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Immune Defense Reactions of Myriapoda —
A Brief Presentation of Recent Results

by

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A b s t r a c t : A short review of immune defense mechanisms of diplopods and chilopods (with special reference to the species *Rhapidostreptus virgator* (SILVESTRI), *Chicobolus* spec., *Scolopendra* spec. and *Lithobius forficatus* (L.)) is given. Myriapods have different types of hemocytes which are able to detect between self and non-self and phagocytize or encapsulate foreign material. As humoral defense systems myriapods have different antibacterial substances, a phenoloxidase system and lectins. At least two antibacterial substances occur in the hemolymph of myriapods: a lysozyme and at least one substance which is different from a lysozyme. The activity of the phenoloxidase in many myriapods is low in comparison to crustaceans and most insects investigated; zymosan and chymotrypsin may act as inductors but they are less efficient as in the lepidopteran *Manduca sexta* (L.). Phenoloxidase uses DOPA and pyrogallol as substrates better than tyrosine and is inhibited by PTU. Lectin(s)/hemagglutinins which specifically agglutinate erythrocytes from different vertebrate species were also found in the hemolymph of the diplopod species tested; they were more efficient in *Chicobolus* than in *Rhapidostreptus* with most of the erythrocytes tested. The lectin(s) are sensitive to heating and could be inhibited by a number of different mono- and disaccharides.

1. Introduction:

An elaborated internal defense system is necessary to resist infections of parasitic organisms (e. g. bacteria, fungi or protozoan and metazoan parasites) and viruses. Bacteria and fungi which are very frequent in the habitat of terrestrial arthropods may invade the hemocoel through wounds, multiply and lead to lethal infections if there is no effective immunological response which can destroy the potential pathogens. Such an internal defense system seems to be an indispensable prerequisite for survival of a species when taking into account the high frequency of wounds in natural populations of various chilopods as shown by FRÜND (1990 and this volume) which normally will lead to infections. The elements responding to such infections belong to the so-called immune system.

Whereas the immune systems of insects and crustaceans have been studied by invertebrate immunologists for nearly half a century and our knowledge on their various parts is comparatively high, only very few investigations have dealt with immune defense mechanisms of other arthropod taxa (for literature see GÖTZ 1988). On the other hand, investigations on natural substances from numerous animal taxa have elucidated their antibacterial, antiviral, antifungal or otherwise pharmacologically interesting features and the non-investigated taxa might represent a pool for such substances. Hence, the lack of knowledge on immune systems in myriapods, and the possible medical relevance of their components, recently inspired our working group to focus on this field of invertebrate immunology.

2. Results and Discussion:

2.1. Hemocytes and Cellular Defense Mechanisms against non-self Material:

Hemocytes have been investigated in Chilopoda (GRÉGOIRE 1957, RAJULU 1971, RAVINDRANATH 1981, NEVERMANN 1989, NEVERMANN et al. 1991), Diplopoda (KRISHNAN & RAVINDRANATH 1973; RAVINDRANATH 1973, 1977, 1981; this paper) and Symphyla (GUPTA 1968). The hemocyte spectrum found in the various species of myriapods is different, but four or five main types of hemocytes could be found throughout most of the taxa investigated:

1. the small prohemocytes,
2. the plasmatocytes which have the capacity to spread on foreign material (Fig. 1 A),
3. the granulocytes which also can spread but less extensively and more slowly, and which bear a higher number of refracting irregularly-formed granules (Fig. 1 A and B),
4. the spherule cells (Fig. 1 A) which also enclose granules which, however, are regularly shaped and yellow in phase contrast; spherule cells tend to spread only after a very long period in vitro (see NEVERMANN et al. 1991), and
5. the adipohemocytes which contain very large granules of varying diameter and are normally very infrequent; this hemocyte type could possibly be released into the hemolymph during preparation i.e. their presence could be an artifact.

SEIFERT (in RAVINDRANATH 1981), NEVERMANN (1989) and NEVERMANN et al. (1991) also gave some ultrastructural characteristics of the hemocytes of Chilopoda.

