Uniwersytet Śląski w Katowicach

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Instytut Biologii, Biotechnologii i Ochrony Środowiska

Wydział Nauk Przyrodniczych

Institute of Biology, Biotechnology and Environmental Protection

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Wpływ tlenku grafenu, nanocząstek srebra oraz kompozytu tlenku grafenu i nanocząstek srebra na funkcje pokarmowe *Acheta domesticus*

The effects of graphene oxide, silver nanoparticles and graphene oxide-silver nanoparticle composite on the nutritional functions of *Acheta domesticus*

Rozprawa doktorska

Doctoral thesis

Rozprawa doktorska wykonana pod kierunkiem:

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STRESZCZENIE

Rozwój nanotechnologii i nanonauki doprowadził do przełomowych osiągnięć w wielu dziedzinach. Dzięki zdolności do manipulacji materią na poziomie atomowym i molekularnym możliwe stało się opracowywanie materiałów o unikalnych właściwościach, które nie występują w skali makroskopowej. Nanocząstki i nanomateriały znalazły zastosowanie w medycynie i farmacji, elektronice, energetyce, produkcji szerokiej gamy materiałów o szczególnych właściwościach, produkcji żywności oraz jej przechowywaniu, monitorowaniu stanu środowiska i oczyszczaniu wody i powietrza. Rozwój ten otwiera nowe możliwości dla wielu branż, jednocześnie stawiając wyzwania w zakresie bezpieczeństwa i etyki, szczególnie jeśli chodzi o wpływ nanocząstek na zdrowie ludzkie i środowisko.

Chociaż nanocząstki mają niezwykły potencjał zastosowania w różnych dziedzinach, ich mały rozmiar i unikalne właściwości chemiczne mogą prowadzić do nieprzewidywalnych interakcji z organizmami żywymi i ekosystemami. Dlatego, przedostawanie się nanocząstek do środowiska, zwłaszcza w sposób niekontrolowany, budzi uzasadnione obawy. Wiedza na temat ich bioakumulacji i biomagnifikacji, wpływu na stan gleby, wody i powietrza, ich toksyczności dla organizmów, w tym zdrowia człowieka, pozostaje ciągle fragmentaryczna i niewystarczająca. Z powyższych względów badania oceniające wpływ nanocząstek na funkcje organizmów zajmują jedno z priorytetowych wyzwań nanonauki.

Niniejsza rozprawa doktorska skupia się na ocenie oddziaływania nanocząstek tlenku grafenu (GO), nanocząstek srebra (AgNPs) oraz kompozytu powstałego na bazie GO i AgNPs na funkcje pokarmowe modelowego organizmu *Acheta domesticus*. Badania obejmowały pomiar wybranych parametrów budżetu pokarmowego oraz aktywności enzymów trawiennych, zakładając, że wszelkie substancje obce (ksenobiotyki) powodują zaburzenia funkcji organizmu, które wymagają alokacji energii w celu neutralizacji efektów ich oddziaływania. Głównym celem badań było określenie zależności między ekspozycją na wyżej wymienione nanocząstki a konsumpcją/asymilacją pokarmu, aktywnością enzymów trawiennych oraz stanem komórek jelita *Acheta domesticus*. Poza rodzajem nanocząstek, uwzględnionymi parametrami były także stężenie oraz czas ekspozycji.

W pierwszej części badania (manuskrypt 1) przeanalizowano konsumpcję pokarmu oraz aktywność enzymów trawiennych u owadów narażonych na GO, AgNPs i kompozyt, zastosowanych w pojedynczych stężeniach, odpowiednio: 20 μg/g (GO), 400 μg/g (AgNPs), oraz 20/400 μg/g (GO/AgNPs). Wczesnym efektem było zmniejszenie asymilacji energii, po którym następowało kompensacyjne zwiększenie konsumpcji. Zmianom tym towarzyszyła

zwiększona aktywności niektórych enzymów trawiennych w pierwszych dniach ekspozycji na nanocząstki. Jakkolwiek, efekt ten zanikał po kilku dniach. Taka odpowiedź organizmu przypominała typową reakcję na stres, z wczesną fazą alarmową i późniejszą fazą oporności. Wyniki zostały przedyskutowane w kontekście teorii hormezy, ze względu na obserwowaną stymulację niektórych parametrów. Ponadto zasugerowano, że efekty hormetyczne mogą być lepiej zauważalne u osobników młodych, u których sumaryczne narażenie na różne ksenobiotyki jest niskie, a czas ekspozycji jest relatywnie krótki.

W drugiej części badań (manuskrypt 2) postanowiono przeanalizować efekty GO i AgNPs na funkcje pokarmowe *A. domesticus*, w szerokim zakresie stężeń oraz w kilku punktach czasowych, reprezentujących ważne etapy życia tego gatunku. W wyniku badań stwierdzono silniejsze efekty AgNPs w porównaniu do GO. Zauważono zwiększoną aktywność α-amylazy, α-glukozydazy i lipazy, przy jednoczesnym hamowaniu aktywności proteazy. Ponadto, stwierdzono, że długotrwałe narażenie na wyższe stężenia AgNPs u dorosłych świerszczy powodowało istotne zmniejszenie spożycia pokarmu i zmianę asymilacji w porównaniu z grupą kontrolną. Co ciekawe, wzrost masy ciała *A. domesticus* był obserwowany jedynie w grupie narażonej na najniższe stężenie AgNPs, co pozwala sądzić, że te nanocząstki nie stosują się do prostoliniowej zasady dawka-efekt, a u podłoża takich efektów mogą leżeć interakcje między nanocząstkami (aglomeracja) i substratami pokarmowymi, które, przy zwiększonym stężeniu nanocząstek, niwelują efekty toksyczne.

W eksperymencie trzecim (manuskrypt 3) skupiono się na ocenie oddziaływania kompozytu GO i AgNPs, w szerokim zakresie stężeń i czasu ekspozycji, na funkcje trawienne *A. domesticus*. Oceniono również zmiany histologiczne w komórkach jelita świerszczy narażonych na kompozyt. W wyniku badań ustalono, że zarówno stężenie kompozytu jak i czas ekspozycji istotnie wpłynęły na badane parametry. Kompozyt generalnie zmniejszał spożycie pokarmu i jego asymilację, a ilość reaktywnych form tlenu (ROS) w komórkach jelita wzrosła w grupach otrzymujących wyższe stężenie kompozytu. Ponadto, najwyższe stężenie kompozytu hamowało aktywność proteazy. Analiza histologiczna ujawniła strukturalne uszkodzenia komórek nabłonka jelita i oznaki autofagii lub martwicy przy wyższych stężeniach kompozytu.

Uzyskane w niniejszej pracy wyniki pokazują, że GO, AgNPs oraz kompozyt wytworzony z tych nanocząstek mogą destabilizować funkcje pokarmowe organizmów i, tym samym, nie mogą być uważane za w pełni bezpieczne dla środowiska, szczególnie gdy są stosowane w wysokich stężeniach lub w długotrwałej ekspozycji.

SUMMARY

The development of nanotechnology and nanoscience has led to groundbreaking advances across multiple fields. The ability to manipulate matter at the atomic and molecular level has enabled the creation of materials with unique properties that are not present on a macroscale. Nanoparticles and nanomaterials have found applications in medicine and pharmaceuticals, electronics, energy, the production of specialized materials, food production and preservation, environmental monitoring, as well as water and air purification. This progress opens new possibilities across various industries but also presents challenges in terms of safety and ethics, especially regarding the impact of nanoparticles on human health and the environment.

Although nanoparticles hold remarkable potential across diverse fields, their small size and unique chemical properties can lead to unpredictable interactions with living organisms and ecosystems. Therefore, the uncontrolled release of nanoparticles into the environment raises justified concerns. Knowledge on their bioaccumulation and biomagnification, impact on soil, water, and air quality, as well as their toxicity to organisms, including human health, remains fragmented and insufficient. For these reasons, research assessing the impact of nanoparticles on biological functions is one of the key challenges in nanoscience.

This doctoral dissertation focuses on assessing the impact of graphene oxide nanoparticles (GO), silver nanoparticles (AgNPs), and a composite made from GO and AgNPs on the digestive functions of the model organism *Acheta domesticus*. The study involved measuring selected parameters of the digestive budget and enzymatic activity, under the assumption that any xenobiotic disrupts the organism's functions and necessitates energy allocation for neutralizing its effects. The primary aim was to determine the relationship between exposure to the aforementioned nanoparticles and food consumption/assimilation, digestive enzyme activity, and intestinal cell health in *Acheta domesticus*. In addition to nanoparticle type, exposure concentration and duration were also considered parameters.

In the first part of the study (manuscript 1), food consumption and digestive enzyme activity were analyzed in insects exposed to GO, AgNPs, and the composite, administered in individual concentrations of 20 μg/g (GO), 400 μg/g (AgNPs), and 20/400 μg/g (GO/AgNPs). An early effect was a reduction in energy assimilation, followed by compensatory increases in consumption. These changes were accompanied by an initial increase in the activity of certain digestive enzymes in the first days of exposure. However, this effect diminished after several days, resembling a typical stress response with an initial alarm phase followed by resistance. The results were discussed in the context of hormesis theory, due to the observed stimulation

of some parameters. Furthermore, it was suggested that hormetic effects may be more noticeable in younger individuals, who have lower cumulative xenobiotic exposure and shorter exposure periods.

In the second part (manuscript 2), the effects of GO and AgNPs on the feeding functions of *A. domesticus* were analyzed over a wider range of concentrations and at different life stages of this species. The study showed stronger effects from AgNPs compared to GO, including increased activity of α-amylase, α-glucosidase, and lipase, accompanied by protease inhibition. Moreover, prolonged exposure to higher concentrations of AgNPs in adult crickets significantly reduced food consumption and altered assimilation compared to the control group. Notably, weight gain in *A. domesticus* was observed only in the group exposed to the lowest AgNPs concentration, suggesting that these nanoparticles may not follow a simple dose-response rule, with underlying interactions between nanoparticles (agglomeration) and food substrates that may mitigate toxic effects at higher concentrations.

The third experiment (manuscript 3) focused on the effects of the GO/AgNPs composite, at various concentrations and exposure durations, on *A. domesticus* digestive functions. Histological changes in intestinal cells were also assessed. Results indicated that both composite concentration and exposure time significantly influenced the evaluated parameters. In general, the composite reduced food intake and assimilation, while reactive oxygen species (ROS) levels in intestinal cells increased in groups exposed to higher composite concentrations. Additionally, the highest composite concentration inhibited protease activity. Histological analysis revealed structural damage to the intestinal epithelium and signs of autophagy or necrosis at higher composite concentrations.

The findings of this study indicate that GO, AgNPs, and their composite can destabilize the digestive functions of organisms and therefore should not be considered fully safe for the environment, especially when used in high concentrations or during prolonged exposure.

A. GENERAL INFORMATION

A.1. LIST OF PUBLICATIONS CONSTITUTING THE DOCTORAL DISSERTATION

Thesis title: "The effects of graphene oxide, silver nanoparticles and graphene oxide-silver nanoparticle composite on the nutritional functions of *Acheta domesticus*"

1. **Seyed Alian R**, Dziewięcka M, Kędziorski A, Majchrzycki Ł, Augustyniak M. Do nanoparticles cause hormesis? Early physiological compensatory response in house crickets to a dietary admixture of GO, Ag, and GOAg composite. Sci Total Environ. 2021; 788:147801. doi: 10.1016/j.scitotenv.2021.147801.

   IF: 8.2; MNiSW: 200 pkt

2. **Seyed Alian R**, Flasz B, Kędziorski A, Majchrzycki Ł, Augustyniak M. Concentration- and Time-Dependent Dietary Exposure to Graphene Oxide and Silver Nanoparticles: Effects on Food Consumption and Assimilation, Digestive Enzyme Activities, and Body Mass in *Acheta domesticus*. Insects. 2024;15(2):89.
   doi: 10.3390/insects15020089.

   IF: 2.7; MNiSW: 100 pkt

3. **Seyed Alian R**, Flasz B, Kędziorski A, Rost-Roszkowska M, Rozpedek K, Majchrzycki Ł, Augustyniak M. Concentration-dependent disturbances of digestive functions in house cricket (Insecta: Orthoptera) exposed to GO-AgNP composite. Scientific Reports. 2025; 15:12699.
   https://doi.org/10.1038/s41598-025-97589-w

   IF: 3.8; MNiSW: 140 pkt

A.2. LIST OF OTHER PUBLICATIONS, CONFERENCES, RESEARCH FUNDING AND INTERNSHIPS

Publications

1. Hayati N, Amirinia S, Parivar K, **Seyed Alian R**. Replacing antibiotics with nanosilver in bull sperm extenders. New Cellular and Molecular Biotechnology. 2013, 3(10), 59-65. http://ncmbjpiau.ir/article-1-335-en.html

   IF: 0.0; MNiSW: 0 pkt

2. Augustyniak M, Babczyńska A, Dziewięcka M, Flasz B, Karpeta-Kaczmarek J, Kędziorski A, Mazur B, Rozpędek K, **Seyed Alian R**, Skowronek M, Świerczek E, Świętek A, Tarnawska M, Wiśniewska K, Ziętara P. Does age pay off? Effects of three-generational experiments of nanodiamond exposure and withdrawal in wild and longevity-selected model animals. Chemosphere. 2022: 135129. doi: 10.1016/j.chemosphere.2022.135129..

   IF: 8.1; MNiSW: 140 pkt

3. Augustyniak M, Ajay AK, Kędziorski A, Tarnawska M, Rost-Roszkowska M, Flasz B, Babczyńska A, Mazur B, Rozpędek K, **Seyed Alian R**, Skowronek M, Świerczek E, Wiśniewska K, Ziętara P. Survival, growth and digestive functions after exposure to nanodiamonds - Transgenerational effects beyond contact time in house cricket strains. Chemosphere. 2024:140809. doi: 10.1016/j.chemosphere.2023.140809.

   IF: 8.1; MNiSW: 140 pkt

Conferences

1. 1st National Conference on New Cellular and Molecular Biotechnology (April, 2012), Parand, Iran.

Title of poster: Antibiotical Nanosilver property in bull semen extenders.

Authors: **Seyed Alian R**, Hayati N, Amirinia S, Parivar K

2 National Conference of Nano Science and Technology (May, 2013), Tehran, Iran.

Title of poster: Toxic effect of Nano silver particles on bull sperm in sperm extenders.

Authors: Hayati N, Amirinia S, Parivar K, **Seyed Alian R**

3 International Conference of NanoTech Poland 2023 (14-16 June 2023), Poznan, Poland.

Title of poster: Time- and dose-dependent digestive enzyme activity in *Acheta domesticus* after exposure to Ag NPs or GO.

Authors: **Seyed Alian R**, Flasz B, Kędziorski A, Augustyniak M

4 International Conference of Nature Polish Scientific Conference (21 May 2022) Lublin, Poland.

Title of poster: Ocena toksyczności tlenku grafenu z różnych źródeł pochodzenia z użyciem standardowych biotestów.

Authors: Ziętara P, **Seyed Alian R**, Dziewięcka M

5 Polymer Meeting 15 (4-7 September 2023), Polymer Institute, Bratislava, Slovakia.

Title of poster: Dose-dependent effects of Quaternized Chitosan, GO and AgNPs on oxidative stress and cell status in house cricket.

Authors: **Seyed Alian R**, Heydari A, Flasz B, Kędziorski A, Augustyniak M

Internships and courses

- PCR Technique Workshop, 1st International Biology Conference, Gilan University, Iran, 2006. (one days)
- Internship: Fertility and Infertility Center, Karaj, Iran, 2014. (one mounth)
- Chromosomal Analysis Workshop, Royan Research Institute, Tehran, Iran, 2014. (three days)
- Internship: Pathobiology Medical Laboratory, Semnan, Iran, 2018. (two week)
- Innovation and Entrepreneurship Event, Tehran, Iran, 2018. (three days)
- Polymer Institute of the Slovak Academy of Sciences, Bratislava, Slovakia (18.09.2022 to 16.10.2022).

  Title of internship: Familiarization with the techniques of biomaterials synthesis and their characteristics. (Projekt pozakonkursowy Narodowej Agencji Wymiany Akademickiej pn. „Międzynarodowa wymiana stypendialna doktorantów i kadry akademickiej", nr projektu POWR.03.03.00-IP.08-00-P13/18, realizowany w ramach Działania: 3.3 Umiędzynarodowienie polskiego szkolnictwa wyższego, POWER (Non-competition project of the Polish National Agency for Academic Exchange

"International scholarship exchange of PhD candidates and academic staff", project No. POWR.03.03.00-IP.08-00-P13/18, implemented under Measure: 3.3 Internationalisation of Polish Higher Education, OP KED).

Grants

1. Master's Dissertation supported by grants from Animal Breeding Center of Iran and Animal Science Research Institute of Iran.

2. PRELUDIUM project

Searching for the power of hormesis - Effects of silver nanoparticles, graphene oxide (GO), and GO-Ag composite on the functions of digestive enzymes in *Acheta domesticus* (W poszukiwaniu podstaw hormezy - wpływ nanocząstek srebra, tlenku grafenu (GO) oraz kompozytu GO-Ag na funkcje enzymów trawiennych u *Acheta domesticus*).

Project ID: 2021/41/N/NZ7/01974

Source of financing: National Science Centre (NCN), Poland.

Duration: 12.01.2022- 11.01.2025

Principal Investigator: **Reyhaneh Seyed Alian**, Supervisor: Prof. dr hab. Maria Augustyniak

Awards

- Ranked 2nd among graduates in the Cellular and Developmental Biology Department, Science and Research University, Tehran, Iran, 2012.
- Best Poster Award: Antibiotic properties of nano silver in bull semen extenders, 1st National Conference on New Cellular and Molecular Biotechnology, Parand, Iran, 2012.
- Award of Iran Nano Committee for supporting research projects with innovative and applied topics in the field of nanomaterials. Iran, 2013.
- Special award from the Doctoral School for the best doctoral students with significant achievements. Funds to increase the doctoral scholarship. Poland, academic year 2020-2021.

B. THESIS DESCRIPTION

B.1. INTRODUCTION

B. 1.1. INTEREST IN NANOMATERIALS AND NANOPARTICLES (NPS)

The growing interest in nanomaterials, particularly nanoparticles (NPs), has seen a dramatic surge over the past few decades. This increased attention is primarily driven by the unique and enhanced physical, chemical, and biological properties that nanoparticles exhibit compared to bulk material counterparts. These nanoscale particles offer a wealth of new opportunities in research and applications due to their distinct behavior, which arises from their reduced size and the associated quantum effects. According to the International Organization for Standardization (ISO), nanoparticles are defined as particles with at least one dimension (length, width, or height) in the range of 1 to 100 nanometers. This size distinction is significant, as it provides the foundation for understanding why nanoparticles exhibit properties that are fundamentally different from those of larger materials. Their high surface-area-to-volume ratio, for example, enables more surface atoms to participate in chemical reactions, leading to enhanced catalytic activity, electrical conductivity, magnetic behavior, and optical properties (Tarafdar et al., 2013).

Nanoparticles possess unique characteristics that can be leveraged across many industries. For instance, their ability to interact with and penetrate biological membranes enables them to access cells and tissues more effectively than larger particles, making them particularly attractive for biomedical applications. Additionally, the distinctive optical, electrical, and magnetic properties of nanoparticles allow for their use in a wide range of technologies, from advanced imaging techniques to drug delivery systems, cancer treatments, and energy storage devices. The quantum effects that become prominent at the nanoscale result in behaviors such as tunable fluorescence, plasmonic resonance, and super Para magnetism, which are not typically observed in bulk materials. Nanoparticles have found applications across various sectors, ranging from medicine and industry to environmental science. In medicine, nanoparticles are being investigated for their potential in diagnostics, drug delivery, and cancer therapy, where their small size allows them to target diseased cells more effectively, minimizing damage to healthy tissue. In industry, nanoparticles are used to enhance the strength, durability, and functionality of materials, contributing to innovations in electronics, coatings, and energy storage systems. Furthermore, their unique properties have opened doors to environmental applications, such as water purification, pollutant removal, and waste treatment, where

nanoparticles can be used to capture contaminants or catalyze the breakdown of harmful substances.

Nanoparticles can be broadly categorized into two main types: organic and inorganic. Organic nanoparticles are typically carbon-based, while inorganic nanoparticles are composed of metals such as silver, gold, and copper; magnetic materials like cobalt, iron, and nickel; or semiconductor materials like zinc oxide (ZnO), zinc sulfide (ZnS), and cadmium sulfide (CdS). These inorganic nanoparticles have become critical components in fields such as electronics, catalysis, and biomedical engineering, where their specific properties—such as high electrical conductivity or magnetic responsiveness—can be exploited for targeted applications (Rafique et al., 2017).

As concerns about sustainability and environmental impact continue to grow, recent advances in nanotechnology have focused on the development of environmentally friendly methods for synthesizing nanoparticles. One such approach is the "biological" synthesis of nanoparticles, which involves using plant extracts or microorganisms to mediate nanoparticle formation. This method is considered more sustainable than traditional physical and chemical synthesis techniques, which are often costly, energy-intensive, and associated with hazardous by-products. Biological synthesis offers a greener alternative that minimizes the need for toxic reagents and harsh conditions, aligning well with the principles of green chemistry and sustainable technology (Joudeh and Linke, 2022).

As the use of nanoparticles continues to grow, there is an increasing need to carefully evaluate their biosafety and environmental impact. The very properties that make nanoparticles so advantageous - such as their high reactivity and ability to penetrate biological membranes - can also pose risks if not properly managed. The potential for nanoparticles to accumulate in living organisms, disrupt cellular processes, or persist in the environment underscores the importance of conducting thorough studies on their toxicity, long-term effects, and safe handling practices. Addressing these concerns through rigorous research and regulation is essential to ensuring that the benefits of nanotechnology can be harnessed safely and sustainably for future generations (Khan et al., 2019).

B.1.2. GRAPHENE OXIDE NANOPARTICLES (GO NPS)

Graphene oxide (GO) is a derivative of graphene, consisting of a single atomic layer of carbon atoms arranged in a hexagonal lattice. What distinguishes graphene oxide from graphene is the

presence of oxygen-containing functional groups, which are covalently bonded to both surfaces of the carbon layer. These functional groups include hydroxyl, carbonyl, carboxyl, and epoxy groups. As a result of these chemical modifications, graphene oxide behaves very differently from pure graphene. GO can exist in both single-layer or multi-layered forms, depending on the number of stacked carbon sheets (Bianco et al., 2013).

In its multi-layered state, the individual carbon sheets in graphene oxide are separated by the oxygen-containing functional groups that are attached to each layer of carbon atoms. These functional groups disrupt the π-π stacking interaction between graphene layers, allowing the material to disperse more easily in water and other solvents, which is one of the key advantages of graphene oxide over pure graphene. Despite its structural similarity to graphene, the introduction of oxygen groups fundamentally changes its properties. For example, graphene oxide does not absorb visible light, has much lower electrical conductivity, and exhibits significantly higher chemical reactivity compared to graphene. These differences stem from the disruption of graphene's highly conjugated carbon network by the oxygen groups, which impede electron mobility (Geim, 2012).

One of the most fascinating aspects of graphene oxide is its versatility as a nanomaterial. Recent research has highlighted its unique chemical, optical, and electronic properties, positioning it as a promising material for a wide range of applications. Its high chemical reactivity makes it ideal for functionalization, allowing for the attachment of various molecules and materials to its surface. This property has made graphene oxide highly desirable in fields like surface chemistry and material science, where it can be used as a platform for further chemical modifications. In addition, GO's optical properties, such as its strong photoluminescence, have opened new possibilities in the field of optics and optoelectronics. Its ability to emit light at different wavelengths depending on its reduction state has made it an interesting candidate for applications in biosensing, imaging, and photodetectors. GO's high surface area and ability to adsorb ions and molecules have also led to its widespread use in energy storage devices, such as supercapacitors and batteries, where it can improve charge storage capacity and stability (Dideikin and Vul, 2019).

Graphene oxide's enhanced chemical reactivity also plays a crucial role in its biological applications. It has been investigated as a drug delivery vehicle due to its ability to interact with biomolecules, its biocompatibility, and its capacity for surface modification. Moreover, its antimicrobial properties, attributed to its sharp edges and oxidative stress-inducing capabilities,

make it a promising material for applications in medicine and biotechnology, including wound healing, antibacterial coatings, and tissue engineering.

Furthermore, GO's dispersibility in water and other polar solvents allows for easier processing in industrial applications. This makes graphene oxide a favorable choice for composite materials, where it can be used to enhance mechanical strength, thermal stability, and conductivity of polymers and other matrices. In the field of environmental science, graphene oxide has shown potential as a water purification agent, thanks to its ability to adsorb pollutants, heavy metals, and organic contaminants from water sources.

The unique combination of properties exhibited by graphene oxide nanoparticles - ranging from high chemical reactivity and tunable optical characteristics to their wide application potential in electronics, energy storage, biology, and environmental science - underscores their importance as a versatile nanomaterial. Continued research is expected to further unlock new possibilities and applications for graphene oxide in both fundamental science and practical technologies.

B.1.3. SILVER NANOPARTICLES (AGNPS)

The advent of nanotechnology has facilitated the development of a wide range of nanomaterials, including inorganic nanoparticles with potent antimicrobial properties, such as silver nanoparticles (AgNPs). These nanoparticles have garnered significant attention as a promising alternative for combating the rising incidence of infectious diseases, as well as addressing the global challenge of antibiotic resistance (Faramarzi and Sadighi, 2012). Silver nanoparticles are particularly notable among antibacterial agents due to their unique properties, including a large surface area-to-volume ratio and the ability to release silver ions slowly over time. These characteristics make AgNPs highly effective in inhibiting the growth of bacteria, which has led to their widespread use in various sectors, including medicine, food safety, and environmental protection (Sanpui et al., 2008; Silvan et al., 2018).

AgNPs have proven effective against a broad spectrum of microorganisms, including both Gram-positive and Gram-negative bacteria. This versatility, combined with their relatively low toxicity to human cells at therapeutic concentrations, has positioned silver nanoparticles as one of the most promising materials for antibacterial applications. Their potential extends beyond healthcare to include uses in coatings, textiles, and water purification systems (Rai et al., 2008; Cui et al., 2012). Multiple studies have demonstrated that the antimicrobial efficacy and

physicochemical properties of silver nanoparticles are influenced by factors such as their size, shape, and surface charge. Smaller AgNPs generally exhibit stronger antibacterial activity due to their increased surface area, which allows for greater interaction with bacterial membranes. Additionally, the shape of the nanoparticles, whether spherical, rod-like, or triangular, also plays a critical role in determining their effectiveness against various pathogens (Pal et al., 2007; Albanese et.al., 2012; Akram Raza et al., 2016).

As research into AgNPs continues to evolve, their potential applications are expanding, with ongoing studies exploring their use in wound dressings, medical devices, and even cancer therapies. However, while silver nanoparticles hold great promise, further investigation is needed to fully understand their long-term environmental impact and their potential for developing resistance in microorganisms.

B.1.4. GRAPHENE OXIDE-SILVER NANOPARTICLES (GO-AG NPS)

Graphene oxide nanocomposites containing silver nanoparticles (GO-Ag NPs) have emerged as a highly popular hybrid material in biomedical research, owing to their unique properties and multifunctional capabilities. The integration of silver nanoparticles onto graphene oxide (GO) sheets offers several advantages. One of the most notable benefits is the prevention of silver nanoparticle aggregation, which enhances the stability of the nanocomposite. This stabilization allows for greater control over the release of silver ions, leading to increased antibacterial and anticancer efficacy (Ahmad et al., 2019; Gurunathan et al., 2015; Gurunathan and Kim., 2017).

In GO-Ag nanostructures, graphene oxide and silver nanoparticles can work synergistically, enhancing several key properties. The synergy between GO and Ag NPs boosts their antimicrobial and catalytic activities, improves thermal conductivity, and increases their overall performance in various applications. This synergistic behavior makes GO-Ag nanocomposites highly effective in fields such as electronics, catalysis, electrochemical biosensing, drug delivery, and the development of advanced antimicrobial agents (Gurunathan et al., 2016; Burnouf et al., 2013).

Moreover, both graphene and silver nanoparticles have been successfully combined with polymer matrices to create materials with enhanced antimicrobial properties. These composite materials are being explored for use in coatings, medical devices, and other applications requiring effective antimicrobial activity (Li et al., 2013; Navalon et al., 2016). The activity of GO-Ag nanocomposites is influenced by various factors, including the concentration, particle

size, and distribution of the silver nanoparticles. These parameters play a critical role in determining the overall effectiveness of the nanocomposites, particularly in terms of their antimicrobial and catalytic activities (Mamata et al., 2023).

One of the most effective methods for applying nanoparticles onto surfaces is ultrasonic technology. Ultrasonic waves can induce structural changes in nanomaterials, which, depending on the conditions, can lead to the separation or aggregation of the particles. This technique has proven useful not only for nanoparticle coating but also for the synthesis of a wide range of nanocomposites that incorporate metal ions and nanoparticles (Perelshtein et al., 2010; Taurozzi et al., 2011). The use of ultrasonic methods has enabled researchers to control the distribution and arrangement of GO-Ag NPs, improving their performance across various applications. Whether used for antimicrobial surfaces, advanced catalysts, or drug delivery systems, GO-Ag nanocomposites represent a highly versatile class of nanomaterials with immense potential for future development (Lu et al., 2006; Tong et al., 2007; Samuel et al., 2018).

B.1.5. TOXICITY OF NANOPARTICLES (NPS)

The rapid development of nanotechnology presents a significant challenge regarding the exposure of living organisms to nanoparticles across diverse ecosystems. One of the key issues is the unknown scale of nanoparticle penetration into various environmental systems, with considerable uncertainty about how these particles disperse and accumulate in the natural world (Malakar et al., 2020). As the use of engineered nanomaterials becomes more widespread, nanoparticles are increasingly being introduced into the environment through various pathways. While some of these routes are controlled, accidental spills are particularly concerning due to the limited ability to regulate and mitigate the release of nanoparticles. Sources of nanoparticle contamination include wastewater treatment discharges, landfills, waste incineration plants, and accidental releases during the production, transportation, or use of nanomaterials (Gottschalk et al., 2015). These unintentional discharges pose a serious risk to both ecosystems and human health, highlighting the need for more robust safety protocols and monitoring systems.

In vivo studies play a crucial role in assessing the biological impact of nanoparticles by examining how these particles affect homeostasis and various physiological systems in living organisms. The liver, kidneys, digestive system, lungs, blood, cardiovascular system, nervous system, and immune system are among the major body systems that need to be evaluated to understand how nanoparticles influence biological function. Although numerous studies have

been conducted using both in vitro and in vivo models to investigate specific nanostructures, there remains a lack of comprehensive, systematic evaluations that can fully capture the complexity of nanoparticle interactions within living organisms (Service, 2004; Chen et al., 2006; Lovern and Klaper, 2006; Moore, 2006; Yang et al., 2016). This gap underscores the urgent need for further research into nanoparticle toxicity and long-term effects.

Nanoparticles, by their very nature, have the potential to leak into the environment at various stages of their lifecycle—from production and processing to use and disposal. This leakage can lead to environmental pollution, posing a threat to all forms of life. Once released, nanoparticles can interact with food, enzymes, or bile acids, potentially disrupting biological processes, including the microbiome (the community of microorganisms living in the gut). In particular, nanoparticles found in food can interfere with the immune system, damage the protective barrier of the gut, and contribute to microbial imbalances. (Fröhlich and Fröhlich, 2016; Lamas et al., 2020). These interactions raise serious concerns about the long-term health effects of ingesting nanoparticles, especially considering their widespread use in food packaging and agricultural products.

Various factors influence how nanoparticles accumulate and disperse in the digestive system. The presence of organic substances, for example, can either promote the aggregation of nanoparticles or help them remain dispersed. Humic acids, for instance, can keep nanoparticles suspended, while other conditions may cause them to stabilize and cluster together. Enzymes, surfactants, and fiber in the gastrointestinal tract also play roles in modifying the behavior of nanoparticles. Surfactants, which are naturally produced in the body, can enhance the dispersion of nanoparticles, while fiber can act as a sponge, absorbing nanoparticles and potentially limiting their bioavailability (Van der Zande et al., 2020). Given the diversity of nanoparticles and their interactions with a wide array of organic substances, it remains difficult to draw general conclusions about their behavior in biological systems. The effects of nanoparticles depend not only on their size, shape, and concentration, but also on the type of food they are ingested with, the digestive strategies of the organism, and the presence of other environmental factors. Clearly, more research is needed to elucidate these complex interactions (Huh and Kwon, 2011; Slavin et al., 2017).

The physicochemical properties of soil also have a profound influence on how nanoparticles behave and impact terrestrial ecosystems. Key factors such as soil acidity (pH), ionic composition, particle size, organic matter content, temperature, solar radiation, and hydrostatic pressure can all affect the fate and toxicity of nanoparticles in soil environments. Nanomaterials

interact with these soil properties in intricate ways, meaning that their environmental behavior and potential for toxicity depend on a combination of these factors along with the physiological state of organisms exposed to them (Babich et al., 1980; Shoults-Wilson et al., 2011). For instance, certain types of nanoparticles can be absorbed by insects and plants, enabling them to enter the food web and potentially bioaccumulate in higher organisms. This raises questions about the broader ecological impacts of nanoparticles, particularly in terms of how they may influence insect populations, which are vital components of many foods' chains (Winter and Streit, 1992; Dauwe et al., 2004).

B.1.6. TOXICITY MECHANISM OF NANOPARTICLES

Nanoparticles (NPs) have the ability to penetrate biological cells through various mechanisms, which can result in significant cellular damage. Among the most common entry pathways are endocytosis, particularly pinocytosis, as well as passive diffusion and mechanical damage to the cell membrane. The latter mechanism is often observed with sharp-edged nanoparticles, such as graphene oxide (GO) sheets, which can physically disrupt the integrity of the cell membrane, facilitating the entry of nanoparticles. (Borm et al., 2006; Liu et al., 2014). Once inside the cell, nanoparticles can interact with various organelles and cellular structures, leading to a cascade of harmful effects (Dziewięcka et al., 2016; Dziewięcka et al., 2017).

One of the primary toxicological effects of nanoparticles is their ability to induce oxidative stress. Damaged cells often show an increased number of autophagosomes - vesicles involved in the degradation of cellular components - as well as structural damage to lysosomes, the organelles responsible for breaking down waste materials. Furthermore, nanoparticles can disrupt the normal function and structure of mitochondria, the powerhouses of the cell, which are critical for energy production (Dziewięcka et al., 2018). Mitochondrial dysfunction is particularly concerning because it is closely linked to the generation of reactive oxygen species (ROS). Under normal conditions, mitochondria produce small amounts of ROS as byproducts of cellular respiration. However, when nanoparticles interfere with mitochondrial processes, ROS production can increase dramatically, leading to elevated levels of intracellular oxidative stress. This oxidative stress can have far-reaching consequences, including the accumulation of free radicals, which are highly reactive molecules that can damage key cellular components such as lipids, proteins, and nucleic acids (Dziewięcka et al., 2017).

Damage to DNA caused by ROS is especially problematic, as it can result in mutations, impaired gene expression, and even cell death. If left unchecked, such damage can initiate carcinogenic processes, potentially leading to cancer. Additionally, nanoparticles may exacerbate these effects by directly interacting with the cell's genetic material, further increasing the risk of genotoxicity. Studies have shown that nanoparticle-induced oxidative stress can trigger various cellular defense mechanisms, including the activation of antioxidant systems and DNA repair pathways. However, in cases where oxidative damage overwhelms these protective systems, irreversible cellular damage and apoptosis (programmed cell death) may occur (Dziewięcka et al., 2017).

The toxicity of nanoparticles is largely driven by their ability to enter cells and disrupt normal cellular functions. By inducing oxidative stress and causing mechanical damage, nanoparticles can interfere with critical organelles, such as mitochondria, and lead to the generation of ROS. These processes, in turn, result in widespread cellular damage, potentially contributing to a range of pathological outcomes, including inflammation, tissue injury, and cell death.

B.1.7. *ACHETA DOMESTICUS* AS A MODEL IN ECOTOXICOLOGY

Acheta domesticus (commonly known as the house cricket, family Gryllidae, order Orthoptera) has emerged as a highly suitable and widely used model organism in physiological and toxicological research. Originating from Southwest Asia, *Acheta domesticus* is now found across the world due to its adaptability to various climates and habitats. House crickets are commonly used in entomological research due to their tractable nature and well-understood biology (Clifford et al.1976; Szelei et al., 2011). Its growing popularity in laboratory studies is largely due to several practical advantages that make it ideal for designing controlled in vivo experiments. These advantages include its relatively large body size, which facilitates experimental handling and measurement, and its relatively short life cycle, which allows researchers to conduct generational studies within a manageable timeframe. Additionally, *Acheta domesticus* is widely distributed across the globe, which makes it a versatile organism not only for laboratory studies but also for field research (Horch et al., 2017).

The cricket's global distribution is particularly valuable for ecotoxicological studies because it enables researchers to evaluate the effects of various environmental pollutants across different ecosystems. By using a species with such wide-ranging adaptability, researchers can gather data from both controlled laboratory settings and natural environments, gaining a deeper

understanding of how nanoparticles and other contaminants affect biological systems on a broader scale. Carefully designed toxicological assays using *Acheta domesticus* provide crucial insights into both individual-level effects and broader environmental impacts of pollutants. These findings are essential for developing future preventive measures and regulatory policies aimed at minimizing ecological damage from emerging contaminants such as nanomaterials (Szelei et al., 2011; Horch et al., 2017).

One of the major challenges in toxicological research, particularly in the context of nanoparticle exposure, lies in understanding how contaminants affect the digestive system of insects. Nanoparticles can interact in complex ways with biological processes in the gut, and these interactions can vary depending on the type of food, the organism's feeding behavior, and the physicochemical properties of the contaminants. The digestive system is a highly dynamic environment, with a wide array of interactions between ingested substances, digestive enzymes, and gut microbiota. This complexity makes it difficult to predict how nanomaterials will behave within an organism's system.

To address this complexity, using a model organism such as *Acheta domesticus*, which consumes a standardized and well-characterized diet, offers an ideal framework for investigating the effects of food contamination by nanoparticles. House crickets provide a controlled environment that minimizes variables associated with natural diets, allowing researchers to isolate and study specific interactions between nanomaterials and the digestive system. This controlled setup helps in simplifying the problem of nanoparticle toxicity, as it reduces the number of confounding factors and allows for more precise assessments of the effects of contaminants on digestive function. Thus, the insights gained from studies on *Acheta domesticus* can form the foundation for understanding broader ecological and physiological impacts of nanoparticle exposure in more complex, natural settings.

B.1.8. Digestive Enzymes in Insects

Insects, the most diverse and abundant group of animals on Earth, owe much of their evolutionary success to their ability to consume a wide range of foods. This dietary adaptability allows insects to thrive in virtually every ecosystem. While many studies have explored the general morphology of the insect gut and the mechanisms underlying digestion, the molecular processes involved in nutrient digestion and absorption are still not fully understood. A deeper

understanding of these processes could provide valuable insights into insect biology, ecology, and potential biotechnological applications.

The insect digestive system runs longitudinally through the body, and is divided into three main sections: the foregut, midgut, and hindgut. Each of these sections plays a distinct role in food processing. Of particular importance is the midgut, which serves as the primary site for nutrient absorption and the secretion of digestive enzymes. Notably, the midgut is the only section of the insect digestive tract with an endodermal origin, meaning it lacks the protective cuticular layer that covers other parts of the digestive system (Persaud and Davey, 1970; Holtof et al., 2019).

The timing and location of digestive enzyme secretion are critical for efficient food breakdown and nutrient absorption in insects. The midgut is the most active site for the release of these enzymes, which play an essential role in the hydrolysis of macromolecules such as proteins, carbohydrates, and lipids. While some insects exhibit amylase activity in their foregut (stomach), the actual production and secretion of amylase, which catalyzes the breakdown of carbohydrates, typically occurs in the midgut (Dow, 1986; Evans and Payne, 1964).

The composition of digestive enzymes in the insect gut is highly complex and often species-specific. This complexity is driven by several factors, including the insect's dietary habits, the nutritional composition of the food it consumes, and the environmental conditions within the gut. For instance, herbivorous insects that feed primarily on plant matter typically produce more cellulases and pectinases, enzymes that break down plant cell walls, whereas carnivorous insects may produce a higher concentration of proteases to digest animal-based proteins. Moreover, the quality and quantity of food consumed can influence enzyme production, with different enzymes being upregulated or downregulated depending on the nutritional needs of the insect. Environmental factors, such as gut pH and temperature, also play a significant role in shaping the digestive enzyme profile. The insect gut is often divided into regions with distinct pH levels, allowing for the sequential breakdown of different types of food. For example, in some insects, the foregut may be more acidic, while the midgut is neutral or alkaline, creating an optimal environment for specific enzymes to function. This regional differentiation in pH, combined with temperature fluctuations, influences enzyme activity and the efficiency of nutrient absorption (Holtof et al., 2019).

B.1.9. Evaluation of Physicochemical Parameters to Determine the Toxicity of Nanoparticles

Understanding the toxicity of nanoparticles (NPs) requires a comprehensive analysis of their impact on biological systems, including food and energy budgets, the activity of digestive enzymes, and the induction of oxidative stress. By evaluating these parameters, one can gain valuable insights into how nanoparticles influence the physiology of organisms, their metabolism, and overall fitness.

Food and Energy Budget

The food budget in an experimental setting is determined by carefully measuring the amount of food consumed, uneaten food residues, and excreted waste. Additionally, the weight gain of the tested organisms, such as insects, during the experimental period is recorded. This data provides a foundation for analyzing the activity of digestive enzymes and understanding how nanoparticles might interfere with nutrient absorption. Maintaining energy balance is essential for an organism's survival, growth, reproduction, and ability to cope with environmental stress. Any disruption - whether due to food scarcity, reduced food quality, or exposure to toxic nanoparticles - can upset this balance, leading to diminished energy efficiency. Such disruptions can manifest in various ways, including slower growth rates, extended developmental periods, smaller adult size, reduced fitness, and impaired reproduction (Parsons, 2007).

During exposure to moderate stressors, such as nanoparticles, metabolic activity may decrease as organisms activate protective mechanisms to repair damage. This may result in reduced food absorption and alterations in ATP-generating aerobic pathways, further influencing the organism's ability to maintain its energy budget (Monaghan et al., 2008; Sokolova et al., 2012).

Activity of Digestive Enzymes

All living organisms produce digestive enzymes to break down complex molecules into simpler forms, facilitating nutrient absorption. It is hypothesized that nanoparticles could form colloids with digestive enzymes or food molecules, potentially affecting their behavior in the digestive tract. While theoretical models suggest that enzyme interactions with nanoparticles could immobilize or inactivate them, experimental confirmation of this hypothesis remains limited (Van der Zande et al., 2020).

In laboratory studies, enzyme activity can be measured using specialized assay kits. For example, a protease assay using FTC-casein has shown moderate to weak protease activity in *Acheta domesticus* (house crickets), suggesting a potential interaction between nanoparticles and protein digestion. Additionally, amylases, crucial for breaking down polysaccharides, play a key role in insect digestion. Specific enzymes like α-amylase, α-glucosidase, and β-glucosidase, essential for digesting polysaccharides and cellulose, can also be analyzed using similar techniques. Lipase activity, critical for lipid digestion, can be accessed via a coupled enzyme reaction, producing methyl resorufin, which is measured spectroscopically or fluorometrically. Each enzyme activity measurement protocol for insect tissues has to be optimized for accuracy and precision (Da Lage, 2018).

ASSESSMENT OF OXIDATIVE STRESS

One of the most significant toxicological effects of nanoparticles is their ability to induce oxidative stress, which is widely recognized as a major mechanism of nanoparticle toxicity (Murray et al., 2009; Guo et al., 2013). Oxidative stress occurs when the production of reactive oxygen species (ROS) in tissues exposed to nanoparticles overwhelms the organism's antioxidant defense mechanisms, leading to the accumulation of harmful free radicals. ROS are highly reactive molecules capable of damaging essential cellular components, including proteins, lipids, and DNA. The resulting oxidative damage can interfere with critical cellular processes such as signal transduction, cell migration, and proliferation. Furthermore, this damage can initiate the activation of apoptotic pathways, leading to programmed cell death, which may disrupt tissue function and compromise the survival of the organism (Deaton et al., 2003).

In this thesis, oxidative stress was quantified by measuring ROS levels in tissues using flow cytometry (Muse™ Cell Analyzer; Millipore, Billerica, MA, USA) following the manufacturer's protocols. This method allows for the precise assessment of oxidative damage caused by nanoparticles. By analyzing the extent of ROS production and correlating it with tissue damage, researchers can evaluate the severity of nanoparticle-induced stress (Klaunig et al., 2011; Chen et al., 2012; Dzięwiecka et al., 2017).

