EcR* and *USP-1* inhibited spiderling development and resulted in non-viable eggs in the egg sacs. *EcR* and *USP-1* responded to changes in ecdysteroid levels, and interference in ecdysteroid biosynthesis led to similar phenotypes as dsEcR and dsUSP-1 treatments. These findings suggest that EcR/USP-1-mediated ecdysteroid signaling regulates *P. pseudoannulata* development and reproduction. The *P. pseudoannulata* females with suppressed ecdysteroid signaling proactively consumed their non-viable egg sacs, resulting in a 7.19 d shorter first reproductive cycle than the controls. Termination of the failed reproductive cycle enabled the spiders to produce a new egg sac more rapidly. This reproductive strategy may partially rescue the reduction in population growth due to non-viable eggs and compensate for the physiological expenditure of wasted maternal care, which would be beneficial for the conservation of *P. pseudoannulata* populations and their natural control of insect pests.

Keywords: *Pardosa pseudoannulata*; Ecdysone receptor; Ultraspiracle; Development; Reproduction

INTRODUCTION

Increasing egg numbers is an effective strategy for population expansion in arthropods. Some arthropods, such as spiders, provide extensive maternal attention to protect and improve the success of their offspring (Yip & Rayor, 2014), including egg sac construction and care and juvenile spiderling care (Ruhland et al., 2016b). For example, web weaving spiders attach their egg sacs to their webs, while Lycosidae females carry egg sacs in their spinnerets and newly emerged juveniles on their notum (Yang et al., 2018; Yu et al., 2022). Egg care is effective for protecting offspring from predators, parasites, and adverse environments (Iida & Fujisaki, 2005; Ruhland et al., 2016a; Vieira & Romero, 2008). *Pardosa pseudoannulata*, a wolf spider species that preys on various pest insects in agricultural fields, also displays meticulous

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maternal care. After egg sac production, the females carry their egg sacs for about 15 d until hatching and then carry the newly emerged spiderlings for 4–5 d before dispersal (Yu et al., 2022). Once they start mating, *P. pseudoannulata* females can produce up to five egg sacs in their lifetime (Yang et al., 2018). Although cannibalism is common in reproductive female arthropods, it is suppressed during maternal care (Yu et al., 2022), with ecdysteroids known to play critical roles in both maternal care and cannibalism (Trabalon et al., 1998; Vancassel et al., 1984).

We previously identified that Halloween genes are involved in ecdysteroid biosynthesis and that ponasterone A (PA) is an endogenous ecdysteroid in *P. pseudoannulata* (Yang et al., 2021). In arthropods, ecdysteroids act on a series of nuclear receptors (NRs) to perform various physiological functions. As a group of ligand-activated transcription factors, NRs are widely present in animals and function to regulate different biological processes (Christiaens et al., 2010). NRs are classified into seven subfamilies (NR0–NR6) containing two typical domains, i.e., a highly conserved DNA-binding domain (DBD) and a less conserved ligand-binding domain (LBD), except for the NR0 subfamily, which lacks the LBD (King-Jones & Thummel, 2005). Insect NRs have received considerable attention due to their roles in embryogenesis, molting, 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 & Thummel, 2005), it has been identified in an increasing number of insects based on whole-genome sequencing, including in *Anopheles gambiae* (21 members) (Bertrand et al., 2004), *Apis mellifera* (22 members) (Velarde et al., 2006), *Tribolium castaneum* (21 members) (Bonneton et al., 2008; Tan & Palli, 2008), *Bombyx mori* (19 members) (Cheng et al., 2008), *Aedes aegypti* (20 members) (Cruz et al., 2009), *Acyrthosiphon pisum* (19 members) (Christiaens et al., 2010), *Nilaparvata lugens* (20 members) (Xu et al., 2017), and *Bactrocera dorsalis* (21 members) (Yang et al., 2020). As important NR members, the ecdysone receptor (EcR) and ultraspiracle (USP)/retinoid X receptor (RXR) form a heterodimer that binds with ecdysteroids to initiate the signaling cascade (Christiaens et al., 2010; Thomas et al., 1993; Yao et al., 1993).

Identification and functional analysis of NRs in arachnids has been slower than work performed in insects. To date, systematic identification of the *NR* family has only been conducted on the *Tetranychus urticae* genome (30 members) (Grbić et al., 2011), with limited studies on other arachnids, but 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 previous research has focused on the functions of EcR and USP/RXR in arachnids. However, although a growing number of genomes have been sequenced, the *NR* family remains poorly investigated in spiders.

In the current study, we investigated the *NR* family in *P. pseudoannulata* based on genomic data. Both EcR and USP-1 were identified as significant members mediating ecdysteroid signaling in *P. pseudoannulata*. When EcR/USP-1-mediated ecdysteroid signaling was suppressed, female spiders produced egg sacs with non-viable eggs. To compensate for the impact on population growth due to the production of non-viable eggs, the females preemptively consumed the non-viable egg sac to promote the generation of a new egg sac within a short period.