The number of circulating hemocytes per volume unit depends largely on diet and rearing conditions (XYLANDER, unpublished). Caution, therefore, is necessary to refer to data of different investigations if the rearing conditions of the specimens investigated are not under control. Nevertheless, the data available show that chilopods have higher numbers of circulating hemocytes than diplopods (NEVERMANN 1989, XYLANDER in NEVERMANN 1989 and unpublished results). Recent investigations furthermore showed that non-sterile injury raises the number of circulating hemocytes of millipedes reared under conducive conditions (XYLANDER unpublished).

Functions of the different hemocytes of Myriapoda are: 1. phagocytosis of bacteria or other pathogens (XYLANDER unpublished results), 2. encapsulation of parasites and larger foreign particles (BOWEN 1967, NEVERMANN 1989, this paper, Fig. 1C), 3. storage and probable controlled discharge of granules or vacuole content (e.g. precursors of the phenoloxidase, see BOWEN 1968, KRISHNAN & RAVINDRANATH 1973, NEVERMANN et al. 1991) in case of infection, 4. wound closure after injury and 5. hematopoiesis as an outstanding role of non- or weakly differentiated hemocytes.

2.2. The Phenoloxidase System:

The phenoloxidase (PO) system is an important defense mechanism in different taxa of Arthropoda. It is responsible among many other features for polymerization and deposition of melanin on foreign particles during the defense reaction; the deposited melanin constitutes a layer which prohibits nutrient uptake of a parasite from, and discharge of toxic material into, the surrounding hemolymph. The PO system involves a complicated stimulating as well as inhibiting enzyme-cascade (recently reviewed by JOHANNSON & SÖDERHÄLL 1989), parts of which have also been considered to be responsible for foreign recognition and to initiate different immunological responses. In two earlier investigations BOWEN (1968) and KRISHNAN & RAVINDRANATH (1973) reported the PO of five different millipede species to occur mainly in granulocytes but also in the free plasma. However, as they did not analyse the activity of the PO quantitatively, these data are not satisfactory for a comparative evaluation.
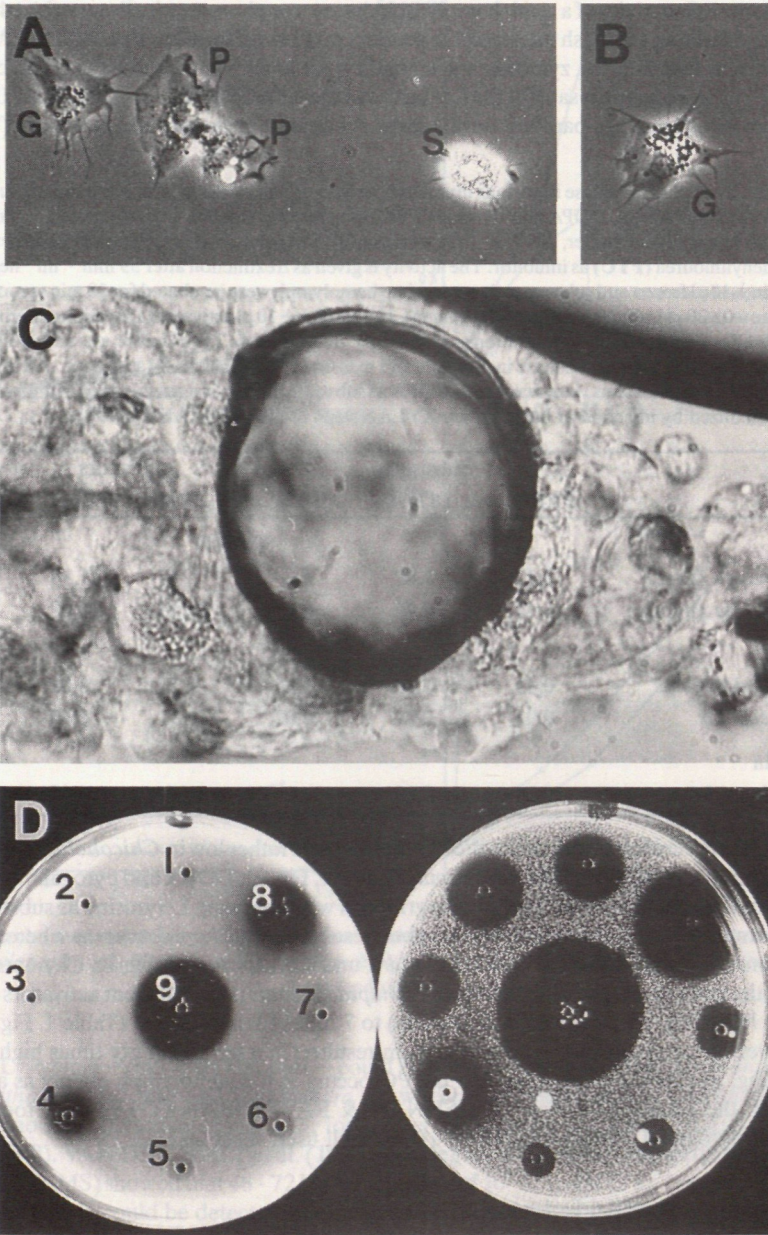
Fig. 1: A - B. Different cell types of *Chicobolus* sp. (Phase contrast): P = Plasmatocyte, G = Granulocyte, S = Spherule cell. C. Encapsulation response in vivo of a polystyrol bead after 4 d in *Scolopendra* sp. D. Test on anti-bacterial substances in the hemolymph of various myriapods as indicated by clear plaques on agar containing murein from *Micrococcus lysodeikticus* (left) or inhibition plaques on living *Micrococcus luteus* (right) (wells 1-4: *Chicobolus* sp., 5-6: *Rhapidostreptus virgator*, 7: *Lithobius forficatus*, 8: *Manduca sexta*, 9: lysozyme-standard (= 0,5 mg ml^{-1} egg-white-lysozyme) (for details of the method see XYLANDER & NEVERMANN 1990). Note: The dark circles around wells 2 and 4 on the left plate are melanin and not lysis plaques.

