

IMUNITNÍ SYSTÉM HMYZU

BUNĚČNÁ IMUNITA

HUMORÁLNÍ IMUNITA

- pohyblivé krevní buňky – hemocyty
(počet a aktivita hemocytů je modulována humorálními faktory a neuroendokrinním systémem)

- molekuly v hemolymfě
- antigenem indukované antibakteriální a regulační faktory

- koagulace
- fagocytóza (oxidativní vzplanutí?)
- nodulace
- enkapsulace

- aglutininy
- koagulační kaskáda
- fenoloxidásová kaskáda
- baktericidní peptidy
- antimikrobiální faktory (lektiny, hemolin)
- lysozym

stresové faktory

Hmyzí hemocyty:

- prohemocyty
- plasmacyty
- granulocyty
- spherulocyty
- oenocyty

Imunitní reakce hemocytů:

- fagocytóza
- enkapsulace
- nodulace
- koagulace

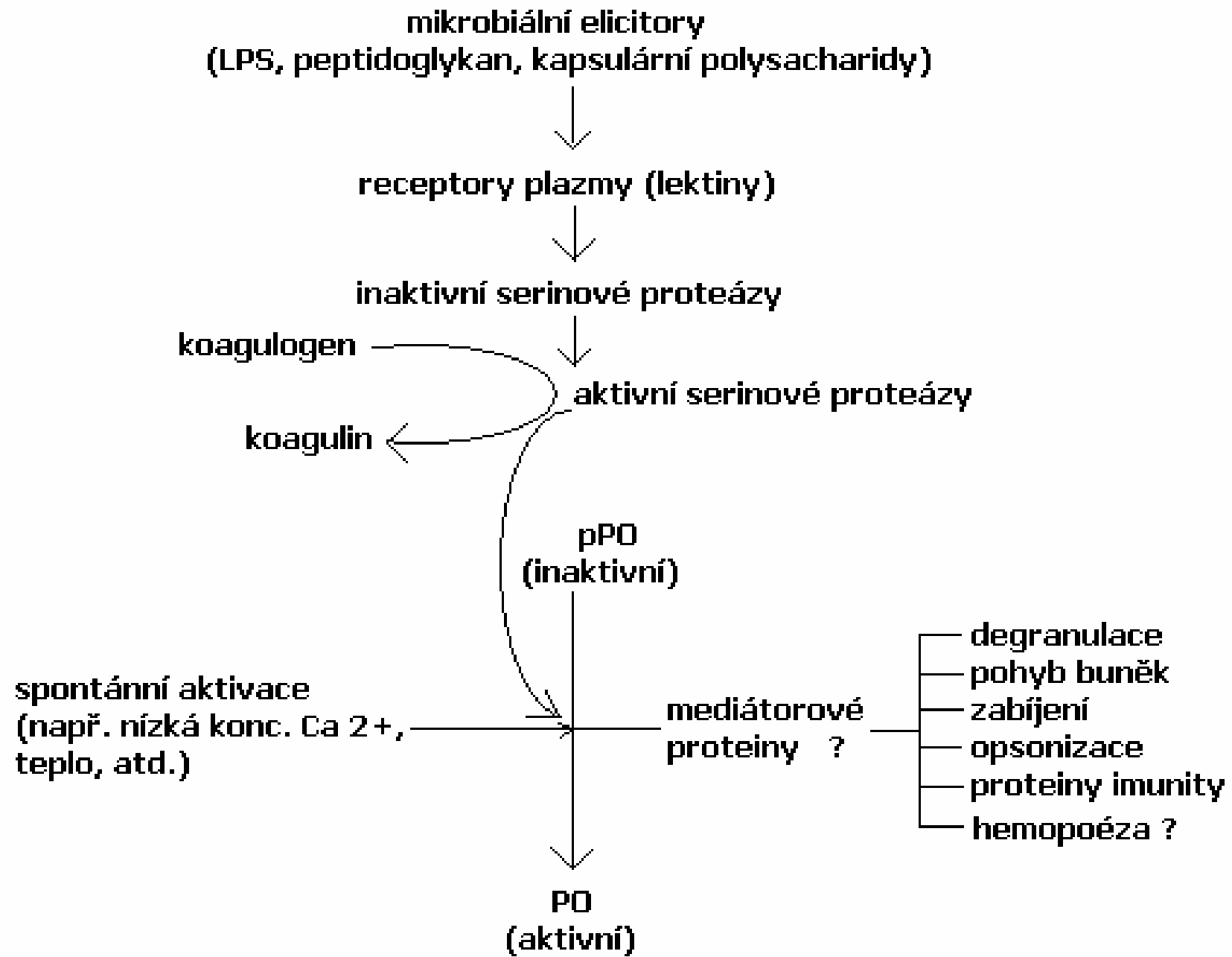
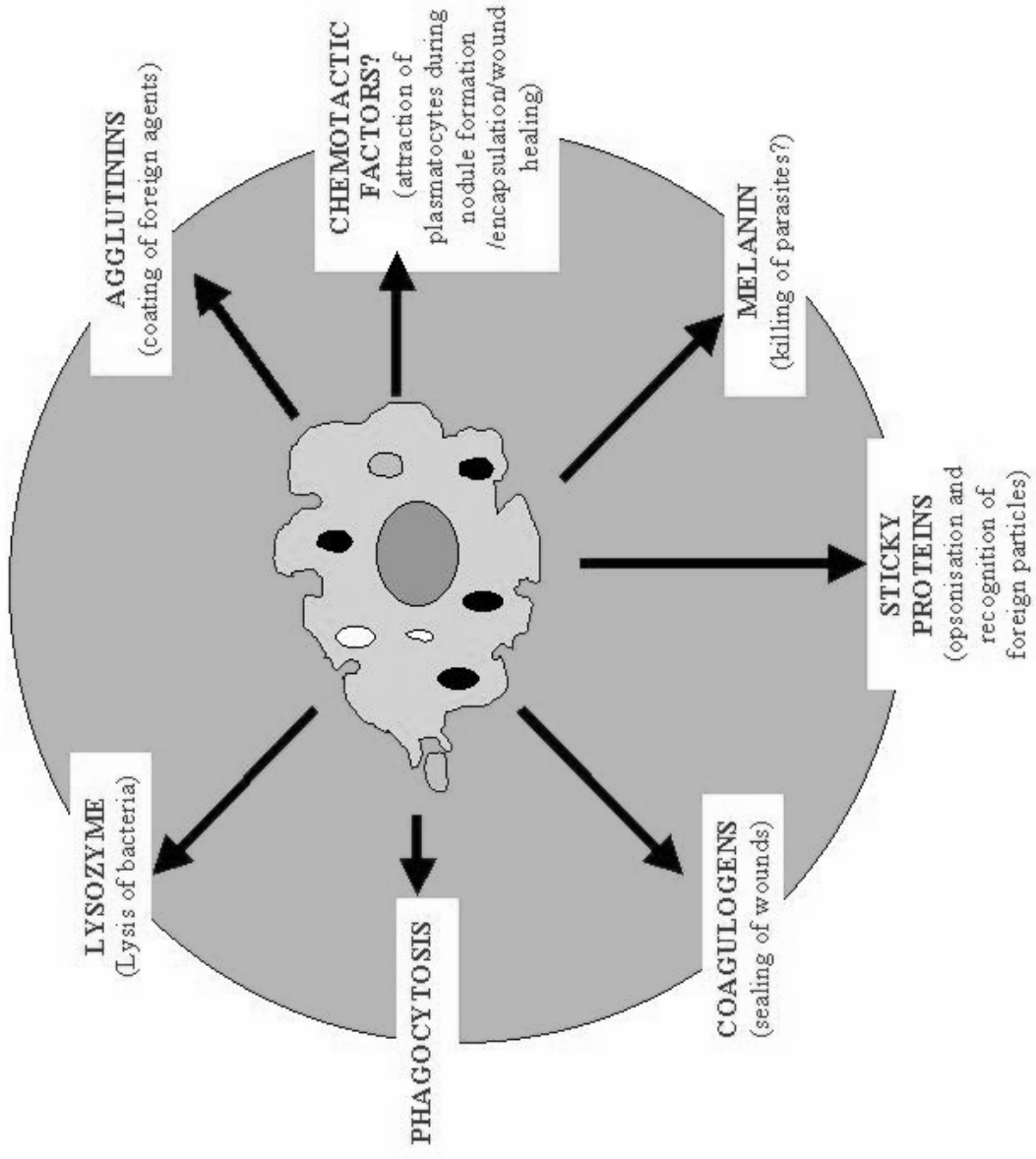


Figure 5: Diagram of an insect granular cell emphasizing its multifunctional role.