MATERIALS AND METHODS

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

Putative *NRs* were retrieved from the chromosome-level *P. pseudoannulata* genome using orthologs of *Drosophila melanogaster* (King-Jones & Thummel, 2005), *Anopheles gambiae* (Bertrand et al., 2004), *Tribolium castaneum* (Bonneton et al., 2008; Tan & Palli, 2008), *Bombyx mori* (Cheng et al., 2008), *Aedes aegypti* (Cruz et al., 2009), *Acyrthosiphon pisum* (Christiaens et al., 2010), *Nilaparvata lugens* (Xu et al., 2017), *Bactrocera dorsalis* (Yang et al., 2020), *Tetranychus urticae* (Grbić et al., 2011), and *Panonychus citri* (Li et al., 2017) as queries using the BLAST tool (v2.7.1, downloaded from ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/). A neighbor-joining phylogenetic tree of NRs was constructed with 1 000 bootstraps in MEGA X (v10.0.5) (Kumar et al., 2018). The conserved domains of NRs were predicted using the NCBI Conserved Domain Database (Lu et al., 2020) and their structures were drawn using the Illustrator for Biological Sequences (IBS, v1.0.3) tool (Liu et al., 2015).

Spiders

We collected *P. pseudoannulata* spiders at multiple stages from paddy fields in Nanjing (China) in May 2020. The spiders were individually maintained in plastic cups (500 mL in volume) at 28±1 °C under a 16 h:8 h light/dark cycle and fed with *Nilaparvata lugens* until adulthood. Samples were collected from 11 developmental stages, including egg (E), spiderling in egg sac (ES), aggregated spiderling (AS), dispersed spiderling (DS), virgin male (VM), virgin female (VF), mated female (MF), early-egg sac-carrying female (EESF), late-egg sac-carrying female (LESF), spiderling-carrying female (SCF), and non-spiderling-carrying female (NSCF), for RNA sequencing (RNA-seq) at the Beijing Genomics Institute (Shenzhen, China) and 10 egg sacs or spiders were pooled as one sample. Newly molted 2nd instar spiderlings (II0), 2nd instar spiderlings 1–5 days post-molting (II1–II5), and newly molted 3rd instar spiderlings (III0) were harvested individually, with 10 spiderlings pooled as one sample. Six tissues, including brain, venom gland, intestine, fat body, ovary, and testis, were dissected from 20 adult females and males. All samples were prepared in three biological replicates. Spider images were taken using a Leica S9i stereomicroscope (Leica Microsystems, Germany).

RNA interference (RNAi)

Primers with T7 RNA polymerase promoter sequences for

double-stranded RNA (dsRNA) synthesis (Supplementary Table S1) were designed using Beacon Designer (v7.92, PREMIER Biosoft International, USA) and synthesized by GenScript (China), with dsRNA against enhanced green fluorescent protein (*eGFP*) used as the negative control. Specific fragments of target genes were amplified using Phanta® Max Super-Fidelity DNA Polymerase (Vazyme, China), then purified using a GeneJET Gel Extraction Kit (Thermo Scientific, USA) following the manufacturer's protocols. The dsRNA was synthesized using the T7 RiboMAX™ Express RNAi System (Promega, USA) according to the manufacturer's instructions. Microinjection of *P. pseudoannulata* was performed as per previous study (Meng et al., 2015). (1) RNAi in spiderlings. The II1 spiderlings were kept on an agar gel plate after anesthetization with carbon dioxide, after which dsRNA (50 ng in 10 nL of RNase-free water) was injected into each spiderling. The injected spiderlings were individually transferred to Petri dishes (3.5 cm in diameter) and fed with 2nd instar *Nilaparvata lugens* nymphs, with injured spiderlings removed within 12 h. The spiderlings were divided into two groups. Group I was used for gene quantification. Ten spiderlings were pooled as one sample at 48 h and three biological replicates were prepared. Group II was used for phenotypic observation. The number of molts and mortality rate were recorded at 12 h intervals until day 10. Each treatment was prepared in three biological replicates and each replicate contained 15–20 spiderlings. (2) RNAi in females. Mated females were kept on agar gel plates after anesthetization with carbon dioxide. Within 6 h of mating, dsRNA (1 μg in 200 nL of RNase-free water) was injected into each female. The injected females were individually transferred to plastic cups and fed with *Nilaparvata lugens* adults. The reproductive periods, including pre-oviposition period, egg sac-carrying period, spiderling-carrying period, and post-reproductive period, were recorded at 12 h intervals. The egg sac was weighed at 24 h after oviposition, then quickly returned to the female. The juvenile spiderlings hatched on day 6 after oviposition and remained in the egg sac for 9 d (Supplementary Figure S1). The egg sac was opened on day 7 after oviposition to check the developmental states of eggs and count the hatched spiderlings (viable) or unhatched eggs (non-viable). Each treatment group contained at least 100 females. Six females that had not oviposited eggs were individually harvested at 72 h for gene quantification.

PA application

Standard PA (purity>95%) was purchased from Cayman Chemical (USA) and dissolved in absolute ethanol to prepare a stock solution of 5 mg/mL. A working solution of 2.5 mg/mL was prepared with sterile water. Ethanol (50%) was used as the negative control. PA solution or ethanol was introduced by microinjection. Briefly, II1 spiderlings were maintained on an agar gel plate after anesthetization with carbon dioxide, after which 10 nL of PA solution or ethanol was injected into each spiderling. The injected spiderlings were individually transferred to Petri dishes and fed with 2nd instar *Nilaparvata lugens* nymphs. Ten spiderlings were pooled as one sample at 48 h and four biological replicates were prepared.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Trizol™ reagent (Invitrogen, USA), then used to synthesize cDNA with a PrimeScript RT Reagent Kit (TaKaRa, Japan) according to the manufacturer's instructions. Primers used for qRT-PCR (Supplementary Table S1) were designed using Beacon Designer and synthesized by GenScript. Elongation factor-1 alpha (*EF-1α*) and glyceraldehyde-3-phosphatedehydrogenase (*GAPDH*) were selected as the reference genes (Meng et al., 2015). QRT-PCR analysis was performed using a TB Green Premix Ex Taq II Kit (TaKaRa, Japan) following the manufacturer's instructions on the QuantStudio Real-Time PCR System (Applied Biosystems, USA). Each reaction was carried out with two technical replicates.

