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Effects of alloferon and its analogues on reproduction and development of the *Tenebrio molitor* beetle

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As the most numerous group of animals on Earth, insects are found in almost every ecosystem. Their useful role in the environment is priceless; however, for humans, their presence may be considered negative or even harmful. For years, people have been trying to control the number of pests by using synthetic insecticides, which eventually causes an increased level of resistance to applied compounds. The effects of synthetic insecticides have encouraged researchers to search for alternatives and thus develop safe compounds with high specificity. Using knowledge about the physiology of insects and the functionality of compounds of insect origin, a new class of bioinsecticides called peptidomimetics, which are appropriately modified insect analogues, was created. One promising compound that might be successfully modified is the thirteen amino acid peptide alloferon (HGVSGHGQHGVHG), which is obtained from the hemolymph of the blue blowfly *Calliphora vicinia*. Our research aimed to understand the physiological properties of alloferon and the activity of its peptidomimetics, which will provide the possibility of using alloferon or its analogues in the pharmaceutical industry, as a drug or adjuvant, or in agriculture as a bioinsecticide. We used alloferon and its three peptidomimetics, which are conjugates of the native peptide with three unsaturated fatty acids with various chain lengths: caprylic, myristic, and palmitic. We tested their effects on the morphology and activity of the reproductive system and the embryogenesis of the *Tenebrio molitor* beetle. We found that the tested compounds influenced the growth and maturation of ovaries and the expression level of the vitellogenin gene. The tested compounds also influenced the process of egg laying, embryogenesis, and offspring hatching, showing that alloferon might be a good peptide for the synthesis of effective bioinsecticides or biopharmaceuticals.

Keywords Alloferon, Peptidomimetic, Gonadotropic activity, Oogenesis, Oviposition, Embryonic development, *Tenebrio molitor*, Beetle

The most numerous group of animals in the world, whose representatives can be found in almost every ecosystem on Earth, providing its broadly understood biodiversity, are insects[1]. Many species, such as bees and silkworms, play useful roles in human life, *e.g.*, from an economic point of view[2,3]. Nevertheless, insects are also harmful to humans because they are vectors of pathogens of serious diseases, damage forestry or cause great losses in agriculture[4]. In addition, some insects, such as mosquitoes and blackflies, are burdensome for human function[5].

To control the number of insects harmful to the human economy, spraying or fumigation with synthetic insecticides is used[6]. They impair functions of nicotinic cholinergic receptors (nAChRs), gamma-aminobutyric acid receptors (GABARs), and glutamate receptors (NMDARs), which act on the nervous system or affect the activity of acetylcholinesterase (AChE), are the most common[7]. However, one of the drawbacks of using synthetic insecticides is increasing level of resistance in pest populations[8]. Moreover, these compounds are often harmful to the environment, *e.g.*, due to their low selectivity and biodegradability, ability to penetrate through different ecosystems, and ability to accumulate in tissues of other organisms, which often negatively affects them[9]. These

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negative effects of synthetic insecticides prompted researchers to search for alternative methods for controlling insect pests and thus to develop new, safe compounds that can affect specific insect species, physiological processes, or cellular and molecular structures other than those of the nervous system[10,11]. Using knowledge about the functionality of compounds of insect origin, a new class of bioinsecticides called peptidomimetics, *i.e.*, appropriately modified insect analogs, was created. The process of creating peptidomimetics begins with the initial selection of the compound. Then, several chemical modifications, which aim to increase the chemical stability of newly designed compounds, *e.g.*, by lowering their sensitivity to UV radiation, high temperature, or strong redox factors, are made, mainly within the core fragment[12–15]. It is also important to increase resistance to biological agents, such as proteases present in the digestive tract of insects, for which new compounds can be used. It also applies the addition of hydrophobic residues, *e.g.*, fatty acids or their derivatives, to facilitate penetration through the insect cuticle[16–19]. The results of studies carried out on mosquitoes, beetles, locusts, and moths thus far are very promising[20]. Nevertheless, despite these studies, the mechanism of action and broader spectrum of biological activity of this large and diverse group are still not fully understood[21].

One promising compound is the thirteen amino acid peptide alloferon (HGVSGHGQHGVHG), which was first isolated from the hemolymph of the blue blowfly *Calliphora vicinia*[22,23]. The name given to the peptide is also not accidental and indicates functional similarity to interferon, which also has an immunostimulating effect[23,24]. Additionally, research conducted by Kuczer et al.[25] showed that it also possesses weak cardiostimulatory properties when applied to the beetle *Zophobas atratus*. However, its exact physiological function has not been described thus far.