B.1.10. IMPORTANCE AND PRACTICAL ASPECTS OF THE RESEARCH

The findings from this research are crucial for advancing our understanding of how nanoparticles (NPs) interact with the digestive system when ingested through food. As

nanoparticles are increasingly used in various fields, including medicine, agriculture, and consumer products, it is essential to comprehend the mechanisms by which they influence biological systems. This research will provide key insights into the physiological responses of the digestive system when exposed to this new class of stress agents.

One of the most significant contributions of this research lies in its relevance to biotechnology and medicine. Nanoparticles are often employed as drug delivery systems due to their unique properties, such as their ability to encapsulate and transport therapeutic agents directly to target sites in the body. These particles are frequently administered orally, meaning they pass through the digestive system before reaching their intended destination. By examining how nanoparticles interact with digestive enzymes, gut microbiota, and other components of the gastrointestinal tract, this research can help improve the design of nanoparticle-based drug carriers, making them more effective and reducing potential side effects.

Furthermore, the results of the study may have important implications for environmental science. The issue of environmental pollution caused by nanoparticles has gained increasing attention in recent years, as these materials are released into ecosystems from various sources, including industrial waste, agricultural runoff, and consumer products. Wild animals, particularly invertebrates, are increasingly exposed to nanoparticles in their habitats, and the long-term effects of this exposure remain largely unknown. Despite growing concern, research on the impact of nanoparticles on the digestive systems of wildlife, especially invertebrates, has been relatively scarce. This study aims to bridge that gap, providing much-needed data on how nanoparticles affect gastrointestinal functions in non-mammalian species.

The significance of this research extends beyond academic and scientific circles. By shedding light on how nanoparticles behave in the digestive systems of invertebrates, the study could inspire broader discussions on environmental policies and regulations concerning nanoparticle use and disposal. The insights gained could contribute to more effective environmental protection strategies, ensuring that the introduction of nanoparticles into ecosystems is better managed and their harmful impacts minimized.

B.2. RESEARCH AIMS AND HYPOTHESES

This study investigates whether graphene oxide (GO), silver nanoparticles (AgNPs), and their composite (GO-AgNPs) can disrupt the functioning of the digestive system and, consequently, impact the available energy for biological processes.

The primary objective of this thesis is to evaluate and characterize the physiological and biochemical responses in the intestines of *Acheta domesticus* crickets following exposure to these nanomaterials. Special attention is given to energy allocation, digestive system function and structure, as well as the stability of stress markers in intestinal cells after treatment with GO, AgNPs, and their composite.

Graphene oxide nanoparticles were chosen due to the extensive prior experience and knowledge of their toxicity within the research team. Additionally, silver nanoparticles were selected because of their widespread use in various human applications and their well-documented antibacterial properties. The GO-AgNPs composite was also investigated to explore the potential interaction between GO and AgNPs, especially given the growing interest in GO-based metal composites due to their novel properties and applications.

The specific **aims** of the research are as follows:

1) To determine the relationship between the concentration of GO, AgNPs, and the GO-AgNPs composite and the activity of key digestive enzymes (protease, amylase, α-glucosidase, β-glucosidase, β-galactosidase, and lipase) in *A. domesticus*.

2) To describe potential changes in digestive enzyme activity as a function of exposure time and nanoparticle concentration, with particular focus on the early exposure stage, where hormetic effects might manifest.

3) To assess the food and energy budget in *A. domesticus* during the initial stages of exposure to GO, AgNPs, and the GO-AgNPs composite across various concentrations and durations in the diet.

4) To evaluate the viability of intestinal cells in crickets exposed to nanoparticles in a time- and concentration-dependent manner.

5) To describe the ultrastructural changes in the intestines of *A. domesticus* after exposure to the GO-AgNPs composite on selected days.

6) To determine the relationships between digestive enzyme activity, food intake, nutrient absorption, growth rate, and the food/energy budget in crickets exposed to GO, AgNPs, and the GO-AgNPs composite.

The following alternative **hypotheses** were tested:

H1.0: The consumption of food contaminated with nanoparticles (GO, AgNPs) at various concentrations will not significantly affect the activity of selected digestive enzymes, including proteases, α-amylase, α-glucosidase, β-glucosidase, β-galactosidase, and lipases, particularly during short-term exposure. This hypothesis is based on the assumption that nanoparticles may interact with food components, thereby limiting the number of reactive sites available on their surfaces. In such cases, these "shielded" nanoparticles could be excreted from the gastrointestinal tract without significant interaction with intestinal cells, potentially only accelerating intestinal transit. As a result, the viability of gut epithelial cells should not be substantially compromised. However, an alternative possibility is that nanoparticles may enter epithelial cells, and once food molecules detach, the nanoparticles could secondarily interact with these cells, leading to increased cell death.

H1.1: Exposure of insects to selected nanoparticles (at various concentrations) through the gastrointestinal tract will lead to a significant increase in the activity of some or all of the measured enzymes. Compensatory and/or hormetic mechanisms, especially in the early stages of exposure, may be triggered. As xenobiotics, nanoparticles can induce multiple changes in cellular or organismal function, aimed at defending against or repairing damage. These processes would require additional energy, which may manifest as increased food consumption and/or nutrient assimilation.

H1.2: Insects consuming food contaminated with nanoparticles will exhibit a significant reduction in digestive enzyme activity compared to control insects. This hypothesis is based on the assumption that nanoparticles, being foreign agents, could deactivate digestive enzymes, which are proteins. Another potential mechanism is nanoparticle-induced damage to the gut epithelium (and possibly effects on the gut microbiome), leading to homeostatic disruption and impaired digestive function. Such responses may be particularly evident in the early stages of exposure. This hypothesis could be partially supported by an observed increase in dead intestinal cells during cytometric analysis.

H2.0: The GO-AgNPs composite will elicit effects comparable to those induced by graphene oxide (GO) and/or silver nanoparticles (AgNPs) at equivalent concentrations with respect to digestive enzyme activity, gut cell viability, and food consumption in Acheta domesticus. The changes triggered by the composite are expected to mirror the impact caused by the more reactive nanoparticle (either GO or AgNPs) within the composite.

H2.1: The presence of the composite in contaminated food will lead to more pronounced changes in the physiological parameters mentioned above. This is due to the possibility that the composite's reactivity results from a simple additive effect of the reactivity of GO and AgNPs, and/or an extended interaction time of nanosilver within the gastrointestinal tract. In this scenario, graphene oxide would act as a carrier, facilitating prolonged exposure of the composite's components, thereby intensifying the observed effects.

H2.2: The composite will exert weaker effects compared to GO and AgNPs at identical concentrations. This attenuation in reactivity could be attributed to the formation of bonds between GO and AgNPs during the composite synthesis, which may deactivate the surface of graphene oxide. As a result, the composite particles may exhibit reduced reactivity.

B.3. RESEARCH METHODOLOGY

To achieve the set aims and approach the verification of the hypotheses, three experiments were planned and conducted (Scheme 1). The studies focused on the food/energy budget, digestive enzyme activity, and the cellular status of the A. domesticus gut. In the first experiment, graphene oxide (GO), silver nanoparticles (AgNPs), and the GO-AgNP composite were tested at single concentrations. The second experiment investigated the effects of varying concentrations of GO and AgNPs as well as different exposure times. In the third experiment, the effects of the GO-AgNP composite were assessed across various concentrations and exposure durations.

Graphene oxide (GO) was purchased from Nanografi (Germany) as an aqueous suspension (10 mg/mL), then diluted, sonicated, and analyzed for morphology and structure. Silver nanoparticles (20–30 nm, 99.9%) were obtained from SS Nanomaterials, Inc. (USA) and prepared as a stable colloidal solution (10 mg/L) using sonication in a citrate buffer (pH 6.5). GO and AgNPs were used to prepare a composite by mixing GO and AgNP stock solutions, citrate buffer, and deionized water, followed by sonication for 4 hours. The resulting composite suspension was homogenous and stable. Rabbit feed with various concentrations of GO or AgNPs or GO-AgNPs was prepared and sterilized for experimental purposes. Nanoparticles were visualized and measured using scanning electron microscopy (SEM) and atomic force microscopy (AFM). The zeta potential of nanoparticle suspensions was measured at 25 °C using a Litesizer 500.

Acheta domesticus was used as a model organism due to its ease of breeding and relevance in physiological and toxicological studies. Crickets were obtained from a laboratory stock colony at the University of Silesia.

EXPERIMENT I

Silver nanoparticles (AgNPs), graphene oxide (GO), and GO-AgNPs composite were chosen due to their potential varying mechanisms of action. The GO-AgNPs composite was prepared in a ratio of 20:400 (GO:AgNPs) to observe potential synergistic effects. GO and AgNPs were separately sonicated in solution, then combined to form a GO-AgNPs composite. The composite was prepared by mixing GO with silver colloid in a citrate buffer and sonicated for homogeneity.

Adult female crickets were divided into control and three experimental groups (GO, AgNPs, GO-AgNPs). The insects were fed food supplemented with nanoparticles, and changes in weight, feed consumption, and feces production were monitored over 10 days.

Samples of food and feces were analyzed for calorific values using a semi-microcalorimeter. The energy budget was calculated by measuring consumption, assimilation rates, and digestibility.

Gut enzyme activity was assessed using the API® ZYM system. The activity of 19 enzymes involved in digestion was measured in crickets from each group, based on a colorimetric reaction.

Data were analyzed for normality and variance. Parametric tests (ANOVA, LSD) were performed to assess differences between groups, with significance set at $p < 0.05$.

EXPERIMENT II

Cricket eggs were collected, hatched, and reared to the adult stage under standard conditions. Seven groups of 1-day-old adults were fed food with different GO (2, 20, 200 μg/g) or AgNPs (4, 40, 400 μg/g) concentrations, with a control group. Food consumption and assimilation were measured, and insects' gut cell health and digestive enzyme activities were assessed over time.

Food and feces were weighed at regular intervals to calculate individual food intake and digestion rates. Each experimental group had six replicates with six individuals each.

The percentage of dead gut cells was assessed on days 3, 5, and 21, and digestive enzyme activities (protease, amylase, α-glucosidase, β-glucosidase, β-galactosidase, and lipase) were measured on days 1-5, 16, and 21. Enzyme activity was determined using commercial kits optimized for *A. domesticus*.

The data were analyzed using ANOVA and PERMANOVA for significant effects and interactions, with post-hoc tests where applicable. Results were presented as mean ± SE or median ± interquartile range.

EXPERIMENT III

One-day-old crickets were divided into five groups: control and groups fed with food containing GO-AgNPs at different concentrations (GO2Ag4, GO20Ag40, GO200Ag400, GO200Ag4000 μg/g of food). The insects were housed in fauna boxes with access to food and water. Three

experimental sets were used for food budget assessment, cell status analysis, and enzymatic/histological analysis.

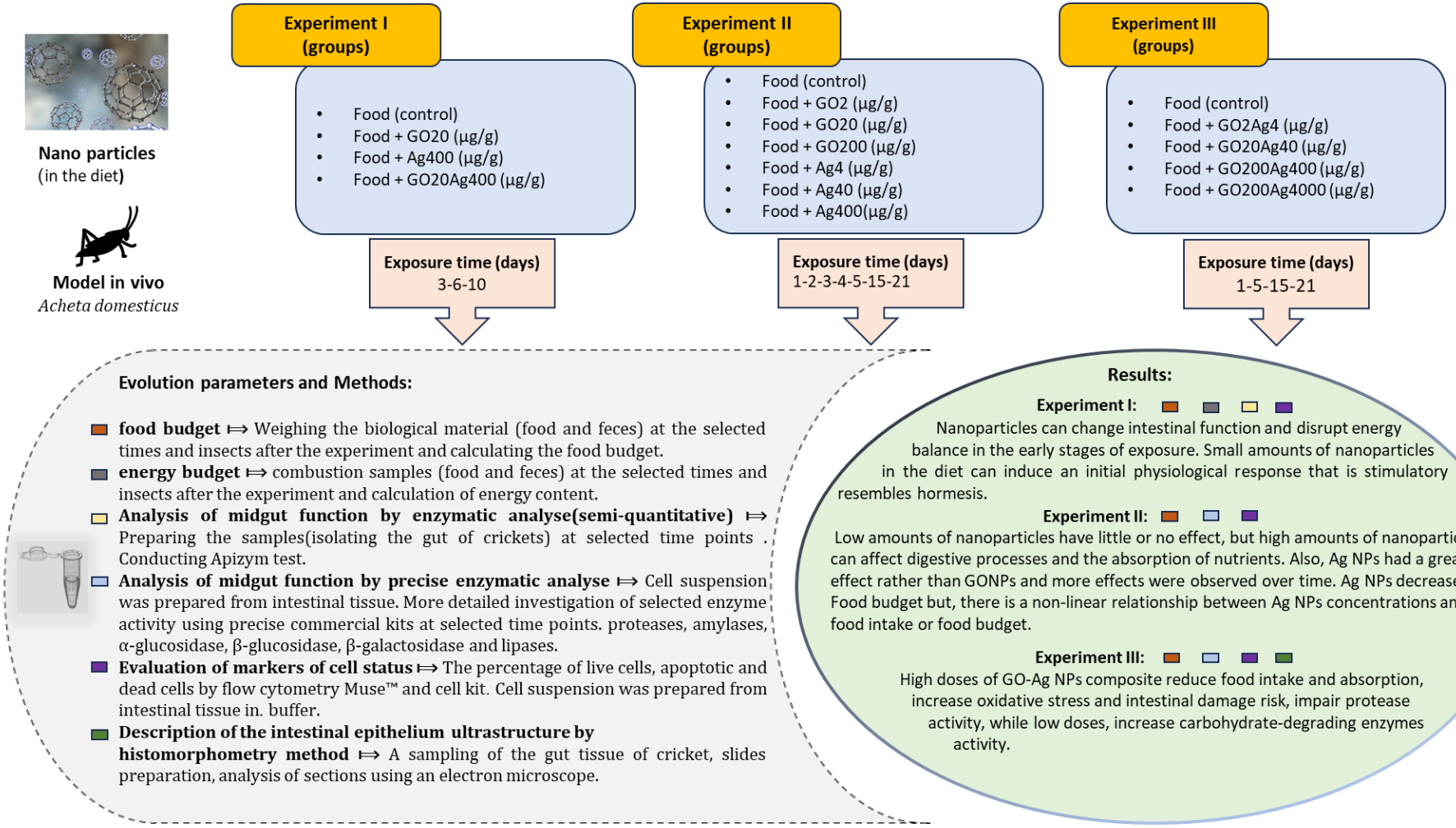
Five replicates of each group (five crickets per box) were monitored. Food and insect weight were measured at intervals to calculate food consumption and assimilation, with dry weights obtained by drying the samples.

Gut cells were analyzed on days 1, 5, and 21 to measure the percentage of dead cells and oxidative stress. Guts were isolated, homogenized, and cell suspensions analyzed by cytometry to assess cell death and oxidative stress.

Enzyme activity was measured at various time points using midgut samples. Commercially available kits were used to measure protease, amylase, α-/β-glucosidase, β-galactosidase, and lipase activities under optimized conditions for *A. domesticus*. Enzyme activity was expressed in standard units.

Midgut samples were collected on days 5 and 21 for transmission electron microscopy (TEM) analysis to observe the effects of GO-AgNPs at the cellular level.

Data were analyzed using multivariate repeated measures ANOVA with post-hoc tests to assess the effects of nanoparticle concentration and exposure time on food consumption, assimilation, and enzyme activity. Statistical assumptions were checked before analysis, and interactions between variables were also assessed.



Scheme 1. Model of the experiments

B.4. DISCUSSION OF THE MOST IMPORTANT RESEARCH RESULTS

EXPERIMENT I

Hormetic factors, including stressors and fluctuations in energy availability, trigger a stress response in cells and organisms, characterized by increased free radical concentrations, disturbed ion distribution, and heightened energy consumption. This moderate stress typically results in improved defense mechanisms, such as enhanced antioxidant enzyme activity, increased chaperone production, and intensified protein synthesis and metabolism, as noted by Mattson (2007). These responses protect cells from more severe stress but require additional energy, which can lead to increased food consumption and digestive efficiency.

In this study, it was observed that nanoparticles (NPs) present in the food temporarily disrupted the energy budget and digestive enzyme activity of *Acheta domesticus*. A significant reduction in food consumption and assimilation was observed in the first exposure period (0–3 days), which may reflect an initial disturbance of homeostasis (Calabrese, 2005). However, by the third day, compensatory mechanisms were triggered, resulting in a smaller decline in consumption and assimilation in the NP-treated groups compared to the control, suggesting a hormetic response. This indicates that the low NP concentrations used in this experiment were sufficient to induce a hormetic effect. The amounts of graphene oxide (GO) and silver nanoparticles (AgNPs) consumed during the first three days were calculated, showing exposure levels lower than those used in other studies (Guo and Mei, 2014; Gomes et al., 2015; Yasur and Usha Rani, 2015; Ferdous and Nemmar, 2020; Malhotra et al., 2020).

The hormetic effect disappeared during the final experimental phase (days 6–10), with food consumption, assimilation, and enzyme activities returning to control levels. This pattern could be explained by factors such as the dose and exposure duration of NPs, as well as the age, health status, and physiological condition of the organism (Calabrese, 2005; Agathokleous and Calabrese, 2020). For *A. domesticus*, the 10-day period corresponds to approximately ⅓ of the adult lifespan, which may have contributed to the reduced hormetic effect as aging and reproductive effort began to accumulate (Augustyniak et al., 2009a, 2009b, 2011).

In contrast to the effects of individual nanoparticles, the GO-AgNPs composite did not cause a significantly stronger response. This could be due to the formation of bonds between GO and AgNPs, leading to the deactivation of part of the nanoparticle surface and the formation of larger agglomerates, thereby reducing the overall active surface area (Jeevanandam et al.,

2018). Consequently, the composite may exhibit lower biological activity than its individual components.

Additionally, the study revealed increased water retention in the body and feces of the composite-treated crickets, particularly between days 3 - 10. This may have been caused by the greater water-holding capacity of the composite, a phenomenon previously reported in plants exposed to carbon nanoparticles (Khodakovskaya et al., 2012; Zhang et al., 2015; He et al., 2018; Park et al., 2020). This is the first time such an effect has been observed in insects, and the implications of long-term composite exposure on water balance should be examined in future research.

EXPERIMENT II

The results of this study suggest that GO in concentrations ranging from 2-200 ppm did not cause a significant reduction in body weight in *Acheta domesticus*, except for a transient decrease in the GO200 group, which was compensated by increased food assimilation. This observation is particularly noteworthy since adult crickets typically exhibit limited body weight gain after their final molting. The GO exposure did not reduce gut cell viability; on the contrary, a decrease in the percentage of dead cells in the GO2 and GO20 groups was recorded by day 21. These findings align with previous studies where a slight reduction in dead cells was noted, but an increase in apoptotic cells, reactive oxygen species (ROS), and DNA damage also occurred, alongside moderate histological changes in midgut cells (Dziewięcka et al., 2017). These compensatory mechanisms may mitigate initial negative effects on body weight, though they may come at the expense of other functions, such as reproduction or longevity, which should be explored in future research.

AgNPs had a more pronounced impact on body weight, food consumption, and assimilation, particularly in the Ag40 group, consistent with earlier research (Raj et al., 2017). Previous studies have demonstrated that AgNPs can cause a reduction in body weight in other insect species (Yasur and Usha Rani, 2013), as well as abnormalities in midgut cells in beetles (El-Samad et al., 2022) In our study, the relationship between AgNP concentration and body weight change was non-linear, but the Ag40 group exhibited a notable decrease in weight, consumption, and assimilation. This observation suggests that AgNPs, particularly at a concentration of 40 ppm, may alter food quality or taste. Further studies are needed to understand these effects.

When comparing GO and AgNPs, it is important to consider their structural differences and how they interact with cells. AgNPs may exhibit delayed effects due to the gradual release of Ag ions, while GO toxicity depends on factors such as flake size and surface functionalization (Dang et al.,2021; Khodaparast et al., 2021; El-Samad et al., 2022; Dziewięcka et al., 2021; Nebol’ sin et al., 2020; Rhazouani et al., 2021).

Digestive enzyme activity showed more pronounced changes under AgNP exposure. Previous studies have documented a reduction in gut enzyme activity, such as amylase, protease, and lipase, in insects exposed to AgNPs (Arvind Bharani and Karthick Raja Namasivayam, 2017). However, our study did not show a clear concentration-dependent inhibitory effect on digestive enzymes. Rather, the activity of enzymes depended on the type of nanoparticle, and exposure time. Hormetic effects, where enzyme activity is stimulated to enhance organismal survival under moderate stress, were also observed (Seyed Alian et al.,2021; Agathokleous et al., 2021; Wang et al., 2023; Yue et al., 2021).

Further research on the effects of nanoparticles, particularly their interaction with digestive enzymes, has demonstrated that nanoparticles can directly or indirectly impair enzyme function by altering food quality or feeding behavior (Wang et al., 2019). In the present study, while AgNPs inhibited protease activity, especially in older crickets, a stimulatory effect on enzyme activity was observed for most enzymes at lower NP concentrations. This outcome aligns with previous findings on the modulation of enzyme activity by metal nanoparticles (Wang et al., 2015; Brennan et al., 2010; Deka and Paul, 2012; Saware et al., 2015; Gole et al., 2001; Lv et al., 2009).

The study also highlights the potential for nanoparticles to form complexes with food components, which could influence nutrient retention and enzyme accessibility. These findings underscore the need for further research to fully elucidate the long-term effects of nanoparticles on digestive function and overall organism health.

EXPERIMENT III

Despite extensive research on the toxicity of GO and AgNPs, studies on their composites remain limited, particularly regarding their effects on the digestive functions of exposed organisms. Previous research (Seyed Alian et al., 2021) indicated that a single concentration of the GO-AgNPs composite elicited a weak to moderate stimulatory response in food digestion and absorption, reflecting stress-induced adaptive reactions.

This study expands on those observations in *Acheta domesticus* by broadening the concentration range of the GO-AgNPs composite and extending exposure to the mean survival time of adults, 22.4 ± 5.2 days. Results showed that food consumption and assimilation were significantly influenced by concentration and exposure time, although feeding patterns were largely unaffected by the composite, except at the highest concentration. The findings align with limited literature suggesting potential impairments in digestive functions (Buffet et al., 2012; Hanna et al., 2013; Lopes et al., 2014; Souza et al., 2018).

Analysis indicated increased oxidative stress (ROS) in aging individuals, with a notable rise in dead cells among one-day-old subjects exposed to higher composite concentrations, suggesting a link to cell cycle arrest (Yin et al., 2022; Takáč et al., 2023). Prior research demonstrated that GO can induce DNA damage and adverse histological changes in *A. domesticus* (Dziewięcka et al., 2017), with both GO and AgNPs likely increasing ROS production, weakening antioxidant defenses, and inducing programmed cell death.

Disruptions in cell structure were accompanied by changes in digestive enzyme activities, with stimulation of carbohydrate-degrading enzymes at low concentrations and significant inhibition of proteases at high concentrations. While stimulation of carbohydrate-degrading enzymes may indicate a compensatory response to increased energy demands, reduced protein utilization in the GO200Ag4000 group raises concerns about amino acid synthesis and development (Lee et al., 2008; Reifer et al., 2018).

The mechanisms by which the NP composite affects digestive enzymes remain unclear. However, insights from nanocatalysis research suggest that optimal NP/enzyme ratios may enhance enzymatic activity, while higher NP concentrations could hinder it.

The observed activity of digestive enzymes following NP composite exposure may arise from structural damage to epithelial gut cells and conformational changes affecting enzyme functionality. Furthermore, the complex interactions of NPs with various nutrients and host molecules in the gut microbiome warrant further investigation.

B. 5. CONCLUSIONS

This study confirms the hypothesis that the nanoparticles employed can significantly alter gut functions and deregulate the energy budget during the early stages of exposure. Even minimal dietary concentrations of nanoparticles provoke early physiological responses characterized by stimulation rather than inhibition, reminiscent of a hormetic effect. This suggests that, despite the diverse deleterious mechanisms of action exhibited by the nanoparticles, the exposed organisms mobilize a "standard" defense system, manifesting as an alarm stage of the stress response to environmental threats. Notably, factors such as the age of the organism and the overall physiological burden due to reproduction or other stressors may modulate the observed hormetic effects.

Furthermore, our results indicate that the subtle physiological changes induced by GO and AgNPs at the tested concentrations appear relatively safe for *Acheta domesticus*. However, it is noteworthy that AgNPs exhibited a more pronounced effect on body weight and food consumption compared to GO. The observed non-linear relationships between AgNP concentration and parameters such as body weight, consumption, and assimilation may be attributed to agglomerate formation, which could affect the reactivity of the nanoparticles.

Generally, the nanoparticles at the employed concentrations stimulated the activity of digestive enzymes, with the exception of protease, aligning with the observed hormetic effects. Nonetheless, whether these changes represent a trade-off in the struggle for survival amid sublethal but prolonged stress induced by the nanoparticles remains an open question.

Finally, the findings indicate that the GO-AgNPs composite can disrupt gut structure and the activity of digestive enzymes, as well as influence food and energy budget parameters. Some of these alterations do not exhibit a clear dose-dependent relationship but instead appear to intensify with prolonged exposure, correlating with the age of the organisms and the increasing concentration of the composite. These results suggest intricate interactions between the composite nanoparticles and the biological constituents of the target organisms. Nonetheless, the current state of knowledge permits only speculative interpretations regarding the underlying mechanisms, particularly concerning in vivo exposures. Consequently, the results underscore the conclusion that the GO-AgNPs composite, akin to its individual components documented in prior studies, cannot be deemed environmentally safe, particularly at elevated concentrations and/or with extended exposure durations.

B. 6. REFERENCES

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C. MANUSCRIPTS INCLUDED IN THE DOCTORAL DISSERTATION

C.1. MANUSCRIPT I

Seyed Alian R, Dziewięcka M, Kędziorski A, Majchrzycki Ł, Augustyniak M. Do nanoparticles cause hormesis? Early physiological compensatory response in house crickets to a dietary admixture of GO, Ag, and GOAg composite. Sci Total Environ. 2021;788:147801. doi: 10.1016/j.scitotenv.2021.147801.

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Do nanoparticles cause hormesis? Early physiological compensatory response in house crickets to a dietary admixture of GO, Ag, and GOAg composite

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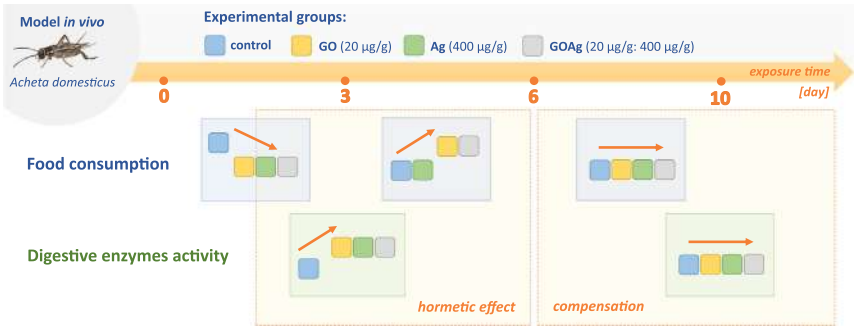
HIGHLIGHTS

- Low amounts of GO, AgNPs, or GO-AgNPs triggers an early stress response in crickets.
- Nanoparticles (GO, AgNPs, or GO-AgNPs) can disturb the energy budget of crickets.
- Initial suppression of energy consumption/assimilation is compensated afterwards.
- Increased activity of digestive enzymes accompanies the compensation response.
- Insects treated with GO-AgNPs composite retained more body water.

GRAPHICAL ABSTRACT



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ABSTRACT

This study aimed to identify the physiological responses of house cricket females following short-term exposure to relatively low dietary doses of graphene oxide (GO, 20 μg · g^{-1} food), silver (Ag, 400 μg · g^{-1} food) nanoparticles (NPs), or graphene oxide-silver nanoparticle composite (GO-AgNPs, 20: 400 μg · g^{-1} food). Energy intake and distribution were measured on the third, sixth, and tenth day. A semi-quantitative API®ZYM assay of digestive enzyme fingerprints was performed on the third and tenth day of continuous treatment. Physicochemical properties of the NPs were obtained by combining SEM, EDX spectrometry, AFM, and DLS techniques.
The obtained results showed decreased energy consumption, particularly assimilation as an early response to dietary NPs followed by compensatory changes in feeding activity leading to the same consumption and assimilation throughout the experimental period (10 days). The increased activities of digestive enzymes in NP-treated females compared to the control on the third day of the experiment suggest the onset of compensatory reactions of the day. Moreover, the insects treated with GO-AgNP composite retained more body water, suggesting increased uptake. The observed changes in the measured physiological parameters after exposure to NPs are discussed in light of hormesis.

1. Introduction

Recent years have brought the dynamic development of nanotechnology and an exponential increase in the synthesis of new nanoparticles. Silver nanoparticles (AgNPs) and graphene (its derivatives

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including graphene oxide (GO)) are widely used nanoparticles with significant potential. Their specific physicochemical properties allow them to be suitable for a wide array of applications (Wu et al., 2012; Dideikin and Vul, 2019; Smith et al., 2019; de Medeiros et al., 2021). Silver nanoparticles, due to their unique optical, catalytic, photo-thermal, and antibacterial properties, are currently the most commonly used nanoparticles (Rai et al., 2009; Taglietti et al., 2012; Katz et al., 2015; Wei et al., 2015; Pulit-Prociak and Banach, 2016; McGillicuddy et al., 2017).

The synthesis of hybrid materials, for example, GO-metal nanocomposites, aims to enhance their functionality, provide novel properties, and consequently, new applications (Zhu et al., 2013; Bhunia and Jana, 2014; de Saravia et al., 2020; de Medeiros et al., 2021). GO and AgNPs are considered to work synergistically, and the GO-AgNP nanocomposite may have better antimicrobial and catalytic activities and higher thermal conductivity than its components (Tang et al., 2013; Cobos et al., 2020). Hence, GO-AgNP composites are of great interest and potential, including anticancer therapy (Gurunathan et al., 2015; Kavinkumar et al., 2017). However, the antibacterial properties of GO, AgNPs, and GO-AgNP composites should be considered due to their effects on microorganisms that constitute the intestinal or gut microbiome (Li et al., 2018). Accidental contamination of the environment/food with such material can lead to impairment of digestive functions, indirectly affecting the nutrients and energy intake and, consequently, the energy budget of an organism.

The production and application of nanoparticles, including GO, Ag, and AGO-Ag composites, is constantly increasing (Pulit-Prociak and Banach, 2016; Inshakova and Inshakov, 2017; Malakar A et al., 2021). Therefore, they can be common environmental contaminants to which innumerable organisms could be exposed. The problem of the unfavorable influence of nanoparticles often manifested in the inhibition/impairment of various physiological processes, is frequently investigated. Less attention has been given to the potential hormetic/compensatory effects of nanoparticle exposure.

Hormesis consists of a two-phase reaction of an organism to a stressor (a chemical compound or an environmental factor) - low doses cause stimulation or beneficial effects, and high doses inhibit numerous processes and cause unfavorable effects (Mattson, 2008). Hormesis is an adaptive compensatory response that follows the initial disturbance of an organism's homeostasis. It is worth considering that hormetic effects are usually subtle/modest changes, which sometimes makes it difficult to distinguish from natural variability within a specific parameter (Hoffmann and Stempsey, 2008; Wang et al., 2017). One of the first hormesis studies was performed on house cricket, *Acheta domesticus* (Luckey, 1968; Cohen, 2005; Guedes et al., 2009; Cutler, 2013).

Toxicity studies on nanoparticles continuously provide new data. However, few studies have focused on doses/concentrations below the toxic threshold. In some studies that have focused on the biological effects of low doses of nanoparticles a hormetic effect has been observed. There are examples of in vitro studies that used carbon nanotubes, nanodiamonds, quantum dots, or metal nanoparticles, mostly silver. A few in vivo studies have described hormetic for various/specific endpoints as an effect of exposure to metal nanoparticles or carbon nanotubes. These studies were performed on selected strains of bacteria and some aquatic biota, including some species of algae and plants, few crustaceans, and few vertebrates (Iavicoli et al., 2010; Iavicoli et al., 2018; Agathokleous et al., 2019). However, the data on this phenomenon, particularly regarding insects and the potential energy costs of the supposed compensation, are limited and very fragmented or even missing.

Changes caused by additional stress factors should be accompanied by an increase in the energy consumption to activate defense, repair, and/or compensatory mechanisms. Increased energy demand should require an increase in food consumption. If the conditions are favorable, an increase in the efficiency of the digestive system is needed. Hence, estimating the energy budget and examining the overall efficiency of digestive enzymes is strongly justified.

The basic parameter of the animal's energy budget is consumption (C). Assimilation (A) is a part of the energy absorbed by the organism that is spent on respiration (R) to maintain life processes and production (P) related to weight gain, body reconstruction, and reproduction. In insects, energy allocation to production is associated with critical stages of development, molting, cocoon formation, and egg production (Schowalter, 2006; Gao et al., 2007; Yates et al., 2011). Adequate energy flow through an organism and its distribution is a basic demand for life, and various stressors may disturb its intake and allocation. However, sufficiently low “stressors' doses” might cause a compensatory response that could increase the chances of coping with unfavorable conditions.

In previous long-term studies on NP effects in crickets, we found mostly toxic effects of varying severity on different tissue/cellular/molecular endpoints (Dziewięcka et al., 2015; Karpeta-Kaczmarek et al., 2016a–c; Dziewięcka et al., 2017; Dziewięcka et al., 2018; Karpeta-Kaczmarek et al., 2018; Dziewięcka et al., 2020: Flasz et al., 2020). During our observations, particularly in the initial stage of exposure, we observed an increased consumption, compared to the control, in crickets provided with food contaminated with low concentrations of NPs (e.g., 20 μg GO/g of food), with no apparent toxic effects at the systemic level. Simultaneously, at high concentrations (e.g., 200 μg GO/g of food), food consumption contaminated with nanoparticles decreased significantly compared with the control group (unpublished data). These observations encouraged us to design an experiment to assess the detailed energy budget and digestive enzymes in the first 10 days of exposure to nanoparticles: GO, Ag, and GO-Ag composite. As we were interested in changes over time (i.e., multiple measurements within the experimental group), we chose only one concentration for each NP that seemed to cause increased consumption in our previous studies. Thus, this study aimed to quantify the expected changes and check whether the changes in food intake and utilization are accompanied by changes in the efficiency of the digestive system. Therefore, we evaluated the intestinal enzyme fingerprints (Kaufman et al., 1989; Boetius and Felbeck, 1995; Doherty-Weason et al., 2019) and the energy budget of *A. domesticus*. We hypothesized that nanoparticles could change gut function and deregulate the energy budget. However, considering the low doses used in this study and the short exposure time, possible activation of compensatory mechanisms could restore the measured parameters to the level of unexposed individuals.

2. Materials and methods

2.1. House cricket

Acheta domesticus (Orthoptera, Insecta) is often used as a model organism in physiological and toxicological research (Szelei et al., 2011; Horch et al., 2017). The species is omnivorous and easy to breed; the life cycle takes approximately 2–3 months. The animals used in the experiment were obtained from our laboratory stock colony.

2.2. Nanoparticles selection

Ag, GO nanoparticles, and their GO-Ag composite were used as exposure factors; their effect on the body, due to their properties, may have a different mechanism. The GO-Ag composite was prepared as a separate nanoparticle solution with a concentration ratio of the working components (GO:Ag = 20:400). This approach allowed the observation of the potential enhancement of the AgNP performance after anchoring to GO sheets or the additive effect of the two types of components. The specific structure of the nanocomposite can provide a unique nanointerface for interaction with the gut microbiome and facilitate the interaction between AgNPs and GO sheets, resulting in a synergistic effect (Tang et al., 2013).

2.3. Preparation of graphene oxide suspension, silver suspension, and Ag-GO composite

Graphene oxide powder (15–20 sheets, Sigma Aldrich) was sonicated in deionised water (10 mL) by ultrasonic homogenizer (cycle: 1, amplitude 100%; model UP-100H, DONSERV) until a homogeneous GO suspension (10 mg/mL).

Silver nanoparticles (99.9%, 20–30 nm, SS Nanomaterials, Inc.) were dispersed in a citrate buffer solution (0.1 M, 10 mL, pH =6.5) by sonication (cycle: 1, amplitude 100%) to form a stable Ag colloid solution (10 mg/mL).

Graphene oxide-silver nanoparticle composite suspension (GO-AgNPs) was obtained due to the mixing of silver nanoparticles with graphene oxide in a 5:1 ratio deionised water and citrate buffer solution (0.1 M; pH = 6.5). Briefly, 2 mg of graphene oxide powder was dispersed in 100 mL (20 ppm) deionised water followed by sonication for 2 h to greater homogeneity. Thereafter, silver colloid in the 0.1 M citrate buffer (400 ppm, 20 mL) was added to the GO suspension and gently heated while sonication for 1 h. The final product was left overnight in the dark at room temperature (Bao et al., 2011; Dinh et al., 2014; Xue-Fei et al., 2015).

2.4. NP characterization

The morphology and structure of the Ag, GO nanoparticles, and GO-AgNP composites were characterized using a scanning electron microscope (SEM) with an energy dispersion X-ray spectrometer (EDX) (Quanta FEG 250; FEI) and atomic force microscopy (AFM)(Agilent 5500). The size distribution profile of the Ag nanoparticles was determined by dynamic light scattering (DLS) equipped with 4 mW He-Ne, 633 nm Malvern Zetasizer Nano-ZS (Malvern Instruments, U.K.).

Samples for analysis were highly diluted and deposited on a silicon wafer (SEM) and fresh cleaved mica for AFM measurements. SEM imaging was performed with a beam accelerating voltage of 2 kV under high-vacuum conditions to ensure good contrast during imaging. Energy-dispersive X-ray spectrometry was used to identify and quantify the elemental composition of the GO-AgNP composite. EDX spectra from individual particles were analyzed using a vector-based algorithm to determine the elements. The AFM measurements were performed in tapping mode with a typical force constant of 40 N/m and a resonant frequency of 350 kHz. The standard scan frequency was 0.2. The DLS measurements were taken at 21 °C for 200 s with a 173° detection angle. The final results were calculated using the size distribution by the number technique.

The results showed well-defined GO structures, mostly single-layered with a typical height of 1.0 nm and an average flake area of approximately 2 μm (Fig. 1 A2, B2). Silver nanoparticles were present as aggregates with a diameter of about 65 nm (Fig. 1 B1) and individual particles in the range of 5–20 nm (Fig. 1 B2). The GO-AgNP composite was in the form of thick GO aggregates between and 5–40 nm coated with silver nanoparticles (Fig. 1 A3, B3, D1).

2.5. Experimental set-up for energy budget and digestive enzyme screening

Adult females (0–1 d after final molting) were randomly divided into control, and three experimental groups provided feed supplemented with NPs: GO (graphene oxide at 20 μg/g of food), Ag (silver nanoparticles, 400 μg/g of food), and GOAg (20 and 400 μg of GO and AgNPs, respectively, per gram of food). Each group consisted of six sub-groups kept in plastic boxes (28 × 20 × 16 cm; 7 individuals in each) under standard conditions (28.8 °C ± 0.88 °C, 20%–45% RH, and photoperiod L:D 12:12). A weighed amount of feed was provided on days 0, 3rd and 6th day (Dziewięcka et al., 2017). The remains and feces were collected separately on 3rd, 6th and 10th day. The increase in female body weight was measured with 1 mg accuracy on days 0, 3rd, 6th and 10th (final). All the collected feed and feces samples were dried at 40 °C for 24 h to obtain their dry weight. The 10th-day females were anesthetized with CO_2, then frozen and lyophilized for approximately 44 h. An additional reference group of 0-day females was frozen and lyophilized to further calculate the initial dry weight of females from the groups.

Separate plastic boxes (2 for each experimental group: control, GO, Ag, and GOAg) were set up to analyze digestive enzyme activity. The insects were fed ad libitum with the same food and kept under the same conditions as for calculation of the energy budget. Each plastic box contained ten 0–1-day-old females. Insects for the digestive enzyme activity assay were collected on the third and tenth day of adult age.

2.6. Calorimetry assay

Samples of the feed and feces were re-dried in a halogen moisture analyzer (HR73, Mettler Toledo, Switzerland) immediately before preparation of a 100 mg pellet for combustion in a 6725 semi-microcalorimeter (Parr Instrument Company, USA) under high oxygen pressure (2.8 MPa). After the combustion of the sample, the vessel was washed with distilled water, and the washing was titrated with 0.0709 N Na_2CO_3 using methyl red. Before sample determination, the device was calibrated using benzoic acid as a thermochemical standard.

Females were combusted in the same way. The unknown calorific value (CV) of the female samples was calculated from their dry weight and an appropriate regression equation ($R^2 = 0.991$).

2.7. Energy budget calculations

Consumption and assimilation rates and approximate digestibility (AD) were calculated for particular intervals and the entire experimental time. For the latter, the growth rate and efficiency of ingested energy conversion into bodyweight (ECI) and efficiency of digested energy conversion into bodyweight (ECD) indices were calculated according to standard formulas (Waldbauer, 1968).

2.8. Enzymatic activities screening by API® ZYM system

Enzymatic activity in the gut was assayed using an API®ZYM kit (BioMérieux, France) designed for cell suspensions and successfully used in assaying enzymes for invertebrate species (Kaufman et al., 1989; Boetius and Felbeck, 1995; Collin and Starr, 2013; Doherty-Weason et al., 2019; Przemieniecki et al., 2020). The API®ZYM test can be used as a general (screening) method of assessing an organism's digestive capacity, concerning the total activity of digestive enzymes derived from microbiota and their host, that is, *A. domesticus* in this case.

The API®ZYM strip test contains dehydrated chromogenic substrates of 19 enzymatic reactions involved in the breakdown of lipids, peptides, phosphoric esters, and polysaccharides. The enzymes and substrates included in this study are listed in Table 1.

A cell suspension of the required protein concentration was prepared for each experimental group by homogenizing the midgut dissected from three ice-anesthetized females. The same mass of intestinal fragments (75 ± 2 mg) was collected to obtain a homogenate with the required volume needed for the test and a similar protein concentration. The homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C.

According to the manufacturer's protocol, the API test plates were placed in moist chambers, and 65 μL of supernatant was added to each well, followed by incubation for 3 h at 37 °C in the dark. After incubation, reagents Zym A and Zym B were added to stop the reaction, and the resulting color was allowed to develop for 5 min. Any excess of unreacted Fast Blue BB was removed by short-term exposure to a strong light source (1000 W, 10 s, 10 cm from the plate), and after aligning the colors, the plates were photographed.

The semi-quantitative assay was based on a visual comparison of the colors on the test strips obtained by enzymatic reactions with a

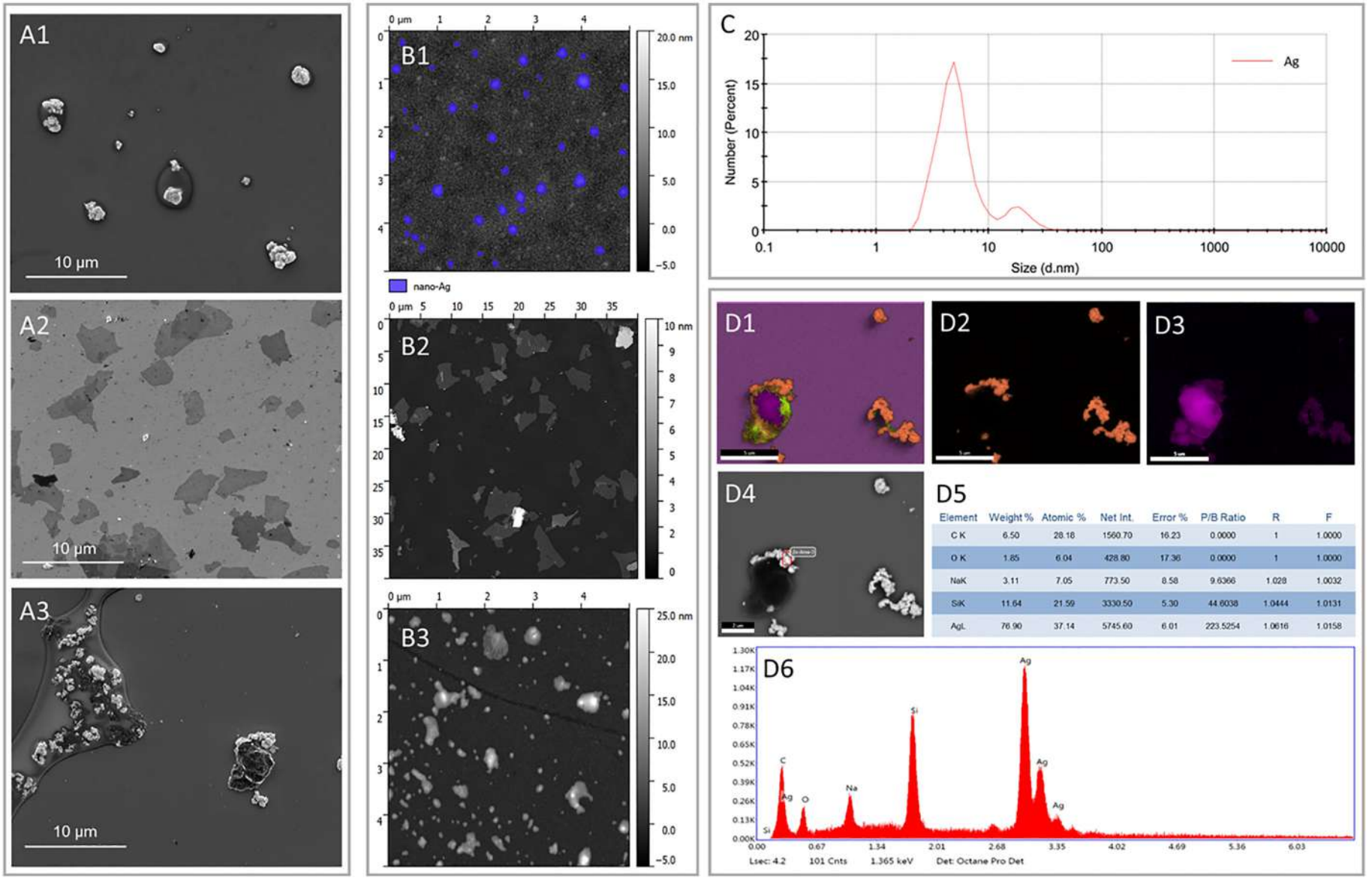

Fig. 1. Physicochemical characteristics of nanoparticles. (A) SEM images of silver nanoparticles (A1), graphene oxide (A2), GO-AgNPs composite (A3). (B) AFM images of silver nanoparticles (B1), graphene oxide (B2), GO-AgNPs composite (B3). (C) Dynamic light scattering (DLS) of silver nanoparticles. (D) SEM-EDS elemental mapping of GO-AgNPs composite (D1) with representative particles: Ag (D2), and carbon (D3). Spot EDS of GO-AgNPs (D4) with table for the atomic and weight percentage of various elements (D5) and area EDS spectrum (D6).

Table 1
Enzymes and substrates in API ZYM test (BioMérieux).

No.	Enzyme	APIZYM substrates	Abbreviation
1	*Negative control*	None	
2	*Alkaline phosphomonoesterase*	2-Naphtyl phosphate	AlP
3	*Esterase (C4)*	2-Naphtyl butyrate	Est
4	*Esterase lipase (C8)*	2-Naphtyl caprylate	EstLip
5	*Lipase*	2-Naphtyl myristate	Lip
6	*Leucine-arylamidase*	L-Leucyl-2-naphthylamide	ALeu
7	*Valine arylamidase*	L-Valyl-2-naphthylamide	AVal
8	*Cystine arylamidase*	L-Cystyl-2-naphthylamide	ACys
9	*Trypsin*	*N*-Benzoyl-DL-arginine-2-naphtylamide	T
10	*α-chymotrypsin*	*N*-Glutatyl-phenylalanine-2-naphthylamide	ChT
11	*Acid phosphatase*	2-Naphthyl phosphate	AcP
12	*Naphthol-AS-BI-phosphohydrolase*	Naphthol-AS-BI-phosphate	NPH
13	*α-Galactosidase*	6-Br-2-naphthyl-αD-galactopyranoside	αGal
14	*ß-Galactosidase*	2-Naphthyl-βD-galactopyranoside	ßGal
15	*ß-Glucuronidase*	Naphthol-AS-BI-βD-glucuronide	ßGlr
16	*α-Glucosidase*	2-Naphthyl-αD-glucopyranoside	αGlu
17	*ß-Glucosidase*	6-Br-2-naphthyl- βD-glucopyranoside	ßGlu
18	*N-acetyl-ß-glucosaminidase*	1-Naphthyl-*N*-acetyl-βD-glucosamide	NAG
19	*α-Mannosidase*	6-Br-2-naphthyl- αD-mannopyranoside	αMan
20	*α-Fucosidase*	2-Naphthyl-αL-fucopyranoside	αFuc

manufacturer's standard. The enzymatic activity (color intensity of the wells) was measured using ImageJ® software using grayscale images. Each enzyme was described as the arithmetic mean of the frequency plot (1D histogram) over the range [0.255] and converted relative to the control well.

2.9. Statistical analysis

The measurements were performed in six replicates. The distribution of the data and homogeneity of variance were checked for all parameters before analysis. The Kolmogorov-Smirnov and Lilliefors tests for normality and Levene's test for variance homogeneity allowed the analysis of variance. As the data fulfilled the analysis of variance assumptions, we performed parametric tests to check for differences between experimental groups. The least significant difference test (ANOVA, LSD test, $p < 0.05$) was performed separately for each parameter and each time point. All parameters are expressed as mean + SE in the figures. Statistical analyses were performed using the Statistica 13.1.

3. Results

3.1. Energy budget

Ten-day-lasting exposure of young female crickets to nanoparticles (NPs) revealed time-dependent changes in food (energy) consumption and utilization.

The average daily consumption per individual was approximately 800 J in all groups except females from the GO group that ingested 24% more energy than the control insects (Fig. 2B). The highest daily consumption in all groups occurred during the first 3 days of the experiment; however, it was significantly lower in the GOAg group's crickets.

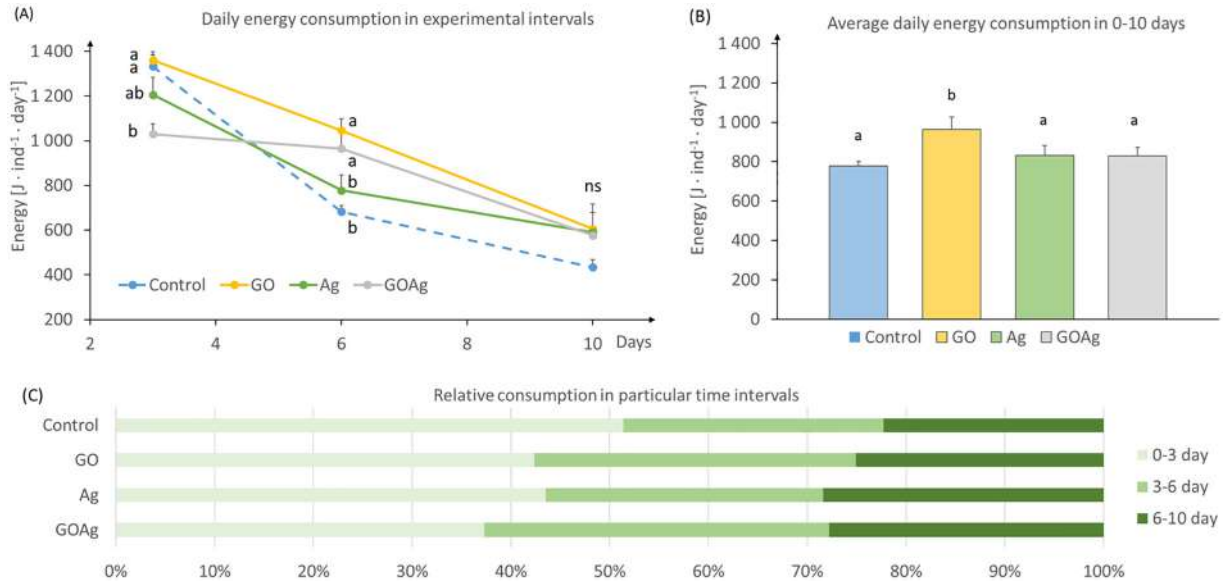

Fig. 2. Daily energy consumption (mean + SE) by young female crickets during the subsequent time intervals (A), the whole experiment (B), and its changes among the intervals (C). The same letter denotes homogenous group (ANOVA, NIR, $p < 0{,}05$). Experimental groups: GO – crickets exposed to graphene oxide admixture at 20 μg · 1 g^{-1} dry weight feed, Ag – crickets exposed silver nanoparticles at 400 μg · 1 g^{-1} dry weight feed, GOAg – crickets exposed to both GO and AgNPs in the given amounts; experimental intervals: 0–3 days, 3–6 days, and 6–10 days.

In the following days, the energy intake decreased, although it was higher in the NP-treated groups than in the control group (Figs. 2A,C). However, during the 6–10 d period, the differences between the experimental groups were not significant.

Average energy assimilation during the whole experiment did not differ among the experimental groups; however, the differences occurred in the subsequent measurement time intervals (Figs. 3A-C). Energy assimilation followed the consumption "pattern," however it was significantly lower in all NPs-treated groups than in control during the first 3 days. The control females assimilated 52.3% of the total assimilated energy in this interval, whereas the NPs-exposed, up to 40% and 37.8% in the Ag and the GOAg groups, respectively (Figs. 3A, C). In the next 3 days, the decrease in assimilation was much lower in the GO and GOAg groups than in the control and Ag groups. After the sixth day, the assimilation in the groups receiving nanoparticles did not differ from that in the control group (Fig. 3A).

The approximate digestibility of ingested energy was significantly lower in NP-exposed crickets (up to 20% in the GO group) than in the control ones during the first 3 days of the experiment. This difference diminished in the following days; therefore, digestibility in the whole experimental period in the NP-treated crickets was only 4%–7% lower than that in the control (Figs. 4A-B). Calculations of ECD values revealed similar efficiency of digested energy conversion into the tissues (28.2%–22.2%). This means that approximately 70% of the assimilated energy was allocated to maintenance costs.

Comparison of water content in the samples revealed that feces of NP-treated crickets contained more H_2O than feces of the control ones by 25%–30% in the 0-3rd day period, and 14.7%–26.5% in the 3th–6th day period. Only the feces of the GOAg crickets had higher water content than that of the control (Fig. 4C). Interestingly, females from this group also retained more water in their tissues than did the control insects (Fig. 4D).

3.2. Gut enzymes' activities

Following quantitative transformation of the API®ZYM test, we observed time-dependent changes in the activity of gut enzymes. The highest relative activity was measured for enzymes digesting carbohydrates, whereas proteolytic and esterolytic enzymes were less important. This difference may reflect the food composition mainly containing carbohydrates, including fiber.

On the third day of the experiment, we observed the increased activity of most assayed carbohydrate-degrading enzymes in NP-exposed crickets, particularly those hydrolyzing β-glycosidic bonds such as β-galactosidase and β-glucosidase. Generally, the highest stimulation of these enzymes versus control was observed in females from the Ag group, while the lowest was observed in females from the GOAg group. The activity of α-mannosidase was lower following NP exposure, and α-galactosidase showed the lowest activity in all the groups (Fig. 5A).

On the 10th day, this stimulatory effect disappeared in all the assayed enzymes, and in the case of β-glucosidase and *N*-acetyl-β-glucosaminidase activity we measured slight inhibition (Fig. 5B).

Both acid (AcP) and alkaline (AlP) phosphatase and naphthol-AS-BI-phosphohydrolase (NPH) activity were higher in NP-treated female crickets than in the control on the third day of exposure (Fig. 5C). At the end of the experiment, the activity was the same in all the groups (Fig. 5D).

We also observed an elevated proteolytic activity on the third day of NP treatment compared to the control, particularly in the case of arylamidases. Higher activity of trypsin was observed only in the GO group (Fig. 6A). On the 10th day, this effect disappeared, and no stimulation or inhibition was observed (Fig. 6B).

The activity of esterolytic enzymes followed the same 'pattern' – stimulated activity in exposed crickets was observed on the third day and the same activity in all the groups – on the 10th day of the experiment.

4. Discussion

Hormetic factors (stressors, also changes in energy availability) trigger a stress reaction in the cell/organism, which is manifested, for example, by changes in the concentration of free radicals, disturbed ion distribution, increased consumption of energy reserves, and others. The most frequently described hormetic effects in response to this moderate stress are increased defense strategies, for example, enhanced activity of antioxidative enzymes, stimulation of chaperones indicating the intensification of protein synthesis/destruction processes,

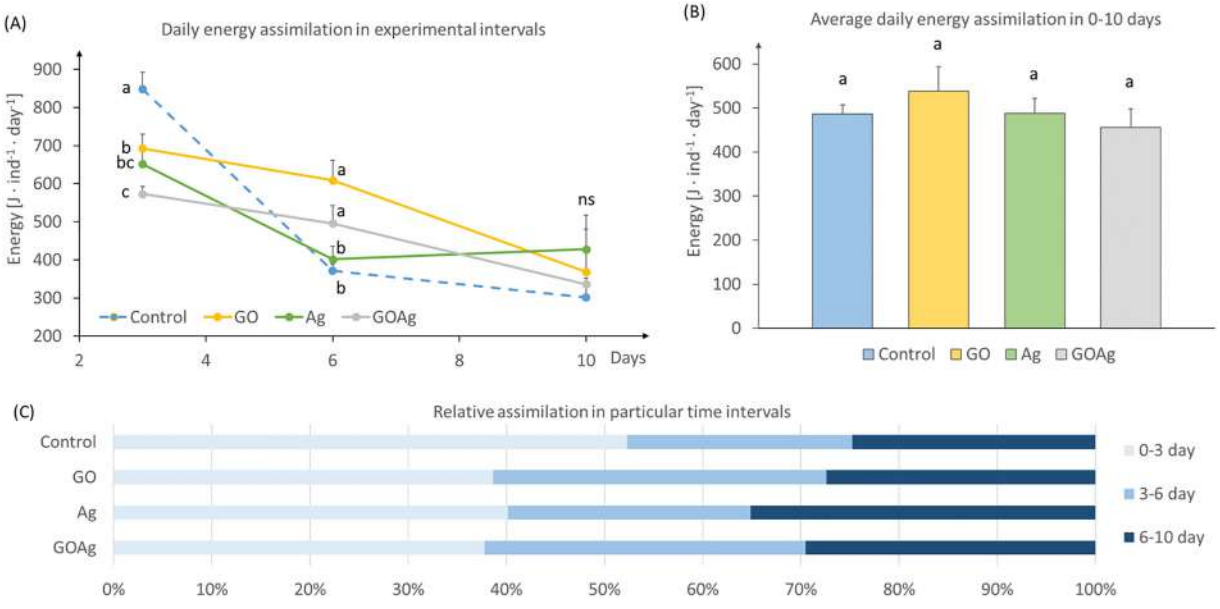

Fig. 3. Daily energy assimilation (mean + SE) by young cricket females during the subsequent time intervals (A), the whole experiment (B), and its changes among the intervals (C). The same letter denotes homogenous group (ANOVA, NIR, $p < 0,05$). Description of the groups as in Fig. 2.

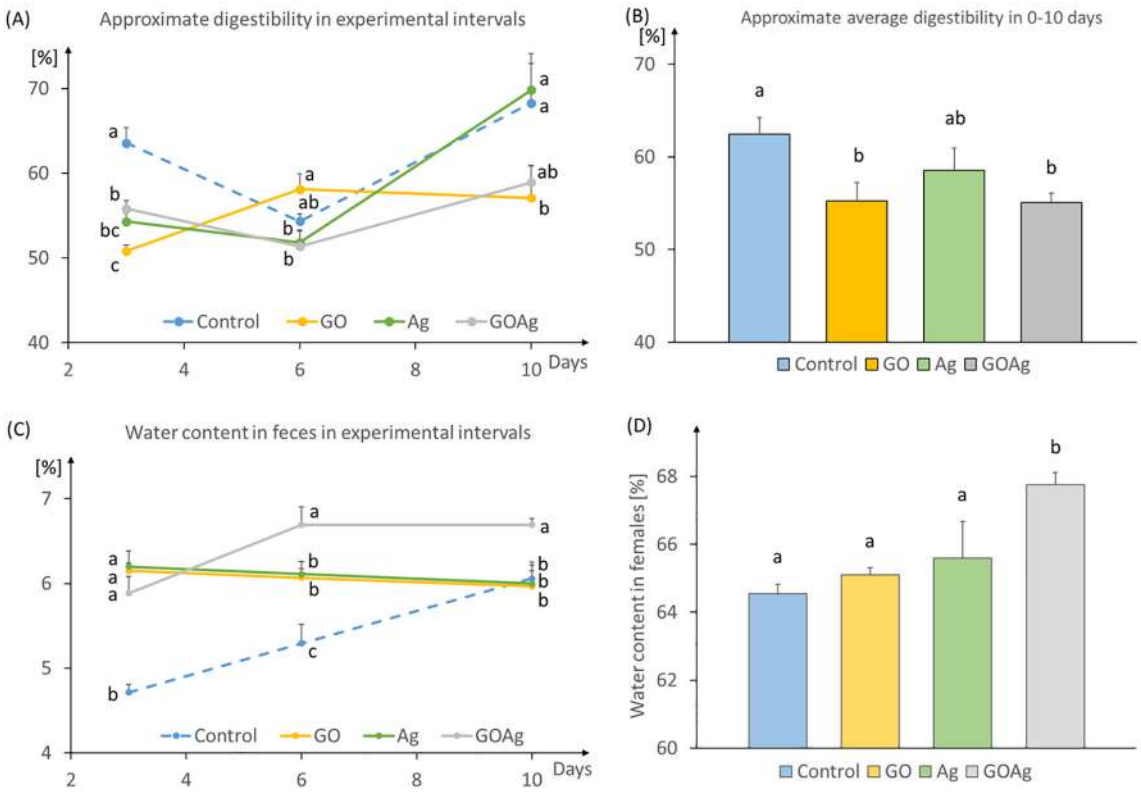

Fig. 4. Digestibility of ingested energy in subsequent time intervals (A), the whole experiment (B), and water content (mean + SE) in the feces (C) and females at the end of the experiment (D). The same letter denotes homogenous group (ANOVA, NIR, $p < 0,05$). Description of the groups and experimental intervals as in Fig. 2.

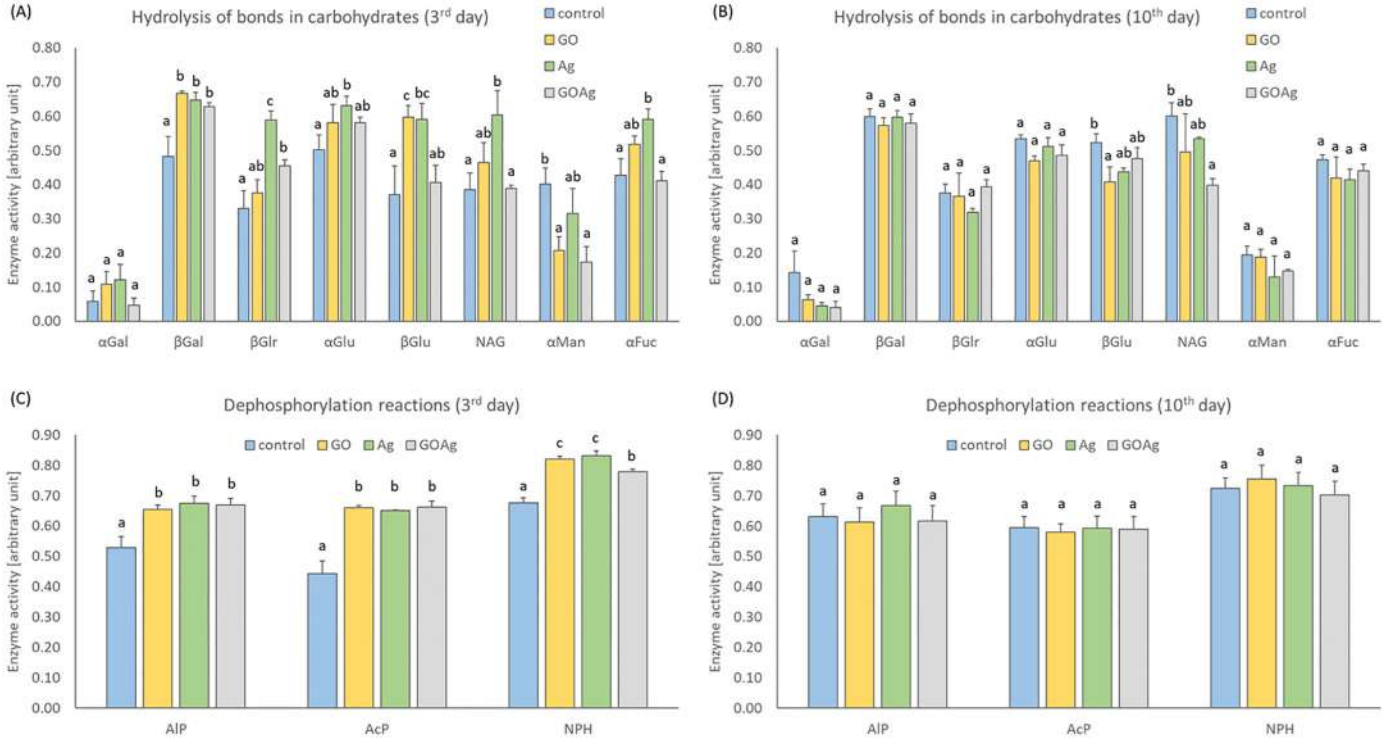

Fig. 5. Relative activity (mean + SE) of glycosidic bond-clevaging (A and B) and dephosphorylating (C and D) enzymes in digestive tract of house cricket females from control and nanoparticles-treated groups at 3rd (A, C) and 10th (B, D) day of continuous treatment. The same letter denotes homogenous group for particular enzyme (ANOVA, NIR, $p < 0,05$). Abbreviations of the groups as in Fig. 2; Glycolytic enzymes: αGal - α-galactosidase, βGal - β-galactosidase, βGlr - β-glucuronidase, αGlu – α-glucosidase, βGlu – β-glucosidase, NAG – *N*-acetyl-β-glucosaminidase, αMan – α-mannosidase, αFuc – α-fucosidase; Dephosphorylating enzymes: AlP – Alkaline phosphatase, AcP – Acid phosphatase, NPH – Naphthol-AS-BI-phosphohydrolase.

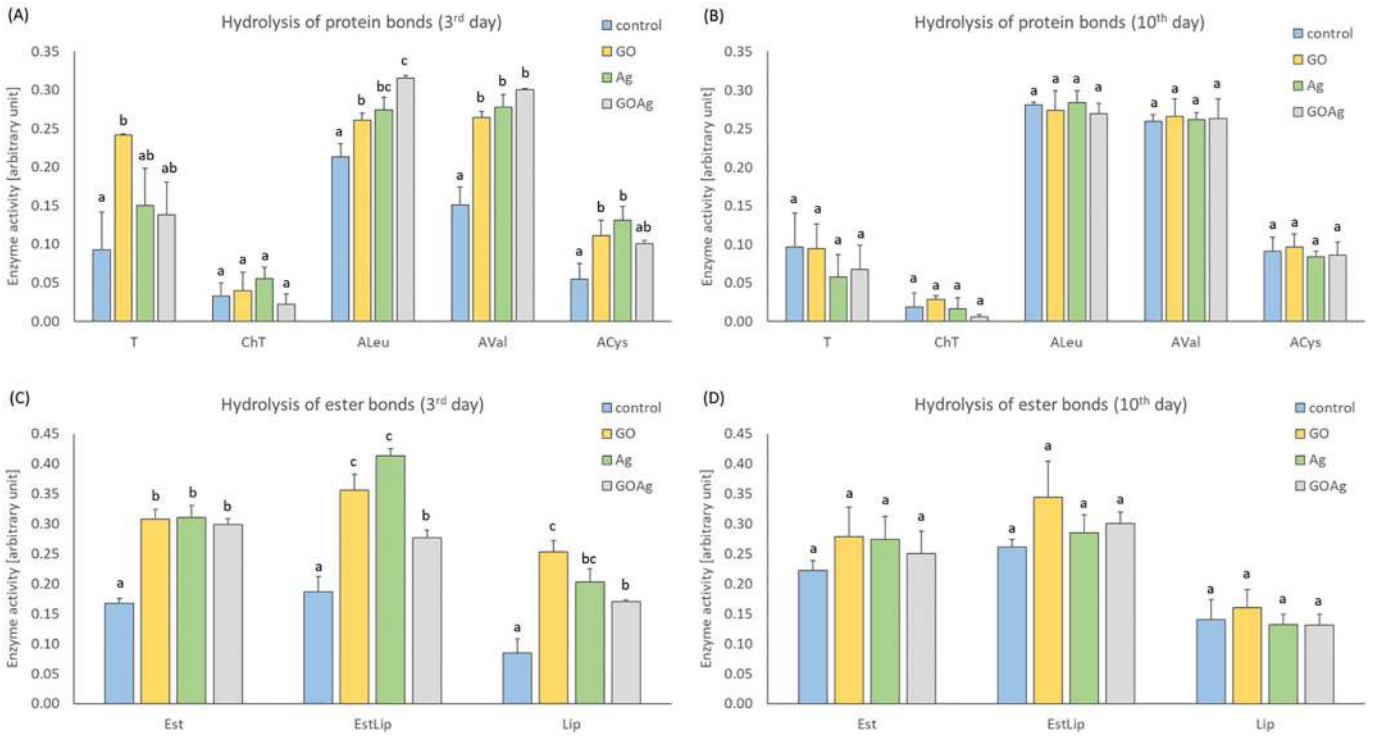

Fig. 6. Relative activity (mean + SE) of proteolytic (A and B) and esterolytic (C and D) enzymes in digestive tract of house cricket females from control and nanoparticles-treated groups at 3rd (A, C) and 10th (B, D) day of continuous treatment. The same letter denotes homogenous group for particular enzyme (ANOVA, NIR, $p < 0,05$). Experimental groups: GO – crickets exposed to graphene oxide admixture at 20 μg · 1 g^{-1} dry weigh feed, Ag – crickets exposed silver nanoparticles at 400 μg · 1 g^{-1} dry weigh feed, GOAg – crickets exposed to both GO and Ag in the given amounts; Proteolytic enzymes: T – trypsin, ChT – α-chymotrypsin, ALeu – Leucine arylamidase, Aval – Valine arylamidase, ACys – Cystine arylamidase; Esterolytic enzymes: Est – Esterase (C4), EstLip – Esterase Lipase (C8), Lip – Lipase (C14).

stimulation of growth factors, and proteins involved in energy metabolism. All these factors protect the cell/organism from more severe stress (Mattson, 2008). However, it should be noted that these defense processes require additional energy, which may increase food consumption and assimilation and presumably improve the digestive system's efficiency.

This study found that nanoparticles present in the food could temporarily disturb the cricket's energy budget and change digestive enzyme activities (Figs. 2–6). During the first exposure period (0–3 days), a significant reduction in consumption and assimilation was observed (Figs. 2A and 3A), which can be a manifestation of the initial disturbance of homeostasis (Calabrese, 2005). However, the analysis of the fingerprints of the gut enzymes on the third day of exposure (Figs. 5A and C, Figs. 6A and C) showed that the compensation mechanisms were triggered. They may be seen as a smaller drop in consumption and assimilation during the following intervals in NP-treated crickets than in control ones (i.e., days 3–6; Figs. 2 and 3 - see GO and GOAg groups). Higher energy intake and absorption than in the control might be a hormetic response triggered by low "pressure" of stressors. This suggests that the nanoparticle concentrations in our experiment were low enough to cause such a hormetic effect. Taking into account the concentration of NPs in the food and the consumption rate, we calculated that in the first 3 days of exposure, *A. domesticus* could take up 4.607 μg GO (GO group), 81.617 μg Ag (Ag group), and 3.488 μg GO and 69.762 μg Ag (GOAg group) with food. In fact, the biological effects of NPs have been studied over a wide range of concentrations, and the amounts used in the present work are among the lowest (Guo and Mei, 2014; Gomes et al., 2015; Yasur and Pathipati, 2015; Ferdous and Nemmar, 2020; Malhotra et al., 2020). The amount of available energy can vary under certain conditions and in a given life stage of an organism. It is a direct consequence of the accessible food resources and the ability to use them, that is, in animals, the efficiency of the digestive system and its supporting microorganisms (Karasov and Douglas, 2013; Celi et al., 2017). Hormesis may not occur if an organism does not have access to sufficient nutrients. In 2017, Wang et al. described an antibiotic-induced hormetic effect in *E. coli* only for bacteria cultured in media rich in utilizable carbon. According to Liebig's law of the minimum, the described regularity can be, with certain limitations (Gorban et al., 2010), extended to other organisms and processes involved in hormesis.

During the experiment, the insects had unlimited access to food. Aside from the unlikely effect of limiting the availability of nutrients by nanoparticles, it can be assumed that the insects did not starve during the experiment. Nevertheless, the effect of hormesis disappeared in the last period of the experiment, when consumption and assimilation (days 6–10), and the activity of the vast majority of the gut enzymes (day 10 of exposure) returned to the values typical for the control group (Figs. 2, 3, 5B, 5D, 6B, and 6D). To explain this phenomenon, we should also consider other factors that can shape the final response of an organism. The relationship between hormesis and dose and exposure time is clear and well documented (Calabrese, 2005; Agathokleous and Calabrese, 2020). However, many factors, partly related to the time, such as the animal's age, health/condition, physiological state, other harmful substances and reproductive effort (general body burden), may also be significant. Previous studies and our results allow to propose a 'hormesis graph' showing hormetic response as a resultant effect of numerous internal and external variables (Fig. 7). It should be noted that exposure time is only partially related to age and depends on the overall length of the animal's life. Aging organisms have a lower chance of surviving adverse conditions, weaker defense/repair mechanisms, and fewer energy reserves (Augustyniak et al., 2009a; Augustyniak et al., 2009b; Augustyniak et al., 2011). For *A. domesticus*, the period of 10 days of imago life was approximately ⅓ of the entire imago stage. Thus, the effect of hormesis can diminish

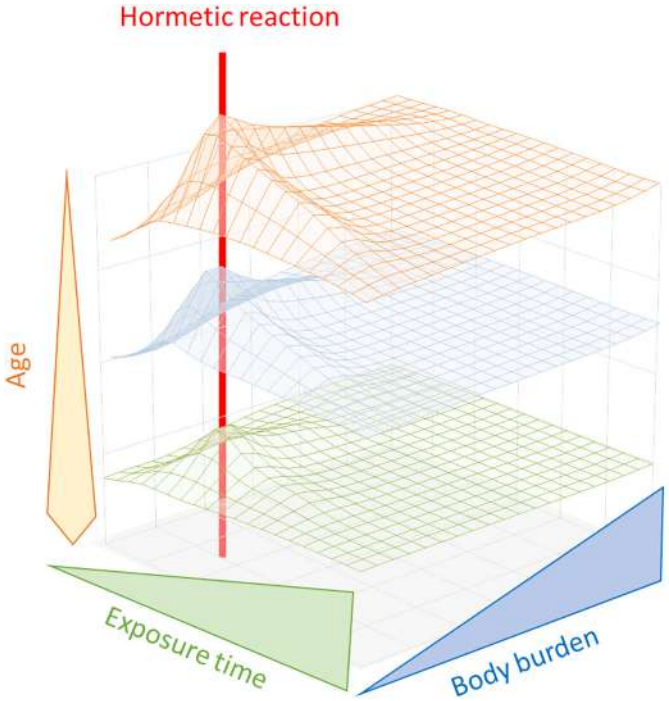

Fig. 7. Graphical presentation of hormetic response that depends on some internal and external variables. Explanations to the figure in the text.

with the exposure time and progressive aging of the animal (Fig. 7). In addition, the accumulation of other stress factors (toxins, metabolites, and degraded molecules) shapes the overall "body burden" and can influence the hormetic response. In *A. domesticus*, there were no additional stress factors. However, essential for the insects' overall "body burden" is the convergence of the disappearance of the hormetic effect on days 6–10 with the physiological need to produce eggs and oviposition. This phenomenon is cyclical and begins around this time (Destephano et al., 1982; Murtaugh and Denlinger, 1985).

Compared to the influence of individual nanoparticles on the tested parameters, it can be concluded that the GO-AgNP composite did not cause a drastically greater reaction than GO or AgNPs separately. The idea of an active nanoparticle surface, which can interact with the biomaterial, can help understand the obtained results. The large surface area of nanoparticles is responsible for their high reactivity (Jeevanandam et al., 2018). In the GO-AgNP composite, bonds between GO and AgNPs were formed, deactivating a part of the NP surface. Moreover, larger agglomerates were observed. This situation can lead to a reduction in the overall active area. Therefore, the GO-AgNP effect is weaker than it can be assumed, taking into account the simplified calculation, that is, the sum of the GO and AgNPs areas, used in this experiment. Thus, the composite may display lower activity with biological structures than its separate components. Further advanced material measurements and functional surface calculations are necessary to address this issue.

In the group treated with the composite, we found an increased amount of water in the feces (during the 3–6 days and 6–10 d intervals) and in the body of *A. domesticus* compared to the other experimental groups (Figs. 4C and D). Thus, the insects retained and possibly drank more water, which might have resulted from the greater water-holding capacity of the composite. Such effects were previously observed in plants grown on substrates with the addition of carbon nanoparticles (Khodakovskaya et al., 2012; Zhang et al., 2015; He et al., 2018; Park et al., 2020). To the best of our knowledge, this is the first observation of the effect of NPs on the water balance in insects. The effects of long-term exposure to the composite and greater water uptake importance will be investigated in the future.

5. Conclusions

This study confirms the hypothesis that used nanoparticles can change gut functions and deregulate the energy budget in the early stage of exposure. Even low amounts of dietary NPs can provoke an early physiological response that is stimulatory rather than inhibitory and resembles hormesis. Therefore, despite the different mode of deleterious action of nanoparticles, the exposed organisms seemed to mobilize a "standard" defense system that manifests itself by the alarm stage of the stress response to environmental threats. The age of the animal and the overall burden of the organism caused by reproduction or other factors may also influence the hormetic effect. However, this aspect should be examined in more advanced experiments, including a more extended period of life, critical stages in developing a given species, and, possibly, varus mixtures of xenobiotics.

CRediT authorship contribution statement

Reyhaneh Seyed Alian: Methodology, Investigation, Formal analysis, Writing – original draft, Visualization. **Marta Dziewięcka:** Methodology, Investigation, Validation, Formal analysis, Writing – original draft, Visualization. **Andrzej Kędziorski:** Methodology, Investigation, Validation, Formal analysis, Writing–original draft, Visualization. **Łukasz Majchrzycki:** Methodology, Validation, Investigation, Writing – review & editing. **Maria Augustyniak:** Conceptualization, Formal analysis, Writing–original draft, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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C.4. DECLARATION OF THE CO-AUTHOR OF THE MANUSCRIPT II

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I declare that my contribution to the preparation of the manuscript:

Seyed Alian R, Dziewięcka M, Kędziorski A, Majchrzycki Ł, Augustyniak M. Do nanoparticles cause hormesis? Early physiological compensatory response in house crickets to a dietary admixture of GO, Ag, and GOAg composite. Sci Total Environ. 2021;788:147801. doi: 10.1016/j.scitotenv.2021.147801,

which is part of my doctoral dissertation, involved participation in research planning, material collection, method optimization and analysis, data processing and interpretation, manuscript preparation for publication, and implementation of revisions.

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I hereby declare that obtaining a dedicated statement regarding the contribution of dr. Marta Dziewięcka was not possible, due to the lack of contact information following dr. Dziewięcka's change of employment. I would also like to note that a detailed description of co-authorship contributions is available in the publication text, within the CRediT authorship contribution statement section.

I declare that contribution of dr. Marta Dziwięcka to the preparation of the manuscript:

Seyed Alian R, Dziewięcka M, Kędziorski A, Majchrzycki Ł, Augustyniak M. Do nanoparticles cause hormesis? Early physiological compensatory response in house crickets to a dietary admixture of GO, Ag, and GOAg composite. Sci Total Environ. 2021;788:147801. doi: 10.1016/j.scitotenv.2021.147801,

which is part of my doctoral dissertation, involved assistance with methodology development for insects' maintenance, support in culture management and partial result analysis, as well as help in preparing the materials description (GO, AgNPs, and GOAg composite).

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which is part of Reyhaneh Seyed Alian doctoral dissertation, involved support in developing the methodology for determining the energy budget, assistance in the analysis and validation of results in this area, and support in preparing the manuscript content.

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which is part of Reyhaneh Seyed Alian doctoral dissertation, involved assistance in developing the research concept, support with data visualization and interpretation, as well as help in manuscript writing and revisions prior to publication.

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Prof. dr hab. Maria Augustyniak

C. MANUSCRIPTS INCLUDED IN THE DOCTORAL DISSERTATION

C.3. MANUSCRIPT II

Seyed Alian R, Flasz B, Kędziorski A, Majchrzycki Ł, Augustyniak M. Concentration- and Time-Dependent Dietary Exposure to Graphene Oxide and Silver Nanoparticles: Effects on Food Consumption and Assimilation, Digestive Enzyme Activities, and Body Mass in *Acheta domesticus*. Insects. 2024;15(2):89.
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Article

Concentration- and Time-Dependent Dietary Exposure to Graphene Oxide and Silver Nanoparticles: Effects on Food Consumption and Assimilation, Digestive Enzyme Activities, and Body Mass in *Acheta domesticus*

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Simple Summary: The increasing presence or contamination of various everyday-life products (including foodstuffs) by graphene oxides and silver nanoparticles (GOs and AgNPs, respectively) raises a risk of their possibly deleterious effects on digestive functions and, consequently, nutrient and energy intake by an organism. The study addresses this issue by considering various NP concentrations and exposure times. The scarcity of relevant data makes such studies necessary for the reliable assessment of NP effects. This study on a model insect species—adult house crickets—revealed a changed profile of digestive enzymes' activities in the gut, mainly when a high content of NPs was present in the food: stimulated digestion of carbohydrates and lipids but inhibited digestion of proteins. These changes were more pronounced in AgNP-treated than in GO-treated insects and increased with exposure time. Disturbed digestion led to decreased food consumption with exposure time in AgNP-treated crickets. Food assimilation was also affected—the cumulative food assimilation (CFA) was higher and lower compared with the control in crickets exposed to the lowest and moderate concentrations of AgNPs, respectively. These findings confirmed weak or no effects of low amounts of NPs in food and revealed that their higher concentrations may adversely influence digestive processes and resulting nutrient and energy intakes, particularly during prolonged exposure of an organism.

Abstract: The advancement of nanotechnology poses a real risk of insect exposure to nanoparticles (NPs) that can enter the digestive system through contaminated food or nanopesticides. This study examines whether the exposure of model insect species—*Acheta domesticus*—to increasing graphene oxide (GO) and silver nanoparticle (AgNP) concentrations (2, 20, and 200 ppm and 4, 40, and 400 ppm, respectively) could change its digestive functions: enzymes' activities, food consumption, and assimilation. We noticed more pronounced alterations following exposure to AgNPs than to GO. They included increased activity of α-amylase, α-glucosidase, and lipase but inhibited protease activity. Prolonged exposure to higher concentrations of AgNPs resulted in a significantly decreased food consumption and changed assimilation compared with the control in adult crickets. A increase in body weight was observed in the insects from the Ag4 group and a decrease in body weight or no effects were observed in crickets from the Ag40 and Ag400 groups (i.e., 4, 40, or 400 ppm of AgNPs, respectively), suggesting that even a moderate disturbance in nutrient and energy availability may affect the body weight of an organism and its overall condition. This study underscores the intricate interplay between NPs and digestive enzymes, emphasizing the need for further investigation to comprehend the underlying mechanisms and consequences of these interactions.

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1. Introduction

The rapid advancement of nanotechnology poses an increasing challenge to numerous species in diverse ecosystems due to exposure to various nanoparticles (NPs). Although the severity of ecosystem infiltration by NPs remains uncertain, one may expect their increasing concentrations due to growing applications in various fields [1–4].

Many NPs, including graphene oxide (GO) and silver nanoparticles (AgNPs), have recently drawn attention as undesirable food contaminants [5–9]. Contemporary applications of GO in the cultivation of edible plants, food production, processing, and packaging, in order to prevent bacterial growth and spoilage, may increase its content in a variety of food products. Other possible sources of their contamination with NPs include biosensors, food quality detectors, food composition analyzers, and NP-containing disinfectants and coatings. Most studies highlighting innovative applications of NPs in the food industry emphasize the need for comprehensive toxicological research to understand their effects on digestive functions and, consequently, the organisms' conditions [8–12].

Nanoparticles can influence food digestion and, consequently, impact nutrient availability and assimilation, which may disturb an organism's development and performance [13–17]. Exposed animals may respond to the stressor by increasing food intake to maintain the efficiency of food utilization or by accelerating food passage through the digestive tract, leading to decreased assimilation [18]. However, under certain conditions, compensatory mechanisms may be activated [19].

There are few in vivo studies on NP effects on digestive enzymes, particularly in invertebrates, and a lack of comprehensive ones that encompass various NP concentrations and exposure durations. Some authors noticed inhibition of the enzymes' activity [13,15,20–23], but we observed, in a semi-quantitative screening approach, increased gut enzyme activity in insects exposed to low concentrations of AgNPs or GO [19]. Therefore, thorough investigations are required to explain these contradictory results, considering a broad range of both NP concentrations and exposure periods.

In this study, we compared the effects on the digestive functions of two types of nanoparticles (AgNPs and GO) that may display somewhat different modes of action. The proposed mechanisms of AgNP action include interaction with the cell membrane and its penetration, interactions with proteins and other cellular components, and enhancement of oxidative stress. The gradual release of silver ions from the NPs' surface can elicit time-delayed and prolonged effects [24–28]. Graphene oxide, an allotrope of carbon occurring in variously sized flakes, primarily affects target molecular structures by provoking oxidative stress [29–31]. Adhesion of GO particles to the cell membrane is also suggested [32]. Such adhesion to the surface of the gut epithelium may impair its functions, even without the internal penetration of GO.

The main aim of the study was to determine which of the selected nanoparticles and what concentration of them could change the activity of digestive enzymes and to establish if there was a causal relationship among the type of nanoparticles, their concentration, exposure time, the activity of selected digestive enzymes, and the amount of food consumption. We focused on detailed, quantitative measurements of the activity of selected digestive enzymes in *Acheta domesticus* exposed to nanoparticles at various concentrations. Furthermore, we performed the measurements over an extended period of time, covering almost the entire life span of the adult insect.

Our research was conducted on *Acheta domesticus* (Gryllidae, Orthoptera), a model organism in physiological and toxicological research that offers numerous advantages for designing in vivo experiments [33]. This species originates from southwestern Asia but is currently distributed worldwide. These insects have well-understood biology, a relatively

short life cycle, and a sufficiently large size for research purposes [34,35]. Recently, it has gained much attention as a possibly edible species, raising hopes for addressing global protein food shortages [36–39].

2. Materials and Methods

2.1. NP Characteristics

Silver nanoparticles (99.9%, 20–30 nm) were purchased from SS Nanomaterials, Inc. (Houston, TX, USA) and prepared as a stable colloidal stock solution (10 mg/L) by sonication (UP-100H, DONSERV, Warsaw, Poland; cycle 1, amplitude 100%) in a citrate buffer solution (0.1 M; 10 mL; pH = 6.5; 30 min). Graphene oxide (GO) was purchased from Nanografi (Ankara, Turkey) and supplied as an aqueous suspension (10 mg/mL). Before use, the material was appropriately diluted, sonicated, and subjected to morpho-structural and physical analysis. Nanoparticles (NPs) were visualized and measured using microscopy techniques: scanning electron microscopy (SEM; Quanta FEG 250, FEI, Hillsboro, OR, USA) and atomic force microscopy (AFM) (Agilent 5500) (Agilent Technologies, Santa Clara, CA, USA). The electrokinetic potential (zeta potential) of the NP water in aqueous suspension was measured at 25 °C using a Litesizer 500 (Anton Paar, Graz, Austria). A detailed description of the sample preparation procedure for the analysis was published previously [19,29].

The silver nanoparticles had a size of ca. 20 nm and tended to form aggregates 50–60 nm in diameter (Figure 1A,B). The zeta potential was −44.5 mV, confirming the good stability of the suspension (Figure 1C). The microscopic analysis of the GO suspension confirmed the presence of flakes, mainly single-layered with a diameter of up to several µm (Figure 1A′,B′). The zeta potential (−30.2 mV) indicated the good stability of the GO suspension used for food preparation (Figure 1C′).

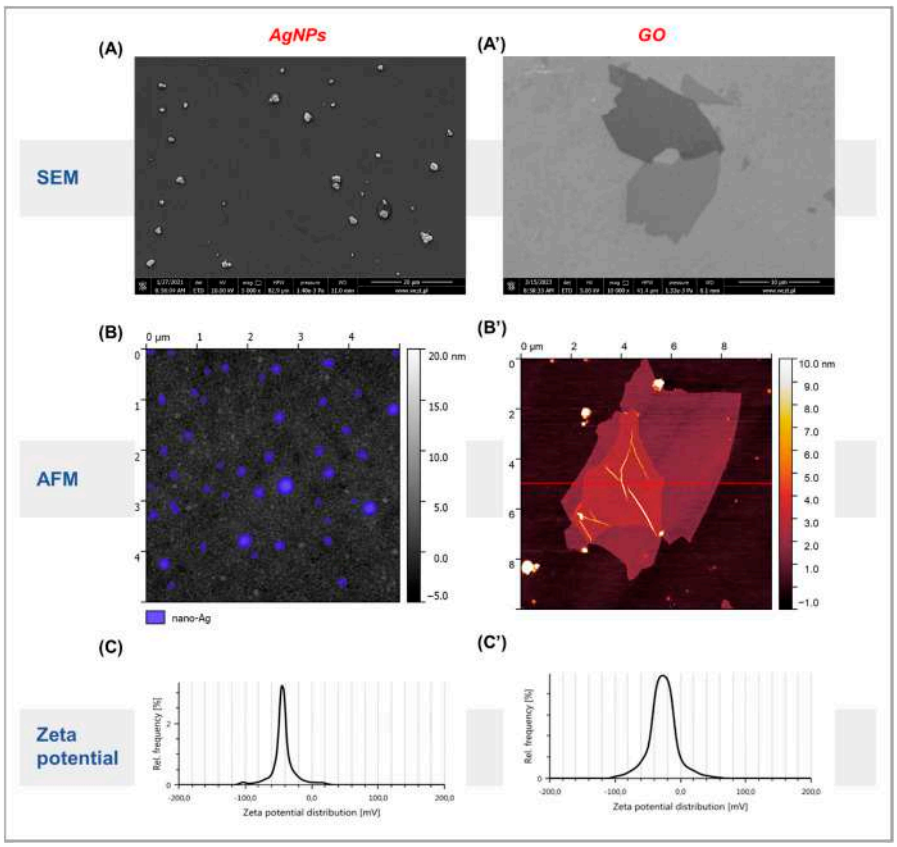


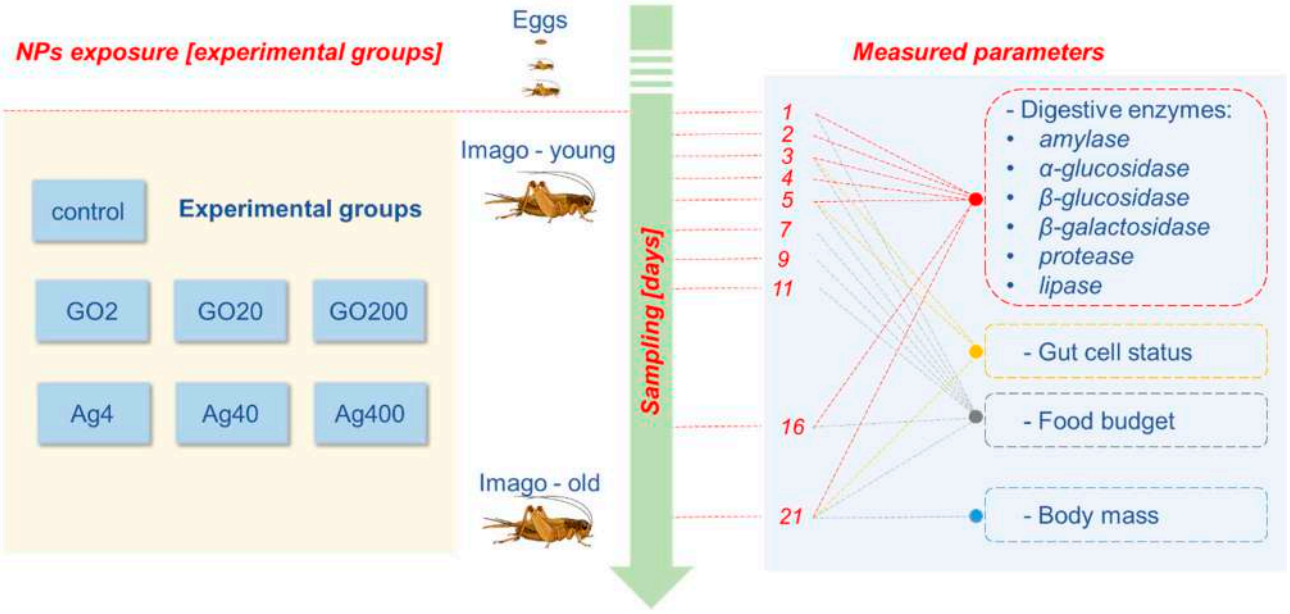
Figure 1. Physicochemical characteristics of silver nanoparticles (AgNPs) and graphene oxide (GO) used in the experiment. (**A**,**A′**) Scanning electron microscopy (SEM) images, (**B**,**B′**) atomic force microscopy (AFM) images, and (**C**,**C′**) zeta potential distribution.

2.2. Acheta Domesticus

The insects used in the experiment were obtained from our stock colony, maintained at the Institute of Biology, Biotechnology, and Environmental Protection at the University of Silesia in Katowice for over 30 years [40]. The breeding conditions were monitored and controlled to maintain optimal ranges of temperature (28.6 ± 1 °C), a photoperiod (L:D 12:12), and humidity (35–47%). The insects were fed the standard pellet food for rabbits (KDT; UNIPASZ, Siemiatycze, Poland; see Supplementary Materials for detailed description), and in the experiment, they were provided with food containing various concentrations of graphene oxide (GO) or silver nanoparticles (AgNPs).

2.3. Experimental Design

Cricket eggs were obtained from the laboratory stock colony and kept on a wet substrate. The hatched nymphs were reared in a plastic fauna box under standard conditions until they reached the imago stage. The 1-day-old adults were randomly assigned to seven experimental groups: a control group and groups fed with the feed with GO or AgNPs admixtures, each at three different concentrations (Scheme 1). The food was prepared following the protocol established in our laboratory and described previously [19,41]. Briefly, ground rabbit pellets were mixed with the appropriate GO or AgNP suspension volume in distilled water. The final concentrations of GO or AgNPs were as follows: 2, 20, and 200 µg GO/g of food (experimental groups GO2, GO20, and GO200, respectively) or 4, 40, and 400 µg AgNPs/g of food (experimental groups Ag4, Ag40, and Ag400, respectively). The feed for the control group was prepared the same way but with distilled water added instead of the nanoparticle suspension. Then, the feed was dried for 48 h in a dryer (Pol-Eko Aparatura, Wodzislaw Slaski, Poland) at 45 °C and sterilized for 48 h in a laminar chamber (UVcleaner, BIOSAN, Warren, MI, USA). The insects were housed in plastic fauna boxes (28 cm × 20 cm × 16 cm) under standard conditions with unrestricted food, water, and shelter access throughout the experiment.



Scheme 1. Experimental design.

2.4. Food Consumption and Assimilation

The assessment of food consumption and assimilation was conducted following the procedure described earlier [19]. Each experimental group comprised six replicates, with six individuals in each (36 individuals per treatment and 252 insects in the whole experiment). The mass of the provided food, food residues, feces, and insects was accurately measured

at 2-day intervals during the first ten days and at 5-day intervals subsequently (Scheme 1). Feed and feces samples were weighed after drying (50 °C for 48 h) with 1 mg accuracy (Semi-Micro Balance EX225D, OHAUS, Parsippany, NJ, USA). From these raw data, food consumption and assimilation (amount of ingested and digested feed) were calculated in mg dry weight per day for each individual and for each time interval [19].

2.5. Gut Cell Status

On days 3, 5, and 21, the percentage of dead cells in the *A. domesticus* gut was examined. Seven groups (one for each treatment) containing fifteen 1-day-old adults were set up and reared as described above. Five insects from each group were collected on the 3rd, 5th, and 21st days and gently anesthetized on ice. Then, the gut was isolated in 0.1 PBS buffer (pH 7.4; 400 µL; 4 °C). Subsequently, the tissue was gently homogenized (Minilys®, Bertin Technologies, Montigny-le-Bretonneux, France), and the resulting cell suspension was used to determine the percentage of dead cells by using flow cytometry (MUSE® Cell Analyzer, Millipore, Billerica, MA, USA), employing the Muse® Annexin V & Dead Cell Kit and following the provided kit protocol. Dead cells were identified as 7-AAD (Aminoactinomycin D—a fluorescent intercalator) positive.

2.6. Digestive Enzyme Measurements

For the measurements of digestive enzymes' activity (protease, amylase, α-glucosidase, β-glucosidase, β-galactosidase, and lipase), gut samples were collected on days 1, 2, 3, 4, 5, 16, and 21 from the start of NP treatment (Scheme 1). Samples were obtained from anesthetized crickets by isolating the midgut and homogenizing the tissue in a phosphate buffer (pH 7.4; 1 mL; 4 °C), followed by centrifugation of the homogenates at 14,000 rpm for 10 minutes at 4 °C. Each sample consisted of midguts isolated from three individuals (100 ± 20 mg). Five homogenates were prepared for each experimental group. All analyses of digestive enzymes' activity were conducted using commercially available kits. Our team has optimized all protocols provided by the manufacturers for *Acheta domesticus* tissues.

To estimate protease activity, the Protease Assay Kit (Calbiochem; Merck KGaA, Darmstadt, Germany; Cat. No. 539125; LOT 3802816) was used, and the activity was measured spectrophotometrically as changes in absorbance at 492 nm per minute. Amylase activity was measured using the Amylase Activity Assay Kit (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. MAK009; LOT 8E24K07110) and expressed in µmol of product/min/mL supernatant. The α-Glucosidase Activity Assay Kit (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. MAK123; LOT 123CA05A04) and β-Glucosidase Activity Assay Kit (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. MAK129; LOT 129CB08A18) were applied to determine α- and β-glucosidase activity, respectively. These assays were based on the substrate-specific product formation reaction rate and were measured spectrophotometrically (TECAN Infinite M200, Männedorf, Austria) in 96-well flat-bottom plates at a wavelength of 405 nm. The activities of α-glucosidase and β-glucosidase were expressed in units/L, with 1 unit equaling the amount of the enzyme that catalyzes the hydrolysis of 1 µmol of substrate/min. The activity of β-galactosidase was assessed using the β-Galactosidase Activity Assay Kit (Abcam, Cambridge, CB2 0AX, UK; Cat. No. ab287846; LOT GR3429797-1). The reaction rate was measured spectrofluorimetrically (HITACHI F-7000 Fluorescence Spectrometer Plate Reader, Tokyo, Japan; Ex/Em = 480/530 nm) for 30 minutes. β-Gal activity was expressed in units/L, with 1 unit representing the amount of enzyme that generates 1.0 µmol of Fluorescein per minute. Lipase activity was measured using the Lipase Activity Assay Kit (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. MAK046; LOT 8H15K07220). The formation rate of the reaction product catalyzed by lipase was measured at 570 nm with glycerol as the standard. Enzyme activity was expressed in µmol/min/mL.

2.7. Statistical Analysis

The analyses of digestive enzyme activity were conducted in five replicates, while the food budget analyses included six replicates. Before proceeding with statistical analyses,

the assumptions of the analysis of variance were checked. The Kolmogorov–Smirnov and Lilliefors tests were used to assess the data distribution. The Levene and Brown–Forsythe tests were applied to evaluate the homogeneity of variances. For parameters meeting the assumptions of ANOVA (body mass, food consumption, and assimilation, as well as cumulative food consumption and assimilation), the main effects and interactions were analyzed using ANOVA/MANOVA. Then, the differences were examined with a post hoc LSD test. For other parameters, due to the ambiguity of the results of these tests for ANOVA assumption, two statistical approaches were used in the subsequent analysis of the results. To assess the main effects and their interactions, PERMANOVA, a non-parametric test, and multivariate repeated measures ANOVA (Tukey test, $p < 0.05$) were performed. The results of both tests were compared. The charts present results as mean ± SE or median ± interquartile range. Statistical analyses were conducted using STATISTICA® 13.3 (StatSoft Inc., Tulsa, OK, USA) and R (Adonis function, vegan package).

3. Results

3.1. Body Mass and Food Budget—Consumption and Assimilation

The average body mass gain of insects in the control group after 5 and 21 days of the experiment was 53.6 and 73.3 mg/individual, respectively, indicating a major increase during the first few days of the adult stage (Figure 2. The ANOVA/MANOVA analysis showed a significant effect of "NP type" and "concentration," as well as both factors' interactions (Table 1). Post hoc analyses revealed that these effects were mainly related to AgNPs (Figure 2). Treatment with GO did not affect the body weight in *A. domesticus*, except for the GO200 group, where significantly lower weight gain, compared with the control, during 1–5 days of adult life was observed (Figure 2A). The effect of AgNPs depended more on the concentration than on the duration of exposure—the lowest concentrations (4 µg AgNPs/g of food) resulted in the highest weight gain among all groups. The intermediate one (40 µg AgNPs/g of food) slowed down the insect's growth (Figure 2B). Despite the slowest growth, increased mortality was also observed in the latter group during the second half of the experiment.

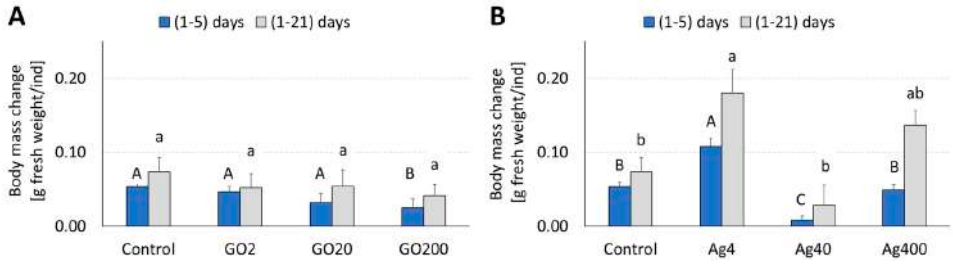


Figure 2. Fresh body mass change (grams of fresh weight per individual; mean + SE) in *A. domesticus* treated with nanoparticles: (**A**) graphene oxide (GO) and (**B**) silver nanoparticles (AgNPs). The changes in body mass after 5 days (blue bars) or 21 days (gray bars) of the experiment are depicted in the charts. Explanation and abbreviations: The same uppercase and lowercase letter indicates no differences among the groups within a particular NP treatment after 5 or 21 days of the experiment, respectively (ANOVA, LSD test, $p > 0.05$).

Table 1. The main effects and interactions of the factors: NP type and concentration on body mass gain of *Acheta domesticus* after 5 and/or 21 days of exposure (ANOVA/MANOVA; F—F ratio; df_1 and df_2—treatment and error degrees of freedom, respectively; *p*—*p* value, n = 6).

Effect	5th and 21st Day				5th Day				21st Day			
	F	df_1	df_2	*p*	F	df_1	df_2	*p*	F	df_1	df_2	*p*
NP type [1]	4.973	2	37	0.012	6.065	1	40	0.018	9.823	1	38	0.003
Concentration [2]	6.564	6	74	<0.001	15.135	3	40	<0.001	3.710	3	38	0.020
[1] × [2]	4.111	6	74	0.001	8.381	3	40	<0.001	5.393	3	38	0.003

Food consumption was measured in 2- and 5-day intervals depending on the expected consumption rate by the crickets and calculated per day per individual for each interval (Figure 3). The effects of "NP type", their "concentration", and duration of exposure, as well as their interactions, were analyzed using repeated measures ANOVA (Table 2) and PERMANOVA (Table S1). The highest consumption occurred during the first 5 days of the imago life in all the groups. The control crickets consumed on average 37.0 and 42.0 mg of dry food per individual per day in the first and second measured intervals, respectively. In the next intervals, consumption in this group decreased and remained in the range of 21.0–25.7 mg per individual per day. The multivariate repeated measures ANOVA analysis revealed that the "NP type" had no significant effect on food consumption. However, the "concentration" and "time" factors significantly influenced it. Interactions between the exposure time and the "NP type" or "concentration" were also significant but not the interaction among all three factors (Table 2), showing that both nanoparticles similarly affected food consumption. However, unlike GO-treated crickets, decreased food consumption in the Ag40 group compared with the control was observed over the entire experiment and in the other AgNP groups in its second half (Figure 3). Detailed results of the post hoc test are provided in supplementary materials (Table S2).

Food assimilation in the control group followed changes in the consumption rate and was the highest during the first 5 days of the experiment, reaching 47.6% of the consumed food. Then, it slowly decreased to 33% in the last measured age interval. All factors included in this experimental model significantly affected food assimilation, with the strongest effect exerted by the "time" factor (Table 2). The main effects analyses revealed significantly higher assimilation in the GO groups treated with the lowest concentration of nanoparticles (55% of the consumed food). Assimilation decreased significantly with the age of the insects but still allowed them to absorb about 50% of the consumed food. A higher assimilation rate than the control was also observed in the AgNP-treated groups, particularly in the Ag4 group. Factor interactions were significant, except for the "time" × "NP type" × "concentration" interaction, suggesting a similar pattern of effects induced by different concentrations of GO and AgNPs throughout the experiment (Figure 4). However, the non-parametric PERMANOVA test showed that all factors and their interactions affected food assimilation (Table S1). Detailed results of the post hoc analyses for the food assimilation are included in supplementary materials (Table S3).

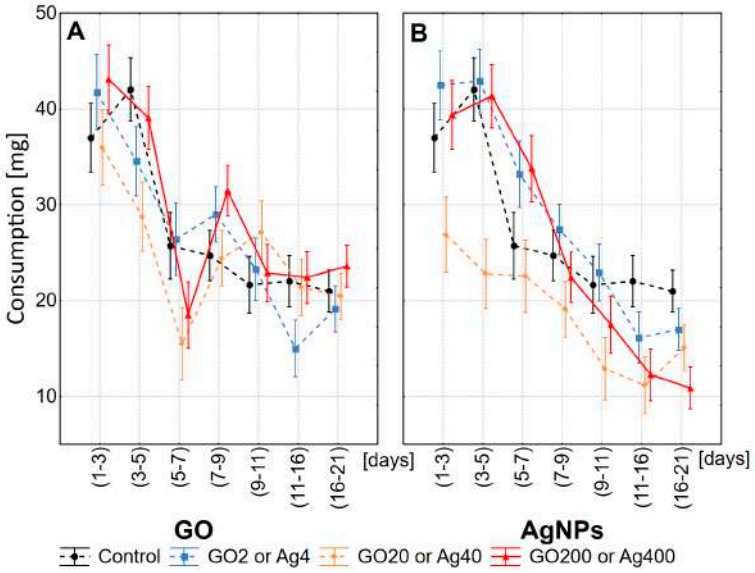


Figure 3. Food consumption (mg dry weight per individual per day; mean + SE) in *A. domesticus* treated with nanoparticles: (**A**) graphene oxide (GO) and (**B**) silver nanoparticles (AgNPs), calculated for particular few-day intervals. For detailed post hoc test results, see Table S2 in Supplementary Materials.

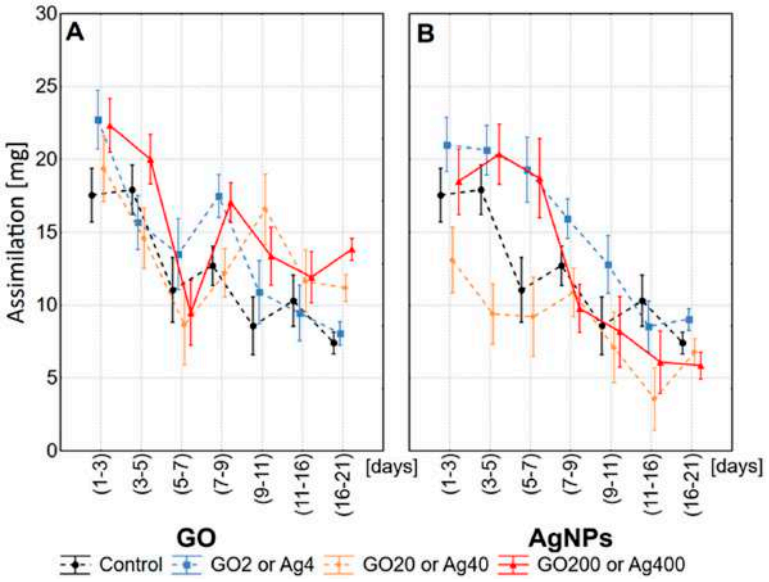


Figure 4. Food assimilation (mg dry weight per individual per day; mean + SE) in *A. domesticus* treated with nanoparticles: (**A**) graphene oxide (GO) and (**B**) silver nanoparticles (AgNPs), calculated for particular few-day intervals. For detailed post hoc test results, see Table S3 in Supplementary Materials.

Table 2. Multivariate repeated measures ANOVA for NP type, concentration, time, and interaction of all factors on food consumption and assimilation measured in time intervals within 21 days of exposure (F—F ratio; df_1 and df_2—treatment and error degrees of freedom, respectively; *p*—*p* value, n = 6).

Effect	Food Consumption				Food Assimilation			
	F	**df_1**	**df_2**	***p***	**F**	**df_1**	**df_2**	***p***
NP type [1]	2.383	1	37	0.131	7.882	1	33	0.008
Concentration [2]	5.235	3	37	0.004	7.819	3	33	<0.001
[1] × [2]	1.952	3	37	0.138	5.697	3	33	0.003
Time [3]	69.177	6	32	<0.001	36.632	6	28	<0.001
[3] × [1]	5.453	6	32	<0.001	4.350	6	28	<0.001
[3] × [2]	2.461	18	91	0.001	1.933	18	80	0.015
[3] × [1] × [2]	1.499	18	91	0.092	1.428	18	80	0.121

The obtained results were consistent with the cumulative food budget parameters—cumulative food consumption (CFC) and cumulative food absorption (CFA)—analyzed on day 21 (Table 3). The type of NPs significantly affected CFA, but their concentration affected both parameters. Again, interactions between the "NP type" and "concentration" appeared significant in the combined analysis and the CFA case (Table 3). Post hoc analysis showed that only in the Ag40 group was there a significantly lower CFC. GO caused a significant increase in CFA only in the GO200 group, while AgNPs caused a significant increase in CFA in the Ag4 group and its decrease in the Ag40 group, compared with the control (Figure 5). A visualization of the averaged CFC and CFA changes throughout the experiment is provided in the supplementary materials (Figures S1 and S2).

Table 3. The main effects and interactions of the factors: NP type and concentration on cumulative food consumption (CFC) or cumulative food assimilation (CFA) of *Acheta domesticus* during 21 days of exposure (ANOVA/MANOVA; F—F ratio; df_1 and df_2—treatment and error degrees of freedom, respectively; *p*—*p* value, n = 6).

Effect	CFC				CFA			
	F	df_1	df_2	*p*	F	df_1	df_2	*p*
NP type [1]	2.555	1	40	0.118	12.366	1	40	0.001
Concentration [2]	4.451	3	40	0.009	8.002	3	40	<0.001
[1] × [2]	2.541	3	40	0.070	9.471	3	40	<0.001

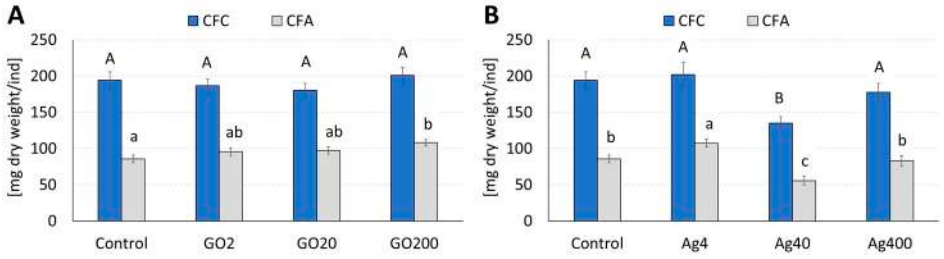


Figure 5. Cumulative food consumption (CFC) and cumulative food assimilation (CFA) in mg dry weight per individual (mean ± SE) in *A. domesticus* treated with nanoparticles for 21 days: (**A**) graphene oxide (GO) and (**B**) silver nanoparticles (AgNPs). Explanation and abbreviations: see Figure 2.

3.2. Gut Cell Status

The gut cell viability significantly depended on the insects' age (Figure 6). In the control group, the percentage of dead cells was below 1% at the beginning of the experiment, 3.98% on average on the 5th day, and 20.72% on the 21st day. Compared with the control, nanoparticles did not change the percentage of dead cells on the third and fifth days of the experiment. On the final day of the experiment, crickets from groups GO2 and GO20 maintained a low percentage of dead cells, significantly below that of the control (Figure 6).

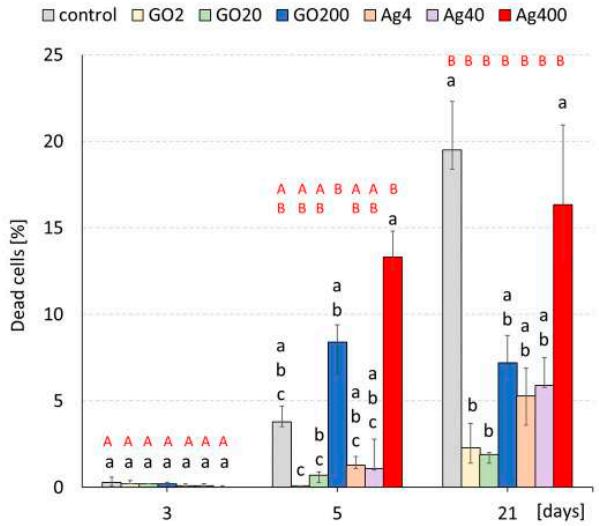


Figure 6. Dead cells (%; median ± interquartile range) detected by MUSE® Annexin V in the gut of *A. domesticus* treated with GO or AgNPs and measured in 3, 5, or 21 days of the experiment. Bars labeled with identical lowercase letters do not differ significantly among experimental groups within a given day; identical uppercase letters denote a lack of differences within the group among particular days (Kruskal–Wallis test, $p > 0.05$).

3.3. Enzyme Activity

Due to the ambiguous results of the tests that verified the assumptions for the analysis of variance, we performed a PERMANOVA analysis, as well as a repeated measures ANOVA (for the latter, see Tables S4A,B in Supplementary Materials), to reveal the effects of experimental factors and their interactions on the activity of digestive enzymes. Both particular factors and their interactions significantly affected the activity of digestive enzymes (Table 4). The analysis of variance with repeated measurements yielded similar results (Tables S4A,B).

Table 4. Permutational analysis of variance (PERMANOVA) test considering the main factors, NP type, concentration, and time, and their interactions, influencing digestive enzymes measured in the gut of *Acheta domesticus* following exposure to GO or AgNPs in the diet (PERMANOVA was performed with 999 permutations, using the adonis2 function in R vegan package R4.2.1; F—F ratio; df—degrees of freedom; *p*—*p*-value, n = 5).

Effect	All Parameters			Amylase			Protease			Lipase		
	F	**df**	***p***	**F**	**df**	***p***	**F**	**df**	***p***	**F**	**df**	***p***
NP type [1]	62.015	1	0.001	413.564	1	0.001	15.880	1	0.001	61.463	1	0.001
Concentration [2]	133.090	3	0.001	455.196	3	0.001	24.528	3	0.001	106.993	3	0.001
Time [3]	14.300	6	0.001	4.727	6	0.001	54.354	6	0.001	4.270	6	0.001
[1] × [2]	21.819	3	0.001	144.079	3	0.001	3.811	3	0.006	15.371	3	0.001
[1] × [3]	3.412	6	0.001	2.409	6	0.009	11.015	6	0.001	4.897	6	0.001
[2] × [3]	2.604	18	0.001	2.540	18	0.001	6.107	18	0.001	3.084	18	0.001
[1] × [2] × [3]	1.738	18	0.008	1.725	18	0.019	9.762	18	0.001	4.037	18	0.001
Residual		211			211			211			211	
Total		266			266			266			266	

Effect	α-Glu			β-Glu			β-Gal		
	F	**df**	***p***	**F**	**df**	***p***	**F**	**df**	***p***
NP type [1]	15.728	1	0.001	47.961	1	0.001	27.483	1	0.001
Concentration [2]	24.852	3	0.001	13.239	3	0.001	16.973	3	0.001
Time [3]	17.558	6	0.001	3.111	6	0.003	14.541	6	0.001
[1] × [2]	4.453	3	0.004	32.356	3	0.001	18.742	3	0.001
[1] × [3]	5.181	6	0.001	2.681	6	0.010	5.717	6	0.001
[2] × [3]	2.625	18	0.001	3.147	18	0.001	4.732	18	0.001
[1] × [2] × [3]	2.231	18	0.003	2.100	18	0.004	2.502	18	0.001
Residual		211			211			211	
Total		266			266			266	

The α-amylase activity typical for adult *A. domesticus* in the control group showed slight fluctuations throughout the experiment, with median values ranging between 3.15 and 6.17 µmol/min/mL. In this group, the highest activity was observed in insects on the second day of the experiment, while the lowest was recorded on the fifth day. GO significantly increased α-amylase activity but only in insects from the GO200 group. Lower GO concentrations did not alter α-amylase activity compared with the control, which was confirmed by both parametric and non-parametric tests (Figures 7A and S3 and Table S5). Treatment with AgNPs significantly increased α-amylase activity in the insects from the Ag400 group, where the activity was about 22–44 times that of the Ag400 group compared with the control (Figures 7B and S3 and Table S5).

The activity of α-glucosidase (α-Glu) in the control group increased slowly with the crickets' age. Both "NP type" and "concentration" had significant effects and indicated a stronger influence of AgNPs than GO (Table 4). The most pronounced increase in α-Glu activity was observed in the Ag4 group. However, on the last day of the experiment, the activity of α-Glu was significantly higher in the Ag40 and Ag400 groups than in the control (Figures 8B and S4 and Table S6). Following GO treatment, the activity of this enzyme did

not change, except for single time points for the used concentrations (Figures 8A and S4 and Table S6).

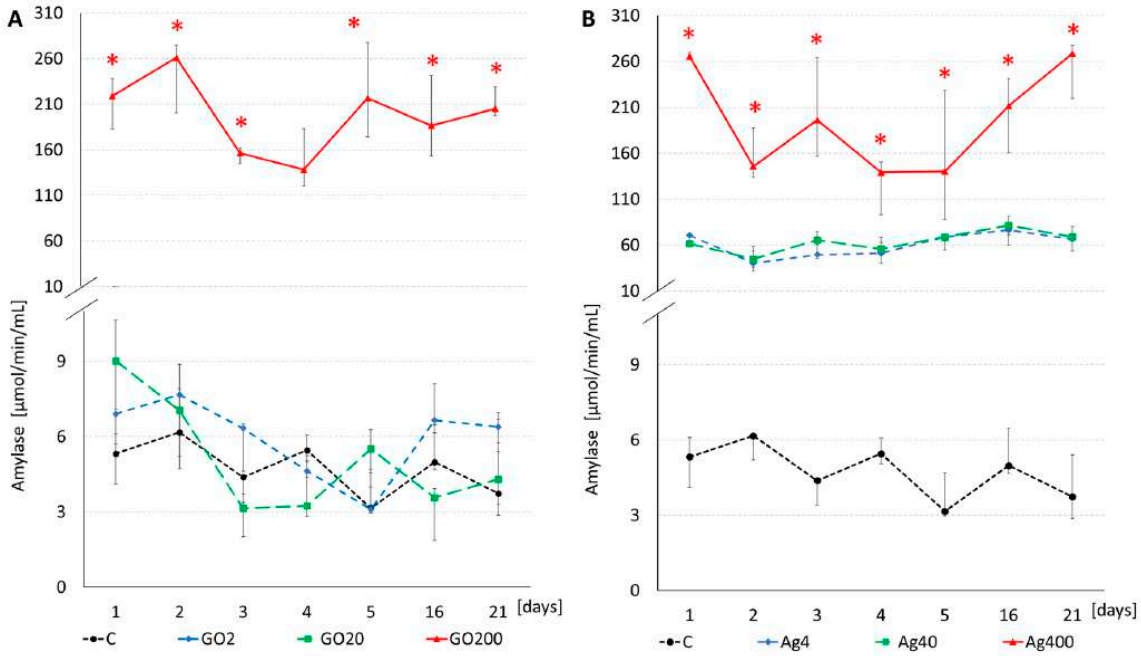


Figure 7. α-amylase activity (median ± interquartile range) in samples from the digestive tract of *A. domesticus* from control and nanoparticle-treated groups. (**A**) Graphene oxide (GO) and (**B**) silver nanoparticles (AgNPs). * indicate significant differences between the NP-treated group and control within each day (Kruskal–Wallis test, $p < 0.05$).

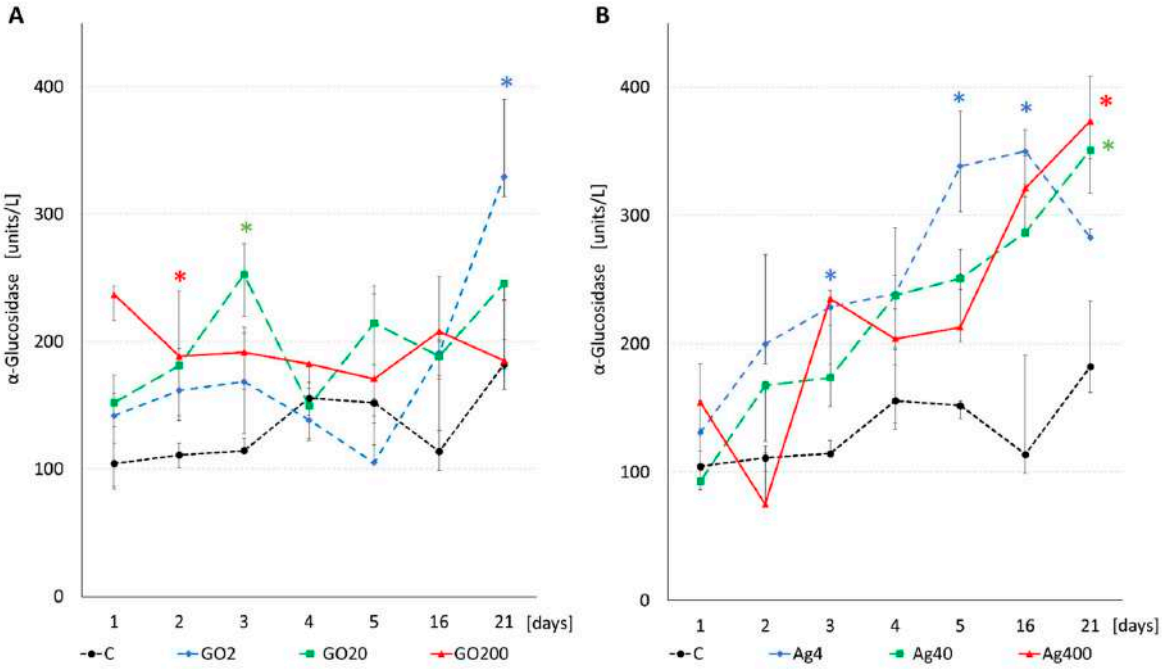


Figure 8. Activity of α-glucosidase (median ± interquartile range) in samples from the digestive tract of *A. domesticus* from control and nanoparticle-treated groups. (**A**) Graphene oxide (GO) and (**B**) silver nanoparticles (AgNPs). Explanation: see Figure 7.

In contrast to α-glucosidase, the basic activity of β-glucosidase (β-Glu) in the control group tended to decrease over time (Table S7). A significant effect on its activity was

stated for both the "NP type" and "concentration" factors (Tables 4 and S4B). GO groups demonstrated higher β-Glu activity than the control, with the highest values observed in the GO20 group. AgNPs at concentrations of 4 and 400 µg/g of food stimulated the activity of β-Glu. In contrast, an intermediate concentration (40 µg/g of food) inhibited this enzyme (Figure 9). "Time" and its interactions with other factors had a significant but weaker effect on β-Glu activity in comparison with "NP type" and "concentration" and their interaction. This was due to a distinct "enzymatic response" to various concentrations of GO or AgNPs, manifested in groups treated with intermediate concentrations (GO20, stimulation of the enzyme, and Ag40, inhibition of enzyme activity). The significance of the main effects and their interactions was confirmed in both analyses (Tables 4, S4B and S7 and Figures 9 and S5).

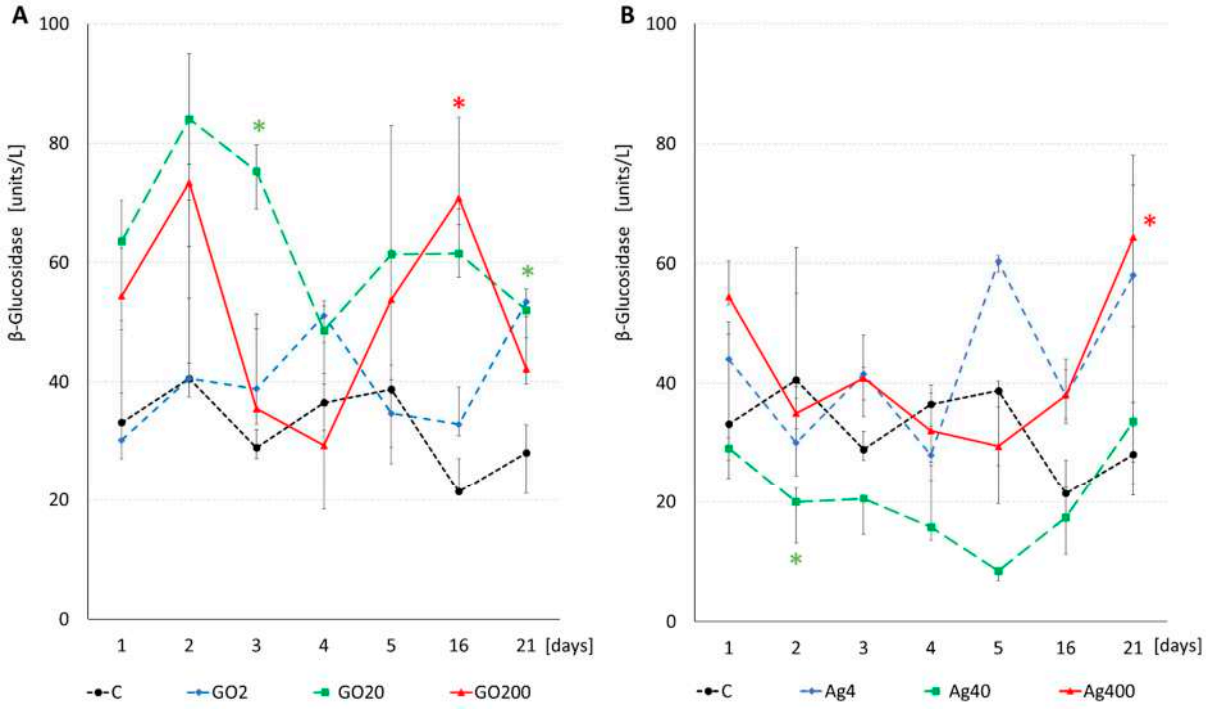


Figure 9. Activity of β-glucosidase (median ± interquartile range) in samples from the digestive tract of *A. domesticus* from control and nanoparticle-treated groups. (**A**) Graphene oxide (GO) and (**B**) silver nanoparticles (AgNPs). Explanation: see Figure 7.

The PERMANOVA analysis revealed that all factors and interactions significantly affected β-galactosidase (β-Gal) activity (Table 4). The most substantial effect was attributed to the "NP type" factor and was manifested in higher overall β-Gal activity in the groups exposed to GO compared with insects treated with AgNPs. The main effect of "concentration" was also significant, albeit modified by other factors in the experiment. In the GO groups, the highest overall β-Gal activity occurred at the highest concentration. AgNPs stimulated β-Gal activity at the lowest and inhibited it at the highest concentration (Figures 10 and S6). The main effect of the "time", analyzed for both nanoparticles and all concentrations simultaneously, indicated an increase in β-Gal activity from the fifth day. "Time" interactions with the other factors were significant, indicating different patterns of changes over time for both nanoparticles and all analyzed concentrations (Figures 10 and S6 and Tables 4, S4B and S8). Figure 10 illustrates only significant differences between the NP-treated groups and the control at each time point. Detailed post hoc analyses are provided in the supplementary materials (Table S8).

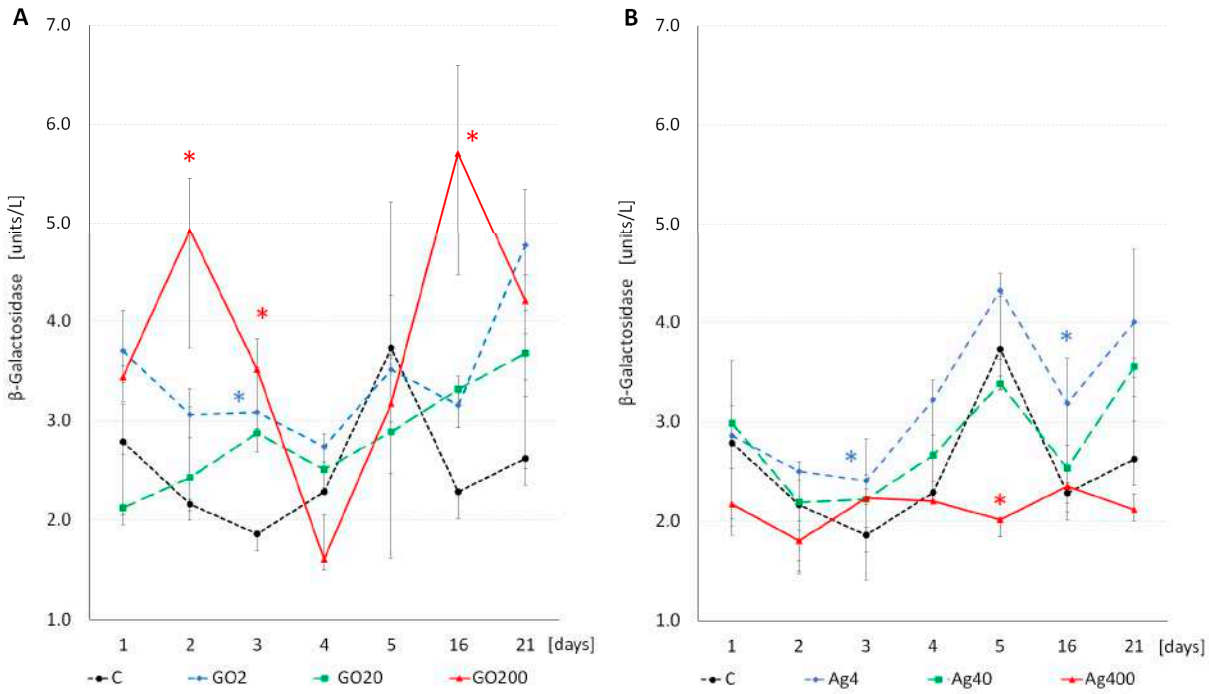


Figure 10. Activity of β-galactosidase (median ± interquartile range) in samples from the digestive tract of *A. domesticus* from control and nanoparticle-treated groups. (**A**) Graphene oxide (GO) and (**B**) silver nanoparticles (AgNPs). Explanation: see Figure 7.

The basic (control) protease activity remained at a relatively constant level during the first five days of the experiment, then decreased with the insect age (Figure 11). The main effect of "NP type" was significant, indicating a greater decrease in protease activity in the AgNP-treated groups compared with the control. A significant "concentration" factor confirmed the general inhibitory effect of NPs on protease activity, which was most noticeable in the Ag40 group, followed by the Ag4, GO20, and GO2 groups. The "time" factor also significantly influenced this enzyme's activity, particularly evidenced by its decrease in the control and NP-treated crickets. This may suggest the diminishing importance of protein digestion in aging insects. However, significant interactions between "time", "NP type", and "concentration" factors indicated a slightly different course of changes in protease activity over time, which varied across all concentrations of both tested nanoparticles (Figures 11 and S7 and Tables 4, S4A and S9).

The lipase activity in the control group remained very low and almost constant throughout the experiment. Adding nanoparticles to the cricket diet increased the activity of this enzyme. The "NP type" effect was significant (Tables 4 and S4A) and indicated stronger enzyme stimulation by AgNPs. This stimulation was dose-dependent, and the "concentration" factor, tested separately for both NPs, showed a stronger positive dependence of the enzyme activity on AgNPs than GO concentration. The observed increase in lipase activity on days 16 and 21 in NP-treated groups was confirmed by a significant "time" effect. The observed effects suggested a potentially increasing importance of fat digestion with the age of the insects when exposed to AgNPs. GO administered at the lowest concentration hardly caused any changes in lipase activity, but other applied concentrations of GO resulted in similar stimulation of lipase activity (Figures 12 and S8 and Tables 4, S4A and S10).

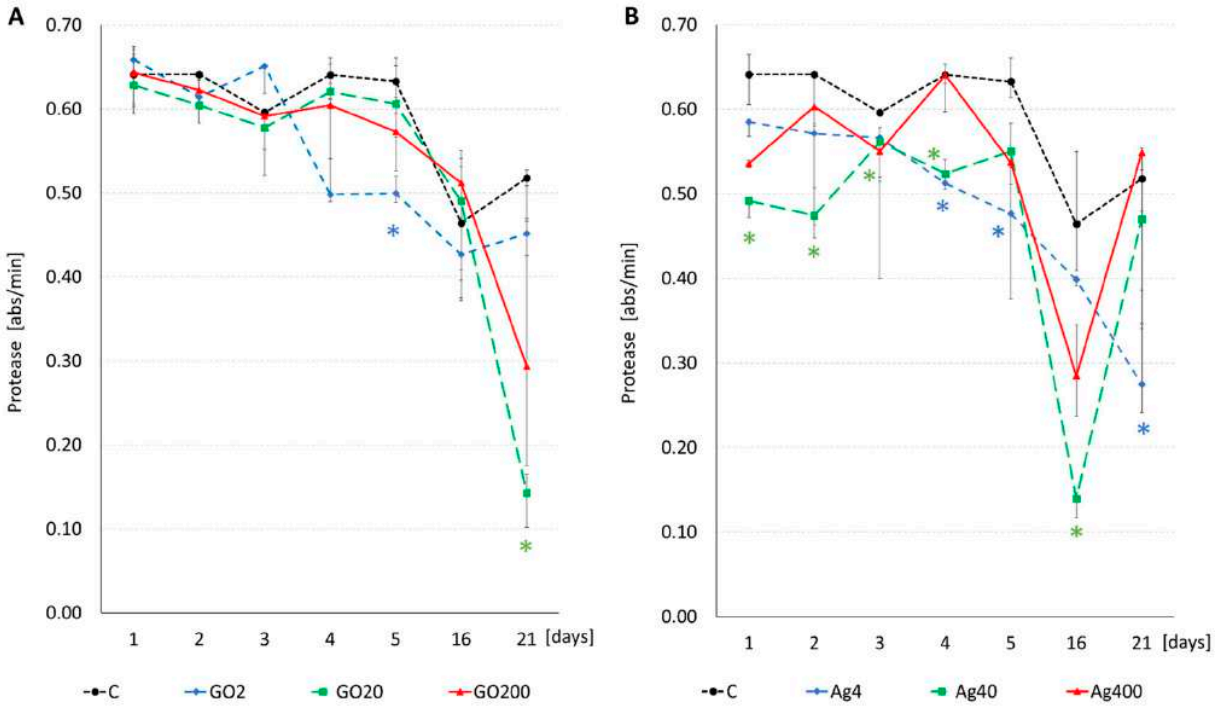


Figure 11. Activity of protease (median ± interquartile range) in samples from the digestive tract of *A. domesticus* from control and nanoparticle-treated groups. (**A**) Graphene oxide (GO) and (**B**) silver nanoparticles (AgNPs). Explanation and abbreviations: see Figure 7.

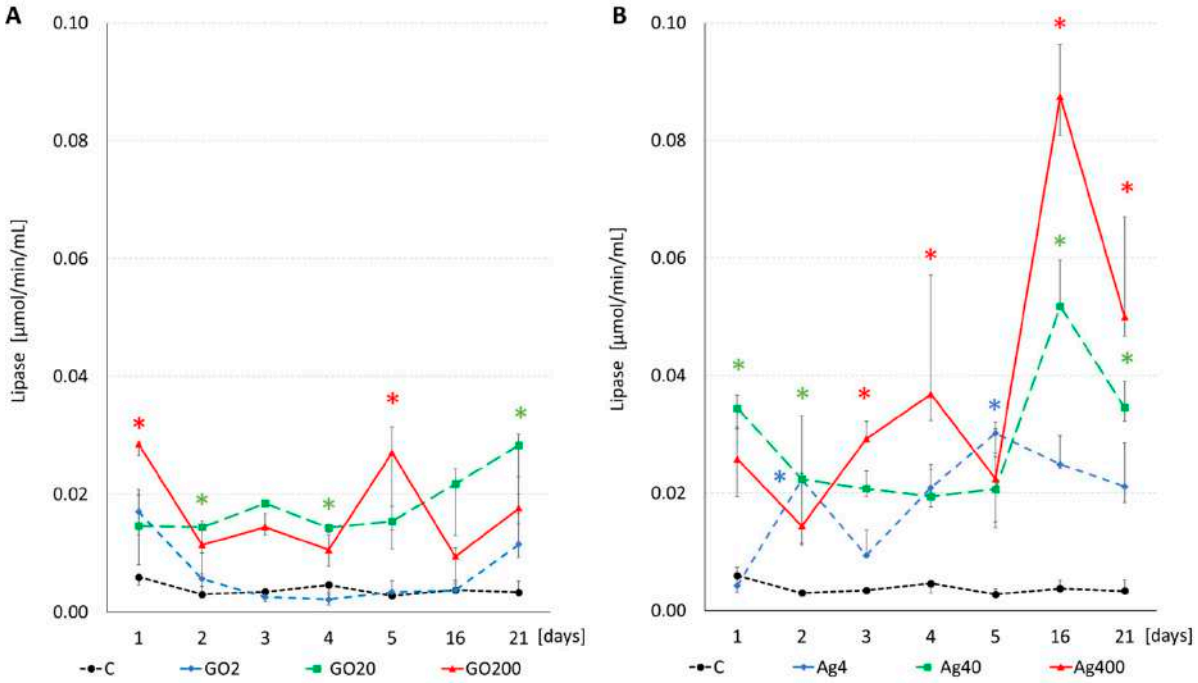


Figure 12. Activity of lipase (median ± interquartile range) in samples from the digestive tract of *A. domesticus* from control and nanoparticle-treated groups. (**A**) Graphene oxide (GO) and (**B**) silver nanoparticles (AgNPs). Explanation: see Figure 7.

4. Discussion

The results obtained in this study indicate that graphene oxide (GO) in the range 2–200 ppm did not result in a remarkable impairment of body weight in *A. domesticus*, except for the GO200 group in which a transient decrease in body weight during the initial

period of the experiment was observed (Figure 2). Greater food assimilation in this group compensated for this effect (Figure 6). This result is interesting because exposed adults have a limited increase in body weight and mainly during the first few days after the final molting. So, this measure aimed to capture any potential adverse effects of NPs at the organismal level as a consequence of physiological and biochemical disturbances. Interestingly, GO did not decrease gut cell viability, and we noticed the opposite—a significantly reduced percentage of dead cells in the GO2 and GO20 groups on day 21 (Figure 6). Hence, we confirmed results from our previous, more comprehensive studies, showing a slight decrease in the percentage of dead cells in crickets exposed to 200 µg GO/g of food. However, simultaneously, a significant increase in the percentage of apoptotic cells, cells with intensified oxidative stress (ROS+), and DNA damage occurred. Additionally, histological examination revealed moderate adverse changes in digestive cells but only in the midgut. Moreover, these changes did not affect regenerative cells [42]. The observed increase in food assimilation in the GO200 group or the decrease in the percentage of dead cells suggested a compensatory mechanism that could alleviate initial adverse changes in the body weight of exposed crickets. However, it cannot be ruled out that this compensatory effort may involve tradeoffs at the expense of other functions, like reproduction, physical activity, or life span. These aspects could be investigated in future studies.

AgNPs had a noticeable effect on body mass, food consumption, and assimilation, especially in the Ag40 group, and these results correspond to previous studies. For example, a reduction in body mass resulting from exposure to AgNPs at the highest applied concentration, 25 mg/L, was observed in *D. melanogaster* [43]. Yasur and Pathipati [13] applied AgNPs in the concentration range of 500 to 4000 µg/mL to lepidopteran pests, *Spodoptera litura* and *Achaea janata,* and noticed a decrease in their body weight proportional to the increase in AgNP concentration. Furthermore, the study revealed that while low doses of AgNPs accumulated in the gut, the majority were excreted in the feces. Also, in the beetle *Blaps polychresta*, a dose of 30 ppm per body weight was sufficient to induce numerous abnormalities in midgut cells [28]. In our study, we did not observe a linear relationship of body weight, consumption, and assimilation change with the concentration of AgNPs in the food, but a notable decrease in body weight in the Ag40 group, concomitant with decreased food consumption and assimilation, remains an intriguing observation. This outcome raises the question of whether AgNPs can alter the quality, nutritional value, or even the taste of food. It is likely that at a concentration of 40 ppm, AgNPs do not form large agglomerates and clusters. However, at a concentration of 400 ppm, such structures may already appear, limiting the total surface area of ion release, the effects of which may be potentially unfavorable. Undoubtedly, such a finding necessitates further investigation.

When comparing the effects of both types of nanoparticles, it is essential to remember the differences in their structure and, consequently, the mechanisms by which they interact with cells. AgNPs may act with a delay as Ag ions are released slowly from the nanoparticle surface over an extended period [26–28]. The toxicity of GO is related to the flake size, the degree of oxidation, and the type of surface and edge functionalization [29,44,45].

In the present experiment, we observed a series of changes in the activity of digestive enzymes. These changes were, again, more pronounced and evident mainly in the case of AgNPs (Figures 7–12). The alterations in digestive enzyme activity under the influence of NPs, especially in insects, remain highly underexplored. The available results are limited and primarily pertain to AgNPs. Nonetheless, it is documented that in *Spodoptera litura* exposed to AgNPs at an LC50 equivalent concentration, the activities of crucial gut enzymes, such as amylase, protease, lipase, and invertase, were significantly lowered. Furthermore, a clear correlation was observed between the concentration of AgNPs and enzyme activity: the higher the nanoparticle concentration, the more pronounced the inhibitory effect. The activities of amylase and invertase were the most significantly reduced [21]. Our study did not reveal such a clear relationship. However, the activity of digestive enzymes in *A. domesticus* depended on "NP type", "concentration", and "time" and the interactions of these factors (Table 4 and Table S4A,B). It is worth noting that Bharani

and Namasivayam [21] used a high AgNP dose ranging from 10^3 to 10^6 µg AgNPs/mL, three orders of magnitude higher than in our study. The concentration of NPs we used caused subtle changes in enzymes' activity that may have caused compensatory reactions, which overlapped with the natural, physiological changes in enzymatic activity occurring throughout the insect's life span. However, within the concentration range applied in our experiment, one cannot infer a distinct inhibitory effect on digestive enzyme activity (except for protease). On the contrary, the observed stimulation of enzyme activity in most groups and enzymes evokes the notion of hormetic effects aimed at enhancing organism survival under moderate stress conditions [19,46–48].

In studies on *Spodoptera litura* and *Achaea janata* exposed to AgNPs, a species-dependent response in β-Glu activity was observed. While an increase in the enzyme's activity was noted in all experimental groups of *S. litura*, *A. janata* showed a reduction in β-Glu activity in almost all groups compared with the control. The exception was the group treated with 2000 µg/mL, where β-Glu activity intensified compared with the control [13]. In another experiment, the influence of AgNPs on the activity of the gut protease was examined in insecticide-resistant *Helicoverpa armigera* caterpillars. A significant reduction in insect body mass and survival was observed, along with the inhibition of protease activity at higher concentrations, and this effect was dose dependent. Further research by the authors demonstrated that AgNPs can bind with high affinity to the protease at higher concentrations, causing its immobilization and inactivity [22]. Indeed, in our study, we observed the inhibition of protease across the entire range of AgNPs and GO concentrations. Additionally, this effect intensified with the crickets' age, indicating that not only the nanoparticle–enzyme orientation but also the age of the insects was essential. This suggests that the quantity of synthesized/available enzymes, as well as the ratio between NPs, enzymes, and substrates, plays a significant role. The effect of AgNPs on digestive enzymes has also been studied in vertebrates. In common carp fed a diet containing AgNPs at concentrations of 0.05 to 0.15 µg/g for 60 days, a significant reduction in protease and lipase activity was observed, while amylase activity increased [49]. On the other hand, the exposure of another fish species, butterfly splitfin (*Ameca splendens*), to AgNPs at low concentrations (0.01 to 1.0 µg/mL) lasting 42 days did not change the activity of acid and alkaline phosphatase, amylase, lipase, and trypsin, although this exposure was sufficient to induce morphological changes in tissues and disrupt liver and gonad functions [17].

To the best of our knowledge, except for our preliminary study [19], there are no articles describing the effects of GO on digestive enzymes in insects. There are several studies on potential mechanisms of action of other nanoparticles, also in species other than insects. In general, the type and structural properties of NPs determine their mode of action and shape the effect—inhibition or stimulation of digestive enzyme activity [20,23,50]. When administered at low concentrations, nanoparticles composed of essential metals may elicit a stimulatory effect on digestive enzyme activity. Conversely, at elevated concentrations, they tend to inhibit enzyme activity [50]. Wang et al. [20] proposed three interesting hypotheses to explain the impairment of digestive functions following exposure to NPs. The first assumes a direct impact of NPs on digestive enzymes or their synthesis. The second posits an indirect impact of NPs on digestive enzymes through changes in feeding behavior and feeding activity. The third one focuses on the quality of food, which may change after contamination with NPs, indirectly impairing digestive functions. Based on collected evidence, the authors advocated for a direct influence of nanoparticles on enzyme activity or their synthesis [20]. Recent studies by Muhammad et al. [15] on *Bombyx mori* exposed to copper oxide or zinc oxide nanoparticles (CuONPs or ZnONPs) resulted in reduced gene expression of α-amylase or both α-amylase and lipase, respectively, that led to reduced α-amylase activity. However, ZnONPs also affected the microbiome, decreasing species richness and diversity and increasing the abundance of pathobionts. Consequently, reduced survival and cocoon production were observed [15].

A slightly different approach to studying metal NPs involves investigating their modulatory effect on the activity of digestive enzymes, focusing on the structures formed

during laboratory-based synthesis [51–53]. These studies help unravel the mechanisms of enzyme–NP conjugate formations, hence the role of NPs in modulating enzyme activity [51,53–55]. For instance, citrate-stabilized gold nanoparticles (cit-AuNPs) modulate α-amylase activity, with the highest increase observed at the lowest concentration, forming enzyme–NP complexes that positively influence the active site orientation. However, higher NP concentrations reduce enzyme activity due to larger agglomerates [52]. Similar findings show increased enzyme activity when immobilized on gold or biosynthesized silver nanoparticles [53]. Metal NPs can also impair the catalytic functions of other digestive enzymes [51,54,55]. Thus, NPs can act as catalysts within specific concentration ranges, facilitating enzyme–substrate interactions. A separate issue requiring further research is the possibility of binding food component molecules delivered with ingested food on the surface of NPs. The formation of such structures could theoretically influence the retention time of specific nutrients in the gut and its accessibility to digestive enzymes.

5. Conclusions

In summary, the subtle changes induced by GO or AgNPs at the used concentrations appear to be safe for *Acheta domesticus*. Notably, AgNPs exerted a more evident influence on insect body weight and consumption than GO. The observed non-linear relationship between AgNP concentration and body weight, consumption, and assimilation may result from possible agglomerate formation that affects the NPs reactivity. However, further detailed studies are necessary to confirm this suggestion. Generally, NPs at the used concentrations stimulated digestive enzymes' activity, except for protease, resembling a hormetic effect. Whether these observed changes are a tradeoff in a struggle to survive the sublethal but prolonged stress caused by NPs remains a question. Future studies should consider these possible and postponed consequences like the reproductive fitness and life span of the exposed organisms.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects15020089/s1, Figure S1: Cumulative food consumption (mg dry weight per individual; mean) in *A. domesticus* treated with nanoparticles: (A) graphene oxide (GO) and (B) silver nanoparticles (AgNPs) measured in intervals., Figure S2: Cumulative food assimilation (mg dry weight per individual; mean) in *A. domesticus* treated with nanoparticles: (A) graphene oxide (GO) and (B) silver nanoparticles (AgNPs) measured in intervals. Figure S3: α-amylase activity (mean ± SE) in samples from the digestive tract of A. domesticus from control and nanoparticles-treated groups. (A) graphene oxide (GO) and (B) silver nanoparticles (AgNPs). Figure S4: α-glucosidase activity (mean ± SE) in samples from the digestive tract of *A. domesticus* from control and nanoparticles-treated groups. (A) graphene oxide (GO) and (B) silver nano-particles (AgNPs). Figure S5: β-glucosidase activity (mean ± SE) in samples from the digestive tract of *A. domesticus* from control and nanoparticles-treated groups. (A) graphene oxide (GO) and (B) silver nano-particles (AgNPs). Figure S6: β-galactosidase activity (mean ± SE) in samples from the digestive tract of *A. domesticus* from control and nanoparticles-treated groups. (A) graphene oxide (GO) and (B) silver nano-particles (AgNPs). Figure S7: Protease activity (mean ± SE) in samples from the digestive tract of *A. domesticus* from control and nanoparticles-treated groups. (A) graphene oxide (GO) and (B) silver nanoparticles (AgNPs). Figure S8: Lipase activity (mean ± SE) in samples from the digestive tract of *A. domesticus* from control and nanoparticles-treated groups. (A) graphene oxide (GO) and (B) silver nanoparticles (AgNPs). Table S1: Permutational analysis of variance (PERMANOVA) test considering the main factors: 'NP type' 'Concentration' and 'Time', and their interaction, influencing Food consumption and Food assimilation measured in Acheta domesticus following exposure to GO or AgNPs in the diet (PERMANOVA was performed with 999 permutations, using the adonis2 function in R vegan package; F—F ratio, df—degrees of freedom, *p*—*p*-value, n = 5). Table S2: Food consumption—Results of the Tukey post-hoc test performed after multivariate repeated measures Analysis of Variance (Homogeneous groups are marked with gray boxes). Table S3: Food assimilation—Results of the Tukey post-hoc test performed after multivariate repeated measures Analysis of Variance (Homogeneous groups are marked with gray boxes). Table S4A. Multivariate repeated measures Analysis of Variance for 'NPstype', 'Concentration', and interaction of both factors, on digestive enzymes activity in *A. domesticus*, measured at 1-5, 16 and 21 day of

exposure (F—F ratio, df1 and df2—treatment and error degrees of freedom, respectively, *p*—*p* value, n = 5). Table S4B. Continuation of Table S4A. Table S5: Amylase—Results of the Tukey post-hoc test performed after multivariate repeated measures Analysis of Variance (Homogeneous groups are marked with gray boxes). Table S6: α-Glucosidase—Results of the Tukey post-hoc test performed after multivariate repeated measures Analysis of Variance (Homogeneous groups are marked with gray boxes). Table S7: β-Glucosidase—Results of the Tukey post-hoc test performed after multivariate repeated measures Analysis of Variance (Homogeneous groups are marked with gray boxes). Table S8: β-Galactosidase—Results of the Tukey post-hoc test performed after multivariate repeated measures Analysis of Variance (Homogeneous groups are marked with gray boxes). Table S9: Protease—Results of the Tukey post-hoc test performed after multivariate repeated measures Analysis of Variance (Homogeneous groups are marked with gray boxes). Table S10: Lipase—Results of the Tukey post-hoc test performed after multivariate repeated measures Analysis of Variance (Homogeneous groups are marked with gray boxes).

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insects

Supplementary materials

Concentration- and Time-Dependent Dietary Exposure to Graphene Oxide and Silver Nanoparticles: Effects on Food Consumption and Assimilation, Digestive Enzyme Activities, and Body Mass in *Acheta domesticus*

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COMPOSITION OF RABBIT FEED

KDT (for growing rabbits) by UNIPASZ Company (Poland) is used routinely for rearing a continuous laboratory colony of cricket, *Acheta domesticus* L.

The feed is rich in easily assimilated mineral nutrients, free of GMO and contains c.a. 55 % carbohydrates of plant origin.

Analytical composition (per 1 kg):

Raw compounds: proteins - 17 %, fibre – 14,5 %, fat - 3,50 %, ash - 7,50 %

Minerals: Ca - 0,95 %, Na - 0,19 %, P - 0,50 %, Zn – 85 mg, Fe – 25 mg, other – c.a. 100 mg

Some additives

Vitamins: A – 12,000 mass units, E – 60 mg, B (complex) – c.a. 60 mg, Biotin – 200 mcg, C – 250 mg;

Aminoacids: Methionine – 750 mg, L-Lysine – 3700 mg, L-Threonine – 1600 mg

Antioxidants: 2,95 mg

Table S1. Permutational analysis of variance (PERMANOVA) test considering the main factors: 'NP type' 'Concentration' and 'Time', and their interaction, influencing Food consumption and Food assimilation measured in *Acheta domesticus* following exposure to GO or AgNPs in the diet *(PERMANOVA was performed with 999 permutations, using the adonis2 function in R vegan package; F – F ratio, df – degrees of freedom, p – p-value, n = 5).*

Effect	Food consumption			Food assimilation		
	F	***df***	**p**	**F**	***df***	**p**
NPs type [1]	13.239	1	0.001	13.795	1	0.001
Concentration [2]	5.444	3	0.001	3.654	3	0.002
Time [3]	43.003	3	0.001	27.066	3	0.001
[1] × [2]	5.506	6	0.001	7.262	6	0.001
[1] × [3]	3.749	6	0.001	2.986	6	0.001
[2] × [3]	1.529	18	0.061	1.830	18	0.003
[1] × [2] × [3]	1.967	18	0.006	1.891	18	0.003
Residual		270			270	
Total		325			325	

Table S2. Food consumption - Results of the Tukey post-hoc test performed after multivariate repeated measures Analysis of Variance (Homogeneous groups are marked with gray boxes).

Multivariate repeated measures Analysis of Variance – Tukey test (Food consumption)

NPs	Conc.	Time	Mean
Ag	C3	(16-21)	10.865
Ag	C2	(11-16)	11.175
Ag	C3	(11-16)	12.245
Ag	C2	(9-11)	12.874
GO	C1	(11-16)	14.994
Ag	C2	(16-21)	15.065
GO	C2	(5-7)	15.489
Ag	C1	(11-16)	16.112
Ag	C1	(16-21)	16.984
Ag	C3	(9-11)	17.485
GO	C3	(5-7)	18.486
Ag	C2	(7-9)	19.082
GO	C1	(16-21)	19.128
GO	C2	(16-21)	20.442
Ag	C0	(16-21)	20.978
GO	C0	(16-21)	20.978
GO	C2	(11-16)	21.373
GO	C0	(9-11)	21.650
Ag	C0	(9-11)	21.650
GO	C0	(11-16)	22.027
Ag	C0	(11-16)	22.027
GO	C3	(11-16)	22.392
Ag	C3	(7-9)	22.405
Ag	C2	(5-7)	22.531
Ag	C2	(3-5)	22.781
GO	C3	(9-11)	22.892
Ag	C1	(9-11)	22.934
GO	C1	(9-11)	23.277
GO	C3	(16-21)	23.578
GO	C2	(7-9)	24.418
Ag	C0	(7-9)	24.701
GO	C0	(7-9)	24.701
Ag	C0	(5-7)	25.717
GO	C0	(5-7)	25.717
GO	C1	(5-7)	26.399
Ag	C2	(1-3)	26.868
GO	C2	(9-11)	27.113
Ag	C1	(7-9)	27.427
GO	C2	(3-5)	28.731
GO	C1	(7-9)	28.991
GO	C3	(7-9)	31.449
Ag	C1	(5-7)	33.171
Ag	C3	(5-7)	33.770
GO	C1	(3-5)	34.536
GO	C2	(1-3)	35.969
Ag	C0	(1-3)	36.979
GO	C0	(1-3)	36.979
GO	C3	(3-5)	39.068
Ag	C3	(1-3)	39.355
Ag	C3	(3-5)	41.333
GO	C1	(1-3)	41.743
GO	C0	(3-5)	42.020
Ag	C0	(3-5)	42.020
Ag	C1	(1-3)	42.470
Ag	C1	(3-5)	42.908
GO	C3	(1-3)	43.077

Homogeneity

Table S3. Food assimilation - Results of the Tukey post-hoc test performed after multivariate repeated measures Analysis of Variance (Homogeneous groups are marked with gray boxes).

Multivariate repeated measures Analysis of Variance – Tukey test (Food assimilation)

NPs	Conc.	Time	Mean	Homogeneity H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11
Ag	C2	(11-16)	3.557	X										
Ag	C3	(16-21)	5.850	X	X	X								
Ag	C3	(11-16)	6.094	X	X	X	X							
Ag	C2	(16-21)	6.774	X	X	X	X	X						
Ag	C2	(9-11)	7.082	X	X	X	X	X						
GO	C0	(16-21)	7.408	X	X			X		X				
Ag	C0	(16-21)	7.408	X	X			X		X				
GO	C1	(16-21)	8.056	X	X	X	X	X		X				
Ag	C3	(9-11)	8.173	X	X	X	X	X	X	X				
Ag	C1	(11-16)	8.537	X	X	X	X	X		X	X			
GO	C0	(9-11)	8.566	X	X	X	X	X		X	X			
Ag	C0	(9-11)	8.566	X	X	X	X	X		X	X			
GO	C2	(5-7)	8.642	X	X	X	X	X	X	X	X	X		
Ag	C1	(16-21)	9.020	X	X	X	X	X		X	X			
Ag	C2	(5-7)	9.196	X	X	X	X	X	X	X	X	X	X	
Ag	C2	(3-5)	9.393	X	X	X	X	X	X	X	X	X	X	
GO	C3	(5-7)	9.457	X	X	X	X	X		X	X			
GO	C1	(11-16)	9.469	X	X	X	X	X	X	X	X			
Ag	C3	(7-9)	9.768	X	X	X	X	X	X	X	X	X	X	
Ag	C0	(11-16)	10.307	X	X	X	X	X	X	X	X			
GO	C0	(11-16)	10.307	X	X	X	X	X	X	X	X			
Ag	C2	(7-9)	10.870	X	X	X	X	X	X	X	X	X	X	X
GO	C1	(9-11)	10.885	X	X	X	X	X	X	X	X	X	X	
Ag	C0	(5-7)	11.052	X	X	X	X	X	X	X	X	X		
GO	C0	(5-7)	11.052	X	X	X	X	X	X	X	X	X		
GO	C2	(16-21)	11.169	X	X	X	X	X	X	X	X	X	X	X
GO	C2	(11-16)	11.646	X	X	X	X	X	X	X	X	X	X	X
GO	C3	(11-16)	11.911	X	X	X	X	X	X	X	X	X	X	
GO	C2	(7-9)	12.204	X	X	X	X	X	X	X	X	X	X	X
Ag	C0	(7-9)	12.696	X	X	X	X	X	X	X	X	X	X	X
GO	C0	(7-9)	12.696	X	X	X	X	X	X	X	X	X	X	X
Ag	C1	(9-11)	12.802	X	X	X	X	X	X	X	X	X	X	X
Ag	C2	(1-3)	13.088	X	X	X	X	X	X	X	X	X	X	X
GO	C3	(9-11)	13.353	X	X	X	X	X	X	X	X	X	X	X
GO	C1	(5-7)	13.513	X	X	X	X	X	X	X	X	X	X	X
GO	C3	(16-21)	13.831	X	X	X	X	X	X	X	X	X	X	X
GO	C2	(3-5)	14.586	X	X	X	X	X	X	X	X	X	X	X
GO	C1	(3-5)	15.674		X	X	X	X	X	X	X	X	X	X
Ag	C1	(7-9)	15.931		X	X	X	X	X	X	X	X	X	X
GO	C2	(9-11)	16.576		X	X	X	X	X	X	X	X	X	X
GO	C3	(7-9)	17.044		X	X	X	X	X	X	X	X	X	X
GO	C1	(7-9)	17.493		X	X	X	X	X	X	X	X	X	X
Ag	C0	(1-3)	17.557			X	X		X		X	X	X	X
GO	C0	(1-3)	17.557			X	X		X		X	X	X	X
Ag	C0	(3-5)	17.911				X		X		X	X	X	X
GO	C0	(3-5)	17.911				X		X		X	X	X	X
Ag	C3	(1-3)	18.469					X	X	X	X	X	X	X
Ag	C3	(5-7)	18.703					X	X	X	X	X	X	X
Ag	C1	(5-7)	19.294						X			X	X	X
GO	C2	(1-3)	19.368						X	X	X	X	X	X
GO	C3	(3-5)	20.005						X			X	X	X
Ag	C3	(3-5)	20.355								X	X	X	X
Ag	C1	(3-5)	20.629									X	X	X
Ag	C1	(1-3)	21.011										X	X
GO	C3	(1-3)	22.339											X
GO	C1	(1-3)	22.728											X

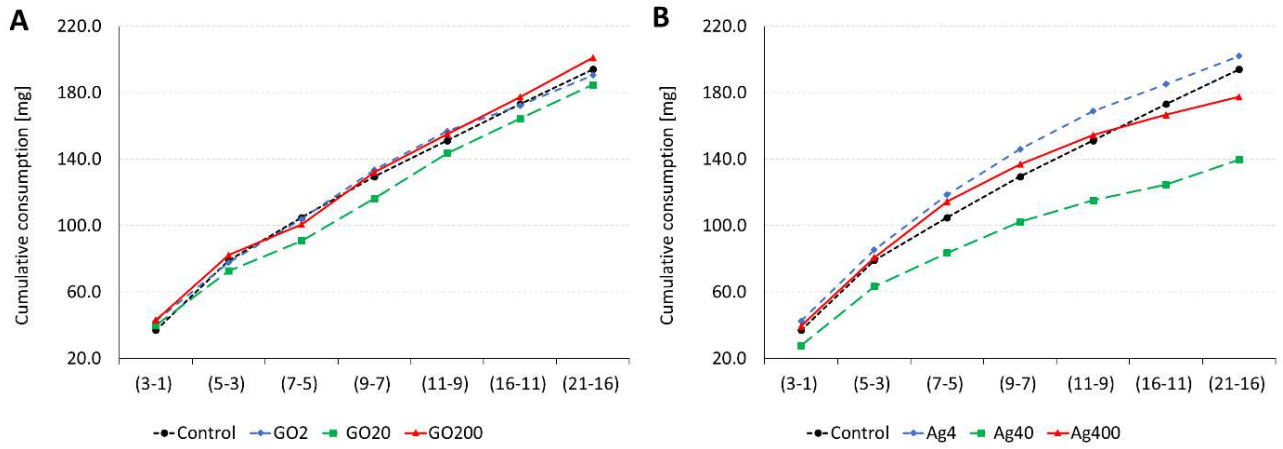


Figure S1. Cumulative food consumption (mg dry weight per individual; mean) in *A. domesticus* treated with nanoparticles: A) graphene oxide (GO) and B) silver nanoparticles (AgNPs) measured in intervals.

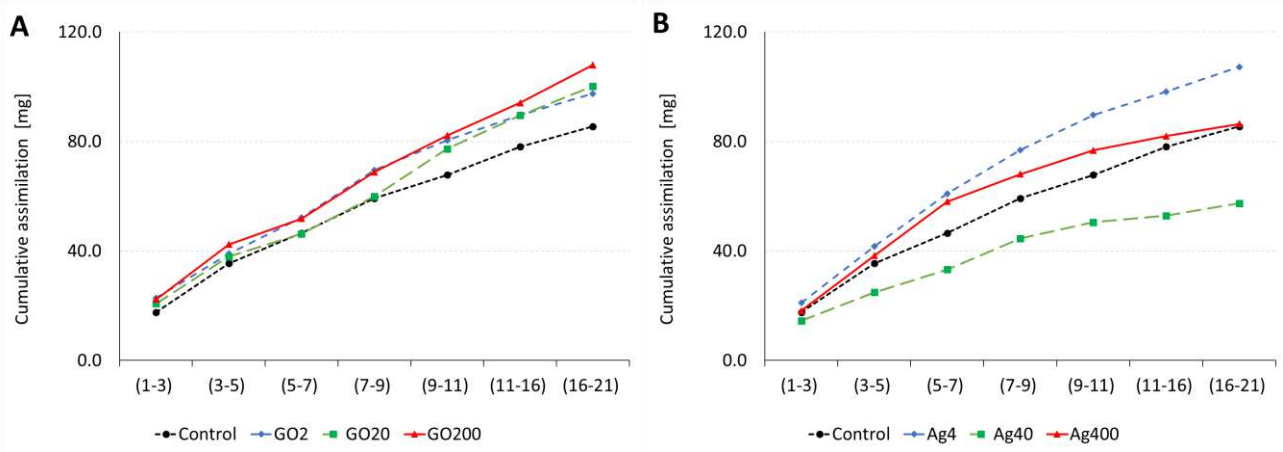


Figure S2. Cumulative food assimilation (mg dry weight per individual; mean) in *A. domesticus* treated with nanoparticles: A) graphene oxide (GO) and B) silver nanoparticles (AgNPs) measured in intervals.

Table S4A. Multivariate repeated measures Analysis of Variance for 'NPstype', 'Concentration', and interaction of both factors, on digestive enzymes activity in *A. domesticus*, measured at 1-5, 16 and 21 day of exposure (*F – F ratio, df_1 and df_2 – treatment and error degrees of freedom, respectively, p – p value, n = 5*).

Effect	Amylase				Lipase				Protease			
	F	df_1	df_2	**p**	**F**	df_1	df_2	**p**	**F**	df_1	df_2	**p**
NPs type [1]	46.113	1	30	<0.001	164.369	1	25	<0.001	23.42	1	30	<0.001
Concentration [2]	427.046	3	30	<0.001	145.687	3	25	<0.001	28.33	3	30	<0.001
[1] × [2]	12.437	3	30	<0.001	27.797	3	25	<0.001	4.72	3	30	0.008
Time [3]	3.544	6	25	0.002	15.253	6	20	<0.001	69.51	6	25	<0.001
[3] × [1]	3.823	6	25	0.001	14.171	6	20	<0.001	6.98	6	25	<0.001
[3] × [2]	2.203	18	71	0.005	4.149	18	57	<0.001	3.84	18	71	<0.001
[3] × [1] × [2]	2.726	18	71	<0.001	7.542	18	57	<0.001	5.68	18	71	<0.001

Table S4B. Continuation of Table S4A.

Effect	α-Glu				β-Glu				β-Gal			
	F	df_1	df_2	**p**	**F**	df_1	df_2	**p**	**F**	df_1	df_2	**p**
NPs type [1]	23.554	1	32	<0.001	63.992	1	31	<0.001	33.647	1	31	<0.001
Concentration [2]	26.301	3	32	<0.001	11.668	3	31	<0.001	14.737	3	31	<0.001
[1] × [2]	3.995	3	32	0.016	46.522	3	31	<0.001	31.149	3	31	<0.001
Time [3]	23.378	6	27	<0.001	3.439	6	26	0.003	11.191	6	26	<0.001
[3] × [1]	6.896	6	27	<0.001	4.345	6	26	<0.001	4.397	6	26	<0.001
[3] × [2]	2.318	18	77	0.003	3.244	18	74	<0.001	3.856	18	74	<0.001
[3] × [1] × [2]	3.854	18	77	<0.001	2.630	18	74	<0.001	2.466	18	74	0.001

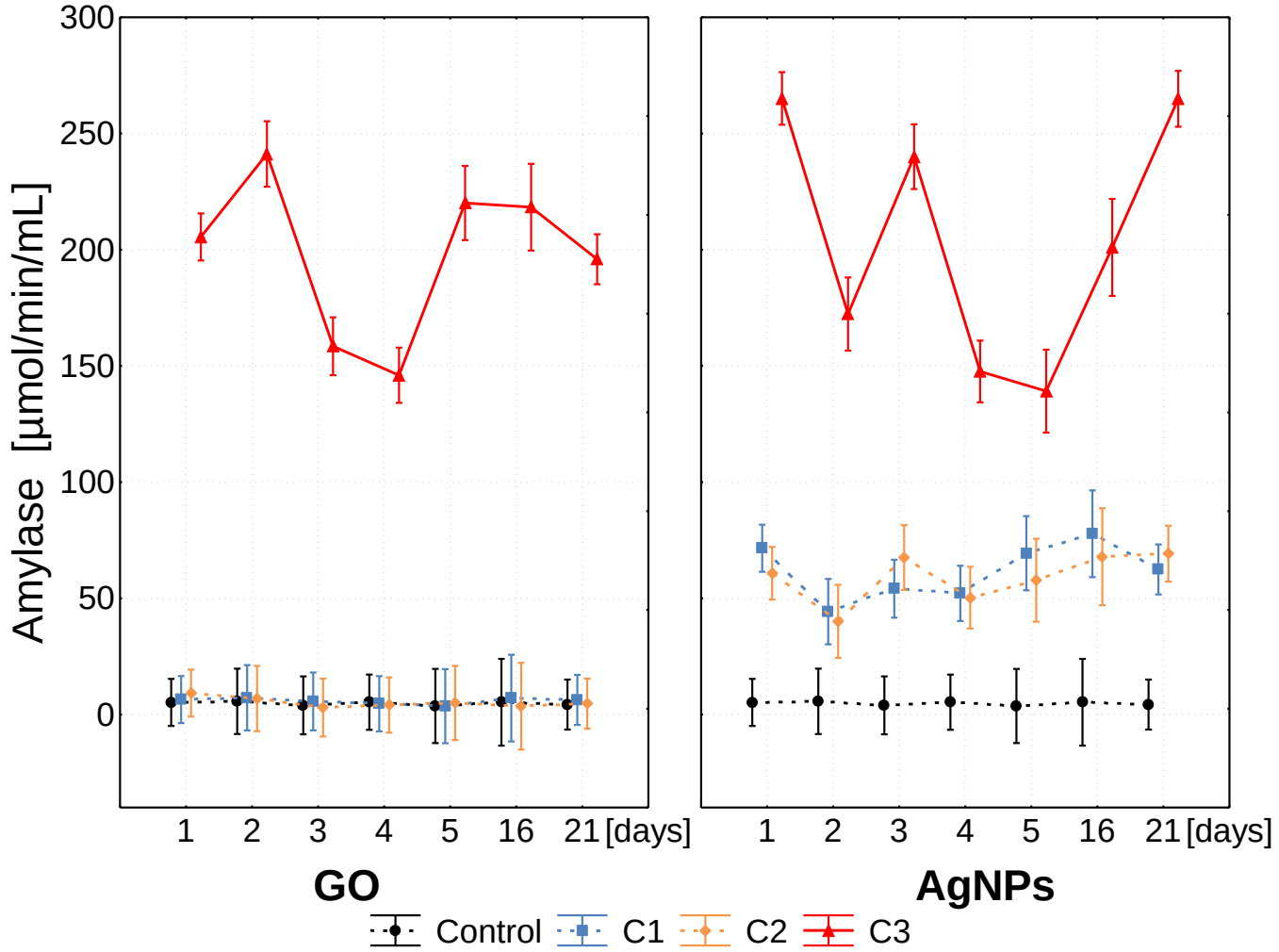


Fig. S3. α-amylase activity (mean ± SE) in samples from the digestive tract of *A. domesticus* from control and nanoparticles-treated groups. A) graphene oxide (GO) and B) silver nanoparticles (AgNPs).

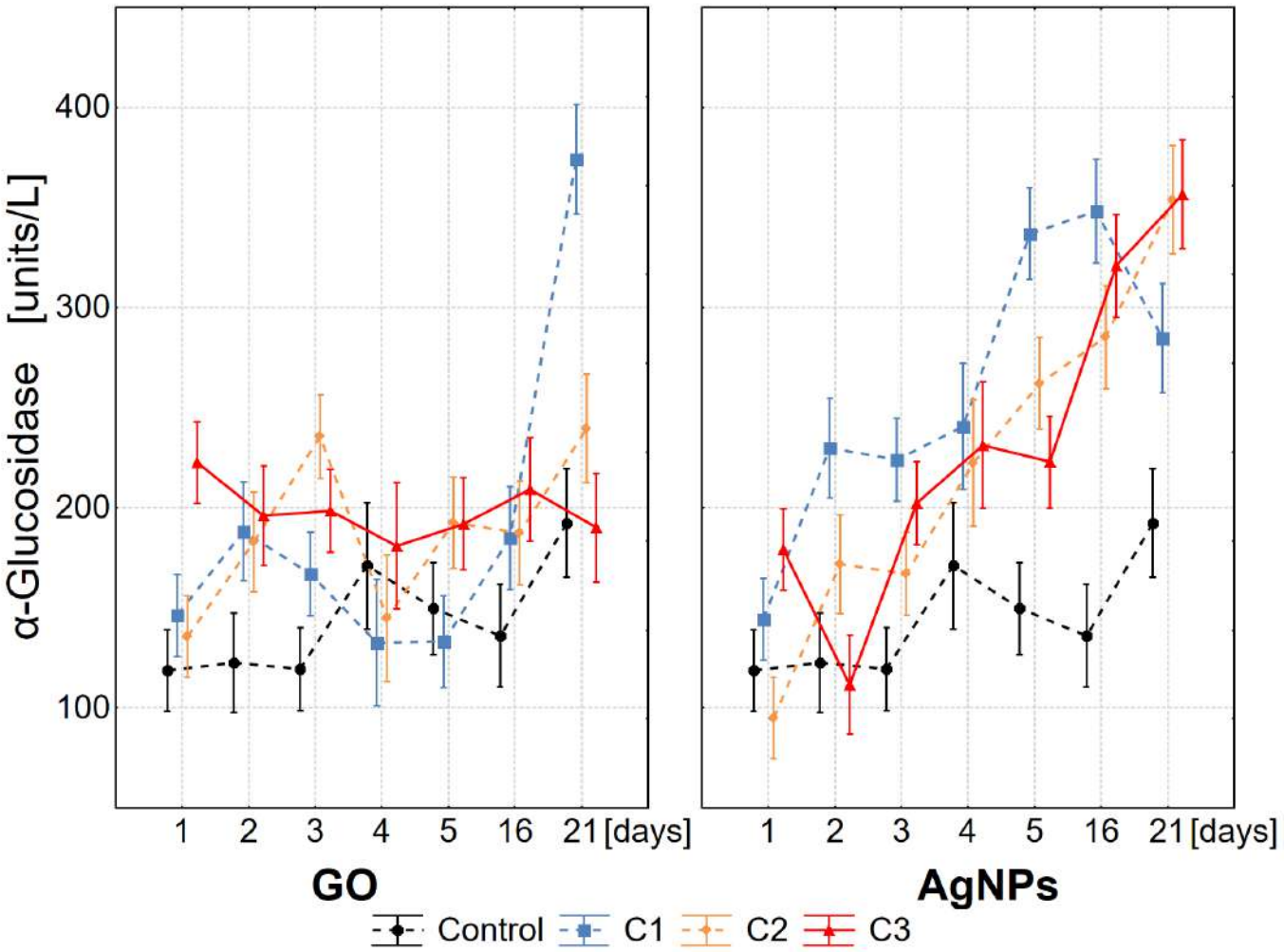


Fig. S4. α-glucosidase activity (mean ± SE) in samples from the digestive tract of *A. domesticus* from control and nanoparticles-treated groups. A) graphene oxide (GO) and B) silver nano-particles (AgNPs).

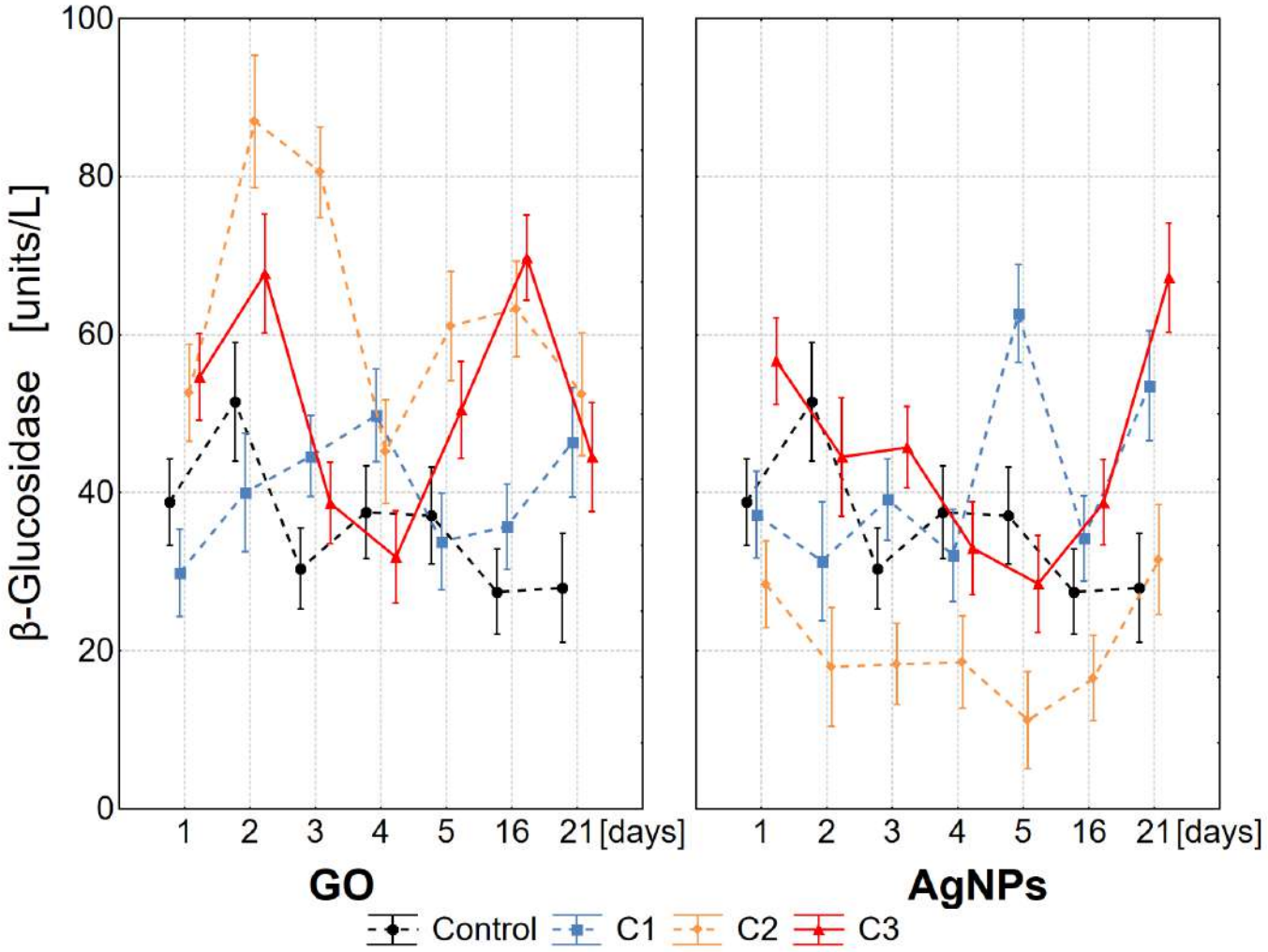


Fig. S5. β-glucosidase activity (mean ± SE) in samples from the digestive tract of *A. domesticus* from control and nanoparticles-treated groups. A) graphene oxide (GO) and B) silver nano-particles (AgNPs).

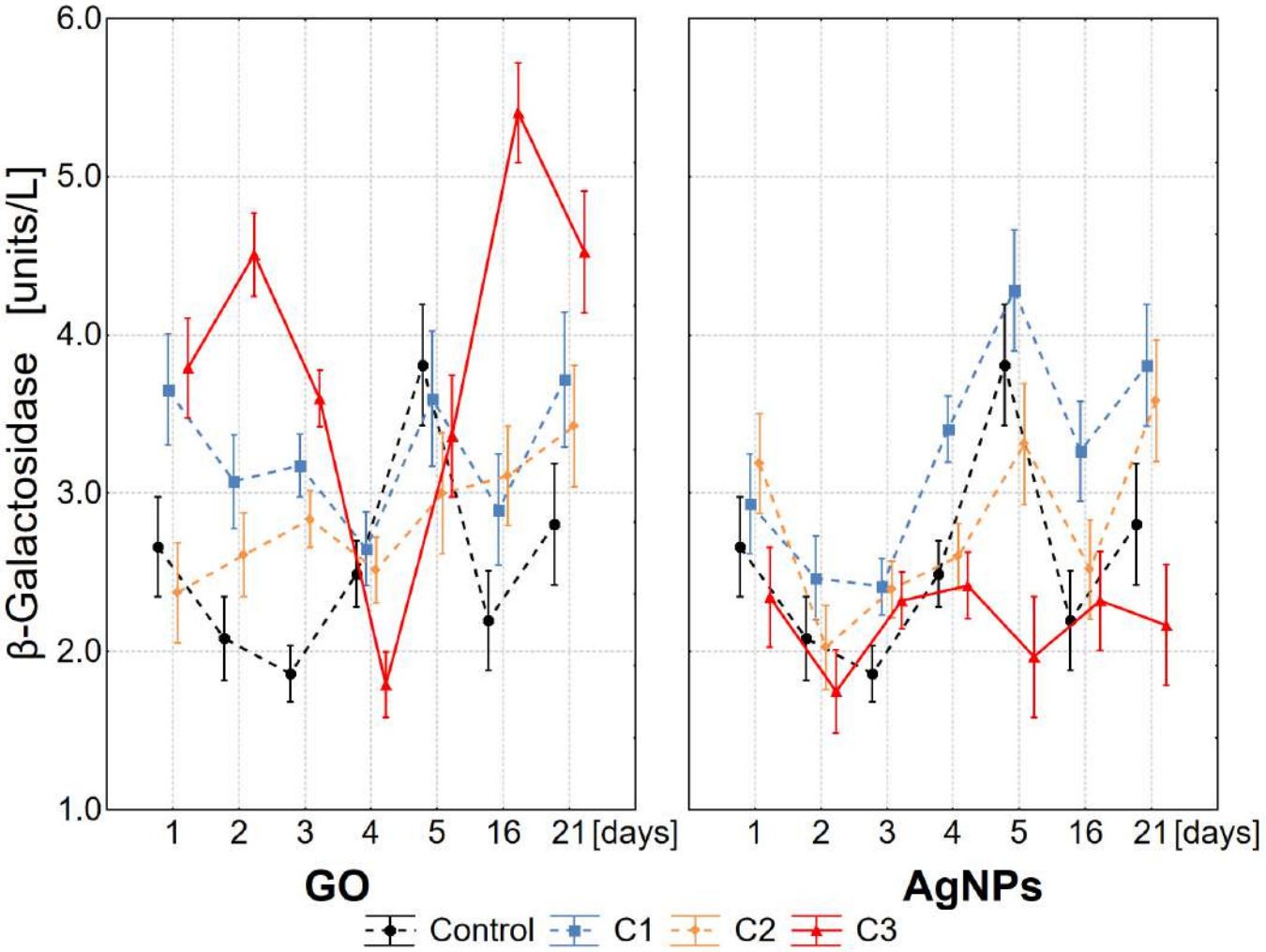


Fig. S6. β-galactosidase activity (mean ± SE) in samples from the digestive tract of *A. domesticus* from control and nanoparticles-treated groups. A) graphene oxide (GO) and B) silver nano-particles (AgNPs).

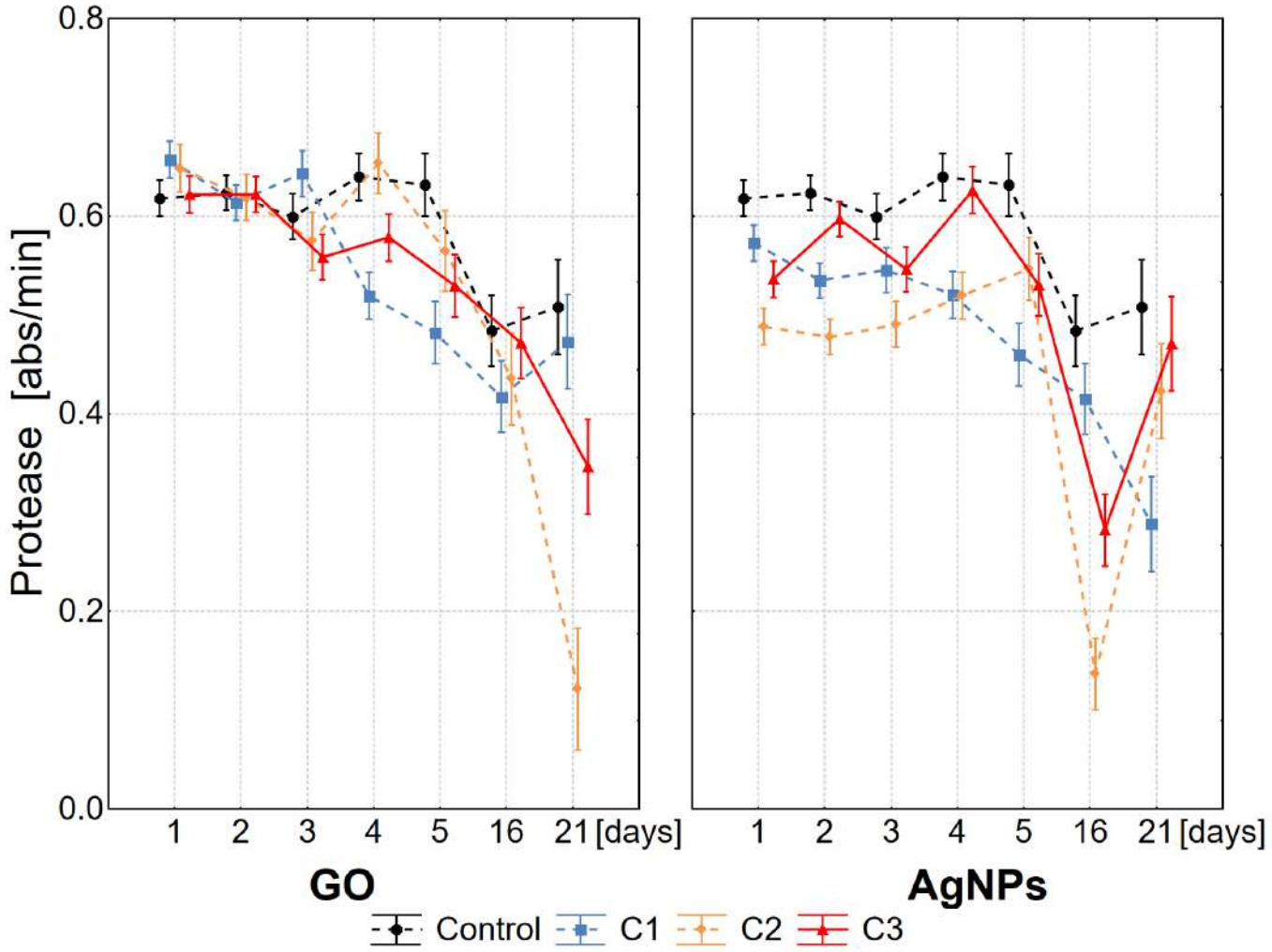


Fig. S7. Protease activity (mean ± SE) in samples from the digestive tract of *A. domesticus* from control and nanoparticles-treated groups. A) graphene oxide (GO) and B) silver nanoparticles (AgNPs).

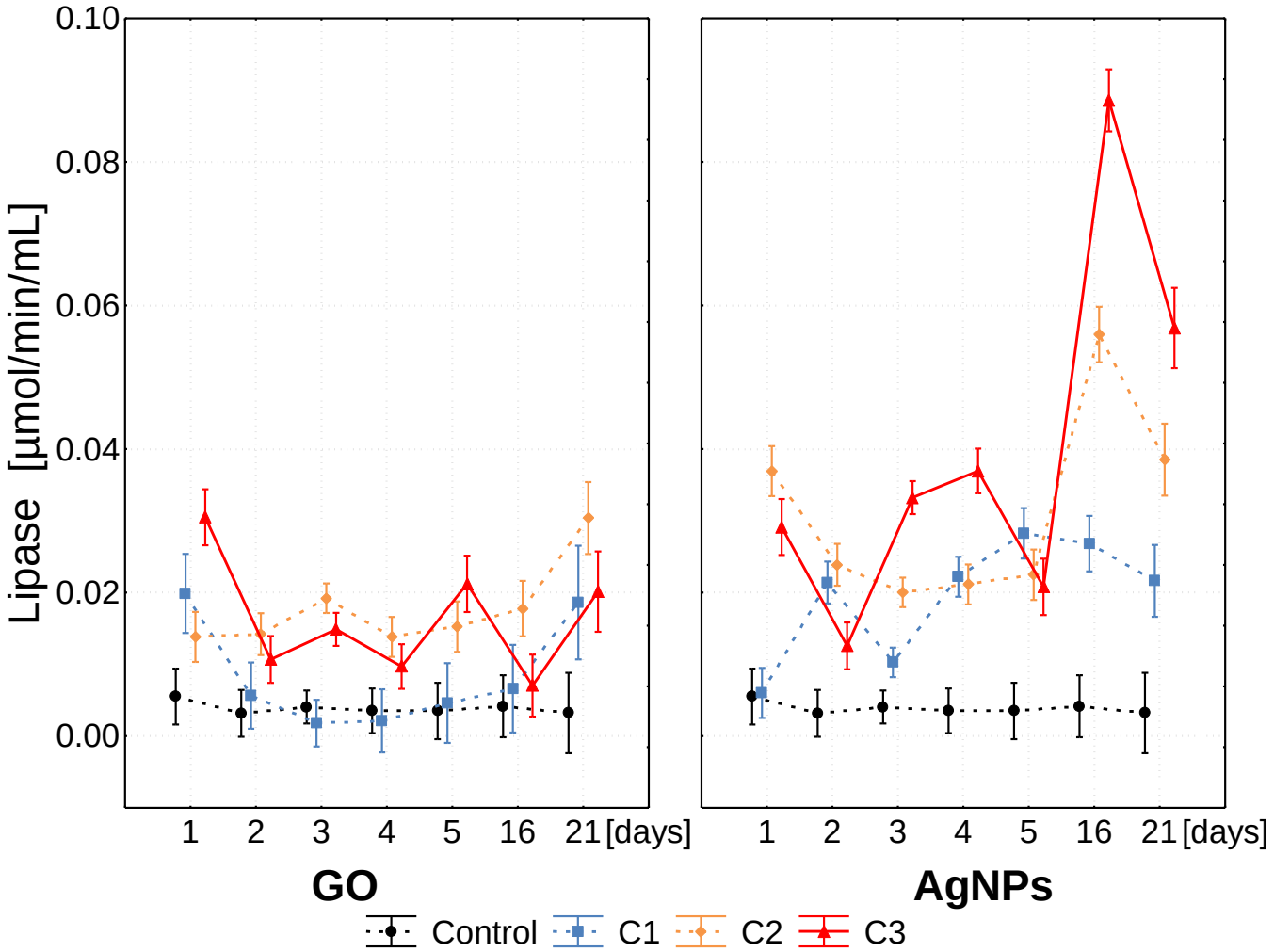


Fig. S8. Lipase activity (mean ± SE) in samples from the digestive tract of *A. domesticus* from control and nanoparticles-treated groups. A) graphene oxide (GO) and B) silver nanoparticles (AgNPs).

Table S5. Amylase - Results of the Tukey post-hoc test performed after multivariate repeated measures Analysis of Variance (Homogeneous groups are marked with gray boxes).

Multivariate repeated measures Analysis of Variance – Tukey test (Amylase)

NPs	Conc.	Time	Mean	Homogeneity 1	2	3	4	5	6	7	8
GO	C2	t3	3.02	X							
GO	C2	t16	3.60	X							
GO	C1	t5	3.61	X							
GO	C0	t5	3.69	X							
Ag	C0	t5	3.69	X							
Ag	C0	t3	3.96	X							
GO	C0	t3	3.96	X							
GO	C2	t4	4.09	X							
GO	C0	t21	4.31	X							
Ag	C0	t21	4.31	X							
GO	C1	t4	4.65	X							
GO	C2	t21	4.70	X							
GO	C2	t5	5.00	X							
Ag	C0	t1	5.23	X							
GO	C0	t1	5.23	X							
GO	C0	t16	5.29	X							
Ag	C0	t16	5.29	X							
Ag	C0	t4	5.32	X							
GO	C0	t4	5.32	X							
GO	C1	t3	5.63	X							
Ag	C0	t2	5.69	X							
GO	C0	t2	5.69	X							
GO	C1	t21	6.30	X							
GO	C1	t1	6.46	X							
GO	C2	t2	6.91	X							
GO	C1	t16	7.08	X							
GO	C1	t2	7.22	X							
GO	C2	t1	9.25	X							
Ag	C2	t2	40.12	X							
Ag	C1	t2	44.30	X							
Ag	C2	t4	50.32	X							
Ag	C1	t4	52.14	X	X						
Ag	C1	t3	54.15	X	X						
Ag	C2	t5	57.81	X	X						
Ag	C2	t1	60.81	X	X	X	X				
Ag	C1	t21	62.44	X	X		X				
Ag	C2	t3	67.57	X	X	X	X				
Ag	C2	t16	67.89	X	X	X	X				
Ag	C2	t21	69.22	X	X	X	X				
Ag	C1	t5	69.42	X	X	X	X				
Ag	C1	t1	71.55	X	X	X	X				
Ag	C1	t16	77.79	X	X	X	X				
Ag	C3	t5	139.17		X	X	X	X			
GO	C3	t4	145.99			X		X			
Ag	C3	t4	147.63			X	X	X			
GO	C3	t3	158.47					X	X		
Ag	C3	t2	172.33					X	X	X	
GO	C3	t21	195.91					X	X	X	X
Ag	C3	t16	200.98					X	X	X	X
GO	C3	t1	205.50					X	X	X	X
GO	C3	t16	218.31					X	X	X	X
GO	C3	t5	220.10					X	X	X	X
Ag	C3	t3	240.04						X	X	X
GO	C3	t2	241.20							X	X
Ag	C3	t21	264.93								X
Ag	C3	t1	265.09								X

Table S6. α-Glucosidase - Results of the Tukey post-hoc test performed after multivariate repeated measures Analysis of Variance (Homogeneous groups are marked with gray boxes).

Multivariate repeated measures Analysis of Variance – Tukey test (α-Glucosidase)

NPs	Conc.	Time	Mean	Homogeneity 1	2	3	4	5	6	7	8	9
Ag	C2	t1	94.77	x								
Ag	C3	t2	111.57	x	x							
GO	C0	t1	118.66	x	x							
Ag	C0	t1	118.66	x	x							
GO	C0	t3	119.41	x	x	x						
Ag	C0	t3	119.41	x	x	x						
GO	C0	t2	122.45	x	x	x						
Ag	C0	t2	122.45	x	x	x						
GO	C1	t4	132.66	x	x	x						
GO	C1	t5	133.07	x	x	x						
GO	C2	t1	135.68	x	x	x						
Ag	C0	t16	136.11	x	x	x						
GO	C0	t16	136.11	x	x	x						
Ag	C1	t1	144.14	x	x	x	x					
GO	C2	t4	144.88	x	x	x	x					
GO	C1	t1	146.18	x	x	x	x					
GO	C0	t5	149.70	x	x	x	x					
Ag	C0	t5	149.70	x	x	x	x					
GO	C1	t3	166.88	x	x	x	x					
Ag	C2	t3	167.14	x	x	x	x					
GO	C0	t4	171.14	x	x	x	x					
Ag	C0	t4	171.14	x	x	x	x					
Ag	C2	t2	171.77	x	x	x	x					
Ag	C3	t1	179.10	x	x	x	x	x				
GO	C3	t4	180.85	x	x	x	x	x				
GO	C2	t2	182.83	x	x	x	x	x				
GO	C1	t16	184.84	x	x	x	x	x				
GO	C2	t16	187.35	x	x	x	x	x				
GO	C1	t2	188.17	x	x	x	x	x				
GO	C3	t21	190.12	x	x	x	x	x				
GO	C3	t5	191.95	x	x	x	x	x				
GO	C0	t21	192.30	x	x	x	x	x				
Ag	C0	t21	192.30	x	x	x	x	x				
GO	C2	t5	192.55	x	x	x	x	x				
GO	C3	t2	196.05	x	x	x	x	x	x			
GO	C3	t3	198.42	x	x	x	x	x	x			
Ag	C3	t3	202.13	x	x	x	x	x	x			
GO	C3	t16	209.19	x	x	x	x	x	x	x		
Ag	C2	t4	222.31	x	x	x	x	x	x	x	x	
GO	C3	t1	222.50	x	x	x	x	x	x	x	x	
Ag	C3	t5	222.83	x	x	x	x	x	x	x	x	
Ag	C1	t3	223.88	x	x	x	x	x	x	x	x	
Ag	C1	t2	229.84	x	x	x	x	x	x	x	x	
Ag	C3	t4	231.26	x	x	x	x	x	x	x	x	x
GO	C2	t3	235.57	x	x	x	x	x	x	x	x	x
GO	C2	t21	239.58		x	x	x	x	x	x	x	x
Ag	C1	t4	240.58		x	x	x	x	x	x	x	x
Ag	C2	t5	262.10			x	x	x	x	x	x	x
Ag	C1	t21	284.70				x	x	x	x	x	x
Ag	C2	t16	285.28				x	x	x	x	x	x
Ag	C3	t16	320.82					x	x	x	x	x
Ag	C1	t5	336.91						x	x	x	x
Ag	C1	t16	348.11							x	x	x
Ag	C2	t21	353.89								x	x
Ag	C3	t21	356.54								x	x
GO	C1	t21	374.10									x

Table S7. β -Glucosidase - Results of the Tukey post-hoc test performed after multivariate repeated measures Analysis of Variance (Homogeneous groups are marked with gray boxes).

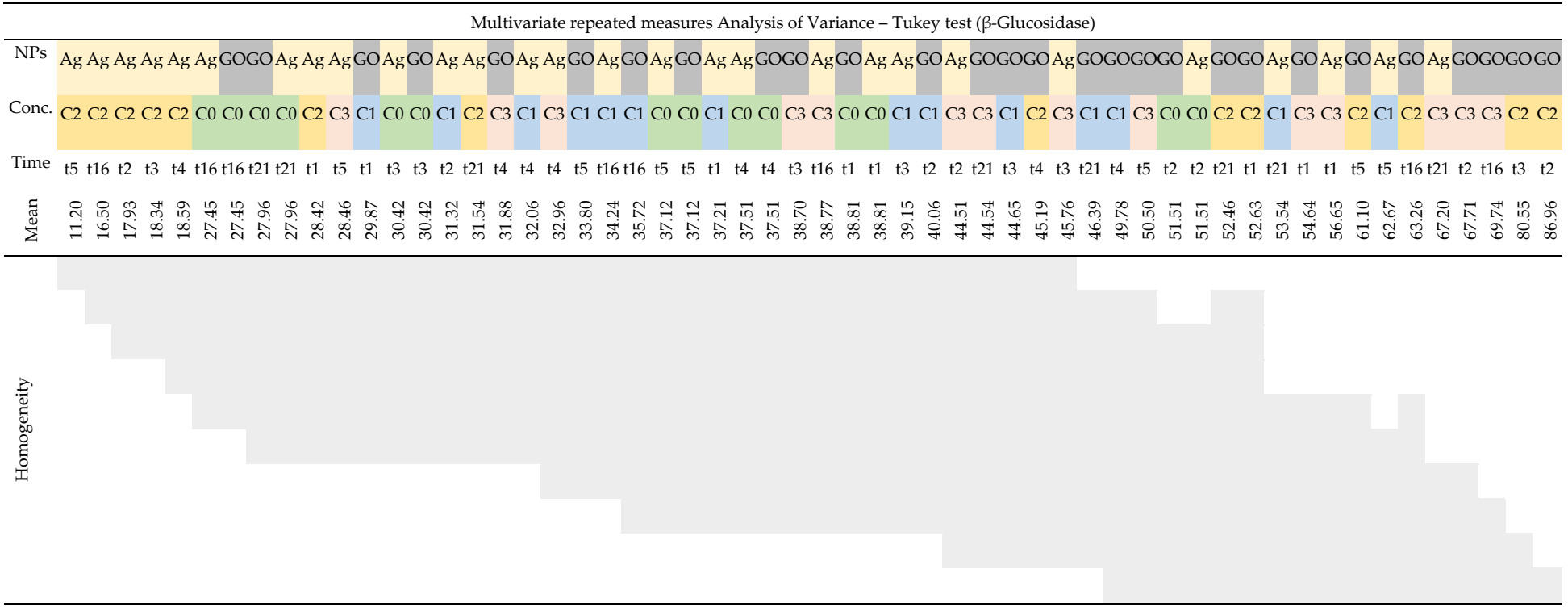


Table S8. β -Galactosidase - Results of the Tukey post-hoc test performed after multivariate repeated measures Analysis of Variance (Homogeneous groups are marked with gray boxes).

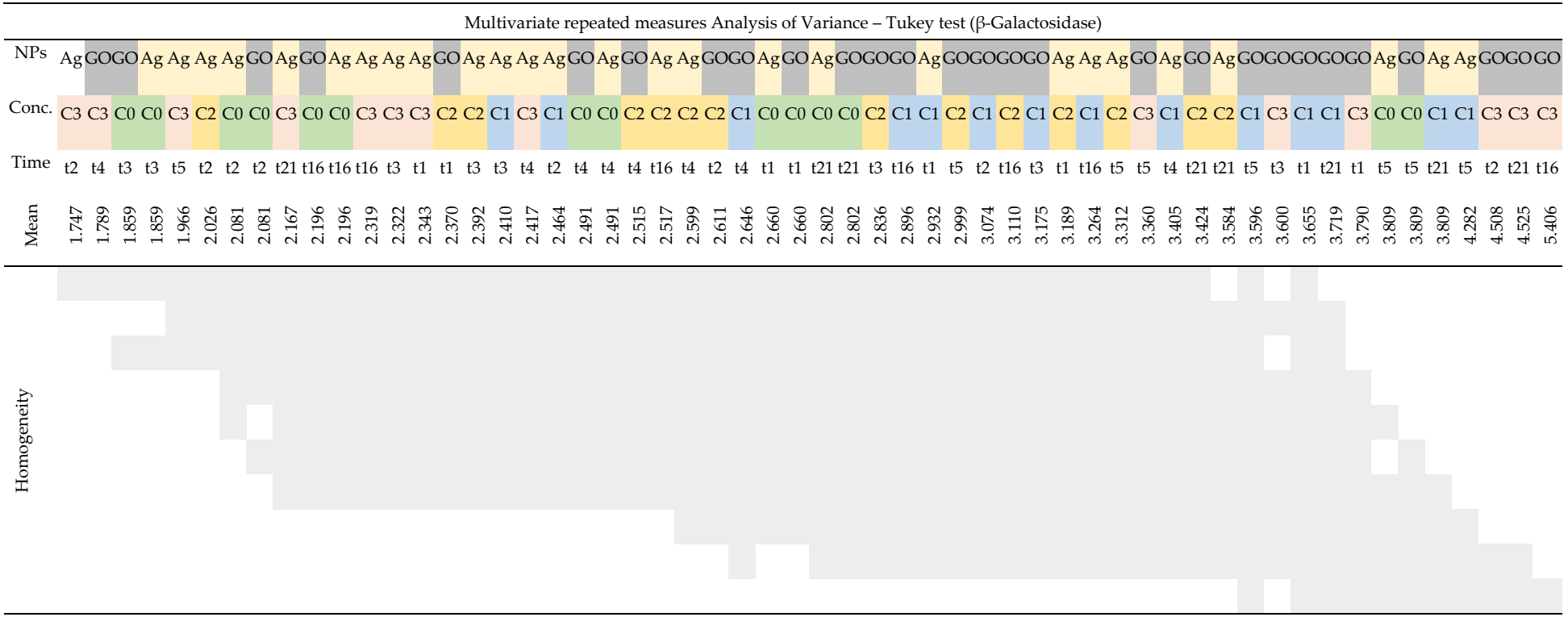


Table S9. Protease - Results of the Tukey post-hoc test performed after multivariate repeated measures Analysis of Variance (Homogeneous groups are marked with gray boxes).

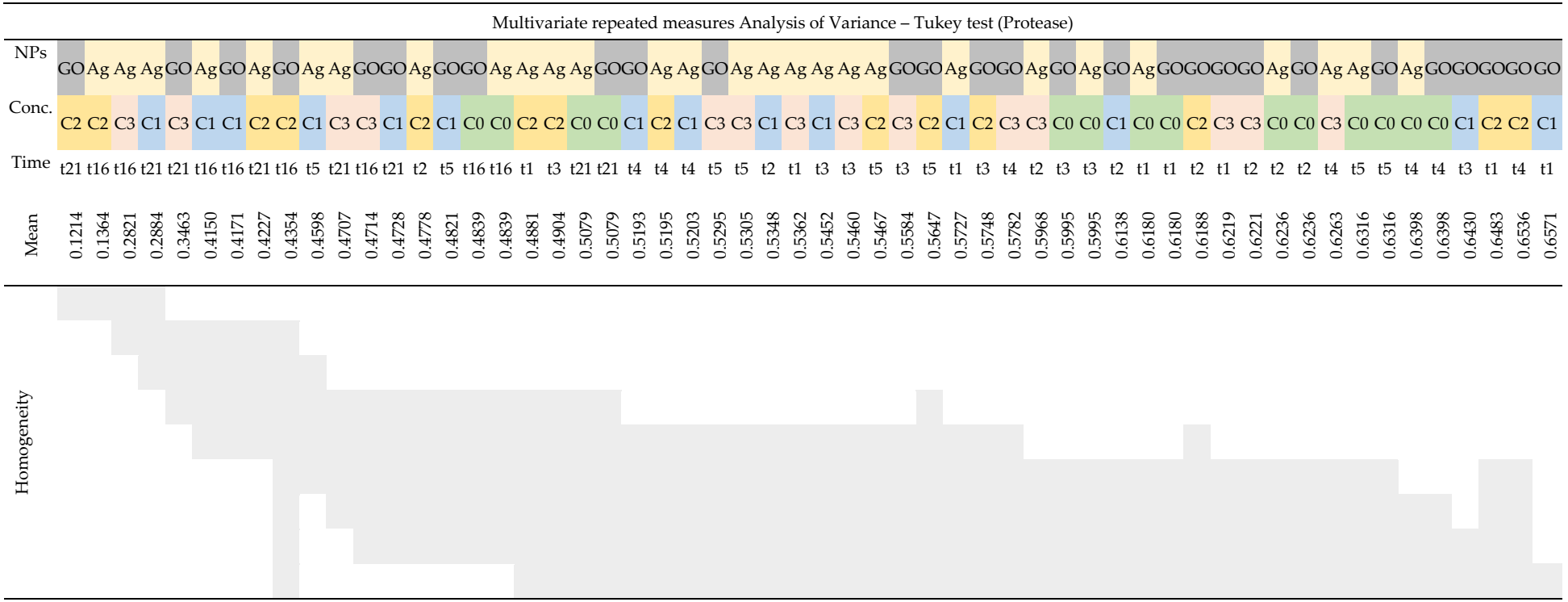
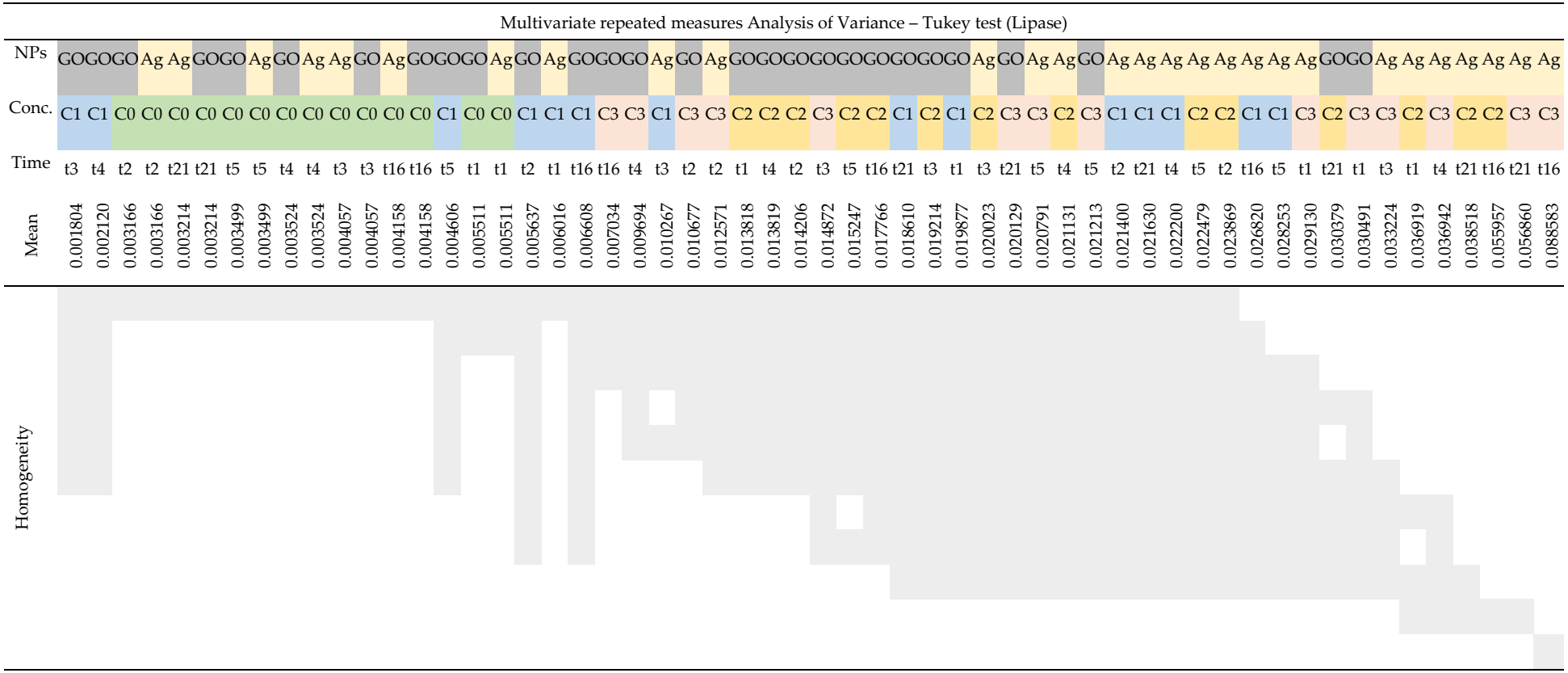


Table S10. Lipase - Results of the Tukey post-hoc test performed after multivariate repeated measures Analysis of Variance (Homogeneous groups are marked with gray boxes).



C.4. DECLARATION OF THE CO-AUTHOR OF THE MANUSCRIPT II

Katowice, 04.11.2024

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Declaration of the co-author of the manuscript

I declare that my contribution to the preparation of the manuscript:

Seyed Alian R, Flasz B, Kędziorski A, Majchrzycki Ł, Augustyniak M. Concentration- and Time-Dependent Dietary Exposure to Graphene Oxide and Silver Nanoparticles: Effects on Food Consumption and Assimilation, Digestive Enzyme Activities, and Body Mass in *Acheta domesticus*. Insects. 2024;15(2):89. doi: 10.3390/insects15020089,

which is part of my doctoral dissertation, involved participation in research planning (conceptualization), material collection, method optimization and analysis, data processing and interpretation (investigation), project administration and funding acquisition, manuscript preparation for publication, and implementation of revisions.

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Mgr Reyhaneh Seyed Alian

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Katowice, 04.11.2024

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I declare that my contribution to the preparation of the manuscript:

Seyed Alian R, Flasz B, Kędziorski A, Majchrzycki Ł, Augustyniak M. Concentration- and Time-Dependent Dietary Exposure to Graphene Oxide and Silver Nanoparticles: Effects on Food Consumption and Assimilation, Digestive Enzyme Activities, and Body Mass in *Acheta domesticus*. Insects. 2024;15(2):89. doi: 10.3390/insects15020089,

which is part of Reyhaneh Seyed Alian doctoral dissertation, involved assistance with methodology development for insects' maintenance, support in culture management and partial result analysis, data curation, and support in preparing the manuscript content.

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which is part of Reyhaneh Seyed Alian doctoral dissertation, involved support in developing the methodology for determining the energy budget, assistance in the analysis and validation of results in this area, and support in preparing the manuscript content.

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which is part of Reyhaneh Seyed Alian doctoral dissertation, involved conducting material characterization, assisting in the preparation of the nanoparticle description, and revising the article prior to publication.

.....

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Seyed Alian R, Flasz B, Kędziorski A, Majchrzycki Ł, Augustyniak M. Concentration- and Time-Dependent Dietary Exposure to Graphene Oxide and Silver Nanoparticles: Effects on Food Consumption and Assimilation, Digestive Enzyme Activities, and Body Mass in *Acheta domesticus*. Insects. 2024;15(2):89. doi: 10.3390/insects15020089.

which is part of Reyhaneh Seyed Alian doctoral dissertation, involved assistance in developing the research concept, support with data visualization and interpretation, as well as help in manuscript writing and revisions prior to publication.

.....

Prof. dr hab. Maria Augustyniak

C. MANUSCRIPTS INCLUDED IN THE DOCTORAL DISSERTATION

C.5. MANUSCRIPT III

Seyed Alian R, Flasz B, Kędziorski A, Rost-Roszkowska M, Rozpedek K, Majchrzycki Ł, Augustyniak M. Concentration-dependent disturbances of digestive functions in house cricket (Insecta: Orthoptera) exposed to GO-AgNP composite. Scientific Reports. 2025; 15:12699.
https://doi.org/10.1038/s41598-025-97589-w.

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Concentration-dependent disturbances of digestive functions in house cricket (Insecta: Orthoptera) exposed to GO-AgNP composite

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This study investigates the effects of graphene oxide (GO) and silver nanoparticle (AgNP) composite (GO-AgNP) on the digestive physiology and gut ultrastructure of *Acheta domesticus* (house cricket) during extended exposure. Various concentrations of GO-AgNP were tested to assess their impact on food consumption, assimilation, cell status (Dead Cells and ROS + cells), gut enzyme activity, and structural damage to gut cells. Concentration and exposure time had significant effects on oxidative stress, enzyme activity, and gut cell structure. The applied composite reduced cumulative food consumption and assimilation efficiency. Enzyme assays showed that lower concentrations enhanced carbohydrate-degrading enzyme activity, while higher concentrations inhibited protease activity. Histological analysis revealed structural damage to gut epithelial cells and signs of autophagy or necrosis at higher concentrations. These results suggest that GO and AgNPs contribute to oxidative stress, cell cycle disruption, and apoptosis, with AgNPs having a potentially stronger effect than GO. The disturbed enzyme activity may result from conformational changes caused by nanoparticle agglomeration. These findings underline potential risks associated with the environmental or agricultural use of GO-AgNP composites.

Keywords *Acheta domesticus*, Food consumption and assimilation, Dead cells and ROS + cells, Gut histology, Digestive enzymes

The rapidly advancing field of nanotechnology continuously introduces new nanomaterials with structural and functional properties that render them desired in a wide array of applications from industry to everyday household life[1–5]. The widespread use of nanomaterials leads to increased environmental exposure to nanoparticles, affecting biota. Since they enter food webs and contaminate food products, there is an urgent need to identify their potentially deleterious effects, both in terms of acute and chronic exposure, encompassing a broad range of concentrations and durations[6,7].

Graphene oxide (GO) is among the most attractive nanomaterials due to its unique properties, which make it valuable for applications in electronics, energy storage, materials engineering (synthesis of nanocomposites and coatings), optics, medicine, environmental technologies, and many other fields[8–11]. Similarly, silver nanoparticles (AgNPs) are among the most commonly used metallic nanoparticles, primarily because of their potent antimicrobial properties, optical characteristics, and excellent thermal and electrical conductivity[12–14]. While the harmful effects of GO and AgNPs have been relatively well-studied, their impact on the digestive functions of organisms remains an area with considerable room for further research[15–18].

Recently, new composite nanomaterials have been synthesized based on GO and metal nanoparticles. They combine the properties of their constituents or exhibit novel features, further expanding their range of applications[19–22]. An example of such nanomaterial is the GO-AgNP composite, whose diverse applications have been clearly presented by de Medeiros et al.[23]. The application of GO-AgNP composites in medicine results from their unique antibacterial properties[24–27]. Additionally, their use has been proposed for various purposes,

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including drug delivery platforms, wound healing, bioimaging materials, and components in sensors for detecting various substances, such as glucose[28–30]. GO-AgNP composites are applied also in agriculture, plant protection, dye degradation, catalysis, electrochemical detection, and even environmental engineering[31–37]. However, using these GO-based nanomaterials raises the question of whether the GO and metal nanoparticles, as components of composite materials, interact within organisms in an additive, synergistic, or antagonistic manner.

Surprisingly, nanoparticles' impact on organisms' digestive functions has not been extensively studied. The available data on the activity of digestive enzymes are fragmentary, indicating the potential inhibition of certain digestive enzymes by some nanoparticles, but also, under certain conditions, their stimulation[38–45]. According to our previous studies, the effect of GO on digestive enzymes is moderate and depends on the concentration and type of enzyme studied. High concentrations of GO primarily stimulated the activity of amylase and lipase, while prolonged exposure inhibited protease activity. Notably, the effects described above were much more pronounced in the case of AgNPs[45].

The impact of nanoparticles on digestive functions is multifaceted, potentially affecting both host enzymes, the gut microbiota and possibly nutrients absorption by the gut cells[17]. These impairments can result in nutrient and energy shortages and disturb proper development, growth, reproduction, and adequate stress responses. In one scenario these may lead to increased mortality of exposed organisms. However, there may be different scenario, particularly following lower exposure - an organism may have triggered an adaptive response to counteract the stressor. In this case, enhancement of digestive functions could be observed[44,46].

Insects, including *Acheta domesticus*, are recognized as valuable model organisms that enable research to be conducted in alignment with the 3R principle (replacement, reduction, and refinement). The sequencing of model insect genomes confirms their genetic and physiological similarities to other animals, including higher vertebrates. These advantages allow for the replacement of mammals in many experiments, particularly in the early stages of research. Insects can thus be used to investigate molecular mechanisms related to nutrition, and study dietary additives for their toxicity or health benefits. Despite species-specific differences, insects serve as robust models that can contribute to understanding human physiology, including nutrition, the genesis of metabolic disorders, and the functioning of the gut microbiome. Consequently, this study may provide universal insights applicable to other insect species and organisms, including humans[47,48].

This study aimed to describe the effects of GO-AgNP composite nanomaterial on the model insect *Acheta domesticus* digestive functions examined from biochemical (activity of selected digestive enzymes), physiological (estimating food/energy budget parameters), and histological (identifying changes in the gut ultrastructure) perspectives. The study considered four different concentrations of GO-AgNP composite and length of exposure times. The tested hypothesis assumed that the possible changes in measured parameters of digestive functions would be proportional to the concentration of GO-AgNP composite and/or might intensify with the length of exposure time.

Materials and methods

Preparation and characterization of GO-AgNP composite

Graphene Oxide Water Dispersion (GO, 99.5%, 2 wt%) was purchased from Nano Graphen (Ankara, Turkey), and Silver Dispersion (Ag, 99.99%, 15 nm, 10000 ppm in water, Tawny) was obtained from US Research Nanomaterials, inc. (3302 Twing leaf Ln, Houston, TX 77084, USA).

By combining graphene oxide and silver nanoparticles with deionized water and citrate buffer solution (0.1 M, pH = 6.5), graphene oxide-silver nanoparticle composite suspension (GO200/Ag400 ppm NPs) was produced. Briefly, 24 mL of silver stock suspension, 6 mL of graphene oxide stock suspension and 100 mL of citrate buffer 0.1 M were pooled, adjusted to final volume with 300 mL of deionized water and sonicated (UP-100 H, DONSERV, Poland) for 4 h (cycle 1, amplitude 100% with gentle heating) and kept overnight in the dark at a room temperature to obtain final homogenous and stable colloidal suspension as described elsewhere[49–51].

Four batches of feed were prepared with increasing content of GO-AgNP composite. 1.5, 15 and 150 mL of the composite suspension were diluted to a final volume of 150 mL with deionized water. Then, each solution was thoroughly mixed with 300 g of finely ground rabbit pellets to obtain dry-weight feed containing GO2Ag4, GO20Ag40 and GO200Ag400 ppm, respectively. For the highest NPs content in the feed (GO200Ag4000 ppm) a mixture of 120 mL of AgNPs and 3 mL of GO stock solutions was prepared and used similarly. Only deionized water (150 mL) was used for control feed preparation. Prepared feed was sterilized for 48 h in a laminar chamber (UVcleaner, BIOSAN, Warren, MI, USA) and dried for 48 h at 45 °C in a dryer (Pol-Eko Aparatura, Poland) (see Refs.[30,36] for the detailed protocol of food preparation). The composite concentrations were selected based on our previous study[44], in which we applied a single composite concentration (20 µg GO/g of food and 400 µg AgNPs/g of food) and observed signs of hormesis. To gain a deeper understanding of the digestive responses of *Acheta domesticus* to the composite and to determine whether it has adverse effects, we decided to expand the concentration range in the present study. The concentrations used ranged from 2 µg GO/g of food and 4 µg AgNPs/g of food to 200 µg GO/g of food and 4000 µg AgNPs/g of food.A scanning electron microscope (SEM) with an energy dispersion X-ray spectrometer (EDX) (Quanta FEG 250; FEI, Oregon, USA) and atomic force microscopy (AFM) (Agilent 5500) were used for the analysis of the shape and structure of the GO-AgNP composites. A more detailed description of the sample preparation method for analysis was previously published[44,53].

The GO-AgNP composite consisted of thick graphene oxide (GO) structures uniformly coated with silver nanoparticles. The average diameter of the nanoparticles was approximately 2 µm. Aggregates were observed sporadically, with diameters reaching 20–30 µm. The height of the structures varied between 1 and 30 nm (Fig. 1).

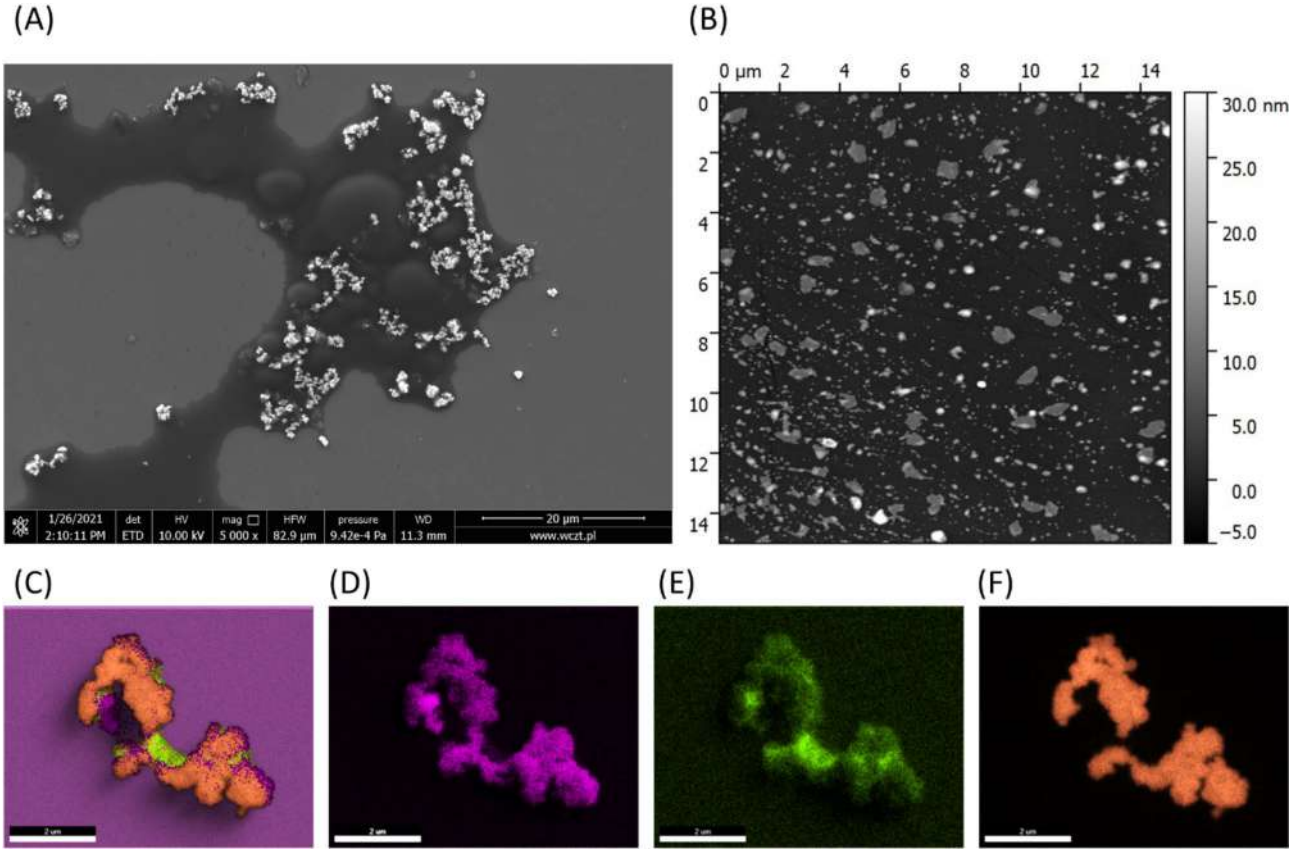

Fig. 1. Physicochemical characteristics of GO-Ag NPs composite. (**A**) SEM, (**B**) AFM imagies. (**C**) SEM-EDS elemental mapping of GO-AgNPs composite with representative particles: (**D**) carbon, (**E**) oxygen, and (**F**) silver.

House cricket (*Acheta domesticus*)

The model organism *Acheta domesticus* (Orthoptera, Insecta) has been used for decades in physiological and toxicological studies[54,55]. Its relatively short life cycle (approximately two months) enables observing the effects of studied compounds across multiple generations in a relatively short period. A high reproductive rate allows the establishment of numerous research groups and a satisfactory number of repetitions. Moreover, crickets are edible in some parts of the world and recently house cricket has been approved by European Commission as a novel food ingredient for humans[56]. Hence, studying the possible adverse effects of new environmental contaminants in the crickets becomes of interest to human health. The insects for the experiments were obtained from a permanent laboratory stock colony held at the Institute of Biology, Biotechnology, and Environmental Protection (Faculty of Natural Sciences, University of Silesia in Katowice, Poland)[57].

Experimental design

The 0–1 day-old adults from the stock cohort were randomly assigned to five experimental groups: control and fed with food containing GO-AgNP composite at the following concentrations: GO2Ag4, GO20Ag40, GO200Ag400, and GO200Ag4000 µg/g of food, prepared as described above. The insects were kept in plastic fauna boxes throughout the experiment, with free access to food, water, and shelter. Three distinct sets of insect groups were established for experimental purposes: (a) food budget assessment, (b) cell status analysis, and (c) enzymatic assays and histological analysis.

Food consumption and assimilation measurements

Each experimental group had five replicates (boxes) containing five individuals in each box. For the first ten days, the dry weight of the supplied food, food remains, faeces, and the insects' fresh weight were measured every two days, and then on the 16th and 21st day with a 1 mg accuracy (Semi-Micro Balance EX225D; OHAUS, Parsippany, NJ, USA). The dry weight of the samples was obtained by drying them at 50 °C for 48 h. These raw data were used to calculate food budget parameters in the experimental groups (see Refs.[44,45] for a detailed description of budget calculations).

Cell status analysis

The percentage of dead cells and oxidative stress in the *A. domesticus* gut was measured on the treatment's first, fifth, and 21st days. Five insects from each group were randomly picked up and slightly anaesthetized on ice.

Subsequently, the whole gut was excised and placed in 0.1 M PBS buffer (400 µL, 4 °C, pH 7.4). The tissue was then gently homogenized (Minilys, Bertin Technologies, France), and cytometry (Muse Cell Analyzer, Millipore, MA, USA) was used to identify the percentage of dead cells and oxidative stress in the resulting cell suspension, according to the protocol for Muse Annexin V & Dead Cell Kit and Muse Oxidative Stress Kit.

Digestive enzyme measurements

Gut samples were taken on the 1st, 5th, 15th, and 21st days of the treatment from anaesthetized crickets by isolation and homogenization of the midgut in a phosphate buffer (pH 7.4, 1 mL, 4 °C), followed by centrifugation of the homogenates for ten minutes at 4 °C at 14,000 rpm. The midguts of three insects were pooled for each sample, weighing 100 ± 20 mg, and five replicates were made for every time point in each experimental group. The activity of digestive enzymes was assayed with commercially purchased kits following the provided protocols previously optimized for *A. domesticus*[45]. Carbohydrates are a key energy source for the house cricket, which is why we selected four enzymes involved in their digestion: α- and β-glucosidases (α-Glu, β-Glu), Amylase, and β-galactosidase (β-Gal). We also included enzymes digesting proteins and lipids to gain a comprehensive understanding of the composite's effects.

Proteolytic activity was measured spectrophotometrically as changes in absorbance at 492 nm per minute using the Protease Assay Kit (Cat. No. 539125; LOT 3802816) from Calbiochem; Merck KGaA, Darmstadt, Germany.

Amylase activity was assayed with the Amylase Activity Assay Kit (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. MAK009; LOT 8E24K07110) and expressed in µmol of product/min/mL supernatant.

Activity of α- and β-glucosidases (assay kits from Sigma-Aldrich, St. Louis, MO, USA; Cat. No. MAK123; LOT 123CA05A04 and Cat. No. MAK129; LOT 129CB08A18, respectively) were measured spectrophotometrically (TECAN Infinite M200, Männedorf, Austria) in 96-well flat-bottom plates, as the substrate-specific product formation reaction rate at 405 nm and expressed in Units/L (one unit represent the quantity of enzyme required to hydrolyze one µmol of substrate per minute).

Activity of β-galactosidase was measured with β-Galactosidase Activity Assay Kit (Abcam, Cambridge, CB2 0AX, UK; Cat. No. ab287846; LOT GR3429797-1). The reaction rate was determined spectrofluorimetrically for 20 min (Ex/Em = 480/530 nm using a Hitachi F-7000 Fluorescence Spectrometer Plate Reader, Tokyo, Japan) and expressed in Units/L, where 1 unit is the quantity of enzyme needed to produce 1.0 µmol of fluorescein per minute[58,59].

The Lipase Activity Assay Kit (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. MAK046; LOT 8H15K07220) was used to measure lipase activity with glycerol as the reference standard of product formation rate at 570 nm. The expressed value of enzyme activity was µmol/min/mL.

Histological analysis

For this analysis, samples were taken from treated adult insects on the 5th and 21st days of exposure. The midgut was isolated and prepared for transmission electron microscopy (TEM) according to the protocol described by Karpeta-Kaczmarek et al.[60,61]. Ultra-thin Sect. (70 nm) were cut on a Leica Ultracut UCT25 and contrasted with uranyl acetate and lead citrate. Tissues were analyzed using a Hitachi H500 transmission electron microscope at 75 kV. The applied technique allows the visualization of cellular structures but not the presumed intracellular localization of the composite.

Statistical analysis

Digestive enzymes activities and food budget analyses included five replicates. Before statistical data processing, Dixon's Q test was applied to identify and reject outliers. Assumptions of the analysis of variance were checked for the obtained data (the Kolmogorov–Smirnov and Lilliefors tests to assess the data distribution and Levene and Brown–Forsythe tests to evaluate the homogeneity of variances) before further analysis.

Multivariate repeated measures ANOVA with Tukey post-hoc test ($p < 0.05$) was applied to assess the effects of NPs concentration and treatment time (= adult age) on food consumption and assimilation, cumulative food consumption and assimilation in crickets, as well as enzyme activity. The main effects of concentration and time, as well as their interactions, were also assessed.

Results

Food budget

Food consumption in control was the highest during the first five days (reaching almost 72 mg dry weight food per individual per day), then lowered significantly in subsequent time intervals to a relatively similar level. The mean daily consumption in this group through 21-day interval (and age-span) was 42.70 mg. Exposure to NPs revealed a significant inhibitory effect of their concentration and exposure time. The joint effect of these factors was also highly significant (Table 1; Fig. 2). The lowest consumption was observed in the group GO200Ag4000 at all time intervals, with the mean daily value reaching hardly 64% of the control (27.36 mg d.w. per individual). The highest difference was observed in 1–3 and 3–5 days intervals when consumption reached 53% and 40% of the control values, respectively (see Fig S1 in Supplementary materials).

Cumulative food consumption (CFC) across the analysed period of adult life revealed similar and even higher significance of NPs-concentration and time-intervals (age) effects in comparison with daily consumption data (Table 2; Fig. 3, and Fig S3 in Supplementary materials).

Assimilation of ingested food in the control was the highest during the first three days of adult life, and it was significantly lower in subsequent time intervals. The mean daily assimilation (calculated as weighted mean) for the analysed period was 19.30 mg of d.w. food per individual. Admixture of GO-AgNP composite in food lowered the assimilation, and both NPs concentration and time intervals, considered separately or jointly,

Effect	Food consumption				Food assimilation			
	F	df_1	df_2	*p*	F	df_1	df_2	*p*
Concentration [1]	13.90	4	20	$<10^{-4}$	11.75	4	20	$<10^{-5}$
Time (age) [2]	85.63	6	120	$<10^{-5}$	118.02	6	120	$<10^{-5}$
[1] × [2]	3.81	24	120	$<10^{-5}$	3.78	24	120	$<10^{-5}$

Table 1. Multivariate repeated measures ANOVA for GO-AgNP concentration [1], time [2], and interaction of the factors [1] × [2] on food consumption and assimilation measured in consecutive time (age) intervals within 21 days of adult crickets exposure. Symbols description: F - F ratio; df_1 and df_2 - treatment and error degrees of freedom, respectively; p - p value, $n = 5$.

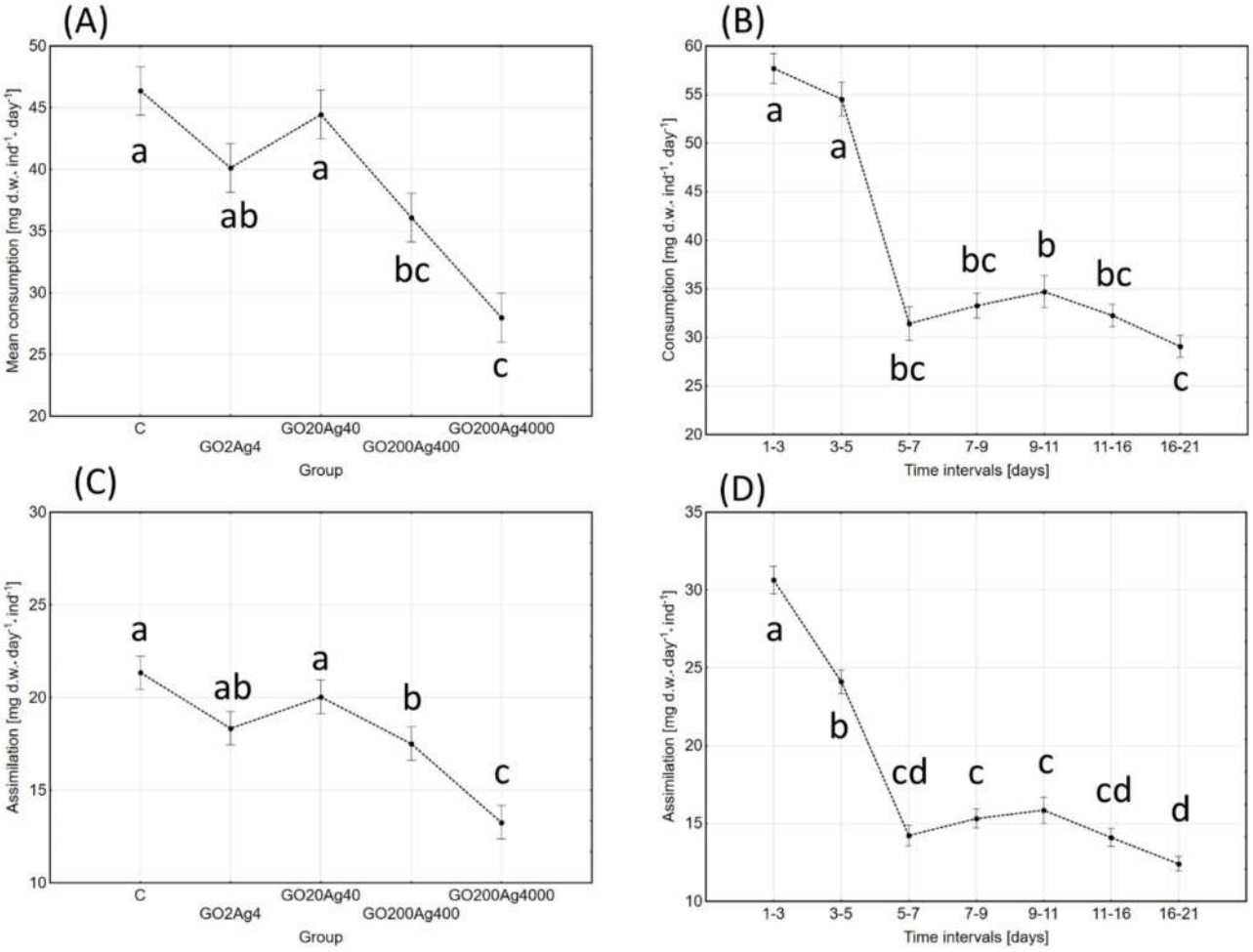

Fig. 2. Effect of group (**A**,**C**) and time-intervals (**B**,**D**) on food consumption (**A**,**B**) and assimilation (**C**,**D**) (mg dry weight per individual per day; marginal means ± SE) in adult *A. domesticus* treated with different concentrations of GO-AgNPs. The same letters denote no significant differences among groups (ANOVA (**A** and **C**) or ANOVA with repeated measurements (B and D); Tukey post-hoc test; $p < 0.05$).

had significant effects (Table 1). The lowest assimilation was observed in the group GO200Ag4000 at all time intervals, with the mean daily value reaching hardly 66% of the control (12.76 mg d.w. per individual). Following the consumption pattern, the most pronounced effect was observed during the first five days (see Fig S2 in Supplementary materials).

Cumulative food assimilation (CFA) followed the CFC pattern in control and experimental groups and confirmed the significance of previous calculations of concentration and time/age effects (Table 2 and Fig S4 in Supplementary materials). It is noteworthy that assimilation efficiency was similar in all the groups and reached values within 44.3–47.8% range. This indicates that NPs primarily affected food consumption, which caused observed changes in its assimilation.

Effect	CFC				CFA			
	F	df_1	df_2	p	F	df_1	df_2	p
Concentration [1]	16.43	4	20	$<10^{-5}$	14.33	4	20	$<10^{-4}$
Time (Age) [2]	1192.60	6	120	$<10^{-6}$	1284.10	6	120	$<10^{-6}$
[1] x [2]	6.08	24	120	$<10^{-6}$	4.54	24	120	$<10^{-6}$

Table 2. Multivariate repeated measures ANOVA for GO-AgNP concentration [1], time [2], and interaction of the factors [1] × [2] on cumulative food consumption (CFC) and cumulative food assimilation (CFA) measured in consecutive time (age) intervals within 21 days of adult crickets exposure. Symbols used as in Table 1.

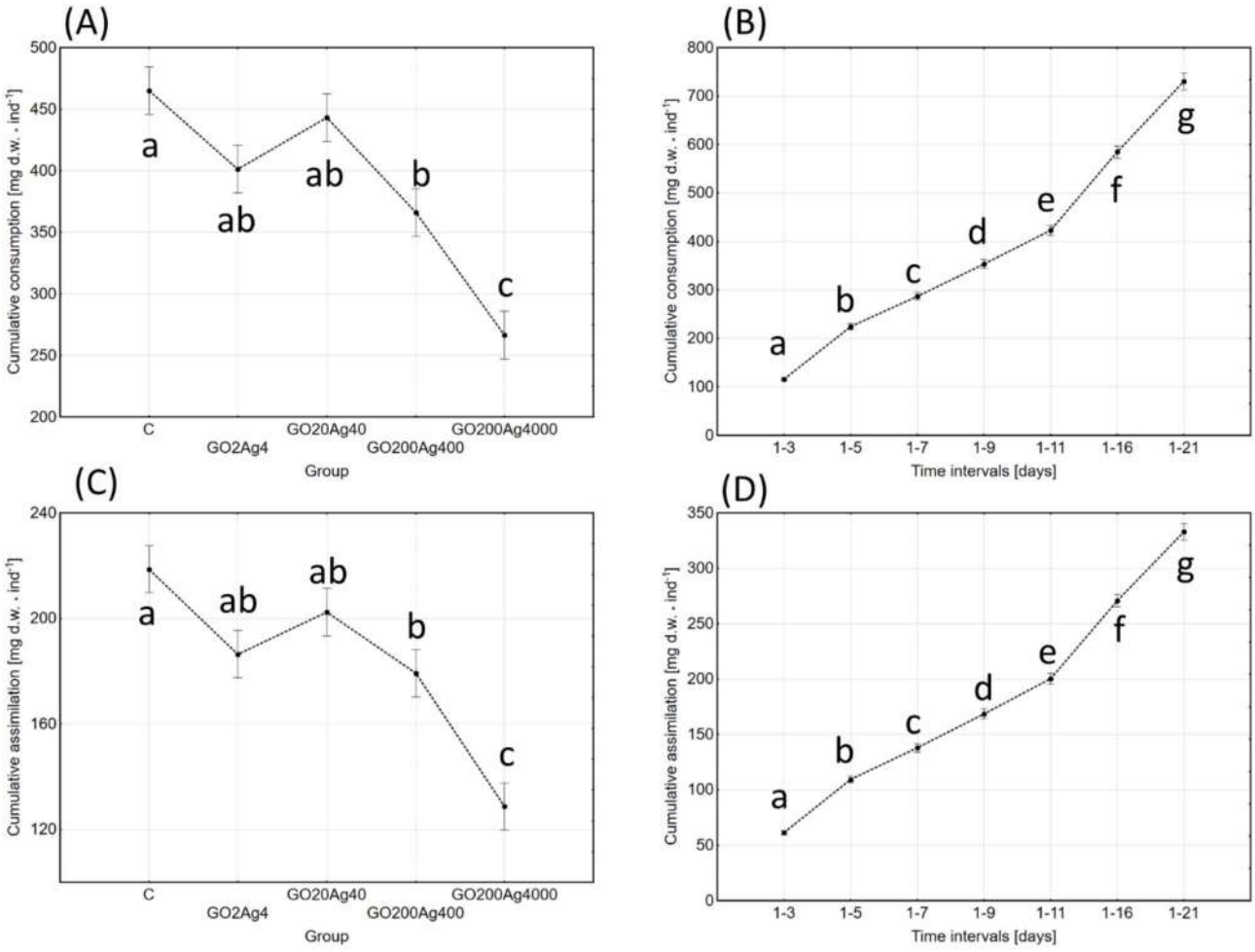

Fig. 3. Effect of group (**A**,**C**) and time-intervals (**B**,**D**) on cumulative food consumption (**A**,**B**) and cumulative food assimilation (**C**,**D**) (mg dry weight per individual per day; marginal means ± SE) in adult *A. domesticus* treated with different concentrations of GO-AgNPs. Abbreviations: see Fig. 2.

Lowered consumption and assimilation resulted in smaller weight gain of adults throughout the experimental period ($F = 3{,}458$, $df_1 = 4$, $p = 0.0265$) (Figs S5-S6 in Supplementary materials).

Gut cell status

The main effects analysis revealed that neither of the used GO-AgNP composite concentrations influenced the percentage of dead cells. In contrast, the exposure time and the interactions between both variables were significant (Table 3; Fig. 4). The percentage of dead cells increased with the exposure time (age of the insects). This effect was particularly pronounced in the control group and the groups treated with lower concentrations of the GO-AgNP composite (GO2Ag4 and GO20Ag40). Interestingly, in the groups treated with higher concentrations of the composite, the percentage of dead cells over time did not change (in GO200Ag400 group) or even decrease (in GO200/Ag4000 group) (Fig. S7).

Effect	Dead cells			Oxidative stress		
	F	df	*p*	F	df	*p*
Concentration [1]	0.39	4	0.815	19.00	4	< 0.001
Time (age) [2]	22.56	2	< 0.001	109.05	2	< 0.001
[1] x [2]	9.93	8	< 0.001	19.60	8	< 0.001
Residual		58			58	

Table 3. The main effects and interactions of the factors: GO-AgNP concentration [1], time [2], and interaction of the factors [1] × [2] on dead cells ratio and oxidative stress level measured in the gut of *A. domesticus* at 1st, 5th and 21st days of exposure. See Table 1 for symbols description.

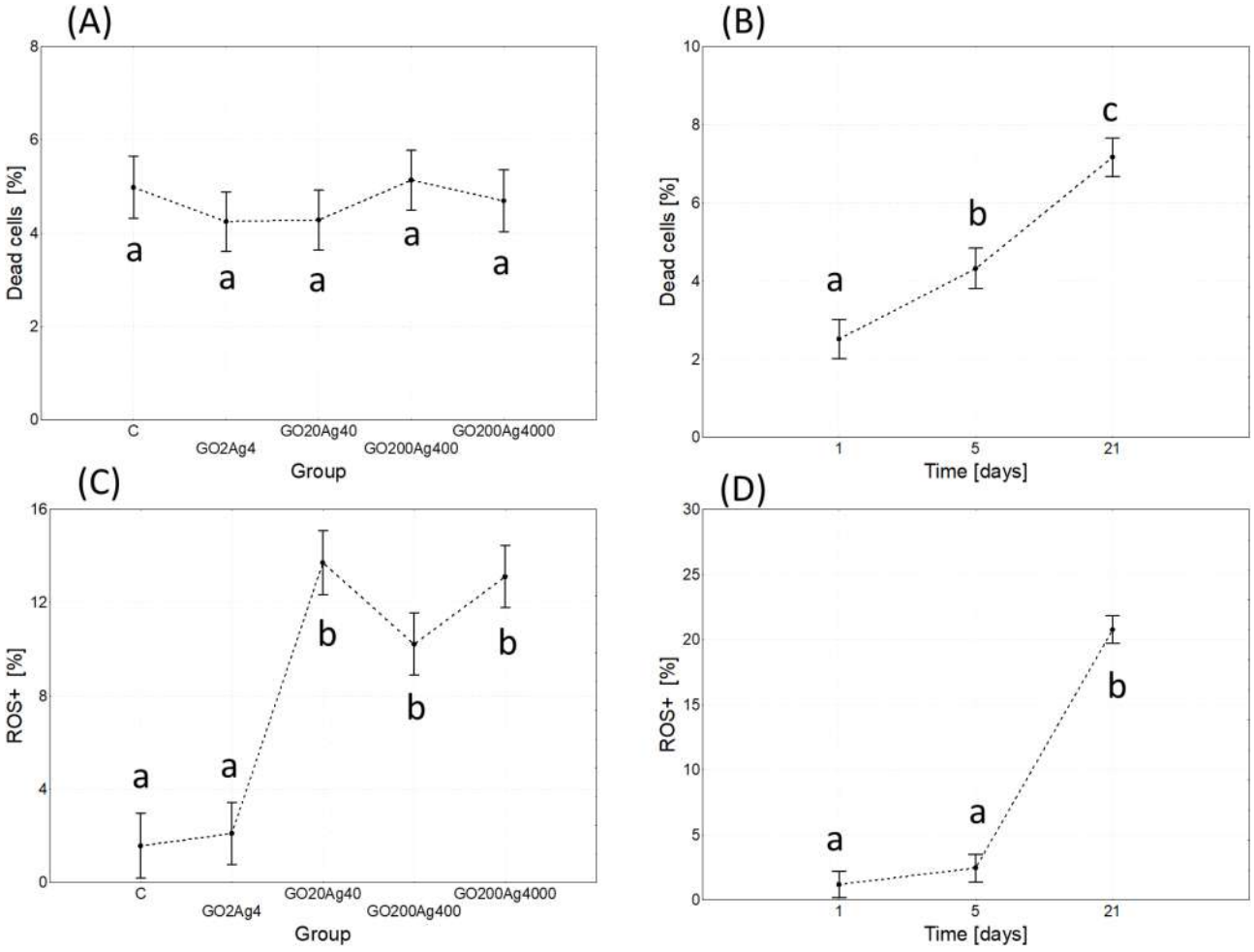

Fig. 4. Effect of group (**A**,**C**) and time (**B**,**D**) on dead cells (**A**,**B**) and ROS + cells (**C**,**D**) (%; marginal means ± SE) in the gut of adult *A. domesticus* treated with different concentrations of GO-AgNPs. Abbreviations: see Fig. 2.

Both the variables of concentration and time and their interactions affected the level of oxidative stress in the gut cells of *A. domesticus* (Table 3). The ROS + level was significantly elevated in the groups treated with higher composite concentrations (GO20Ag40, GO200Ag400, GO200Ag4000) compared to the control and the GO2Ag4 group, which did not differ. Additionally, the ROS + level was significantly higher on day 21 compared to days 1 and 5 (Fig. 4). A more detailed analysis (Fig. S7) showed that this effect was due to the increase in ROS + in the groups treated with higher composite concentrations.

Digestive enzyme activity

Multivariate repeated measures ANOVA revealed an ambiguous effect of the composite concentration on the hydrolytic activity in the gut. It was significant only for β-Glu, β-Gal, amylase, and protease, and the first two enzymes had higher activity in composite NP-exposed cricket vs. control, except the highest composite

concentration used. The activity of the assayed enzymes in the latter was as in control, and protease activity was the lowest (Table 4; Fig. 5).

With exposure time, the activity of glucosidases and protease increased, while other enzymes did not change their activity. However, concentration and exposure time interaction revealed the affected activity of all assayed enzymes except α-glucosidase (Table 4; Fig. 6 and S8).

Gut histology

The midgut of *A. domesticus* comprises two distinct regions: the anterior and posterior parts. It is lined with the simple epithelium formed by two types of cells: the digestive and regenerative cells (midgut stem cells), which form characteristic regenerative crypts. In the five- and 21-day-old control crickets, three regions could be distinguished in the cytoplasm of the digestive cells: basal, perinuclear, and apical, while the cytoplasm of regenerative cells was poor in organelles and did not show any regionalization in organelles distribution (Figs. 7A-B). This description is consistent with earlier analysis of digestive and regenerative cells ultrastructure[58,59].

In five-day-old crickets from GO2Ag4 and GO20Ag40 groups, no changes were observed in the digestive and regenerative cells of the midgut epithelium (Figs. 7C-D). In crickets of the same age from GO200Ag400 and GO200Ag4000 groups, the apical cytoplasm of digestive cells often showed necrotic features: the cytoplasm was electron-lucent and poor in organelles. Vacuoles with electron-lucent interiors and autophagic structures appeared, and many mitochondria were damaged (Figs. 7E-H). In the perinuclear cytoplasm, disrupted nuclear envelopes could be observed. The chromatin formed electron-dense patches in numerous digestive cells (Fig. 7E). The cytoplasm of regenerative cells showed no changes compared to the control group. Similar changes were observed only in the digestive cells of 21-day-old individuals (GO2Ag4, GO20Ag40, GO200Ag400, and GO200Ag4000; Figs. 8A-G) compared to 5-day-old adult crickets. However, more autophagic structures (autophagosomes, autolysosomes, residual bodies) could be observed in all these experimental groups (Fig. 8B-D and G). In addition, necrosis was intensified in the cells of 21-day-old individuals in GO200Ag400 and GO200Ag4000 groups. Thus, cells with electron-lucent cytoplasm and poor organelles appeared, and the apical cell membrane lost the microvilli (Fig. 8D and F).

Discussion

Despite extensive research on the toxicity of GO and AgNPs, studies on GO-AgNP composites remain limited, particularly regarding their effects on the digestive functions of exposed animals. Our previous study[44] with a single concentration of GO-AgNPs composite has shown weak or moderate stimulatory response in food digestion and absorption, similar to the effects of these NPs applied separately. Observed changes resembled stress-induced adaptive reactions.

The current study explores previous observations in the same model insect (*Acheta domesticus*) by broadening the composite concentration range and elongation of the exposure time, almost to the mean adults' survival time, that is 22.4 ± 5.2 days for both sexes (unpublished data). Results indicated that food consumption and assimilation depended significantly on both concentration and exposure time (Table 1; Fig. 2). While GO-AgNPs did not disrupt the typical feeding patterns of *A. domesticus*, (except for the group receiving the highest composite concentration, Figs S1-S2), cumulative consumption increased less towards the end of the feeding period across all treated groups (Fig. S3). Sparse relevant literature data suggests potential impairments of digestive functions and supports our findings[62–67]. Regarding nutrients and energy assimilation, no significant differences were observed in protein, lipid, glucose, and glycogen levels in *Gammarus roeseli* exposed to silica nanoparticles (30–1000 nm). However, the authors ascribed a lack of significant differences between groups to small sample sizes[68]. A 21-day exposure of *A. domesticus* to GO alone showed no impact on cumulative food consumption and a moderate, yet significant, increase in food assimilation at concentration of 200 µg/g food. In contrast, exposure to AgNPs at concentrations of 4, 40, and 400 µg/g food tended to decrease both parameters, although the effect was not concentration-dependent. High concentrations of AgNPs may promote their aggregation and clustering, thus reducing the total surface area of NPs and Ag ions release[45]. Hence, it seems reasonable to suppose that the observed concentration-dependent effects of the GO-AgNP composite were caused merely by AgNPs. GO flakes probably maintained the dispersion and stabilization of silver nanoparticles by preventing their agglomeration[69].

Effect	F	df_1	df_2	*p*	F	df_1	df_2	*p*	F	df_1	df_2	*p*
	α-Glu				β-Glu				β-Gal			
Concentration [1]	2.702	4	16	0.068	3.987	4	14	0.023	4.839	4	14	0.012
Time [2]	3.478	3	48	0.023	11.295	3	42	< 0.001	2.265	2	42	0.095
[1] × [2]	0.869	12	48	0.582	2.767	12	42	0.007	2.687	12	42	0.009
	Amylase				Lipase				Protease			
Concentration [1]	2.937	4	18	0.049	0.946	4	11	0.473	48.524	4	20	< 0.001
Time [2]	0.959	3	54	0.419	0.334	3	33	0.801	3.284	3	60	0.028
[1] × [2]	4.220	12	54	< 0.001	3.548	12	33	0.002	1.963	12	60	0.044

Table 4. Multivariate repeated measures ANOVA for GO-AgNP concentration [1], time [2], and interaction of the factors [1] × [2] on digestive enzymes in the gut of *A. domesticus* following exposure to GO-AgNP composite, measured in consecutive time (age) within 21 days of adult crickets exposure (F - F ratio; df_1 and df_2 - treatment and error degrees of freedom, respectively; p - p value, $n = 5$).

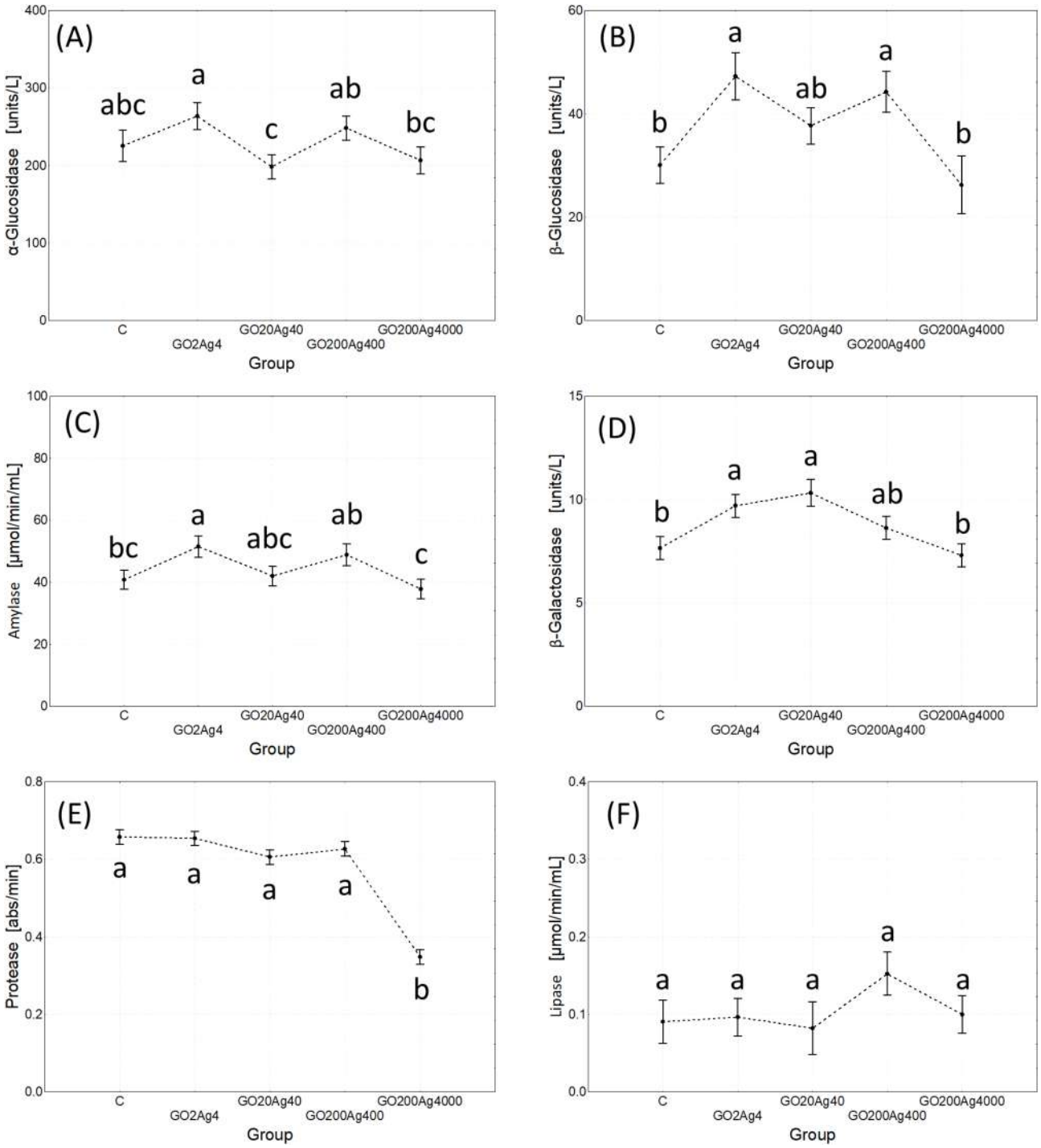

Fig. 5. Effect of treatment (experimental group) on digestive enzyme activity (marginal means ± SE) in adult *A. domesticus* treated with different concentrations of GO-AgNPs. The same letters denotes no significant differences among groups (Tukey post-hoc test; $p < 0.05$).

The behavior of the GO-AgNP composite in the gut, specifically whether it aggregates or dissociates into GO and AgNPs, remains unclear. Nanoparticles interact with nutrients, host-derived compounds, and gut microbiota. Changing conditions within the gut, including pH fluctuations, enzyme concentrations, and the formation of new compounds, may influence nanoparticle stability. However, evidence from prior studies suggests that the GO-AgNP composite exhibits high stability. Zhu et al.[70] attributed its sustained antibacterial activity to the exceptional stability of its nanostructures. Similarly, Bao et al.[35] highlighted the hydrophilicity and stability of GO, which effectively prevent composite aggregation and AgNP dissociation.

Possibly stable, the composite's particles in the gut milieu may cause damage to epithelial cells. The number of dead cells and cells with elevated oxidative stress (ROS+) increased with cricket's age (Figs. 4B and D) and also in

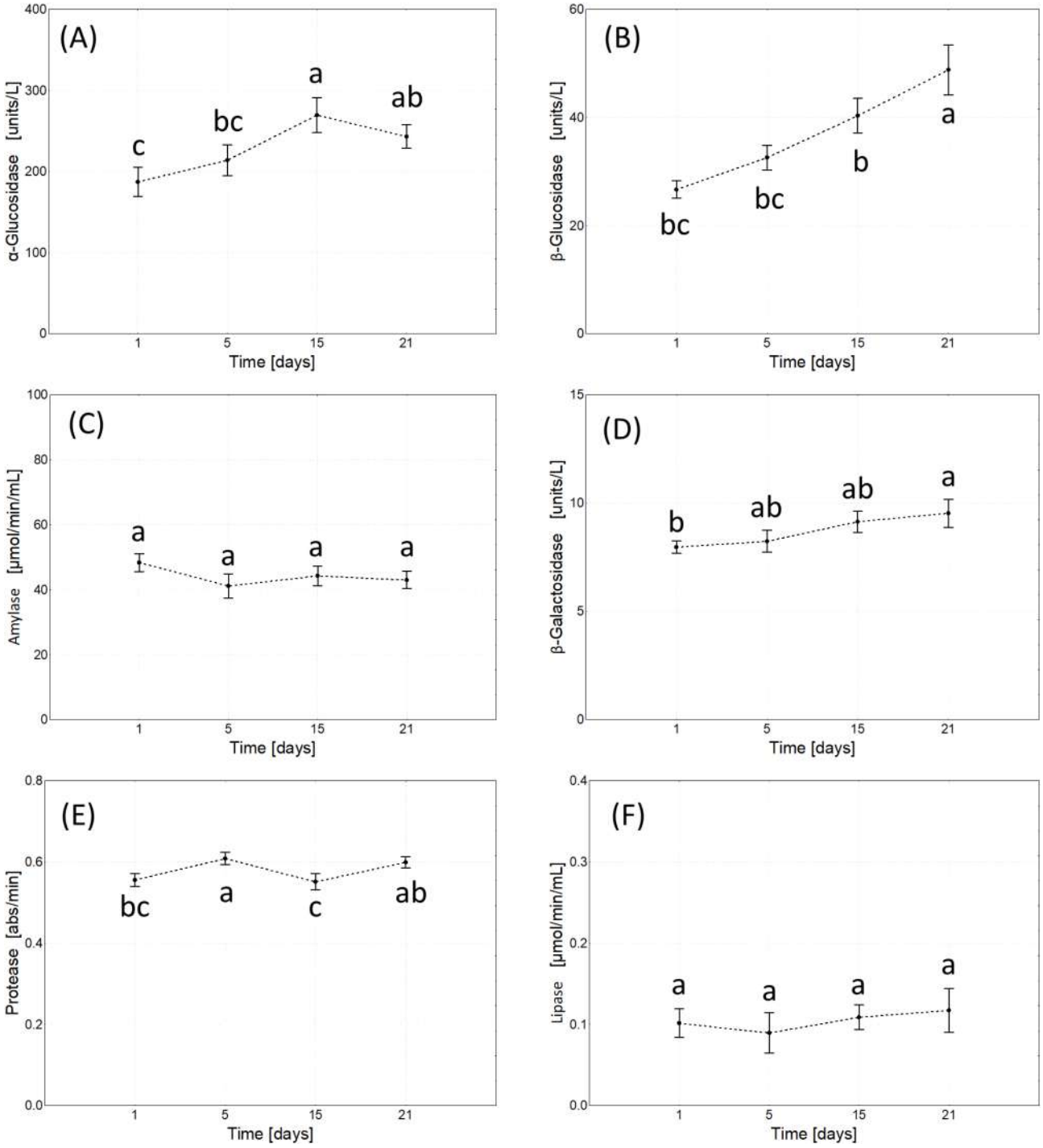

Fig. 6. Effect treatment duration (time) on digestive enzyme activity (marginal means ± SE) in adult *A. domesticus* treated with different concentrations of GO-AgNPs. The same letters denotes no significant differences among groups (Tukey post-hoc test; $p < 0.05$).

one-day-old individuals exposed to higher concentrations of the composite (Fig. S7). Interestingly, in the latter, the percentage of the dead cells did not change with adults' age. This finding suggests a possible link to cell cycle arrest, as seen in various cells exposed to different NPs[71,72]. Observed increased ROS + cells at higher composite concentrations (Fig. S7B) may suggest their elevated molecular damage, including DNA damage. The arrest of the cell cycle, increasing the likelihood of repairing damage and preventing its irreversibility could be potential cellular response to such stress[73]. Such cells may re-enter the cell cycle, potentially reducing the number of dead cells. In case of nutrient deprivation (e.g. following decreased food consumption caused by xenobiotic's presence), cell cycle arrest may save energy resources and avoid cell death[74,75]. Moreover, it was-documented that cell cycle arrest can postpone cellular senescence with retaining metabolic activity for an extended time[76].

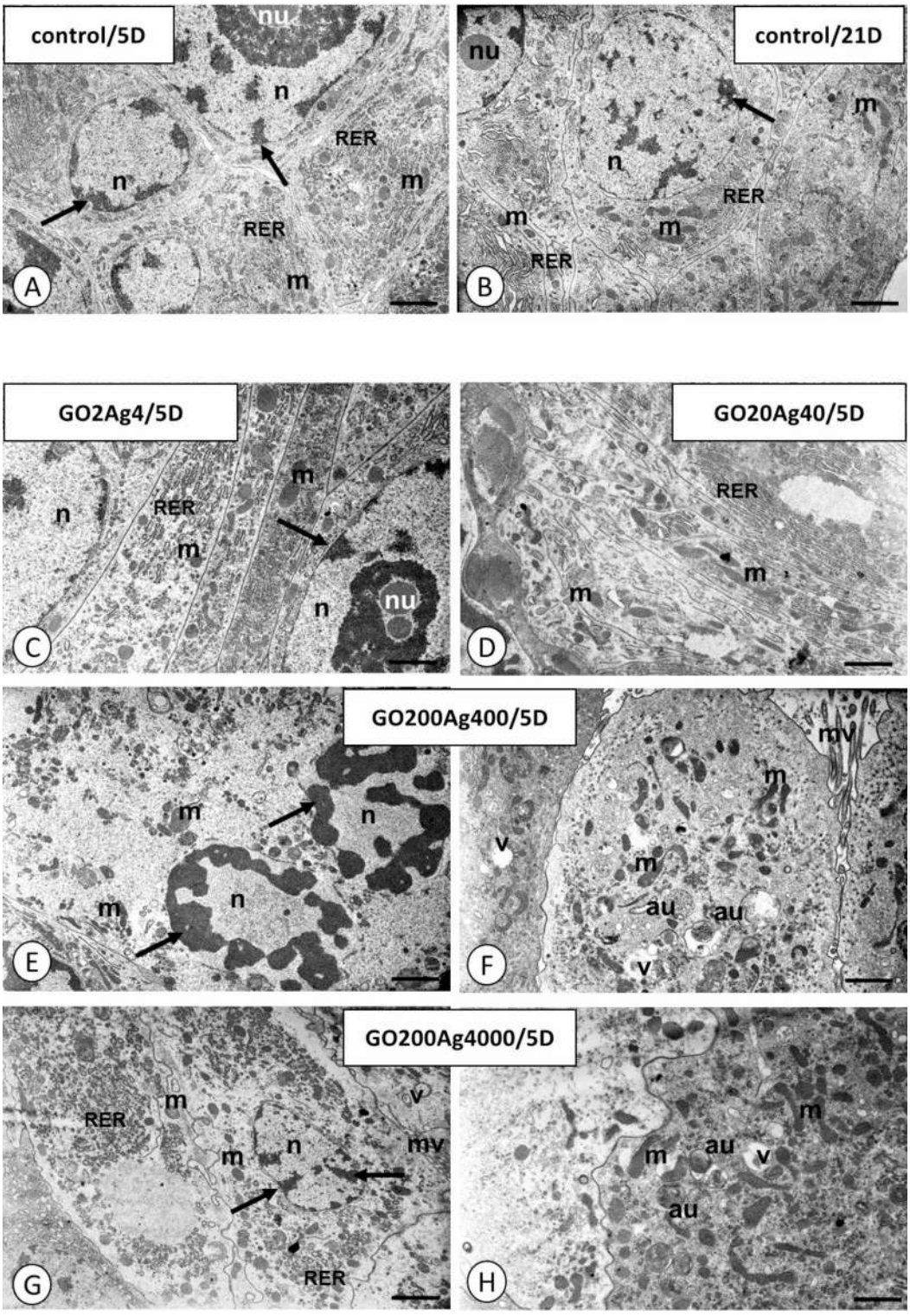

Fig. 7. *Acheta domesticus* midgut epithelium in control (**A**,**B**) and experimental groups GO2Ag4 (**C**), GO20Ag40 (**D**), GO200Ag400 (**E**,**F**), and GO200Ag4000 (**G**,**H**) after 5 days (/5D) of exposure to GO-AgNPs. Mitochondria (m), nuclei (n), nucleoli (nu), patches of heterochromatin (arrows), cisterns of RER (RER), autophagic structures (au), vacuoles (v). TEM. (**A**) Scale bar = 0.8 µm. (**B**) Scale bar = 1.2 µm. (**C**) Scale bar = 0.9 µm. (**D**) Scale bar = 0.7 µm. (**E**) Scale bar = 1 µm. (**F**) Scale bar = 0.9 µm. (**G**) Scale bar = 0.9 µm. (**H**) Scale bar = 0.7 µm.

