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Detection of toxic metabolites in the hemolymph of *Beauveria bassiana* infected *Spodoptera exigua* larvae

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Abstract. Highly active metabolites have been detected in the hemolymph of the lepidopteran *Spodoptera exigua* infected with the mycopathogen, *Beauveria bassiana*. A combination of phenyl sepharose and CM ion exchange chromatography was utilized to extract the active metabolites from infected hemolymph samples. The active in vivo metabolites, having a molecular mass greater than 10 KDa, were thermolabile and were inactivated by proteinase K. These metabolites were characterized by their ability to disrupt metamorphosis, killing treated larvae at the wandering or pupal stage. Additionally, injection of *S. exigua* larvae with 'active' samples caused a reduction in the number of filopodial-producing hemocytes. The biological activities and biochemical properties suggest that novel compounds are produced during *B. bassiana* mycosis.
Key words. Insect mycopathogen; *Beauveria bassiana*; *Spodoptera exigua*; in vivo metabolites; metamorphosis; insect hemocytes.

The mycopathogen, *Beauveria bassiana* capable of infecting a wide range of insect pests, is being investigated for its potential as a microbial pest control agent[1]. Like other insect mycopathogens, the infection cycle of *B. bassiana* is initiated by conidial adhesion to host cuticle, followed by the production of germ tubes which breach the host cuticle and produce hyphal bodies in the host hemocoel. The hyphal bodies produced in the hemolymph, unlike the in vitro produced cells, have been demonstrated to lack a formal cell wall[2]. The development of these in vivo hyphal bodies are known to disrupt the cellular defense response of *S. exigua* hemocytes[3,4]. After extensive replication hyphal bodies convert to a filamentous mycelial stage which ramify throughout host tissues resulting in the production of external conidiophores in mummified hosts[5,6].

At present, very little is known about the metabolites produced during the in vivo development of *B. bassiana*. As with many insect mycopathogens, a spectrum of biologically active components have been detected from culture filtrates[7–13]. Low molecular weight components, such as beauvericin, its analogues the enniatins, and bassianolide have been demonstrated to be insecticidal[7–10]. The red pigment, oosporein, detected in culture filtrates and observed in infected insects, has been reported to have antibacterial activity[11]. More recently, cyclosporin A and C, metabolites with immunosuppressive activities against vertebrate cells, have been detected in *B. bassiana* mycelia[12]. Kucera and Samsinakova[13] separated high molecular weight compounds from *B. bassiana* and demonstrated their toxic effect by injection to the larvae of *G. mellonella*. Whether or not any of these metabolites are produced at physiologically active concentrations during in vivo growth is not clear.

In this paper, we have assessed the effects of hemolymph extracted from late stage *B. bassiana* infected *S. exigua* on the hemocytes of healthy *S. exigua* larvae. We have determined that infected cell-free hemolymph contains metabolite(s) which are highly toxic to healthy *S. exigua*. Challenge of healthy larvae with these hemolymph preparations is shown to cause immediate and long-term alterations in the circulating hemocyte populations which mimic the observations made during the in vivo growth of *B. bassiana* in *S. exigua*[3,4].

Material and methods

Fungus and hemolymph collection. Cultures of *B. bassiana* blastospores were produced as described previously[3]. Larvae of *S. exigua* were reared on an artificial pinto bean diet at 26 °C under a 16 h light: 8 h dark photoperiod. Early sixth-instar larvae of *S. exigua* were immobilized at 4 °C for 1 h and then injected with 1×10^3 *B. bassiana* blastospores in 5 µl of 0.85% saline. Heavily infected larvae (60 h postinjection) were bled from the base of the second proleg by needle puncture, and infected hemolymph was collected in ice-cold microcentrifuge tubes containing several crystals of phenylthiourea to inhibit melanization. Hemolymph samples were also collected and pooled from healthy larvae. Hemocytes and fungal cells were removed from the hemolymph samples by centrifugation (13,000 g, 2 min) at 4 °C. Infected and healthy sera were filtered through a 0.45 µm filter and stored at −70 °C until used. Additional aliquots of infected

and healthy sera were partitioned into low (< 10 KDa) and high (> 10 KDa) molecular mass fractions (LMW, HMW) using a Centricon-10 filter (Amicon Corp.) by centrifugation (5,000 *g* at 4 °C for 2 h).

