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Profiling of proteins and proteases in the products of the salivary gland, digestive tract and excretions from larvae of the camel nasal botfly, *Cephalopina titillator* (Clark)

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Abstract: Proteins and proteolytic activities in the contents of the salivary gland (SGc), digestive tract (DTc) and excretory-secretory products (ESP) from larvae of the camel nasal botfly *Cephalopina titillator* were separated electrophoretically, and characterized. The protein profiles of the different samples were qualitatively quite similar in the larval stages L2 and L3. Zymogram analysis of proteases in the samples indicated that the digestive tract contained a greater variety of proteases than the salivary gland or the excretory-secretory products. They are mainly serine proteases. Proteases of ESP and DTc (especially of 3rd instar) contain trypsin- and chymotrypsin-like serine proteases, while the serine proteases of SGc are not of the trypsin- or chemotrypsin-type.

Keywords: *Cephalopina titillator*; digestive tract; excretory-secretory products; proteases; salivary glands.

1 Introduction

Nasopharyngeal myasis is caused by some dipterous obligatory parasitic larvae, belonging to the family Oestridae, including the camel nasal botfly, *Cephalopina titillator* [1]. These parasites are widely distributed in Egypt [2] and many other countries of the world. *Cephalopina titillator* is an obligate parasite of camels. The female fly deposits its larvae directly into the nasal cavity. The larvae crawl up to the nasopharynx and sometimes to the paranasal sinuses, causing extensive irritation and tissue damage (nasal myiasis), leading to impairment of the animals' welfare. Host physiological functions are reduced, the animals breathe with difficulty, snort, sneeze, and behave abnormally, often become restless and may even stop feeding, thereby causing significant economic losses [3–5].

Larval feeding activity of the Oestridae involves secretion of enzymes into the upper respiratory mucosal substrate, where immunoglobulins and defense cells are present. These proteolytic enzymes degrade the substrates (mucosa and mucus secretion) into smaller units which are taken up and digested for larval growth and development [6, 7]. External protein digestion plays an important role in the biology and acquisition of nutrients by parasitic fly larvae, such as *Hypoderma lineatum* (Oestridae), *Lucilia cuprina* and *Chrysomya bezziana* (Calliphoridae). These proteolytic enzymes are important for larval migration, establishment, feeding, growth, and development [8, 9].

Excretory-secretory products (ESP) are mainly derived from the salivary gland (SGc) and the digestive tract DTc [10] and are of diagnostic value. This study aimed to separate and characterize the proteins and proteolytic activities in the products of salivary gland, digestive tract and excretions from *C. titillator* larvae.

2 Materials and methods

2.1 Collection of larvae

Cephalopina titillator larvae were recovered from naturally infested camel heads. The heads were collected from the local slaughterhouse of Elwaraq (Giza, Egypt), and split longitudinally. Second (L2) and 3rd (L3) instars were washed several times in phosphate buffered saline (PBS).

2.2 Preparation of larval proteins

2.2.1 Excretory-secretory products (ESP): A pool of 20 larvae of each L2 and L3 were washed with phosphate buffered saline (PBS), and

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incubated in sterile PBS (500 µl/larva) for 24 h at room temperature in the dark. The solution was collected by centrifugation at 10,000 *g*, filtered through 0.8/0.2 µm sieves and lyophilized prior to a test to provide the ESP preparation.

2.2.2 Contents of digestive tract and salivary gland: Twenty larvae of each L2 and L3 were dissected in cold PBS (4–10 °C). The whole digestive tracts and the salivary glands were collected and centrifuged at 10,000 *g* at 4 °C for 15 min to expel their contents (DTc and SGc). The supernatants were stored at –70 °C until use.

2.3 Protein concentration

Protein concentrations in ESP, SGc and DTc were determined according to the method of Bradford [11], using bovine serum albumin as standard.

2.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed under denaturing conditions according to Laemmli [12]. The separating gel (12%) was stained with Coomassie brilliant blue (COBB), for 0.5 h. The relative concentration and molecular mass of each characteristic band was deduced by densitometry, using a marker kit (GenDEPOT) containing 12 proteins, their molecular masses ranging from 11 to 245 kDa.

