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**Regulation of extracellular superoxide dismutases in the
leaf beetle *Phaedon cochleariae* after treatment with the
entomopathogenic fungus *Metarhizium anisopliae***

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III List of Abbreviations and Symbols

*	significant difference between 2 groups
~	similar
Δ	deviation
λ	wavelength
A	absorption
BCA	bicinchoninic acid
BLAST	Basic Local Alignment Search Tool
bp	base pair oft nucleobases
BSA	bovine serum albumin
cDNA	complementary DNA
Cq	quantification cycle
C-terminus	carboxy-terminus
DNA	desoxyribonucleic acid
DNase	deoxyribonuclease
dsRNA	double-stranded RNA
GFP	green fluorescent protein
HRP	horseradish peroxidase
i	infected larvae
kDa	kilo Dalton
M	Molar mass
Mn	manganese
mRNA	messenger RNA
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
ni	non-infected larvae
Nox	NADPH oxidase
N-terminus	amino-terminus

<i>P</i>	significance
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
qPCR	quantitative real-time PCR
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
ROS	reactive oxygen species
rpm	revolutions per minute
SD	standard deviation
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
<i>Taq</i>	<i>Thermus aquaticus</i>
TCA	2,4,6-Trichloroanisole
U	unit
UV-radiation	ultraviolet radiation
WST-1	Dojindo's highly water-soluble tetrazolium salt
XO	xanthine oxidase

1 Introduction

Some billion years ago (current estimates: from ~3.8 to 2.35 billion years) oxygen appeared along with the metabolism of oxygenic photosynthesis in the earth atmosphere [1]. The generated oxygen provided new ways to produce energy for the former organisms. An evolutionary advantage emerged, since the newly developed aerobic metabolism delivers more energy per glucose than for example glycolysis in anaerobic organisms does [2]. However, as a metabolic by-product also a new family of highly reactive oxygen species (ROS) appeared causing oxidative stress by damaging almost all cellular molecules [3-7]. Hence, aerobic organisms adapted to the circumstances and evolved antioxidants to protect themselves from ROS, but also developed mechanisms to use ROS advantageously as vital components in cellular signalling pathways or in immune response [8-10]. The ROS level essential for proceeding and at the same time to prevent damage is, under normal physiological conditions, balanced by antioxidant activity.

1.1 Superoxide dismutase

One of the first enzymatic defences against ROS in the cellular defence system are the antioxidant superoxide dismutases (SOD, E.C. 1.15.1.1) [11, 12]. These metalloproteins which can dispose intracellular ROS are found in all kingdoms of life and evolved before differentiation of eubacteria from archaea [13].

The antioxidant enzyme converts superoxide anions ($\cdot\text{O}_2^-$) into dioxygen (O_2) and hydrogen peroxide (H_2O_2) (see enzyme reaction below). The specific binding to the negatively charged $\cdot\text{O}_2^-$ molecules is imparted by the metals in the active site, which have a positive charge.

Enzyme reaction:

1. $\text{M}^{3+} + \cdot\text{O}_2^- + \text{H}^+ \rightarrow \text{M}^{2+}(\text{H}^+) + \text{O}_2$
2. $\text{M}^{2+}(\text{H}^+) + \text{H}^+ + \cdot\text{O}_2^- \rightarrow \text{M}^{3+} + \text{H}_2\text{O}_2$ (M stands for the metallic cofactor)

As the first reaction is thermodynamically favourable, the free released energy can be used for the extremely unfavourable reduction in the second reaction [14]. Further, other enzymes like catalases and peroxidases convert the strong oxidiser H_2O_2 into water [15, 16]. SODs with copper and zinc in their active sites though have been reported to act as slight CO_2 -dependent peroxidases as well. Because SODs are

rapidly reduced and slowly inactivated by H₂O₂, this peroxidase activity protects them against this inactivation mechanism [17]. Furthermore, SODs exhibit one of the highest known catalytic efficiencies of about 10⁹ M⁻¹ s⁻¹ which is the reason why the reactions are only diffusion limited [18]. An increased number of salt bridges and hydrogen bonds in Cu,Zn-SODs additionally leads to a very high biophysical stability to physicochemical stress (e.g. freeze-thaw cycles, high temperatures and urea) [15].

Several isoforms of SODs, evolved from different ancestor genes and they are classified according to their metal centres into Cu,Zn-, Fe-, Mn- and Ni-SODs [15]. In animals three isoforms have been identified. Cu,Zn-SODs (SOD1) are localised in cytoplasm, nucleus and the mitochondrial inter-membrane space, whereas Mn-SODs (SOD2) are found exclusively in the inner matrix of mitochondria and differ from Cu,Zn-SODs in protein sequence and structure [19]. The most recently discovered members of the SOD family are the Cu,Zn-SODs functioning predominantly in the extracellular matrix (SOD3) [20-22]. Previous phylogenetic analyses of SODs from different phyla suggested that extracellular SODs have evolved independently multiple times by addition of a signal peptide to cytoplasmic Cu,Zn-SODs [23].

1.2 Extracellular Cu,Zn-SODs

The SOD3 subfamily was studied comprehensively in mammals and was characterized as a tetrameric slightly hydrophobic glycoprotein with a molecular weight of ~135 kDa [21]. It occurs in a tetramer of identical subunits, linked through disulphide bonds, in most species but is found as a dimer as well [24]. Each monomer contains a copper and a zinc atom, which are both essential for the enzymatic activity [15]. The eukaryotic Cu,Zn-SOD shows a conserved three dimensional folding consisting antiparallel β -sheet strands formed to a flattened Greek key motif [25, 26] (Fig. 1).

The central sequence part of SOD3s contains a catalytic domain with high similarity to this of SOD1, but it is distinguished from the intracellular Cu,Zn-SODs at its N- and C-terminus [27, 28]. Each subunit of SOD3 enzymes is synthesized with an N-terminal signal peptide, which after cleavage leaves a mature protein [29]. The N-terminus of this mature protein appears to be important for tetramerisation and harbours a glycosylation site [30].

The C-terminal region is highly rich in positively charged amino acid residues [31] and involved in extracellular matrix binding. It anchors to heparan sulphate proteoglycans like collagen type1 or fibulin-5, located on cell surfaces and in the connective tissue matrix [32, 33]. This domain can be cleaved proteolytically, and hence, facilitate the transport in the inside of the nucleus [34] or the entrance of the protein to extracellular fluids [31, 35], where it may protect the tissues against antioxidants.

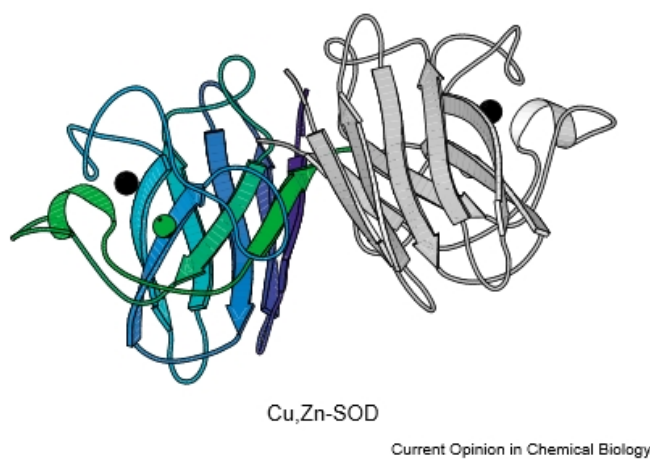


Figure 1: Structure of a Cu,Zn-SOD dimer: Cu,Zn-SODs have monomers with ~ 150 amino acids and mostly appear as dimers, Cu ion is illustrated in green and Zn in black in the coloured monomer [14].

Changes in the activity of SOD3 of human have been shown to contribute to pathogenesis of a number of diseases, especially in tissues with essential high levels of SOD3 expression [22]. For example, the risk of ischemic heart disease has been shown to increase with a decreased tissue antioxidant level, caused by a natural mutation in the extracellular matrix-binding region of SOD3 [36]. Also the pathogenesis of diabetes occurred due to changes in SOD3 activity, through a lower binding to the endothelial surface [20]. Then, there is a controversial hypothesis which says that an increased Cu,Zn-SOD may lead to an increased chance of mothers having a Down syndrome child [16]. Furthermore, recent research on Cu,Zn-SODs indicate a relationship between a misfolded Cu,Zn-SOD and familial amyotrophic lateral sclerosis [37, 38]. As in humans, also in insects a crucial role of extracellular SODs can be suggested. Especially in the immune system of the insects SOD3 is mentioned.

1.3 Insect immune response to pathogens

The innate immune response of insects includes melanisation leading to the physical encapsulation of microbial or parasitoid intruders in a dense melanin coat, and during phagocytosis toxic metabolites such as ROS are generated [39-42]. Circulating haemocytes recognise foreign organisms (pathogens and parasites), adhere to the foreign surface and form a multi-layered capsule by secreting components necessary for the melanisation of the invading organism (Fig. 2) [43].

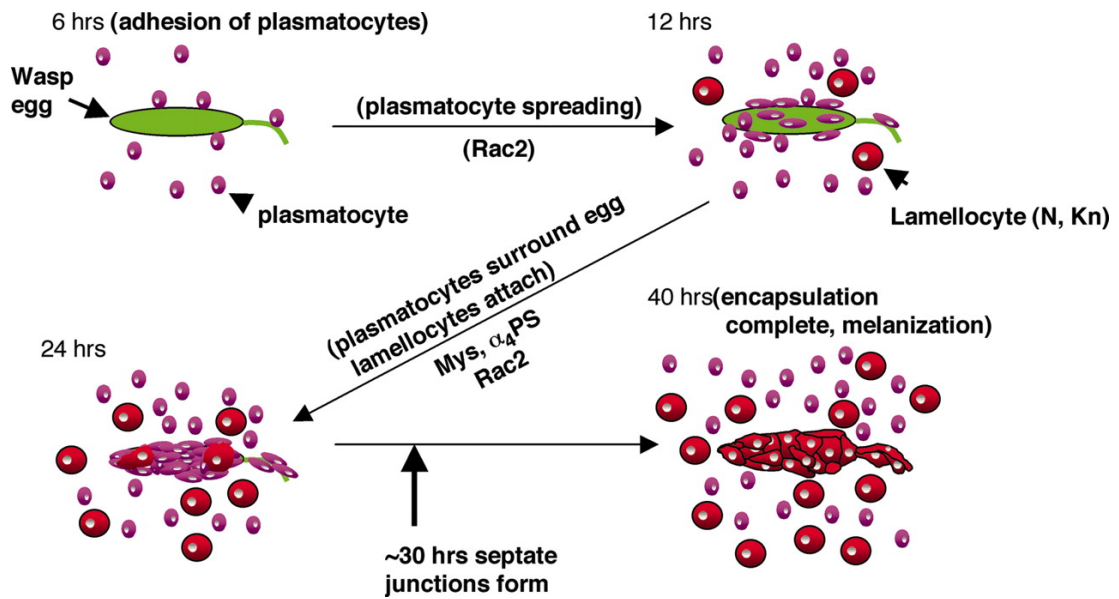


Figure 2: Diagram of the encapsulation of a parasitoid wasp egg: By 6 h postparasitization, plasmatocytes have started to attach to the wasp egg. Once attached, the plasmatocytes begin to spread around the egg, requiring the small GTPase Rac2. By 12 h postparasitization, lamellocytes begin to appear; this requires the Notch (N) signaling pathway and the transcription factor Collier (also known as Kn). By 24 h postparasitization, the plasmatocytes have completely surrounded the wasp egg, and lamellocytes are beginning to adhere. The β_2 -integrin Myospheroid (Mys) and α -integrin α_4PS are required for lamellocyte adherence to the wasp egg. Rac2 is required for the lamellocytes to maintain a spread conformation once attached to the wasp egg. After the plasmatocytes surround the egg, they form septate junctions. Finally, the lamellocytes completely surround the egg and the cellular capsule melanizes.

- transferred unchanged from Williams, 2007 [43]

During these insect immune cascades cytotoxic ROS are produced. For instance, elevated levels of superoxide anions had been examined during the melanotic encapsulation response of *Drosophila melanogaster* to the parasitization by *Leptopilina boulardi* [41]. In the activated phagocytic cells $\cdot O_2^-$ radicals are

produced via plasma membrane NADPH oxidase (Nox) [44]. In context to this Nox mediated signal cascades stands the H₂O₂ production, which also indicates a role of SODs as a mediator between Nox and H₂O₂ signals in the immune response of insects [45].

Although SOD3 has been conserved among metazoans, the first functional evidence for a SOD3 in insects has not been reported before the 21th century [28]. And still, the understanding of the function of extracellular SOD variants in insects is incomplete and only few examples have been published to date.

