Does Only Egg Sac Protect Spider Embryos From Pathogens? Detection Of Antibacterial Proteins In Embryos of Theridiidae And Lycosidae Representatives

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Research Article

Keywords: antibacterial proteins, lysozyme, defensins, spider, embryos

Posted Date: April 28th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-447883/v1

Abstract

Cocoons covering spider embryos may constitute a physical barrier, protecting eggs from microbial infections. The aim of the study was to find out if the embryos have their own immune potential. We test the effect of cocoon deprivation on the level of antimicrobial proteins (AMPs) produced by spider embryos of *Parasteatoda tepidariorum* and *Pardosa* sp. Eggs in the age from 24 to 168 hours were divided in two experimental groups: C (closed, in untouched cocoon) and O (open, embryos isolated from the egg sac). Results indicate that the tested spiders embryos produce lysozyme, defensins and potentially other low-molecular-weight proteins with antimicrobial activity. Level of AMPs increased with the age of spider embryos. Lysozyme in both species was produced at a higher level than defensins. Deprivation of cocoon results in increased production of lysozyme only in *Pardosa* sp., which may be related to the specific type of parental care of lycosids.

Introduction

The spider's immune system is innate and nonspecific. It consists of hemocytes - hemolymph cells derived from myocardial cells of the heart wall. Pathogens are combated through cellular responses as phagocytosis, melanization, encapsulation and via humoral reaction - antibacterial proteins production [1,2].

Several types of hemocytes are distinguished: prohemocytes, plasmatocytes, granulocytes, spherulocytes, leberidocytes, oenocytes and cyanocytes. The percentage ratios of the hemocytes types vary, depending on the stage of development and the species of spider [1,3,4]. In adult spiders, granulocytes are the most common type of hemocytes [1]. Mature hemolymph cells are capable of phagocytosis, but only granulocytes have the ability to produce antimicrobial proteins (AMPs). The production of antimicrobial proteins is the most precise tool of the spider's immune system [1,2].

There are 35 known antibacterial peptides in spiders [5]. These proteins are low (several kilodaltons) molecular weight [6]. Some AMPs occur in venom, while other are present in the hemolymph. Antimicrobial proteins expression in spider`s hemocytes is constitutive and, in contrast to the insects, they do not have pathogen recognition molecules (bGRP) [7].

Out of 42 known spider AMPs, 35 have antibacterial properties. The antifungal proteins are also a large group, and there are 20 known of them. In addition, 3 detected proteins have antiparasitic properties, 2 are anticancer and 1 is antiviral [5].

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anticancer and 1 is antiviral [5]. Antibacterial proteins were detected in the bodies of adult representatives of various species of spiders. These proteins are named after their species names. There are, among others, lycotoxins [8] from *Lycosa carolinensis*, gomesin [9] and acanthoscurrin [10] from *Acanthoscurria gomesiana*, as well as oxyopinin [11] from *Oxyopes kitabensis* or cupiennin 1 from *Cupiennius salei*[12].

The most widespread antimicrobial peptides are lysozyme and defensins. They are found in both vertebrates and invertebrates [13]. Importantly, from the point of view of our study, they have been detected in adult forms of spiders [14,15].

Lysozyme is a 14.4 kDa protein, capable of hydrolyzing β-1,4-glycosidic linkages between N-acetylmuraminic acid and N-acetylglutaminase. It is particularly effective against Gram-positive bacteria due to their lack of an external cell membrane, which facilitates the enzyme reaching the appropriate bonds. The range of action of lysozyme is not limited to Gram-positive bacteria. It also has a lytic effect against Gram-negative bacteria, fungi and viruses [13]. Lysozyme was detected in digestive fluids from adult individuals of *Stegodyphus mimosarum* spiders [15].

Defensins (3–5 kDa) are a large group of antibacterial proteins found in spiders. They were isolated so far from 5 species of spiders: *Cupiennius salei*, *Phoneutria reidyi*, *Polybetes pythagoricus*, *Tegenaria atrica* and *Meta menardi*. Expression of genes encoding defensins occurs not only in hemocytes, but also in the ovaries, midgut gland and muscle tissue. Most defensins are mainly active against Gram-positive bacteria. Defensins in the *C. salei* species are constitutively produced at a very low level. Increased expression begins only after the pathogen invasion [14].

Adult spiders immune responses are based on hemocyte responses, but spiders in early developmental stages have a physical barrier against pathogens. This function is performed by the cocoon (egg sac) made of spider silk, which is characterized by its unique properties, becoming a universal and multifunctional tool. Spiders use silk to make cobwebs, to wrap preys, belay during moving or to protect eggs [16]. The protective function performed by cocoons relies on both camouflaging the embryos and protecting the offspring against mechanical injuries [17]. The properties and purpose of the spider silk depend on the type of silk gland by which it was made. There are seven types of the glands: major, minor, pyriform, aggregate, tubulliform, flagelliform and aciniform. Each gland produces fibers that differ in viscosity, stiffness and extensibility. Egg sacs are produced by tubulliform and aciniform glands [18]. Despite such an important role, data on this subject are scarce, and our previous work is one of the few available sources. Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) showed two different layers that protect eggs, in the egg sacs of spider *Parasteatoda tepidariorum.* The inner layer, in direct contact with the embryos, is composed of loosely woven threads of silk. The outer layer has got significantly densely packed threads, and an amorphous disks and droplet-like spherites appear between them. Furthermore, no bacterial cells were observed on either layer [19]. Research by Wright and Goodacre, (2012) indicates that the web produced by the spider *Tegenaria domestica* has an inhibitory effect on the growth of Gram (+) bacteria *Bacillus subtilis*. The lack of observed

microorganisms in the cocoon structure confirms the antibacterial silk property and bacterial growth tests showed that no cultivable bacteria had grown in spider eggs samples [19].

One of the main role of the cocoon is to provide an antibacterial barrier to developing spider offspring. This can be important for the development of the immune defense of embryos, shifting energy costs to, for example, growth and organogenesis. However, at the moment, there is a lack of data on the formation and activity of the immune system of spider embryos.

There are several papers on the immunology of invertebrate embryos. Gorman et al. (2004) checked whether early staged eggs of tobacco hornworm *Manduca sexta* have the ability to generate an innate responses. It has been shown that even at very early stages the developing eggs produce many proteins involved in the processes of defense against pathogens. Among others, the detected peptides included lysozyme, pro-phenoloxidase, pro-paralytic enzyme and peptidoglycan recognition protein.

Antibacterial enzyme production, phagocytosis, encapsulation or coagulation are sufficiently effective methods to fight harmful microorganisms in the case of imago forms, however, there is no data on the immune response of spider embryos.

The protective properties of the cocoon may not be sufficient, so probably developing embryos must also have their own resistance. Therefore, the primary purpose of this work is to try to answer the question of whether embryos produce lysozyme and/or defensins.