Elektroforéza SDS – PAGGE

(sodium dodecylsulfat polyacrylamide gradient gel electrophoresis)

- gely vznikají polymerací akrylamidu a N',N'-metylenbisakrylamidu
- SDS (sodium dodecylsulfát) se váže shodně na všechny proteiny v poměru 1,4 g / 1g proteinu a předává jim silný záporný náboj
- mercaptoethanol - rozštěpí disulfidické můstky v molekulách proteinů
- vertikální elektroforéza SE 600 (Hofer Scientific Instruments)
- 5 % koncentrační gel a separační gel (14 x 10,5 cm) s gradientem akrylamidu 7,5 – 20,0 %
- dělení proteinů probíhalo ve dvou gelech, každý z nich pro 15 vzorků
- standard (SDS-PAGE Molecular Weight Standard Broad Range, Bio-Rad; 6,5 - 200 kDa)
- gely byly barveny stříbrem podle Kirkeby et al. (1993)

Vyhodnocování gelů:

- videodensitometr GS-670 a software Molecular Analyst (Bio-Rad)

Profilová analýza:

- **kvalitativní:**

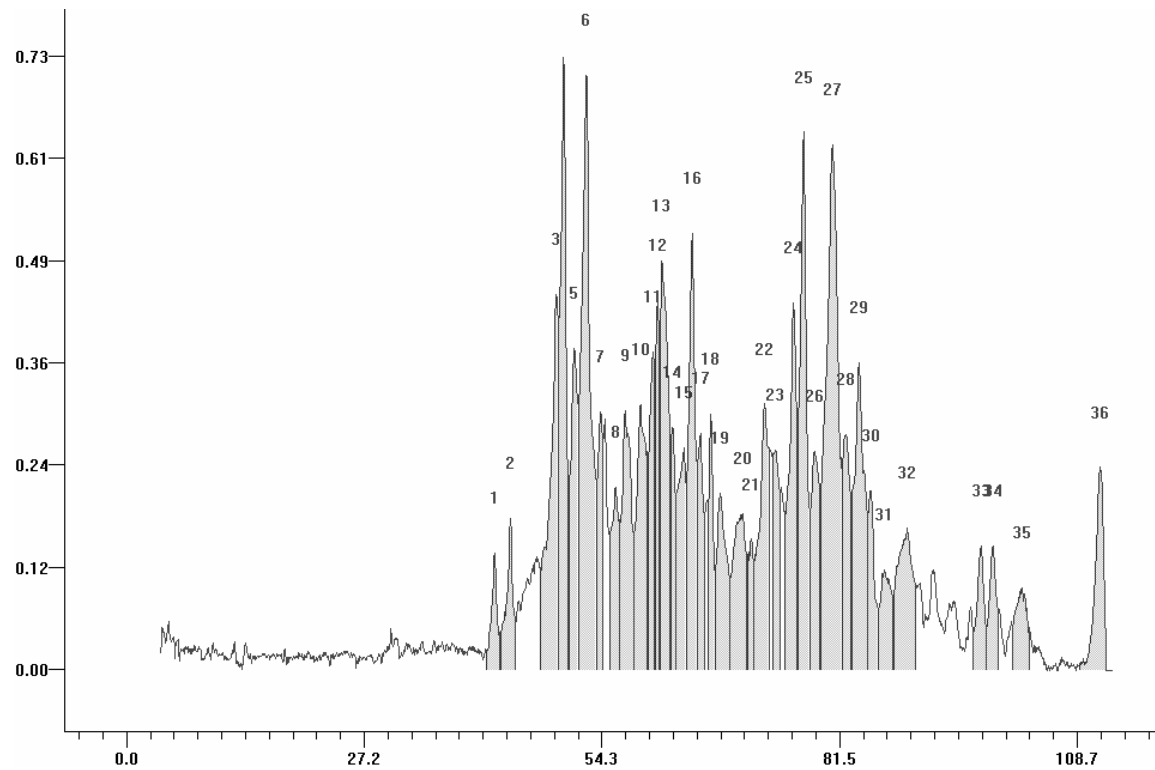
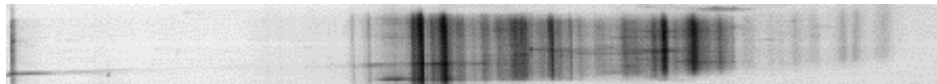
MW jednotlivých proteinových frakcí hemolymfy

- **kvantitativní:**

poměr zastoupení jednotlivých proteinových frakcí
mezi samci a samicemi během vývoje

Profilová analýza:

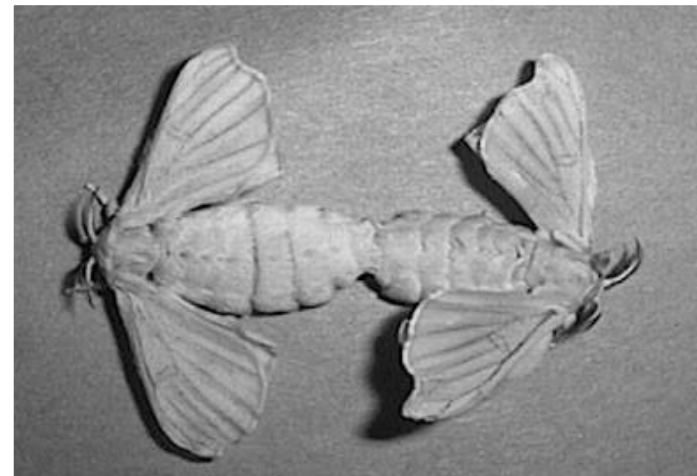
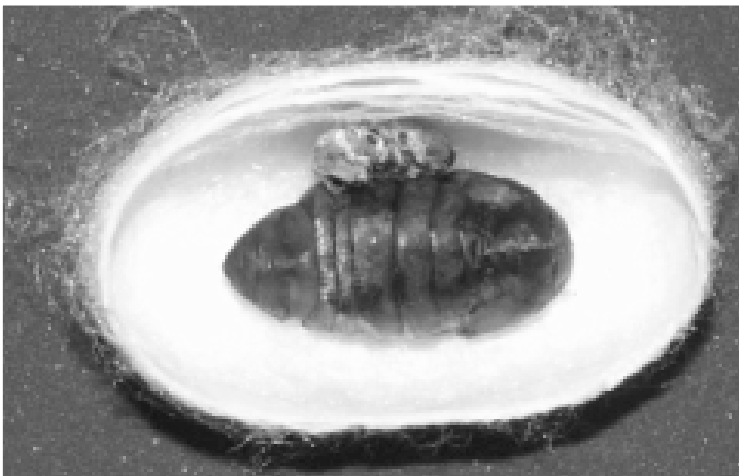
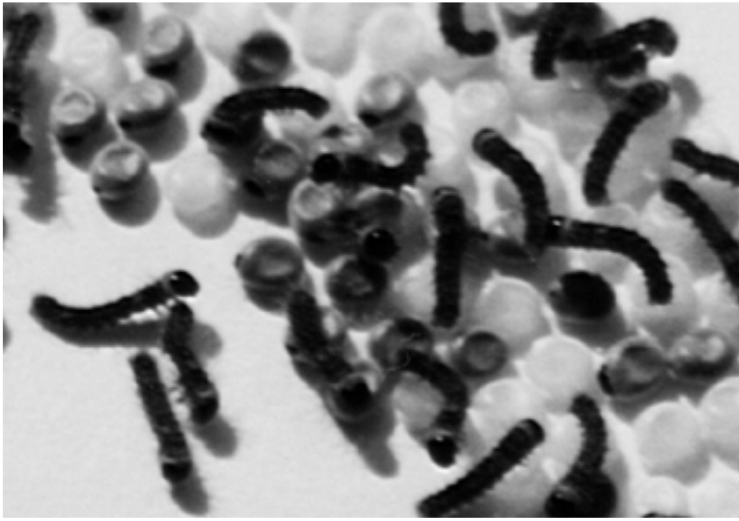
- rozdělené vzorky na polyakrylamidovém gelu
- absorpční křivka (osa x – vzdálenost od startu, osa y – optická hustota)
- tabulka s určenou molekulovou hmotností frakcí (MW) a plochou píku