2.5 Zymograms (gelatin substrate-polyacrylamide gel electrophoresis [GS-PAGE])

Zymograms for the separation and identification of protease activities were made in gels copolymerized with gelatin (0.1% separating gel, gelatin-PAGE) based on the method described by Manchenko [13].

Thirty mg/ml of each of DTc, SGc and ESP from L2 and L3 were mixed with sample buffer (1:1, v/v), and applied to the gels. After electrophoresis at 120 V and 4 °C, gels were renatured with 2.5% Triton X-100 for 30 min at room temperature and washed with distilled water, then incubated for 15 h at 37 °C in developing buffer (pH 5.5) containing 1 mM DTT. Zymogram gels were soaked in a fixing-stain containing Coomassie brilliant blue R-250.

2.6 Protease inhibition

To determine the inhibition of protease activities towards gelatin, DTc, SGc and ESP samples from L2 and L3 (30 µg/ml) were incubated for 1 h at 37 °C with the following inhibitors at the mentioned concentrations: Phenylmethylsulfonyl fluoride (PMSF, 20 mM), N-tosyl-L-phenylalanyl chloromethylketone (TPCK, 150 mM), ethylenediamine tetraacetic acid (EDTA, 10 mM), trypsin inhibitor soybean (SBTI, 100 µg/ml). All inhibitors were purchased from Sigma-Aldrich (St. Louis, MO, USA). After incubation, each sample was mixed 1:1 with sample buffer.

3 Results

3.1 Protein profiles of SGc, DTc and ESP of 2nd and 3rd instars of *C. titillator*

The protein profiles of the contents of the salivary gland (SGc) and digestive tract (DTc) as well as the excretory secretory products (ESP) of L2 and L3 of *C. titillator* were analyzed by SDS-PAGE (Figure 1 and Table 1). SGc both from L3 and L2 showed 9 prominent polypeptide bands with molecular masses ranging from 161 to 10 kDa in L3 (Figure 1, lane 1), and from 168 to 10 kDa, (Figure 1, lane 2). The protein band no.13 of approximately 21 kDa was detected only in SGc. The protein bands no. 2 and 4 (148 and 108 kDa) were detected only in SGc of L3, while the protein band no. 3 (116 kDa) was unique to SGc of L2 (Table 1). Digestive tract contents from L3 and L2 were separated into 6 polypeptide bands with molecular masses ranging from 165 to 10 kDa, in L3 (Figure 2, lane 3), and from 173 to 10 kDa in L2, (Figure 1, lane 4).

ESP from L3 presented 6 polypeptide bands (65–18 kDa), while ESP from L2 showed 3 bands (51–18 kDa) (Figure 1, lanes 5, 6). The protein band no. 1 (161 kDa) was common to all samples, except ESP of L2. Its percentage of the total proteins was in the range of 18–30%, except in ESP of L3, where it was very low (about 1.8%). The protein band no. 6 was the only protein band detected in all samples of L3. The protein bands no. 7 and 15 were detected in DTc and SGc of L3 and L2, but not in the corresponding ESP (Table 1).

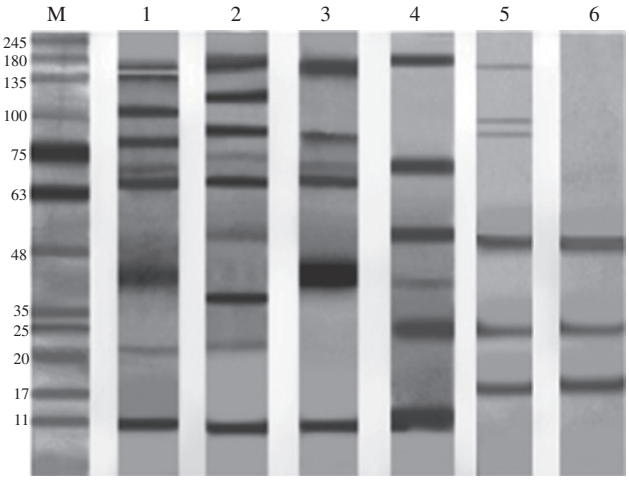

Figure 1: SDS-PAGE profile of *C. titillator*. M: molecular weight marker (kDa), SGc of L3 (lane 1); SGc of L2 (lane 2); DTc of L3 (lane 3); DTc of L2 (lane 4); ESP of L3 (lane 5) and ESP of L2 (lane 6).