Thus, the PO-activity of a total-hemolymph-lysate was photometrically tested (at 490 nm detecting Dopachrome, a pinkish intermediate product of melanin formation) using DOPA, pyrogallol and tyrosine as substrates, zymosan and chymotrypsin as activators and PTU as inhibitor of the PO (Table 1). Hemolymph lysates of the L5-larvae of the lepidopteran *Manduca sexta* (L.) (Sphingidae) were investigated in parallels on the same substrates, inductors and inhibitors (Table 1).

Table 1: Activity of phenoloxidase in various myriapods and L5-larvae of the lepidopteran *Manduca sexta* using Pyrogallol **(PYRO)**, DOPA and tyrosine **(TYR)** as substrates (each at a concentration of 4 mg · ml⁻¹ in 0,01 M cacodylate buffer, pH 7,0 - 7,2), zymosan **(ZYM)** and chymotrypsin (CHY) as activators and phenylthiourea **(PTU)** as inhibitor. The activity is given as Δextinction after 59 min · ml⁻¹ hemolymph · min⁻¹. 10 μl frozen and subsequently warmed up hemolymph were incubated for 10 min at room temperature with 20 μl buffer (substrate experiments), 10 μl buffer + 10 μl activator (activation experiments) or 10 μl buffer + 10 μl inhibitor (inhibition experiments). Subsequently 220 μl buffer was added and the whole mixed up with 1000 μl substrate in a cuvette and measured photometrically at 490 nm. All activation and inhibition experiments were made with DOPA as substrate. (**n.t.** = not tested; **untr.** = untreated; **imm.** = immunized by injection of bacteria, see XYLANDER & NEVERMANN (1990).

	Substrates			Activators		Inhibitor
	PYRO	DOPA	TYR	ZYM	CHY	PTU
Insecta						
Manduca (untr.)	0,22	1,82	n.t.	2,10	21,38	0,06
Manduca (imm.)	0,42	0,46	n.t.	22,00	20,08	0,03
Diplopoda						
Rhapidostreptus	0,43	2,60	0,25	1,29	5,08	0,18
Chicobolus	0,91	0,21	0,02	0,31	0,40	0,07
Chilopoda						
Scolopendra	0,26	0,09	n.t.	n.t.	0,65	0,17

Without application of inductors, the PO-activity was rather low in *Chicobolus* and *Scolopendra* but comparatively high in *Rhapidostreptus* (Table 1, Fig. 2). DOPA and pyrogallol were shown to be appropriate substrates for the PO of myriapods whereas using L-tyrosine as substrate dopachrome-formation was very low. DOPA was a better substrate in *Rhapidostreptus* whereas pyrogallol was more effectively converted in *Scolopendra* and *Chicobolus* (Table 1). Chymotrypsin and zymosan (the latter in all species tested except *Rhapidostreptus*) were efficient activators of the PO, increasing its activity about twice (in diplopods) to 7 times (*Scolopendra*) (Table 1, Fig. 2). In *M. sexta*, however, the application of chymotrypsin resulted in a ten to twenty times higher activity (Table 1, Fig. 2). These results show that the PO occurs in the hemolymph mainly as an inactive proenzyme, the prophenoloxidase. KRISHNAN & RAVINDRANATH (1973), too, reported DOPA and pyrogallol (but also catechol) to be a well used substrate.

2.3. Humoral Antibacterial Defense:

Antibacterial substances occur in the hemolymph of insects without treatment (lysozyme). By injection of e.g. bacteria, bacterial cell wall components or inert particles, the lysozyme-titre can be raised and new substances with a wider range of antibacterial effectivity (cecropins, attacins, diptericins, apidaecins) are synthesized. Recently, three papers have been published showing that antibacterial substances can be found also in the hemolymph of two chilopod and three diplopod taxa (VAN DER WALT et al. 1990, XYLANDER 1989, XYLANDER & NEVERMANN 1990). The hemolymph of all myriapods tested contained substances which are bacteriostatic against various
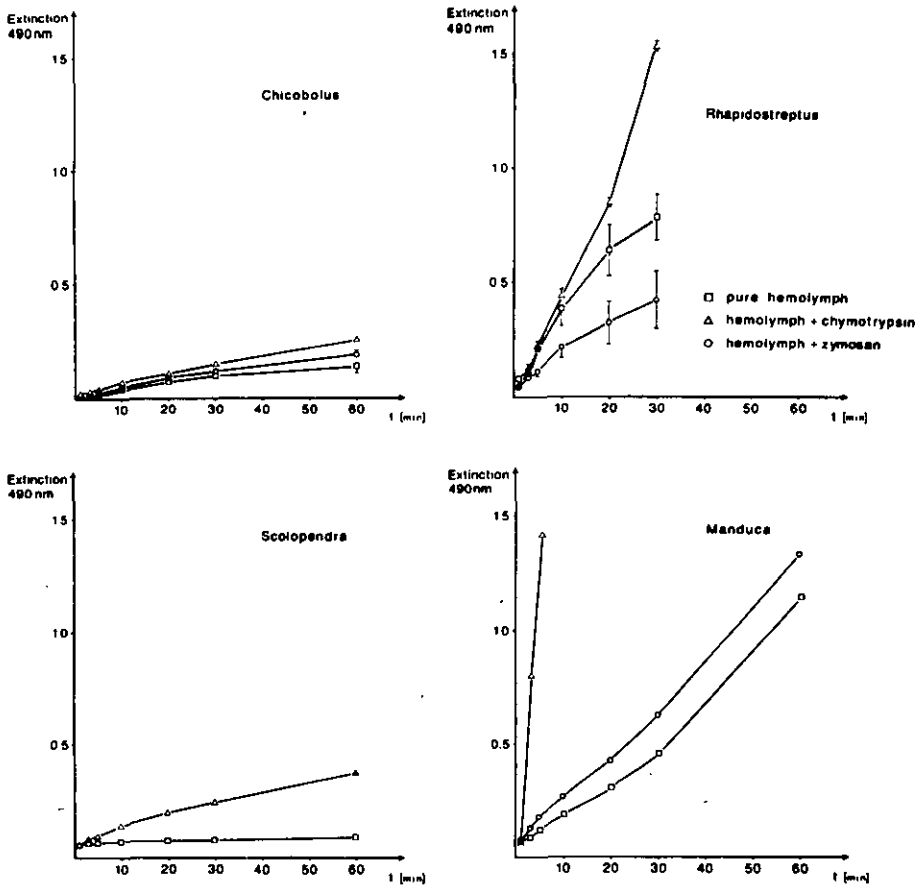
Fig. 2: Dopachrome formation by the PO-system measured photometrically at 490 nm in *Chicobolus*, *Rhapidostreptus*, *Scolopendra* and *Manduca* using pure hemolymph (incubation with buffer) and after incubation with zymosan and chymotrypsin.

bacteria. Lysozyme — only little amounts — could be found in the diplopod *R. virgator* and the chilopods *Lithobius forficatus* and *Scolopendra* sp., whereas the hemolymph of *Chicobolus* lacks detectable amounts of lysozyme (Fig. 1 D; for further information see XYLANDER & NEVERMANN 1990). VAN DER WALT et al. (1990) working on the Kalahari millipede *Triaenostreptus triodus* (ATTEMS) showed that 48 - 72 h after injection of 10^7 *E. coli* K 12 ml^{-1} an antibacterial protein against *E.coli* could be detected when extremely large amounts of hemolymph (30 μl) were used in petri dishes tests; this protein is presumably a basic one indicating a probable homology to the cecropins of insects (VAN DER WALT et al. 1990). In other myriapod species investigated, no antibacterial response on *E. coli* could be found but on gram-negative *Enterobacter cloacae* by XYLANDER & NEVERMANN (1990). However, recent investigations showed very little amounts of substances against *E. coli* in *Rhapidostreptus*, too (XYLANDER, unpublished results). A Western blot stained with antibodies against cecropin from *Hyalophora cecropia* L. (Lep. Saturniidae) showed no staining effect on *Galleria mellonella* L. (Lep., Pyralidae), *Rhapidostreptus* and *Chico-*

bolus hemolymph but reacted with *Hyalophora* cecropins (XYLANDER & TRENCZEK, unpublished results). This indicates that whatever these antibacterial substances in *Chicobolus* and *Rhapidostreptus* might be, they are not very closely related to the cecropins of *Hyalophora*.

The titre of the bacterial substances of myriapods against bacteria as well as the total hemolymph protein content can be raised by injury and injection of bacteria and, therefore, is inducible (VAN DER WALT et al. 1990, XYLANDER 1989 and unpublished results, XYLANDER & NEVERMANN 1990); the period after injection when the antibacterial effect reached its maximum (3 to 5 days) corresponded in all taxa investigated (VAN DER WALT et al. 1990, XYLANDER & NEVERMANN 1990). Only a few proteins found in immunized millipedes are "really antibacterial" as shown in an overlay-test (VAN DER WALT et al. 1990) but may have other functions in immune response (e. g. bacterioagglutinins, opsonins, cellular bacteriolysins of phagocytes — if they do not originate from the injected bacteria).

2.4. Lectins/Hemagglutinins:

Lectins are sugar-specific proteins which occur on the surface of viruses, bacteria, as well as of plant and many animal cells, where they play important roles e. g. in foreign recognition, infection, fertilization and differentiation processes. They bind specific sugars e. g. on the surface of erythrocyte membranes and agglutinate the erythrocyte. Thus, the presence of lectins can be demonstrated in agglutination tests using erythrocytes carrying the specific sugar component(s) on their surface.

Lectins of arthropods have been shown to occur as free hemolymph proteins as on the hemocyte surface. Their function in arthropod hemolymph is not completely clear. However, it is likely that they are responsible for opsonization, foreign recognition (e. g. of cell-wall components of invading bacteria and fungi), for clotting bacteria and fungi, for initiating capsule formation by aggregation of hemocytes and for stimulating phagocytosis (RENWRANTZ 1986). Furthermore, lectins synthesis can be induced by injection of bacteria or bacterial cell wall components and injury (MINNICK et al. 1986, SARBADHIKARY & BHADRA 1990). Thus, among the "non-antibacterial" proteins which were found to be newly built after bacterial challenge in *Triaenostreptus* (VAN DER WALT et al. 1990), could be one (or some) lectin(s) which are non reactive in an overlay-test.

As first approach to investigate the lectins in myriapods, the agglutination capabilities of untreated millipedes on the erythrocytes of different vertebrate species were tested. This investigation showed that 1. the hemolymph of *Rhapidostreptus* showed a weaker level of average agglutination of erythrocytes ($\bar{x} = 5.83 \pm 1.95$) than *Chicobolus* ($\bar{x} = 7.5 \pm 4.83$) and *Manduca* ($\bar{x} = 8.33 \pm 2.84$) with the erythrocytes tested; 2. the erythrocytes of some vertebrate species were more strongly agglutinated than others but this effect also depended on the millipede species (Table 2): *Chicobolus* reacted best with rabbit-, pig-, dog- and rat-erythrocytes, whereas *Rhapidostreptus* showed comparably good agglutination of human (B) and rat (Table 2).

To determine the sugar specificity of the lectins, hemolymph samples were incubated with different mono- and disaccharids for at least 30 minutes. Subsequently, dog-erythrocytes-suspension was added and after 60 min, the lowest dilution of hemolymph in which agglutination occured was determined (for experimental conditions see Table 3). The hemagglutination capabilities of lectins of *Chicobolus* were inhibited best by saccharose, rhamnose, fucose and trehalose as strongest inhibition in *Rhapidostreptus* occured with rhamnose and lactose (Table 3). Freezing only slightly affected the agglutination capabilities of the diplopod hemolymph whereas heating resulted in a strong reduction of reactivity (Table 4).

Table 2: Hemagglutinins against erythrocytes of different vertebrate species in the hemolymph of myriapods and untreated *Manduca sexta* (Erythrocytes were washed three times in NaCl-ringer [0,9 % + 0.02 % NaN_3], centrifuged for 10 min at 1000 U · min⁻¹; 30 µl of the pellet was subsequently resuspended in 1 ml pure NaCl-ringer (0,9 %) and 25 µl of this erythrocyte suspension was added to each 25 µl of a 1 : 1 dilution series of hemolymph and NaCl-ringer. Agglutination capabilities were estimated after 1 h from the lowest dilution in which agglutination could be observed). Results are given as $1 : 2^x$.

	Rhapidostreptus	*Chicobolus*	*Manduca*
Man A Rh+	6	6	11
Man B Rh+	8	6	8
Man AB Rh+	7	8	11
Man O Rh+	7	8	11
Rabbit	2	13	10
Rat	8	11	8
Pig	7	> 12	10
Sheep	4	0	4
Cow	3	2	3
Dog	6	> 12	7
Chicken	5	0	6
Dough	7	10	11

3. Immune Defense in Myriapods and other Arthropods — a Preliminary Comparative Evaluation:

In comparison to the insects and decapod crustaceans the immune system of myriapods has not been very well investigated. Moreover, the strengths of immune reactions in the myriapods and the main elements differ between the various taxa and significant dissimilarities between specimens of a single species were observed. Furthermore, the interactions of the different components which are involved in the immune defense of myriapods are far from being completely understood. Some elements of the system, however, have been investigated and these results were shown and discussed above. This is a preliminary attempt to compare and evaluate the "effectivity" of these isolated components — at our present status of knowledge.