Peak No.	Area (OD*mm)	MW (Da)
1	0.1031	84013
2	0.1521	79290
3	0.4767	66371
4	0.5198	64548
5	0.3230	61972
6	0.8325	59245
7	0.1905	56035
8	0.2044	52885
9	0.3650	50993
10	0.3637	48126
11	0.2294	45910
12	0.1872	45130
13	0.4452	44363
14	0.1340	42593
15	0.2568	40719
16	0.4203	39515
17	0.1859	38265
18	0.1684	36739

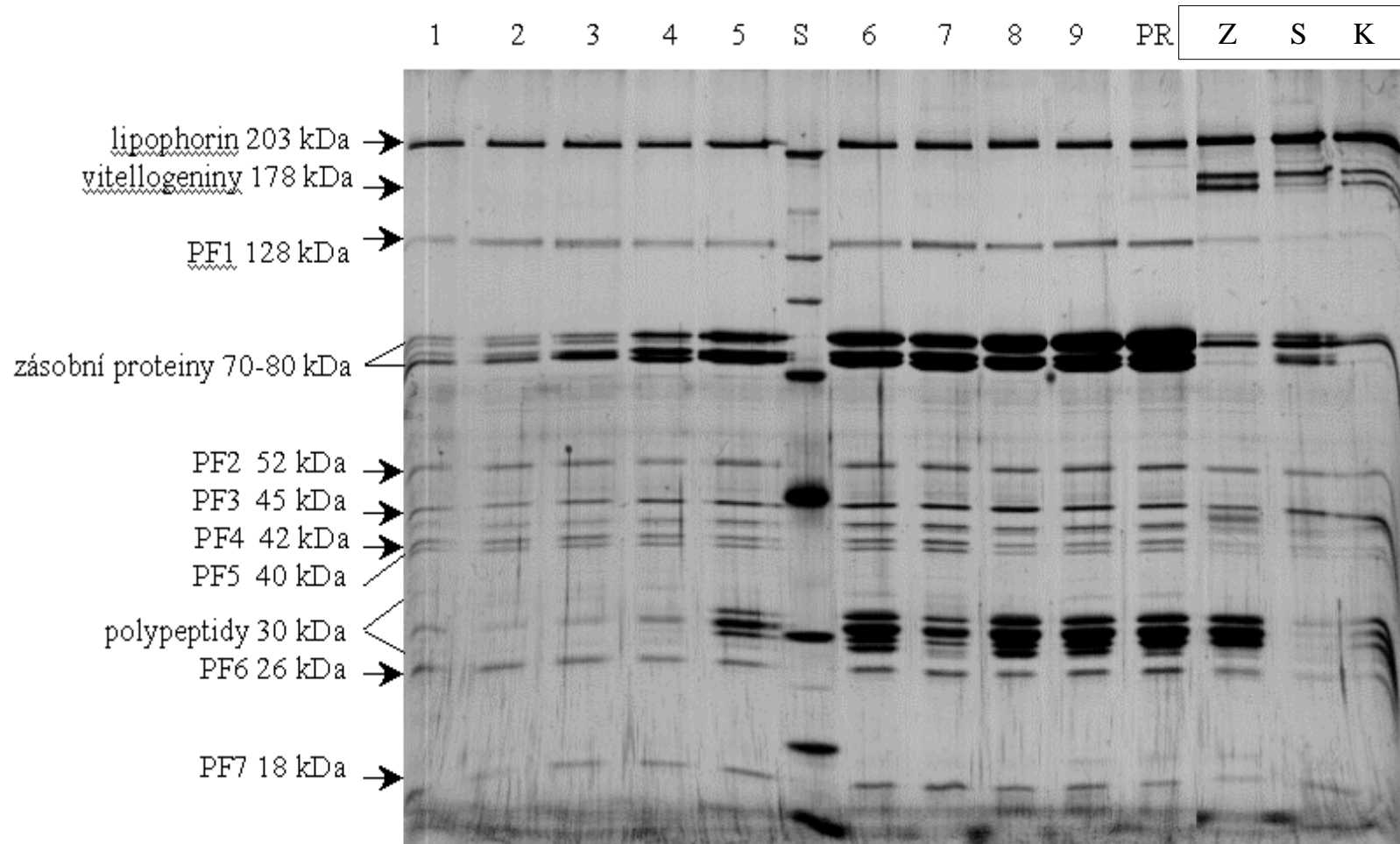
Bombyx mori (Lepidoptera, Bombycidae)

bulharský monovoltinní hybrid AS x KK

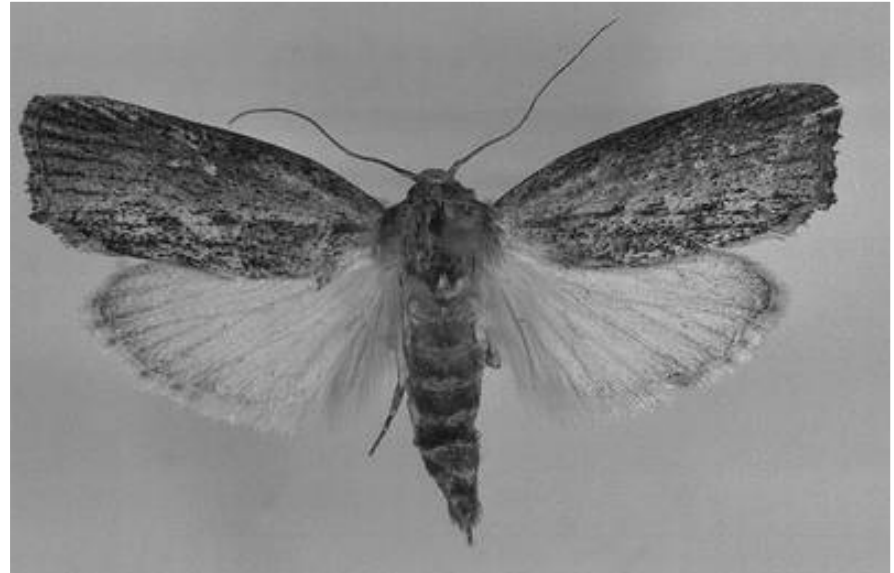


Bombyx mori – samice

S – standard; 1. - 9. den V. instaru; PR – prepupa 1. den;
Z – kukla začátek; S – kukla střed; K – kukla konec.