To explain the nature of any changes in the level of possible humoral defense, we tested these four working hypotheses:

1. The level of lysozyme and defensins increases with age of the spider embryo, due to the progressive process of organogenesis, including an increasing volume of hemolymph, midgut glands and venom glands. There are time points in which the synthesis of AMPs starts and intensifies.
2. The antibacterial proteins level differ between embryos enclosed in the cocoon and those that have been isolated from the egg sac. Isolating eggs from a cocoon results in higher exposure to microorganisms, including potential pathogens.
3. The lysozyme and defensins levels in embryos differ between representatives of the Theridiidae and Lycosidae. This is due to the difference in the way of life, primarily the way of care for the offspring, but also the hunting strategy and habitat.
4. Based on high variability of antimicrobial peptides in animals (and within the group of spiders), lysozyme and defensins are not the only antimicrobial peptides detectable in the embryos of the selected species.

Results

SDS-PAGE electrophoresis

Parasteatoda tepidariorum:

Bands at about 14,2 kDa are visible on the polyacrylamide gel. That protein mass corresponds to the molecular weight of lysozyme – 14,4 kDa. The protein profile of *P. tepidariorum* demonstrate also the presence of proteins with a molecular weight about 3 kDa, which may indicate presence of defensins, as the mass of these proteins is from 2–4 kDa (Fig. 1a).

In tested samples also other low molecular weight peptides have been shown. Noticeable bands are observed at the level of 22–23 kDa, 18–19 kDa, 16 kDa, 7 – 6 kDa and around 1 kDa (table 1).

Pardosa sp.:

Protein profile of *Pardosa* sp. demonstrate bands at 14,2 kDa and around 3,5 kDa, corresponding respectively to lysozyme and defensins masses. Bands of those two masses differ in colour intensity. Hypothetically lysozyme stripes are more intense (Fig. 1b).

Apart from the bands at 14,2 and 3,5 kDa, there is a presence of other low-molecular weight peptides at weights of: 26 kDa, 22–23 kDa, 17–18 kDa, 15–16 kDa and, in some samples, around 1 kDa. Those peptides may potentially have an antimicrobial activity, due to their low-molecular weight.

Table 1
Molecular masses of unidentified proteins found in profiles protein of studied spiders' species.

Spider	*Parasteatoda tepidariorum*	*Pardosa* sp.
Molecular masses of protein profile bands	22–23 kDa	26 kDa
	19 – 18 kDa	22–23 kDa
	16 kDa	17–18 kDa
	14 kDa	15–16 kDa
	7 – 6 kDa	14 kDa
	3 kDa	10 kDa
	1 kDa	3 kDa
		1 kDa

PCR

As a result of the PCR reaction, the presence of the gene encoding lysozyme was observed in all samples apart from the youngest, freshly laid eggs. Expression of the gene encoding lysozyme was confirmed in both tested species.

In the case of defensins, the results are ambiguous. Bands of greater intensity were observed in the species *Pardosa* sp. In *P. tepidariorum* in both age groups of younger and older embryos no defensin

encoding gene was detected (Fig. 2).

ELISA test

In the *P. tepidariorum* species (a), differences in protein levels are observed only in the case of lysozyme. There is a visible increase in the level of lysozyme with the age of the embryos. In 168 h embryos and juveniles, lysozyme was at the same level. In the case of defensins, no statistically significant differences were found. In the examined embryos, the level of these proteins is the same, regardless of embryo age (Fig. 3a).

In the case of *Pardosa* sp. (b), both lysozyme and defensins show statistically significant differences between the studied age groups of embryos. The level of both proteins increases with the age of the spiders and the highest results are obtained in the oldest age groups. In the 168h eggs and in juveniles, the level of defensins is comparable (Fig. 3b).

Statistically significant differences of the lysozyme level between *P. tepidariorum* and *Pardosa* sp. are observed only in juvenile experimental group (Fig. 4a). Embryos groups produce this protein in a similar level in both *P. tepidariorum* and *Pardosa* sp. In the case of defensins, all experimental groups in both tested spiders are statistically different (Fig. 4b).

In *P. tepidariorum* there are significant differences between O and C embryos in the age of 72 h and 168 h in the case of lysozyme level (Fig. 5a). Defensis are on a low level and no significant differences was observed (Fig. 5b).

In *Pardosa* sp. embryos in O and C cocoons there are significant differences between levels of both detected antimicrobial proteins: in 96 h and 168 h age groups as for the presence of lysozyme and in 48 h and 96 h in defensins case (Fig. 5c,d).

Discussion

Despite the very effective protection, the egg sac provides that a universal and efficient immune system must be formed during the development of embryos. Therefore, it was assumed that embryos might produce antimicrobial peptides early enough to be ready for contact with pathogens immediately after leaving the cocoon.

The results of our research confirm that spider embryos have the ability to produce antibacterial proteins, at least lysozyme and defensins. Their response to pathogens relies largely on the physical protection of the cocoon, but embryos of the studied species also have their own immune potential. So far, the presence of several antibacterial proteins in arachnid juveniles has been detected. The importance of an efficient immune system in non-adult spiders can be seen in a study performed on wolf spider *Schizocosa ocreata* (Lycosidae). Bacterial infection of subadult spiders resulted in stronger immune responses after they reached maturity. Moreover, it resulted in a weaker body condition and lower mating success. This suggests that infections at the juvenile stages are partially consuming developmental

energy resources to defend against microbes [22]. However, there is a lack of such data on the impact of bacterial infections in the embryonic stages of spider development. The invasion of pathogens may occur in the case of mechanical injuries to the egg-containing cocoon or the already hatched juvenile spiders.

According to [23], spiders reproduce only once in their lifetime, such as *Pardosa agrestis*, as mature individuals invest more energy in reproduction than in immune responses. In contrast, subadults of that species have stronger bacterial cell wall lytic capacity compared to adults. It proves that the spiders' immune system is more active before it reaches maturity. The greater the need for a detailed study of the defense potential of earlier stages of spider ontogenesis seems to be. Probably during the development of all organ systems, necessary for the proper functioning of the body, a large part of energy resources will be allocated to these processes. However, there are no data available showing immune system changes at an earlier stage of development than the subadult stage.

Multistage tests showed neurotoxic properties in *L. tredecimguttatus* eggs and spiderlings. Several latroeggtoxins, biologically active neurotoxins that work against mouse and insect tissues, have been isolated. Moreover, one of the proteinaceous components - Latroeggtoxin-IV, with a molecular weight of 3.6 kDa, has antibacterial properties against 5 species of bacteria: *Staphylococcus aureus*, *Salmonella typhimurium*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* [24]. This toxin inhibits the growth of both G (-) and G (+) bacterial colonies, which means that it has a fairly broad spectrum of activity. Homogenates made from black widow spider eggs are rich in proteins, both high-molecular-mass and low-molecular peptides. Our study also confirmed the presence of the latter. According to research by Li et al. (2012), some of them may be toxic components analogous to the venom of adult spiders. It includes neurotoxic compounds and proteins with antimicrobial properties [26].