The *Drosophila melanogaster* SOD3 was reported in 2011 to exhibit a protective effect against oxidative stress caused by UV-radiation and an impact to life span regulation [46]. In the same year Colinet *et al.* discovered that parasitoid wasps also produce extracellular Zn,Cu-SODs and even inject them within the venom while oviposition. [27]. The extracellular SODs are suggested to act as a virulence factor to counteract the host immune response. The exogenous SODs interfere most likely with the hosts' ROS formation and increase the survival rate of the parasitoid eggs in the host. Hence a function of extracellular Cu,Zn-SOD in the protection against oxidative stress and thus, in immune response of insects, can be suggested.

2 Aim of the bachelor thesis

The first insect described to have three SOD3 isozymes is the leaf beetle *Phaedon chochleariae* [47]. This work focuses on the role of SOD3s and their regulation, with regards on the larval pathogen response of this Chrysomelina species. Therefor expression level in different tissues is to be assigned by utilizing quantitative real-time polymerase chain reaction (qPCR). SOD activity shall be examined *in vitro* and *in vivo* in different tissues and haemolymph by protein activity assays. Furthermore, the defence reaction is to be explored by exposing the larvae to the entomopathogenic fungus *Metarhizium anisopliae* and subsequently, the involvement and importance of each SOD3s in the immune response shall be detected by preceding RNA interference (RNAi). By proteomics further conclusions about extracellular SODs in the larvae should be achieved.

3 Material and methods

All used material is listed in the supplement.

3.1 Biological methods

3.1.1 Breeding of *P. cochleariae*

P. cochleariae were lab-reared on fresh Chinese cabbage (*Brassica rapa chinensis*) in a light/dark cycle of 16 h light with $14^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and 8 h darkness at $12^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Adults and eggs were separated and larvae were grouped with regard to their day of hatching (pictures in the supplement). Before pupation the larvae are going through three instars.

3.1.2 Dissection of *P. cochleariae* larvae

Dissection of the larvae was done under a Carl Zeiss stereomicroscope. Haemolymph was extracted by twitching off a leg and absorbing the fluid with a glass capillary (inner diameter of 0.5 mm). Head, fat body, Malpighian tubules, gut and defence glands were dissected using tweezers and dissection scissors purchased from Fine Science Tools. To prevent the drying-out of the tissues, dissection was performed in 0.9% NaCl-solution. Mostly third-instar larvae were dissected.

For protein analyses tissues were stored snap-frozen at -80°C . Haemolymph was kept in Ringer's solution (recipe in the supplement), centrifuged 5 min at 5,000 rpm and 4°C to pelletize the haemocytes. The supernatant was checked for cells under a Carl Zeiss Axioskope and the cell-free haemolymph was stored at -20°C overnight.

Tissues for qPCR were stored in Lysis Buffer [RNAqueous-Micro Kit] with ExpressArt NucleoGuard at -20°C until needed. For whole larvae samples, larvae were snap-frozen in liquid nitrogen, pestled with sea sand in Lysis Buffer with NucleoGuard. After centrifugation the supernatant was stored at -20°C until needed.

3.1.3 Induction of RNAi in *P. cochleariae* larvae

RNAi is a version of endogenous gene regulation. Injected double-stranded RNA (dsRNA) binds to the complementary RNA and leads to degrading of the target RNA. Hence, the synthesis of the corresponding protein gets inhibited [48].

Injection of dsRNA was carried out on early second-instar larvae of 2 – 3 mm length (approx. 9 days after hatching). 100 ng of dsRNA was injected dorso-median in the gap between pro- and mesothorax into the haemocoel of the larvae with a Nano2010 injector and self-prepared glass needles driven by a micromanipulator. During the procedure the larvae were kept calm on ice.

3.2 Mycological methods

3.2.1 Cultivation and harvesting of *M. anisopliae* conidia

The entomopathogenic fungus *M. anisopliae* was maintained and harvested from liquid culture (Potato-Dextrose Broth, 200 ml in a 500 ml Erlenmeyer flask), which was incubated at room temperature for several months without shaking. The culture with sufficient conidia was agitated with a 10 cm magnetic stirring bar at 4°C overnight. Fine gauze was then used to separate the mycelium from the supernatant containing the conidia. The supernatant was centrifuged for 10 min at 10,300 rpm and 4°C and washed twice with approx. 20 ml of 0.1% aqueous Tween-20 solution by centrifuging under the same conditions. The pellet of conidia was diluted in 10 ml of 0.1% aqueous Tween-20 solution. Using the Neubauer improved haemocytometer, the concentration of conidia was determined and adjusted to 10^6 conidia/ml afterwards.

3.2.2 Infection of *P. cochleariae* with *M. anisopliae*

To expose the larvae to the fungi they were dabbed on lab tissue to remove the defence secretions first and then submerged in 1 ml of well mixed 10^6 conidia suspension, which mimics the natural way of infection [49]. After 30 sec the suspension was poured on filter paper to let the larvae air dry for 1 min. After infection the larvae were kept on Chinese cabbage in small plastic pots at room temperature to allow the fungus to develop. To eliminate a difference in the results caused by the stress of the procedure between uninfected and infected larvae a

control group was generated. The control group larvae were also submerged in 0.1% aqueous Tween-20 solution, but without conidia and stored at room temperature the same way.

Infection was performed 5 days after RNAi and after 2 days of exposure dissection of the larvae was done. In a later experiment larvae were dissected only 4 days after infection to check if more SOD protein is built after some more days of exposure. Since the larvae begin to pupate after less than a week when they are kept at room temperature (table in the supplement), the distance between RNAi and infection had to be reduced to 2 days for these experiments.

3.3 Molecular methods

3.3.1 Total RNA isolation and DNase I digestion

The tissues of several (mostly 3) larvae were pooled in a 1.5 ml tube with 100 μ l Lysis Solution (RNAqueous Mikro Kit) and 1 μ l ExpressArt NucleoGuard, a universal nuclease and RNase inhibitor. RNA isolation procedure, DNase I treatment and DNA inactivation were conducted according to the manual of the RNAqueous Mikro Kit. The concentration and purity of isolated RNA was measured at $\lambda = 260$ and 280 nm with a NanoVue spectrophotometer against nuclease free water. The purity of the RNA can be calculated by building the quotient of A_{260} and A_{280} . To estimate the RNA concentration following formula was used:

$$c(\text{RNA}) = A_{260} \times 40 \mu\text{g/ml.}$$

Formula 1: Calculation of RNA concentration

The quality of RNA was determined with a RNA Nano Chip and Agilent 2100 Bioanalyzer.

3.3.2 Complementary DNA synthesis

To obtain the template for quantitative PCR (qPCR), complementary DNA (cDNA) was reverse transcribed from the isolated RNA by using SuperScript III enzyme and oligo(dT)₂₀ primer (50 μM) (SuperScript III Reverse Transcriptase Kit) according to the manufacturer's instruction. The reaction contained up to 5 μg of RNA and was incubated for 2 h at 50°C. Enzyme reaction was inactivated by heating the reaction at 70°C for 15 min subsequently. Formed cDNA was stored at - 20°C until needed.

3.3.3 Quantitative real-time PCR

qPCR was done to assess the expression levels of the extracellular SODs in the different larval tissues of *P. cochleariae*. In a PCR DNA is amplified *in vitro*. To quantify the mRNA/cDNA through a qPCR, a fluorescent dye is added additionally to the reaction. SYBR green, which was used as fluorescent dye intercalates with the built double-stranded DNA. Accordingly, there is a linear correlation between the fluorescence and the DNA amount. The CFX96 Real-Time System thermal cycler contains sensors to measure the fluorescence at each PCR cycle (Figure 3: "Plate Read").

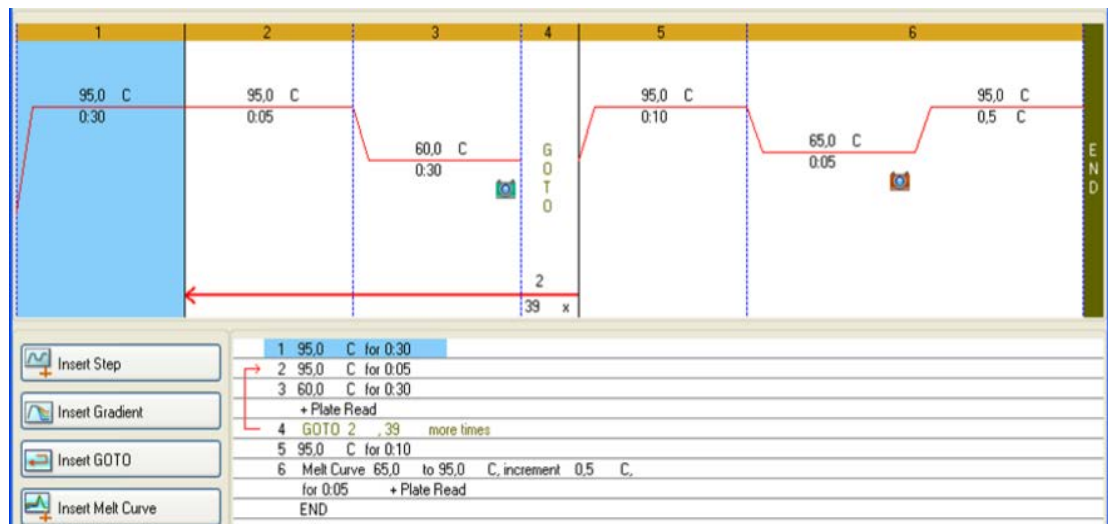


Figure 3: qPCR program in CFX Manager Software: After the initial denaturing at 95°C, 40 cycles of the denaturing, hybridisation and elongation with appropriate temperatures were conducted. The thermal cycler measures the absorbance after each cycle (Plate Read) to quantify the relative expression level. At the end a melt curve is generated by heating the samples from 65 to 95°C and measuring the absorbance after each 0.05°C increment.

Besides the three extracellular Cu,Zn-SODs (*SOD3.1*, *SOD3.2* and *SOD3.3*) also the intracellular Cu,Zn-SOD (*SOD1.1*) were examined in terms of their expression level. The household genes *rps3* and *rpl6* (coding for ribosomal proteins) were chosen as reference genes to normalise the PCRs for the amount of present cDNA template.

In a 25 µl-PCR reaction 12.5 µl SYBR Premix ExTaq II (containing buffer solution, magnesium ions, *Taq* DNA polymerase, dNTPs and the fluorescent dye SYBR-Green) 1 µl of each primer (10 µM) and 1µl of cDNA template were combined in double distilled RNase free water. Primers were designed by René R. Gretscher using primer3PLUS: <http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi> [50] and sequences can be found in the supplement. For each biological replicate three technical replicates were performed. Gene expression was estimated and analysed with CFX Manager Software.

3.3.4 Synthesis of dsRNA for RNAi

Template DNA, which was constructed by René R. Gretscher using pIB-plasmids and T7-polymerase [50], was used to generate dsRNA. The synthesis of dsRNA was made from the PCR fragment (primer sequences in the supplement) corresponding to the instructions of the MEGAscript RNAi Kit. To get higher yields, the incubation time of the linear template DNA with the T7 Enzyme Mix and NTPs was extended to 8 hours at 37°C. Instead of the provided Elution Solution, 3 × 50 µl double-distilled water was used to elute the dsRNA. The water was preheated to 65°C. After applying the first 50 µl of water to the filter cartridge, the closed tube incubated for 1 hour in a heat block at 50°C. Second and third incubation time was approx. 10 min The concentration of the dsRNA was measured spectrophotometrically with a NanoVue spectrophotometer and adjusted to 1 µg/µl with injection buffer (recipe in the supplement). The concentration was calculated with following equation:

$$c(\text{dsRNA}) = A_{260} \times 45 \text{ mg/ml.}$$

Formula 2: Calculation of dsRNA concentration

The quality of dsRNA was checked by TAE-agarose-electrophoresis with the resulting dsRNA size of 238bp for *PcSOD1.1*, 193bp for *PcSOD3.1*, 508bp for *PcSOD3.2* and 172bp for *PcSOD3.3* respectively. Green fluorescent protein (*GFP*) dsRNA shows a length of 523bp.

3.3.5 Membrane protein extraction

With the Native Membrane Protein Extraction Kit membrane proteins can be extracted under mild non-denaturing conditions.

To fat body and Malpighian tubules of 6 larvae 500 µl Wash Buffer (Native Membrane Protein Extraction Kit) was added. Tissue samples were then centrifuged for 10 min at 5,000 rpm. The supernatant was discarded and the pellets stored at - 20°C overnight. Further proceeding was done after the instructions 7.3.6 Frozen tissue. Briefly, the pellet was snap frozen in liquid nitrogen and crushed in a precooled homogenizer, like recommended in the manual. Since there was a little amount of tissue per probe, only ¼ volumes of the reagents were used.

3.4 Protein analysis

3.4.1 Protein quantitation

3.4.1.1 BCA protein assay

To assess the protein concentration a BCA protein assay (Pierce BCA Protein Assay Kit), was conducted after the given instructions. Bovine serum albumin (BSA) standards were used and diluted for a working range from 20 to 2,000 µg/ml. The assays were measured in a Spectra Max 250 microplate reader at 562 nm after 30 min incubation time at 37°C.