Table 1: Molecular mass (M, kDa) of protein bands and percentage (%) of total of proteins separated by SDS-PAGE from SGc, DTc and ESP of 2nd and 3rd instar of *C. titillator*.

Lane	SGc of 2nd instar		SGc of 3rd instar		DTc of 2nd instar		DTc of 3rd instar		ESP of 2nd instar		ESP of 3rd instar	
Band	M	%	M	%	M	%	M	%	M	%	M	%
1	168	18	161	18	173	30	165	16	–	–	165	2
2	–	–	148	3	–	–	–	–	–	–	–	–
3	117	20	–	–	–	–	–	–	–	–	–	–
4	–	–	108	6	–	–	–	–	–	–	–	–
5	92	10	–	–	–	–	–	–	–	–	97	2
6	–	–	81	15	–	–	88	6	–	–	87	2
7	74	5	70	2	71	17	71	5	–	–	–	–
8	67	11	65	17	–	–	67	21	–	–	–	–
9	52	13	–	–	53	10	–	–	51	63	52	36
10	–	–	41	21	41	8	42	40	–	–	–	–
11	37	9	–	–	35	20	–	–	–	–	–	–
12	–	–	–	–	–	–	–	–	26	26	28	46
13	22	2	21	1	–	–	–	–	–	–	–	–
14	–	–	–	–	–	–	–	–	18	11	18	13
15	10	12	10	18	10	16	10	12	–	–	–	–

3.2 Zymogram of protease activities

3.2.1 Zymogram analysis of SGc of 2nd and 3rd instar of *C. titillator*

The zymogram analysis of L2 SGc (Figure 2A, lane 1) revealed partially hydrolyzed broad zones in the mass range from 75 to 180 kDa, from 55 to 63 kDa, and from 20 to 35 kDa. This activity was notably inhibited by the synthetic irreversible serine protease inhibitor PMSF in the zone from 75 to 180 kDa (Figure 2A, lane 2). Treatment with the protease inhibitors EDTA, TPCK and SBTI did not suppress proteolysis (Figure 2A, lanes 3, 4 and 5, respectively).

In the L3 SGc profile, proteolytic activity was visible in ranges between 75 to 245 kDa, 44 to 63 kDa and 20 to 40 kDa (Figure 2B, lane 1). PMSF inhibited proteolytic activity in the zone from 75 to 245 kDa, and partially in the zone from 44 to 63 kDa (Figure 2B, lane 2). Pre-incubation of L3 SGc with the metallo-protease inhibitor EDTA or the chymotrypsin-like serine protease inhibitor, TPCK (Figure 2B, lanes 3 and 4), inhibited the gelatinolytic activity of L3 SGc in the range from 44 to 55 kDa. Treatment with the trypsin inhibitor SBTI suppressed partially the proteolytic activity in the zone from 43 to 56 KDa (Figure 2B, lane 5).

3.2.2 Zymogram analysis of the DTc of the 2nd and 3rd instar of *C. titillator*

The zymogram analysis of L2 DTc revealed proteolytic activity in every mass range with the exception of the range between 17 and 44 kDa (Figure 2C, lane 1). This activity was notably inhibited by PMSF in zones from 158 to 245, and from 44 to 80 kDa. Partial inhibition was also observed in zones from 105 to 158 and from 80 to 95 kDa (Figure 2C, lane 2). EDTA and SBTI (Figure 2C, lanes 3 and 5, respectively) suppressed proteolysis in the same zones from 158 to 245 kDa, and partially inhibited in the zone from 44 to 63 kDa. Preincubation of L2 DTc with TPCK (Figure 2C, lane 4) inhibited fully in the zone from 158 to 245 kDa and partially in the zone from 44 to158 kDa.