Data analysis

The relative expression levels of target genes were related to the geometric mean of two reference genes using the $2^{-\Delta CT}$ method (Livak & Schmittgen, 2001; Vandesompele et al., 2002). The gene expression level, molting rate, and mortality rate were presented as mean±standard error of the mean (*SEM*). The FPKM (fragments per kilobase of exon model per million mapped fragments) values of each gene in samples from the 11 developmental stages were retrieved from the normalized transcriptomes and normalized with scale function. The heatmap was constructed using R (v4.1.1, downloaded from https://cran.r-project.org/). Differences were analyzed by *t*-test, one-way analysis of variance (ANOVA) with Tukey test, and Fisher's exact test using GraphPad Prism (v7) (Swift, 1997), with P<0.05 considered significant.

RESULTS

Characterization of NRs in *P. pseudoannulata*

In total, 23 *NRs* were identified in the *P. pseudoannulata* genome. Phylogenetic analysis showed that they were distributed in six subfamilies, including seven in NR1, 10 in NR2, one in NR3, one in NR4, two in NR5, and two in NR6 (Figure 1A; Supplementary Table S2). Interesting, two *NR* duplicates were found in the 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*) genes (Figure 1A; Supplementary Table S2). All NRs contained the critical DBD and LBD, expect for SVP-2, which lacked the DBD (Figure 1B).

Spatiotemporal expression of *EcR* and *USPs*

We studied the functions of EcR and two USPs. Results showed consistent expression patterns between *EcR* and *USP-2*, with higher expression in spiderlings and lower expression in adults. Except for the late-egg sac-carrying females, *USP-1* exhibited marked expression in reproductive females, showing the opposite pattern to *EcR* and *USP-2* (Figure 2A; Supplementary Table S3). In 2nd instar spiderlings, the expression levels of *EcR* and the two *USPs* were relatively stable in the first four days but increased sharply on day 5 and then declined to a much lower level than that at the beginning once molting was completed (Figure 2B).