Principally, the assay works in two steps. First, the reduction of Cu^{2+} to Cu^{1+} is taking place which is also known as the Biuret reaction. In an alkaline environment, copper and protein form a chelate. Then the reaction of Bicinchoninic acid (BCA) with the developed cuprous cation from the first step follows and a chelate of two BCA molecules with one cuprous ion occurs (Fig. 4). The intense purple-coloured and water-soluble BCA/copper-complex shows a strong linear absorbance with the increase of

protein concentration at a wavelength of 562 nm. The presence of the amino acids tryptophan, tyrosine and cysteine, and the number of the peptide bonds are responsible for the intensity of the colour. As the peptide backbone also contributes to the colour formation, this method exhibits less protein-to-protein variation than Coomassie dye-binding methods [51].

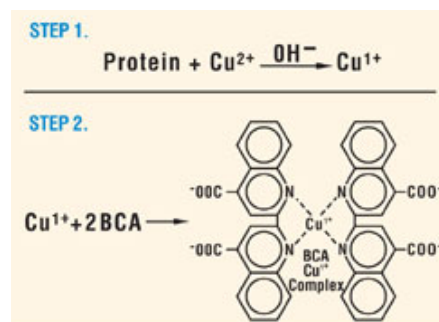


Figure 4: Schematic reaction of BCA protein assay:

Step 1: reduction of Cu^{2+} with protein, Step 2: chelation of cuprous ion and two molecules of BCA.

- Illustrations from Pierce BCA Protein Assay Kit manual

3.4.1.2 UV-measurement

1 μl of the sample solution was applied to the NanoVue spectrophotometer and measured at $\lambda = 280 \text{ nm}$. The concentration of total protein was determined by subtracting the absorbance value of the medium in which the tissue was dissolved from the absorbance of the probe.

For that method much less of a sample is needed, which is advantageous for working with samples with little volume. Hence, although many disruptive factors, like nucleic acids, buffer solutions or distinct colour in the sample can adulterate the results of spectrophotometric measurements, UV-measurements were chosen for most examinations.

3.4.2 SOD activity determination

3.4.2.1 Preparation of samples

Samples were prepared as described in 3.2.2. Here the haemolymph of 4 - 10 larvae was added to 50 μ l of Ringer's solution. After centrifugation and cell-check, the supernatant was stored at -20°C . Snap frozen tissues (2 per sample) were also placed into 50 μ l Ringer's solution with 0.5 μ l Protease-Inhibitor-Mix and grinded with a sterile pestle. After centrifugation at 4°C and 5,000 rpm for 10 min the supernatant was transferred into a new reaction tube and stored at -20°C . The protein concentration was measured spectrophotometrically (see 6.5.1.2) and diluted to the needed concentration with Ringer's solution.

3.4.2.2 SOD-assay

To determine the SOD activity in a probe the 19160 SOD Determination Kit was used. A very suitable SOD assaying is possible by utilising the Dojindo's highly water-soluble tetrazolium salt, WST-1. The reduction of oxygen by the enzyme xanthine oxidase (XO) is linearly related to the XO-activity. WST-1, however, reduces the formed superoxide anions and produces a water-soluble yellow formazan dye simultaneously. The SOD inhibits this reaction, as shown in Figure 5. Since the absorbance at 440 nm is proportional to the amount of superoxide anions, the inhibition rate of SODs in the probe can be determined by a colorimetric method.

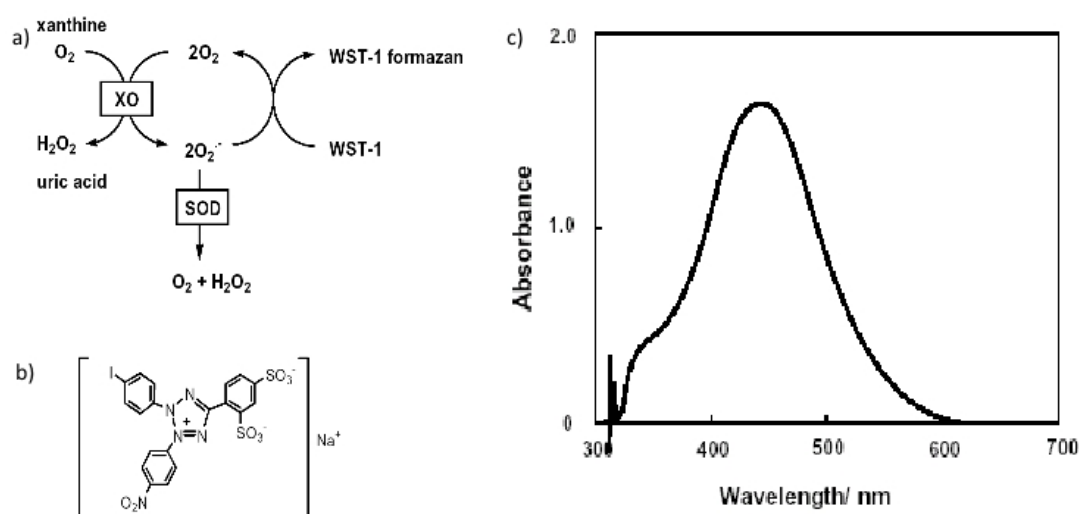


Figure 5: Principle of the SOD Assay Kit: a) reactions involved after addition of XO enzyme, b) structure of WST-1, c) absorption spectrum of WST-1 formazan at 440 nm - illustrations from Sigma-Aldrich SOD Assay Kit manual.

The SOD activity (inhibition rate) was calculated with the following equation:

$$\text{Inhibition rate (\%)} = \frac{(A_{\text{blank 1}} - A_{\text{blank 3}}) - (A_{\text{sample}} - A_{\text{blank 2}})}{(A_{\text{blank 1}} - A_{\text{blank 3}})} \cdot 100$$

Formula 3: Calculation of SOD activity

„A” describes the measured absorbance at 450 nm. All samples contain WST working solution. Blank 1 additionally contains XO enzyme working solution and water instead of protein solution. Blank 3 contains the same, but instead of the enzyme working solution it contains dilution buffer. In case of visible colour of the sample solution, blank 2 needed to be set up additionally. Blank 2 contains the sample protein solution but no enzyme solution, so the colour of the sample solution can be subtracted of the total absorbance. The SOD standard inhibition curve was determined according to the manual with bovine erythrocyte SOD (15000 U/ml) and an incubation time of 20 min.

3.4.3 Polyacrylamide gel electrophoresis (SDS-PAGE)

To check the presence and the amount of SOD-proteins the wells of a protein gel (BIO-RAD Mini-PROTEAN TGX) were filled with protein samples (preparation similar to 3.5.2.1). Therefore 5 × SDS loading buffer (recipe in the supplement) was applied to a solution with a definite amount (25 or 75 µg) of total protein and heated at 95°C for 5 min. Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures and linearizes proteins and grants a negative charge to the proteins. Embedded into a tank containing running buffer (recipe in the supplement) with an electric field the proteins in the gel can migrate from the negative charged electrode towards the positive electrode. Small proteins migrate faster through the gel pores than larger ones. After running the gel 20 min at 220 V it was ready to use for further processing. As a marker 2 µl of Precision Plus Protein Dual Colour Standards was applied in one slot.

A staining for one hour with Roti-Blue quick was performed to view the bands of stained protein. The band for SODs should appear at the size of ca. 18 kD.

3.4.4 Western blot

A Western blot is used as a method to detect a specific protein after gel electrophoresis. Briefly, the proteins were transferred with the Trans-Blot Turbo Transfer System from the gel to a Trans-Blot Turbo Transfer Pack membrane. After blocking with 5% Non Fat Dry Milk (NFDM) in TBS-T (recipe in supplement) for 2 hours the membrane was probed with the *Homo sapiens* SOD1 rabbit polyclonal antibody (1:1000) in 2.5% NFDM in TBS-T at 4°C overnight on a rotating roll mixer. Colinet *et al.* already showed in their paper from 2011 [27], that the antibody against human SOD1 also binds to insect SODs. The membrane was washed with TBS-T 3 times for 10 min and reblocked with 10% NFDM in TBS-T at room temperature. Afterwards the membrane got incubated with a goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate (1:25000) in 2.5% NFDM in TBS-T for one hour and washed again as described above. Chemiluminescence signal detection was performed by pouring chemiluminescent Pierce ECL Western Blotting Substrate over the membrane which reacts with the HRP, and picturing the luminescence on a sensitive CL-XPosure film.

3.4.5. In gel digestion of proteins

P. cochleariae was researched in relation to its proteome. For the analysis, protein solution of Malpighian tubules, fat body (soluble and membrane fraction) and haemolymph from the size 5 kD to 25 kD were cut out (9 pieces each) of a stained SDS-PAGE gel, loaded with 20 µg total protein. Further processing like destaining, digestion and mass spectrometric analyses was conducted by Dr. N. Wielsch and Y. Hupfer from the proteomics department.

3.4.6 TCA precipitation of proteins

For TCA precipitation two replicates were assembled by pooling haemolymph of 2-3 *P. cochleariae* larvae respectively in 25 µl Ringer's solution. The protein solution was kept on ice and Sodium deoxycholate was added to 0.02% final concentration. After vortexing and adding 10% TCA it was vortexed again and incubated on ice for 1 hour. Afterwards the probe was centrifuged at maximum speed and 4°C for 10 min. The supernatant was removed and the pellet was washed twice with 100% acetone and then air dried. Following shotgun proteomics was conducted by Dr. N. Wielsch and Y. Hupfer from the proteomics department.

3.5 Proteome analysis

Mass spectrometry data were processed with the software tool ProteinLynx Global SERVER (PLGS) v.2.5. All yield peptide sequences were dedicated to a protein by means of the Protein Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI).

3.6 Statistics

Statistical tests were performed with Excel 2013. Standard deviation (SD) was calculated to determine the level of statistical dispersion from the average. A two-tailed test was performed to test the significance between two different groups. Values were accepted as significant when $P \leq 0.05$.

4 Results

4.1 Examinations of Cu,Zn-SODs in untreated *P. cochleariae* larvae

4.1.1 Relative expression in several tissues

Four SODs were examined in qPCR experiments: the dominant cytosolic Cu,Zn-SOD (SOD1.1) and three extracellular SODs (SOD3.1, SOD3.2, SOD3.3). For my initial studies I performed qPCR analyses to reveal presence of the four SODs in the different tissues of third-instar larvae of *P. cochleariae*. All four cDNA sequences encode functional proteins which have been assayed as purified recombinant proteins by Gretscher [47]. To minimise the standard deviation of expression levels the expression data Gretscher collected for his work were added to my data set. His data acquisition procedure was comparable to mine.

Visibly in Figure 6, the three extracellular SODs are differently expressed in the tested tissues. In the glands, for example, *sod3.1* showed a high relative expression level (ΔCq -value ≈ 30) compared to the other *sod* genes. In fat body, gut and head *sod3.1* appeared to be dominantly expressed as well. In fat body tissue additionally *sod3.2* exhibited a high expression level. Malpighian tubules showed a relatively high expression of *sod3.3*. As *sod1.1* exhibited a comparable expression level (ΔCq -value between 0.1 and 0.4) in every tested tissue, it seems to play a role in every tissue.

The expression level of the *sod*'s was also measured in haemolymph, since Arbi *et al.* adjudged the SOD in haemocytes of insects to take an active part in pathogen defence [52]. Extracellular SODs are likely to be secreted into the haemocoel to protect all tissues against oxidative stress. Consequently *sod3* RNA may be present in haemocytes as well. The results show that haemocytes indeed express extracellular SODs but the standard deviation of the data is very high (ΔCq -value $\pm SD(sod3.1) = 0.77 \pm 0.91$).