Galleria mellonella (Lepidoptera, Pyralidae)



RESULTS

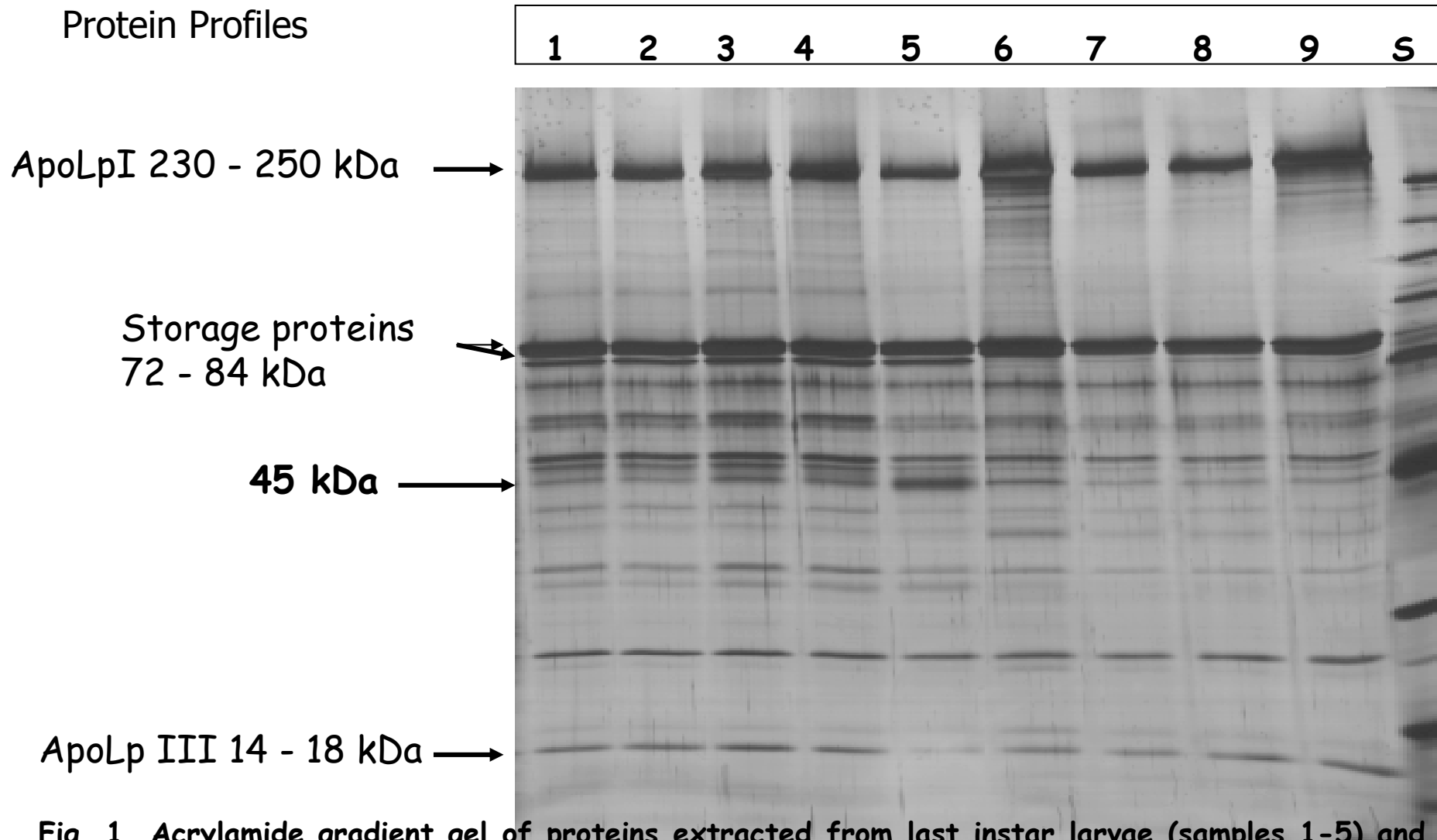
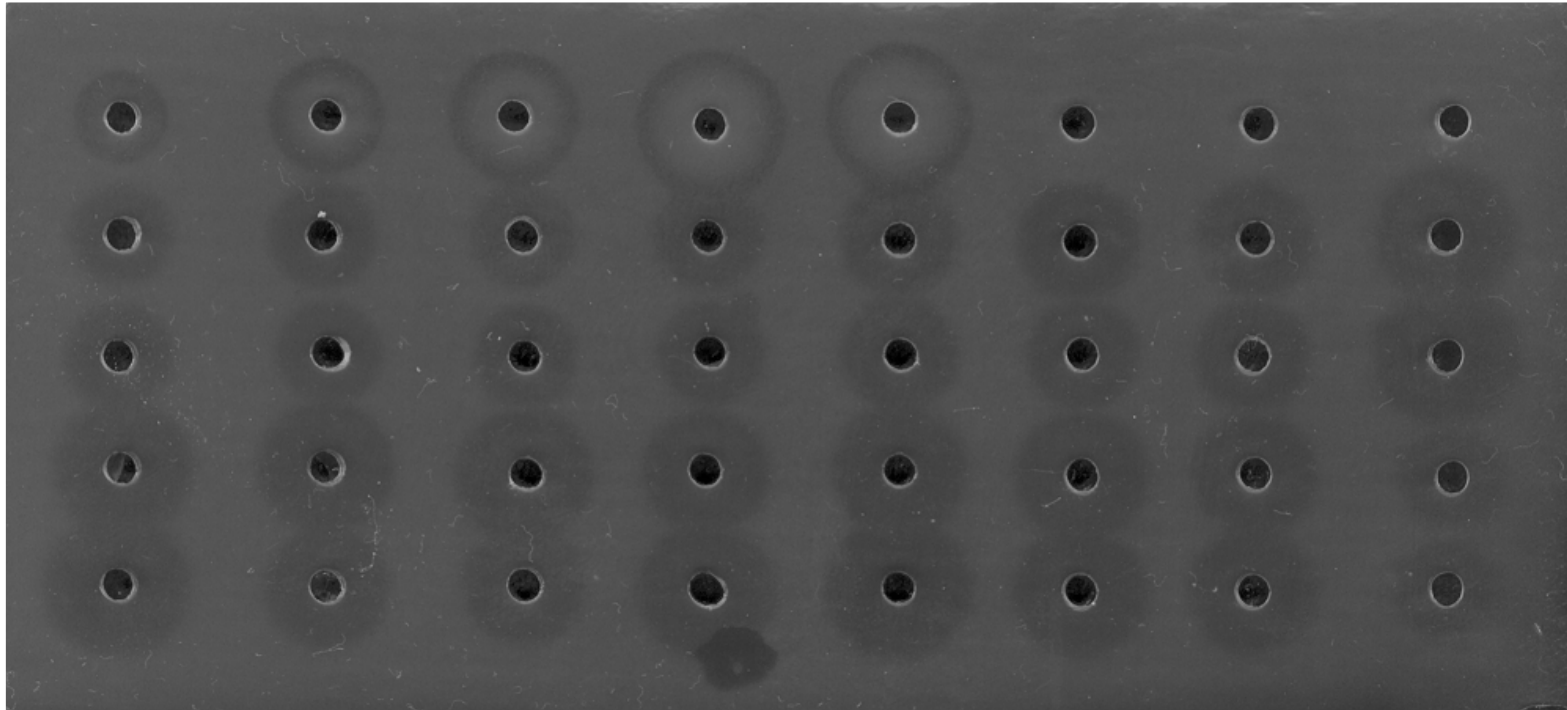
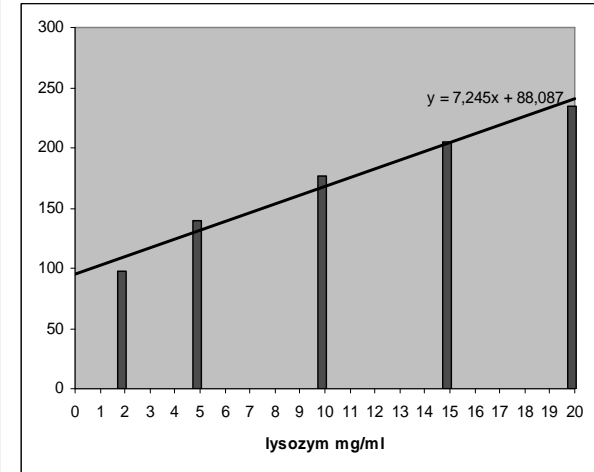
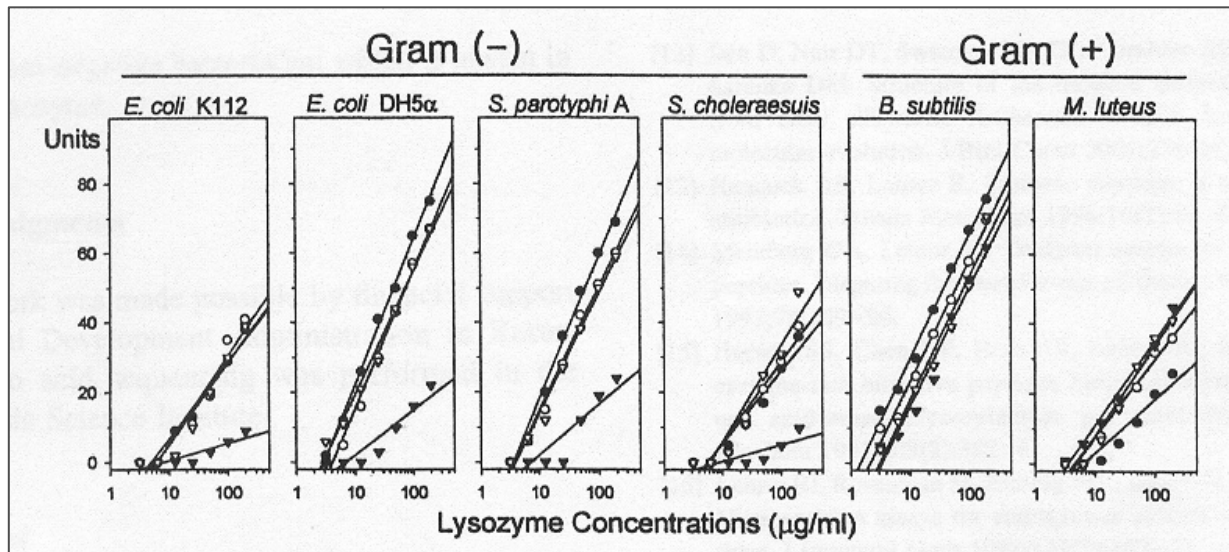


