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EGG SAC STRUCTURE OF *ZYGIELLA X-NOTATA* (ARACHNIDA, ARANEIDAE)

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ABSTRACT. A detailed examination of the egg sac of *Zygiella x-notata* (Clerck 1757) revealed its structure, composition and different fibers. All egg sacs were composed of a basic layer, an insulation layer and an outer layer. The insulation layer consisted of two layers of cylindrical (or tubuliform) fibers with different diameters and probably with different mechanical properties. Knowing the complete structure of the egg sac allows us to locate and extract the needed fibers for further research and to observe how the egg sac composition alters in relation to the habitat.

Keywords: Cylindrical (tubuliform) fibers, sticky thread, major ampullate fiber, attachment disc, adhesion droplet.

Of all natural fibers, silk is the most promising for bioengineering because of its biological and mechanical properties. Much is already known about the molecular and mechanical properties of dragline silk of *Araneus diadematus* (Clerck 1757) and *Nephila clavipes* (Linnaeus 1767) (Shao et al. 1999; Vollrath 1999; Vollrath et al. 1998; Vollrath & Knight 2001); still, regarding the other spider silks, these properties have not yet been explored, especially the properties of egg sac silk. Egg sac threads can be very useful for biomedical applications, like sutures, cell support and scaffolds (Gellynck et al. 2003; Van Nimmen et al. 2003). Before one can analyze these properties, the morphology of the egg sac must be investigated to locate these fibers. The primitive role of egg sacs is in giving protection against predators and parasites. Furthermore, the egg sac must create a good microclimate for embryological development, hatching and it must protect the spiderlings until they leave the egg sac (Hieber 1985). As observed by De Bakker et al. (2002), there appears to be a big difference in egg sac structure between families. Since egg sac threads are possibly the earliest silk used by spiders, it is clear that a detailed analysis of these structures can contribute to spider phylogeny. Further research on egg sacs of other families can perhaps determine, in time, the ancestral construction of the egg sac.

In this study, only egg sacs of *Zygiella x-notata* (Clerck 1757) were investigated. *Zygiella x-notata* is iteroparous and females make most egg sacs in late autumn (Western Europe; November–December). The spiderlings emerge around May of the following year. The egg sacs are elliptical and have a white to yellowish brown color. In addition, they have complex airy structures composed of different layers of silk that enclose and protect the eggs (De Bakker et al. 2002). In the present article, a more detailed description of the egg sac of *Z. x-notata* will be given in which its structure, composition and different fibers will become clear. Knowing the complete egg sac structure will allow us to locate and extract the needed fiber types to investigate their usefulness in several biomedical applications, and to investigate the alteration of egg sac composition in relation to the habitat choice of *Z. x-notata*.

METHODS

Egg sacs ($n = 20$) of *Z. x-notata* were used to analyze their structure and composition. The spiders were collected in Ghent (Belgium) at Coupure Right (lat. 51°5′53″, long. 3°71′11″), in the beginning of November (±

Table 1.—Types of fibers measured by one spider (*Z. x-notata*).

Type of fiber	Average diameter (μm)	St. Dev.
MA threads		
Basic layer (n = 11)	2.63	0.13
Outer layer (n = 13)	2.41	0.35
Dragline (n = 10)	1.54	0.06
TU fibers		
1st insulation layer (n = 231)	3.29	0.30
2nd insulation layer (n = 324)	3.84	0.24

80 spiders). They were kept in the laboratory in small, round, plastic (PS) cups, with plastic (PVC) lids (diameter: 50mm, hight: 25mm). The lids were pierced for aeration and to make them an easier surface for walking. All spiders were fed fruit flies (*Drosophila* sp.). A high level of air humidity was provided by a water-saturated piece of plaster placed on the bottom of the cups. It was moistened every 4 days with a mixture of water and nipagine (Alpha Pharma) to prevent fungal growth. The temperature was kept constant (23 ± 1 °C) and light was regulated following a constant day-night period (16h–8h). In this way, a high uniformity in egg sac structure was obtained which simplified the observations. Draglines, for comparison, were collected from the spider while she was hanging. Voucher specimens have been deposited in the ''Evolutionary Morphology of Vertebrates & Zoology Museum'', Ghent University in Belgium (UGMD 104091).

