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X-ray microtomography for imaging of developing spiders inside egg cocoons

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ABSTRACT

Embryogenesis is especially sensitive to external factors. The changes in its course are often used as biomarkers of environmental impact. Since spider embryogenesis takes place inside cocoons, it is crucial to find a reliable tool to analyze this developmental phase with no intrusion into the cocoons. The aim of this study was to verify the efficacy of X-ray microtomography for non-invasive analysis of embryonic morphology and egg quantity in the cocoons of *Xerolycosa nemoralis* and *Agelena labyrinthica* from polluted and reference sites. X-ray microtomography slice images as well as 3D images and animations obtained from digital visualization of those slides were used to study the morphology of embryos and egg arrangement in the cocoons. Any disorders in embryogenesis or malformation of embryos in relation to site of origin have not been found, but inside an egg cocoon of *X. nemoralis* from the polluted site embryos differing form each other by one developmental stage were identified. Egg calculation revealed a K- reproductive strategy of *X. nemoralis* from polluted sites. Finally, future prospects and benefits, and weaknessess of this method for the study of spider cocoons have presented.

1. Introduction

Embryogenesis is a crucial period of the development of an organism. Numerous physical, chemical or biological factors may negatively influence the duration or proper course of this stage. This phase of development is usually the most sensitive to various internal and external stressors. Invertebrates inhabiting anthropogenically changed ecosystems, exposed to a number of chemicals, often experience developmental disorders. Specific stress-induced disturbances in ontogenetic development and/or in life history parameters were well recognized in earthworms (Spurgeon and Hopkin, 1996; Siekierska and Urbanska-Jasik, 2002), land snails (Notten et al., 2006), polychetes (Durou et al., 2007), mussels (Tsangaris et al., 2010) or grasshoppers (Augustyniak et al., 2008). Disturbances in reproductive and developmental processes were also recognized in spiders inhabiting polluted areas. Negative effects of heavy metals on egg production and egg quality were described in the 1980s. Zimakowska-Gnoińska and Tarwid (1984), Tarwid (1987) stated that the number of eggs laid to the cocoons by the spider *Enoplognatha ovata* (Theriidae) decreased with increased levels of metal pollution. Not only was the mortality rate of hatched spiderlings higher than in spiders from the reference site, but also some cocoons produced by the females from heavily polluted areas were empty. Lower body mass was also found in another spider (*Argiope trifasciata*, Araneidae) inhabiting heavily polluted areas (Ramirez et al., 2011). The authors supposed that the reduced body mass reflected a decrease in the pool of energy allocated for growth and hunting activity due to higher energy expenses paid for the elimination of excess of metals from the spiders' body.

The pressure of environmental factors also interferes with other life history parameters of spiders, such as the duration of ontogenesis, growth rate, number and mass of eggs, etc. The duration of ontogenesis of the wolf spider, *Pardosa astrigera* (Lycosidae) exposed to lead and zinc was longer than that identified in unexposed spiders (Chen et al., 2011). Moreover, the growth rate of these spiders and the number of eggs laid by the females were lower than in spiders unexposed to metals. Eraly et al. (2011) found similar correlations between the pollution levels and reproductive efforts

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of another wolf spider, *Pardosa saltans*. Adult females from polluted sites were smaller, their reproductive period was delayed and they produced less eggs than the females from the reference site. The mass of the eggs, however, was positively correlated with the body loads of cadmium. Body mass and fecundity of mature individuals were the highest in the area with the lowest Cd and Zn pollution (Eraly et al., 2011). Also, the recent studies of Babczyńska et al. (2012) demonstrated that spiders inhabiting polluted areas differ in their reproductive strategy from the individuals of the same species living in unpolluted habitat. The strategy, however, is species specific. Females of actively hunting spiders, *Xerolycosa nemoralis* (Lycosidae), from polluted sites produced less eggs, allocating less or the same amount of energy to the reproduction as these from the reference site (K-strategy). In contrast, web building *Agelena labyrinthica* (Agelenidae) from the polluted site laid more eggs of lower calorific value in comparison with the females from the reference site (r-strategy). Hatching success of the actively hunting spiders from the polluted sites was higher than in these from the reference area. The same parameter calculated for the web building spiders from the polluted sites was slightly lower than for spiders from the reference site (Babczyńska et al., 2012). All the disturbances mentioned above resulted from the limited energy resources that each organism has to its disposal for its functioning. Taking into account the trade-off between reproduction/development and detoxification it can be therefore expected that the deficiency may result in possible embryo malformation. We thus intended to develop a method to analyze the morphology or, possibly, the course of embryogenesis, preferentially *in situ* and in a non-invasive manner. The description of the whole embryonic development is available only for a few spider species (Akiyama-Oda and Oda, 2003; Chaw et al., 2007; Wolff and Hilbrant, 2011). The general view of this process can be found in Foelix (2011). Also, the data on the character of eggs in cocoons [viable (developing into young spiders) *vs* trophic (laid as food source)] are limited to several spider species (Valerio, 1974; Downes, 1985; Kim and Roland, 2000). Generally, our recent knowledge on embryogenesis of spiders is still insufficient. It is not known whether, and if so, to what extent the spider's embryogenesis is disturbed by environmental pressure.