Fig. 1. Acrylamide gradient gel of proteins extracted from last instar larvae (samples 1-5) and pupae (samples 6-9) hemolymph of *G. mellonella*. Samples with increasing concentration of boric acid: 0.00 ppm (1, 6); 156 ppm (2, 7); 620 ppm (3, 8); 1250 ppm (4, 9) and 2500 ppm (5). Main protein groups of proteins are described on the left margin as well as 45 kDa protein fraction according to protein standards (S).

Aktivita lysozymu v hemolymfě



radiální difúze v agaróze

Silkworm (*Bombyx mori*) Hemocytes Do Not Produce Reactive Oxygen Metabolites As a Part of Defense Mechanisms

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ABSTRACT. To investigate whether hemocytes of *Bombyx mori* (Lepidoptera) larvae produce reactive oxygen species (ROS) as part of the oxidative killing of invading pathogens, the production of ROS was measured as a luminol- and lucigenin-enhanced chemiluminescence of unstimulated or stimulated (zymosan particles, phorbol myristate acetate, calcium ionophore, rice starch or *Xenorhabdus nematophila*) hemolymph. No detectable ROS production was found. The spontaneous and activated ROS production measured with hemocytes, i.e. under the conditions when the antioxidative potential of hemolymph plasma was eliminated, was again undetectable. Likewise, ROS production by isolated hemocytes was observed by spectrophotometric (NBT test, cytochrome *c* assay) and fluorimetric (using dihydrorhodamine and hydroethidine probes) methods. Hence none of the experimental approaches used indicated the production of ROS by hemocytes of *B. mori* larvae as part of their immune response.

Hemocytes are basic to the invertebrate innate immune system that is divided into cellular and humoral defense responses. The most common types of hemocytes reported in the literature are prohemocytes, plasmatocytes, granulocytes, spherulocytes and encytes (Yamashita and Iwabuchi 2001; Lavine and Strand 2002). These have been identified by their morphology and histochemical and functional reactions (e.g., Gardiner and Strand 1999). Four basic types of hemocyte immune reactions have been described: phagocytosis, encapsulation, modulation and coagulation. These activities are always connected with a particular type of hemocytes. During phagocytosis, plasmatocytes and granulocytes are mainly activated while other types of hemocytes have mostly no possibility to phagocytose. Mechanisms participating in the recognition of foreign material are still under study (Lavine and Strand 2002).

The production of reactive oxygen species (ROS) seems to be an important microbicidal factor in both invertebrate hemocytes and vertebrate phagocytes since an increase in ROS production by activated hemocytes of some invertebrates such as *Bivalvia*, *Citellata*, *Malacostraca*, *Arachnida*, *Echinoidea* or *Ascidacea* has been reported (e.g., Nakamura *et al.* 1985; Ito *et al.* 1992; Bell and Smith 1993; Valembos and Lassegues 1995; Lambert and Nicolas 1998; Ordas *et al.* 2000; Pereira *et al.* 2001; Azumi *et al.* 2002). However, reports about similar mechanisms in insects are controversial. The aim of the present study was to investigate whether stimulated hemocytes of *Bombyx mori* can produce ROS as part of oxidative killing invading pathogens. Understanding of the defense mechanisms of *B. mori* has a high significance for providing a better insight into the evolution of animal immune systems; it also has a great impact on silk production sericulture.

MATERIALS AND METHODS

Sample preparation. The hemolymph of the Japan polyvoltinne NO2 × CO2 hybrid of the silkworm, *Bombyx mori* LINNÉ 1758 (Lepidoptera, Bombycidae), was obtained from larvae of the 5th instar. Larvae were reared on mulberry leaves (*Morus alba*) *ad libitum*. The sex was not determined. After the collection of the hemolymph from the first-pair proleg (approximately 150 µL per larva), phenylthiourea was added to protect the hemolymph from melanization. (It was verified that this agent does not have a significant effect

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Reactive oxygen metabolites detection:

- chemiluminescence
- spectrophotometry
 - NBT test
 - cytochrome c reduction
- fluorometry
 - DHR 123 staining
 - HE staining
- total radical-trapping antioxidative potential (TRAP)

Entomopatogenní hlístovky (EPN)

třída: Nematoda (Nematoda)

řád: Hád'ata (Anguilata)