Antibacterial proteins are found mainly in the hemolymph of spiders [27]. With embryonic development and progressive organogenesis, the number of prohemocytes and then the mature forms of hemolymph cells capable of producing AMPs also increases. Antibacterial proteins level can therefore increase during ongoing embryos development. The results of our studies carried out on various age groups of *P. tepidariorum* and *Pardosa* sp. eggs show that the level of antibacterial proteins increases with the age of spider embryos. These results are consistent with studies carried out on other arthropods. Research carried out on *Litopenaeus vannamei* shrimps during their ontogenetic development shows that immune-related genes are transcribed already in freshly laid eggs of these crustaceans. However, the translation of these genes occurred to varying degrees at the ontogenetic stages studied. The genes of the antimicrobial substances appeared to be translated in the shrimp larvae and thus in the early stages of development. It should also be noted that some transcripts were detected at significant levels in eggs 0–4 hours after laying. It indicates those genes to be vertically transmitted from parents [28].

However, it is possible that in the female reproductive tract, during the laying of eggs, a certain amount of immunological molecules is passed on to the offspring. Such immunity passed on through maternal transfer, exists in the invertebrate *Daphnia magna*. Offspring of mothers exposed to a specific strain of

pathogenic bacteria *Pasteuria ramosa* showed high survival after exposure to this strain. When juveniles were contacted with another strain of this bacterium, a greater percentage of the offspring did not survive [29]. Therefore, there is a possibility that in the early stages of development, spider embryos rely to some extent on the immunological resistance acquired by maternal transfer.

In our research, in spiders of the genus *Pardosa* sp., there is a correlation between the age of the embryos and the increasing level of lysozyme and defensins. However, in the case of *P. tepidariorum*, no such trend was observed. This may be related to the lifestyle and parental care of these species belonging to two very different Araneae families in these respects. In the studied groups, in the case of *P. tepidariorum* (Theridiidae), the presence of lysozyme was demonstrated, but no defensins were detected. On the other hand, in *Pardosa* sp. (Lycosidae), both tested proteins were present. This indicates the possible influence of the extremely different types of parental care of the studied spiders.

Pardosa sp. belongs to the genus Lycosidae, which is characterized by exceptionally strong parental care. The female does not part with her offspring from the moment of laying eggs. Mother attaches the cocoon to the spinnerets and does not disconnect from it, even while actively hunting or escaping from predators. Lycosidae genus usually forms only one cocoon after mating in the breeding season [30].

Parasteatoda tepidariorum (Theridiidae) is not strongly committed to caring for the offspring. The female hangs the egg sac close to her hunting net and protects the cocoon from predators for the first few days. *P. tepidariorum* lays eggs periodically, often forming consecutive cocoons before the previous young hatch [31]. After hatching, the young are fully independent of the mother.

In addition, the greater effort put into caring for offspring in lycosids than in theridiids may be due to the fact that wolfspiders usually lay eggs only once during their lifetime. In the case of the *P. tepidariorum* spider, females form many cocoons with lots of eggs. Therefore, the reproductive strategy is devoting energy resources to producing a huge number of offspring, but not getting involved in caring for them.. On the other hand, the opposite is the case with *Pardosa* sp., where the female uses all her energy resources to guard the offspring from the moment of laying the eggs until the day the juveniles decide to leave her abdomen.

Such a large discrepancy in the care of offspring in representatives of the Theridiidae and Lycosidae families may induce differences in embryo immunocompetence. The offspring of the wolf spider *Pardosa* sp. are definitely more vulnerable to damage to the cocoon - a physical barrier to microorganisms. In the case of cocoon breakage the probability of infection of microorganisms increases. Perhaps it may cause a higher level of both tested enzymes in *Pardosa* sp. embryos which can be additionally secured with a larger arsenal of AMPs.

Accordingly, few differences between the experimental groups O and C were observed in the studied spiders. The most statistically significant differences concerned the embryos of *Pardosa* sp. In the case of defensins in *P. tepidariorum*, no differences in the level of produced protein were observed, which is

consistent with the work of Bechsgaard et al. (2016), confirming constitutive expression of proteins related to immune responses in spiders.

In our previous studies, we checked whether the eggs isolated from the egg sac are free of microorganisms. *P. tepidariorum* eggs were sprinkled onto Petri dishes with lysogens broth agar directly from the cocoon and the samples were incubated for 3 days. The empty cocoon from which the embryos were taken was also examined in this way. After the incubation period, the study showed that no cultivable bacteria had grown on the Petri dishes with only eggs. Samples with empty cocoons were overgrown with several colonies of bacteria and fungi. Tests suggest that eggs inside the cocoon may be sterile [19]. Moreover, young spiders hatched from the eggs used in this study. This indicates the existence of a functioning immune system already at the beginning of the ontogenetic development of spiders.

In the protein profiles of the studied spider species, proteins of weight matching lysozyme (14.2 kDa) and defensins (2–4 kDa) weights have been shown. Besides these antibacterial peptides, the presence of other low-molecular compounds was also found. Antimicrobial peptides usually weight 25–30 kDa [6], so there is a high probability that visualized low-molecular compounds belong to the group of AMPs.

In both species, proteins weighing about 23 kDa were found. No antimicrobial substance with such a mass was found in the review of the scientific literature. Further studies will be required to isolate and sequence the visualized proteins in SDS-PAGE polyacrylamide gels.

The embryos are constantly exposed to a variety of pathogens. During development inside the egg sac, the cocoon-independent immunity of embryos must be formed so that it is fully functional after the hatching of the spiderlings. Although the egg sac is a very effective physical barrier against microorganisms, it can be mechanically damaged. Therefore, the conducted research shows that embryos of *P. tepidariorum* and *Pardosa* sp. have got the ability to produce antibacterial proteins, which is a form of the humoral response. However, this is not the only form of arachnids immune response. Further research is necessary to gain a deeper understanding of the functionality of the spider embryos immune system, including the potential for cellular defense.

Conclusions

I. Spider embryos are capable of producing antibacterial proteins – lysozyme and defensins. Their level increases with the age of the embryo.

II. The isolation of *tepidariorum* embryos from the cocoon does not cause statistically significant changes in the level of produced lysozyme and defensins. *Pardosa* sp. embryos reacted by increasing the level of AMPs due to the lack of protection provided by the egg sac. Such a reaction occurred in the few analyzed age groups of spiders, which may indicate that the embryos rely mainly on the protective role of the cocoon.