In the L3 DTc profile, proteolytic activity was visible at a range varying from 55 to 135 kDa, and >17 (nearly 18) kDa (Figure 2D, lane 1). These activities were inhibited by PMSF in zones from 55 to 80 kDa and from 80 to 95 kDa, and partially in the zone from 105 to 135 kDa, (Figure 2D, lane 2). EDTA (Figure 2D, lane 3) inhibited in the zone from 55 to 70 kDa. TPCK and SBTI partially inhibited the gelatinolytic activity of L3 in a range varying from 55 to 135 kDa (Figure 2D, lanes 4 and 5 respectively).

3.2.3 Zymogram analysis of the ESP of 2nd and 3rd instar of *C. titillator*

The zymogram analysis of L2 ESP (Figure 2E, lane 1) revealed proteolytic activity from ~160 to 245 kDa and from 25 to 55 kDa. This activity was inhibited by PMSF at a range from 25 to 48 kDa (Figure 2E, lane 2), and completely inhibited by TPCK or SBTI (Figure 2E, lanes 4 and 5, respectively). EDTA suppressed proteolysis in the range from 48 to 55 kDa (Figure 2E, lane 3).

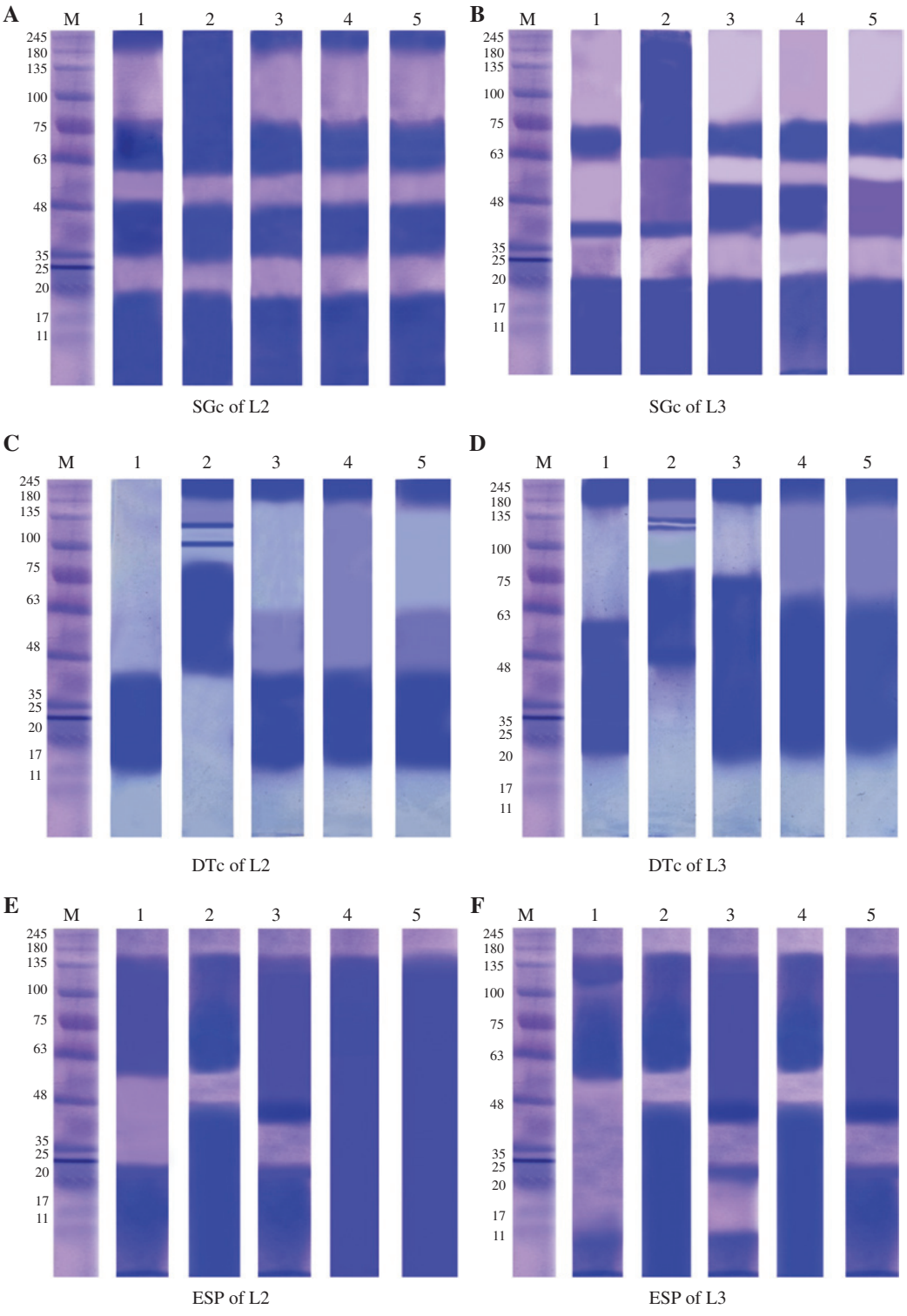

Figure 2: Gelatin-containing SDS-PAGE gels showing the effect of protease inhibitors on the protease activities of the samples from *C. titillator*. Proteolytic assays were performed in the absence (control-lane 1) or presence of the following protease inhibitors: PMSF (lane 2); EDTA (lane 3); TPCK (lane 4); SBTI (lane 5). M: molecular weight marker (kDa).
(A) SGc of L2. (B) SGc of L3. (C) ESP of L2. (D) ESP of L3. (E) DTc of L2. (F) DTc of L3.

In the L3 ESP profile, proteolytic activity was visible in the ranges from 11 to 55 kDa, and >180 kDa (Figure 2F, lane 1). These protease activities were inhibited by PMSF and TPCK in zones from 11 to 48 kDa, (Figure 2F, lanes 2 and 4). Treatment with EDTA suppressed proteolysis in the ranges from 48 to 55 kDa and from 20 to 25 kDa (Figure 2F, lane 3). Preincubation with SBTI inhibited proteolysis in zones from 48 to 55 and from 11 to 25 kDa (Figure 2F, lane 5).

4 Discussion

By SDS-PAGE and zymography, proteolytic activities of the ESP, SGc and DTc of 2nd and 3rd larval instars of *C. titillator* were separated and partially characterized by inhibition studies. Although, L2 and L3 feed on the same nutrients and live in the same environment, having nearly the same proteases to digest their food, qualitative changes were observed between the two instars.

ESP, SGc and DTc of L3 are similar in 3 bands of the masses 164, 87 and 12 kDa. ESP, SGc and DTc of L2 are similar in one band of the mass 51 kDa. The proteases of ESP originate mainly from the DTc and SGc that are exported to the camel's nasal or sinusal mucosa [7, 10].

The salivary glands of larval *Rhinoestrus* spp. induced an intense immune response in the host (horse) compared to that stimulated by whole larval extract, and thus contain the major immunogens [14, 15].

The results of the zymographic analysis and the use of inhibitors of various protease classes showed that, in case of SGc, DTc and ESP, most of the proteolytic enzymes belong to the serine protease subclass. Similar results were observed in the SGc of *O. ovis* [9], and in the ESP of other agents of myiasis [7, 8, 16, 17].

Serine proteases have previously been reported in larvae of various parasitic fly species: *Chrysomya bezziana* [8]; cattle grub, *H. lineatum* [18], *Oestrus ovis* DTc, SGc and ESP [7, 9]. They seem to be essentially involved in larval feeding activity and are possibly involved in immunomodulation, as was demonstrated in other myiasis flies [7]. Serine proteases are known to degrade serum albumin and mucin which are present in the direct environment of the larvae due to their position in the sheep mucosa infected with *O. ovis*. Serine proteases secreted by *O. ovis* larvae are able to degrade collagen, and to facilitate plasmatic protein leakage induced by the inflammatory process [7]. Also, they are essential for penetration of *Dermatobia hominis* larvae [17].