The morphological study was performed by means of a stereomicroscope (Wild M5), a light microscope (Olympus CH-2) and a Scanning Electron Microscope (JEOL JSM-5600 LV, SEM). For light microscopy, slides of strands and connections were prepared with glycerine to prevent air bubbles. Photographs taken by the light- and stereomicroscope were made with a camera (Nikon coolpix 900) mounted on the microscope. For the SEM, samples (connections, fiber types) and a completely dried egg sac were mounted on stubs (standard and large (32mm)) and coated with gold (JEOL JFC−1200 Fine coater, 8nm). An image processing system (Lucia System for Image Processing and Analysis version 4.51), made it possible to process and analyze real color photographs, like measuring the thickness of the draglines and egg sac fibers of one egg sac (Table 1). In order to compare the thickness of the egg sac fibers of the first and second insulation layer, a student t-test (P = 0.05) was performed supposing a normal distribution of the measurements using the Statistica program (Statsoft 2001 Release 6.0). Terminology, except as defined here, is from earlier studies (Peters & Kovoor 1991; Zschokke 1999, 2000; Benjamin et al. 2002).

RESULTS

All 20 egg sacs were analyzed and a great uniformity in their structure was visible. Figure 1 shows a scheme of this uniform egg sac structure.

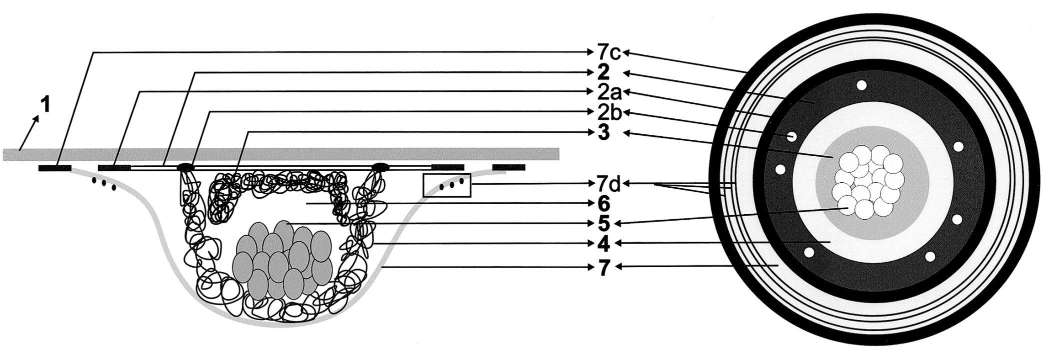
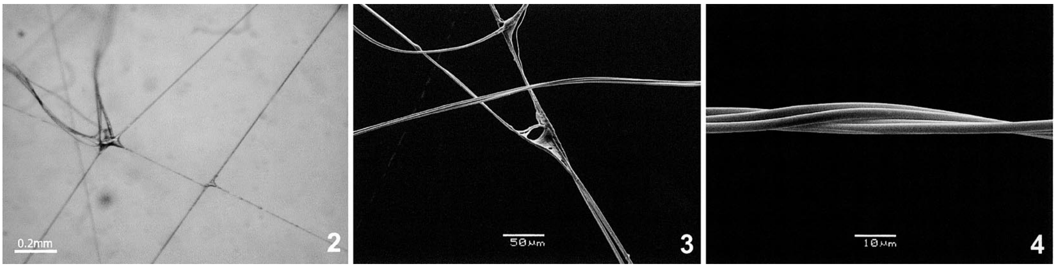


Figure 1.—Older egg sac structure of *Z. x-notata*: Left: side-view; right: top view, most common placement of the different structures. 1 = substrate (lid), 2 = basic layer (a = attachment discs of the basic layer, b = adhesion droplets), 3 = first insulation layer, 4 = second insulation layer, 5 = eggs forming the egg chamber, 6 = egg sac space, 7 = outer layer (c = attachment discs of the outer layer, d = sticky threads).



Figures 2–4.—Basic layer. 2. Basic layer with an attachment of egg sac fibers of the first insulation layer on the left side (stereomicroscope); 3. Basic layer in more detail with some major ampullate (MA-MA) connections (SEM); 4. Two major ampullate (MA) threads of the basic layer (SEM).