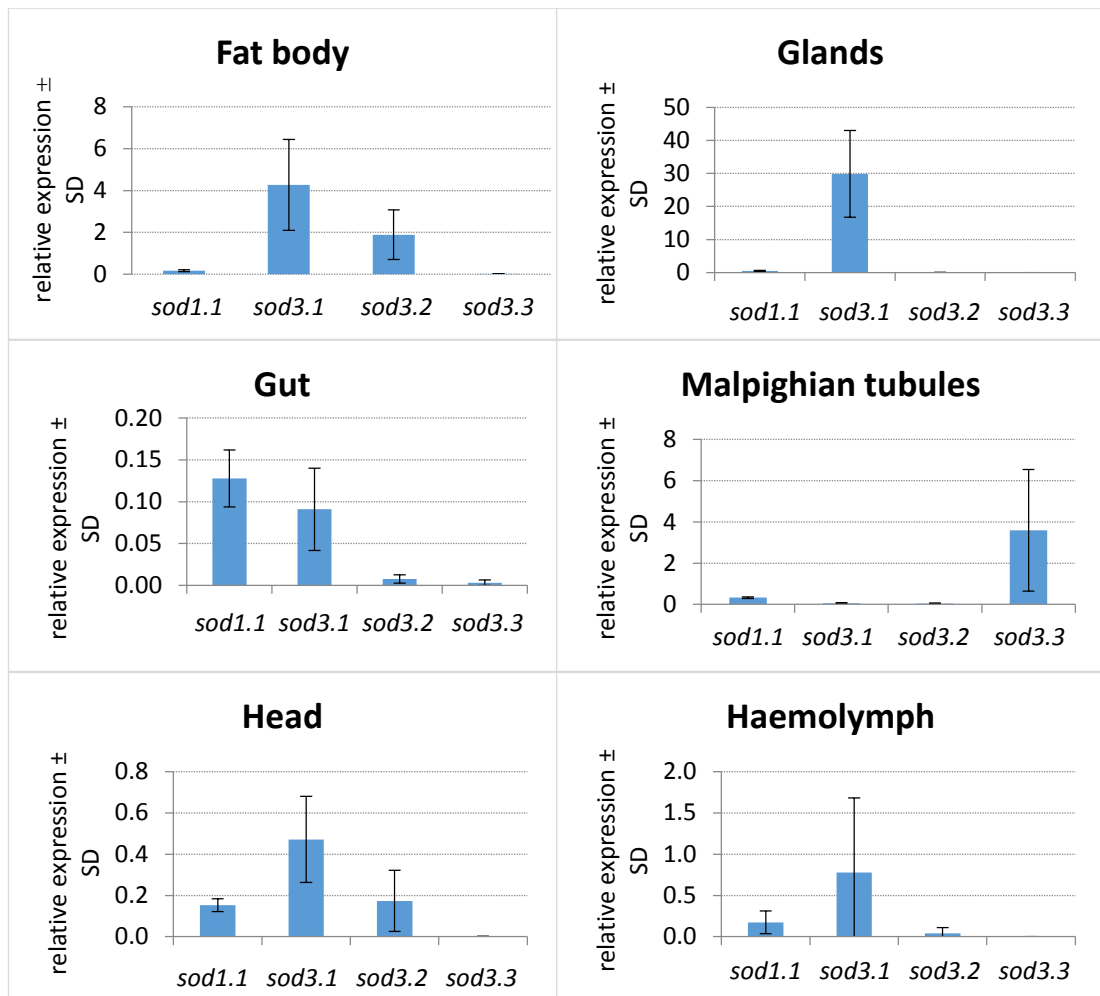


Figure 6: expression level of *sod*'s in several tissues of juvenile *P. cochleariae*: Tissues of 3 larvae were pooled for one sample. 4-12 biological replicates were examined. Reference genes were *rps3* and *rpl6*.

4.1.2 SOD activity after membrane protein isolation of several tissues

Not only the RNA, coding for the SODs, is available in those tissues, *in vivo* experiments also allocated functionality for each SOD isozyme [47]. To determine SOD activity in the tissues the SOD Determination Kit was used. An inhibition curve was performed with bovine erythrocyte SOD to observe the perfect incubation time with a good linearity of the slope (Inhibition curve in the supplement).

From human is known that extracellular SODs are bound to the extracellular matrix and the protein can be secreted to protect tissues against antioxidants upon proteolytic cleavage [27, 28, 34].

To see, if there is an activity of extracellular SODs in the membrane fraction of *P. cochelariae* tissues an isolation of the membrane proteins had to be performed before assaying the SOD activity. Hence, the cytosolic and membrane fraction of fat body and Malpighian tubules got separated, followed by protein concentration determination and SOD activity measurement.

In both tissues an activity in the membrane samples is visible (Fig. 7). However, activity was higher in the cytosolic fractions compared to the membrane enriched fraction.

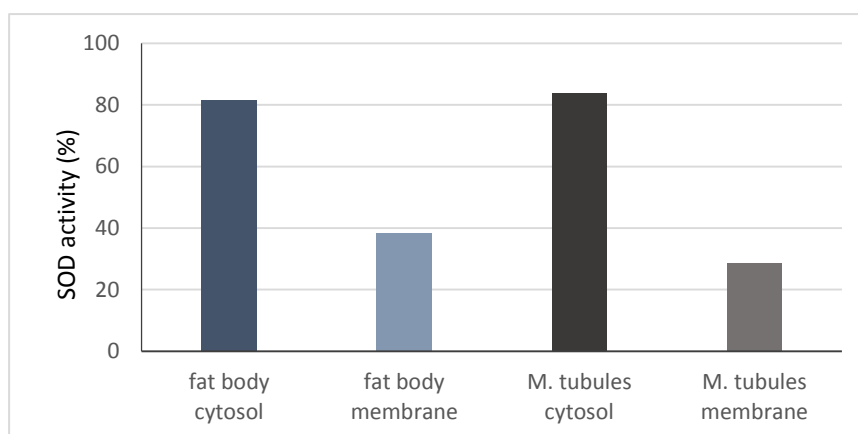


Figure 7: SOD activity in cytosol- and membrane-fraction of fat body and Malpighian tubules: tissues from 6 larvae were pooled in one probe and proteins got separated into cytosolic and membrane proteins, protein measurement was performed with BCA protein assay, 6 μ g of total protein was used.

4.1.3 Proteomics of different tissues and haemolymph

In gel digestion and following mass spectrometric analyses were conducted to identify SODs in the complex protein mixture of fat body, Malpighian tubules and haemolymph (identified SOD peptides in the supplement). Proteomics revealed that there is SOD1 in fat body and Malpighian tubules. Even though extracellular SODs were not detected in those two tissues by mass spectrometry SOD3.1 and SOD3.2 were found in haemolymph. Thus, existence of those extracellular proteins in the haemolymph could be shown.

4.2 Activity of overexpressed Cu,Zn-SODs

In order to demonstrate *in vitro* activity of extracellular Cu,Zn-SODs in *P. cochleariae* all enzymes have been expressed heterologously in *E. coli* and purified subsequently from René R. Gretscher. The SOD activity assay, conducted by myself, revealed interesting results. Whereas cellular SOD1.1 and extracellular SODs 3.1 and 3.3 show a visible activity, SOD3.2 shows an activity by only ~ 1% (Fig. 8).

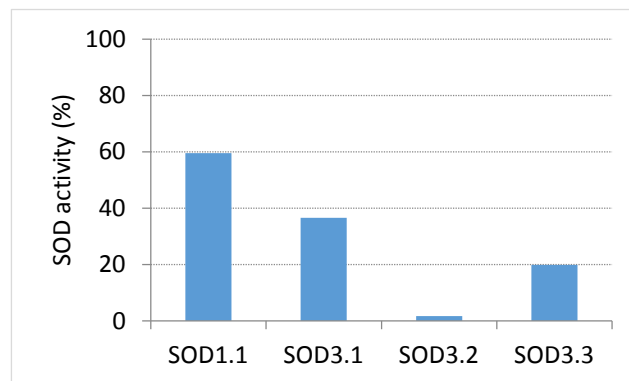


Figure 8: SOD activity of overexpressed protein from *P. cochleariae*: samples were produced from René R. Gretscher, 4 µg protein was used for activity determination, respectively.

4.3 Cu,Zn-SODs in *P. cochleariae* larvae infected by *M. anisopliae*

4.3.1 Relative expression after varying time of fungal exposure

Based on the infection experiments by Gretscher [47] demonstrating implication of extracellular SOD3.1 in the defence against fungal pathogens by using *M. anisopliae*, my first intention was to test if the fungal infection causes an induction of SOD activity on the transcript level of the extracellular SOD3.1, SOD3.2, SOD3.3 and the cytosolic SOD1.1. Therefore, the effects of infection were examined first on whole larvae 1 to 6 days after infection of early third-instar larvae (Fig. 9).

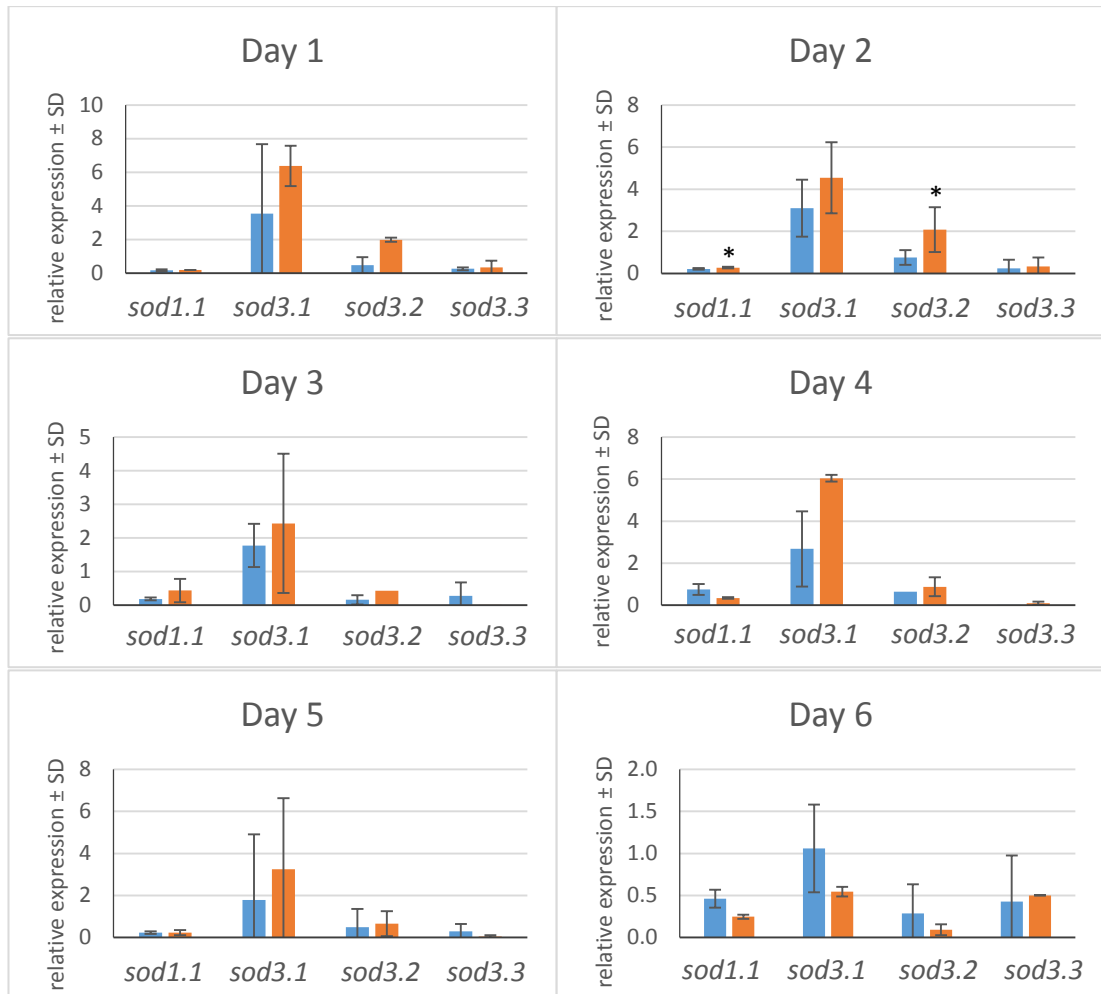
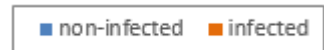


Figure 9: expression level of *sod*'s in whole *P. cochleariae* larvae 1 to 6 days after infection with *M. anisopliae*: uninfected and infected



larvae in comparison, 1 to 6 biological replicates of whole larvae were examined.

* = significantly different to their non-infected group

As can be seen from the graph above a difference in expression levels exists. Noticeable is that *sod*'s in infected larvae often show a higher expression than in uninfected larvae, as expected. From day to day the difference between values of control and infected group seems to decrease and the standard deviation of the data increases until the mean value of relative expression in infected larvae on day 6 is lower in all examined *sod*'s, beside *sod3.3*, than the mean expression level of the control group.

Since there is no statistical significant differences between the non-infected and infected groups ($P > 0.05$) except in the results of *sod1.1* ($P = 0.045$) and *sod3.2* ($P = 0.048$) on day 2, further investigation was done with larvae which were exposed to *M. anisopliae* for 2 days.

4.3.2 Relative expression in several tissues 2 days after infection

Following these results, expression levels were also tested in tissues 2 days after infection (Fig. 10). The analysis of the expression level, however, revealed high standard deviations of the data, so that no significant difference could be detected between infected and non-infected larvae, except for *sod3.1* in the glands where infected larvae show a significantly lower expression level than uninfected. Even though the expression level in whole larvae seemingly increases after infection (Fig. 9), this effect could not be confirmed in the examined tissues.