Lee et al.[26] showed that alloferon reduces the process of replication of herpesvirus type 8, which causes Kaposi's sarcoma, increasing the effectiveness of NK lymphocytes, which is marked by more effective eradication of virus-infected cells. It also has activity against H1N1 type A influenza virus[27]. Antiviral properties were also described by research on alloferon and its *N*-terminally truncated analogs conducted by Kuczer et al.[28]. Studies have shown that these peptides inhibit the replication of human herpesvirus type 8, Coxsackie virus type B2 in Vero cells, a laryngeal epidermoid carcinoma cell line (HEp-2), and an epithelial cell line (LLC-MK2) obtained from monkey kidneys. The anticancer activity of the peptide was also tested in a mouse model designed to determine the activity of cytostatic drugs; mice of the DBA/2 strain were inoculated with P388 leukemia cells. It has been shown that 20 days after the administration of an alloferon, a longer latency was observed[23]. A few years later, Chernysh and Kozuharova[29] showed that emerging tumors were significantly smaller than tumors in a mouse control group.

With respect to potential, less toxic, and more specific insecticides, attempts have been made to use, with greater or lesser success, bacteria and their toxins, viruses, entomopathogenic fungi, secondary plant metabolites, and transgenic plants producing "repellents". Based on the knowledge about peptidomimetics and the described cytotoxic action of alloferon against intensively and rapidly dividing cancer cells, we started research on the possibility of using it as a potential bioinsecticide directed at the reproductive system, which, similar to cancer cells, is a place of intensive and rapid cell division. Considering the above, the following research goals were set in this work: to determine the effect of alloferon and its three peptidomimetics, which are conjugates of the native peptide with three unsaturated fatty acids of various chain lengths—caprylic (C8-alloferon), myristic (C14-alloferon) and palmitic (C16-alloferon)—on the morphology and function of reproductive system insects and to understand the foundation for the process of embryonic development of beetles. As a research model, we chose the beetle *Tenebrio molitor* L. as a cosmopolitan, synanthropic pest of stored grain products occurring in grain warehouses, mills, barns, etc., against which an approach using bioinsecticides could be applied.

Materials and methods

Insects

Tenebrio molitor beetle stock cultures were maintained at the Department of Animal Physiology and Developmental Biology at Adam Mickiewicz University in Poznań, as described by Rosiński et al.[30] and Walkowiak-Nowicka et al.[31]. Briefly, insects were bred in transparent, clear plastic containers with a diameter of 10 cm, and volume of 549.5 cm^3. The containers were kept in breeding chambers with a constant temperature of + 28 °C, a relative humidity of 65%, 8 h of access to light, and, importantly, constant access to food, which included flour (10 g per container) and carrots, which are also a source of water (5 slices per container).

Insects from the test and control groups were provided with identical living conditions. The sex of the insects was determined at the pupal stage, and the males were marked[32]. Both sexes were kept in one container at a 1:1 ratio (10 insects per container). In experiments, only virgin females in the first gonadotropic cycle were used, which is dictated by the biology of this beetle and the resulting asynchrony of its ovarian cycles, which makes it impossible to properly assess the ongoing processes in subsequent ovarian cycles. Insect pupae for breeding appropriate for the experiment were selected randomly. Research ethics committee approval was not required to achieve the objectives of this study.

Tested compounds

Alloferon and its analogues were synthesised and purified in the Peptide Engineering Research Group at the Faculty of Chemistry, University of Wrocław. Solutions of alloferon and its analogues with caprylic, myristic, and palmitic acids (C8-, C14-, and C16-alloferon respectively) were always prepared under sterile conditions by dissolving the peptides in physiological saline solution (PS) for *T. molitor* (containing NaCl at a concentration of 274 mM, KCl—19 mM, $CaCl_2$—9 mM, pH 7.0) as described by Walkowiak-Nowicka[31]. The prepared stock solutions were stored at − 80 °C. Only in the case of experiments with local application of compounds to eggs (on the surface) were the peptides dissolved in ultrapure water before use to avoid salt crystallization on the egg surface, which could disturb the results.

Peptide applications and timeline of experiments

The peptide solution was made according to the procedure described by Walkowiak-Nowicka et al.[31]. Before injection, insects were anaesthetized with CO_2, disinfected, washed with double-distilled water, and dried. Through the ventral membrane, a Hamilton syringe (Model 87908, Hamilton Company) was used between the thorax and abdomen for injection. The insects in the control group were injected with 2 µL of physiological saline (PS), and those in the tested group were injected with 2 µL of peptide solutions at three concentrations of peptides, 10^{-11}, 10^{-7}, and 10^{-3} M[11].

In the experiments with topical application, we used 10^{-12}, 10^{-8}, and 10^{-4} M compounds to compare the results with those obtained after injection. The development of ovaries, as they work asynchronously, was assessed after injecting females, which was conducted on the day following the ecdysis of females. The day of ovary isolation to assess the degree of development and the degree of development of the follicular epithelium were selected based on Ullmann's data[32] on the development of the ovary of the beetle *T. molitor* and observations of our own culture.