Analysis of biological activities. Sixth instar *S. exigua* larvae were challenged with either 5 µl of infected and healthy cell-free hemolymph or samples fractionated from hemolymph. For dose response studies, mortality at wandering and pupal stage, and adult moth emergence were recorded within 2 weeks postinjection. For studies on hemocytes, 5 µl of hemolymph were collected as described previously from injected larvae at 3, 24, and 48 h postinjection and diluted in 95 µl of 0.1 M Hepes buffered saline (HBS), pH 6.8. Fifty microliter aliquots of hemolymph dilution were applied to the wells of ceramic ring slides (1.3 cm diameter, Clay Adams). Hemocyte monolayers were allowed to form at room temperature for 10 min, and the relative number of spreading and the total hemocytes in 3 random microscope fields (500 ×) were counted using phase contrast microscopy. The production of the distinct pseudopodia by hemocytes was the criterium used for hemocyte spreading. To test the viability of hemocytes, hemolymph (5 µl/larva) was collected as described previously at 3, 24, 48 h postinjection and applied to wells of ceramic ring slides containing 45 µl of HBS, pH 6.8. Resulting hemocyte monolayers were rinsed 3 times with 100 µl aliquots of HBS, and 50 µl of 0.2% Trypan Blue (Sigma) was added to the monolayers. Hemocyte viability was then determined using a phase contrast microscopy (500 ×). The influence of infected serum on the phagocytic capability of hemocytes was evaluated by injecting groups of sixth instar larvae with either 5 µl of 0.85% saline, healthy or infected sera. At 3 h postinjection, larvae were reinjected with 2.5×10^6 FITC-labeled *B. bassiana* blastospores in 5 µl of 0.85% saline[3]. One hour after injection of FITC-labeled blastospores, the number of hemocytes, the number of hemocytes associated with FITC-labeled blastospores, and the number of FITC-labeled blastospores per hemocyte were quantitated. Percentage of hemocytes containing FITC-blastospores and mean number of FITC-blastospores per hemocyte were used to evaluate the phagocytic competence of hemocytes.

Fractionation of S. exigua *hemolymph samples.* Cell-free infected hemolymph was first centrifuged at 100,000 × *g* for 2 h with a Sorvall OTD-65B refrigerated ultracentrifuge. The supernatant mixed 1:1 with 50 mM sodium acetate buffer, pH 5.0 was fractionated by hydrophobic interaction chromatography (HIC) through a preparative column of Phenyl-Sepharose® CL-4B (Pharmacia) pre-equilibrated and washed with the Na acetate buffer. The HIC fraction was concentrated using a Centriprep-10 (Amicon) and applied to a CM Bio-Gel A (Bio-Rad) column (1 × 15 cm) pre-equilibrated with 50 mM sodium acetate buffer, pH 5.0. The column was washed and then bound material was eluted with 1 M NaCl in the same buffer at a flow rate of 25 ml/h. Two ml fractions were collected and the absorbance at 280 nm was measured. All chromatography was done in a cold room at 4 °C. Cell-free healthy hemolymph samples were also fractionated following the same procedure.

Electrophoresis and Western blotting. Hemolymph proteins and protein samples fractionated from hemolymph were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 5 to 15% gradient gels and the buffer system described by Laemmli[14]. Low molecular weight standards were run concurrently and protein bands visualized by either Coomassie blue R-250 or silver staining. Additional samples of sera and chromatographic fractions were electrophoresed on 12% SDS-PAGE minigels and transferred to Immobilon-P membranes (Millipore Corp.) according to the protocols of Towbin et al.[15] The efficiency of protein transfer was evaluated by prestaining Western blots with Ponceau red. Strips, blocked with 5% milk in PBS-0.05% tween 20 were incubated with either control rabbit sera or anti-*B. bassiana* rabbit sera (1/400) for 1 h followed by incubation in goat-antirabbit IgG-alkaline phosphatase conjugate (Sigma Chemical).

Determination of total protein concentration. Total protein concentration was determined with the Bio-Rad DC Protein Assay based on the method of Lowry et al.[16] Bovine serum albumin was used as the standard.