čeleď: *Steinernematidae*

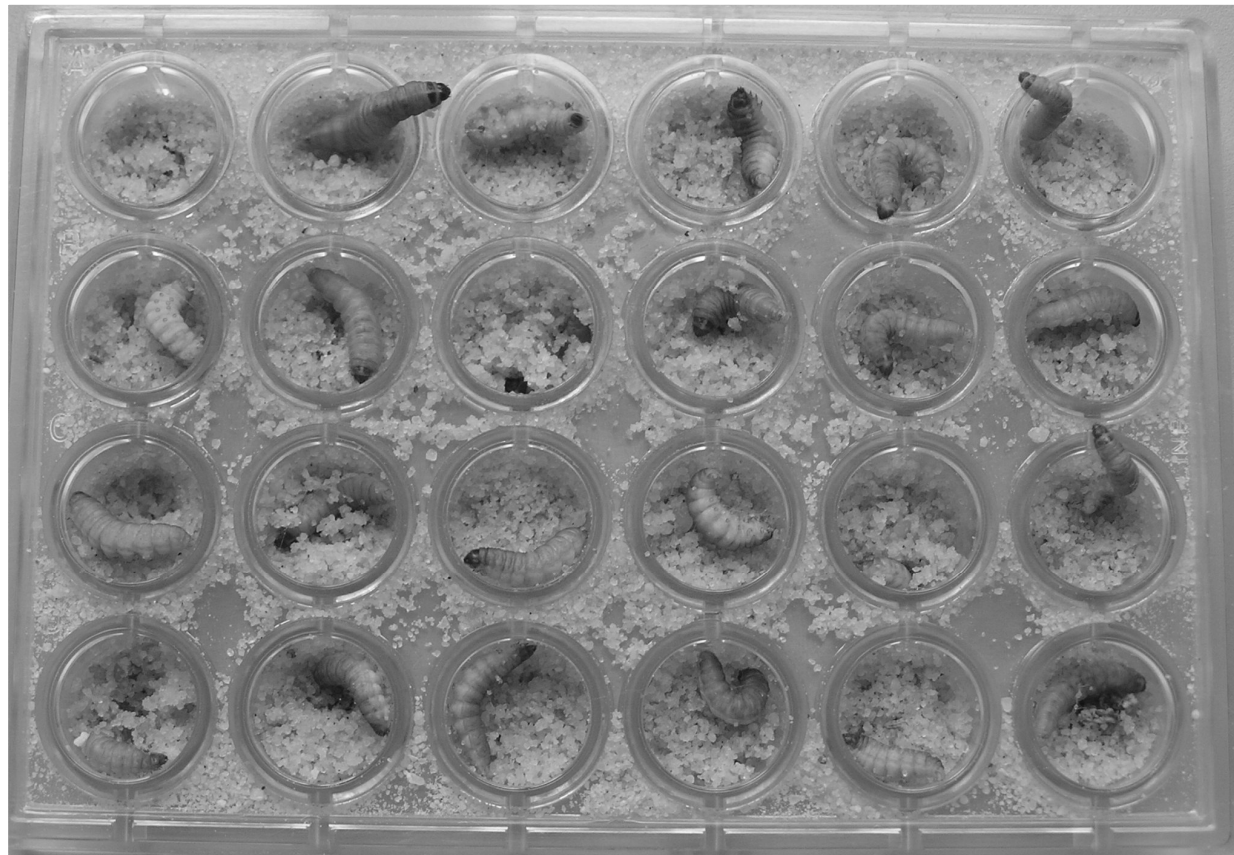
Heterorhabditidae

- vyskytují se volně v půdě
- selektivita - výhradně entomopatogenní
- nízké teplotní optimum - do 20°C
- využívají se jako prostředek biologického boje

METODY

1. Přirozená invaze EPN:

- larvy *G. mellonella* nebo larvy *Amphimallon* sp., *Hoplia* sp. + 25 EPN, 10% vlhkost
- inkubace 20 – 24 hodin za laboratorní teploty
- celkem bylo použito 70 ks *G. mellonella*, 23 ks *Amphimallon* sp. a 23 ks *Hoplia* sp.



Shrnutí:

Enkapsulace: ANO / NE

A / přirozená invaze

	<i>G. mellonella</i>	<i>Amphimallon sp.</i>	<i>Hoplia sp.</i>
<i>H. bacteriophora</i>	ANO	NE	NE
<i>S. glaseri</i>	NE	NE	NE
<i>S. scarabaei</i>	NE	NE	NE

B / injikace EPN

	<i>G. mellonella</i>	<i>Amphimallon sp.</i>	<i>Hoplia sp.</i>
<i>H. bacteriophora</i>	ANO	ANO	NE
<i>S. glaseri</i>	NE	NE	NE
<i>S. scarabaei</i>	NE	ANO	NE