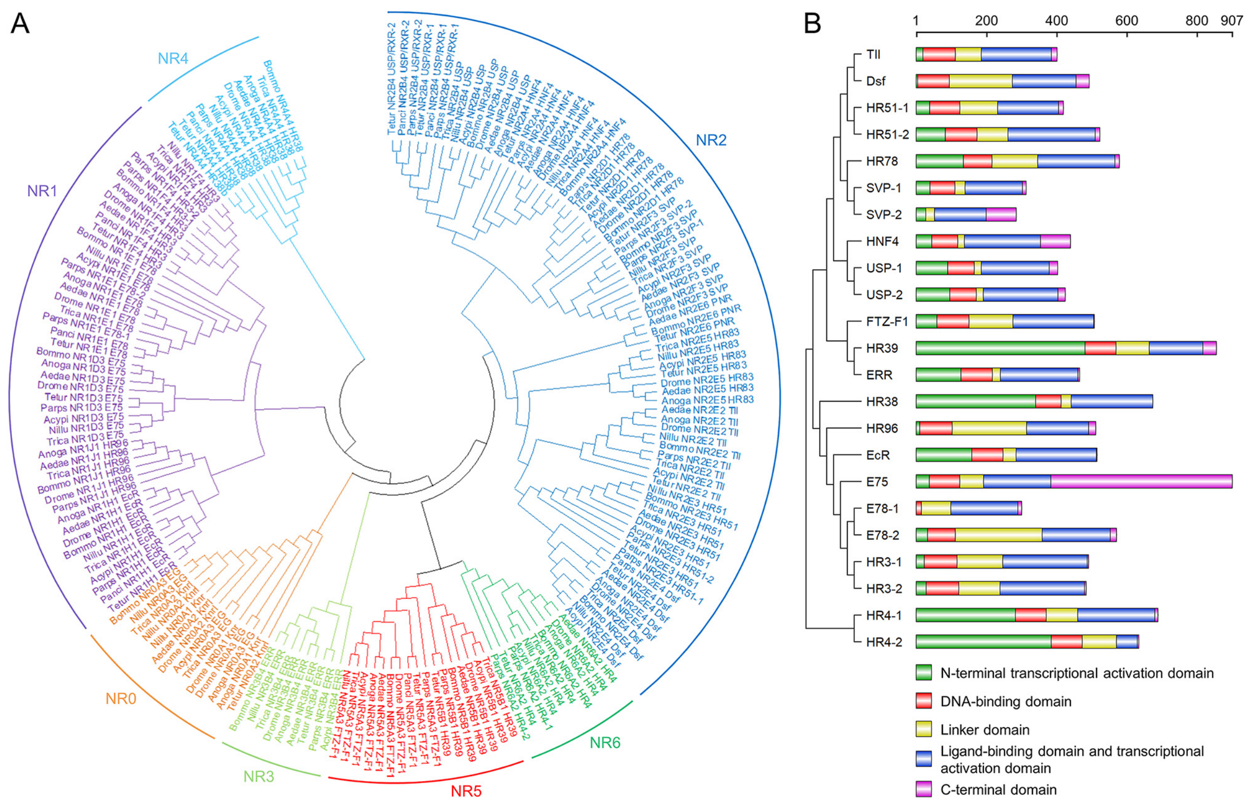


Figure 1 Phylogenetic tree (A) and domain organization (B) of NRs

Drome, *Drosophila melanogaster* (King-Jones & Thummel, 2005); Anoga, *Anopheles gambiae* (Bertrand et al., 2004); Trica, *Tribolium castaneum* (Bonneton et al., 2008; Tan & 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* (Grbić et al., 2011); Panci, *Panonychus citri* (Li et al., 2017); Parps, *Pardosa pseudoannulata*. Scale represents NR protein length (aa).

Spatially, *EcR* and two *USPs* were specifically expressed in all tested tissues, except for a small trace of *USP-2* in the fat body, ovary, and testis (Figure 2C).

Roles of EcR and USPs in *P. pseudoannulata* development

The *EcR* and two *USPs* were perturbed in the dsRNA-treated *P. pseudoannulata* spiderlings, and Halloween genes involved in ecdysteroid biosynthesis were synchronously down-regulated (Supplementary Figure S2A). In total, 55% of dsEcR-treated spiderlings died within 10 d from unsuccessful molting (Figure 3A, B). No significant mortality was observed in spiderlings injected with the dsRNA of the two *USP* genes (Figure 3A). Both *EcR* and *USP-1* silencing markedly affected molting in the spiderlings. The dsEcR-treated spiderlings showed reduced molting, while the dsUSP-1-treated spiderlings exhibited delayed molting (Figure 3C). *USP-2* knockdown did not affect spiderling development (Figure 3C).

Roles of EcR and USPs in *P. pseudoannulata* reproduction

The dsRNA targeting the *EcR* and two *USPs* significantly down-regulated target genes as well as Halloween genes in the mated females (Supplementary Figure S2B). The *EcR* and *USP-1* knockdown females consumed their own egg sacs (Figure 4A), which contained non-viable eggs (dsEcR treatment group) and both non-viable and partially developed eggs (dsUSP-1 treatment group) (Figure 4B). Both *EcR*- and *USP-1*-silenced females produced significantly higher proportions of non-viable egg sacs than the control group (Figure 4C) but performed as well as control females in terms of pre-oviposition (Figure 4D), egg number (Figure 4E), and egg weight (Figure 4F). *USP-2* knockdown significantly reduced egg number (Figure 4E) but had no effect on egg quality (Figure 4B, C).

Transcriptional response of *EcR* and *USPs* to changes in ecdysteroid

To verify whether the *EcR* and two *USPs* respond to ecdysteroid, we manipulated ecdysteroid levels in *P. pseudoannulata* by application of exogenous PA and RNAi of *CYP307A1*, a key gene involved in PA biosynthesis. Results showed that the transcriptional levels of *EcR* and *USP-1* were significantly up-regulated by PA application (Figure 5A) and remarkably down-regulated by *CYP307A1* knockdown (Figure 5B). However, *USP-2* did not respond to changes in ecdysteroid (Figure 5).

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

We also tested the effects of ecdysteroid biosynthesis suppression on *P. pseudoannulata* development and reproduction. The expression levels of *CYP307A1* and downstream Halloween genes in the ecdysteroid biosynthesis