III. Differences in parental care can affect the production of AMPs by the embryos. In Pardosa sp. spiderlings, the presence of statistically significant differences between embryos from closed and opened experimental groups suggest that this species probably supplements the protective function of the cocoon with its own immune reactions. This difference may be due to the fact that *tepidariorum* leaves the cocoon in a safe place. At the same time *Pardosa* sp. females constantly carry the cocoon, exposing it to mechanical damage that may cause the invasion of pathogens.

IV. Apart from lysozyme and defensins, the embryos of *tepidariorum* and *Pardosa* sp. also produce other low molecular weight proteins that may have antimicrobial properties.

Material And Methods

Experimental groups. Two spider species/genus, belonging to different families and characterized by different parental and hunting strategies have been selected for this study:

- *Parasteatoda tepidariorum* (Theridiidae) - a cosmopolitan species, lives in anthropogenic environments. Hunts with irregular hunting nets usually located quite high, on the walls, near roofs or on fences. During a whole life, a female lays up to a dozen cocoons, each of them contains up to 300 eggs. The egg sac is attached to the hunting net. Parental care is limited to staying near the cocoon and possibly guarding it against predators (Fig. 6a). Juveniles, after hatching, are fully independent [31].

- *Pardosa* sp. (Lycosidae) - a species inhabiting sunny, dry areas, mainly edges of forests and grasslands. This species does not create huntable webs. Spiders hunt actively, moving on a forest litter. After laying a cocoon, containing up to several dozen eggs, the female attaches it to spinners an constantly carries egg sac that way (Fig. 6b). After hatching, the juveniles spend a few days on the mother's abdomen [32].

Due to the fact that several species of the genus *Pardosa* are often difficult to distinguish without the dissection of genital organs. In this work, it is limited only to providing a generic name. In the vast majority of cases, they are representatives of the species *Pardosa lugubris*. However, other representatives of this type belonging to the *lugubris* group have comparable morphology, lifestyle, cocoon size and number of offspring.

Lycosids were caught in the sunny edge of the forest in Katowice, southern Poland (50°11'47.2"N 19°03'44.1"E). Mature females with the clearly large abdomen were manually caught in plastic tubes during the breeding period, which fell in May-June.

P. tepidariorum individuals came from long-term lab culture at the Institute of Biology, Biotechnology and Environmental Protection at the University of Silesia in Katowice. Spiders are kept in constant environmental conditions: 25°C, photoperiod 16D: 8N and about 70% humidity.

All spiders used in this study were kept in plastic containers adapted to the size of the species. They were watered and fed every three days with *Acheta domestica* hatchling, *Calliphora* sp larvae or adult

Drosophila hydei flies.

In the case of ELISA and SDS-PAGE gel electrophoresis, the spiders' embryos in the age of 24, 48, 72, 96, 144 and 168 hours were divided into two experimental groups: C (closed, remained in an untouched cocoon) and O (open, without the cocoon – Fig. 7) to check whether the deprivation of the protective layer of spider silk will result in changes in the level of produced antibacterial proteins – lysozyme (Lys) and defensis (Def). The embryos deprived of the protective cocoon were thus exposed to common bacteria in the air and on the surfaces of the laboratory, including possibly pathogenic species.

The exact list of age groups is shown in Fig. 8. The age of the embryos was calculated from the day the female laid eggs in the cocoon. The 24 h group means 1 day after laying eggs. The following days are determined in the same way. Focused on the first days of an embryo's life during progressive organogenesis, embryos were tested up to the first week of life. Fully developed spiderlings, 24 hours after leaving the cocoon, were examined as the control.

Cocoons belonging to the closed group (C) were taken from females when the embryos reached the required age (0 -168 h), while the cocoons from the opened group (O) were collected one day earlier. Then egg sacs were carefully opened and eggs were spread on a Petri Dish for 24 hours. As for the juvenile form (juv.) - spiders were picked up the day (24 h) after hatching.

In PCR gel electrophoresis, the expression of genes encoding lysozyme and defensins was checked in freshly laid eggs (0 h), fully developed embryos (144 h), freshly hatched spiders (juv.) and mature females (♀).

Samples preparation. For SDS-PAGE and ELISA, a single sample was created from the content of one cocoon. Eggs or spiderlings were added to 500µl Sorensen buffer (0.95 M, pH 7.4) and homogenated on ice by hand-operated Pellet Pestle Cordless Motor (Sigma-Aldrich, Merck, Darmstadt, Germany). Homogenized samples were centrifuged at 4°C for 10 minutes at 10,000 rpm. The supernatant was taken and stored at -70°C.

For PCR method, isolation of total RNAfrom tested individuals was performed by using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) according to the standard method [33]. The content of one cocoon corresponded to one sample. Remaining genomic DNA contamination was removed using the TURBO DNA-*free* kit™ (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The quality and quantity of RNA were assessed for using a NanoDrop 2000 spectrometer (Thermo Scientific, Wilmington, USA). Aliquots of 1 µg of total RNA were retrotranscripted for cDNA synthesis using the Reverse Transcription System (Promega, Madison, WI, USA) and random primers according to the recommendations of the manufacturer. The reverse transcription products were then diluted with 80 µl of nuclear-free water. Prepared cDNA was used as the PCR template.

SDS-PAGE Electrophoresis. To check if selected AMPs: lysozyme and defensins are present in the tested embryos, a SDS-PAGE electrophoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

revealing a protein profile in the low-molecular range - from 26.6 kDa to 1.06 kDa was performed. The electrophoresis procedure was started by determining the total protein concentration according to Bradford, (1976) using bovine serum albumin (BSA, protein content > 95%, Fluka) as the standard. Dilutions were made with PBS buffer, while Sample Buffer (according to Laemmli, 1970: 62.5 mM, Tris-HCl, pH 6.8, 2 % SDS, 10 % glycerol, 5 % β-mercaptoethanol and 0.001 % bromophenol blue) was used for the last two-fold dilution. The samples prepared in this way were denatured by boiling for 5 min. SDS-PAGE electrophoresis was conducted using a 5 % stacking gel, 10 % separating gel and a vertical slab gel apparatus. The next stages of the study were carried out on ice, at 4°C. The Running Buffer was flooded in a Tris-Glycine-SDS system and pre-electrophoresis was carried out at 4°C, 50 V for 10 minutes. Electrophoresis was initially carried out at 70 V, then at 90 V. The gels were fixed in methanol: glacial acetic acid: water solution in a ratio of 5: 1: 4 for 30 min. The next step was placing the gel in the CBB250 staining solution (Coomassie brilliant blue) for 15 min and in the decolorant (methanol: acetic acid: water 4.5: 1: 4.5) for about 1 hour. The bands were quantified using densitometric image analysis (Gelscan 2.0 program, Kucharczyk, Poland) compared with a molecular mass marker (Color Marker Ultra-low Range, Sigma-Aldrich).