Experimentální model – modulace imunity *Drosophily*

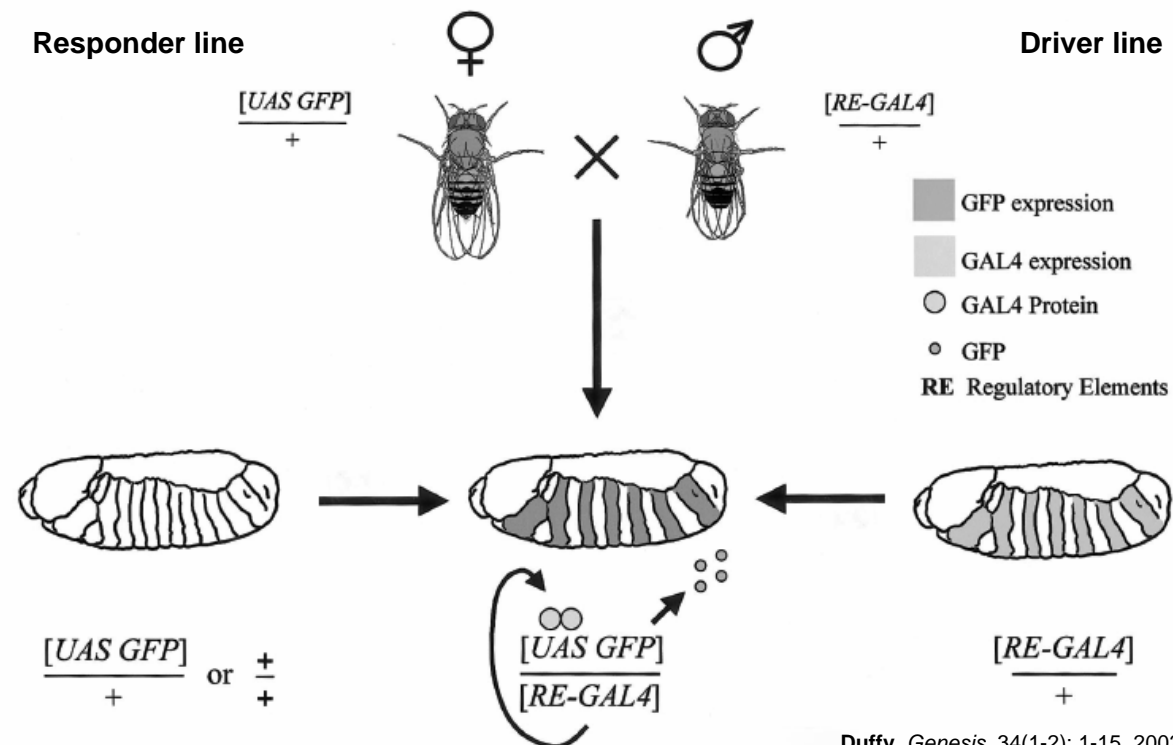
Gal4/UAS systémem řízená exprese genů

„*driver line*“ – specifický promotor určující místo a čas syntézy Gal4

- Gal4 – váže se na DNA a aktivuje transkripci

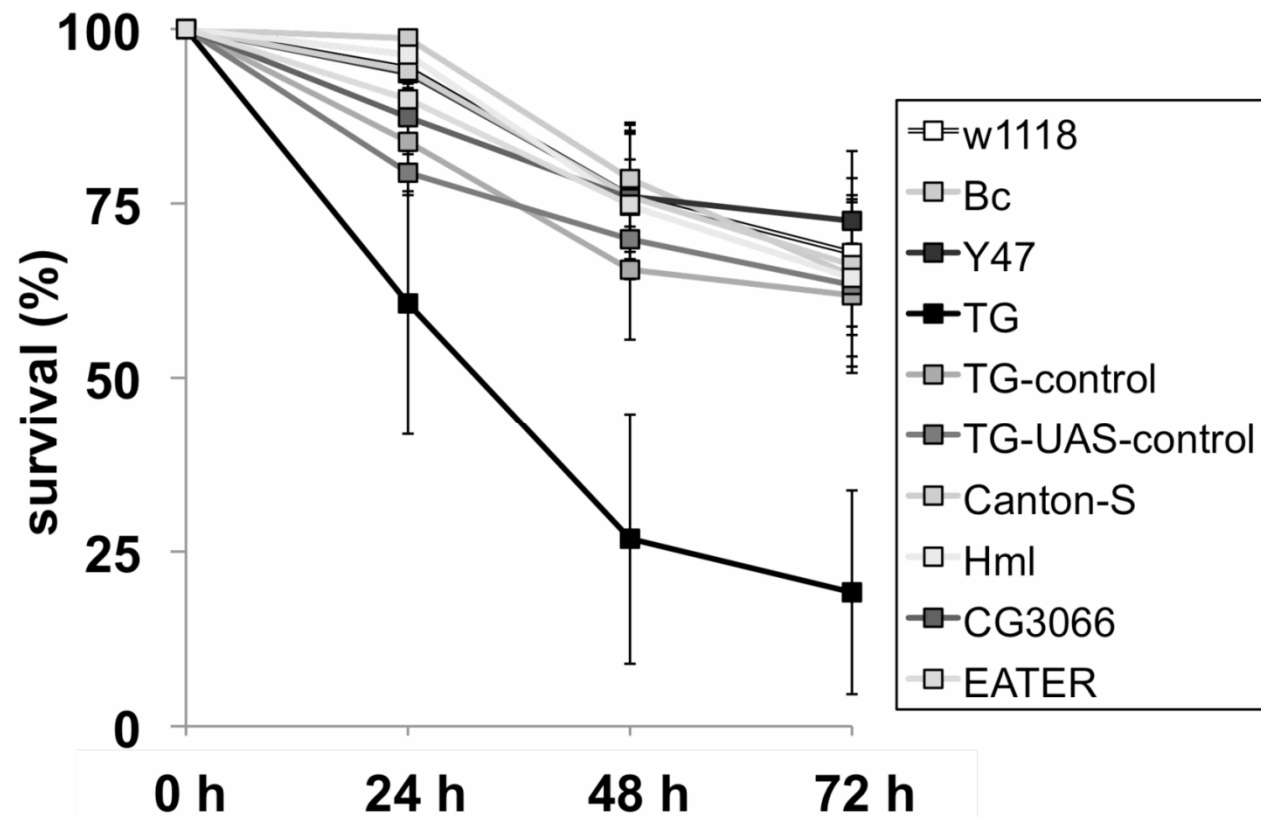
„*responder line*“ – **Upstream Activating Sequences** místem pro vazbu Gal4

- RNAi konstrukt – jeho transkripce je pod vlivem UAS



Transglutamináza má také imunitní funkci

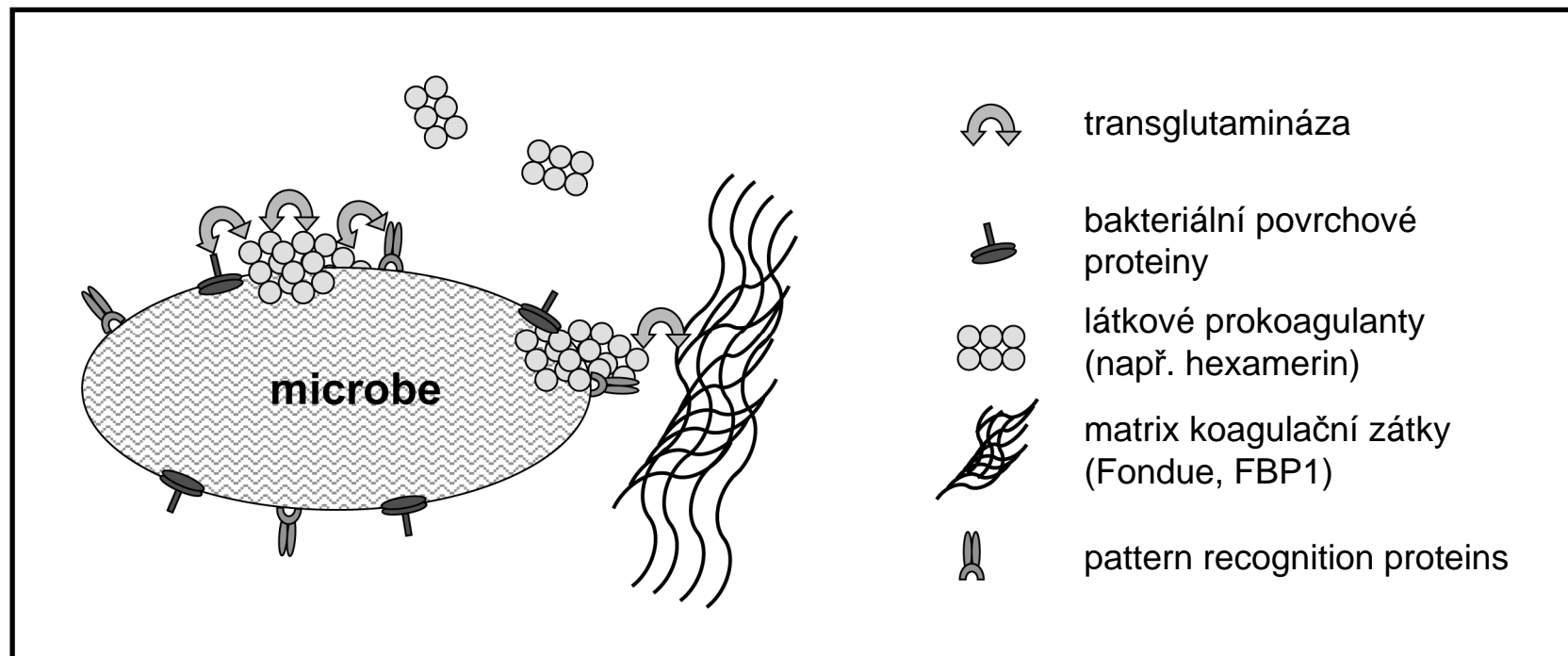
- centrální enzym koagulační kaskády
- homolog Faktoru XIIIa obratlovců
- infekce *H. bacteriophora*, 100 IJ na larvu, 22 °C:



Wang *et al.* PLoS Pathogens, 6(2): 1-9, 2010.