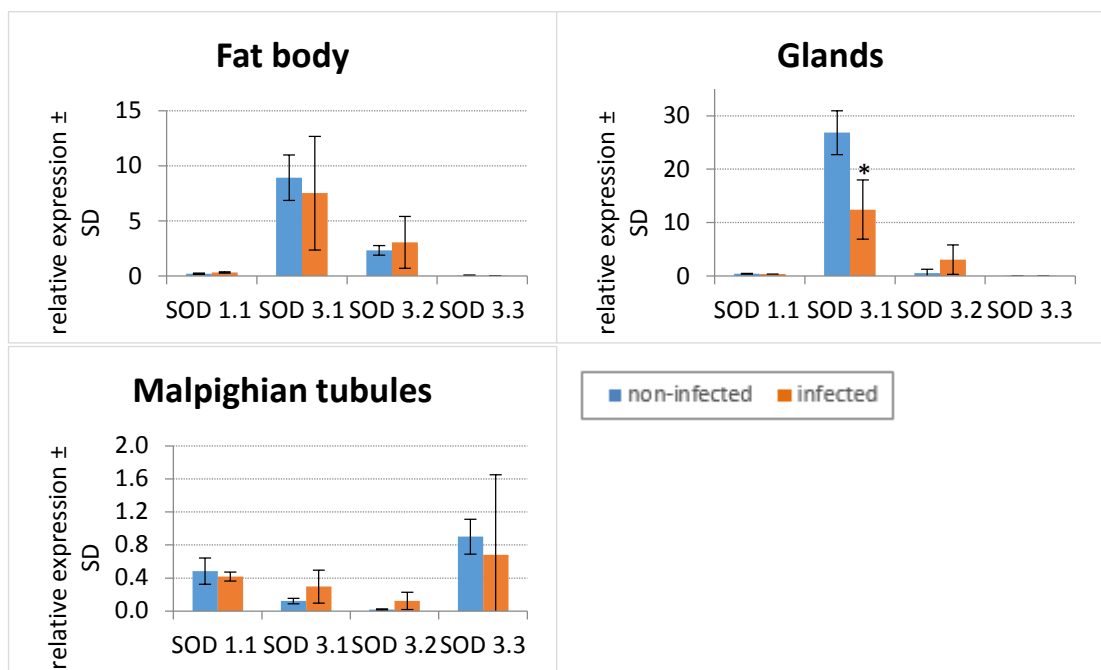


Figure 10: expression level of *sod*'s in several tissues of *P. cochleariae* 2 days after infection with *M. anisopliae*: Tissues of 3 larvae were pooled for one sample. Whole larva samples contain one larva. 3 replicates were examined.

* = significantly different to its non-infected group ($P = 0.026$).

4.4 Extracellular SODs after RNAi in infected *P. cochleariae* larvae

To investigate if extracellular SODs actually play a role in defence against the pathogen in *P. cochleariae*, as supposed from Gretscher [47], I conducted further SOD silencing experiments. For example, it has not been clarified before, whether other SODs can take over the function of a silenced SOD when needed.

4.4.1 Silenced SOD3.1 via RNAi

Western blot, SOD activity assays and proteomics were conducted to view the impacts of RNA mediated silencing of SOD3.1. Five days after injection of the dsRNA the larvae got infected with the entomopathogenic fungus. 7 days after injection of *sod3.1*-dsRNA the protein shouldn't be present in the haemolymph anymore. Haemolymph was taken from larvae a week after RNAi and same samples were used for both assays.

Since there is a significant difference between SOD activities of non-treated larvae and larvae which suffered the injection of dsRNA (graph in the supplement), a control group had to be raised for the group of silenced SOD3.1 larvae. Hence, as a control for the influence of activating the RNAi machinery, dsRNA according to the GFP nucleotide sequences was injected with corresponding amounts into the control group. GFP works well for that, due to the fact that it has no counterpart in the *P. cochleariae* transcriptome [47, 48]. Accordingly, four groups of *P. cochleariae* larvae were arranged for this experiment. In the first place an infected group and a non-infected control group which were inoculated with *gfp*-dsRNA (GFP ni, GFP i), in the second place infected larvae and control with injected *sod3.1*-dsRNA (SOD3.1 ni, SOD3.1 i).

Using 75 µg of total haemolymph protein for Western blot the fluorescent antibodies show a big amount of SOD at the expected size of ~ 18 kDa in the GFP-silenced samples (Fig. 11). For the assay a primary antibody was selected, which originally was developed against human SOD1 but also was successfully used against insect SODs from Colinet *et al.* [27]. Since an immunoblotting with overexpressed Cu,Zn-SOD samples (Western blot in the supplement) revealed no binding of this antibody to extracellular SODs 3.2 and 3.3 of *P. cochleariae*, SOD3.1 is the only extracellular SOD what binds with the antibody. Hence, samples of larvae, where SOD3.1 was silenced, show no signal for SOD, as expected. Also the results of proteomics confirmed that there is no left SOD3.1 in the silenced groups.

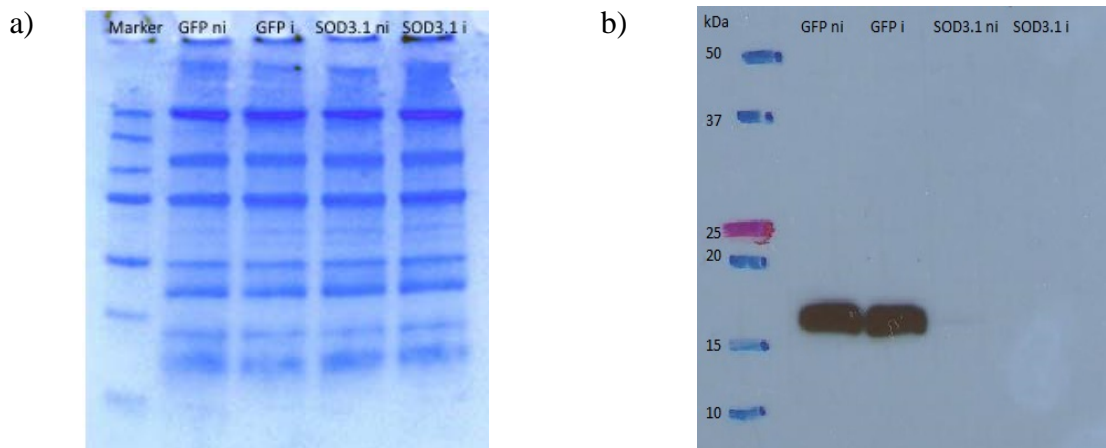


Figure 11: Immunoblotting experiment after RNAi: a) SDS-gel with total haemolymph protein; b) Western blot of SODs: infection 5 days after injection of dsRNA, dissection 2 days after infection, 75 μ g of total haemolymph protein was evenly applied.

The decrease of SOD3.1 can also be shown via SOD activity assay (Fig. 12). There is only a small residual SOD activity which may result from the other active SOD3s that weren't silenced, for example from SOD3.3. Though it hasn't been found in haemolymph by proteomics it could be present in the fluid in small amounts and generate this activity. Likely SOD3.2 causes only a small part of the residual activity, since the activity of this protein revealed a very low activity *in vitro* (Fig. 8).

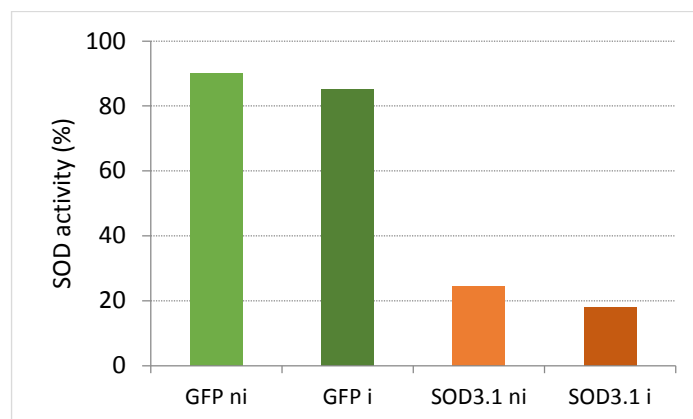


Figure 12: Impact of RNAi and infection to SOD activity in haemolymph: 5 days after injection of dsRNA the infected-group got exposed to *M. anisopliae* conidia, 2 days after infection dissection was done, haemolymph of 10 larvae was pooled in one probe, total protein measurement via UV-measurement and 10 μ g total protein was used.

The loss of any SOD signal and protein and the decrease in SOD activity reveals a successfully executed protein silencing in the course of a week. The difference between the infected (i) larvae and the non-infected (ni) larvae of each group wasn't though statistically distinguishable in both cases (GFP and SOD3.1). Due to the material decrease of SOD activity after RNAi (Fig. 12) it can be suggested that the dominant extracellular SOD in *P. cochleariae* is the SOD3.1.

4.4.2 Effects of RNAi of extracellular SOD isozymes in infected *P. cochleariae* larvae

To find out if SOD3.1 is the most important extracellular SOD responsible for the activity in the haemolymph, the impact of an infection in *P. cochleariae* larvae on silenced extracellular SODs was examined in a final experiment. In addition to the 4 groups (GFPni, GFPi, SOD3.1ni and SOD3.1i) arranged in the experiments before, 2 more groups were generated. One group of juvenile *P. cochleariae* with silenced *sod3.2*-RNA (SOD3.2) and one with silenced *sod3.3*-RNA (SOD3.3). The SOD-activity was measured in different tissues by activity assay, respectively.

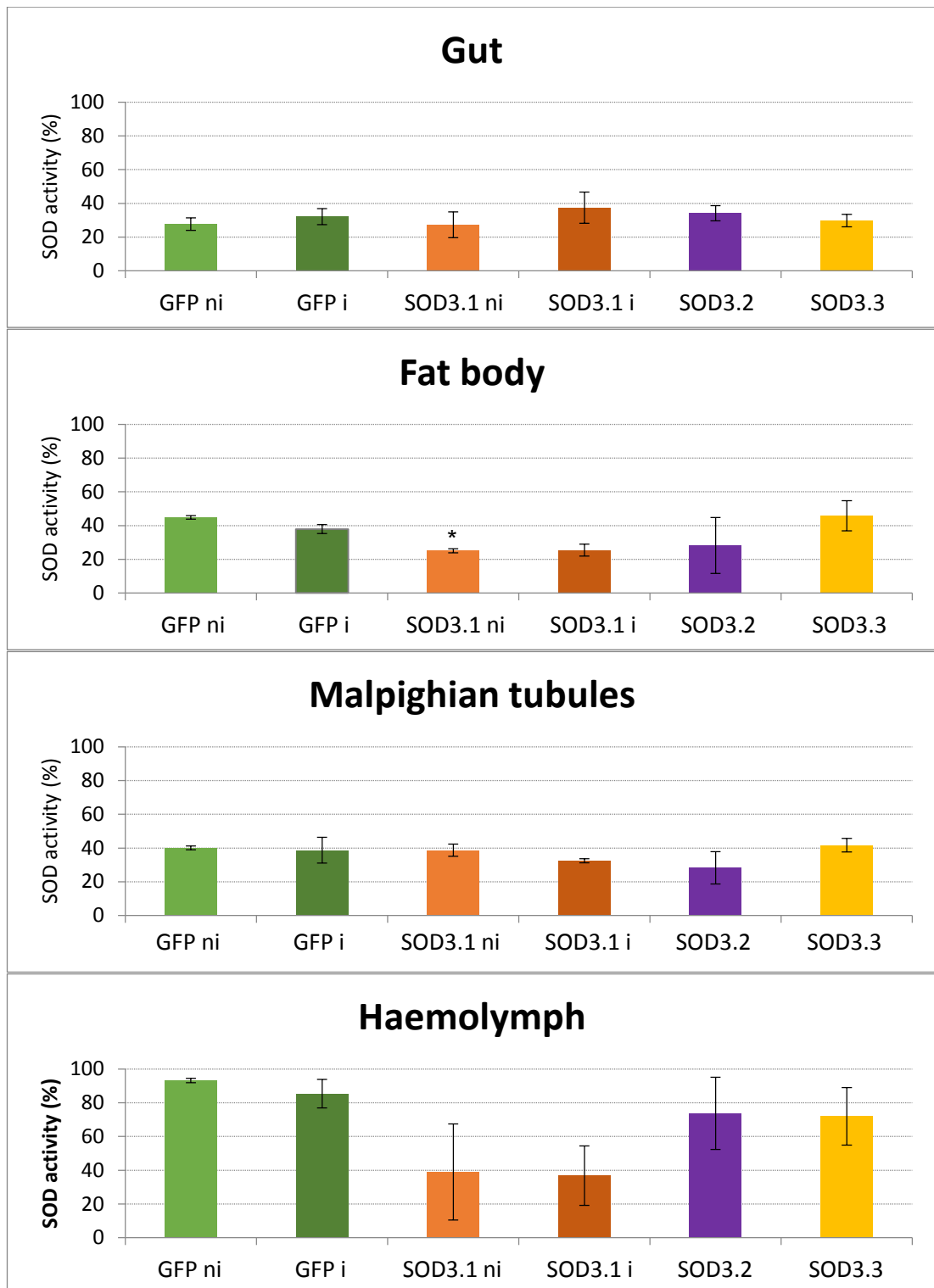


Figure 13: SOD activity in several tissues after RNA interference: 2 days after injection of dsRNA the infected-group got exposed to *M. anisopliae* conidia, 4 days after infection the dissection was done, tissues of 2 larvae were pooled for one sample and 2 replicates were constructed per group, protein concentration was determined by UV-measurement and 10 µg total protein was used.