Proteases and temperature effects. Samples of the biologically active CM fraction, containing 2 mg of protein/ml, were incubated for 2 h at 37°C with solutions (100 µg/ml) of either sequencing grade trypsin (Boehringer) or a Proteinase K (Sigma). Additional samples of biologically active CM fraction were incubated 30 min at 60 °C and 10 min at 100 °C.

Results

Cell-free hemolymph samples extracted from *B. bassiana* larvae were highly toxic to healthy *S. exigua* larvae (table 1). Injection of 5 µl infected serum caused 100% mortality of challenged 6th instar larvae whereas no mortality was detected in the larvae treated with healthy serum. Throughout the last larval stadia these insects fed and developed at rates comparable to control larvae. As they approached the wandering stage melanized spots, not present in control larvae (fig. 1A), appeared randomly over the larval cuticle (fig. 1B). The majority of these larvae died in the wandering stage (fig. 1B) or as larval-pupal intermediates (fig. 1C). Thirty-six percent of the larvae challenged with infected sera died as pharate adults before emergence from the pupae. Challenge with lower dosages of infected serum resulted in an increase in the percentage of the challenged larvae dying in the pupal stage.

Table 1. Effect of serum samples from *B. bassiana* infected *S. exigua* larvae and CM fraction samples on the mortality of healthy *S. exigua* larvae

Treatments		% Mortality[a]		
		Wandering stage	Pupal stage	Total
Healthy serum	5.00 µl	0	0	0
Infected serum	5.00 µl	64	36	100
	2.50 µl	31	69	100
	0.62 µl	0	90	90
	0.32 µl	0	80	80
	0.08 µl	0	60	60
Healthy CM fraction	5.00 µl	0	0	0
Infected CM fraction	5.00 µl	70	30	100
	2.50 µl	40	60	100
	0.62 µl	30	60	90
	0.32 µl	0	60	60
	0.08 µl	0	0	0

[a]At each treatment a total of 10 larvae were examined.

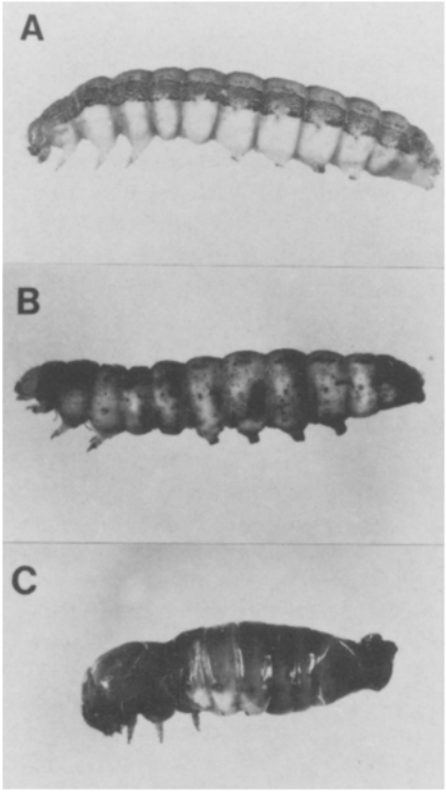

Figure 1. Early sixth instar larvae injected with healthy serum (*A*) or *B. bassiana* infected serum (*B*, *C*). The healthy wandering stage is shown in *A*. Note the presence of melanized spots in *B* which are formed at the wandering stage. Treated larvae may die as wandering larvae or as larval-pupal intermediates (*C*).

Injection of either cell-free infected or healthy hemolymph caused an immediate reduction in the percentage of spreading hemocytes of *S. exigua* larvae compared to that of untreated larvae (table 2). The hemocytes of insects injected with control serum were able to recover; within 3 h after injection these larvae possessed a spreading cell population comparable to naive larvae. However, challenge of *S. exigua* with infected serum resulted in a permanent reduction ($p < 0.001$) in spreading hemocytes. Only 0.8 to 1.2% of the hemocytes were able to produce filopodia throughout the 48 h sampling interval (fig. 2, table 2). The viability of hemocytes sampled at 3, 24, and 48 h from larvae challenged with the infected sera (96–98%) was comparable to that calculated for control larvae (98%). The phagocytic competence of hemocytes from *S. exigua* challenged with infected sera ($35 \pm 10\%$ hemocytes containing 1.7 ± 0.2 FITC blastospores/cell) was not significantly different ($p > 0.05$) from larvae injected with either saline ($43 \pm 10\%$ containing $2.2 \pm 0.7\%$ FITC blastospores/cell) or with healthy serum ($42 \pm 12\%$ containing 2.3 ± 0.5 FITC blastospores/cell).