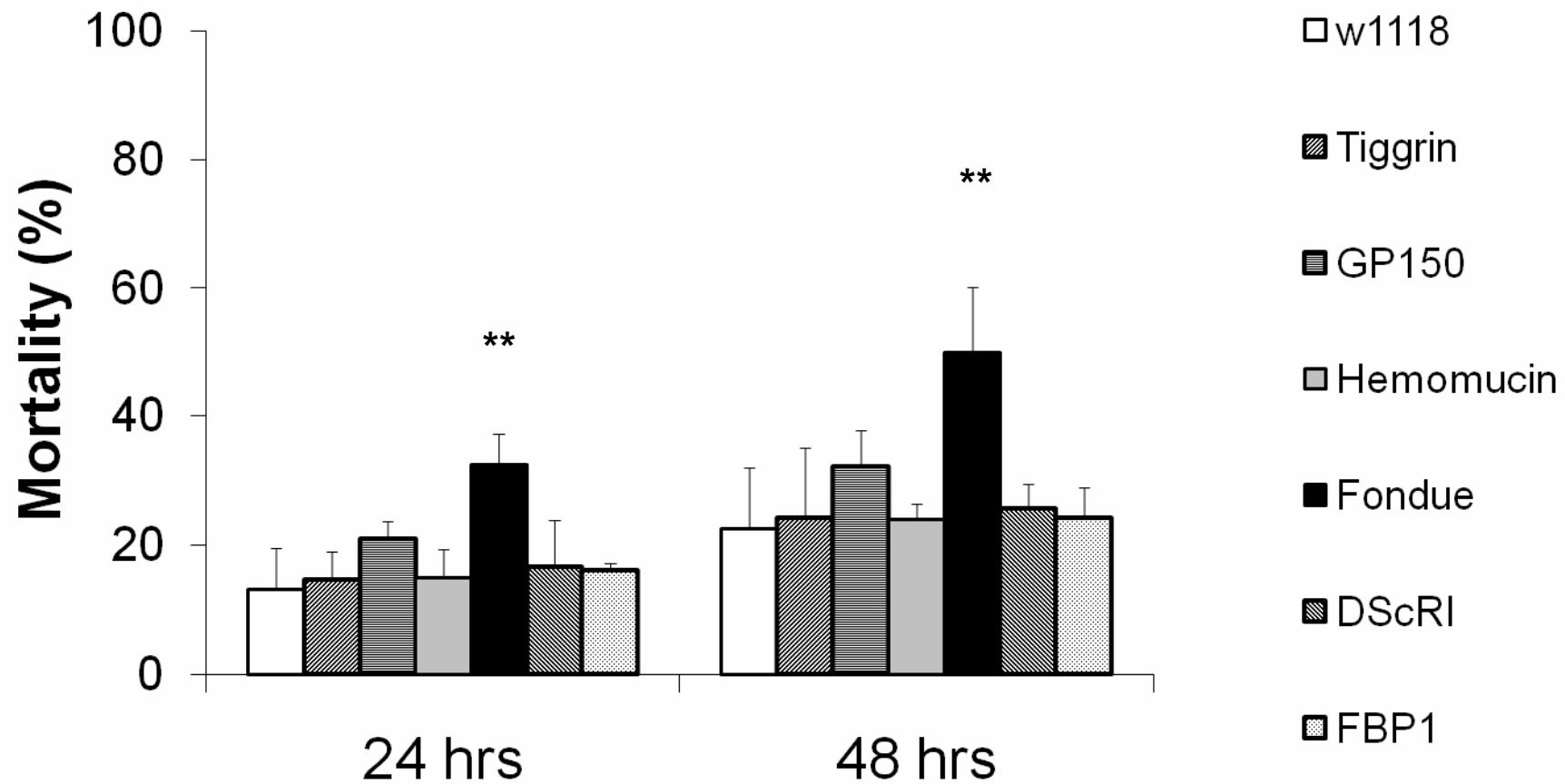
Transglutamináza má také imunitní funkci

- transglutamináza zprostředkuje zachycení bakterií prostřednictvím tvorby koagulační matrix



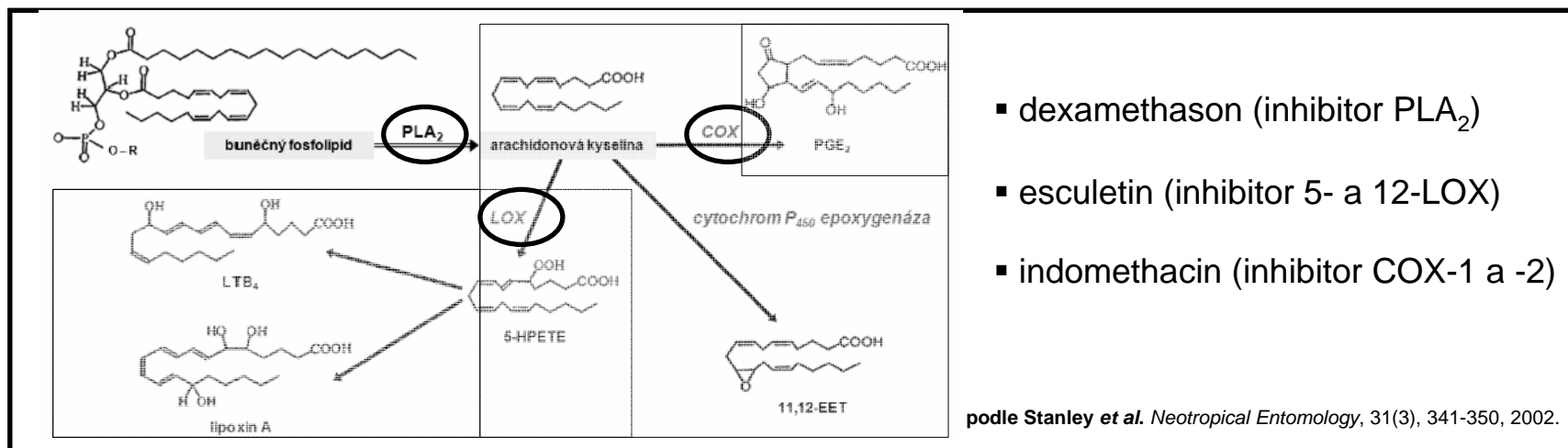
Potřebné substráty transglutaminázy...

- RNAi linie, PPL driver (cílem tukové těleso a slinné žlázy)
- *H. bacteriophora*, 25 IJ na larvu, 29 °C

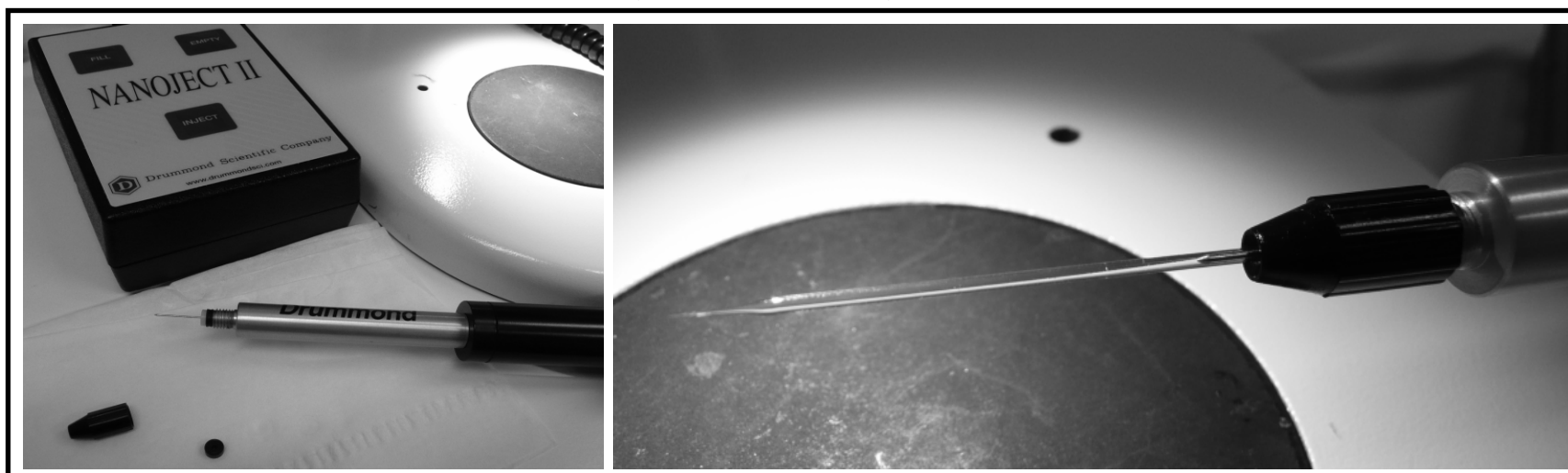


Eikosanoidy zprostředkují obranu proti infekci hlístovkami

- ovlivnění metabolismu kyseliny arachidonové pomocí inhibitorů biosyntézy eikosanoidů (IBE)



- přímá aplikace pomocí nanoinjektoru Nanoject II (Drummond Scientific)



Eikosanoidy zprostředkují obranu proti infekci hlístovkami

- larvy *Drosophily* (w^{1118}) injikované 50,6 nl IBE v PBS
- infekce *H. bacteriophora*, 100 IJ na larvu, 22 °C:

