MUSEUM AND INSTITUTE OF ZOOLOGY
POLISH ACADEMY OF SCIENCES

SUMMARY OF PROFESSIONAL ACCOMPLISHMENTS

Attachment no. 3

dr inż. Anna Katarzyna Wrońska

Warsaw, September 2024

Contents

1. Full name

Anna Katarzyna Wrońska

2. Diplomas, scientific or artistic degrees held - with the name of the entity awarding the degree, the year of obtaining them and the title of the doctoral dissertation

- 15.01.2014 – Ph.D. at the Faculty of Human Nutrition and Consumer Sciences (currently Faculty of Human Nutrition) of the Warsaw University of Life Sciences (SGGW). Defence completed with Distinction. Title of the dissertation: "The influence of maternal and child nutrition on the development of *Candida* infections in newborns", supervisor: dr hab. Wanda Kawecka, prof. Warsaw University of Life Sciences (SGGW).
- 19.07.2010 – Master's degree in Food Technology and Human Nutrition at the Warsaw University of Life Sciences (top grade with Distinction), thesis title: "Assessment of the nutritional habits and microbiological studies of milk of a selected group of women", supervisor: dr hab. Wanda Kawecka, prof. WULS-SGGW.
- 19.02.2009 – Batchelor's degree in Food Technology and Human Nutrition at the Warsaw University of Life Sciences (SGGW) in the field of Human Nutrition and Consumer Sciences (top grade with Distinction from the Faculty Council for very good academic results), thesis title: "Antibacterial properties of selected spice plants", supervisor: dr hab. Wanda Kawecka, prof. SGGW.

3. Previous employment in scientific or artistic institutes

- 2022 to present – Museum and Institute of Zoology PAN, Parasitology Laboratory (adjunct).
- 2015 to 2022 – Institute of Parasitology PAN, Department of Molecular Interactions in the Host-Parasite Relationship (adjunct).
- 2010 to 2014 – Doctoral Studies, Faculty of Human Nutrition and Consumer (currently Faculty of Human Nutrition) SGGW.

4. Discussion of achievements referred to in art. 219 section 1 point 2 of the Act of 20 July 2018 - The Law on Higher Education and Science (Journal of Laws of 2021, item 478, as amended)

4.1. Main Scientific Achievements

The main scientific achievement of Dr. Anna Katarzyna Wrońska comprises a series of thematically-related scientific articles, constituting a significant contribution to the development of the discipline of "Biological Sciences", submitted under the common title :

***Galleria mellonella* (Lepidoptera) as a model organism in research into the role of the immune system during fungal infection**

The publication series submitted for evaluation includes seven scientific articles published in journals with an Impact Factor (IF) found in the Journal Citation Reports (JCR) database. In each of the above-mentioned articles, Dr. Anna Katarzyna Wrońska is the leading author and in each of them her contribution to the preparation of the work was dominant (declarations of co-authors regarding the percentage contribution to the preparation of the articles are included in Attachment 6). In six of the seven articles, Dr. Wrońska is the first author and corresponding author.

The total number of points for the articles representing the scientific achievement is as follows: **MNiSW = 780 points**, **IF = 26.94** and **IF_{2023} = 25.3**. As dated 30.09.2024, the total number of cited articles, representing the scientific achievement is as follows: Google Scholar 114,Web of Science 85, Scopus 90.

IF_{2023}: current Impact Factor
IF: Impact Factor of the date of publication
P_{MNiSW}: point according to MNiSW guidelines dated 05.01.2024
$LC_{GoogleScholar}$: total citations Google Scholar
LC_{WoS}: total citations Web of Science
LC_{Scopus}: total citations Scopus
* corresponding author

- **H1. Wrońska, A. K.***, Boguś M. I., Kaczmarek A. and Kazek M. (2018). Harman and norharman, metabolites of entomopathogenic fungus *Conidiobolus coronatus* (Entomopthorales), disorganize development of *Galleria mellonella* (Lepidoptera) and affect serotonin-regulating enzymes. *PLoS One* 13(10): e0204828.

IF_{2023} = 2.9; IF = 2.76; P_{MNiSW} = 100; $LC_{GoogleScholar}$ = 19; LC_{WoS} = 14 ; LC_{Scopus} = 16

The candidate's contribution to the publication consisted of proposing the research topic, formulating the research concept, scope, objective and hypothesis, preparing a review of the literature on the effect of alkaloids on the regulation of serotonin levels, formulating the Conclusions and Discussion. In addition, the habilitation candidate independently performed all laboratory experiments, developed a method for determining harman and norharman in the filtrates of the fungus *Conidiobolus coronatus* by GC-MS and analyzed and described all results. The candidate was also the corresponding author in the publishing process.

The co-authors assisted in cultivating insects and fungi, provided substantive advice on the developed methods, formulated objectives and hypotheses, and consulted on the first version of the manuscript. The contribution of the candidate to the publication was 85%.

- **H2. Wrońska, A. K.*** and Boguś M. I. (2019). Harman and norharman, metabolites of the entomopathogenic fungus *Conidiobolus coronatus* (Entomophthorales), affect the serotonin levels and phagocytic activity of haemocytes, insect immunocompetent cells, in *Galleria mellonella* (Lepidoptera). *Cell and Bioscience* 9:29.

IF_{2023} = 6.1; IF = 5.03; P_{MNiSW} = 100; $LC_{GoogleScholar}$ = 33; LC_{WoS} = 23 ; LC_{Scopus} = 24

The candidate's contribution to the publication consisted of formulating the topic and concept of the study, and its aim and hypothesis, preparing a review of the literature on the effect of serotonin on the immune system, developing the results of the study and their discussion. The candidate also developed all the research methods used in the study, *viz.* determining serotonin in larval haemocytes, and examining the effect of fungal metabolites on serotonin level and on the phagocytic activity of the studied cells. She conducted the laboratory experiments independently. The candidate was also the corresponding author in the publishing process.

The contribution of the second author included substantive consultations on the study objectives, methods developed, and the first version of the manuscript.

The contribution of the candidate to the publication was 90%.

- **H3. Wrońska, A. K.*** and Boguś M. I. (2020). Heat shock proteins (HSP 90, 70, 60, and 27) in *Galleria mellonella* (Lepidoptera) haemolymph are affected by infection with *Conidiobolus coronatus* (Entomophthorales). *PLoS One* 15(2): e0228556.

IF_{2023} = 2.9; IF = 3.24; P_{MNiSW} = 100; $LC_{GoogleScholar}$ = 37; LC_{WoS} = 27 ; LC_{Scopus} = 30

The candidate's contribution to the publication consisted of proposing the research topic, and formulating its purpose and hypothesis, and the scope of the research. She was responsible for

developing all research methods used in the work concerning the determination of heat shock proteins in insect haemocytes and haemolymph. The candidate performed all laboratory experiments, interpreted the results, and prepared a literature review of the influence of the studied proteins on the fungal infection process. She also prepared the text of the manuscript, including the Discussion and Conclusion. The candidate was also the corresponding author in the publishing process.

The contribution of the second author consisted of substantive consultations on the research objectives, developed methods, and the first version of the manuscript.

The contribution of the candidate to the publication was 90%.

- **H4. Wrońska, A. K.***, Kaczmarek A., Kazek M. and Boguś M. I. (2021). Infection of *Galleria mellonella* (Lepidoptera) larvae with the entomopathogenic fungus *Conidiobolus coronatus* (Entomophthorales) induces apoptosis of haemocytes and affects the concentration of eicosanoids in the haemolymph. *Frontiers in Physiology* 12: 774086.

IF_{2023} = 3.2; IF = 4.75; P_{MNiSW} = 100; $LC_{GoogleScholar}$ = 17; LC_{WoS} = 15 ; LC_{Scopus} = 15

The candidate's contribution to the preparation of the publication consisted of formulating the topic and concept of the study, and its aim and hypothesis, and preparing a literature review of the effect of fungal infection on haemocyte apoptosis and eicosanoid levels. The habilitation candidate developed methods for determining eicosanoids in the insect – fungus research model (*Galleria mellonella – Conidiobolus coronatus*) and conducted all laboratory experiments in this area. In addition, she participated in the development of other research methods and the development of all results. She prepared the content of the manuscript, including the Discussion and the Results. The candidate was also the corresponding author in the publishing process.

The participation of the other authors included performing laboratory experiments on the effect of fungal infection on haemocyte apoptosis, developing these results, providing assistance in conducting insect and fungus cultures, and providing substantive consultations on the first version of the manuscript.

The contribution of the candidate to the publication was 70%.

- **H5. Wrońska, A. K.***, Kaczmarek A., Sobich J., Grzelak S. and Boguś M. I. (2022). Intracellular cytokine detection based on flow cytometry in haemocytes from *Galleria mellonella* larvae: A new protocol. *PLoS One* 17(9): e0274120.

IF_{2023} = 2.9; IF = 3.7; P_{MNiSW} = 100; $LC_{GoogleScholar}$ = 8; LC_{WoS} = 6 ; LC_{Scopus} = 5

The contribution of the candidate to the publication consisted of proposing the research topic, formulating its aim and hypothesis, determining the scope of the research and preparing a literature review. The candidate developed an innovative protocol for determining cytokine-like proteins in insect haemocytes using flow cytometry. She independently conducted all laboratory experiments, leading to the preparation of a repeatable and precise protocol. In addition, she developed research methods in the field of proteomics and fluorescence microscopy. She participated in performing all laboratory experiments, developed the results and prepared the text of the manuscript. The candidate was also the corresponding author in the publishing process.

The participation of the other authors included assistance in performing laboratory experiments in proteomics and breeding the insects and fungi, providing substantive consultations on the developed protocol and other research methods, and introducing comments to the first version of the manuscript.

The contribution of the candidate to the publication was 70%.

- **H6.** Sobich, J.*, **Wrońska A.K.**, Kaczmarek A., Boguś M. I. (2023). Changes in histamine, HSF1, Cysteinyl leukotriene, TLR1 and TLR2 in *Galleria mellonella* haemolymph after *Conidiobolus coronatus infection. The European Zoological Journal* 90(2): 762-774.

IF_{2023} = 1.6; IF = 1.6; P_{MNiSW} = 140; $LC_{GoogleScholar}$ = 0; LC_{WoS} = 0 ; LC_{Scopus} = 0

The contribution of the candidate to the publication consisted of proposing the research topic, formulating its purpose and hypothesis and the scope of the research, and developing the research methods. She also performed laboratory experiments on the determination of histamine, HSF1 and cysteine leukotrienes in insect haemolymph and developed the obtained results. She also participated in interpreting the results from the determination of Toll-like receptors. The candidate also provided substantive supervision over the preparation of the manuscript text, including providing the main outline of the discussion of the results, and over the conduct of the article through the publishing process.

The first author's contribution to the publication consisted of the following: determining the levels of Toll-like receptors, participating in interpreting the results, preparing the manuscript text under the supervision of the candidate and guiding it through the publishing process.

The participation of the other authors consisted of assistance in conducting insect and fungus breeding and substantive consultations on the first version of the manuscript.

The contribution of the candidate to the publication was 60%.

- **H7. Wrońska, A. K.***, Kaczmarek A., Sobich J., Boguś M. I. (2024). The effect of infection with the entomopathogenic fungus *Conidiobolus coronatus* (Entomopthorales) on eighteen cytokine-like proteins in *Galleria mellonella* (Lepidoptera) larvae. *Frontiers in Immunology* 15: 1385863.

IF_{2023} = 5.7; IF = 5.7; P_{MNiSW} = 140; $LC_{GoogleScholar}$ = 0; LC_{WoS} = 0 ; LC_{Scopus} = 0

The contribution of the candidate to the preparation of the publication consisted of formulating the topic and concept of the study, and its aim and hypothesis, preparing a literature review of cytokine-like proteins in insects and other invertebrates, and developing all research methods used in the work. The candidate played a dominant role in conducting all laboratory experiments and interpreting the results. She prepared the content of the manuscript, including the section related to the Discussion and the Results. The candidate was also the corresponding author in the publishing process.

The participation of the other authors consisted of co-performing laboratory experiments and analyzing the results, helping to conduct insect and fungus cultures, and providing substantive consultations on the first version of the manuscript..

The contribution of the candidate to the publication was 75%.

4.1.1. Introduction

Insects as model organisms in the pathogenesis of infection

To be used in the study of the pathogenesis of viral, bacterial or fungal infections, a model organism must accurately reproduce the processes associated with the course of the disease in the human organism, from the stage of colonization to defence reactions. The most commonly-used models in biomedical research are vertebrates such as mice, rats, guinea pigs and rabbits, due to the anatomical and immunological similarity. However, the use of large numbers of these animals in experiments gives rise to various logistical, economic and ethical difficulties. Fortunately, invertebrates can be an alternative to mammalian models in many areas of scientific research. [1, 2].

Comparative analyses have found insects to possess numerous homologues of human genes encoding proteins involved in pathogen recognition or signal transduction. Due to these similarities, organisms such as *Drosophila melanogaster, Blattella germanica, Galleria mellonella, Culex quinquefasciatus* and *Bombyx mori* are used in studies on microbial virulence,

host immunity and in assessing the efficacy of biologically-active substances such as antibiotics and fungicides *in vivo* [3].

The model organism used in the research presented in this scientific achievement is *Galleria mellonella* (greater wax moth, wax moth).

The insects are easy to breed in laboratory conditions and can be fed both natural food (wax combs) and artificial media [4]. The female greater wax moth is capable of laying about 1500 eggs. The development cycle of *G. mellonella* is strictly dependent on the ambient temperature and lasts from four to five weeks to six months, in optimal conditions (30°C, constant darkness, 70% humidity), although this time is typically six weeks [5].

One of the most important features of these insects which makes them good models for pathogenesis studies is the ability to be cultured in a wide temperature range. Unlike many other alternative models, e.g. *B. mori*, *G. mellonella* larvae can develop at temperatures ranging from 18 to 37°C. This feature is particularly important during studies conducted using human pathogens, which mostly require incubation at 37°C. In contrast, some entomopathogenic fungi require lower temperatures for optimal growth, i.e. 20 to 25°C [6].

Another advantage of using this organism as a research model is that the larvae are significantly larger than other species, such as *D. melanogaster*. Wax moth larvae reach a length of 12 to 25 mm, allowing easier manipulation, and more precise application of pathogens or active substances in specific doses. Their larger size allows for easier sampling, such as haemolymph, fat body and other tissues. An additional advantage is that results can be obtained in a relatively short time, even within 24 to 48 hours [7]. The effects of the conducted experiments are often easy to observe and manifest as morphological changes. In order to systematize the results obtained from research, Loh et al. propose the *G. mellonella* Health Index Scoring System, which assesses the health status by assigning points according to found main observations: larval motility, cocoon formation, melanization and mortality [8].

It is also worth emphasizing that such insect models reinforces the importance of applying the 3R principle (replacement, reduction, refinement) in animal experiments in biological and biomedical research. This principle advocates reducing the number of vertebrate animals used for scientific research in favour of using other techniques and improving breeding conditions and experimental procedures in order to alleviate pain, suffering and stress among experimental animals. Moreover, as *G. mellonella* is an invertebrate, its use does not require the consent of the bioethics committee [5].

Most studies employing the greater wax moth are virulence studies of bacterial infection. Among these, the most commonly-used Gram-positive bacteria include *Streptococcus pyogenes, Streptococcus pneumoniae, Enterococus faecalis, Enterococcus faecium, Staphylococcus aureus* and *Listeria monocytogenes*, and the most common Gram negative are *Pseudomonas aeroginosa, Escherichia coli, Klebsiella pneumonia, Legionella pneumophila, Francisella tularensis* and *Acinetobacter baumanii* [9]. Studies have shown that infections with some bacterial strains can be as lethal in *G. mellonella* as in mammals. [10, 11]. In addition, *S. aureus* infection causes similar histological changes in larvae and humans: infection results in the formation of nodules in insects, with similar structures observed in mammals, i.e. in abscesses [12].

Due to the increasing resistance of bacterial strains to available antibiotic therapies, it is important to search for new therapeutic substances or create combinations of existing ones. *G. mellonella* is also used as a model organism in such studies. Li et al. showed that the combination of linezolid and fosfomycin has a synergistic effect against *S. aureus* [13], and Thieme et al. found the combination of ampicillin with ceftriaxone to be effective in the treatment of *E. faecalis* infections [14]. Using wax moth larvae, epigallocatechin gallate has been shown to have strong antimicrobial potential against *S. aureus* and may be a promising new drug to help combat infections [15]. In addition, research on potential bacteriophage therapies is being conducted using *G. mellonella* larvae. [16, 17].

G. mellonella larvae were first described as a model for studying fungal infection in experiments aimed at distinguishing pathogenic from non-pathogenic strains of *Candida albicans* [18]. Since then, in line with the increasing frequency of fungal infections among humans, this insect has become increasingly popular as a model for studying the virulence of various species of fungi, whose optimum growth temperature is in the range of 20–37°C. Such infections are increasingly caused by multidrug-resistant strains, which are particularly dangerous for patients with reduced immunity. In addition, substances produced by insects (mainly proteins and lipids) have a broad spectrum of antifungal activity and may become an alternative to existing medicinal preparations. The wax moth has also been used to study the virulence of fungi pathogenic to humans from the genera *Aspergillus, Candida, Fusarium, Cryptococcus, Penicillium, Rhizopus* and *Trichosporon*, and entomopathogenic fungi such as *Beauveria, Metarhizium* or *Conidiobolus* [19, 20].

The research system used in the studies included in the discussed scientific achievement, was based on *G. mellonella* and the entomopathogenic fungus *Conidiobolus coronatus*. *C.*

coronatus (*Entomophthorales*) was first described by Constantin in 1897 in France and isolated by Emmons and Bridges in 1961 in Australia. It occurs widely throughout the world in soil and decaying organic matter, and is particularly prevalent in tropical and subtropical areas [21]. The characteristic features of this microorganism are its high entomopathogenic potential and its ability to infect humans. This fungus attacks insects selectively: some species, such as flies from the order *Diptera*, are resistant, while others, such as *G. mellonella*, are highly sensitive. The differences in sensitivity are most likely due to differences in the composition of the lipids in the insect cuticle, which contains antifungal substances and prevents the fungus entering the body cavity [22].

C. coronatus also causes chronic nasofacial zygomycosis in humans, i.e. conidiobolomycosis. The first case of human infection was diagnosed in the Grand Cayman Islands and described in 1965 by Bras et al. [23]. The highest number of human infections occurs in Nigeria, Cameroon, Zaire and the Ivory Coast [24]; however, conidiobolomycosis has also recently been diagnosed in patients from temperate climate regions, including Europeans [25], which has been attributed to global warming. The gateway to *C. coronatus* infection is probably the respiratory system: inhaled fungal spores penetrate the nasal mucosa, then germinate and the hyphae penetrate the subcutaneous part of the face, nasal cavity and sinuses. Clinically, conidiobolomycosis occurs in two forms, either involving the nasopharynx or the nasofacial area. The former occurs more frequently and concerns an infection located mainly in the ethmoid region, while the latter is much rarer and affects the vestibule and skin of the nose. In both forms, the clinical symptoms include the formation of serous or mucous nasal discharge, fever, apathy, anorexia, weight loss and marked respiratory failure. In the nasofacial form, the inflammation may extend to one of the eye sockets, which most often results in unilateral proptosis, marked asymmetry of the facial skeleton and ulcerative keratitis. [26]. In addition, inflammation of the frontal lobe of the brain, can give rise to neurological symptoms, including drooping, circulation, abnormal posture, and head compression. Rapid progression has been reported in immunocompromised patients: in such cases, the fungus invades all the way to the blood vessels. In disseminated infection, the fungus inhabits multiple organs, including the lungs, heart, kidneys, spleen, and/or brain. [27]. In addition, conidiobolomycosis can also occur in horses, sheep, dogs and pigs. [28]. **Therefore, it is important to understand the functioning of the immune system in response to infection with *C. coronatus*, and *G. mellonella* larvae represent a suitable model.**

• Similarities between the immune systems of insects and mammals

An important feature of the insects used as models in infection studies is that their immune mechanisms demonstrate a close similarity with those of vertebrates, including humans. The immune system of *G. mellonella* shows functional and structural similarity to the innate immune response of mammals. The insect cuticle acts as a physical barrier to pathogens, like the skin, and the haemolymph can be compared to blood due to the presence of immunocompetent cells. Although insects have not developed the acquired immunity found in mammals, they are able to produce a number of antimicrobial substances (antimicrobial peptides - AMPs) [29, 30]. The humoral immune response of the wax moth is also carried out through the process of melanization and the production of reactive oxygen species. Phagocytosis, nodulation, and encapsulation are the basis of the cellular response, in which five classes of haemocytes (cells present in the haemolymph) participate. Plasmatocytes and granulocytes, like neutrophils, monocytes, and macrophages in mammals, are classified as phagocytes. The number of haemocytes in *G. mellonella* changes during the course of infection, like leukocytes in mammals, and hence can sometimes be regarded as an indicator of the pathogenicity of the microorganism [31].

The factors regulating the immune response are widely described in mammals and analogues can often be found in insects. In *G. mellonella*, AMP production is a major part of the humoral response, and is regulated by three main pathways: Toll, Imd (immune deficiency) and JAK-STAT (Janus kinase-signal transducer and activator of transcription). Infection with Gram-negative bacteria activates the Imd pathway, while the Toll pathway is activated by Gram-positive bacteria and fungi. In turn, the JAK-STAT pathway regulates many biological processes related to immunity, including participation in hematopoiesis and cellular immunity, regulation of defense against viral infections, intestinal immunity, general stress response and wound healing [32].

In mammals, Toll-like receptors (TLRs) present on the surface of dendritic cells, macrophages and granulocytes recognize molecular patterns of pathogens. As a result of an extensive cascade, they activate NF-κB, which leads to the induction of many innate response genes and genes responsible for cytokine production. In insects, the best known proteins from the NF-κB family are Dorsal and Dif. After translocation to the nucleus, Dif activates the transcription of genes encoding immune peptides, while Dorsal plays a role in embryonic development [33].

The insect Imd pathway is very similar to the mammalian TNF-α (tumor necrosis factor α) pathway, the main role of which is to maintain the balance between apoptosis and cell survival. TNF-α-induced apoptosis involves the activation of caspase cascades that cause cell death; in

addition, TNF-α activates two transcription factors: NF-κB and activating protein 1 (AP-1), the first of which prevents apoptosis [34]. The JAK-STAT pathway is one of the best-known signal transduction cascades in the animal kingdom. Its activation stimulates cell proliferation, differentiation, migration and apoptosis, and its factors are known to regulate the production of over 40 cytokines in various organisms to varying degrees [35].

The use of insects as models in research on fungal infection requires a clear understanding of their immune mechanisms. **The research constituting the basis of this scientific achievement examines the influence of infection by the pathogenic fungus *C. coronatus* on selected elements of the immune system of *G. mellonella*.** The innate immune mechanisms of the wax moth are given in Figure 1, highlighting the processes and factors studied in the presented publication series.

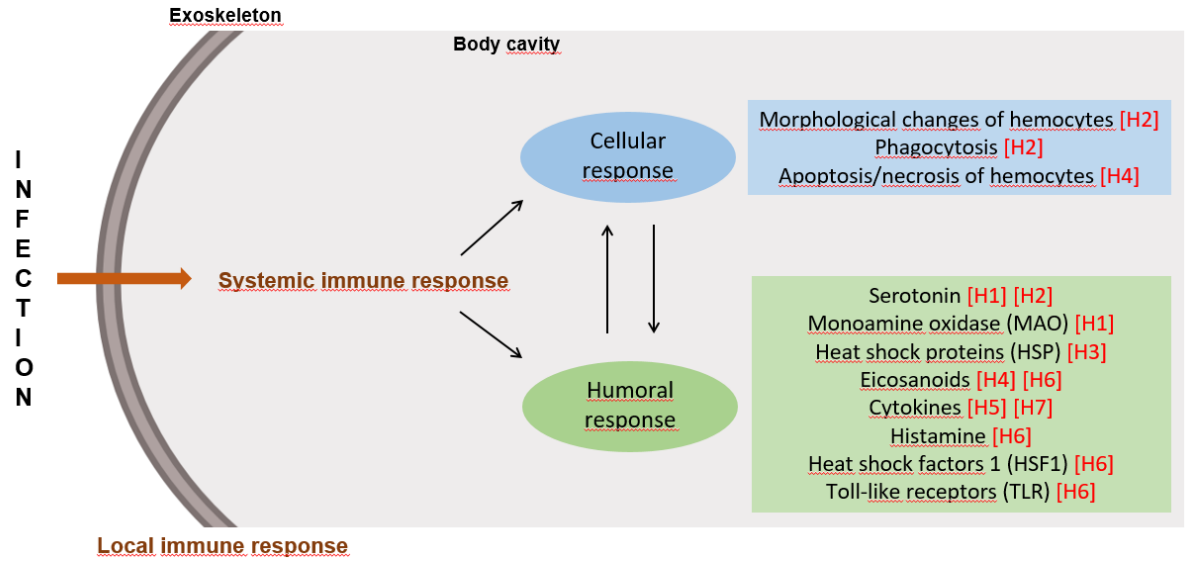

Figure 1. The innate defence mechanisms of *Galleria mellonella* activated in response to fungal infection. The red color indicates which of the presented publications describe a given element of the immune response.

4.1.2. Aims and Research Methods

The main aim of the research constituting the basis of the presented scientific achievement was to analyze selected elements of the innate immune response to fungal infection using *Galleria mellonella* (Lepidoptera) as an alternative to mammalian research models.

Achieving this goal required the use of numerous research methods, most of which were developed by the candidate. The studies analysed various elements of the systemic immune response of *G. mellonella*, including the cellular response (morphological changes of

haemocytes, the process of phagocytosis, apoptosis/necrosis of haemocytes) and the humoral response (serotonin levels, heat shock proteins, eicosanoids, cytokines, histamine, heat shock factor, monoamine oxidase activity, Toll-like receptors). To achieve the main objective of the research, specific objectives were formulated. The selected research methods and specific objectives for the publications are summarised in Table 1.

Table 1. Specific objectives of each publication and research methods used.

Publication	Detailed aims	Research method
[H1] [H2]	• -To test whether alkaloids are among the metabolites released by *C. coronatus* • To investigate whether the identified metabolites affect serotonin levels in *G. mellonella* by acting as monoamine oxidase (MAO) inhibitors, thereby affecting the phagocytic activity of haemocytes.	• Gas chromatography-mass spectrometry (GC-MS) for the identification of alkaloids • ELISA for the determination of serotonin and MAO • Luminescent measurement of MAO activity • Immunolocalisation using fluorescence microscopy • Phagocytic activity test using *Escherichia coli* (strain K-12).
[H3]	• - To test whether infection with the entomopathogenic fungus *C. coronatus* causes changes in the levels of selected heat shock proteins (HSP90, HSP70, HSP60 and HSP27) in the haemolymph and haemocytes of *G. mellonella* larvae.	• Immunolocalisation using fluorescence microscopy • Flow cytometry • ELISA tests.
[H4]	• To investigate whether infection with *C. coronatus* induces apoptosis of haemocytes of *G. mellonella* larvae and affects caspase activity • To determine the effect of fungal infection on the levels of selected eicosanoids in the haemolymph of *G. mellonella*	• Apoptosis/necrosis detection test using fluorescence microscopy and flow cytometry. • Caspase activity test using fluorescence microscopy and fluorimetry. • Colorimetric caspase 1 activity test. • ELISA tests. • Colorimetric phospholipase A2 activity test..
[H5]	• Development of a reproducible and efficient protocol for the detection of cytokines in insect haemocytes using flow cytometry. • Detection of IFN-γ in haemocytes of *G. mellonella* larvae using fluorescence microscopy, flow cytometry and proteomic analysis.	• Immunodetection using flow cytometry • Immunolocalisation using fluorescence microscopy • 2D electrophoresis • Western Blot

		• Protein identification using LC-MS-MS/MS.
[H6]	• To investigate the effect of infection with *C. coronatus* on the levels of histamine, heat shock factor 1 (HSF1), cysteine leukotrienes and two Toll-like receptors (TLR1 and TLR2) in the haemolymph and haemocytes of *G. mellonella*.	• Immunolocalisation using fluorescence microscopy • Flow cytometry • ELISA tests.
[H7]	• To test whether infection with *C. coronatus* causes changes in the levels of selected eighteen cytokine-like proteins in the haemolymph and haemocytes of *G. mellonella* larvae.	• Immunolocalisation using fluorescence microscopy • Flow cytometry • ELISA tests • Preliminary proteomic analysis using the BLASTp tool.

The element that connects all publications in the discussed scientific achievement is the use of the insect-entomopathogenic fungus system. All experiments used *G. mellonella* larvae on day 3, instar 7 stage; the age of the larvae was selected based on the fact that larvae stop feeding before pupation. Therefore, all insects used in the experiments were bred under standardized conditions. All were fed with artificial medium according to Sehnal [4] *ad libitum*, and kep in darkness at 30°C in 70% humidity.

The research used *C. coronatus* isolate 3747 from the collection of prof. S. Bałazy (Department of Agricultural and Forest Environment Research, Polish Academy of Sciences, Poznań). The *C. coronatus* was cultivated on solid Sabouraud medium (SAB).

In order to maintain the virulence of the fungus, this medium was additionally enriched with *G. mellonella* larva homogenate to a final weight-volume concentration of 10% (SABG medium). The cultures were carried out in sterile Petri dishes, 90 mm in diameter, with ventilation. After seven days of culture, the fungus was passaged to a dish with sterile SABG medium. Cultures on liquid medium were carried out on minimal medium (MM) and on rich Luria Broth medium (LB). The fungus was stored in an incubator with continuous air circulation, at a temperature of 20ºC, with a 12-hour light: 12-hour dark photoperiod.

In the discussed experiments, a uniform method of infecting larvae with the fungus was used. Individuals were transferred to a plate with a 7-day spore-forming culture of the *C. coronatus*. Then, after 24 hours, the insects were removed from the plates. Some of them were used for research (test F24). The rest were incubated for another 24 hours, in conditions optimal for insect development (test F48) and then used for research. A control group was also used, consisting of insects incubated on a plate with sterile SABG medium.

4.1.3. Synthesis of research results

This section presents the most important results, referring to the key research areas (Figure 1). A summary of the results and the conclusions are presented in Table 2.

- **Harman and norharman – newly-discovered metabolites of the fungus *Conidiobolus coronatus***

The mechanism of infection by entomopathogenic fungi is a multi-stage process. Physical contact of the spore with the insect is necessary for infection to occur. Spores most commonly settle on the insect cuticle, especially in places of injury, and less often in the mouth or anus or in spiracles. Adhesion occurs on a fragment of the cuticle containing hydrophilic molecules. Conidia contain a hydrophobic envelope, the main protein components of which are

hydrophobins. Adhesins also play an important role in the process of spore attachment to the surface of the cuticle. They contain a region mediating adhesion rich in proline and threonine. This protein is probably anchored in the C-terminal part by glycosylphosphatidylinositol, which allows proteins to be located on the surface of the insect [36, 37].

The spore is equipped with enzymes that break down antifungal substances on the insect cuticle, such as cysteine and subtilisin proteases and chitinases, whose activity causes the release of substances that stimulate spore germination. These are accompanied by enzymes that cause the degrade fatty acids or alkanes with antifungal properties. The newly-formed hyphae also synthesise proteolytic, lipolytic and chitinolytic enzymes [38].

After overcoming the barrier of the insect cuticle, and penetrating the body cavity, the fungi produce a number of metabolites. *C. coronatus* is able to produce metabolites from various chemical groups. The coronatin-1 protein (36 kDa) exhibits chitinolytic and elastinolytic activity, while coronatin-2 (14.5 kDa) does not; however, both proteins cause haemocyte disintegration and disrupt the formation of networks between plasmatocytes in *G. mellonella* [39, 40]. Another metabolite, octanoic acid, damages haemocytes, induces late apoptosis and/or necrosis in these cells; it also activates caspases and influences the increase in the level of 8-hydroxyguanosine, a marker of oxidative stress associated with DNA damage. [41]. In turn, dodecanol has a negative effect on the haemocytes of *G. mellonella* and *C. vicina*, causing their disintegration. [42].

Literature data indicates that alkaloids are common fungal metabolites, being present in the genera *Penicillium, Aspergillus* and *Trichoderma* [43]. It is also known that plant alkaloids affect the physiology of insects and may be potential bioinsecticides [44-46].

Therefore, publication [H1] checked whether alkaloids are also among the metabolites of the fungus *C. coronatus*: the article presents the first description of the extraction and identification of alkaloids from filtrates after culture on minimal (MM) and rich (LB) media. Solid phase extraction was used on Waters OASIS MCX 8 columns, and methanol was used as the eluent. The efficiency of the extraction method was 85%. The alkaloids were identified using gas chromatography coupled with mass spectrometry (GC-MS) on a GCMS-QP2010 device (Shimadzu). The procedure used an appropriate chromatographic column (ZB-5 MS Zebron, Phenomenex), temperature, helium flow and detector parameters. Initial identification of metabolites was carried out using a mass spectral library, and their presence was confirmed using an internal standard.

The publication [H1] showed for the first time that *C. coronatus* produces two β-carboline alkaloids: harman and norharman. Their concentration depends on the culture medium used. A significantly higher concentration of both alkaloids was found in the MM post-culture medium than in LB: this may be caused by the stress conditions resulting from cultivation on minimal medium. In order to check the effect of harman and norharman on the development of *G. mellonella*, the compounds were administered to larvae on the third day of the last (seventh) larval instar, both topically and with food, at three concentrations: 750, 1000 and 1250 ppm. While topical administration did not affect pupation or imaginal moulting, food administration delayed these processes compared to controls.

The effects of β-carboline alkaloids on the nervous and immune systems of mammals are well described. Harman and norharman are reversible inhibitors of monoamine oxidase (MAO): the former preferentially inhibits the action of MAO-A, and the latter MAO-B. MAO catalyzes the oxidative deamination of 5-hydroxytryptamine (5-HT; serotonin), converting it to 5-hydroxy-3-indolacetaldehyde (5-HIAL), which is further processed to 5-hydroxy-3-indoacetic acid (5-HIAA) by aldehyde dehydrogenase. In addition, β-carboline alkaloids are able to bind to serotonin receptors in tissues. Binding of harman and norharman to these receptors increases the levels of 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA) in the brain of rats [47]. It is known that both serotonin and monoamine oxidase are highly evolutionarily conserved substances and also occur in insects.

Publication [H1] examined the influence of harman and norharman on the level and activity of serotonin and MAO, in the head capsules *G. mellonella* larvae. As in the previous experiment, the larvae were given the metabolites in three concentrations topically or with food, and tissue samples were collected after one hour or 24 hours. Both topical and food administration caused an increase in serotonin concentration. No changes in the MAO level were observed in the head capsules of *G. mellonella* larvae 24 hours after the topical administration. However, a significant decrease in MAO concentration in the tissues was observed one hour after the topical administration. The same effect was observed after food administration. However, neither harman nor norharman affected the activity of MAO-A or MAO-B isoforms in the head capsules of the wax moth 24 hours after topical administration. Food treatment reduced the activity of both enzymes. **The findings indicate that harman and norharman may be monoamine oxidase inhibitors, which affect the level of serotonin in insects. Moreover, it is worth emphasizing that these alkaloids delay pupation and imaginal moulting, which may be related to the increased level of serotonin.**.

Serotonin is a neurotransmitter that is considered to play a significant role in regulating the immune system. In mammals, 5-HT acts as a potent chaemoattractant, causing the influx of immune cells to sites of inflammation. It also influences the production and release of cytokines and the activation and proliferation of immunocompetent cells. It is also known that some immune cells, including mast cells and T lymphocytes, have the ability to synthesize and release serotonin. Mammalian immune cells are able to express the serotonin receptors 5-HT1, 5-HT2, 5-HT3, 5-HT4 and 5-HT7, and the serotonin transporter (SERT), and produce key enzymes of neurotransmitter metabolism, such as tryptophan hydroxylase (TPH) and monoamine oxidase (MAO) [48]. Literature reports also indicate that 5-HT may play a role in the immune response of insects. Qi et al. report that insect haemocytes contain TPH and are able to synthesize serotonin, and present 5-HT1B, 5-HT2B and 5-HT7 receptors [49]. This neurotransmitter is also involved in such cellular response processes as phagocytosis, nodulation and regulation of the activity of individual classes of haemocytes in *Spodoptera exigua* [50].

Publication [H2] checked whether harman and norharman, i.e. MAO inhibitors, affect the serotonin level in the haemocytes of *G. mellonella* and their phagocytic activity. The same research system was used as in article H1: larvae on the third day of the last (seventh) instar were given harman or norharman (in concentrations of 750, 1000, 1250 ppm) topically or with food, and the material for testing was collected after one hour or 24 hours. In addition, an *in vitro* experiment was conducted in which the three tested concentrations of harman or norharman were added directly to the haemocyte cell culture. The effects of these factors were assessed 1 and 24 hours after addition.

This publication presents the first description of this method of immunodetecting serotonin in *G. mellonella* haemocyte cultures using fluorescence microscopy. Suitable conditions were determined for cell fixation and permeabilization, antibody concentration, cytoskeleton and nucleus staining, and visualization. Additionally, it describes the first use of specialized µ-Slide VI 0.4 microplates from Ibidi in insect haemocyte cultures.

It was found that *G. mellonella* haemocytes demonstrate strong adherence to these plates, and the small volumes of the wells allow for economical use of reagents. These plates were used in further experiments using microscopy, as described in subsequent publications. In addition, the concentration of serotonin in the whole haemolymph was determined using commercial ELISA tests. A method for testing the phagocytic activity of haemocytes was also developed using *Escherichia coli* (K-12 strain) BioParticles labelled with fluorescein. The experiment was

performed in cell culture, with cytoskeleton and cell nuclei staining, and fluorescence microscopy.

It was found that 24-hour exposure to harman and norharman, administered with food, caused a significant increase in the level of serotonin in *G. mellonella* haemocytes and their phagocytic activity. A similar reaction was observed in larvae one hour after topical administration or in the haemocyte culture *in vitro*. This effect decreased after 24-hour exposure, which may suggest that the larvae are able to metabolize the tested alkaloids..

Our findings indicate that harman and norharman affect the level of 5-HT in insect haemocytes and their phagocytic activity. It is therefore possible that entomopathogenic fungi modulate the immune response through metabolites which inhibit insect enzymes.

The microscope observations reported in publication [H2] showed that harman and norharman at concentrations of 1000 and 1250 ppm cause negative changes in haemocyte cytoskeletons. The following were observed: difficulties in network formation by plasmatocytes and granulocytes, formation of cell aggregates, disruption of cell membrane continuity and presence of fragments of disintegrated cells. Other findings not included in the discussed series of publications showed that infection with the *C. coronatus* causes significant morphological changes in wax moth haemocytes, with changes in the cytoskeleton and cell shape, and difficulties in network formation being observed in cell cultures taken from insects after 24-hour infection. After 48 hours from infection, many cells were destroyed, were unable to form networks and displayed the presence of 'naked' cell nuclei [51]. **It can therefore be concluded that damage to immunocompetent cells during fungal infection may also be related to the action of fungal metabolites.**.

The observed morphological changes in *G. mellonella* haemocytes may be indicative of apoptosis or necrosis. **Therefore, publication [H4] investigated whether *C. coronatus* infection induces apoptosis of haemocytes of *G. mellonella* larvae and affects the activity of caspases**. Apoptosis/necrosis and caspase activity were examined in haemocytes collected from larvae after 24- and 48-hour fungal infection and from healthy insects (control group). Apoptosis/necrosis was confirmed using fluorescence microscopy and flow cytometry. A commercial reagent kit (GFP CERTIFIED Apoptosis/Necrosis Detection Kit (Enzo Life Sciences)) was used for microscope detection.

The kit contained annexin V, which selectively recognizes phosphatidylserine (PS), conjugated to green fluorescent protein (GFP). In physiological conditions, PS is present on the cytosolic side of the cell membrane. In the early phase of apoptosis, PS located in the cytosol is removed from the cell through the cell membrane. Late apoptosis and necrosis can be detected using a necrosis detection reagent similar to 7-AAD (7-aminoactinomycin D), a red dye.

Detection was performed by flow cytometry using the Dead Cell Apoptosis Kit with Annexin V FITC and PI (Thermo Fisher Scientific), where annexin V was used to identify apoptotic cells, and propidium iodide (PI) to identify necrotic cells. Positive controls were used in both assays, where haemocyte apoptosis was induced by the addition of staurosporine (1 μM) or actinomycin D (10 μg/ml) to cultures or by exposing cells to UV radiation for 15 min. **The results show that 24-hour infection with *C. coronatus* causes apoptosis and early necrosis of *G. mellonella* haemocytes, while 48-hour infection results in severe necrosis**.

Caspase activity was measured using the Carboxyfluorescein MultiCaspase Activity Kit (Enzo Life Sciences). This kit contained FAM-VAD-FMK, a carboxyfluorescein derivative of valylalanylaspartic acid fluoromethyl ketone (VAD-FMK), which is a potent inhibitor of caspase activity. The FAM-VAD-FMK reagent enters the cell and covalently binds to a reactive cysteine (Cys 285) on the large subunit of the caspase heterodimer. The VAD multicaspase substrate enables detection of caspases 1–9.

Caspase activity in haemocytes was determined by fluorescence microscopy and fluorometry. Caspase-1 activity was measured using the Caspase-1 colorimetric assay kit (Enzo Life Sciences). The test is based on spectrophotometric detection of the p-nitroaniline (pNA) chromophore after cleavage from the labelled YVAD-pNA substrate. The results are presented as a percentage of activity relative to the control. Our analysis of the obtained results showed that *C. coronatus* infection induces caspase activation in *G. mellonella* haemocytes, with a significant increase in enzyme activity observed after 48 hours of infection. **The findings confirm the presence of proteins capable of cleaving the FAM-VAD-FMK complex, resulting in the release of carboxyfluorescein (FAM). It can therefore be assumed that enzymes with properties similar to human caspases are active in *G. mellonella* after fungal infection.**

• Heat shock proteins in insects in response to fungal infection

In order to use *G. mellonella* larvae as model organisms, it is necessary to know the factors of the immune response which are evolutionarily conserved. One such group of factors is the heat

shock proteins (HSPs), which have similar amino acid sequences in different groups of organisms, and co-create one of the oldest survival mechanisms. [52]. HSPs were first described in the 1960s in *D. melanogaster*, and later in mammals. They perform similar functions in all living organisms and play an important role in normal cellular homeostasis. Heat shock proteins are produced constitutively in the cell, but their expression increases when cells are exposed to stress factors, e.g. elevated temperature, toxins, UV radiation, starvation, hypoxia, inflammation and infections [53, 54]. On the other hand, stressful environmental factors such as changes in ambient temperature also have a strong impact on the immune system in insects. [55].

The role of HSPs in the immune response has been well described in mammals. They are a linking factor between innate and adaptive immune mechanisms. They participate in antigen presentation (especially HSP70 and HSP90), macrophage and lymphocyte activation and dendritic cell maturation and stimulate proinflammatory cytokines such as TNF-α or Il-6. However, the participation of HSPs in insect immune responses is not well understood. [56].

Publication [H3] investigated whether infection with the *C. coronatus* causes changes in the level of selected heat shock proteins (HSP90, HSP70, HSP60 and HSP27) in the haemolymph and haemocytes of *G. mellonella* larvae. Insects at day three of the last larval stage from the three groups F24, F48 and the control group were used in the experiments. Three test methods were used to detect selected heat shock proteins: immunolocalisation using fluorescence microscopy, immunodetection using fluorescence microscopy and commercially-available ELISA. The first two methods were used for the first time in this context in the present experiment. Conditions were chosen for the preparation of haemocyte samples, analysis and processing of the results.

The results confirmed the presence of all four tested heat shock proteins in uninfected *G. mellonella* larvae, with the highest concentrations recorded for HSP60 and HSP90; in contrast, HSP70 and HSP27 were found in trace amounts. Infection with *C. coronatus* resulted in increased levels of HSP60 and HSP27 both 24 and 48 hours after infection. Higher levels of HSP90 were noted in the F48 larvae compared to controls; however, no significant change in HSP70 levels was observed in the infected insects. **The results indicate that HSPs, as evolutionarily-conserved proteins, are also present in *G. mellonella* and may play a role in the immune response to fungal infection.**

HSP protein gene expression and production require appropriate HSF transcription factor (Heat Shock Transcription Factor) activity. It is present in cells as an inactive monomer. It is

considered the main factor of the stress response, and is activated by stress factors such as infection [57]. **Publication [H6] investigated whether infection with *C. coronatus* is a factor influencing the level of active HSF1 in the haemolymph of *G. mellonella***. The determinations in the F24, F48 and control groups were performed using commercial ELISA tests. It was found that 48-hour fungal infection caused a significant increase in HSF1 in the tested insects compared to the control group. No such increase was observed in the F24 group. **It can therefore be assumed that in insects, fungal infection is a stress factor causing the activation of HSF**.

Eicosanoids and histamine - mediators of inflammation in fungal infection

Due to their diversity and the presence of a number of specific receptors in target (effector) cells, eicosanoids play an extremely important role in maintaining homeostasis. These lipid mediators have both pro-inflammatory and anti-inflammatory effects, as well as pro-extinction properties [58]. Their biosynthesis is associated with calcium ion-dependent phospholipase A2 (PLA2), leading to the release of arachidonic acid and other polyunsaturated fatty acids (PUFAs) PUFAs from the sn-2 position of cell membrane phospholipids. PLA2 activation is caused by inflammation, under the influence of various hormones, cytokines, growth factors, protein kinase C (PKC), mitogen-activated kinase (MAPK), or immune complexes [59].

In insects, eicosanoids mediate various physiological processes and play a key role in the immune response. Stanley et al. showed that eicosanoids are involved in microaggregation and nodulation during bacterial infection, and to activate prophenyl oxidase (PPO) and induce phagocytosis [60]. The detailed molecular action of eicosanoids was analysed in *S. exigua*, where they induced lysis of the oenocytoid cell membrane, causing the release of PPO, which was activated through a number of catalytic serine proteinase activities [61]. The eicosanoids participating in the humoral immune response mediate the induction of AMP gene expression. In *Tribolium castaneum*, both the Toll and Imd signalling pathways, which induce specific AMP expression, activate the biosynthesis of eicosanoids in response to bacterial infections [62]. Furthermore, it is noteworthy that in *S. exigua*, serotonin can regulate phagocytosis and nodulation by influencing eicosanoid levels [63].

Publications [H4] and [H6] investigated the effect of infection with *C. coronatus* on the levels of selected eicosanoids in the haemolymph of *G. mellonella* larvae. Determinations were performed in haemolymph collected from insects of groups F24, F48 and controls using commercial ELISAs. Publication [H4] examined the levels of prostaglandins (PGE1, PGE2,

PGA1, PGF2α, 8-iso-PGF2α), thromboxanes (TXA2, TXB2, 11-dehydro-TXB2) and leukotriene (LTB4), while article [H6] focussed on the cysteine leukotriene group (LTC4/D4/E4). Fungal infection was found to affect levels of most studied eicosanoids in haemolymph, with the exception of LTB4, TXA2 and 11-dehydro-TXB2. Significant increases in prostaglandins PGE1, PGA1 and PGE2 were noted in both F24 and F48 compared to the control group. In contrast, significant increases in PGF2α and 8-iso-PGF2α levels were only observed in the F24 group. Only thromboxane B2 levels were significantly lower in both F24 and F48 groups compared to controls. Fungal infection further caused a decrease in cysteine leukotrienes in the haemolymph of *G. mellonella*, which was described in the publication [H6].

PLA2, the main enzyme of lipid metabolism that results in eicosanoid biosynthesis, also occurs in insects. Tunaz et al. showed that bacterial infection significantly induces PLA2 activity in haemocytes of *Manduca sexta* [64]. The enzyme was also found to have significant activity on immunosuppression in *S. exigua* following infection with the entomopathogenic bacterium *Xenorhabdus nematophila* [65]. **The effect of infection with *C. coronatus* on PLA2 activity in the haemolymph of G. mellonella was investigated in publication [H4]**. The determination was performed using a commercially-available colorimetric assay. The results showed a statistically significant increase in PLA2 activity in the haemolymph of infected larvae infection (groups F24 and F48) compared to healthy controls.

To summarise, it can be concluded that eicosanoids are involved in the pathogenesis of fungal infection in insects, which is associated with an increase in phospholipase A2 activity.

The aim of publication [H6] was also to determine the effect of fungal infection on histamine levels in wax moths. The results of the commercial ELISA assays showed a statistically significant increase in histamine in the haemolymph of infected *G. mellonella* larvae.

- **Cytokine-like proteins in response to fungal infection in *Galleria mellonella***

In vertebrates, responses to external and internal damaging factors, tissue repair mechanisms and the restoration of tissue homeostasis are controlled by cytokines, secreted primarily by immunocompetent cells. These proteins are therefore considered the main regulators of immune processes. Their function is regulated by interactions with specific cytokine receptors [66]. Studies on functional similarities between innate defence mechanisms of vertebrates and invertebrates suggest that invertebrates possess cytokine-like mediators that regulate inflammatory responses to infection [67].

Immunocytochemical studies have confirmed the presence of cytokine-like proteins similar to IL-1, IL-6 and TNF in various invertebrate species belonging to the Mollusca (34), Nematoda (35), Annelida (36), Tunicata (37) and Insecta (38). However, few reports of the presence of cytokine-like proteins in insects can be found in the literature. A cytotoxic molecule (Gallysin 2), which is an analogue of mammalian TNF, has been isolated from G. mellonella [68]. Unactivated wax moth granulocytes and haemocytes of *Estigmene acraea* larvae showed strong positive responses to polyclonal antibodies (pAb) against IL-1α and TNF-α [69]. In addition, TNF-like molecules have been identified in plasmatocytes and granulocytes of *Calliphora vomitoria* [70]. Little is known about the role of cytokine-like proteins in immune mechanisms in insects, although in *D. melanogaster*, exposure to recombinant human IL-8 was found to stimulate phagocytic cells and increase their number [71]. In addition, a TNF-like molecule was found to be expressed in *C. vomitoria* haemocytes during encapsulation, suggesting that this protein may be a chaemoattractant in these insects [70]. In contrast, a defence complex consisting of an IL-1-like molecule and phenol oxidase, the enzyme responsible for melanisation, has been described in the haemolymph of the insect *M. sexta* [72].

In mammals, cytokine production is regulated by a number of factors, including serotonin, heat shock proteins, or eicosanoids [73], whose role in fungal infection in *G. mellonella* is described in the included articles. It is important to gain an thorough understanding of the cytokine-like proteins in *G. mellonella* to be able to use this insect as a research model in immunology.

Accordingly, publication [H5] developed protocols for the determination of cytokine-like proteins in *G. mellonella*, and publication [H7] examined the effect of infection with *C. coronatus* on the levels of these proteins in the insect. As the study examined 18 cytokine-like proteins, it was first necessary to develop reproducible research protocols. Publication [H5] describes the detection of these proteins, using IFN (interferon)-gamma as an example; the methods included fluorescence microscopy and intracellular cytokine detection by flow cytometry (ICCS), the development of the latter being the main objective of this article.

The presence of IFN-gamma in *G. mellonella* haemocytes was confirmed using bidirectional 2-D electrophoresis and proteomic analysis, with the method described in detail in the manuscript. Protein was extracted from haemocytes using a commercial CytoBuster Protein Extraction Reagent kit (Merck). Isoelectric focusing was performed using the PROTEAN i12™ IEF system (Bio Rad). The hydration conditions of the gel strips (7 cm strips) and the electrical voltage gradient of the protein separation were determined. Vertical electrophoresis (SDS-PAGE) was performed in a 12% polyacrylamide gel in a Mini-PROTEAN Tetra cell apparatus

(Bio Rad). The proteins were stained after electrophoretic separation using Pierce Silver Stain Kit (Thermo Scientific), and detected by Western Blot using the same primary antibody used for fluorescence microscopy and flow cytometry, and a goat anti-rabbit IgG (H+L) antibody, HRP (Invitrogen) as the secondary antibody. Protein identification by LC-MS-MS/MS was performed by the Mass Spectrometry Laboratory of the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw.

Proteomic analysis revealed the presence of an IFN-gamma-like protein in the haemolymph of *G. mellonella* larvae. This is the first report of the identification of this protein in a wax moth. Analysis using the NCBI Blast tool confirmed that the identified protein shared 33% sequence homology with the IFN-gamma sequence in *Homo sapiens*. In comparison, human IFN-gamma shows approximately 40% sequence homology to mouse IFN-gamma, and 30-35% homology with chicken IFN-gamma (ChIFN-gamma) [74]. The positive identification of IFN-gamma formed the basis for the development of immunolocalisation protocols using fluorescence microscopy and ICCS.

The study aimed to develop a method for the immunodetection of cytokine-like proteins in *G. mellonella* haemocyte cultures, using fluorescence microscopy. Hence, the conditions for cell fixation and permeabilisation, antibody concentration, cytoskeleton and cell nuclei staining, and visualisation were determined. Studies were carried out on µ-Slide VI 0.4 microplates from Ibidi, thus optimising the volumes of reagents used.

The ICCS protocol was developed using haemocytes collected from *G. mellonella* larvae on the third day of the last (seventh) larval stage: the same age of the larvae used in all the experiments described. Due to the ability of plasmatocytes and granulocytes to adhere, it was necessary to develop an optimal anticoagulant buffer: eventually, PBS with the addition of 10mM EDTA, 30mM sodium citrate and 0.1mM phenylthiourea (PTU) was selected.

As haemocytes are prone to disintegration, it was necessary to select optimal conditions for centrifugation, which were eventually determined to be 300 x g for 5 minutes. In addition, the cell fixation buffer was selected as 4% paraformaldehyde (PFA) in PBS, and 0.1% Triton X-100 in PBS as the permeabilising buffer. The best results were obtained with an overnight incubation at 4°C with the primary antibody and a 90-minute incubation at room temperature with the secondary antibody. In developing the protocol, Mouse Cytokine Positive Control Cells (Invitrogen) were used as a positive control. The detailed protocol has been deposited in the protocols.io database and is available at dx.doi.org/10.17504/protocols.io.b3xxqppn. **In conclusion, it is important to highlight that publication [H5] developed innovative,**

reproducible and efficient protocols for the determination of cytokine-like proteins in *G. mellonella* haemocytes.

Using the protocols developed in publication [H7], the study examined the effect of infection with *C. coronatus* on the levels of 18 selected cytokine-like proteins in the haemocytes and haemolymph of *G. mellonella*. The levels of IL-1α, IL-1β, IL-2, IL-3, IL-6, IL-7, IL-8, IL-12, IL-13, IL-15, IL-17, IL-19, IFN-γ, TNF-α, TNF-β, GM-CSF (granulocyte-macrophage colony-stimulating factor), M-CSF (macrophage colony-stimulating factor) and G-CSF (granulocyte-macrophage colony-stimulating factor) were examined using immunolocalisation. The levels of macrophage colony-stimulating factor (M-CSF), G-CSF (granulocyte colony-stimulating factor) were examined by immunolocalisation using fluorescence microscopy, ICCS and commercially-available ELISA. Haemolymph for testing was collected from larvae on day three of the last (seventh) larval stage from groups F24, F48 and the control group.

The presence of thirteen proteins (G-CSF, GM-CSF, M-CSF, TNF-β, IFN-gamma, TNF-α, IL-1 β, IL-3, IL-6, IL-7, IL-15 , IL-17, IL-19) was confirmed in the haemolymph and haemocytes of *G. mellonella*; however, the results differed depending on the test method used. Immunolocalisation using fluorescence microscopy showed an increase in the levels of G-CSF, GM-CSF, M-CSF, IL-3, IL-15, IL-1 β, IL-6, IL-19 in both F24 and F48 compared to controls. Cytometric analysis indicated an increase in M-CSF, GM-CSF, IL-1 β, IL-8 and IL-19 in both F25 and F48 compared to controls. In contrast, the ELISA tests showed higher levels of M-CSF, GM-CSF and IL-1 α in haemolymph from groups F24 and F48. These differences may be due to the way the sample was prepared. While flow cytometry allows the determination of all haemocyte subpopulations, fluorescence microscopy can only identify adherent cells, i.e. plasmatocytes and granulocytes, due to the frequent washes, and ELISA uses the whole haemolymph, i.e. homogenised cells and plasma. Therefore, the use of a variety of test methods appears justified.

Preliminary proteomic analysis using the BLASTP tool revealed potential homology between three human cytokines (IL-1α, IL-17 and M-CSF) and uncharacterised *G. mellonella* proteins, the sequences of which have been deposited in databases. A full proteomic analysis will be required to further assess the homology between human cytokines and insect cytokine-like proteins. Proteomic analysis of the larval haemolymph is currently underway, using FAIM (high-field asymmetric ion mobility spectrometry) and protein sequence comparisons with

Swissprot Eukaryota databases. Preliminary identification results have been obtained for IL-17 and IL-19, which are being further analysed and prepared for publication.

It can therefore be concluded that cytokines are highly evolutionarily-conserved proteins whose counterparts are present in insects. Furthermore, it is possible that they play a role in the immune response to fungal infection in this group of animals.

Toll-Like Receptors (TLR) in G. mellonella during fungal infection

Toll-like receptors (TLRs) were first identified in a study of ventrodorsal polarity in *D. melanogaster* larvae. Since then, extensive phylogenetic analyses have demonstrated the presence of the Toll pathway in many organisms, from protozoa to mammals. The pathway is activated in response to infections by Gram-positive bacteria or fungi, and also controls the expression of genes encoding proteins with antibacterial and antifungal properties. In the case of fungal infection, the Gram-negative binding protein 3 (GNBP3) senses beta-1,3-glucan, a component of the fungal cell wall, and triggers a three-step serine protease cascade to activate the Toll pathway [75]. A probable ligand for Toll is Spaetzle (Spz). This protein occurs in the haemolymph as an inactive cytokine pro-Spz. Its activation is based on the cleavage of this pro-protein by the serine protease SPE (Spaetzle processing enzyme). Binding of Spz to the transmembrane Toll receptor, present on the surface of fat body cells, activates an intracellular signalling cascade, the effect of which is the production of AMP [76].

Therefore, publication [H6] examined the effect of C. coronatus infection on TLR receptors in *G. mellonella* haemocytes. Immunolocalization of TLR1 and TLR2 was performed using fluorescence microscopy and flow cytometry; haemocytes were taken from larvae (day three, seventh instar) from groups F24, F48 and control. Both methods confirmed a significant increase in the level of both tested receptors in haemocytes in response to the ongoing infection process. **This suggests that Toll-like receptors are involved in the response to fungal infection in *G. mellonella***.

4.1.4. Summary and Conclusion

The last 20 years has seen a steady increase in the incidence of fungal infections in humans. Paradoxically, this is partly due to advances in medicine: patients receiving organ transplantation, cancer treatment, myelosuppressive and immunosuppressive therapy and with impaired immunity are more at risk of infection. In addition, the introduction of more invasive

diagnostic and therapeutic methods and broad-spectrum antibiotics has increased the rate of infection. In recent years, there has also been a more frequent emergence of drug-resistant strains, or new or previously rare pathogens, and an increase in mortality due to fungal infections [77]. Of the approximately 250,000 species of fungi known to share the environment with humans, over 200 cause infections. Only a few types of fungi (*Histoplasma, Blastomyces*) cause infections in people with a normal immune system: most are opportunistic infections that develop in people with impaired immunity. It is also possible that, due to global warming, fungal species that have so far caused infections only in tropical zones may become increasingly dangerous in temperate areas [78]. It is therefore important to learn as much as possible about the immune mechanisms in response to fungal infections. Insects can be useful as research models in this type of study due to the similarity between their innate immune system components and those found in mammals. This series of publications describes the effect of infection with *C. coronatus* on selected elements of the immune system in *G. mellonella*. Three aspects of the research carried out are worth noting.

Firstly, our findings show for the first time that *C. coronatus* produces β-carbonyl alkaloids (harman and norharman), through which it influences the physiology and immune mechanisms of the wax moth. These alkaloids modulate serotonin levels in both the head capsules and haemocytes of the insect. In particular, they are inhibitors of monoamine oxidase (MAO) and delay pupation and imaginal moulting. They also negatively affect haemocyte morphology and stimulate their phagocytosis.

Secondly, fungal infection in *G. mellonella* was found to affect the levels of a number of factors known to be involved in the immune response in mammals, including heat shock proteins, heat shock factors and cytokines, which are highly evolutionarily conserved. In addition, the fungus is able to modulate levels of inflammatory factors such as eicosanoids by stimulating phospholipase A2 activity. The results also indicate that Toll-like receptors (TLRs) also play a role in entomopathogenic fungus infection. The similarity between the innate immune system of the wax moth and that of mammals suggests that this insect could be successfully used as a model in immunological studies.

Thirdly, numerous research methods were developed in the course of the experiments, including efficient and reproducible protocols for the determination of cytokine-like proteins in haemocytes using fluorescence microscopy and flow cytometry. Our findings also confirm that *G. mellonella* is a promising research model due to its ease of culture, low cost, the possibility

of obtaining a large number of specimens for study at one time, the ease of tissue collection and the use of small volumes of reagents.

A summary of the research results, published in the articles forming part of this scientific achievement, is summarised in Table 2.

Table 2. Summary of the results and conclusions of publications included in the main scientific achievement.

Publication	Key findings	Conclusions
[H1]	• The entomopathogenic fungus *C. coronatus* produces two β-carbonyl alkaloids, harmane and norharmane • Both alkaloids given with food delayed pupation and imaginal moult in *G. mellonella* • Administration of these metabolites to larvae both topically and with food increased serotonin concentrations and decreased MAO-A and MAO-B activity in head capsules.	• The secondary metabolites of entomopathogenic fungi are involved in infection and pathogenesis • In insects, as in mammals, alkaloids can be monoamine oxidase (MAO) inhibitors and thus modulate serotonin levels in neural tissues.
[H2]	• Twenty four-hour exposure to harman and norharman with food caused a significant increase in serotonin levels and phagocytic activity in haemocytes of *G. mellonella* larvae • A similar response was observed one hour after the alkaloids were administered topically to larvae or added to haemocyte cultures *in vitro*. • The alkaloids harman and norharman caused changes in haemocyte morphology, i.e. impaired networking by plasmatocytes and granulocytes, formation of cell aggregates, disruption of cell membranes and presence of fragments of damaged cells.	• Entomopathogenic fungi can modulate the host immune response through their metabolites, which are inhibitors of insect enzymes • Damage to immunocompetent cells during fungal infection may be related to the action of fungal metabolites.
[H3]	• Four heat shock proteins (HSP90, HSP70, HSP60, HSP27) were found in uninfected *G. mellonella* larvae, with the highest concentrations recorded for HSP60 and HSP90, while HSP70 and HSP27 were found in trace amounts.	• Heat shock proteins, being highly evolutionarily conserved, are found in insects and may be involved in the pathogenesis of fungal infection.

	• Infection with *C. coronatus* resulted in increased levels of HSP60 and HSP27 both 24 and 48 hours after infection.	
[H4]	• Fungal infection affected the levels of PGE1, PGE2, PGA1, PGF2α, 8-iso-PGF2α, TXB2 in the haemolymph of wax moths. • A statistically significant increase in PLA2 activity was noted in the haemolymph of infected insects compared to the control group. • 24-h infection with *C. coronatus* induces apoptosis and early necrosis of G. mellonella haemocytes, while 48-h infection results in severe necrosis • *C. coronatus* infection induces caspase activation in haemocytes of wax moth larvae, with a statistically significant increase in enzyme activity observed 48 h after infection.	• Eicosanoids are involved in the pathogenesis of fungal infection in insects which is associated with an increase in phospholipase A2 activity • Fungal infection can model caspase activity in insects and activate apoptosis pathways dependent on these enzymes.
[H5]	• Proteomic analysis revealed the presence of an IFN-gamma-like protein in the haemolymph of G. mellonella larvae. A 33% homology of the detected protein with the IFN-gamma sequence in *Homo sapiens* was confirmed. • Innovative, reproducible and efficient protocols were developed for the determination of cytokine-like proteins in *G. mellonella* haemocytes.	• Cytokines are evolutionarily conserved and show strong amino acid sequence similarity between insects and mammals. • *G. mellonella* larvae appear to be promising models for biomedical research because of their potential to be used to develop efficient and reproducible laboratory protocols.
[H6]	• Infection with *C. coronatus* caused a decrease in cysteine leukotrienes and an increase in histamine and HSF1 in the haemolymph of *G. mellonella.* • Fungal infection resulted in an increase in TLR1 and TLR2 receptors in larval haemocytes compared to healthy insects.	• Fungal infection can induce inflammation in insects involving an increase in histamine and leukotriene levels. • Toll-like receptors are involved in the insect immune response to fungal infection.

[H7]	• Thirteen of the proteins tested (G-CSF, GM-CSF, M-CSF, TNF-β, IFN-gamma, TNF-α, IL-1 β, IL-3, IL-6, IL-7, IL-15, IL-17, IL-19) were found in *G. mellonella* haemolymph and haemocytes. • - Immunolocalisation using fluorescence microscopy showed increased levels of G-CSF, GM-CSF, M-CSF, IL-3, IL-15, IL-1 β, IL-6, IL-19 in insects infected with the fungus *C. coronatus*. • Cytometric analysis indicated an increase in M-CSF, GM-CSF, IL-1 β, IL-8 and IL-19 in both samples taken from larvae after infection. • ELISA analysis showed higher levels of M-CSF, GM-CSF and IL-1α in haemolymph samples from infected larvae	• Cytokines are highly evolutionarily conserved proteins whose counterparts are present in insects. Furthermore, it is possible that they play a role in the immune response to fungal infection in this group of animals.

Novelty of research

This scientific achievement represents a significant contribution to 'Biological Sciences'. It is the first examination of the innate immune response of *G. mellonella* to infection with the pathogenic fungus *C. coronatus*. It investigates numerous elements of humoral and cellular immunity, both in terms of infection and the action of fungal metabolites. It also shows for the first time that *C. coronatus* produces alkaloids (harman and norharman) that can act as MAO inhibitors in insects, like in mammals, and thus affect the immune system. In addition, the presence in *G. mellonella* of evolutionarily conserved key immune factors, such as heat shock proteins, eicosanoids, histamine or TLRs, demonstrates the similarity in response to infection between insects and mammals.

These studies are the first to comprehensively examine the effects of fungal infection on cytokine-like proteins. The results confirm the high similarity between IFN-gamma isolated from wax moth haemocytes to those from *Homo sapiens* protein using proteomic methods. Therefore, the results suggest that *G. mellonella* larvae are a good model for studying the innate immune response. The use of model insects in immunology experiments may contribute to the faster development of the discipline due to a significant reduction in costs, while the elimination of ethical problems will significantly increase research opportunities.

The novelty of the research is also related to the research techniques and methods used. In particular, it uses a new method for extracting and determining the alkaloid content of culture filtrates of *C. coronatus* using GC-MS. The proposed method, with minor modifications, is certainly feasible for determining alkaloids as metabolites of other fungi.

In addition, methods for immunodetection of various proteins in cell cultures of wax moth haemocytes were developed based on fluorescence microscopy. The studies also formulate and publish an efficient and reproducible protocol for the detection of cytokine-like proteins in haemocytes using flow cytometry. Undoubtedly, these methods can be the basis for the development of further research techniques using immunocompetent cells from other insect species.

4.2. Second scientific achievement

A series of thematically-related scientific articles under the common title were submitted as the second scientific achievement of Dr Anna Katarzyna Wronska, making a significant contribution to Biological Sciences:

The role of lipids in the insect immune response

The second scientific achievement consists of a series of two thematically-related scientific articles (an original paper and a review paper), with the subject matter corresponding to its title. Both papers were published in journals listed in the Journal Citation Reports (JCR) database. In each of the mentioned articles, Anna Katarzyna Wrońska, PhD is the first author, and she played a dominant role in the preparation of the work (co-authors' statements concerning the percentage of participation in the preparation of the articles are included in Attachment 6).

The total number of points for the articles representing the scientific achievement is as follows: **MNiSW = 200 points**, **IF = 5.576** and **IF_{2023} = 5.7**. As dated 30.09.2024, the total number of cited articles, representing the scientific achievement is as follows: Google Scholar 45,Web of Science 28, Scopus 34.

IF_{2023}: current Impact Factor
IF: Impact Factor of the date of publication
P_{MNiSW}: point according to MNiSW guidelines dated 05.01.2024
$LC_{GoogleScholar}$: total citations Google Scholar
LC_{WoS}: total citations Web of Science
LC_{Scopus}: total citations Scopus
* corresponding author

- **A1.** Wrońska A. K.*, Boguś M. I., Włóka E., Kazek M., Kaczmarek A., Zalewska K. (2018) Cuticular fatty acids of *Galleria mellonella* (Lepidoptera) inhibit fungal enzymatic activities of pathogenic *Conidiobolus coronatus*. PLoS One 13(3): e0192715.

IF_{2023} = 2.9; IF = 2.776; P_{MNiSW} = 100; $LC_{GoogleScholar}$ = 36; LC_{WoS} = 23 ; LC_{Scopus} = 29

The candidate's contribution to the publication consisted of proposing the topic of the study, formulating the study concept, scope, objective and hypothesis, preparing the literature review on cuticular lipids in insects, formulating the study conclusions and their discussion. She also developed the methods for the determination of fatty acids in *G. mellonella*, participated in all laboratory experiments, processed the findings and prepared the content of the manuscript. She was also the corresponding author in the publication process.

The participation of the other authors consisted of co-performing laboratory experiments and analysing their results, providing substantive consultation in the development of the research methods, assisting in the management of the insect and fungus cultures, and providing comments on the first version of the manuscript. The lack of a statement regarding participation in the publication by one of the co-authors is due to the lack of contact possibilities.

The contribution of the candidate was 60%.

- **A2.** Wrońska A. K., Kaczmarek A.*, Boguś M. I., Kuna A. (2023) Lipids as a key element of insect defense systems. Frontiers in Genetic 14: 1183659.

IF_{2023} = 2.8; IF = 2.8; P_{MNiSW} = 100; $LC_{GoogleScholar}$ = 9; LC_{WoS} = 5 ; LC_{Scopus} = 5

The contribution of the candidate to the publication consisted of proposing the topic of the article, formulating the concept, scope, hypothesis and structure of the article, preparing the literature review and the text of the manuscript.

The other authors provided consultation in the formulation of the concept, hypothesis and structure of the article, preparation of the manuscript text excerpts and comments on the first version of the publication.

The contribution of the candidate was 55%.

4.2.1. Introduction

Since the Nobel Prize in Medicine was awarded in 1964 for research into lipid metabolism, their the function and biochemistry has received increasing attention. Although most of this research has been conducted on mammals, insects are growing in popularity as research models.

Lipids are present in many tissues and organs in insects, but the main storage and metabolic site is the fat body (FB). The amount of lipids in tissues varies and depends on various factors, such as developmental stage, nutritional status, sex and environmental temperature. In most species, females accumulate more fat than males, which is related to their use for egg production. Lipid metabolism begins in the midgut after food intake. Lipid compounds are first digested by lipases and the products are transported by lipophorins to the FB, ovaries and muscle. From here, fatty acids enter the target cells via proteins that bind and transport them [79].

The lipids comprise a diverse group of chemical compounds that include fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterols and prenols. They have many physiological and pathological functions in insects. They are the main energy reserve material for many processes such as embryogenesis, growth, development, metamorphosis, diapause, reproduction and prolonged flight, and play a key role in overwintering and enabling survival during periods of food shortage [80]. In holometabolous insects, the lipid content of the body increases steadily during larval development, although this increase is not proportional between

larval stages. Approximately 95% of the energy required for metamorphosis comes from the oxidation of fatty acids. In migratory insects, during intensive lipogenesis, carbohydrates from the diet are converted to lipids and stored in the body as triglycerides [81]. The lipid layer covering the cuticle serves as the main barrier preventing water loss in insects, allowing them to live in a variety of environments [82].

Lipids also play a key role in immunological processes. The first barrier protecting the insect body from pathogen entry is the cuticle. Its lipid components have protective and antimicrobial properties. Lipids are also important for the functioning of cellular and humoral response mechanisms, and the fat body is of key importance in insect immunity. Also important is the role of apoliporoteins (Apo) and lipid metabolites such as eicosanoids in the body's defence against infection [31]. These mechanisms are discussed in the publications forming this scientific achievement.

4.2.2. Aims and methods of research

The main aim of the research is to understand of the role of lipids in the immune response of insects. To achieve this , specific objectives were formulated for each of the included publications.

The aim of publication [A1] was to determine the relationship between cuticular lipid composition in *G. mellonella* and the susceptibility of its cuticle to digestion by enzymes produced by the entomopathogenic fungus *C. coronatus*.

Galleria mellonella was reared under optimal conditions, i.e. temperature 30°C, humidity 70%, constant darkness, with *ad libitum* access to food. The insects were fed with artificial medium according to Sehnal [4]. The experiments used larvae on the fifth day of the last (seventh) larval stage, pupae on the second day after pupation, and imagoes on the second day after imaginal moulting. The study used *C. coronatus* isolate 3747 from the collection of Prof. S. Bałaze (Department of Agricultural and Forest Environment Research, Polish Academy of Sciences, Poznań) cultured on SABG solid medium. In order to obtain a mixture of fungal enzymes hydrolysing the insect cuticle, *C. coronatus* was cultured at 20°C in 500 ml Erlenmeyer flasks containing 250 ml minimal medium (MM), as described by Bania et al. After three weeks of culture, the mycelium was removed by filtration using Whatman No. 1 filter paper. For cuticle hydrolysis studies, cell-free filtrates were used.

The insect cuticle obtained at different larval stages was isolated by precise separation from the muscle and fat body in ice-cold 10 mM Tris-HCl buffer, pH 7.0. All isolated fragments were washed three times with the buffer and stored at -20°C until further experiments.

Elastase, N-acetylglucosaminidase (NAGase), chitinase and lipase activities were measured in cell-free *C. coronatus* filtrates. Enzyme activity was measured spectrophotometrically or spectrofluorimetrically using appropriate synthetic substrates. The publication describes in detail the buffers used and the methods for measuring enzyme activity.

Cuticle samples (detailed in the publication) were incubated for eight hours at 30°C with *C. coronatus* filtrate with known activity of the tested enzymes. Two negative controls were used: the first containing 1 mg of cuticle in reaction buffer without fungal filtrate (C1); the second containing fungal filtrate without insect cuticle (C2). The cuticle samples were hydrolysed by the fungal proteases, giving free amino acids whose concentration was measured by reaction with picryl sulphonic acid. The amounts of N-glucosamine released from cuticle hydrolysed by fungal chitinases were measured using the D-glucosamine Assay Kit (Megazyme), according to the manufacturer's instructions. The concentrations of free fatty acids released by lipases were determined using an EnzymChrom™ Free Fatty Acid Assay Kit (BioAssay Systems).

The composition of the cuticular lipids of larvae, pupae and imago of *G. mellonella* was determined using GC-MS. A lipid extraction method was developed using petroleum ether and dichloromethane and derivatisation of fatty acids to trimethylsilyl esters. The analysis was performed on a GCMS-QP2010 chromatograph (Shimadzu). The chromatographic column (DB-5 MS Zebron, Phenomenex), analysis temperature, helium flow rate and detector parameters were selected during the study design. All compounds were identified on the basis of mass spectra and mass-to-charge ratio of ions using NIST library 11. The mass spectrum of trimethylsilyl esters of fatty acids showed the presence of ions: M+ (molecular ion), [M-15]+ and fragment ions at m/z 117, 129, 132 and 145. 19-methylarachidic acid was used as an internal standard.

The aim of publication [A2] was to review the literature, and discuss the effects of lipids on insect immune mechanisms with particular emphasis on those involved during fungal infection. This review paper focuses on the most recent literature in this topic. Extensive use was made of online publication databases such as PubMed, Google Scholar, Web of Science Core Collection and Scopus.

4.2.3. Synthesis of study results

The cuticle plays a key role in insect defence processes against entomopathogens, and is mainly composed of proteins, which make up about 70% of its total components. The remainder is made up of chitin and lipids such as free fatty acids (FFA), fatty acid esters and acylglycerols. The thickness and hardness of the cuticle provide considerable resistance to infection, particularly the thickness of the cuticle, its degree of cross-linking with proteins and the hardness of the body cover [83].

The first layer of the cuticle exposed to pathogens is the epicuticle. It contains hardened lipoproteins with phenolic compounds and phenoloxidases. These proteins are extracellular enzymes that hydroxylate tyrosine and oxidise o-diphenols to quinones. The quinones then undergo a series of enzymatic and non-enzymatic reactions leading to polymerisation, and the synthesis of melanin. In the presence of entomopathogens, the phenolic compounds oxidise to dihydroxyphenylalanine, resulting in brown-black pigmentation. Melanin protects the cuticle from enzymes and toxins produced by fungi [84].

The presence of specific lipids in the cuticle can inhibit or promote spore adhesion, thus reducing or increasing the efficacy of various entomopathogens. Although the hydrophobic nature of the epicuticle favours spore adhesion, insects have evolved a number of adaptations that inhibit spore attachment. For example, *Liposcelis bostrychophila* deposits fatty acid amides on the surface of the cuticle, which prevent the attachment of conidia [85]. The free fatty acids present on the cuticle surface of various insect species have also been found to inhibit the effects on particular entomopathogens [86].

Accordingly, publication [A1] investigated the ability of enzymes produced by *C. coronatus* to hydrolyse the *G. mellonella* cuticle and correlate this process with the free fatty acid composition of the cuticle. It indicates that *C. coronatus* produces chitinolytic, proteolytic and lipolytic enzymes that are capable of digesting the cuticle of wax moths. The cuticle from the thorax of adult individuals was the most susceptible to digestion by chitinases, while that of the larvae was the least susceptible. Proteinases and lipases showed the greatest ability to digest the larval and imago thorax cuticles. It can therefore be concluded that the composition of the cuticle in *G. mellonella* varies between developmental stages.

GC-MS analysis confirmed differences in the composition of free fatty acids in the cuticle of *G. mellonella* between stages. Eleven FFAs were detected in larvae, 10 in pupae and 14 in adults. Nine acids (C8:0, C9:0, C12:0, C14:0, C15:0, C16:1, C16:0, C18:1 and C18:0) were

present in all stages. It was found that C16:0, C18:0 and C18:1 fatty acids predominated in adults, C16:0 and C18:0 (to a lesser extent C18:1) in pupae, and C16:0 and C18:1 in larvae.

An r-Pearson correlation coefficient was also calculated for the relationship between the concentration of identified FFAs and the efficiency of cuticle digestion by proteases, chitinases and lipases produced by C. coronatus. Proteolytic degradation of cuticle was positively correlated with concentrations of C10:0, C13:0, C15:0, C17:0 and C18:0 and negatively correlated with concentrations of C12:0, C16:1 and C20:1. The activity of chitinolytic and lipolytic enzymes was negatively correlated with concentrations of C10:0 and C16:0.

In publication [A2] reviewed the role of the cuticle as the first defence barrier of the insect organism and extensively discusses the antimicrobial properties of cuticular lipids. It focuses on the composition of cuticular lipids in different insect species and the mechanisms by which entomopathogens break the cuticular barrier. A detailed literature review was also carried out showing which cuticular lipids exhibit antimicrobial properties. Particular attention was paid to FFAs possessing strong antibacterial and antifungal properties present in various insect species.

This paper describes the mechanisms of cellular and humoral immunity in insects with a particular focus on lipids. It discusses *inter alia* the role of triacylglycerols, fatty acids (including arachidonic acid and octanoic acid) and cholesterol and its metabolites, including the hormone ecdysone.

It also examines the fat body, a tissue with a key role in insect immunity, with particular focus on its structure and constituent cells, and on the role of lipids in immune pathways such as IMD/NF-κB and Toll/REL1. One of the most important functions of the fat body is the production of antimicrobial peptides (AMPs). The paper discusses the division of these proteins, their occurrence and role in different insect species, and their antibacterial and antifungal properties. It also highlights the role of ecdysone in the Toll and Imd pathways, whose activation leads to the production of AMPs.

The paper also describes the role of lipid droplets (LDs) in insect immunity. These organelles are markers of inflammation in bacterial infections in insects, discussed using *D. melanogaster* as an example. It also examines the role of LDs in insect social immunity, as described in *Nipponaphis monzeni*.

The role of eicosanoids as metabolites of arachidonic acid is also highlighted. The occurrence and role of prostaglandins, leukotrienes and troboxanes in the immunity of different insect species is extensively discussed with reference to the author's own publications.

Furthermore, the paper discusses the role of apolipoproteins (Apo) such as diacylglycerol, phospholipids or sterols in cellular immunity; these are known to act as lipid transport proteins in insects. ApoLP-III stimulates *G. mellonella* plasmatocytes to phagocytose, encapsulate, nodulate and form haemocyte networks. As the role of these proteins in the activation of the Toll and Jak/STAT pathways and the production of lysozyme is also important, the study discusses the role of Apo in these processes and in antibacterial and antifungal resistance in different insect species.

Finally, lipid peroxidation was presented as a marker of inflammation. It was noted that in insects, lipid peroxidation correlates with inflammation during both bacterial and fungal infection.

4.2.4. Summary and conclusions

The relationship between entomopathogenic fungi and insects is a classic example of a coevolutionary arms race between pathogen and host: pathogens evolve mechanisms that increase their advantage over the host, and the host increasingly strengthens its defences. The present scientific achievement aims to describe the direct and indirect roles of lipids as an important defence mechanism during infections, especially those caused by fungi. Such defence mechanisms include anatomical and physiological barriers, as well as cellular and humoral response mechanisms. Entomopathogenic fungi have the unique ability to digest insect cuticle material by producing hydrolytic enzymes with chitin-, lipo- and proteolytic activities.

A key factor determining whether an insect species/developmental stage will be susceptible or resistant to fungal infection is the composition of the lipids covering the cuticle. Certain lipids may promote the adhesion of the invasive structures of a given fungus to the cuticle, while others may prevent it. In addition, some lipids show antifungal properties, while others can be used by the fungus as an energy source. **The results of [A1] confirm that the cuticular fatty acids influence the pathogenicity of entomopathogenic fungi. In addition, the different developmental stages of *G. mellonella* demonstrate qualitative and quantitative differences in FFA profiles, and these may be responsible for the heterogeneous efficiency of fungal enzymes in degrading cuticles from these stages. This phenomenon may explain the differential susceptibility of the insects to fungal infection.**

In addition, lipids play a key role in innate humoral immunity, participating in the biosynthesis of lysozyme as well as various other bactericidal proteins and polypeptides. The energy derived from lipid metabolism is used by haemocytes to migrate to the site of infection and for phagocytosis, nodulation and encapsulation, the main processes in the cellular response. One polyunsaturated fatty acid, arachidonic acid, is a substrate in the synthesis of eicosanoids, which play a key role in insect physiology and immunology. One important antifungal compound that can modulate the cellular response of insects is apolipoprotein III, which is also considered an important signalling molecule.

The literature review presented in [A2] indicates that lipids are among the key factors involved in all insect immune mechanisms, viz. the local, cellular and humoral responses.

4.3. Other achievements which are not included in the two main scientific achievements

In addition to the research work presented in the main and second scientific achievement, the candidate was involved in other scientific research, summarised below.

Analysis of the lipid composition of cuticles of different insect species

Włóka E., Kaczmarek A., Kamut M., Wrońska A. K., Zalewska K., Gołębiowski M., Boguś M. I. (2014) Susceptibility versus resistance of insects to fungal infection may result from differential lipolytic rates of their cuticles by fungal lipases affected by some epicuticular lipids. Acta Biochimica Polonica 61 Supplement 1/2014, 105. **IF_{2023} = 1.4; IF = 1.7; P_{MNiSW} = 70**

Boguś M. I., Włóka E., Wrońska A. K., Kaczmarek A., Kazek M., Zalewska K., et al. (2017) Cuticle hydrolysis in four medically important fly species by enzymes of the entomopathogenic fungus *Conidiobolus coronatus*. Medical and Veterinary Entomology 31(1):23-35. **IF_{2023} = 1.6; IF = 1.9; P_{MNiSW} = 140**

Kazek M.*, Kaczmarek A., Wrońska A. K., Boguś M. I. (2019) Diet influences the bacterial and free fatty acid profiles of the cuticle of *Galleria mellonella* larvae. PloS One 14(2): e0211697. **IF_{2023} = 2.9; IF = 2.74 P_{MNiSW} = 100**

Kaczmarek A., Boguś M. I., Włóka E., Wrońska A. K., Krawiel A., Kazek M., et al. (2020) The interaction between cuticle free fatty acids (FFAs) of the cockroaches *Blattella germanica* and *Blatta orientalis* and hydrolases produced by the entomopathogenic fungus *Conidiobolus coronatus*. PLoS One 15(7): e0235785. **IF_{2023} = 2.9; IF = 3.24 P_{MNiSW} = 100**

Kaczmarek A., Wrońska A. K., Kazek M., Boguś M. I. (2020) Metamorphosis-related changes in the free fatty acid profiles of *Sarcophaga* (*Liopygia*) *argyrostoma* (Robineau-Desvoidy, 1830) Scientific Report 10, 17337. **IF_{2023} = 3.8; IF = 4.379; P_{MNiSW} = 140**

Kaczmarek, A., Wrońska, A.K., Bogus, M.I., Kazek, M., Gliniewicz, A., Mikulak, E., et al. (2021). The type of blood used to feed *Aedes aegypti* females affects their cuticular and internal free fatty acid (FFA) profiles. PLoS One 16(4): e0251100. **IF_{2023} = 2.9; IF = 3.752; P_{MNiSW} = 100**

Włóka E., Boguś M. I., Wrońska A. K., Drozdowski M., Kaczmarek A., Sobich J., et al. (2022) Insect cuticular compounds affect *Conidiobolus coronatus* (Entomopthorales) sporulation and the activity of

enzymes involved in fungal infection. Scientific Reports 12(1), 13641. **IF_{2023} = 3.8; IF = 4.6; P_{MNiSW} = 140**

Boguś M. I., Kazek M., Drozdowski M., Kaczmarek A., Wrońska A. K. (2023) The Entomopathogenic Fungus *Conidiobolus coronatus* Has Similar Effects on the Cuticular Free Fatty Acid Profile of Sensitive and Resistant Insects. Insects 14 (11): 895. **IF_{2023} = 2.7; IF = 2.7; P_{MNiSW} = 100**

The cuticle, particularly its lipid layer, plays a key role in insect defence against infection. Therefore, it would be valuable to compare the cuticular lipid composition between in insect species that are susceptible and resistant to infection. The resistant species *Calliphora vicina, Calliphora vomitoria, Lucilia sericata, Musca domestica, Blattella germanica, Blatta orientalis, Sarcophaga (Liopygia) argyrostoma, Aedes aegypti* and the susceptible species *Galleria mellonella* were used in the above experiments. The results showed that some of the fatty acids present in the cuticle of insects resistant to *C. coronatus* infection have the ability to inhibit its growth, sporulation, virulence and toxicity; they also affect the activity of fungal enzymes responsible for the degradation of the cuticle. Differences in cuticular lipid composition also depend on the developmental stage of the insect. Furthermore, it has been shown that in *G. mellonella* and *A. aegypti*, the fatty acid profile is influenced by the food used.

In the present study, the candidate assisted in the development of methods for the extraction and chromatographic determination of free fatty acids in insect cuticle. She participated in the laboratory experiments and in interpreting the results. In addition, she developed the method and identified the microflora of *G. mellonella* larvae fed artificially and naturally. In all publications, she consulted individual versions of manuscripts.

Dodecanol and octanoic acid - metabolites of the entomopathogenic fungus *C. coronatus*

Kazek M., Kaczmarek A., Wrońska A. K., Boguś M. I. (2021) Dodecanol, metabolite of entomopathogenic fungus *Conidiobolus coronatus*, affects fatty acid composition and cellular immunity of *Galleria mellonella* and *Calliphora vicina*. Scientific Reports 11(1), 15963. **IF_{2023} = 3.9; IF = 4.996; P_{MNiSW} = 140**

Kaczmarek A., Wrońska A. K., Kazek M., Boguś, M. I. (2022) Octanoic Acid-An Insecticidal Metabolite of *Conidiobolus coronatus* (Entomopthorales) That Affects Two Majors Antifungal Protection Systems in *Galleria mellonella* (Lepidoptera): Cuticular Lipids and Haemocytes. International Journal of Molecular Science 23(9). **IF_{2023} = 4.9; IF = 5.6; P_{MNiSW} = 140**

Kaczmarek A., Wrońska A. K., Boguś M. I. (2024) Octanoic acid kills *Lucilia sericata* (Diptera: Calliphoridae) by affecting two major defence systems: Cuticular free fatty acids and immunocompetent cells. Journal of Invertebrate Pathology 206:108165. **IF_{2023} = 3.6; IF = 3.6; P_{MNiSW} = 140**

The entomopathogenic fungus *C. coronatus* produces a number of toxic metabolites that affect the insect immune system. Chromatographic analysis confirmed the presence of dodecanol and octanoic acid in the post-culture fungal filtrates. The results showed that topical administration of dodecanol to *G. mellonella* and *C. vicina* larvae caused significant changes in cuticular lipid composition. In addition, the alcohol negatively affected the morphology and function of haemocytes isolated from both insect species and caused complete breakdown of cells from the Sf9 line 48 hours *in vitro*. Analogous observations were made after the administration of octanoic acid to *G. mellonella* and *L. sericata* larvae at LD50 and LD100 doses. This acid was also found to induce apoptosis of wax moth haemocytes, activation of caspases 1-9 and elevation of 8-OHdG levels in the haemolymph. These findings indicate that some metabolites of entomopathogenic fungi may be used to damage the insect immune system.

In the above-mentioned studies, the candidate helped develop the methods and performed the chromatographic determination of fatty acids in the insects. She also consulted on the methodology of the other experiments, participated in the interpretation of the results and made comments on the different versions of the manuscripts.

Apoptosis and autophagy of haemocytes of infected insects

Kazek M., Kaczmarek A., Wrońska A. K., Boguś M. I. (2020) *Conidiobolus coronatus* induces oxidative stress and autophagy response in *Galleria mellonella* larvae. PLoS One 15(2): e0228407. **IF_{2023} = 2.9; IF = 3.24; P_{MNiSW} = 100**

Kaczmarek A., Wrońska A.K., Boguś M. I. (2023) The Changes in Mitochondrial Morphology and Physiology Accompanying Apoptosis in *Galleria mellonella* (Lepidoptera) Immunocompetent Cells during *Conidiobolus coronatus* (Entomophthorales) Infection. International Journal of Molecular Science 24(12): 10169. **IF_{2023} = 4.9; IF = 4.9; P_{MNiSW} = 140**

Infection with *C. coronatus* causes morphological changes in haemocytes and the breakdown of these cells in *G. mellonella* larvae. It was therefore important to identify the pathological mechanisms taking place in the immunocompetent cells. The above publications indicate that fungal infection causes oxidative stress, peroxidation of cell membrane lipids and autophagy of wax moth haemocytes. In addition, apoptosis is associated with mitochondrial damage. Fungal infection has been found to induce loss of mitochondrial membrane potential, megacanal formation, impaired intracellular respiration, reduced extracellular and intracellular oxygen consumption and increased extracellular pH. Immunocompetent *G. mellonella* cells were also shown to exhibit increased Ca^{2+} concentrations in the mitochondria, translocation of cytochrome c-like protein from the mitochondrial to cytosolic fraction and higher activation of caspase-9-like protein after *C. coronatus* infection. In summary, the findings suggest that an

important mechanism of action of entomopathogenic fungi is their ability to cause destructive changes in haemocytes, making it difficult for insects to activate multiple immune pathways.

The candidate's contribution to the above publications consisted of substantive consultation on research methods, participation in laboratory experiments and inputting comments on individual versions of the manuscripts.

Effects of European mistletoe extracts on the nervous system of the model insect *G. mellonella*

Szurpnicka, A., Wrońska A. K., Bus K., Kozinska A., Jabłczyńska R., Szterk A., et al. (2022) Phytochemical screening and effect of *Viscum album L.* on monoamine oxidase A and B activity and serotonin, dopamine and serotonin receptor 5-HTR1A levels in *Galleria mellonealla* (Lepidoptera). Journal of Ethnopharmacology 298, 115604. **IF_{2023} = 4.8; IF = 5.4; P_{MNiSW} = 140**

Viscum album L. (European mistletoe) has been used in traditional and folk medicine to treat central nervous system disorders such as epilepsy, hysteria, insomnia, nervous excitability, neuralgia, headache, dizziness and fatigue. This article investigates its neuropharmacological activity using *G. mellonella* larvae as a research model. In total, 88 compounds were identified in the mistletoe extracts using UPLC-DAD-ESI-MS/MS analysis, with flavonoids, hydroxycinnamic acids and lignans predominating. Aqueous and hydroethanolic mistletoe extracts inhibited the enzymatic activity of MAO-A and MAO-B and increased serotonin and serotonin receptor 5-HTR1A levels in wax moth head capsules. None of the tested extracts had a significant effect on dopamine levels. The results confirmed that *G. mellonella* can be used as a model in the study of new active substances in Pharmacology.

The candidate’s participation consisted of developing the methods, performing the experiments and interpreting the results. In addition, she was involved in the preparation of the content of the manuscript and consulted on the hypotheses and assumptions of the work.

4.4. Literature

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5. Information on the demonstration of significant scientific or artistic activity in more than one university, research institution or cultural institution, particularly abroad

The candidate began her scientific activity as a doctoral student at the Faculty of Human Nutrition and Consumer Sciences of the Warsaw University of Life Sciences. She then became an Assistant Professor at the Institute of Parasitology of the Polish Academy of Sciences, and later at the Museum and Institute of Zoology of the Polish Academy of Sciences. She has also collaborated with other research units from Poland and abroad.

- Before receiving the title of Doctor

<u>The scientific activity within the doctoral studies at the Faculty of Human Nutrition and Consumer Sciences of the Warsaw University of Life Sciences included:</u>

- Co-authorship of five scientific articles. Two publications were literature reviews: the first on deoxynivalenol mycotoxin in raw materials and food products, and the second on the benefits and risks of minimally-processed food production. The others were experimental papers: one on the evaluation of the microflora of the air of household refrigerators, and two others on the antibacterial properties of nanosilver particles.
- Participation in four scientific conferences, including lecturing in a panel session and leading workshops.
- The realisation of a project for young researchers funded by the School of Life Sciences entitled: „Influence of maternal and child nutrition on the development of *Candida albicans* infections in the neonatal period".

- After obtaining a doctoral degree

<u>Scientific activities within the work at the Institute of Parasitology, Polish Academy of Sciences:</u>

- Co-authorship of articles published in scientific journals. Publications not included in the main and second scientific achievements were related to the following:
    - analyses of the composition of cuticular lipids of insects of the species *Calliphora vicina, Calliphora vomitoria, Lucilia sericata, Musca domestica, Blattella germanica, Blatta orientalis, Sarcophaga* (*Liopygia*) *argyrostoma, Aedes aegypti;*
    - effect of the diet on the microflora in *G. mellonella* larvae*;*

- *in vitro* screening of 65 mycotoxins for insecticidal potential;
- effects of octanoic acid and dodecanol (metabolites of *C. coronatus*) on insect immune function;
- oxidative stress and autophagy in *G. mellonella* during fungal infection;
- a literature review on insect resistance mechanisms and their overpowering by entomopathogenic fungi.

- Authorship or co-authorship of reports at eight national and international conferences.
- Realisation and management of the project " The influence of selected metabolites of the fungus *Conidiobolus coronatus* belonging to the β-carboline alkaloid group on the nervous system of *Galleria mellonella* (*Lepidoptera*) larvae". funded from the grants for young scientists of the Institute of Parasitology, Polish Academy of Sciences, Warsaw.
- Realisation and management of the Miniatura 1 project "The effect of harman and norharman, metabolites of the entomopathogenic fungus *Conidiobolus coronatus*, on the phagocytic activity of haemocytes of *Galleria mellonella* larvae" funded by the National Science Centre. The findings were published in two articles and formed the basis for applying for the Sonata project:

  Wrońska, A. K., Boguś M. I., Kaczmarek A. and Kazek M. (2018). Harman and norharman, metabolites of entomopathogenic fungus *Conidiobolus coronatus* (Entomopthorales), disorganize development of *Galleria mellonella* (Lepidoptera) and affect serotonin-regulating enzymes. *PLoS One* 13(10): e0204828.
  IF_{2023} = 2.9; IF = 2.776 P_{MNiSW} = 100 (publication included in the main achievement)

  Wrońska, A. K., Boguś M. I. (2019). Harman and norharman, metabolites of the entomopathogenic fungus *Conidiobolus coronatus* (Entomophthorales), affect the serotonin levels and phagocytic activity of haemocytes, insect immunocompetent cells, in *Galleria mellonella* (Lepidoptera). *Cell and Bioscience* 9: 29.
  IF_{2023} = 6.1; IF = 5.026; P_{MNiSW} = 100 (publication included in the main achievement)
- Realisation and management of the Sonata 15 Project entitled: "Use of *Galleria mellonella* larvae as an alternative to mammalian research models - determination of selected cytokine-like proteins in haemocytes after infection with the fungus *Conidiobolus coronatus* and after exposure to its metabolites", funded by the National Science Centre. The findings were presented at international conferences and published in six scientific articles:

  Wrońska, A. K., Kaczmarek A., Kazek M. and Boguś M. I. (2021). Infection of *Galleria mellonella* (Lepidoptera) larvae with the entomopathogenic fungus *Conidiobolus coronatus*

(Entomophthorales) induces apoptosis of haemocytes and affects the concentration of eicosanoids in the haemolymph. *Frontiers in Physiology* 12: 774086.
IF_{2023} = 3,2; IF = 4,75; P_{MNiSW} = 100 (publikacja wchodząca w skład głównego osiągnięcia)

Wrońska, A. K., Kaczmarek A., Sobich J., Grzelak S. and Boguś M. I. (2022). Intracellular cytokine detection based on flow cytometry in haemocytes from *Galleria mellonella* larvae: A new protocol. *PLoS One* 17(9): e0274120.
IF_{2023} = 2,9; IF = 3,70 P_{MNiSW} = 100 (publikacja wchodząca w skład głównego osiągnięcia)

Włóka E., Boguś M. I., **Wrońska A. K.,** Drozdowski M., Kaczmarek A., Sobich J., et al. (2022) Insect cuticular compounds affect *Conidiobolus coronatus* (Entomopthorales) sporulation and the activity of enzymes involved in fungal infection. *Scientific Reports* 12(1), 13641.
IF_{2023} = 3,8; IF = 4,6; P_{MNiSW} = 140

Wrońska A. K., Kaczmarek A., Boguś M. I., Kuna A. (2023) Lipids as a key element of insect defense systems. *Frontiers in Genetic* 14: 1183659.
IF_{2023} = 2,8; IF = 2,8; P_{MNiSW} = 100 (publikacja wchodząca w skład drugiego osiągnięcia)

Kaczmarek A., **Wrońska A.K**., Boguś M. I. (2023) The Changes in Mitochondrial Morphology and Physiology Accompanying Apoptosis in *Galleria mellonella* (Lepidoptera) Immunocompetent Cells during *Conidiobolus coronatus* (Entomophthorales) Infection. *International Journal of Molecular Science* 24(12): 10169.
IF_{2023} = 4,9; IF = 4,9; P_{MNiSW} = 140

Sobich, J., **Wrońska A.K.**, Kaczmarek A., Boguś M. I. (2023). Changes in histamine, HSF1, Cysteinyl leukotriene, TLR1 and TLR2 in *Galleria mellonella* haemolymph after *Conidiobolus coronatus* infection. *The European Zoological Journal* 90(2): 762-774.
IF_{2023} = 1,6; IF = 1,6; P_{MNiSW} = 140 (publikacja wchodząca w skład głównego osiągnięcia)

Wrońska, A. K., Kaczmarek A., Sobich J., Boguś M. I. (2024). The effect of infection with the entomopathogenic fungus *Conidiobolus coronatus* (Entomopthorales) on eighteen cytokine-like proteins in *Galleria mellonella* (Lepidoptera) larvae. *Frontiers in Immunology* 15: 1385863
IF_{2023} = 5,7; IF = 5,7; P_{MNiSW} = 140 (publikacja wchodząca w skład głównego osiągnięcia)

- Participation as a worker and coordinator in the project entitled: "Development of an insecticide preparation based on identified metabolites of the entomopathogenic fungus *Conidiobolus coronatus*", funded by the National Centre for Research and Development (project leader Prof. Dr. Mieczysława Boguś). The candidate conducted studies on chromatographic determination of metabolites of the fungus *C. coronatus* and their effects on various physiological processes in insect species *in vitro* and *in vivo*. The project has resulted in 17 scientific publications

- Participation as a worker in the project entitled: “Development of methods for comprehensive analysis of the content of biological and chemical contaminants posing a threat to consumer health in herbal products”, funded by the European Regional Development Fund (project leader Prof. Dr. Mieczysława Boguś). The candidate analysed microbiological contaminants and the developed a molecular diagnostic test for the detection of parasites in herbal products. She also participated in the study of chemical pollutants such as pesticides, dioxins, polychlorinated biphenyls, aromatic hydrocarbons and heavy metals. The project has resulted in eight scientific publications.
- Cooperation with Dr Marek Gołębiewski (University of Gdansk, Faculty of Chemistry, Laboratory of Natural Compounds Analysis) on the development of chromatographic methods for the determination of lipids in insects and chemical impurities in herbal products, resulting in joint publications:

  Włóka E., Kaczmarek A., Kamut M., **Wrońska A. K**., Zalewska K., Gołębiowski M., Boguś M. I. (2014) Susceptibility versus resistance of insects to fungal infection may result from differential lipolytic rates of their cuticles by fungal lipases affected by some epicuticular lipids. *Acta Biochimica Polonica* 61 Supplement 1/2014, 105.
  IF_{2023} = 1.4; IF = 1.7; P_{MNiSW} = 70

  Boguś M. I., Włóka E., **Wrońska A. K.,** Kaczmarek A., Kazek M., Zalewska K., i in. (2017) Cuticle hydrolysis in four medically important fly species by enzymes of the entomopathogenic fungus *Conidiobolus coronatus*. *Medical and Veterinary Entomology* 31(1):23-35.
  IF_{2023} = 1.6; IF = 1.9; P_{MNiSW} = 140

  Boguś M. I., **Wrońska A. K.,** Kaczmarek A., Drozdowski M., Laskowski Z., Myczka A., Cybulska A., Gołębiowski M., Chwir-Gołębiowska A., Siecińska L., Mokijewska E. (2023) A comprehensive analysis of chemical and biological pollutants (natural and anthropogenic origin) of soil and dandelion (Taraxacum officinale) samples. *PLoS One* 18, e0280810.
  IF_{2023} = 2.9; IF = 2.9; P_{MNiSW} = 100

- Collaboration with Dr Aleksandra Gliniewicz (National Institute of Public Health - National Institute of Hygiene) to investigate differences in fatty acid composition in artificially and naturally-fed mosquitoes. Collaboration resulted in a scientific article:

  Kaczmarek, A., **Wrońska, A.K.,** Bogus, M.I., Kazek, M., Gliniewicz, A., Mikulak, E., i in. (2021). The type of blood used to feed *Aedes aegypti* females affects their cuticular and internal free fatty acid (FFA) profiles. *PLoS One* 16(4): e0251100.
  IF_{2023} = 2.9; IF = 3.752; P_{MNiSW} = 100

- Collaboration with Prof. Dr. Arkadiusz Szterek and Anna Szurpnicka MSc. (National Institute of Medicines) in studies of the effects of *Viscum album* L. on monoamine oxidase

A and B activity and serotonin, dopamine and serotonin receptor 5-HTR1A levels in *G. mellonella*, resulting in a joint publication:

> Szurpnicka, A., **Wrońska A. K.,** Bus K., Kozinska A., Jabłczyńska R., Szterk A., i in. (2022) Phytochemical screening and effect of *Viscum album L.* on monoamine oxidase A and B activity and serotonin, dopamine and serotonin receptor 5-HTR1A levels in *Galleria mellonealla* (Lepidoptera). *Journal of Ethnopharmacology* 298, 115604.
> **IF_{2023} = 4.8; IF = 5.4; P_{MNiSW} = 140**

- Collaboration with Prof. Gianluca Tettamanti (University of Insubria, Varese, Italy) to study the morphology and function of haemocytes of *Hermetia illucens* larvae. The candidate determined PLA2 activity and prostaglandin E2 concentration in the cells studied, compiled the results of these experiments and discussed them in the manuscript. While the candidate was due to undertake a planned internship at the University of Insubria as part of the collaboration, it could not be realised due to the COVID19 virus pandemic. Despite the obstacles related to the global epidemic, collaboration yielded a publication:

> Bruno D., Montali A. Gariboldi M., **Wrońska A. K.,** Kaczmarek A., Mohamed A., Tian L., Casartelli M. Tettamanti, G. (2022) Morphofunctional characterization of haemocytes in black soldier fly larvae. *Insect Science* 30(4): 912-932.
> **IF_{2023} = 2.9; IF = 4.0; P_{MNiSW} = 70**

<u>Scientific activities within the framework of work at the Museum and Institute of Zoology of the Polish Academy of Sciences:</u>

- Co-authorship of articles published in scientific journals. The publications included some not included in the main and second scientific achievements. These were related to the following:

  - comprehensive analysis of chemical and biological contaminants, of natural and anthropogenic origin, of soil and dandelion (*Taraxacum officinale*) samples;

  - changes in mitochondria and apoptosis in haemocytes of *G. mellonella* larvae after fungal infection;

  - the effect of fungal infection on lipid composition in different insect species;

  - effects of octanoic acid (a metabolite of *C. coronatus*) on the immune system of *Lucilla sericata;*

  - literature reviews on: the role of minerals in immunity, the antimicrobial properties of propolis and *Candida albicans* in the oral cavity.

- Co-authorship of a report at an international conference.

- Co-authorship of a chapter in a scientific monograph:

  Kaczmarek, A., **Wrońska, A.K.,** Sobich, J., Boguś, M.I., (2024): Insect Lipids: Structure, Classification, and Function. In: Advances in Experimental Medicine and Biology. Springer, Cham. https://doi.org/10.1007/5584_2024_805.

- - Continuation of the Sonata 15 Project "Use of *Galleria mellonella* larvae as an alternative to mammalian research models - determination of selected cytokine-like proteins in haemocytes after infection with the fungus *Conidiobolus coronatus* and after exposure to its metabolites" funded by the National Science Centre.
- Participation as a contractor in the Prelude 20 Project "Pharmacological potential of mistletoe (Viscum album L.) in the prevention of diseases of the central nervous system" funded by the National Science Centre in cooperation with the National Institute of Medicines. The candidate's participation consisted of studies on the effects of common mistletoe on the nervous system, using *G. mellonella* larvae as a research model.
- Collaboration with Prof. Magdalena Rost-Roszkowska (University of Silesia, Faculty of Life Sciences) on research into the digestion of polypropylene by *G. mellonella* larvae. The joint research resulted in a publication:

  Rost-Roszkowska M., Mermer P., Chajec Ł., Sosińska A., Wilczek G., Student S., **Wrońska A. K.,** Karnówka O. (2024) Consumption of polypropylene caused some ultrastructural and physiological changes in some tissues of *Galleria mellonella* (Lepidoptera: Pyralidae) larvae. The European Zoological Journal 91(1): 213-234.
  IF_{2023} = 1.6; IF = 1.6; P_{MNiSW} = 140.

6. Information on achievements in teaching, organisation and popularisation of science or arts

6.1. Teaching achievements

Prior to obtaining a doctoral degree:

- Conducting laboratory classes at the Faculty of Human Nutrition and Consumption Sciences of the Warsaw University of Life Sciences in the following subjects: Biology, general Food Technology, food for special nutritional purposes, convenience and functional food, and sensory analysis of food.

After obtaining a doctoral degree:

- Associate Supervisor in the doctoral dissertation of Dr Agata Kaczmarek; dissertation title: „Effects of metabolites of the entomopathogenic fungus Conidiobolus coronatus:

octanoic acid, 2-octenoic acid and 2,6-dimethylphenol on selected physiological processes of *Galleria mellonella* and *Lucilia sericata*". Date of defence: 11.12.2018

- Supervision of summer internship students at the Institute of Parasitology, Polish Academy of Sciences.
- Conducting lectures on Allergology for full-time and part-time students of Dietetics and Cosmetology at the Academy of Economics and Humanities in Warsaw.

6.2. Organisational achievements

Prior to obtaining the doctoral degree:

- Member of the organising committee on behalf of doctoral students at the "Human Nutrition Science - Achievements and Challenges" International Jubilee Conference on the occasion of the 35th anniversary of the Faculty of Human Nutrition and Consumer Sciences.
- Member of the scientific committee of the national "Breastfeeding – straight talking,, conference. Organisers: Department of Human Nutrition and Consumer Sciences and Medela Polska.

After obtaining the doctoral degree:

- Member of the Scientific Council of the Institute of Parasitology of the Polish Academy of Sciences and the Commission on Degrees and Titles.

6.3. Achievements in popularising Science

Prior to obtaining the doctoral degree:

- Conducting workshops on convenience and functional foods at the Faculty of Human Nutrition and Consumer Sciences stand during the SGGW Days in 2012 and 2013.
- Providing content consultation on the Medela (breast pump manufacturer) website regarding the benefits of breastfeeding newborn babies.
- Content support for the creation of information brochures for the Breastmilk Banks Foundation.
- Lectures on the benefits of breastfeeding for mother and child at the birthing school, Ursynów Health Centre.

After obtaining the doctoral degree:

- Conducting workshops as part of the Festival of Science at the Institute of Parasitology of the Polish Academy of Sciences for high school students. The workshops were entitled: “Mammalian blood and insect haemolymph - similarities and differences” and classes for primary school children entitled: “Insects - our allies or enemies” and „Insects on our side” in 2015, 2016 and 2017.
- Conducting workshops on detecting contaminants in herbal products and providing consultations for entrepreneurs on the subject as part of the 9th Mazovia Forum.
- Participation in a live report on social media (Facebook) on the profile of the European Funds for Mazovia concerning methods for the determination of chemical and biological contaminants.

7. Other career-related information

The candidate has received the following honours and awards:

- Distinction from the Council of the Faculty of Human Nutrition and Consumer Sciences for academic performance and for engineering and undergraduate work.
- Distinction of the Council of the Faculty of Human Nutrition and Food Sciences for the defence of the doctoral dissertation.
- Scholarship for the best doctoral students, awarded academic years 2011/2012, 2012/2013, 2013/2014.
- Scholarship from the grant for subsidising pro-quality tasks, awarded for academic achievements in the academic year 2012/2013 and 2013/2014.
- Awards from the Director of the Institute of Parasitology of the Polish Academy of Sciences for publications in high-impact scientific journals in 2017, 2018, 2019, 2021.

During the her research career, the candidate has attended the following training courses:

- Training in the operation of a gas chromatograph kit with Shimadzu GC-MS 2010 mass detector and LabSolution software and NIST spectra library.
- Training in the operation of a liquid chromatograph kit with Shimadzu LC-MS 2020 mass detector and LabSolution software.
- Training in the operation of the Smart Prep automated SPE and XcellVap sample concentration device.

- Training covering the principles of the Sysmex CyFlow Cube 8 flow cytometer and its CyView software, the CyFlow Rubby 8 sample feeder, the CyFlow Sorter cell sorter and, the FSC Express cytometric data analysis software; Warsaw, Poland.
- X Workshop for users of confocal and fluorescence microscopes, M. Nencki Institute of Experimental Biology, Polish Academy of Sciences.
- Training in the use of 2-D bi-directional electrophoresis kit from BioRad, Institute of Parasitology, Polish Academy of Sciences.
- Training in the analysis of flow cytometry results using FCS Express software.
- Training in the use of ECIS Model Z Theta device for the study of cell movements.
- On-line training „Western blotting - practically".

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(Applicant's signature)