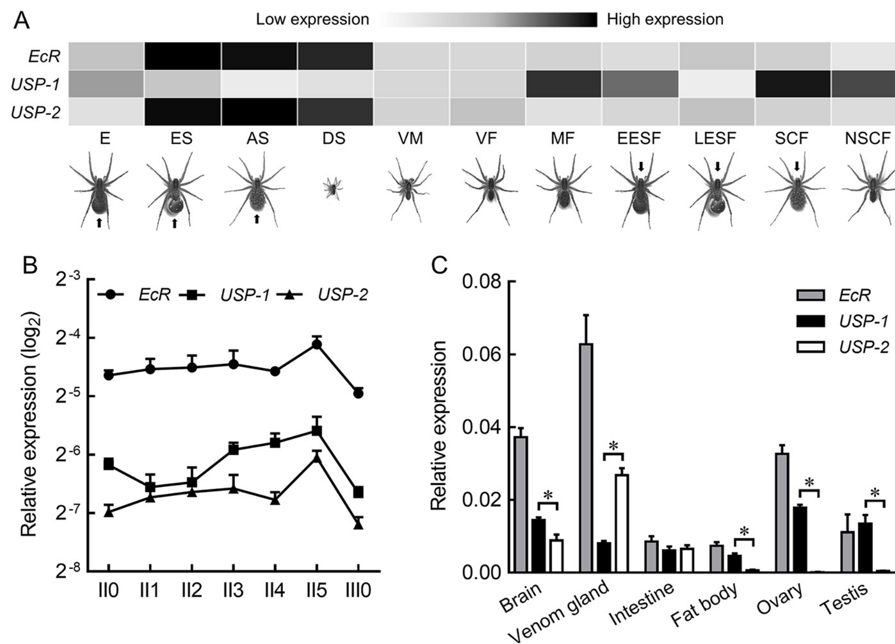


Figure 2 Spatiotemporal expression of *EcR* and *USPs* in *P. pseudoannulata*

A: Spiders at 11 developmental stages. E, egg (early-egg sac); ES, spiderling in egg sac (late-egg sac); AS, aggregated spiderling (spiderling carried by female); DS, dispersed spiderling (actively moving spiderling); VM, virgin male; VF, virgin female; MF, mated female; EESF, early-egg sac-carrying female; LESF, late-egg sac-carrying female; SCF, spiderling-carrying female; NSCF, non-spiderling-carrying female. Transcriptional levels (i.e., FPKM values) of *EcR* and two *USPs* in A were derived from average of three biological replicates (Supplementary Table S3). Upward arrows indicate egg sac or juvenile spiderling samples. Downward arrows indicate female samples. B: Whole 2nd and newly molted 3rd instar spiderlings. II0 and III0 represent newly molted 2nd and 3rd instar spiderlings, respectively. II1–II5 represent 2nd instar spiderlings on days 1–5, respectively. C: Six tissues from adult spiders. "*" indicates significant difference in expression between two *USPs* analyzed by *t*-test at $P<0.05$.

pathway were markedly suppressed in the dsCYP307A1-treated spiderlings (Supplementary Figure S3A) and females (Supplementary Figure S3B). The spiderlings molted from 96 h and plateaued at 180 h. From 96 to 144 h, the molting rate of the *CYP307A1*-silenced spiderlings was significantly lower than that of the control group (Figure 6A). Similar to the *EcR*- and *USP-1*-silenced females, the *CYP307A1*-silenced females consumed their non-viable egg sacs (Figure 6B), with a significantly higher proportion of non-viable egg sacs found in the dsCYP307A1 treatment group compared to the control (Figure 6C). In addition, *CYP307A1* knockdown did not affect the pre-oviposition period (Figure 6D), egg number (Figure 6E), or egg weight (Figure 6F).

Duration of reproductive cycle in *P. pseudoannulata* females

In the first reproductive cycle, the *P. pseudoannulata* females produced a new egg sac, including the egg sac-carrying females from the control group (dseGFP), which laid viable egg sacs, and the egg sac-eating females from the treatment groups (dsEcR, dsUSP-1, and dsCYP307A1), which laid non-viable egg sacs. In the control group, females carried their first egg sac for 14.72 d followed by their spiderlings for 4.40 d. After 5.94 d of post-reproductive preparation, the females laid a new egg sac (Table 1). In the treatment group, the mothers consumed their egg sacs after carrying them for 9.36 d and remained in the post-reproductive stage for 8.51 d before the next oviposition period (Table 1). Thus, the intervals between the two egg sacs were 25.06 and 17.87 d in the control and treatment groups, respectively.

DISCUSSION

In the present study, we identified 23 *NRs* in the *P. pseudoannulata* genome, with their deduced proteins found to contain the critical NR domains. Based on phylogenetic analysis, the *NRs* were grouped into six subfamilies (excluding the NR0 subfamily). This is the first complete identification and characterization of the *NR* family in spiders. The number of *NRs* in *P. pseudoannulata* is close to that reported in insects (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 & Thummel, 2005; Tan & Palli, 2008; Velarde et al., 2006; Xu et al., 2017; Yang et al., 2020). Interestingly, each *NR* has been found with a single copy in investigated insects, except for duplicates of the tailless gene (*Tll*) in *Anopheles gambiae* (Bertrand et al., 2004) (Supplementary Table S4), whereas six *NRs* in *P. pseudoannulata* were found with two duplicates, including *E78*, *HR3*, *USP*, *HR51*, *SVP*, and *HR4*. *NR* duplication may be relatively common in arachnids. For example, *Tetranychus urticae* contains eight hormone receptor-like in 96 genes (*HR96s*), two *USP/RXRs*, and two hormone receptor-like in 38 genes (*HR38s*) (Grbić et al., 2011) (Supplementary Table S4), while both *Amblyomma americanum* (Guo et al., 1998) and *Panonychus citri* (Li et al., 2017) contain two *USP/RXRs*.