Proteases from L2 and L3 instar larvae of *C. titillator* were inhibited by TPCK, an inhibitor of chymotrypsin-like serine proteases, and by SBTI, an inhibitor of trypsin. These results indicate the presence of different types of serine proteases, among which trypsin-like serine proteases predominate.

Most of the ESP proteases are trypsin-like serine proteases, which participate in extracorporeal pre-digestion of proteins used for larval nutrition. ESP contain mixed secretions from the larval gut and salivary glands [9].

In case of L3 DTc, TPCK and SBTI both reduced the gelatinolytic activity, which may be due to the similarity between chymotrypsin-like serine protease and trypsin [18]. Trypsin and chymotrypsin-like serine proteases were found to be the dominant digestive proteases of *C. titillator* and several fly species [7, 20–22]. These larval chymotrypsin-like proteases are of digestive origin and required for larval nutrition [7, 19].

Trypsin- like serine proteases play an important role in numerous physiological processes of the insect, such as digestion, immunity, reproduction, development, signal transduction and wound healing [22, 23]. In case of ESP and DTc of *C. titillator,* EDTA and SBTI inhibited proteolysis in the same mass ranges of the zymograms. It was suggested that trypsin-like proteases are likely to have Ca^{++} as a co-factor, which may be sequestered by EDTA, thus leading to inhibition of the enzyme [24].

Zymographic analyses of SGc, ESP and DTc revealed quantitative differences between L2 and L3 of *C. titillator.* This could be due to changes in different larval instars through the course of development and infection, as in larvae of *L. cuprina* [25] and *D. hominis* [17], in which the proteolytic activities of secreted proteases changed dramatically over the time of development. These changes have also been observed by other authors, specifically in the intestine of *Aedes* larvae [22, 26]. The proteolytic activities in SGc, ESP and DTc from the 2nd and 3rd instar of *C. titillator* larvae appear to be related to the feeding activity, and thus growth rate, mainly of L3, since the high growth rate is a consequence of the increased nutrient intake during this period. Similar results were found in *O. ovis* and various other dipterous species [6, 7, 17, 27].

Several authors suggested the involvement of ESP in additional aspects of larval development in many myiasis-causing dipterous larvae, such as in larval settling and development, or in the escape from the host's immune system [7, 8, 17, 28].

Proteases have been shown to be important in the establishment of some parasite infections through facilitating skin penetration [29]. Besides, they can function as immunodominant antigens by stimulating a host protective immune response, or serve as potential targets for chemotherapy [30]. In the case of myiasis-causing

organisms, interest has focused on several proteases from the point of view of the immunological response, acquired immunity and vaccine development [3, 7–9, 17].

Tabouret et al. [10] found that the serine proteases in the digestive tract of *O. ovis* were poor antigens in sheep. Kumar [31] explains the lack of antigenicity of some insect digestive proteases due to the highly conserved amino-acid sequences in serine proteases during the course of evolution. In contrast, the SGc, which have low enzymatic activity, are highly immunogenic in sheep and may be good targets for a protective immunological response [7, 10].

ESP released in the mucosa might be able to degrade the components of the extracellular matrix and the lamina of the epithelium [16]. ESP and SGc, may also stimulate the host immune system for mucus production to enhance the larval production in a feedback mechanism [32], assuming that the immunoglobulins of the host are degraded by the excretory-secretory proteolytic enzymes of the larvae and that the resulting amino acids may be utilized in larval growth and cellular regeneration [7]. These data are supported by the curves of larval growth [6]. The depletion of lymphocytes and the degradation of immunoglobulins are used as immunosuppressive strategies to evade defensive attacks from the host [33].

5 Conclusion

The protein spectra of SGc, DTc and ESP were found to be more or less similar in L2 and L3. Serine proteases were clearly the main proteases in all samples. These must now be characterized and their functions either in larval settling and development, or in immunological escape mechanisms, must be established.

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