Basic layers.—When in the cup, the spiders generally walked on the lid (Fig. 1 (1)), and constantly produced draglines (major ampullate (MA) thread), which they fixed on the substrate by means of attachment discs (Fig. 1 (2a)). In this way a parallel, sheet-like layer was formed and was here named the ''walk plate''. The MA thread of this layer was always doubled or sometimes fourfold (Figs. 3, 4). Before egg sac construction, an additional network of fibers was fixed on the center of the walk plate, which was here named the ''stitch plate'' (observed in 7 egg sacs), because it was on this layer the tubuliform (TU) fibers were attached. In contrast with the walk plate, the stitch plate was a tightly woven network that was attached to the substrate by ''adhesion droplets'' (Fig. 1 (2b)). The threads resembled MA threads such as those of the walk plate but they had a smaller diameter.

Attachment discs.—An attachment disc (Fig. 1(2a)) consisted of an MA thread (Fig. 5, a) and a big sheet of finer fibers (= the disc; Fig. 5, b). The dragline was continuous and did not stop in the disc. The area of the disc varied and appeared to depend on the importance of the attachment. The number of attachment discs per basic plate (Fig. 1(2a)) was relatively small according to the number of attachment discs of the outer layer (Fig. 1(7c)).

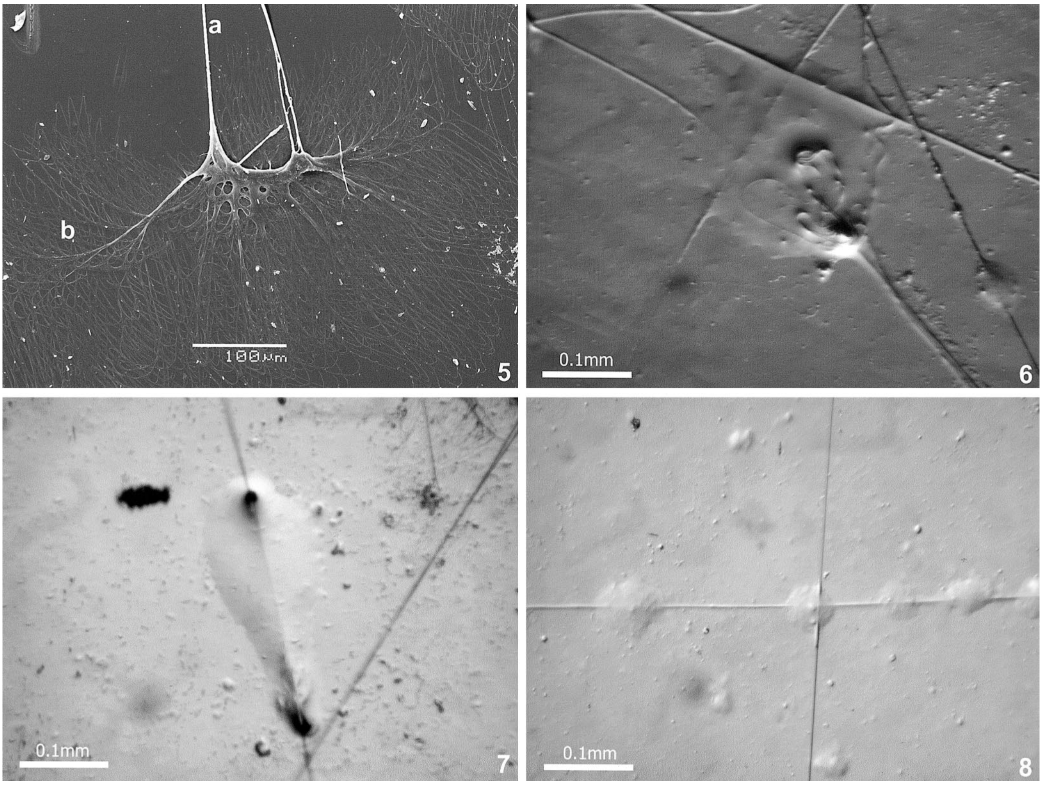
Adhesion droplets.—Adhesion droplets (Fig. 1 (2b)) consisted of a glue-like droplet in which a fiber was placed without the presence of a disc (Figs. 7, 8). These attachments were less abundant than attachment discs, they were more centrally placed in the egg sac (Fig. 1(2b)) and seemed to attach the stitch plate to the substrate (observed in seven egg sacs).