The insecticidal activity in the infected sera was inactivated by heat treatment (60 °C, 30 min). The >10 KDa fraction of infected sera contained components which significantly reduced ($p < 0.001$) the percentage of spreading hemocytes (from 26.3% to 3.3%). Furthermore, this fraction of infected sera resulted in 100%

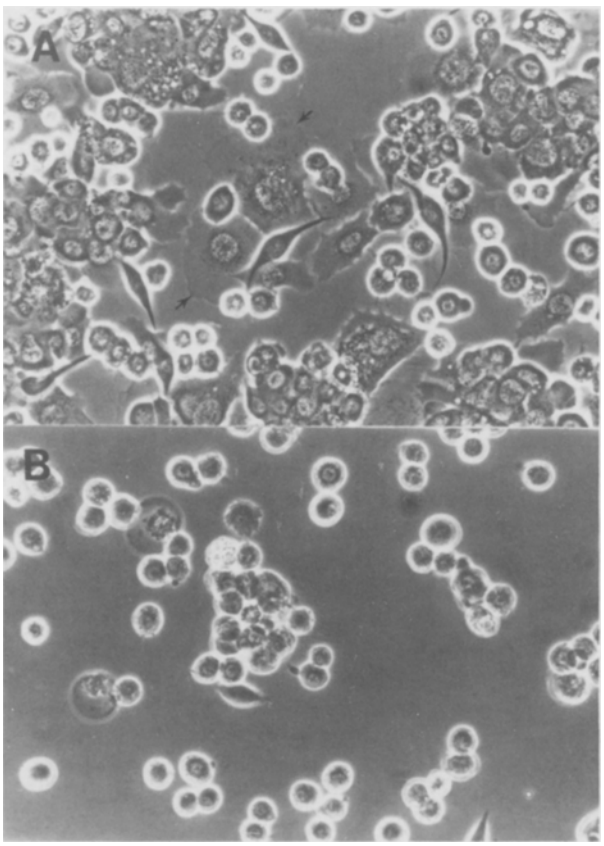

Figure 2. Phase contrast micrography of hemolymph collected after 24 h of injection with (*a*) healthy serum and (*B*) *B. bassiana*-infected serum of *S. exigua* larvae. Arrows denote spreading hemocytes. 460 ×.

Table 2. Effect of serum samples from *B. bassiana* infected *S. exigua* larvae and CM fraction samples on the percentage of spreading hemocytes of *S. exigua* larvae.

Time (h)	% of spreading hemocyte[a]				
	Untreated	Healthy serum	Infected serum	Healthy CM fraction	Infected CM fraction
0.5	25.6 ± 3.4 (2942)	1.7 ± 3.7 (456)	0 (284)	-	-
3	26.4 ± 3.3 (2764)	22.4 ± 7.4 (3538)	0.8 ± 1.2 (1738)	22.1 ± 8.6 (1885)	9.5 ± 6.4 (1723)
24	28.3 ± 4.7 (2895)	33.7 ± 13.7 (2870)	2.1 ± 3.8 (857)	27.9 ± 6.2 (1416)	2.7 ± 3.6 (703)
48	28.6 ± 3.7 (3251)	26.4 ± 10.2 (2386)	0.9 ± 2.2 (767)	32.7 ± 11.1 (1239)	0.7 ± 1.4 (781)

[a]Mean percent of spreading hemocytes ± SD. Number in parenthesis represents total hemocytes observed in 10 or 15 larvae for each treatment interval.