ELISA. The ELISA procedure was started with placing 100 µl of samples (diluted to 30 µg protein per 1 ml) in wells of the microtiter plate and it was incubated for 16 hours at 4°C. Then wells was rinsed three times with PBS-Tween 20 solution. The next step was to fill the wells with 100 µl 1 % BSA solution and incubate 1 hour at 37°C. Another three times were flushed. 100 µl of mouse anti-lysozyme primary antibody (Abcam, Cambridge, United Kingdom) in 1:5000 solution or rabbit anti-B-defensin 3 antibody (Sigma-Aldrich, Missouri, United States) in 1: 1000 solution were added. Incubation with primary antibodies lasted 2.5 hours at 37°C. The wells were washed 3x with PBS with Tween 20. A solution of a secondary antibody conjugated with alkaline phosphatase was then added. For lysozyme diluted 1: 1000 (goat anti-mouse IgG, pAb, AP conjugate, Bio-Rad, California, United States), same for defensins (goat anti-rabbit IgG, Sigma-Aldrich, Missouri, United States). The incubation lasted 2 hours at 37°C. The wells were rinsed three times again. A color reaction buffer was prepared: para-nitrophenyl phosphate dye (pNpp, Phosphatase substrate, Sigma-Aldrich, Missouri, United States) was added to the diethanolamine buffer pH 9.5, with a 1mg dye ratio and 1 ml buffer. The prepared solution was added to the wells and incubated 30 min at room temperature. After this time, the absorbance of the samples was measured in a Tecan Infinite M200 Microplate reader at 405 nm. The level of AMPs was expressed as the absorbance value. The more intense the color, the higher the protein (lysozyme or defensin) content in the sample.

PCR. Primers for lysozyme and defensin gene (Table 2) were designed with available online Primer3Plus software (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) based on the sequences available in the NCBI (XM_016066117, XM_016074084). The PCR reaction was performed using the PPP Master Mix (Top-Bio, Vestec, Czech Republic). Reactions were performed in 25 µl total volume with 4 ng DNA, 2.5 U Taq Purple DNA polymerase and 20 mM each of the primers under the following thermal conditions: 95°C for 5 min, 36 cycles at 95°C for 30 s, 55°C for 35 s, 72°C for 45 s and termination at 72°C for 10 min. Amplification products were separated by size by agarose gel electrophoresis. Data analysis relied on confirming the presence of a band at the appropriate height on the agarose gel.

Table 2
The forward and reverse sequences for the primers.

Target genes	Forward primer sequence	Reverse primer sequence	Product size (bp)
Lysozyme	TCTAGCCCCCGGAGCTATTT	CAGGCTCCGAATCCCAACTT	533
Defensin	TCACGCAAATTGTGGGGAA	AGCAATCAGAGCAATTGGAATTGA	430

Statistics. The results are reported as mean values ± SD. Normality was checked with the Kolmogorov-Smirnov test. The data were tested for the homogeneity of variance using Levene's test of the equality of error variances. Whenever a significant effect was observed, Tukey's multiple comparisons test was used for a post hoc one-way analysis of variance (ANOVA). The analysis of variance for the level of defensins and lysozyme for *P. tepidariorum* and *Pardosa* sp. spiders was performed using two-way ANOVA with the experimental groups (opened and closed egg sacs) and the spider embryos age as the sources of differences. Results with $p \leq 0.05$ were considered to be significant. The data were analysed using GraphPad Prism® ver. 6.

Data Availability

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

Declarations

Author information

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Contributions

Conceptualization: AC, methodology: AC, AB, software: MS, validation: AB, formal analysis: AB, MS, investigation: AC, MS, resources: AC, MS, AB, data curation: AC, writing: AC, AB, MS, original draft preparation: AC, MS, writing—review & editing: AC, MS, AB, visualization: AC, MS, supervision: AB, project administration AC, AB.

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Competing interests

The authors declare no competing interests.

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Figures

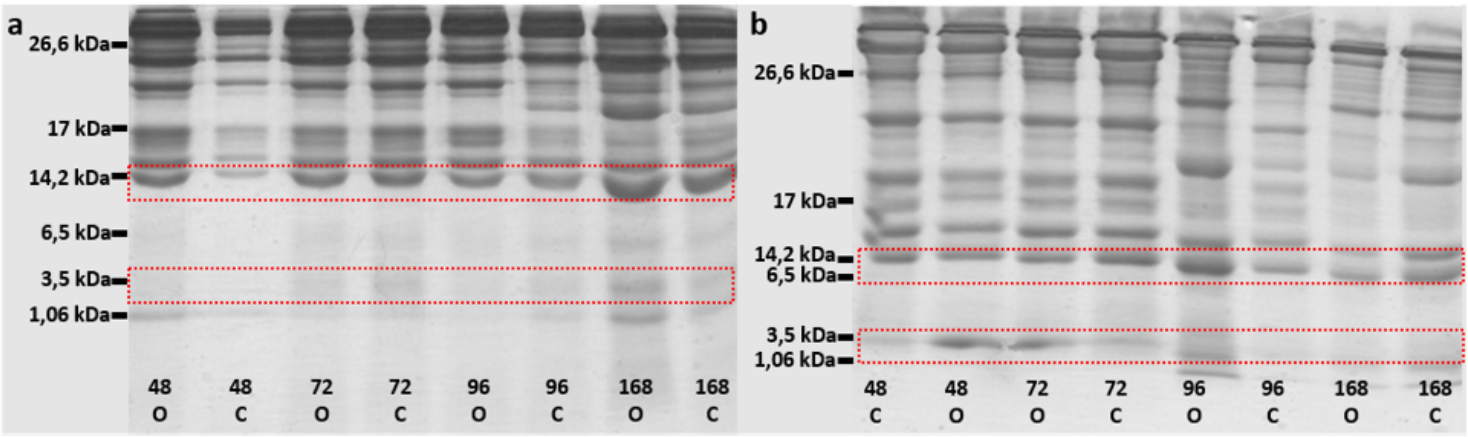

Figure 1

SDS-PAGE electrophoresis gel with P. tepidariorum samples (a) and Pardosa sp. samples (b). Colour Marker Ultra-low Range band heights, in the range 26.6 - 1.06 kDa, are marked in a schematic form on the left side of the gel. C - closed egg sacs, O - opened egg sacs.

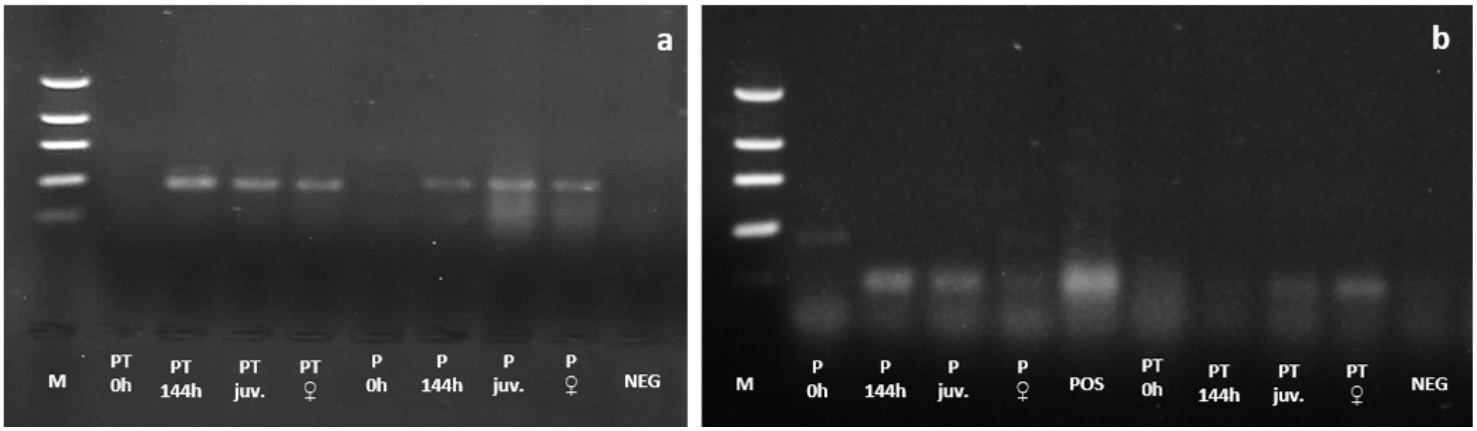

Figure 2

Lysozyme (a) and defensins (b) genes expression in P. tepidariorum (PT) and Pardosa sp. (P) spiders at various age. NEG - negative control without any nucleic acid, M – molecular-weight size marker.

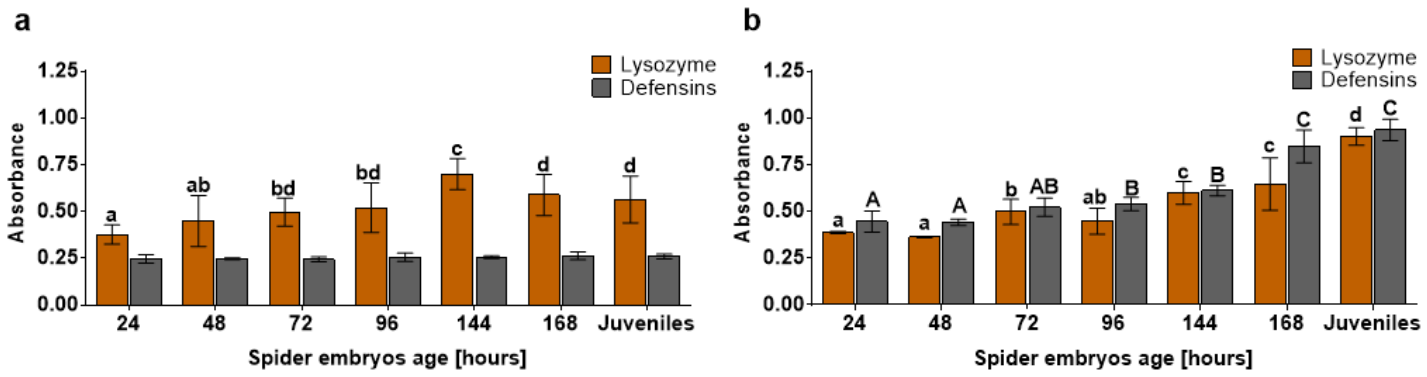

Figure 3

Lysozyme and defensins levels in P. tepidariorum (a) and Pardosa sp. (b) spider embryos. Different letters mean statistically significant differences between embryo groups of different ages. Lowercase letters are for lysozyme and uppercase are for defensins (Tukey's multiple comparisons test, $p \leq 0.05$).

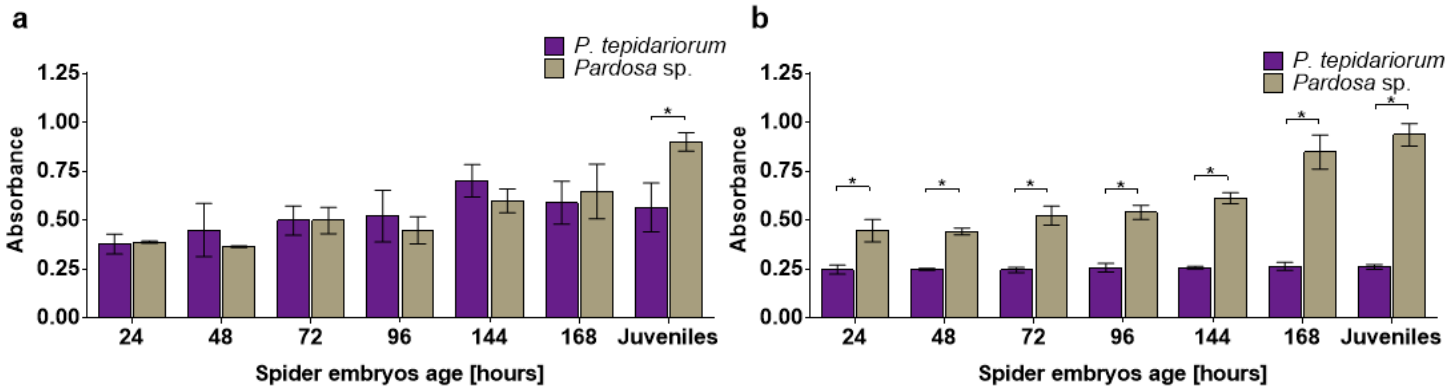

Figure 4

Lysozyme (a) and defensins (b) levels in P. tepidariorum and Pardosa sp. spiders embryos. Different letters mean statistically significant differences between embryo groups of different ages. Lowercase letters are for lysozyme and uppercase are for defensins. (Tukey's multiple comparisons test, $p \leq 0.05$).

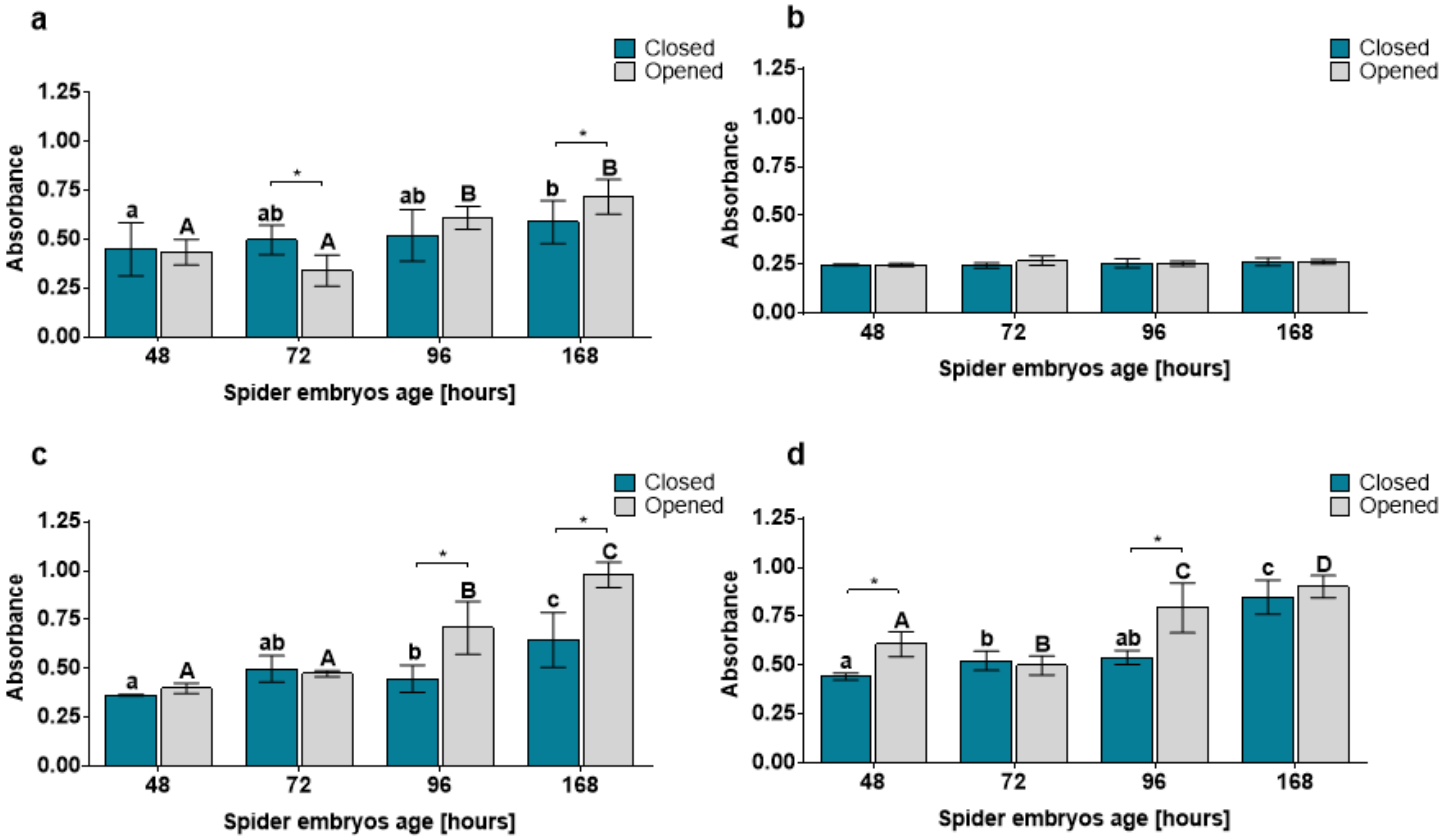

Figure 5

Comparison of detected AMPs levels in closed and opened cocoons. Lysozyme (a) and defensins (b) level in P. tepidariorum. Lysozyme (c) and defensins (d) levels in Pardosa sp. Different letters mean statistically significant differences between embryo groups of different ages. Lowercase letters are for lysozyme and uppercase are for defensins. An asterisk sign means statistically significant differences between O and C experimental groups inside one age group (Tukey's multiple comparisons test, $p \leq 0.05$).

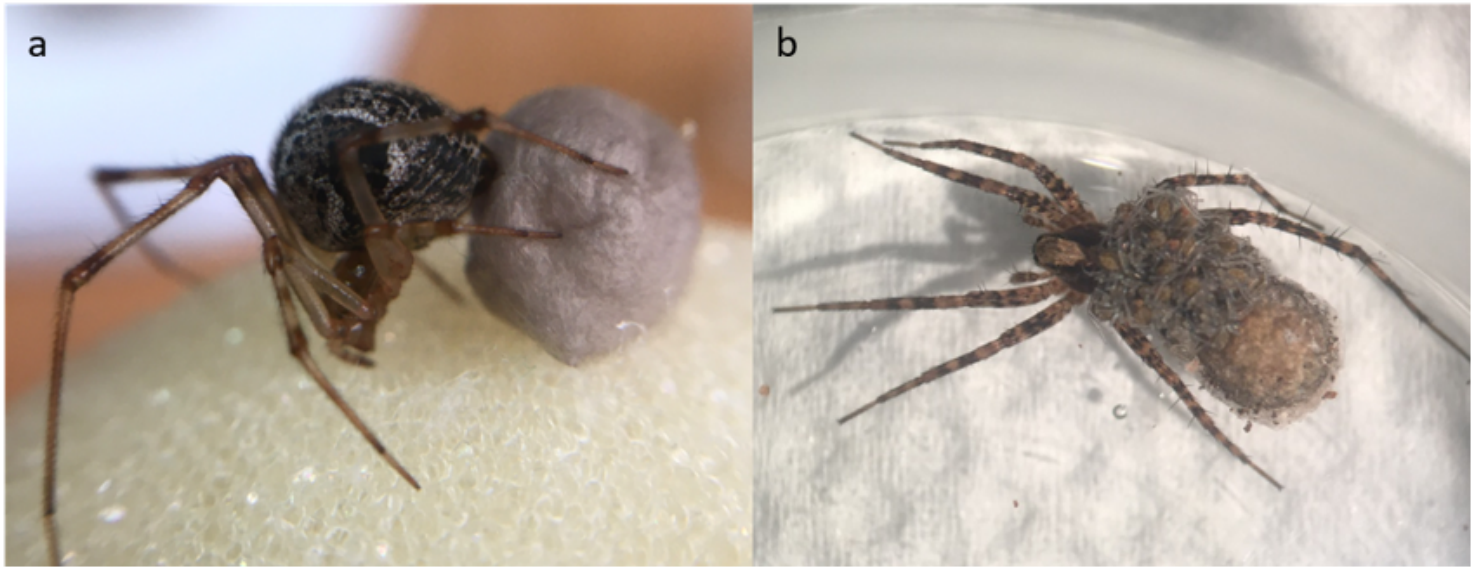

Figure 6

Parasteatoda tepidariorum with cocoon (a) and Pardosa sp. with spiderlings at the moment of emerging the egg sac (b).

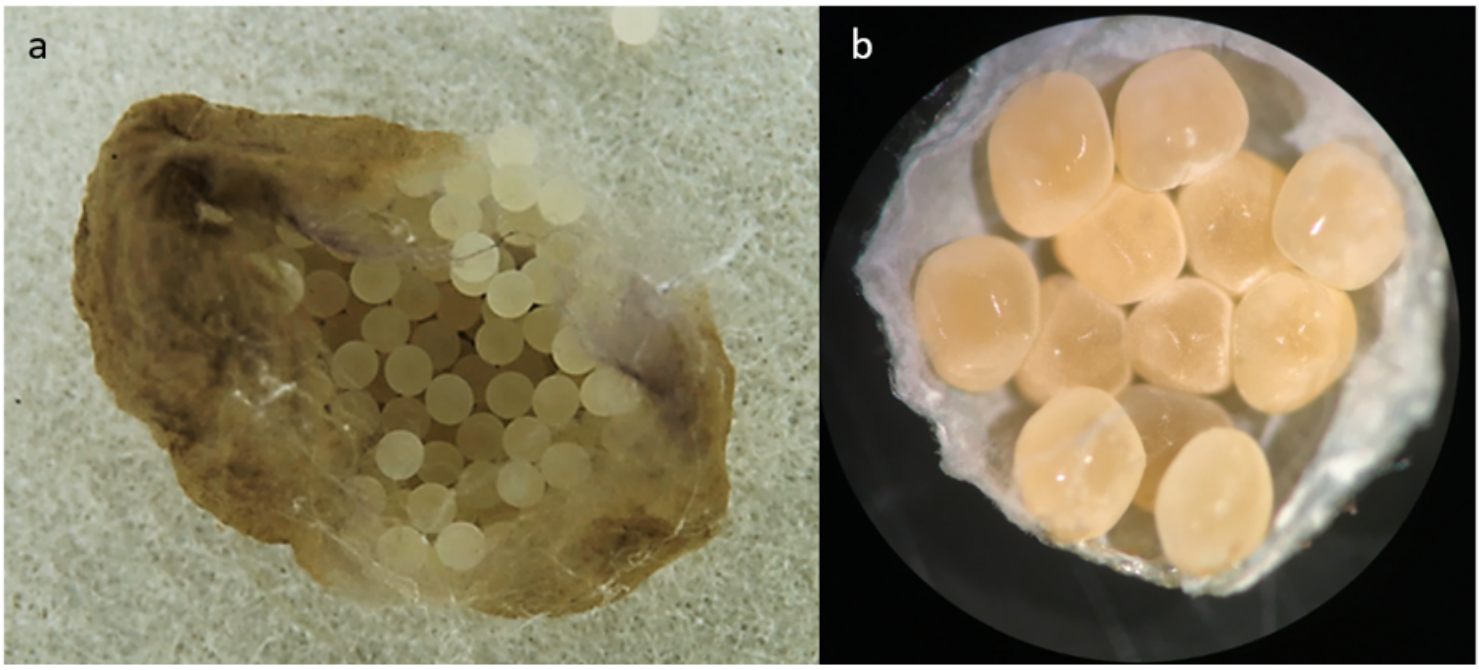

Figure 7

Opened egg sacs of P. tepidariorum (a) and Pardosa sp. (b).

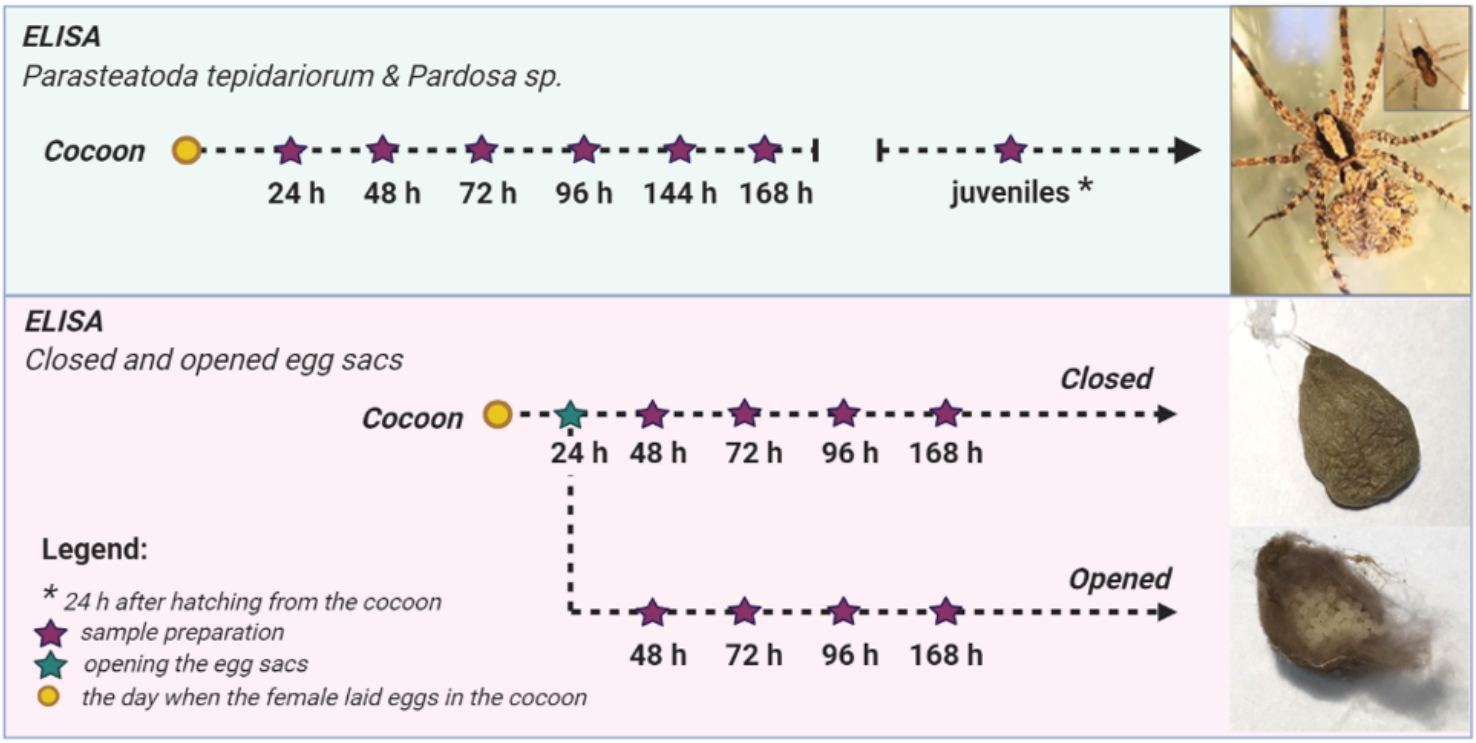

Figure 8

Experimental groups (n=6).