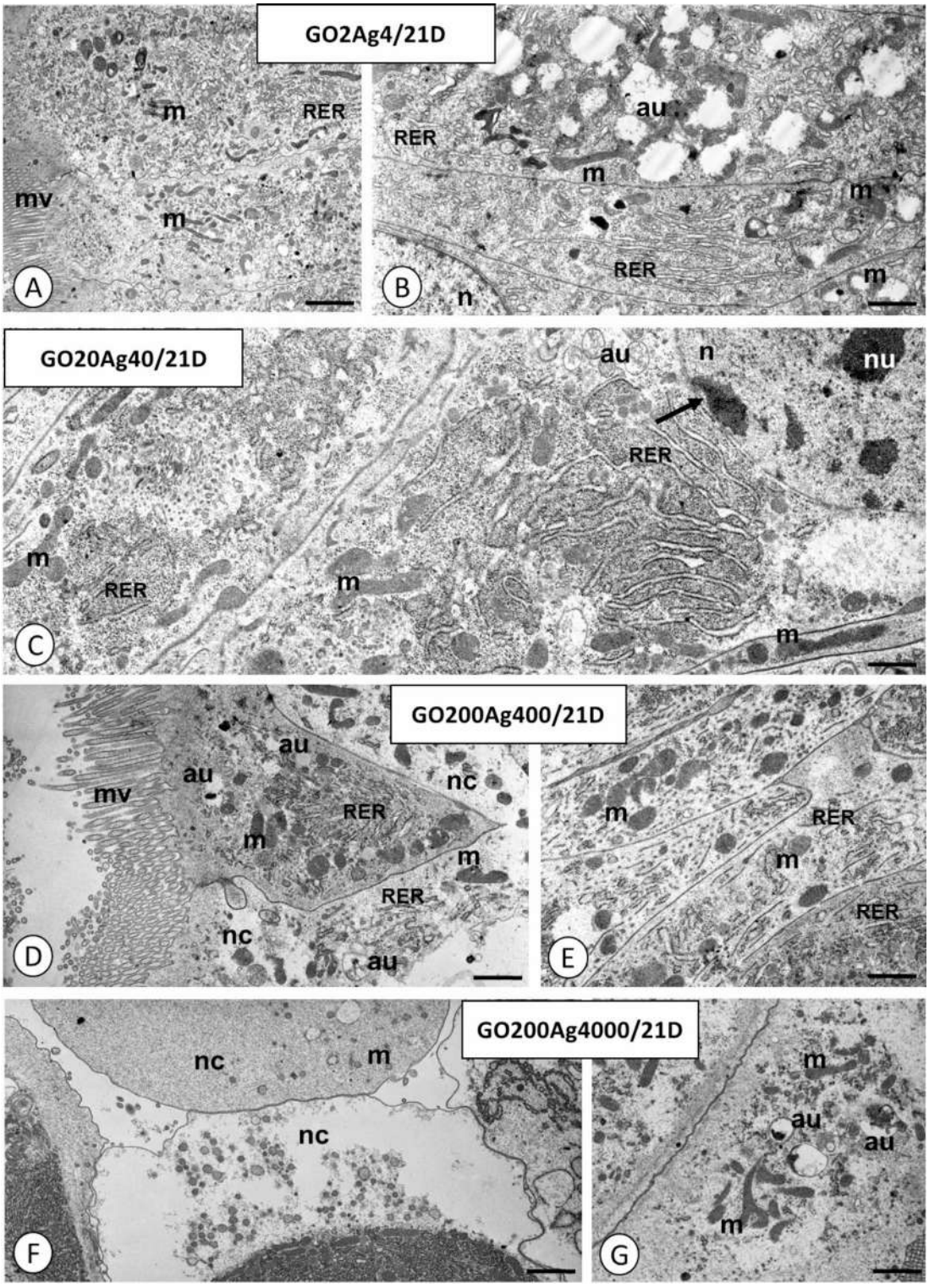

Fig. 8. *Acheta domesticus* midgut epithelium in experimental groups GO2Ag4 (**A**,**B**), GO20Ag40 (**C**), GO200Ag400 (**D**,**E**), and GO200Ag4000 (**F**,**G**) after 21 (/21D) days of exposure to GO-AgNPs. Mitochondria (m), nuclei (n), nucleoli (nu), patches of heterochromatin (arrows), cisterns of RER (RER), autophagic structures (au), necrotic cells (nc). TEM. (**A**) Scale bar = 1.5 µm. (**B**) Scale bar = 0.7 µm. (**C**) Scale bar = 0.5 µm. (**D**) Scale bar = 0.9 µm. (**E**) Scale bar = 0.7 µm. (F) Scale bar = 1.6 µm. (**G**) Scale bar = 0.9 µm.

This may contribute to decreased number of dead cells in the high-stress exposed tissue, compared to the control. Following these assumptions, it could be hypothesized that higher NPs composite concentrations cause cellular damage, while cell cycle arrest provides a window for damage repair or adaptation to stressful conditions. A similar delay in the progression of percentage of dead cells, typical of aging individuals, was observed in crickets exposed to AgNPs at concentrations of 4 and 40 µg/g of food and GO[45].

We previously showed that despite DNA damage, increased ROS + cells, and induced apoptosis, GO can cause adverse histological changes in the midgut of *A. domesticus*[77]. Symptoms of autophagy or necrosis in insects' still-living cells exposed to high GO-AgNP composite concentrations (Figs. 7 and 8) suggest activation of defense mechanisms, attempts at cell repair, or initiation of programmed cell death. While the mechanism of GO-AgNPs action in intestinal cells is unknown, recent studies by Feng et al.[78] revealed that GO toxicity in these cells involves the p53 protein. They also observed structural damage to small intestine cells in GO-exposed rats, suggesting that the adverse effects of oral GO exposure are significant[78]. Additionally, GO can cut mitochondrial membranes causing organelle damage and impairing ATP production[79]. The toxicity mechanism of AgNPs is also linked to the p53 protein, cell cycle arrest, and apoptosis induction[71,80–82]. We can hypothesize on these fragmentary data that both components of GO-AgNP composite exert similar effects by increasing ROS production, weakening the cell's antioxidant defenses, arresting the cell cycle, and then inducing programmed cell death or, potentially, cell regeneration. A similar mechanism for GO-AgNPs was postulated in studies on human liver normal (CHANG) and cancer (HepG2) cells[83], caprine fetal fibroblast cells[84], and U87 cancer cell lines and also in the fungus *Alternaria alternata* cells[85]. However, at this research stage, we cannot confirm whether the GO-AgNP composite enters digestive and/or regenerative cells. Recent studies conducted by Lange et al.[25] on bacterial cells suggest that the GO-AgNP composite may accumulate around cells or on their surface, primarily interacting with the cell wall and membrane. However, the authors of this study do not rule out other points of interaction with cells, including membrane damage, ROS generation, and inhibition of cell growth[25]. Apart from oxidative stress, other common symptoms included the accumulation of autophagosomes and autophagic vacuoles[84]. Furthermore, there is evidence that the nanocomposite's cytotoxic effects are stronger than AgNPs and GO given separately[22].

Disruptions in the cell cycle and gut cell structure caused by the GO-AgNP composite were accompanied by changes in digestive enzyme activities, albeit in a limited range. The main effects showed stimulation of some carbohydrate-degrading enzymes at low nanocomposite concentrations and significant inhibition of protease at the highest concentration (Fig. 5). The joint effect of exposure time and treatment provided a more complex picture (Fig. S8). Crickets, like some other insects, can regulate protein and carbohydrate intake and metabolism[86]. Stimulation of carbohydrate-degrading enzyme activities, the most readily available energy source, may indicate a compensatory adjustment to meet increased energy demands under stress. Reduced protein utilization in the GO200Ag4000 group raises concerns, as protein is an essential source of amino acid for endogenous molecule synthesis and proper development[87,88]. However, in this study, impaired protein utilization in the GO200Ag4000 group did not significantly hinder fresh weight gain of adult crickets (Figs S5-S6), although it might have affected other functions related to protein synthesis (e.g. nutrients allocation to eggs production in females).

The mechanism underlying the observed effects of NPs composite on digestive enzymes remains enigmatic. However, stimulation of carbohydrate-degrading enzymes and inhibition of proteases have been noticed in earlier studies in various species, including insects exposed to AgNPs[45]. Studies by Muralisankar et al.[89] demonstrated that the freshwater prawn *Macrobrachium rosenbergii* exhibited improved growth parameters, survival rates, and increased activity of digestive enzymes, including proteases, when dietary zinc nanoparticles (ZnNPs) were provided at concentrations up to 60 µg/g. However, exceeding this concentration resulted in toxic effects, including inhibiting digestive enzymes. Similarly, protease inhibition was observed in juvenile *Epinephelus coioides* exposed to copper nanoparticles (CuNPs) at concentrations of 0.02–0.1 µg/mL for 25 days[38]. The authors proposed that this inhibition might result from a direct effect of nanoparticles on enzyme activity or synthesis, ruling out changes in feeding behaviour, feeding activity, or food quality due to nanoparticle contamination.

We believe that insights into the interaction between nanoparticles and digestive enzymes come from research on nanocatalysis and the use of enzyme-NP conjugates in industry for rapid decomposition of complex molecules[90–92]. Although these studies focus mainly on the structures created in a laboratory (ex vivo), they shed light on the mechanisms of enzyme-NP conjugate formation and the role of NPs in modulating enzyme activity. For example, Deka et al.[91] observed a nine-fold increase in α-amylase activity when a low concentration (0.175 µg/mL) of citrate-stabilized gold nanoparticles (cit-AuNPs) was added to the reaction milieu. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) revealed enzyme molecules attached to cit-AuNPs. The α-amylase molecule, which contains two cysteine thiol groups away from the active site, bound to NPs, forming a complex with a more effectively oriented active center. However, these structures can form varying levels of NP agglomeration, affecting enzyme activity.

Following this, it seems reasonable that optimal NPs/enzyme molecules ratios may enhance enzymatic activity due to conformational changes, while higher NP concentrations forming larger agglomerates may reduce enzyme functional group availability. Saware et al.[92] found that immobilizing α-amylase on gold nanoparticles and biosynthesized silver nanoparticles increased enzyme activity one- to two-fold compared to the 'free' enzyme.

Metal nanoparticles also affect other catalytic properties of digestive enzymes'[90,93,94]. For example, gold nanoparticles coated with PEG-biotin and lipase digested substrates differently than 'free' lipase, with significant dependency on enzyme orientation, decreasing activity when the active site faced the nanoparticle[90]. Therefore, NPs can act as catalysts within specific concentration ranges, facilitating enzyme-substrate interaction.

The above leads to a tentative conclusion that the observed activity of digestive enzymes following exposure to NPs composite may result from structural damage to the epithelial gut cells producing the enzyme molecules and conformational changes of these molecules caused by NPs that affect the activity. Moreover, in the gut

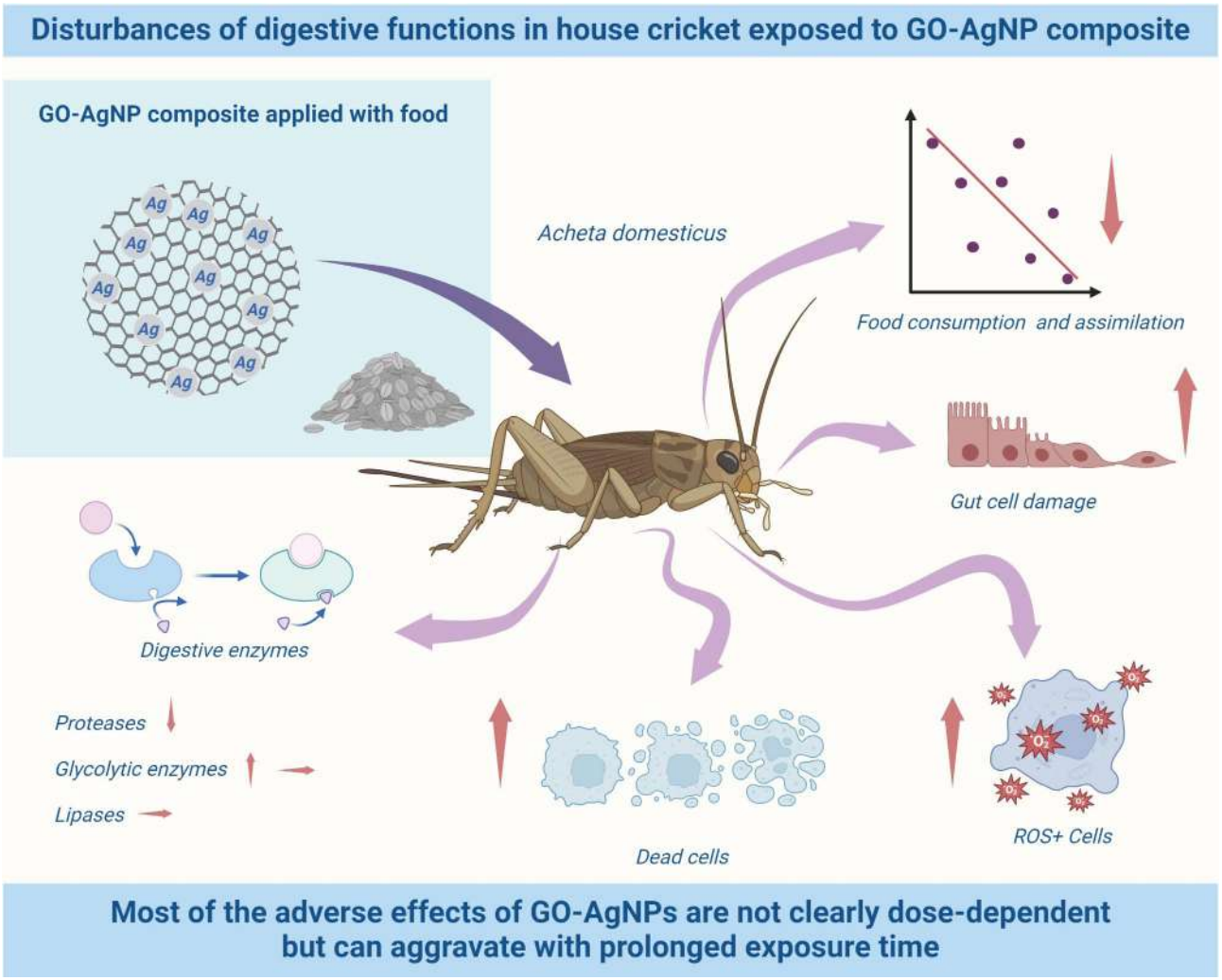

Fig. 9. Disturbances of digestive functions in house cricket exposed to GO-AgNP composite.

milieu, NPs are surrounded by various nutrients, host-produced molecules, and the gut microbiome, which makes these molecular interactions far more complex.

Conclusions

The results of this study can support the hypothesis that the GO-AgNP composite can disrupt gut structure, digestive enzymes' activity and food/energy budget parameters (Fig. 9). Some of these changes were not dose-dependent. However, they became more pronounced with prolonged exposure (age of organisms and indirectly with increasing composite concentration). Such results suggest complex interactions among composite NPs and target organism's constituents. However, current state-of-the-art allows speculative rather than definitive explanations of their mechanisms, particularly concerning in vivo exposure. Nevertheless, our results point out that the composite of GO-AgNPs, like its constituents (described in our previous papers), cannot be considered harmless, particularly in higher concentrations and/or long-term exposure.

Our research has limitations, notably in understanding how the GO-AgNP composite affects digestive functions, including enzyme activity. Adding the composite to the feed and the required sterilization and drying processes may have affected its organoleptic properties and altered (slightly) its composition. Future studies should investigate the composite's fate after ingestion, including whether its particles penetrate gut cells or if AgNPs dissociate in the gastrointestinal tract. Another interesting question to be studied is the role of gut microbiota in the NPs effects within the gut tissue and milieu.

Data availability

Raw data are provided on the RepOD database (DOI: 10.18150/XKOIEP; https://repod.icm.edu.pl/dataset.xhtml? persistentId=doi%3A10.18150%2FXKOIEP).

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Supplementary Materials

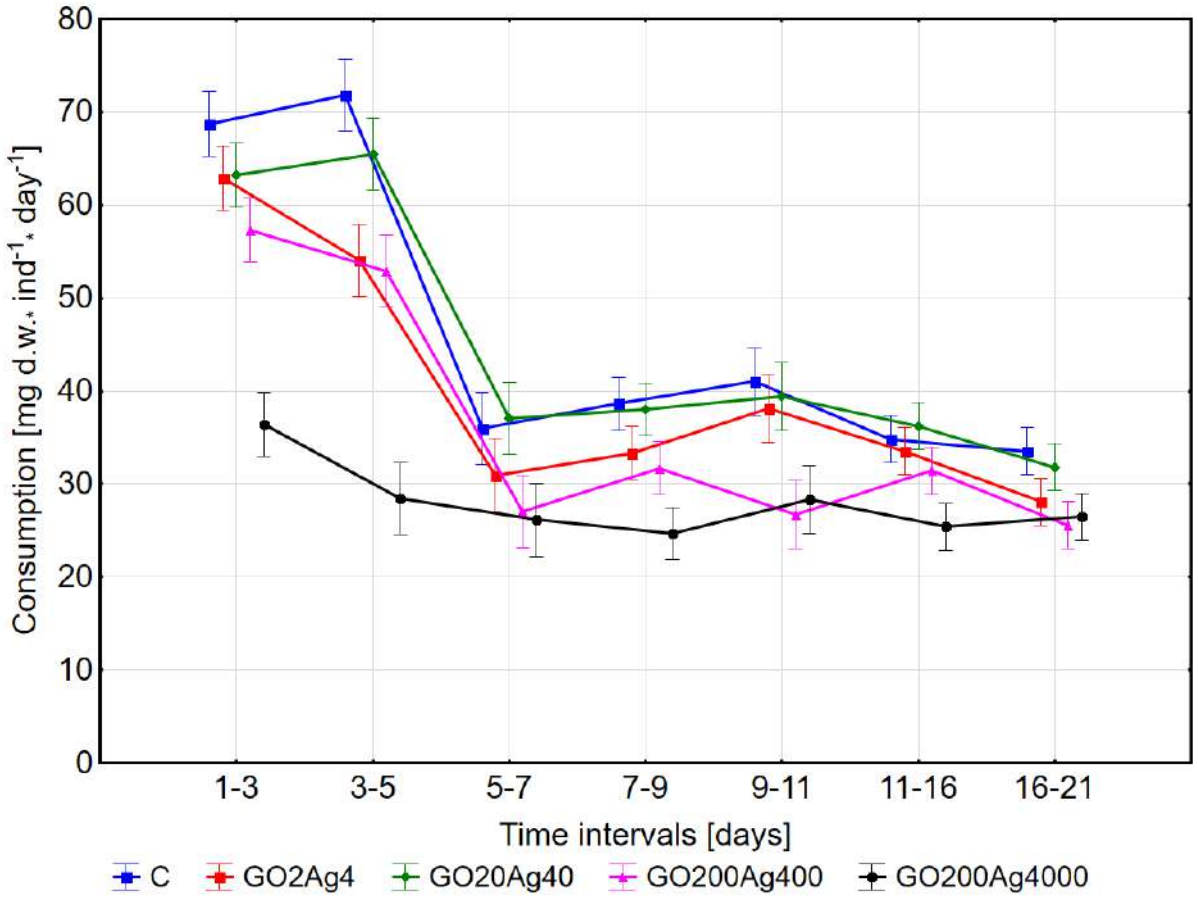

Figure S1. Food consumption (mg dry weight per individual per day; mean + SE) in consecutive time-intervals (days) by adult *A. domesticus* treated with different concentration of nanoparticles. See M&M for detailed groups description.

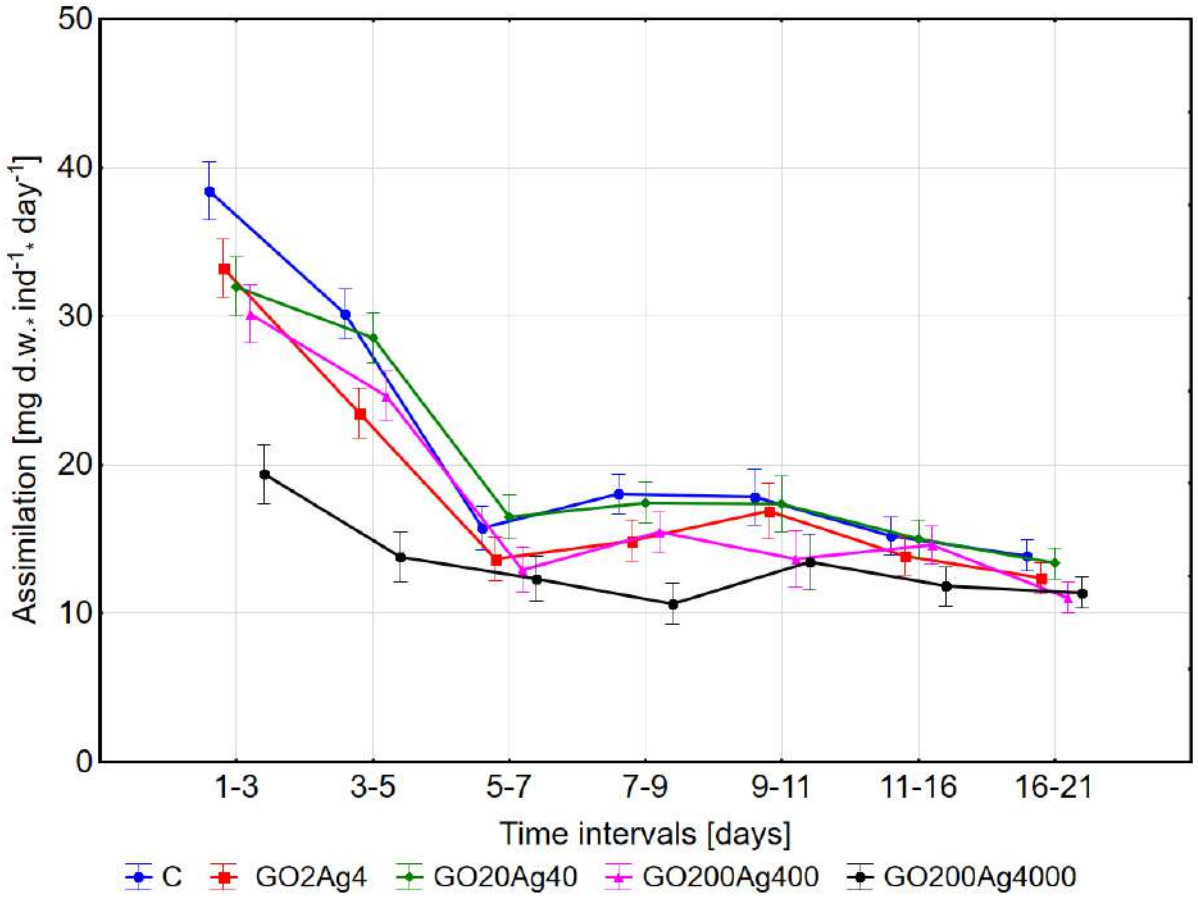

Figure S2. Food assimilation (mg dry weight per individual per day; mean + SE) in consecutive time-intervals (days) by adult *A. domesticus* treated with different concentration of nanoparticles. See M&M for detailed groups description.

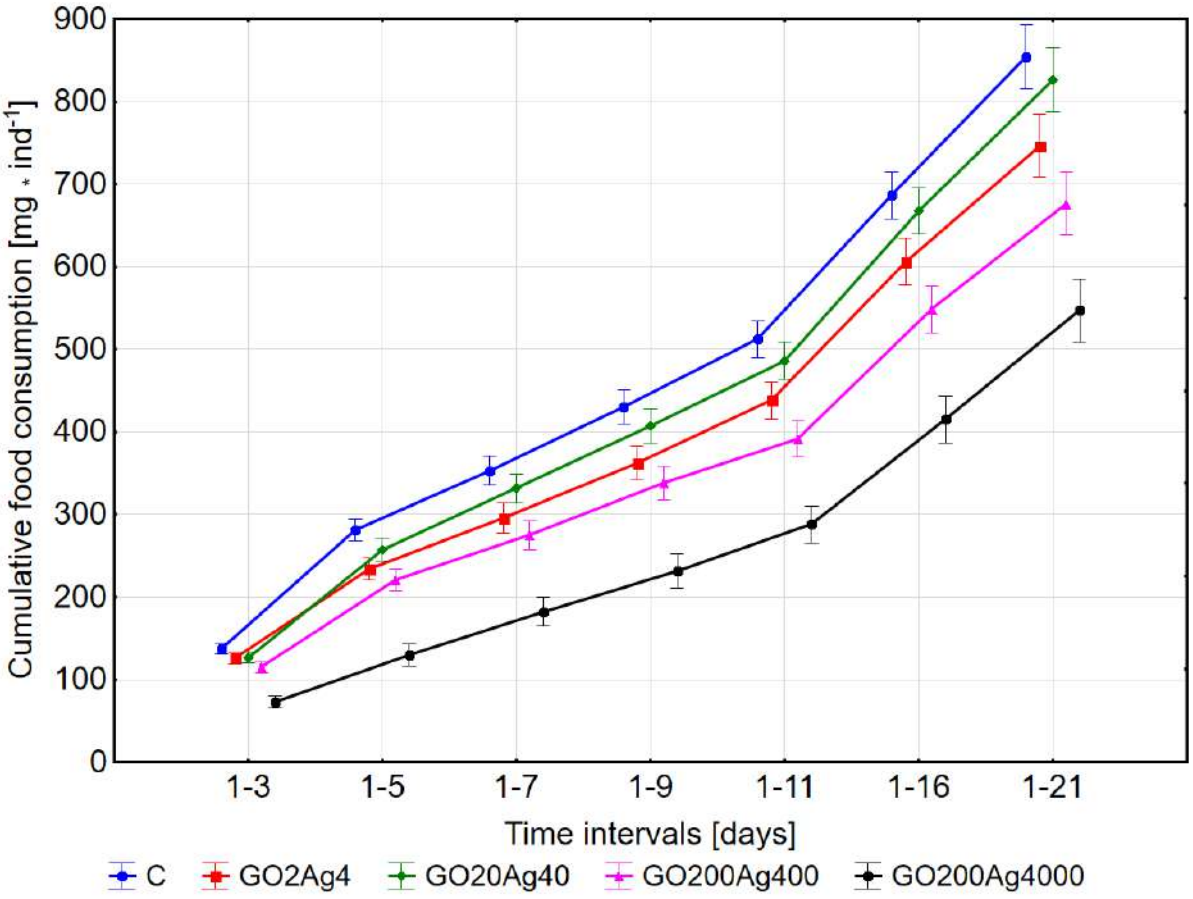

Figure S3. Cumulative food consumption (CFC, mg dry weight per individual per day; mean + SE) in consecutive time-intervals (days) by adult *A. domesticus* treated with different concentration of nanoparticles. See M&M for detailed groups description.

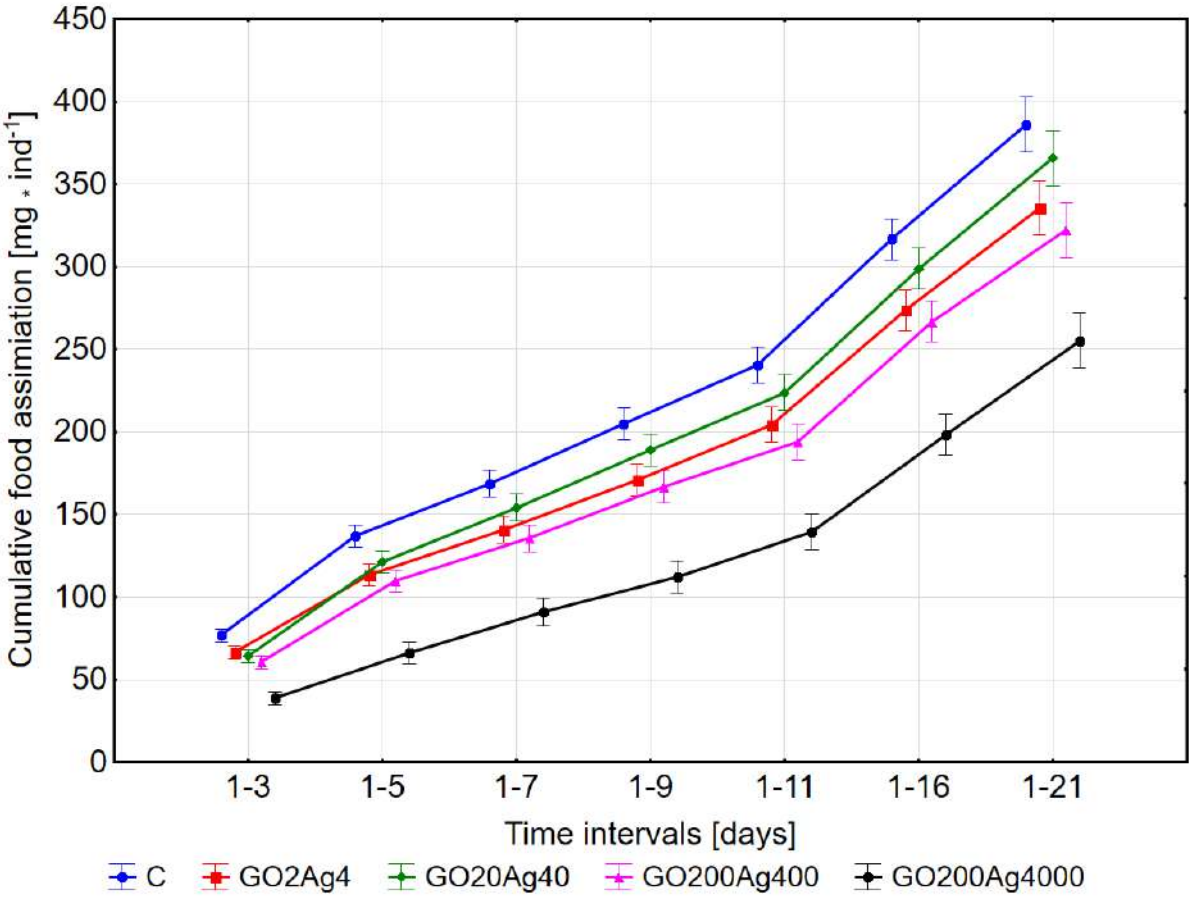

Figure S4. Cumulative food assimilation (CFA, mg dry weight per individual per day; mean + SE) in consecutive time-intervals (days) by adult *A. domesticus* treated with different concentration of nanoparticles. See M&M for detailed groups description.

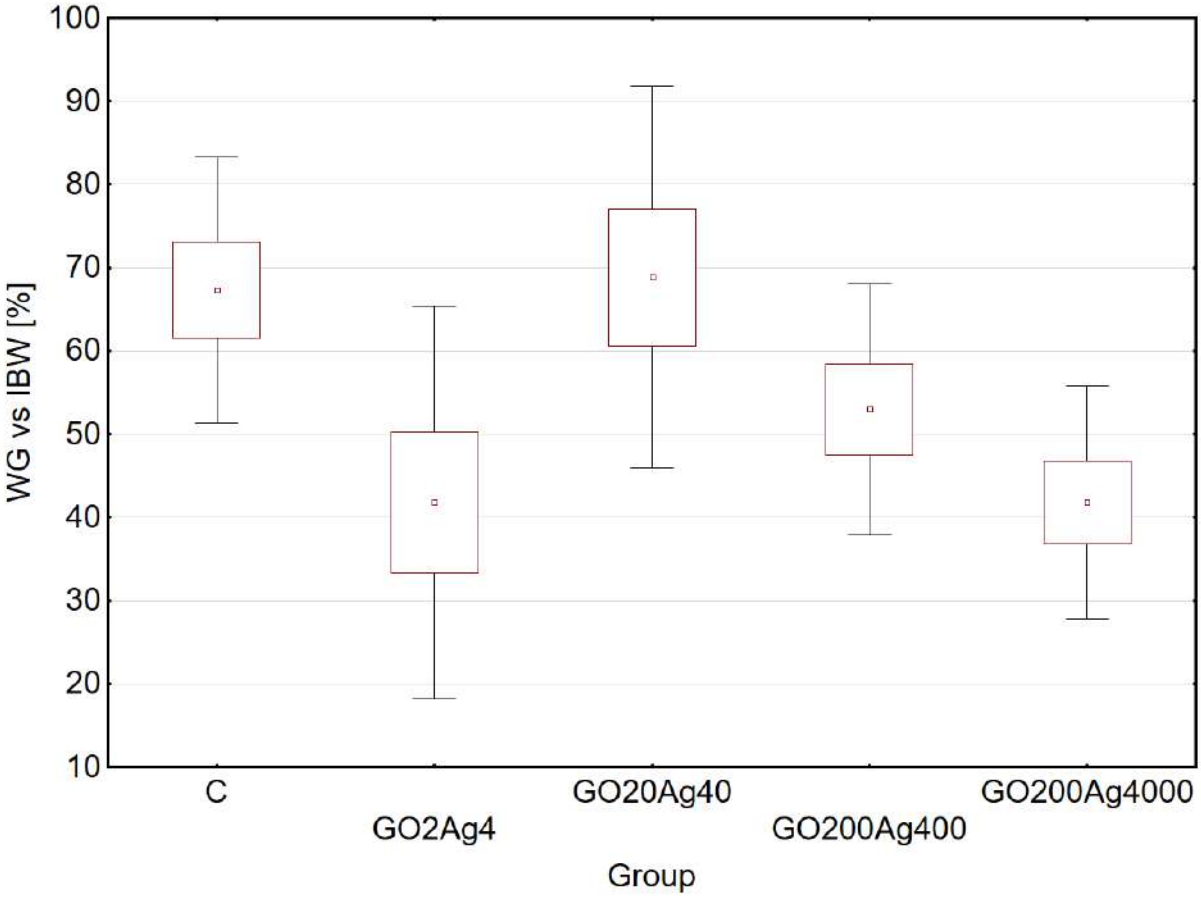

Figure S5. Percentage of fresh weight gain (WG) relative to initial weight (IBW) of adult crickets during the experiment in the control and groups treated with different concentration of GO-AgNPs.

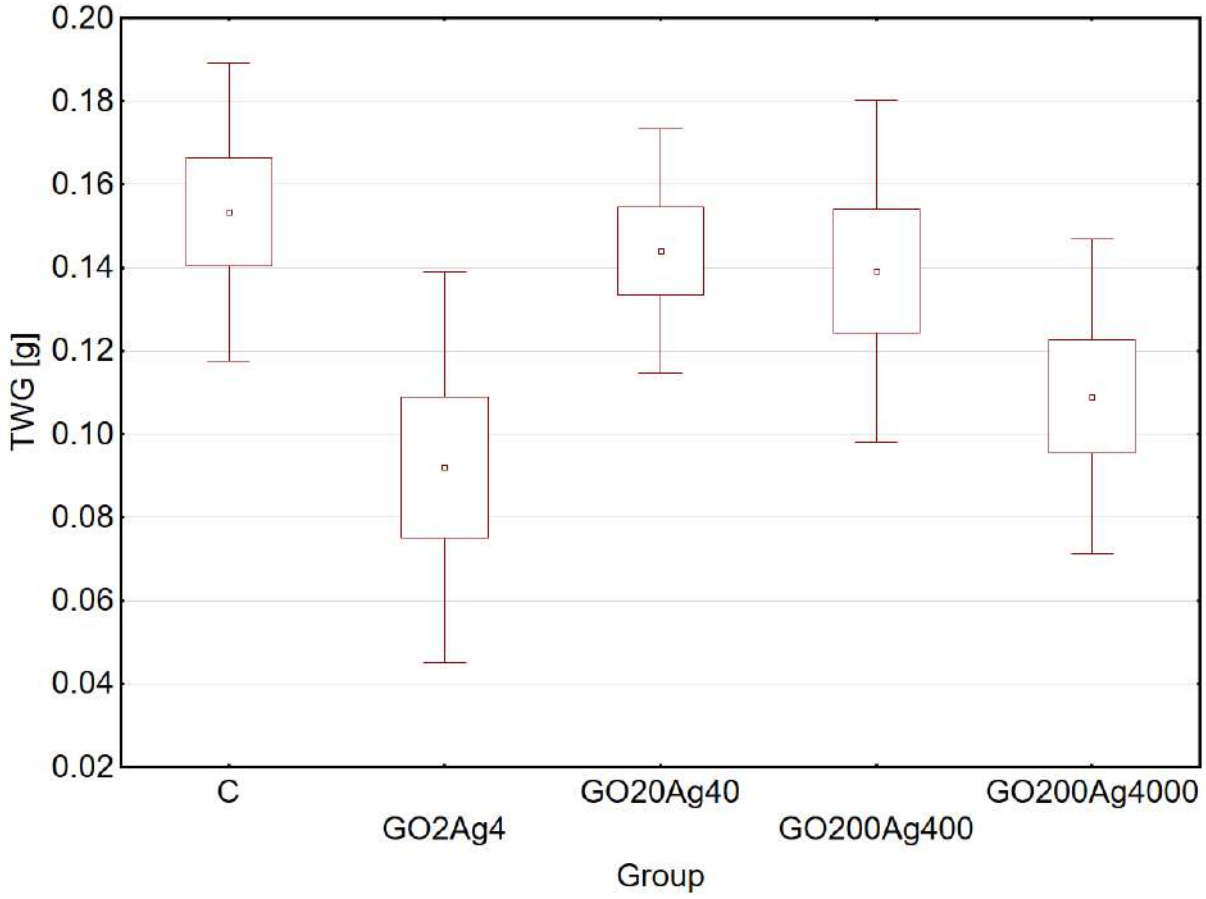

Figure S6. Total fresh weight gain [g] in adult crickets from the control and experimental groups groups treated with different concentration of GO-AgNPs.

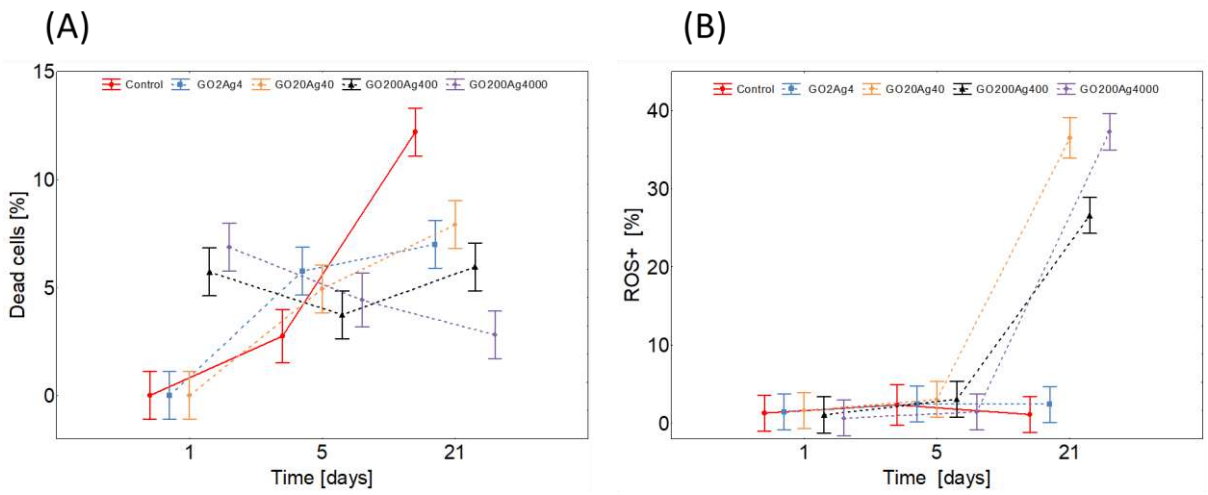

Figure S7. Dead cells (A) and ROS+ cells (B) (%; mean ± SE) in the gut of adult *A. domesticus* treated with different concentrations of GO-AgNPs. See M&M for detailed groups description.

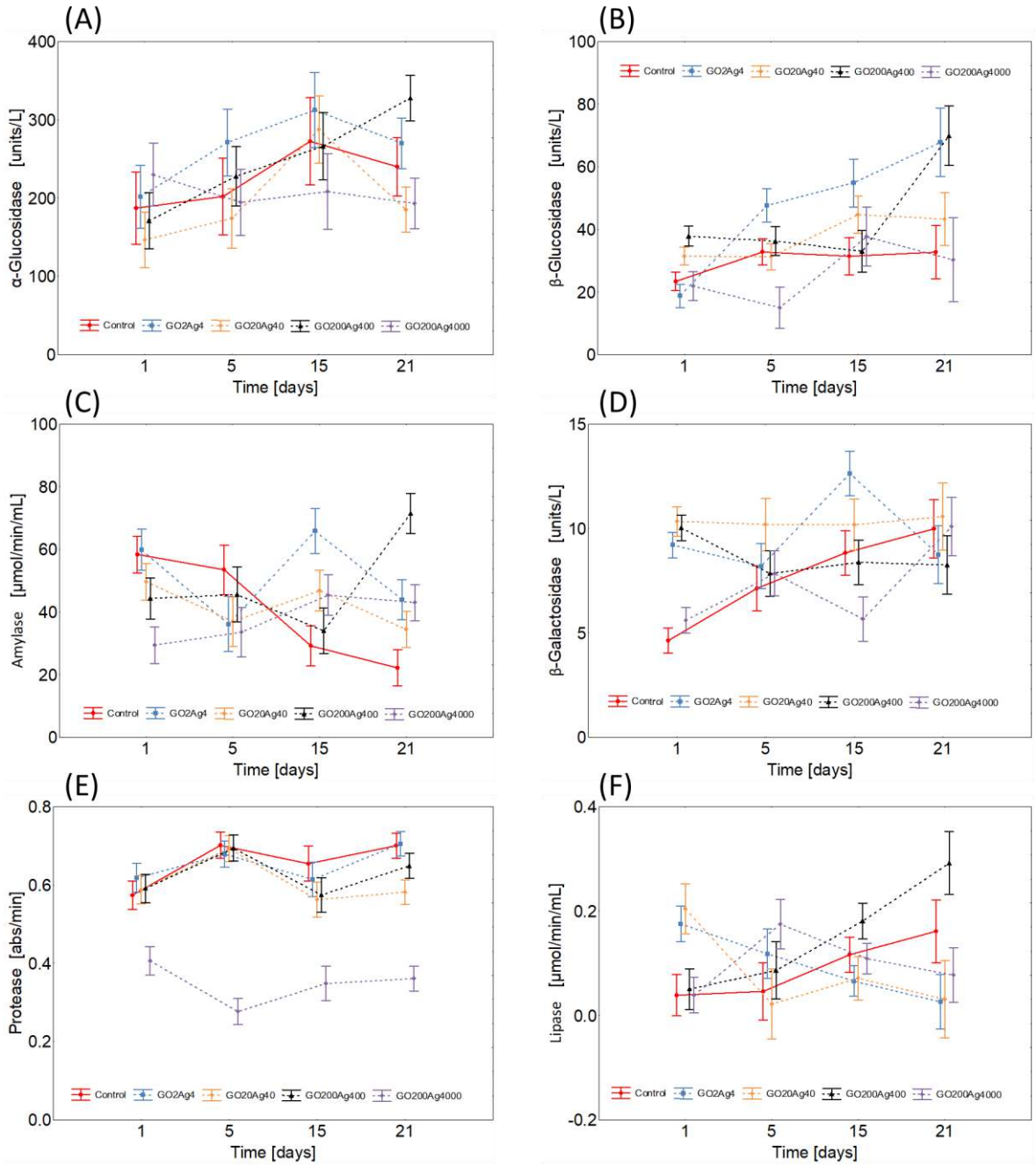

Figure S8. Digestive enzyme activity: (A) α-Glucosidase; (B) β-Glucosidase, (C) Amylase, (D) β-Galactosidase, (E) Protease, and (F) Lipase (mean ± SE) in the gut of adult *A. domesticus* treated with different concentrations of GO-AgNPs. See M&M for detailed groups description.

C.6. DECLARATION OF THE CO-AUTHOR OF THE MANUSCRIPT III

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Prof. dr hab. Maria Augustyniak