Figure 6 shows in detail an ending fiber in an adhesion droplet. The thread was double stranded and once in the droplet, it spread out in many finer fibers. This seemed to be different from the other fibers (Fig. 7, 8) which were only connected to the substrate by means of a glue droplet.

Major ampullate (MA-MA) thread connection.—The basic layer contained many fixations between MA threads. These connections could be complex and consisted of thread-like glue secretions (Figs. 9, 10). The secretions enveloped the two MA threads individually and there was a stretch zone present in the secretion between the two MA threads (Fig. 10, a).

Insulation layer.—This layer formed the actual egg sac and consisted of TU (tubiliform) fibers (Figs. 11, 12). The insulation layer could be subdivided into two layers: a ''first'' and a ''second'' insulation layer. These layers consisted of crisscrossed, tufted fibers with few or no connections (Fig. 11). Sometimes TU fibers were found doubled (Fig. 11, a). They were lying next to each other in close contact, pointing in the same direction and seeming to adhere to one another along a fine line.

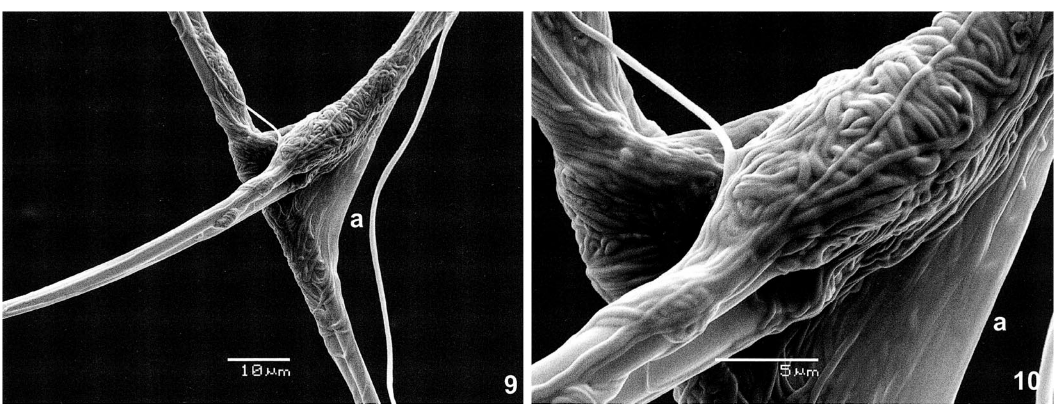
TU fibers of the first insulation layer (Figs. 1 (3), 13) were attached to the basic layer (Fig. 2), after which the spider pulled the fibers out of her spinnerets and attached them again to the basic layer a bit further. In this way she spun around attaching the TU fibers, making a cup in which to put the eggs. After the eggs were laid (Fig. 1 (5)), a second insulation layer (Figs. 1 (4), 14) was placed over the eggs and the first insulation layer. This layer was also attached to the basic layer in



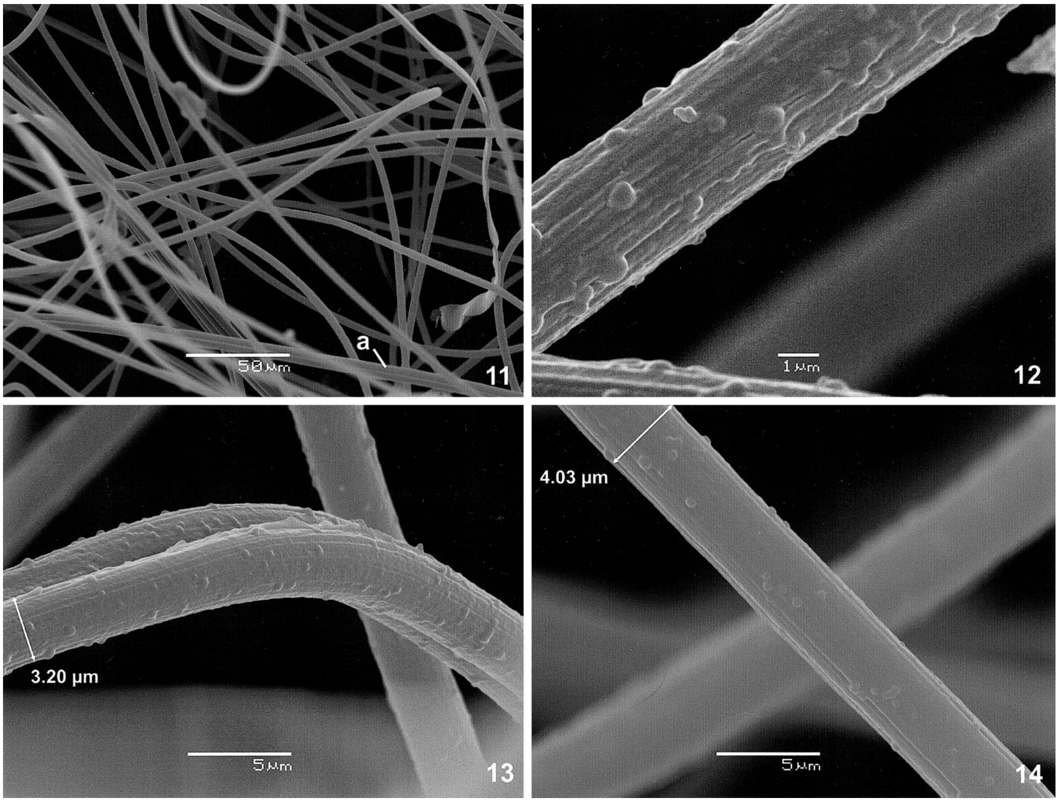
Figures 5–8.—Fiber-substrate connections. 5. Attachment disc, a = dragline, b = disc of finer fibers (SEM); 6. One adhesion droplet fixing an ending thread (stereomicroscope); 7. Fixation of a continuous fiber with one glue droplet (stereomicroscope); 8. Fixation of a continuous fiber with several glue droplets (stereomicroscope).

the same way as the first insulation layer, but was more peripheral.

Statistical analysis showed a highly significant difference in thickness between the TU fibers of the first and the second insulation layer within the same egg sac. (df = 230, $p<0.0001$). The first insulation layer (3.29 µm ± 0.30 µm) had finer fibers than the second



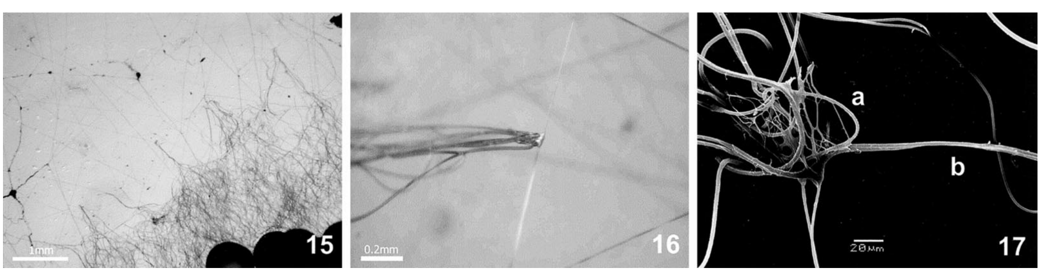
Figures 9–10.—SEM pictures of a basic layer MA-MA thread connection. 9. Overview of an attachment; 10. In more detail; a = stretch zone in the connecting secretion.



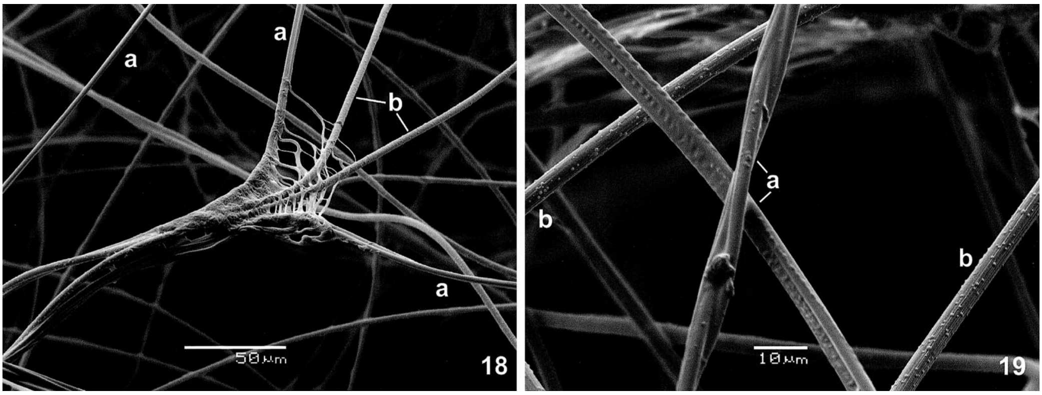
Figures 11–14.—SEM pictures of the insulation layer. 11. Overview of the fibers of the insulation layer, a: doubled TU fiber; 12. Detail of a egg sac fiber; 13. Detail of a egg sac fiber of the first insulation layer; 14. Detail of a egg sac fiber of the second insulation layer.

insulation layer (3.84 μm $\pm$ 0.24 μm) (Table 1). Although they differed in thickness, they did not differ in surface structure and appearance (Figs. 13, 14). Egg sac fibers of the first and second insulation layer were connected to the basic layer in the same way (Fig. 15, 16). These connections consisted of four to six continuing TU fibers (Fig. 17, a) attached together with a kind of glue to one MA thread (Fig. 17, b) of the basic layer.

Egg sac chamber.—In recently placed egg sacs, only fibers surrounding the egg mass (Fig. 1 (5)) were found, and no fibers were found between the eggs. In older egg sacs, a



Figures 15–17.—Egg sac fiber-basic layer connection. 15. Attachment of the first insulation layer to the basic layer (stereomicroscope); 16. Multiple egg sac fibers attached at one place on a basic layer thread (MA) (stereomicroscope); 17. Detail of multiple egg sac fibers (a) attached to a basic layer thread (b), somewhat torn apart (SEM).



Figures 18–19.—SEM picture of the outer layer. 18. Attachment of the outer layer thread to some egg sac fibers of the second insulation layer; 19. Detail of some outer layer threads. a = outer layer threads, b = egg sac fibers.

space (Fig. 1 (6)) was found between the eggs and the first insulation layer.

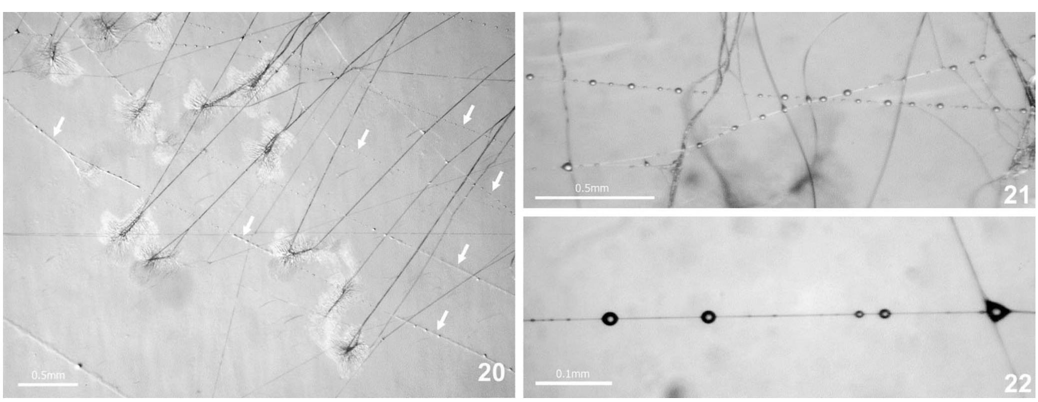
Outer layer.—The outer layer (Fig. 1(7)) was placed over the insulation layers and basic layer. It was made up of an airy network of threads attached to the substrate by means of attachment discs (Figs. 1 (7c), 20). These attachment discs were numerous and peripherally situated, forming the edge of the egg sac. They were similar in structure like those found in the basic layer. The threads were doubled and contained a kind of glue on their surface (Fig. 19, a) in contrast to the MA threads of the basic layer (Fig. 4).

There were also connections found between the fibers of the outer layer (Fig. 18, a) and the second insulation layer (Fig. 18, b). These connections consisted, like MA-MA thread connections, of a kind of glue and finer fibers (Fig. 18). Although there was much more variation in the number of fibers included in this connection type, there were always two MA fibers (one MA thread), whereas the number of TU fibers was variable.

In five egg sacs, a sticky thread was observed on the outer layer which resembled the sticky threads of orb webs (Figs. 1 (7d), 20–22). The sticky threads were winded several times around the egg sac beginning from the attachment discs of the outer layer, going inwards with an almost constant interval (Fig. 20, arrows).

DISCUSSION

Egg sacs in spiders vary substantially in structure interspecifically but usually display



Figures 20–22.—Stereomicroscope pictures of the outer layer. 20. Outer layer with attachment discs and diagonally on it the sticky threads indicated with arrows; 21. Sticky threads with glue droplets; 22. Detail of the glue droplets.

consistent similarities intraspecifically, that is, most families have egg sacs of only one or two structural types (Austin 1985). The egg sacs of *Zygiella* were fairly uniform and consisted of a basic layer, a double insulation layer and an outer layer. This structural composition is consistent among most spiders belonging to the family Araneidae (unpub. data).

Basic layer.—The basic layer is composed of a walk plate and a stitch plate and forms the basis for egg sac construction. The MA threads (draglines) of the walk plate are stronger and stiffer than TU fibers (Van Nimmen et al. 2003). Their presence in the basic layer and the outer layer is probably to fix the egg sac well to the substrate and to support the egg mass. The fact that the fibers of the stitch plate look like MA threads and have a smaller diameter suggests that they originate from the minor ampullate (MI) glands. The stitch plate is probably present in all egg sacs but due to the difficult observation of these fibers it was only detected in seven egg sacs.

In nature however, one can sometimes observe that a new, fresh egg sac is attached to an older one. In this case it can be expected that the ‘‘outer layer’’ of the older egg sac serves as a basic layer for the new one what costs the spider less energy.

Attachment discs.—It is known that the piriform spools (Pi) produce the disc while the MA thread (dragline) is extruded by the MA spigots on the anterior spinnerets (Foelix 1996). The attachment discs used for the basic and outer layer strongly resemble those used for walking and web building described by Schütt (1996), so it is not so surprising that they are used for the egg sac construction as well.

Adhesion droplets.—These adhesion droplets were no artifacts of oviposition, because no ‘‘glue droplets’’ were observed without fibers and the fixation of the fibers to the substrate was too specific. We suggest that the MI fibers of the stitch plate are fixed to the substrate in this way. The Pi spools are probably not involved in this fixation type because the MI spigots are located to far from them. A glue spool/spigot located closer to the MI spigot is more likely.

We hypothesize that the ending thread seen in Fig. 6 does probably not originate from the minor ampullate spigots but rather from the MA spigot and that it is the beginning or ending of an MA thread, which would explain their low abundance.

Major ampullate (MA-MA) thread connection.—The thread-like glue secretions make us suspect that these connections originate from the Pi spools of the anterior spinnerets, like the attachment discs. Most of the connections and supporting structures in araneoid webs are also made up of attachment discs: sticky spiral thread to radius, auxiliary spiral to radius, radius to frame and some connections to the hub (Peters & Kovoor 1991; Peters 1993; Benjamin et al. 2002). The stretch zone indicates that the fibers were laid parallel while fixing and reorientated afterwards.

Insulation layer.—*Zygiella x-notata* simultaneously produces six TU fibers by six TU spigots, two on the median spinnerets and four on the posterior spinnerets (Foradori et al. 2002), forming the two insulation layers in the egg sac. Measuring the thickness of the fibers of the two insulation layers was only executed on one egg sac but recent experiments (unpub. data) indicate that the found difference in thickness is generally applicable. The difference in thickness between the fibers of the two layers is most likely due to the difference in volume of the TU glands before and after placing the eggs. Before oviposition, the glands are limited in space for expansion. Probably a smaller lumen is causing a smaller secretion which results in a finer fiber of the first insulation layer. After oviposition, more space is available in the abdomen causing a bigger lumen and secretion, resulting in a thicker fiber of the second insulation layer. This difference in diameter will probably also be reflected in the mechanical properties of these fibers.