Spider embryogenesis as well as the stage of postembryo take place inside the egg cocoons (Wolff and Hilbrant, 2011). The structure of spider cocoons is highly diverse, from several silk threads wrapped around the eggs to complex cocoons consisting of the basal layer and external covering of the silk that protect the eggs (Foelix, 2011). The cocoons are round or ellipse-shaped. According to Foelix (2011), due to a specific behavior accompanying the egg sac construction and egg laying, the size of cocoons depends on the size of the female but not on the number of the eggs inside. The cocoon is crucial for maintaining appropriate humidity, for protection against parasites and to ensure thermal insulation. Moreover, inter-individual contacts, probably of a chemical character (e.g. pheromones) are very likely in cocoons, although there are no studies that would give precise confirmation of this statement. In general, opening the cocoon is a strong stress, resulting in significant developmental disorders at various organization levels, e.g. switching to enhanced apoptotic or even necrotic changes in developing embryos (Giesy et al., 1983).

Studying the developmental processes through undestroyed cocoon layers requires specific tools and reliable methods of registration. X-ray microtomography (XMT) has been used in 3D visualization of the structure of diverse biological human, mouse and insect samples (Mizutani and Suzuki, 2012). This technique is also applied in evolutionary studies – the ability to investigate fossils (Tafforeau et al., 2006; Błażejowski et al., 2011, 2013; Sutton et al., 2014) or unique specimens in a non-invasive way enables the identification of evolutionary tendencies (Jeffery et al., 2011). XMT is expected to be commonly applied in the studies of tooth and bone microstructure (Nowaczewska et al., 2013). The technique is also claimed necessary to construct artificial biomaterials on the basis of 3D structure of living tissues (Mizutani and Suzuki, 2012). There are two reasons why the usage of XMT can be significantly limited. The first one results from the fact that the majority of soft tissues do not differ in their X-ray attenuation, causing difficulties in distinguishing the details of their structure (Greco et al., 2008). Saito and Murase (2012, 2013) and Saito et al. (2012), concluding from their *in vivo* and *ex vivo* studies of soft tissues, suggested that the use of phase contrast may partly eliminate this obstacle. Harmful effects of radiation, as well as the heat emitted during the measurement, constitute the other possible limitations in such studies (Westneat et al., 2008).

Despite numerous disturbances in spider developmental processes, these predatory invertebrates successfully colonize polluted areas where they create relatively stable populations (Łuczak, 1984; Majkus, 1988, 2003). Their proper density is crucial for the regulation of insect populations (Nentwig, 1987; Foelix, 2011). Some spider species were often used as biomonitors, especially in post-industrial environments (Maelfait, 1996; Maelfait and Hendrickx, 1998; Jung et al., 2005). The aim of the present study was therefore the examination of the applicability of computed microtomography for the quantitative and qualitative study of the early stages of spider development and for vital monitoring of the embryogenesis in relation to the pollution level of their habitats. This technique was applied herein to study the morphology of eggs and embryos, and to determine the developmental stage of the embryos in two spider species: *X. nemoralis* and *A. labyrinthica*. The number of eggs in cocoons of *X. nemoralis* were also counted and compared. For this study, spiders from variously polluted areas were chosen to observe possible differences in their developmental course. Pollution here was treated as a natural factor potentially changing the embryogenesis.

2. Material and methods

All specimens used in the experiments were captured according to Polish regulations concerning the protection of wild species and do not encroach any property rights (Dz.U. 1984, No. 2 item 11, Dz.U. 1991, No. 114 item 492, Dz. U. 1991 No 101 item 444). Collected spider species are not included into the list of protected species, according to Polish regulations (Dz. U. 2011, No 237 item 1419). All study sites were localized outside of any protected areas and do not infringe related regulations (Dz. U. 2009, No 151 item 1220).

2.1. Spider species

Agelena labyrinthica (Clerck, 1758) (Agelenidae) is a web building spider, abundant in various types of habitats. They attach their webs to plants and low bushes close to, or within 1 m above the ground. Their life span covers a year. Adult, sexually mature individuals appear in June/July. About one month later they lay eggs and wrap them in several layers of silk. The egg cocoons are about 1 cm in diameter. Then the cocoons are attached to the main web and hidden within the vegetation. Young individuals hatch in late August/September. They function as self-sufficient predators and overwinter as immature spiders (Nentwig, 1987; http://srs.britishspiders.org.uk/portal.php/p/Summary/s/Agelena+labyrinthica).

Xerolycosa nemoralis (Westring, 1861) (Lycosidae) is an actively hunting spider frequent in various habitats, either open or covered with vegetation. Mature individuals can be found from May until September. Females lay eggs in May and carry them in egg cocoons