The immune system of the Myriapoda involves cellular and humoral components. The cell types and their capabilities to destroy or exclude probable pathogens correspond — as far as they have been investigated — to the system found in insects (see GÖTZ 1988), although encapsulation processes seem to take more time in myriapods (NEVERMANN 1989). In Chilopoda, the hemocyte counts are higher than in Diplopoda. Thus one could speculate that the immune system of Chilopoda depends more on a cellular defense. However, as the investigations by FRÜND (1990 and this volume) have shown, the predatory chilopods are more endangered by injury and might, therefore, need higher numbers of circulating hemocytes to prevent microbial infection by phagocytosis and for wound closure.

In general, the humoral defense system of myriapods (including lectins, phenoloxidase and antibacterial substances) is similar and of comparable heterogeneity to that of insects and other Articulata. Lectins seem to be equivalently developed in insects and millipedes although the capabilities to agglutinate the erythrocytes tested were higher on average in *Manduca* than in *Chicobolus* and *Rhapidostreptus*. In comparison to most insects, the "in-vitro-effectivity" of the antibacterial substances of Myriapoda is low. Lysozyme, which is an important antibiotic agent in insects, seems to be less important in myriapods. Antibacterial substances other than lysozyme have a lower potency or a smaller range of bacteria on which they are bacteriostatic: The substances found in

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Table 3: Sugar specifity of hemolymph lectins from different millipedes and *M. sexta* mono- and disaccharides using dog erythrocytes. Preparation of erythrocytes was done as described above. 25 µl of a 0,2 M sugar solution in 0,05 NaCl was added to 25 µl of a 1 : 1 hemolymph dilution series on agglutination plates. The plates were shaken for 30 min at room temperature and then the erythrocyte suspension was added, mixed up and left on the shaker for another 1 h. Agglutination capabilities were estimated as described above). **n.t.** = not tested.

	Rhapidostreptus	*Chicobolus*	*Manduca* (treat.)	*Manduca* (untreat.)
Monosaccharides				
(+)-Glucose	6	8	6	6
D(-)-Fructose	6	9	8	7
D-Galactose	5	7	7	2
D(+)-Mannose	7	9	8	2
α-Rhamnose	2	5	7	6
L-Fucose	6	5	7	2
N-Acetyl-Glucosamine	7	9	8	n.t.
N-Acetyl-Galactosamine	6	7	8	n.t.
Disaccharides				
Sucrose	6	4	3	n.t.
D(+)-Trehalose	5	5	7	n.t.
α-D-Lactose	3	8	6	n.t.
Control	7	9	9	n.t.

Table 4: Effect of freezing (several days at − 17° C) and heating (15 min at 70° C in a water bath) on hemolymph samples of different millipede species and *Manduca* using dog erythrocytes. **n.t.** = not tested.

	Rhapidostreptus	*Chicobolus*	*Manduca* tread.
Freshly collected hemolymph	7	11	n.t.
Frozen hemolymph	6	9	9
Heated hemolymph	3	3	2

Triaenostreptus by VAN DER WALT et al. (1990) show effects on growing *E. coli* corresponding to that found in insects only after injection of very high amounts of the rather pathogenic *E. coli* (see XYLANDER & NEVERMANN 1990) and applying large volumes of hemolymph to the test agar plates (30 µl instead of about 2 - 4 µl normally used when testing insect hemolymph; see GÖTZ et al. 1987, KEPPI et al. 1986). The titre of antibacterial substances needs about 3 to 5 days after inoculation to reach its maximum; this is longer than in many insects which only need 6 - 24 h (e.g. CHADWICK & DUNPHY 1986, KEPPI et al. 1986, MOHRIG & MESSNER 1968). The activity of the inactivated phenoloxidase of most myriapod species investigated was lower than in insects. After activation, however, in all cases the effectivity of the PO was lower in myriapods than in *Manduca*. This corresponds to the observation that melanization processes in myriapods (e. g. at wound margins or of hemolymph droplets exposed to air) are much slower than in insects.

Thus, the immune system of myriapods comprises the same elements — as far as they have been investigated till now — as have been found in other taxa of Arthropoda, especially insects; it seems, however, to react generally more slowly. Nevertheless, it is sufficient to protect myriapods against potential pathogens under natural conditions.

4. Acknowledgements:

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