mortality of challenged larvae. The LMW filtrate fraction (< 10 KDa) did not alter the ability of hemocytes to produce pseudopodia nor kill challenged *S. exigua*. Infected sera was subsequently fractionated using conventional chromatographic methods, adjusted to original volume, and assayed against *S. exigua* larvae. The biologically active components did not bind to the Phenyl-Sepharose® CL-4B column and were eluted during washing with the loading buffer. The active HIC fraction was loaded onto a CM Bio-Gel A column. At pH 5.0, the majority of the serum proteins were adsorbed to the column and eluted with high-salt buffer. However, the bioactivity was detected in the low-salt buffer eluant containing unbound material. After the ion exchange step, the active fraction contained ~1.25 mg protein/ml corresponding to a >40-fold increase in specific activity. The fractionation of healthy serum gave a similar yield of protein. SDS-polyacrylamide gel electrophoresis of the various chromatographic fractions demonstrated that both major high molecular proteins and several minor proteins (20–30 KDa) detected in parental fractions were absent in the CM fraction (fig. 3). However, comparisons of peptide profiles visualized with either Coomassie blue R-250 or silver stain did not reveal differences between the infected or control chromatographic fractions. The CM fractions of both sera preparations contained qualitatively and quantitatively similar proteins. Probing Western blots of the infected parental serum and the CM infected fraction versus the control parental serum and the CM control fraction with an anti-*B. bassiana* rabbit polyclonal antibody revealed the presence of multiple antigens in the infected samples (fig. 4).

Injecting *S. exigua* larvae with dilutions of CM fraction derived from infected larvae resulted in a decrease in the percentage of treated larvae dying as prepupae as compared to the mortality patterns of infected serum challenged larvae (table 1). At dosages less than 0.62 µl/larva equivalent to 1 µg of protein/larva, active CM fraction caused partial pupal mortality. Finally, at 0.08 µl/larva no mortality was detected. The insecticidal activity in the CM fraction was inactivated by either heat or proteinase K treatments. Treatment with trypsin

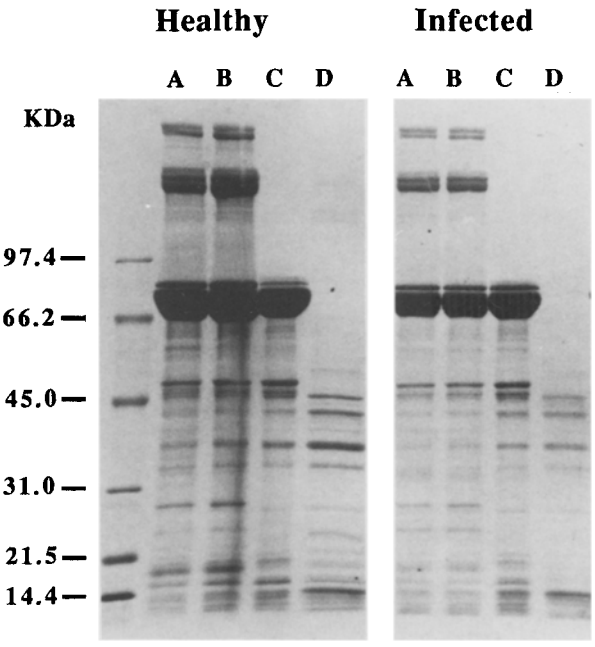

Figure 3. SDS-PAGE of samples from the different fractionation steps of healthy and *B. bassiana* infected hemolymph stained with Coomassie blue: *A* whole hemolymph, *B* ultracentrifugation supernatant, *C* phenyl-Sepharose® buffer eluant, *D* CM buffer eluant. Left end lane represents SDS-PAGE molecular weight standards.

caused only a slight decrease in activity. Injection of trypsin-treated CM fraction caused 80% mortality with 30% of the larvae dying in the wandering stage. Injection of control trypsin and proteinase K preparations did not cause mortality. The effects of toxic CM fraction on the hemocytes of healthy *S. exigua* larvae were similar to those of infected serum. Challenge with CM fraction suppressed significantly ($p < 0.001$) the ability of hemocytes to spread compared to that of untreated larvae, larvae injected with either healthy serum or healthy CM fraction (table 2).