MA and TU fibers from *Z. x-notata* are morphologically very different. MA fibers have a smaller diameter (Tabel 1) and no underlying structures (Fig. 4), unlike TU fibers (Fig. 12) (Van Nimmen et al. 2003). The fact that TU fibers are another type of fiber means that they have some advantages compared to MA fibers: 1. In water, TU fibers only increase in diameter without longitudinal shortening (pers. obs.), unlike draglines which supercontract (Bell et al. 2002). This observation suggests that TU fibers can play a role in moisture regulation in the egg sac. This would also confirm the suggestion of Hieber that TU fibers can serve as a regulator of the relative humidity by taking up water if the relative humidity is too high and releasing water if it is too low. It would also explain why fibers of the second insulation layer are thicker than the first, because the second insulation forms the actual barrier with the environment.2. The fact that TU fibers do not supercontract is probably also favorable for the survival of the eggs. If TU fibers should supercontract, the eggs would probably be killed if egg sacs are placed in humid environments.3. It has been suggested that the egg sacs of *N. clavipes* protect the egg mass against micro-organisms (Austin 1985). Like sticky threads, it is possible that TU fibers possess a high concentration of potassium dihydrogen phosphate to prevent the eggs from bacterial and fungal degradation (Schildknecht et al. 1972).4. The tufted nature of egg sac fibers protects the eggs against mechanical damage, predation or parasitism (Guarisco 2001) and can also save the eggs and spiderlings from drowning and physical damage (Hieber 1992a, b).

Egg sac fibers are always attached to the threads of the basic layer or, in nature, the MA threads of webs or the outer layer of egg sacs and never to the substrate. Apparently they can only be attached to other fibers. The TU fibers (of the two layers) are also attached to other fibers with glue-like fibers, which includes both. The origin of these secretions is however unknown.

Egg sac chamber.—In recently placed egg sacs, the eggs are encircled by the first and second insulation layer which forms the egg chamber (Fig. 1 (5)), which is here the same as the egg sac chamber. By older egg sacs however, the mass of the eggs and the upside-down position (horizontal or vertical) causes the egg sac to sag out due to gravity, forming a space (Fig. 1 (6)) between the eggs and the first insulation layer. So here the egg sac chamber is the total of the ‘‘egg chamber’’ (Fig. 1 (5)) and the ‘‘egg sac space’’ (Fig. 1 (6)). It is possible that this egg sac space is vital for the hatching and the survival of the young spiderlings till the first ecdysis.

Outer layer.—The threads of the outer layer are double stranded, attached to the substrate with attachment discs and have a similar morphology like draglines. All these observations suggest that these threads originate from the MA spigots. If the MA threads used for the egg sac are compared with the dragline of the same spider, a remarkable difference ($\cong 1 \mu m$) in thickness can be seen (Table 1). Because both fibers are from MA gland origin, this difference can only be explained by the way they were produced. As found by Vollrath et al. (2001), both thread extension and reeling speed affect the diameter of the thread by a constant temperature. Draglines in this experiment were collected from hanging spiders which resulted in a bigger thread extension as well as a higher reeling speed resulting in a fine thread. Threads used for the egg sac structure are never stretched in this way because the spider is at all time attached to the substrate with her legs and the reeling speed was like the walking speed of the spider, resulting in a thicker thread. The difference in thickness between the fibers of the basic layer and the outer layer (Table 1) is probably due to a greater thread extension in the fibers of the outer layer caused by a bigger load on the thread from the mass of the spider. The connections of the outer layer threads to the second insulation layer fibers have probably the same origin as the MA-MA thread connections.

In contrast with *A. aurantia*, the outer layer of *Z. x-notata* is not as dense, which would suggest that it is rarely or never attacked by generalist predators. This, however, is not so. These egg sacs were protected against predators by use of a defensive layer made up of sticky threads. These sticky threads were only found on the outer layer of the egg sac in a very specific arrangement. It is very likely that these sticky threads are the same sticky thread as those used in the sticky spiral of webs and that they fulfill the same function. The fact that *Z. x-notata* is iteroparous means that she can replace the sticky thread of the egg sac when it dries out. This sticky thread was only observed in five egg sacs where mites were present in the cup, which may explain the extra security in contrast with the other observed egg sacs.

In conclusion, egg sacs are built of four layers; a basic layer, a first insulation layer, a second insulation layer and an outer layer. This study shows for the first time the details of the different fibers involved in the egg sac, their possible function, their connection types and the role of the different structures they form in the egg sac. The basic- and outer layer are formed of MA threads which are for support and attachment of the TU fibers. In contrast, the insulation layers are made up by TU fibers and arranged in two layers. The fibers of the first insulation layer are finer than those of the second insulation layer which could indicate that the second insulation layers has a moisture regulation function in the egg sac. In some egg sacs there was an additional fiber type present, namely sticky threads. These sticky threads were found on the outer layer and probably protect the egg sac against generalist predators, such as mites.

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