* = significantly different from GFP ni ($P = 0,004$).

As shown in Figure 13 the trend in gut-samples is, that the activity in SOD3.1 i larvae is higher than in uninfected larvae. In the other tissues this effect could not be shown. There is no big difference between the SOD activities of the three silenced SOD groups in gut, which leads to the assumption, that the extracellular SODs have no distinguished activities in the gut. Only in fat body a significant difference ($P = 0,004$) between GFPni - and SOD3.1 ni - SOD activity could be calculated. The other SOD3s, however, show no decreased SOD activity. In Malpighian tubules no significant difference could be recognised. Even though in qPCR a high *sod3.3* expression was discovered the SOD activity in SOD3.3 larvae seems to be equal or even higher than in the control. In haemolymph a very high SOD activity (~85 %) could be shown in larvae which were inoculated with GFP-dsRNA. Whereas activity is visibly lower in SOD3.1 larvae, SOD3.2 and SOD3.3 don't show such a big change in activity.

With regard to the increased time of exposure to the entomopathogenic fungus, no significant differences in SOD activity could be shown, but a tendency to an elevated activity is visible (cf. Fig. 12 and 13).

5 Discussion and Conclusions

SOD is an enzyme in the cellular defence system in *P. cochleariae* larvae. According to previous research [27, 46, 47] extracellular SODs play an important role in the defence against pathogens in insects, which in this work was examined for *P. cochleariae*.

5.1 Complex *Cu,Zn-sod* expression and SOD activity in tissues of juvenile *P. cochleariae*

For the first time expression of Cu,Zn-SODs was analysed in *P. cochleariae*. Zhu *et al.* already did that in 2014 for the yellow mealworm beetle, *Tenebrio molitor* and found an expression of intra- and extracellular Cu,Zn-SODs in cuticle, fat body and haemocytes in all major developmental stages [53].

My performed qPCR analysis to reveal localisation of all Cu,Zn-SODs also indicate a complex expression pattern in the *P. cochleariae* larvae. While the cytosolic SOD1.1 is expressed in every examined tissue in comparable amounts, the extracellular SOD3s are not expressed similarly in all tissues.

In glands, for example *sod3.1* is expressed solely and especially high. High expression of extracellular Cu,Zn-*sod*'s can also be found in fat body (*sod3.1* and *3.2*) and Malpighian tubules (*sod3.3*). For the haemocytes no statement was possible in terms of the expression level of *sod*'s, but as haemocytes are the main cellular element in the immune system [28] it is still supposable that haemocytes express extracellular Cu,Zn-SODs, as it was recorded in *T. molitor* [53].

It is referred, that expression of *sod3* is restricted to a few cell types in diverse tissues in mammals [54]. My results point out that the expression of extracellular Cu,Zn-*sods* in *P. cochleariae* is ubiquitous in the whole larvae. Only *sod3.3* is likely to be expressed tissue specific in Malpighian tubules. The proteins rather demonstrate a constitutive expression in the larvae and hence, a complex SOD defence reaction with all parts of their body.

5.2 Extracellular SODs in the haemolymph

Studies exist where insect Cu,Zn-SOD activity was examined via recombinant proteins in transfected or transformed cells [27, 46] *in vitro*. In my work, besides *in vitro* examinations, haemolymph of the living *P. cochleariae* larva was tested for its SOD activity and the total protein was analysed by shotgun proteomics.

The activity determination of heterologously expressed *P. cochleariae* Cu,Zn-SODs revealed a strongly reduced activity of SOD3.2 compared to the other SODs what most likely is caused by amino acid exchanges in their active centre. The histidine which is important for metal cation binding and catalysis got replaced by proline and asparagine (H85→P85, H102→N102) (alignment in the supplement). Activity assay though, revealed a high SOD activity in haemolymph. Hence, although proteomics proved the existence of the extracellular SODs 3.1 and 3.2 in the fluid (without haemocytes) the shown SOD activity derives most likely only from SOD3.1.

Jung *et al.* revealed 2011, that the SOD3 protein from *D. melanogaster* is secreted as soon as it is synthesised [46], what could be applied for those in *P. cochleariae* as well. The SOD activity demonstrated an activity for the membrane fraction in fat body and Malpighian tubules. Extracellular SODs though couldn't be detected by proteomics, what could be due to very low SOD3 protein concentrations in the membrane which might have been below the detection limit of the proteome analysis. It is also possible that the shown activities in the membrane fractions result from cytosolic SODs which probably occur because of an incomplete membrane separation by the method used.

Thus, and because the extracellular SOD from *P. cochleariae* has no matrix-binding element, it seems most likely that the extracellular variants are not associated with the membrane but directly excreted into the extracellular fluid, to give an protection for the whole body of the larvae. The high expression levels in fat body suggest that synthesis of the SODs 3.1 and 3.2 which occur in the haemolymph appears in this tissue.

Even though expression level of *sod3.3* was very high in Malpighian tubules, the protein couldn't be found in any examined tissue by proteomics. It could be, that the *sod3.3* mRNA isn't translated into protein or rather very low concentrations of the protein exist in the tissues so that it wasn't detectable by proteomics. It's also possible that SOD3.3 is routed directly into the nucleus of the Malpighian tubules cells to protect genomic DNA and nuclear proteins against antioxidants [34]. Because of a sample preparation which couldn't break the nucleus the SOD3.3 might not have been found via proteome analyses. If this is the case, a different sample preparation should be considered to also gain all nuclear proteins in the sample.

Based on my data I suggest that extracellular SOD3.1 and SOD3.2 are secreted into the haemolymph and are most likely not attached to the extracellular matrix as known from the homologous human SOD3s [35]. SOD3.2 though doesn't show much activity what points out that this protein hasn't a crucial role in *P. cochleariae* and SOD3.1 is the most important extracellular SOD in the haemolymph.

5.3 SOD defence reaction to parasitization by *M. anisopliae*

There is evidence that SOD3 plays a crucial role in the immune response of arthropods like crustacean [55] and insects [27, 46, 53, 56]. During the phagocytosis of a pathogen by haemocytes ROS is built in insects [39-42] and extracellular SODs are supposed in those studies to exhibit a protective effect against this oxidative stress.

Also Gretscher [47] showed, that mortality in *P. cochleariae* was increased and pupal weight was reduced after exposure to the entomopathogenic fungus *M. anisopliae*, indicating a major role of the extracellular SOD3.1 in the defence against this fungus. To pursue these results I examined the expression level of the *sods* after infection and studied the impact of infection to the SOD activity of SOD-silenced larvae.

5.3.1 Effects on transcriptional level

My qPCR experiments on whole infected larvae revealed the strongest effects two days after infection with *M. anisopliae* as the expression levels were elevated in the infected larvae. Research on different tissues two days after infection with the entomopathogenic fungus, however, couldn't prove this effect because the standard deviation was very high (especially in the tissues of the infected larvae). That every larva reacts slightly different to the procedure of infection with the fungus is supposable. In 2 days the defence reaction of each larva might be differently advanced, so that this variance of the data appears.

As the expression level still is increased in the whole infected larvae, I assume the larvae exhibit a complex SOD defence reaction with all parts of their body.

5.3.2 Effects on SOD protein activity

According to Gretscher [47] *P. cochleariae* SOD3.1 is implicated in the defence against this fungus. To follow these results up I invented SOD silencing experiments, where SOD3.1, SOD3.2 or SOD3.3 got silenced. That should also clarify the role of the other extracellular SODs in *P. cochleariae* in the pathogen defence and show if SODs are capable to complement the function of a silenced SOD.

Due to RNAi SOD proteins can be silenced, which was important for further experiments. The procedure of silencing *sod3.1* indeed led to nearly no residual SOD protein in haemolymph (Fig. 11, 12). With the considerable decrease of SOD activity after RNAi with *sod3.1* dsRNA (Fig. 12) it can be suggested, that the dominant extracellular SOD in *P. cochleariae* is the SOD3.1. Also, it seems that no other SOD is inducible after silencing SOD3.1 and fungal challenging of the immune response. Since SOD3.2 doesn't show an activity (Fig. 8) the role of this SOD is not understood to date.

In my experiments SOD activity reflected the results of the qPCR in almost all groups. Expression levels of SOD3s were low in the gut what explains why there is no distinguishable difference in the SOD activities of the silenced groups. The assumption is that extracellular SODs don't have an active part in the gut.

In fat body a significantly lower SOD activity was shown in SOD3.1 ni-larvae than in GFP ni-larvae. Also in haemolymph this effect could be exhibited. Thus, SOD3.1 apparently has a great influence in both, fat body and haemolymph, what

can be verified by qPCR results. On the other hand the SOD3.3 group showed no decrease of SOD activity in fat body, which is evidence that SOD3.3 is not crucial in this tissue, which confirms the data from the qPCR as well.

In Malpighian tubules the SOD3.3 silenced group showed a slightly higher SOD activity than the GFP group, although the silenced gene was expressed in a high level in Malpighian tubules of untreated larvae and a huge decrease of the activity would have been expected. On one hand is possible that other SODs took over the function of SOD3.3 and compensate the loss. On the other hand, as described above, it is more likely that the protein isn't built in the first place or that it is routed directly into the nucleus of the cells. When the nucleus couldn't be broken by the sample preparation used SOD3.3 might not show its activity in the assay and thus, the little difference between the activities only derives from natural physiological differences between the larvae.

Since SOD activity assay only shows activity of SODs in general, research on the amount of the different SOD3 proteins in the samples should be done by proteomics. Pilot proteomic analyses did not identify an additional SOD enzyme (for example SOD3.3) after silencing mentioned SODs and *M. anisopliae* treatment. This led to the hypothesis, that the extracellular SOD3.1 is the most important functional extracellular SOD in the haemolymph of *P. cochleariae* whose function couldn't be complemented by another SOD.

In the RNAi experiments SOD activity between uninfected and infected larvae was compared, but no significant difference could be ascertained, although expression level was shown to be inducible upon fungal infection. It is possible that differences in protein activity after infection only appear after a few more days, what remains to be examined.

6 Future directions

The results gained in this thesis already deliver a good insight into the role and the regulation of extracellular SODs in *P. cochleariae*, but still there are many issues to handle before a total understanding of the pathogen defence processes regarding extracellular SODs in the leaf beetle larvae.

For the start an infected group of larvae could be designed also for SOD3.2- and SOD3.3-silenced larvae, to examine if there are any effects of infection with *M. anisopliae* on those SODs. Especially for haemolymph samples, more than the fluid of 2 larvae should be pooled in one sample to have enough protein for all required tests.

Although expression of *sod*'s was induced in whole larvae following parasitization by *M. anisopliae*, no significant increase of protein activity from non-infected to infected larvae could be detected neither after 2 days of exposure nor after 4 days. To see if the induced expression still leads to an increased protein amount it would be expedient to research the SOD activity after a few more days of exposure. For this purpose younger larvae would needed to be infected with the fungus, since the pathogen needs room temperature to develop, but also development of the larvae proceeds more quickly and pupation seemed to start earlier when they were exposed to the fungus.

The results also revealed that SOD3.2 shows no activity *in vitro*. Hence, the function of this extracellular SOD is not clear and should be examined in future researches. It would be interesting to find out if the *sod3.3* mRNA gets translated into a protein in the first place and if so, where the protein occurs in the larva.

A great finding is the fact that extracellular SODs get secreted into the haemolymph and were proven to actually exist in the fluid by proteomics. For better quantification of the proteins the program should be checked with regard to its ability to distinguish between the sequences. Then proteomics could reveal the amount of actual produced protein and disclose the question from which tissue secretion occurs.

To gain a holistic pattern of the *sod* expression a differential gene expression analyses between all components of untreated and infected larvae could be conducted after RNAi of each SOD3s. With the results it will get more clearly in which physiological processes SODs are involved in the *P. cochleariae* larvae.

7 Summary

Since insects encounter lots of pathogens in nature, they developed strategies to defend themselves from intruders [57]. Often during the defence reaction of the host reactive oxygen species are built which, in turn, is highly cytotoxic [39-42, 44]. Here superoxide dismutases (SODs) serve as a protective enzymes by turning superoxide anions into harmless compounds [14]. These enzymes are metalloproteins and can be found in nearly all parts of a cell [19, 22]. A particular role has the extracellular Cu,Zn-SOD (SOD3) which has a signal peptide and can be secreted into the nucleus or extracellular fluids [34, 35]. In insects the function of these proteins is not completely understood and there is only a few examples published to date. Still there is evidence that SOD3 has a crucial role in the immune response in insects, for example in the leaf beetle *Phaedon cochleariae* [47].