Discussion

This study demonstrated that hemolymph extracted from *B. bassiana* infected *S. exigua* larvae contained toxic metabolites. These metabolites, partitioned from

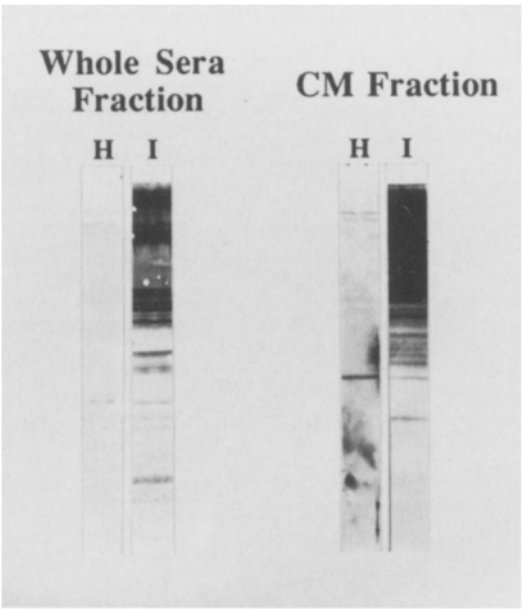

Figure 4. Western blots of whole sera and CM fraction (H: healthy, I: infected) probed with anti-*B. bassiana* rabbit polyclonal IgG then incubated in goat antirabbit-IgG-alkaline phosphatase. Note the presence of high molecular weight antigens in both infected samples.

major sera proteins using conventional chromatographic methods, caused an immediate and permanent reduction in the number of spreading hemocytes when injected into *S. exigua*. The remaining hemocytes were viable and were able to phagocytosize injected FITC-blastospores. Similar observations have been made during the in vivo development of *B. bassiana*, i.e., the total hemocyte number[3,17] and the percentage of spreading hemocytes[4] decreased as infection progressed.

These observations suggested that the suppression of hemocyte function by *B. bassiana* observed in earlier studies[3,4] is due to soluble components which are released into the hemocoel. Dose-response studies demonstrate that these components are present in relatively high titer with as little as 80 nanoliters of infected sera giving a detectable response. Our studies suggested that the biological activity is not due to low molecular weight metabolites such as the cyclic peptides, destruxins, beauvericin, bassianolide, or cytochalasin-B which have been detected in either culture filtrates or detected in infected insects[18–21]. First, biological activity was always detected in the retentate after the 10 KDa ultrafiltration. Second, the biological activity was inactivated by heat treatment. Third, attempts to extract freeze-dried hemolymph samples with conventional organic solvents used to extract cyclic peptides[22] failed to produce active fractions. Fourth, the infected sera samples did not produce the mortality response observed with lethal challenges of cyclic peptides. For example, *S. exigua* larvae injected with either destruxins A or E were immediately paralyzed and died within 1–2 days postinjection (unpublished data). Lastly, in vitro assays involving hemocyte monolayers have shown that the aforementioned fungal toxins all cause detectable cytopathic effects[20,23,24]. However, incubating established *S. exigua* hemocyte monolayers in insect media containing 50% infected sera caused no observable changes in hemocyte morphology (Hung et al., unpublished).

Our attempts to extract the active metabolite from infected sera have only been partially successful. The active CM fraction (1.2 mg protein/ml) produced a peptide profile identical to the profile of CM fraction of control sera (0.7 mg protein/ml) when electrophoresed on SDS-gels and stained with either silver or Coomassie blue R-250. These results suggest that the active component, which is sensitive to proteinase K digestion is either a highly active protein or is a heavily glycosylated protein. Immunostaining the Western blots of the active CM fraction with the anti-*B. bassiana* rabbit polyclonal antibody revealed an abundance of high molecular weight antigens. The staining pattern was similar to that achieved with Western blots of culture filtrate of *B. bassiana* (Boucias et al., unpublished). The antigenicity in these blots could be greatly reduced by Na-periodate treatment suggesting that carbohydrates are serving as major epitopes for antibody recognition.

In summary, we have demonstrated that *B. bassiana* infected serum disrupted the metamorphosis of *S. exigua* larvae. Although the hemocyte population was dramatically altered, challenged larvae maintained normal growth patterns throughout the last larval instar. No immediate paralysis, which is characteristic of certain fungal metabolites, was observed in the challenged larvae. The majority of the treated larvae died in the wandering stage with extensive melanized cuticle. Possibly, the active sera component by activation of granulocytes-plasmatocytes and non-specific induction of the phenoloxidase cascade resulted in a condition which disrupted metamorphosis. Alternatively, debilitation of the hemocytes by challenge with infected sera disrupted the tissue degradation and/or assimilation events associated normally with metamorphosis.

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SCIENTIFIC CORRESPONDENCE

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