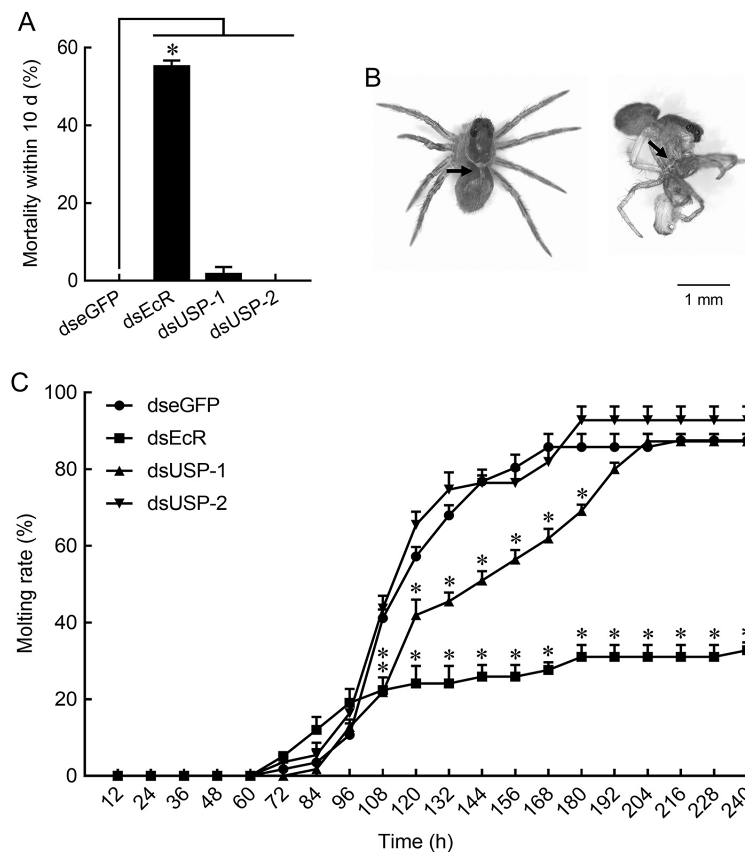
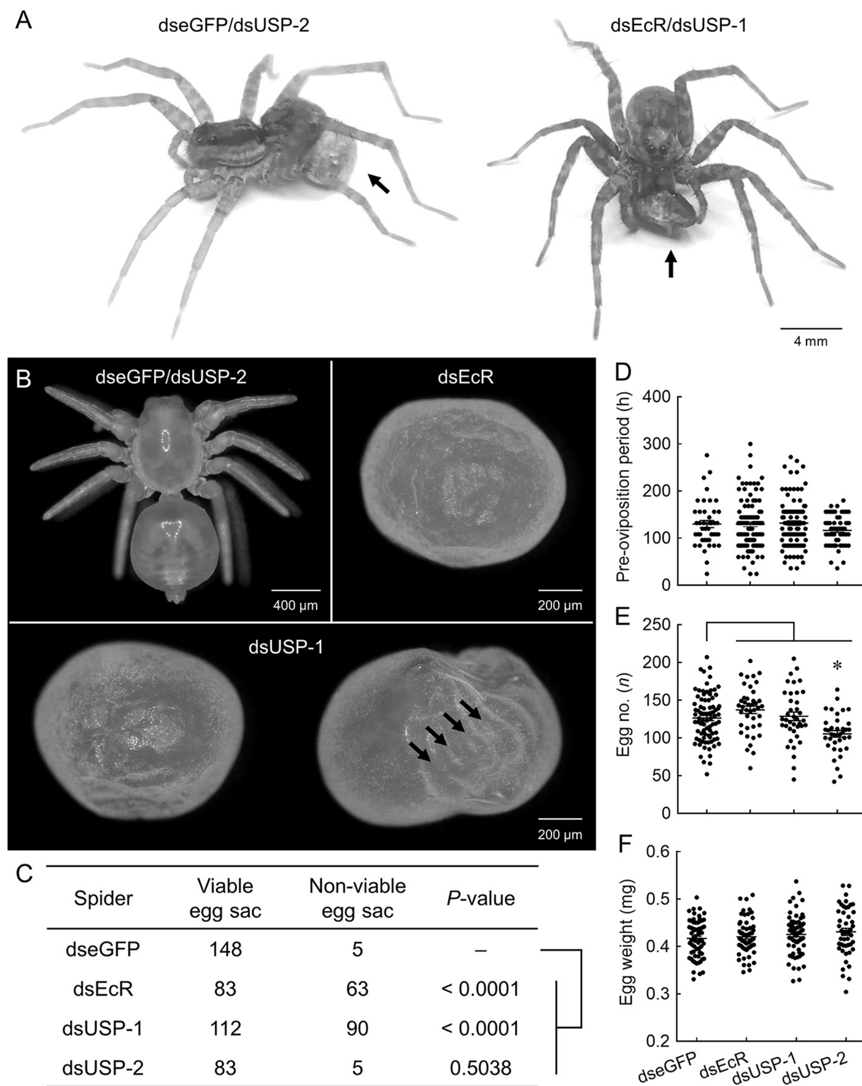


Figure 3 Effects of *EcR* and *USPs* knockdown on *P. pseudoannulata* development

A: Mortality. B: Dead spiderlings by *EcR* silencing. Arrows indicate abnormal molts. C: Molting rate. "*" indicates significant difference in mortality and molting rates between dsEcR and dsUSP treatment and control groups, analyzed by one-way ANOVA with Tukey test at *P*<0.05.