Following up the work of Gretscher, the regulation of SOD3s was examined in the larvae of *P. cochleariae* following parasitization with the entomopathogenic fungus *Metarhizium anisopliae in vivo*.

My studies revealed that *sod3s* are ubiquitous expressed in the whole larvae and as Tang *et al.* 2012 and Zhu *et al.* 2014 [53, 56] already indicated in other insects, the expression in the *P. cochleariae* larvae is inducible upon fungal infection. Since that induction couldn't be confirmed in the selected tissues a complex SOD defence reaction with all parts of the larval body can be suggested.

Furthermore, in this work extracellular SODs could be shown to exist in the haemolymph of an insect through proteomics for the first time, to my knowledge. This enzyme is most likely not attached to the extracellular matrix, but secreted as soon as it is synthesised, as Jung *et al.* also revealed 2011 for *Drosophila melanogaster* [46]. As production site of the secreted SODs fat body is suggested. From my results I can also assume, that SOD3.1 is the most important functional SOD3 in the haemolymph of *P. cochleariae* and that its function is not complemented by another SOD. The function of SOD3.2 though is not understood to date since it doesn't show an activity *in vitro*. Especially protein quantification by proteomics and differential expression are now owing and would contribute to a better understanding of extracellular SODs in the physiology of insects and their regulation after treatment with pathogens.

8 Zusammenfassung

Da Insekten in der Natur vielen Pathogenen ausgesetzt sind, entwickelten sie im Laufe der Evolution Strategien um sich gegen diese zur Wehr zu setzen [57]. Oft werden während solchen Abwehrreaktionen jedoch reaktive Sauerstoffspezies freigesetzt, welche wiederum stark zytotoxisch wirken [39-42, 44]. Superoxiddismutasen (SODs) dienen dann als schützende Enzyme indem sie die Superoxidanionen in ungefährliche Stoffe umwandeln [14]. Diese Metalloproteine sind in fast allen Bestandteilen einer Zelle zu finden [19, 22]. Neben den zytosolischen SODs gibt es auch extrazelluläre Cu,Zn-SODs (SOD3), die durch ein Signalpeptid in den Zellkern oder ins extrazelluläre Medium sekretiert werden können [34, 35]. In Insekten ist die Funktion dieser Proteine noch nicht komplett verstanden. Dennoch gibt es schon Hinweise darauf, dass die SOD3 eine wichtige Rolle in der Immunantwort von Insekten spielt, wie zum Beispiel in dem Blattkäfer *Phaedon cochleariae* [47].

Auf die Arbeit von Gretscher aufbauend, wurde hier die Regulation der SOD3s in den Larven von *P. cochleariae* nach Infektion mit dem entomopathogenen Pilz *Metarhizium anisopliae* *in vivo* untersucht.

Meine Untersuchungen enthüllten, dass *sod3*-Gene universell in der gesamten Larve exprimiert werden und, wie Tang *et al.* 2012 und Zhu *et al.* 2014 [53, 56] schon für andere Insekten berichteten, die Expression auch in *P. cochleariae*-Larven durch eine Pilzinfektion angekurbelt wird. Da dieser Effekt jedoch nicht in ausgewählten Geweben der Larve bestätigt werden konnte, wird eine komplexe SOD-Abwehrreaktion über den gesamten larvalen Körper vermutet.

Des Weiteren konnte, meines Wissens nach, zum ersten Mal mit dieser Arbeit über Proteomik gezeigt werden, dass extrazelluläre SODs in der Hämolymphe eines Insekts vorhanden sind. Die SOD3.1 spielt dabei die wichtigste Rolle. Das Enzym ist sehr wahrscheinlich nicht an der extrazellulären Matrix gebunden, sondern wird, sobald es synthetisiert wurde, von der Zelle sekretiert, wie auch Jung *et al.* 2011 für *Drosophila melanogaster* zeigen konnten [46]. Als Produktionsort der sekretierten SODs ist der Fettkörper wahrscheinlich.

Durch meine Ergebnisse kann man annehmen, dass die SOD3.1 die wichtigste funktionelle SOD3 in der Hämolymphe von *P. cochleariae* ist und dass deren Funktion nicht durch eine andere SOD vervollständigt werden kann. Die Funktion der SOD3.2 bleibt hier unverstanden, da diese *in vitro* keine Aktivität aufzeigt.

Besonders die Quantifizierung der Proteinnengen durch Proteomik und eine differentielle Expression stehen noch aus und würden zu einem besseren Verständnis der extrazellulären SODs in Insekten und deren Regulation nach Behandlung mit einem Pathogen beisteuern.

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B Supplement

List of used materials

Devices

Agilent 2100 Bioanalyzer	Agilent technologies, Waldbronn
CFX96 Real-Time System	Bio-Rad Laboratories GmbH, München
Electrophoresis Power Supply- EPS 600	Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA
Eppendorf centrifuge 5417R	Sigma Laborzentrifugen GmbH, Osterode am Harz
Eppendorf Thermomixer comfort	Eppendorf AG, Hamburg
LIM-3P-A Puller	List Medical Electronic, Darmstadt
Microscope Axioskop	Carl Zeiss Microscopy GmbH, Jena
Mini Protean Tetra Cell	Bio-Rad Laboratories GmbH, München
Multi-Axle-Rotating-Mixer RM5	Ingenieurbüro CAT, M. Zipper GmbH, Staufen
Nano2010 Injector	World Precision Instruments Inc., Sarasota, FL, USA
NanoVue Spectrophotometer	GE Healthcare Europe GmbH, Freiburg
pH-Meter InoLab	Wissenschaftlich Technische Werkstätten, Weilheim
Spectra Max 250 microplate reader	Molecular Devices GmbH, Biberach an der Riss
Trans-Blot Turbo Transfer System	Bio-Rad Laboratories GmbH, München
Zoom stereomicroscope Stemi DV4	Carl Zeiss AG, Jena

Organisms

<i>Brassica rapa chinensis</i>	purchased at a commercial supermarket
<i>Metarhizium anisopliae</i>	DSM-1490, DSMZ, Braunschweig
<i>Phaedon cochleariae</i> – larvae	lab-reared in the Max-Planck-Institute for chemical ecology, Jena

Consumables

96-well flat bottom with lid	Sarstedt, Inc., Newton, NC, USA
CL-XPosure Film 5x7 inches	Thermo Fisher Scientific GmbH, Bonn
Dissecting set	Fine Science Tools GmbH, Heidelberg
Glas capillary (Ø inside 0.5 mm)	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt
Hard-shell PCR plates, 96-well WHT/CLR	Bio-Rad Laboratories GmbH, München
Microscope slide & cover glas	Carl Roth GmbH & Co. KG, Karlsruhe
Mini-Protean TGX Precast Gels	Bio-Rad Laboratories GmbH, München
Multiple Well Plate,	
Neubauer improved haemocytometer	Brand GmbH & Co. KG, Wertheim
Safe-Lock Tubes 1.5 ml	Eppendorf AG, Hamburg
Trans-Blot Turbo Transfer Pack	Bio-Rad Laboratories GmbH, München

Commercial kits

19160 SOD Determination Kit	Sigma-Aldrich Chemie GmbH, Steinheim
MEGAscript RNAi Kit	Ambion Applied Biosystems, Hamburg
Native Membrane Protein Extraction Kit	Calbiochem Proteo Extract, Darmstadt
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific GmbH, Bonn
RNA Nano Chip	Agilent technologies, Waldbronn
RNAqueous-Micro Kit	Ambion Applied Biosystems, Hamburg
SuperScript III Reverse Transcriptase	Invitrogen, Darmstadt

Chemicals

2,4,6-Trichloroanisole (TCA)	Sigma-Aldrich Chemie GmbH, Steinheim
β-Mercaptoethanol	Sigma-Aldrich Chemie GmbH, Steinheim
Bromphenol Blue	Sigma-Aldrich Chemie GmbH, Steinheim
Calcium chloride (CaCl ₂)	Merck KGaA, Darmstadt
Dithiothreitol (DTT)	Sigma-Aldrich Chemie GmbH, Steinheim
Ethanol	Merck KGaA, Darmstadt
Express Art NucleoGuard	AmpTec GmbH, Hamburg
Glycerol	Carl Roth GmbH & Co. KG, Karlsruhe
Glycine	Serva Elektrophoresis GmbH, Heidelberg
Hydrogen chloride (HCl)	Carl Roth GmbH & Co. KG, Karlsruhe
Magnesium chloride hexahydrate (MgCl ₂ x 6H ₂ O)	Carl Roth GmbH & Co. KG, Karlsruhe
Monopotassium phosphate (KH ₂ PO ₄)	Carl Roth GmbH & Co. KG, Karlsruhe
Monosodium phosphate (NaH ₂ PO ₄)	Sigma-Aldrich Chemie GmbH, Steinheim
Non Fat Dry Milk (NFDF)	Biomol GmbH, Hamburg
Nuclease-Free Water	Qiagen GmbH, Hilden
Pierce ECL Western Blotting Substrate	Thermo Fisher Scientific GmbH, Bonn
Pipes	Sigma-Aldrich Chemie GmbH, Steinheim
Potassium chloride (KCl)	Carl Roth GmbH & Co. KG, Karlsruhe
Potassium hydroxide (KOH)	Carl Roth GmbH & Co. KG, Karlsruhe
Potato-Dextrose Broth (2% glucose)	Sigma-Aldrich Chemie GmbH, Steinheim
Protease-Inhibitor-Mix HP (100x)	Serva Elektrophoresis GmbH, Heidelberg
Roti-Blue quick	Carl Roth GmbH & Co. KG, Karlsruhe

Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium deoxycholate	Thermo Fisher Scientific GmbH, Bonn
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH & Co. KG, Karlsruhe
Sucrose	Carl Roth GmbH & Co. KG, Karlsruhe
SYBR Premix ExTaq II	Takara Bio Inc., Otsu, Shiga, Japan
Tris(hydroxymethyl)aminomethane (Tris)	Serva Elektrophoresis GmbH, Heidelberg
Tween-20	Carl Roth GmbH & Co. KG, Karlsruhe

Antibodies

1. Rabbit anti-human SOD1 polyclonal IgG Abcam plc, Cambridge, UK
2. Goat anti-rabbit IgG with HRP Sigma-Aldrich Chemie GmbH,
Steinheim

Standards

Bovine erythrocyte SOD standard	Sigma-Aldrich Chemie GmbH, Steinheim
Bovine serum albumin standards (BSA)	Serva Elektrophoresis GmbH, Heidelberg
Precision Plus Protein Dual Color Standards	Bio-Rad Laboratories GmbH, München

Software

Basic local alignment search tool	http://blast.ncbi.nlm.nih.gov/
CFX Manager 3.1	Bio-Rad Laboratories GmbH, München
Excel 2013	Microsoft Corporation, Redmond, WA, USA
ProteinLynx Global SERVER v.2.5	Waters Corporation, Manchester, UK

Buffer recipes

2x RNAi injection buffer (pH 7.4):	1.4 mM	NaCl
	0.07 mM	NaH ₂ PO ₄
	0.03 mM	KH ₂ PO ₄
	4 mM	KCl
In 100 ml Ringer's solution (pH 7.0):	0,184 g	NaCl
	0,298 g	KCl
	0,017 g	CaCl ₂
	0,030 g	MgCl ₂ X 6H ₂ O
	3,350 g	Sucrose
	0,605 g	Pipes
	➔	Sterile filtration
10x SDS-PAGE running buffer (pH 8.6):	3.03%	Tris (w/v)
	14.4%	Glycine (w/v)
	1%	SDS (w/v)
5x SDS loading buffer:	50%	Glycerol (v/v)
	30 mM	Tris (pH 6.8)
	25%	β-mercaptoethanol (v/v)
	10%	SDS (m/v)
	0.002%	Bromphenol blue (w/v)
	0.1 M	DTT
For 1 l 10x TBS (pH 7.6):	24.08 g	Tris
	80 g	NaCl
	13 ml 6 M	HCl
TBS-T:	add 1 ml of Tween-20 to 1 l of 1x TBS	

Primer sequences

qPCR:

sod1.1 fwd. ATCATGATCTGGGCCACCATGC
rev. ACAACACCAACGGGTGCATTTCC

sod3.1 fwd. AAACATCGTAGCCGACGCAACAG
rev. TGCCGATGATGTTGTGGTTGC

sod3.2 fwd. TCAATGTGAGCGACGCCGAAAC
rev. TGGACCTTTCAATCCCCACAACG

sod3.3 fwd. GCGCGATGCCGTATTCGTCGG
rev. ATTGGATCAGGATGTCTGGCA

rps3 fwd. AAGGCTGTGAAGTTGTGGTATCTGG
rev. ATGGCGGGTGGCAGTGTCTAC

rpl6 fwd. CAAGACAGGATCAGACCTAGAC
rev. CACTCTCTTTCCTTTGTGGGAAC

dsRNA:

sod1.1 fwd. TAATACGACTCACTATAGGGAGACTCCAATGTGGCGAATA
rev. TAATACGACTCACTATAGGGAGATGCCTACAAAAGCAGTT
total length 243

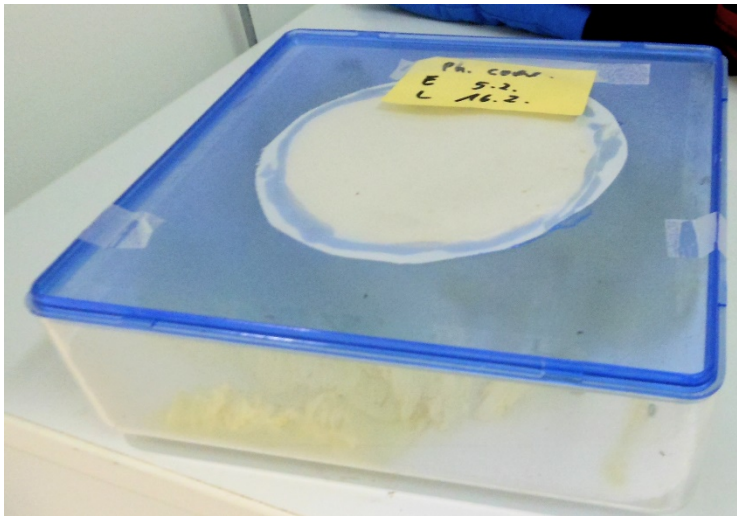
sod3.1 fwd. TAATACGACTCACTATAGGGAGAACATCGTAGCCGACGCA
rev. TAATACGACTCACTATAGGGAGACCGATGACCCCGCAA
total length 193

sod3.2 fwd. TAATACGACTCACTATAGGGAGAGACTCCGCAAGCTATAC
rev. TAATACGACTCACTATAGGGAGACAAGTTTGTAGTAGCCG
total length 508

sod3.3 fwd. TAATACGACTCACTATAGGGAGATCGCGCTCCTGCGTG
rev. TAATACGACTCACTATAGGGAGACATCGAGGCCGACGAAT
total length 172

gfp fwd. TAATACGACTCACTATAGGGAGACCATGGCCAACACTTGT
rev. TAATACGACTCACTATAGGGAGATAATCCCAGCAGCAGTT
total length 523

Pictures of larvae and their breeding



Boxes where larvae were kept for rearing



Third-instar *P. cochleariae* larvae with extended defence glands and in peace, pictures were taken from Dr. S. Frick and Dr. A. Burse



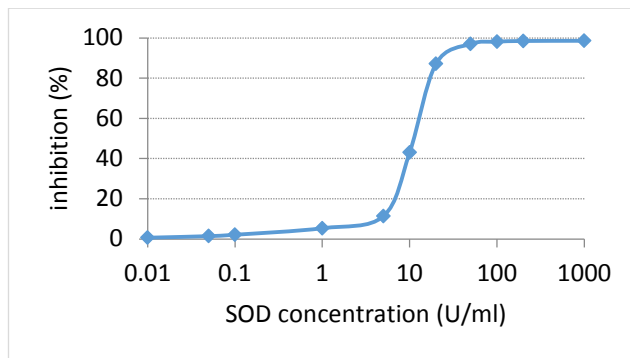
P. cochleariae adults, pictures were taken from T. Faasen (www.koleopterologie.de/gallery/) and Dr. A. Burse

Pupation rate of *P. cochleariae* after 5 days in comparison

All larvae kept at 11 to 15 °C stay larval while at room temperature 3 pupate, more than half of infected larvae pupate when kept at room temperature.

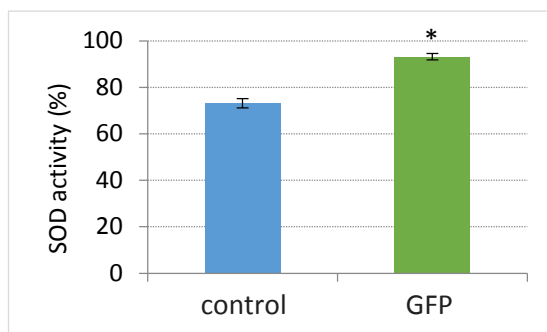
Temperature	Cold	Room temperature	Room temperature + infection
Total number of examined larvae	19	19	19
Still larval after 5 days	19	16	8
Pupated after 5 days	0	3	11

SOD inhibition curve



SOD standard inhibition curve: bovine erythrocyte SOD was used as a standard, 20 min incubation time.

Comparison between untreated and dsRNA injected group



SOD activity in haemolymph of untreated larvae and larvae which suffered dsRNA injection: 6 days after injection the dissection was done, tissues of 2 larvae were pooled for one sample and 2-3 replicates were constructed per group, protein concentration was determined by UV-measurement and 10 µg total protein was used. * = significantly different from control ($P = 0.001$)

Proteome analysis

Peptides identified from PCO_Comb-A_C18136_antisense (SOD1) in Malpighian tubules and fat body

Peptide sequence ^{a, b}	theor. [M+H] ⁺ _c	exp. [M+H] ⁺ _d	mass error (ppm)	Peptide score	retention time (min)
TVVVHADPDDLGGGHELSK	2074.0231	2074.02	1.5275	6.3604	28.96
FISLEGENSIIGR	1434.7636	1434.7587	3.4281	6.1879	40.59
LAC*GVIGLAK	1001.5863	1001.5813	4.9952	6.2426	33.78
GTVFFNQANSTSPVEVTGEITGLQK	2624.3185	2624.3202	-0.6708	5.6012	42.78
HIGDLGNIQATDNGVAK	1722.893	1722.8769	9.2947	5.4164	43.38
IYC*IVELGTESFIIGNYK	2233.118	2233.121	-1.3226	5.6918	40.36

→ Exemplarily table of identified Peptides from SOD1 in Malpighian tubules. Results for fat body are similar

Peptides identified from PCO_Comb-A_C1927 (SOD 3.1) in haemolymph

Peptide sequence ^{a, b}	theor. [M+H] ⁺ _c	exp. [M+H] ⁺ _d	mass error (ppm)	Peptide score	retention time (min)
DSQIQISGEVHGLTPGK	1765.9177	1765.9156	4.3852	7.9729	33.07
HVGDLGNIVADATGVAHVHIEDDVIALQGNHNIIGR	3739.9205	3739.9205	-1.5512	7.761	43.86
AMVVHAGEDDLGR	1369.6487	1369.6507	-1.5572	7.6913	26.37
GGQSDSLTTGHAGGR	1400.6741	1400.6707	13.8785	6.3154	25.34
AM _{ox} VVHAGEDDLGR	1385.6495	1385.6515	2.6843	7.9804	23.16

Peptides identified from PCO-UC_C2881 or PCO_Comb-A_C11450 (SOD 3.2) in haemolymph

Peptide sequence ^{a, b}	theor. [M+H] ^{+c}	exp. [M+H] ^{+d}	mass error (ppm)	Peptide score	retention time (min)
NTGDLGNVVGDR	1216.6014	1216.5986	5.6779	7.9112	28.06
FGVAHIDVVDKR	1355.7344	1355.7367	-4.6809	7.7084	30.36
ITGEIHGIHEGK	1290.6637	1290.6663	-10.6695	7.5699	21.63
GIDGIIGR	800.4588	800.46	-3.0527	7.5104	31.62
GC*IGAGGPFNPHNVR	1552.7408	1552.7418	-1.3087	7.2528	29.52
GNNAESGKTGNSGGR	1405.6543	1405.6495	5.7156	6.1109	29.74

^a C*, C-carbamidomethylation; Mox, M-oxidation

^b unique peptides are underlined

^c theoretical monoisotopic mass of the peptide

^d calculated monoisotopic mass of the lock-mass corrected peptide ion cluster

Cu,Zn-SOD alignment

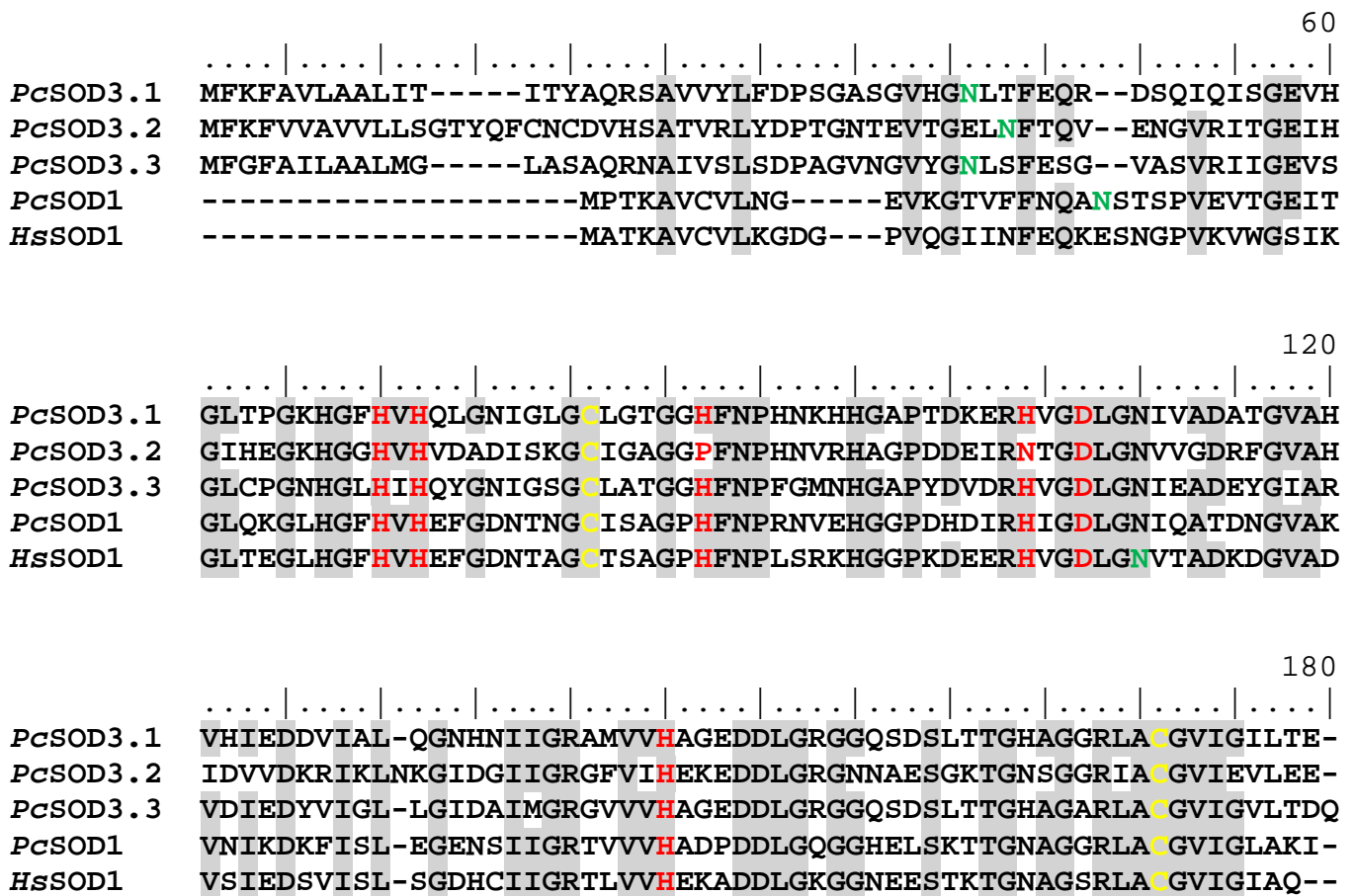


Figure X: Multiple sequence alignment of Cu,Zn-SODs from *P. cochleariae* and *H. sapiens*: red amino acids residues: metal cation binding and catalysis; yellow residues: cysteines involved in disulfide bridges; green residues: N-glycosylation sites

Western blot of overexpressed SODs

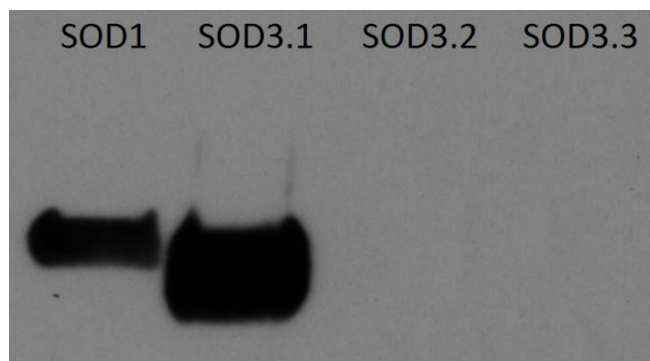


Figure X: Western blot with overexpressed Cu,Zn-SOD samples: 200µg/ml of protein was applied, respectively, antibody doesn't bind to SOD3.2 and SOD3.3

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D Declaration of authorship

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Ort, Datum

Unterschrift der Verfasserin