Functional differentiation among NR duplicates in arachnids, especially USP/RXRs, has received growing attention. Earlier research on *Amblyomma americanum* was the first to explore the functions of USP/RXR (Guo et al., 1998). Previous studies have also shown that although the transcript levels of *EcR* and *USP/RXRs* are correlated with ecdysteroid titers at developmental stages, EcR/USP/RXR-1 but not EcR/USP/RXR-2 exhibits broad DNA binding specificity (Palmer et al., 2002). In *Panonychus citri*, *EcR* shows similar temporal expression patterns to *USP/RXR-2* but different temporal patterns to *USP/RXR-1*, and differential expression genes in deutonymphs indicate that EcR/USP/RXR-2 and USP/RXR-1 may regulate different physiological processes to control molting (Li et al., 2022). Furthermore, based on *EcR* and *USP/RXR* cloning in *Liocheles australasiae*, EcR is reported to show high binding ability to PA, while USP/RXR does not enhance binding ability (Nakagawa et al., 2007). USP/RXR is required for the binding of EcR to ecdysteroids (Thomas et al., 1993; Yao et al., 1993); however, whether another USP/RXR in *Liocheles australasiae* plays this role remains to be explored. We thoroughly quantified the spatiotemporal expression of *EcR* and two *USPs* in *P. pseudoannulata*. The *EcR* and two *USPs* showed expression patterns consistent with Halloween genes, with high expression in 2nd instar spiderlings and a rapid decline after molting (Yang et al., 2021), suggesting potential involvement in the molting process. The *EcR* and *USPs* exhibited distinct expression patterns in the 11 developmental stages, with *EcR* and *USP-2* showing significant expression in spiderlings and *USP-1* showing significant expression in adult females. Furthermore, the *EcR* and two *USPs* were highly expressed in the six tissues obtained from adults, except for *USP-2*, which was almost undetectable in the fat body, ovary, and testis. These results strongly suggest functional differentiation between the two USPs in *P. pseudoannulata*. Based on RNAi, *EcR* and *USP-1* knockdown significantly inhibited molting in the spiderlings and increased the number of non-viable egg sacs in females, while development and reproduction in *P. pseudoannulata* were not affected by dsUSP-2 treatment, except for a reduction in egg number. In the present study, the two USPs exhibited differential functions in the reproduction and development of *P. pseudoannulata*, while the *NR* duplications provided a comprehensive network for gene expression regulation in arachnids.

In addition, expression responses of *EcR* and two *USPs* were detected by changes in ecdysteroid. *EcR* and *USP-1* were up-regulated by PA application and down-regulated by *CYP307A1* silencing, while *USP-2* expression was not disturbed by ecdysteroid changes. Knockdown of *CYP307A1* delayed molting in spiderlings and caused non-viable eggs in females. The phenotypes in spiderlings and females treated with dsCYP307A1 were similar to the dsEcR and dsUSP-1



Spider	Viable egg sac	Non-viable egg sac	*P*-value
dseGFP	148	5	–
dsEcR	83	63	< 0.0001
dsUSP-1	112	90	< 0.0001
dsUSP-2	83	5	0.5038

Figure 4 Effects of *EcR* and *USPs* knockdown on *P. pseudoannulata* reproduction

A: Egg sac-carrying and egg sac-eating females. Arrows indicate dseGFP- or dsUSP-2-treated female carrying her egg sac (left) and dsEcR- or dsUSP-1-treated female eating her egg sac (right). B: Egg development. Arrows indicate developed appendages. C: Number of viable and non-viable egg sacs. Significant difference in number of viable/non-viable egg sacs between dsEcR and dsUSP treatment and control groups were analyzed by Fisher's exact test. "–" represents not available. D: Pre-oviposition period. E: Egg number. "*" indicates significant difference in egg number between dsEcR and dsUSP treatment and control groups, analyzed by one-way ANOVA with Tukey test at $P<0.05$. F: Egg weight.

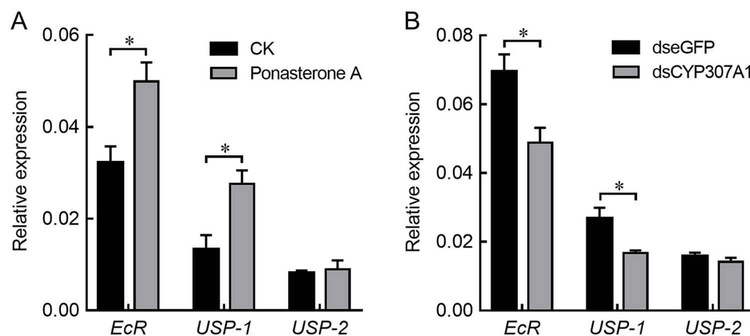
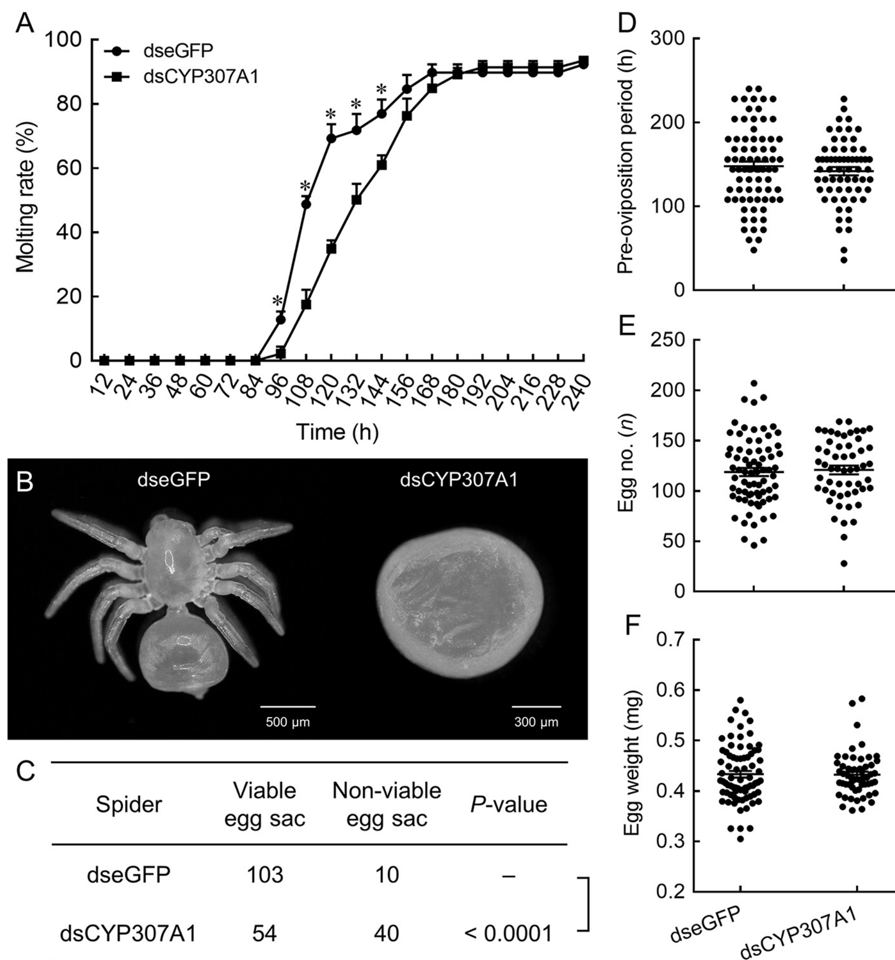


Figure 5 Transcriptional level of *EcR* and *USPs* in *P. pseudoannulata* treated with ponasterone A (A) and dsCYP307A1 (B)

CK, 50% ethanol. "*" indicates significant difference in expression of *EcR* and two *USPs* between treatment and control groups, analyzed by *t*-test at $P<0.05$.



Spider	Viable egg sac	Non-viable egg sac	*P*-value
dseGFP	103	10	–
dsCYP307A1	54	40	< 0.0001

Figure 6 Effects of *CYP307A1* knockdown on *P. pseudoannulata* development and reproduction

A: Molting rate. "*" indicates significant difference in molting rate between dsCYP307A1 treatment and control groups, analyzed by *t*-test at P<0.05. B: Egg development. C: Number of viable and non-viable egg sacs. Significant difference in number of viable/non-viable egg sacs between dsCYP307A1 treatment and control groups was analyzed by Fisher's exact test. "–" represents not available. D: Pre-oviposition period. E: Egg number. F: Egg weight.

treatment groups. Therefore, we propose that EcR/USP-1 mediated ecdysteroid signaling to regulate development and reproduction in *P. pseudoannulata*. The ecdysteroid 20-hydroxyecdysone can up-regulate vitellogenin in the *Parasteatoda tepidariorum* spider (Bednarek et al., 2019). However, the non-viable eggs produced in the ecdysteroid signaling-suppressed *P. pseudoannulata* females may not be associated with vitellogenesis, as the expression levels of vitellogenin and its receptor are not affected by ecdysteroid application (Yang et al., 2022). Thus, further exploration of the mechanisms underlying non-viable egg production is required. Pesticides used in farm fields, including ecdysteroid analogue pesticides, may disrupt EcR/USP-mediated ecdysteroid signaling, leading to the generation of non-viable eggs (Borchert et al., 2005; Zhang et al., 2021). Therefore, reasonable selection of pesticides should be considered to protect natural enemies, i.e., spiders, during insect pest control.

To reduce unnecessary maternal care, *P. pseudoannulata* females in the dsEcR, dsUSP-1, and dsCYP307A1 treatment groups, in which EcR/USP-1-mediated ecdysteroid signaling was suppressed, ingested their non-viable egg sacs. Wolf spiders, including *P. pseudoannulata*, express maternal care by carrying egg sacs and juvenile spiderlings (Ruhland et al., 2016b). Spider mothers can detect the status of their juveniles and open the egg sac to release the spiderlings at specific times, with the exact time of opening partially dependent on stimuli from inside the egg sac (Ruhland et al., 2019; Viera et al., 2007). In this study, the ecdysteroid signaling-suppressed *P. pseudoannulata* females ate their non-viable egg sacs 9.36 d after egg production, when the control group spiderlings were 3 days post-hatching. In addition, egg sac exchange between the dseGFP and dsUSP-1 treatment groups showed that dseGFP-treated females consumed the non-viable egg sacs from the dsUSP-1-treated females, but not vice versa (data not shown). These results suggest that *P. pseudoannulata* mothers actively eat their non-viable egg sacs, possibly due to a failure to receive accurate signals from the eggs rather than to changes in the females themselves. As non-viable eggs are of no reproductive benefit, the mothers

Table 1 Reproductive periods of egg sac-carrying and egg sac-eating females

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

may have selected to eat the non-viable egg sacs to preemptively terminate the non-reproductive cycle and generate a new egg sac, thereby shortening the time spent caring for non-viable eggs and partially compensating for reproductive loss due to the suppression of EcR/USP-1-mediated ecdysteroid signaling. Ingestion of inactive eggs may also partially recover energy costs otherwise spent on costly maternal care (Ruhland et al., 2016b). In conclusion, inhibition of ecdysteroid signaling severely constrains *P. pseudoannulata* population growth by generating non-viable eggs. In this case, female spiders proactively consume non-viable eggs to initiate the production of a new egg sac, thus compensating for reduced population growth.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Z.M.Y. and Z.W.L. designed and supervised the study. Z.M.Y., Y.Y.Y., and Y.W. performed the experiments. Z.M.Y. analyzed the data and wrote the manuscript. N.Y. and Z.W.L. revised the manuscript. All authors read and approved the final version of the manuscript.

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