In experiments involving the topical application of compounds (20 µL), we used only fertilized eggs (10 eggs per repetition in each variant; repeated 10 times) before reducing the number of false-negative effects. Because *T. molitor* beetles start to copulate on the 2nd day after ecdysis and considering that the time needed for the development of eggs is approximately 6 days, we collected eggs on the 8th day after the imago transition (Supplement Figure S1).

Peptide synthesis

Individual stages of synthesis and purification of alloferon, as well as its caprylic (C8-), myristic (C14-), and palmitic (C16-) conjugates, were performed manually using the solid phase method involving a standard 9-fluorenylmethoxycarbonyl (Fmoc) procedure[33]. Briefly, Fmoc-Gly-Wan resin was used to assemble amino acids. 2(1Hbenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), in the presence of 1-hydroxybenzotriazole (HOBt), was used as a coupling reagent. The Fmoc protecting groups were removed using 20% piperidine in *N*,*N*-dimethylformamide (DMF). The Kaiser test was used to assess the completeness of each coupling reaction. Peptides separated from the substrate were obtained after 2 h of treatment with a trifluoroacetic mixture (trifluoroacetic acid/water/triisopropylsilane 95:2.5:2.5) at room temperature. The obtained peptides were then dissolved in water, lyophilized, and purified using reversed-phase high-performance liquid chromatography (RP-HPLC, Varian ProStar chromatograph, TOSOH Bioscience C18 column [21.5 mm × 300 mm, Tosoh, Tokyo, Japan], UV detector – λ = 210/254 nm). For cleaning, a mixture of water and acetonitrile with 0.1% trifluoroacetic acid (TFA) was used in a gradient at a flow rate of 7 ml/min. After purification, lyophilized peptides with a purity > 95% were obtained. Purity was assessed using HPLC (Thermo Separation Product, column: Vydac Protein RP C18 [4.6 mm × 250 mm, [Grace, Deerfield, IL, USA]; conditions: linear gradient from 0 to 100% B in 60 min, a flow rate of 1 mL/min^{-1}, solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in 80% acetonitrile/water, and UV detection at λ = 210 nm). The chemical composition of the peptides was confirmed by electrospray ionization–mass spectrometry (ESI–MS) using a MicrOTOF-Q mass spectrometer or Apex-Qe Ultra 7T Fourier transform–ion cyclotron resonance (FT-ICR) (Bruker Dalton, Bremen, Germany).

Isolation of ovaries with oviducts

Ten female insects from each tested group were anesthetized. The ovaries with oviducts were isolated under a stereomicroscope using microsurgical instruments. Dissected reproductive organs were cleaned from fat body tissue residues, trachea, and Malpighian tubules were removed, and finally, the cleaned organs were placed in physiological saline or 4% paraformaldehyde (PFA) as described in Walkowiak-Nowicka et al.[11].

Gonadotropic effects of alloferon and its analogs on the morphology of reproductive organs

The general morphology, maturation, growth, and follicular epithelium development of the organs were assessed using an Olympus SZX12 stereomicroscope on the 3rd day after injection (Supplement Figure S1). The assessment of volume was preceded by the formula in which it was assumed that the terminal oocyte takes the shape of a rotational ellipsoid; therefore, to calculate its volume, the formula for the volume of this solid was used. Observation of the intercellular spaces between follicular cells was possible following the procedure described by Czarniewska et al.[34], with the use of the Stereo Lumar. V12 stereomicroscope (Zeiss). In the test, 10 insect females per tested variant were used.

Vitellogenin expression level

The expression level of the vitellogenin gene (*Vg*) was assessed in samples of fat body tissue obtained from females previously injected with alloferon and its analogs on the 1st day after ecdysis. The concentration of 10^{-7} M was chosen because it had the strongest effect on the development of terminal oocytes. PS was used as a control. Tissue was isolated 24 h after injection. Fat body tissue from 10 females was pooled to obtain one biological sample. The tissues were placed in a lysis buffer (Zymo Research) and homogenized with an automatic pestle at room temperature. RNA isolation, according to the manufacturer's protocol, was subsequently conducted using a ready-made Quick-RNA™ MiniPrep kit (Zymo Research). After the remaining genomic DNA was removed from the RNA samples with DNase I (Thermo Scientific), the purity was checked, and the concentration was measured with a Synergy H1 Hybrid Plate Reader spectrophotometer (BioTek). The samples were frozen and stored at -80 °C before use. A ready-made RevertAid™ Reverse Transcriptase kit from Thermo Scientific was used to obtain cDNA. For PCR, we used primers that were designed based on NCBI public databases (accession number: AY714212.2) with Primer3 software (Geneious 9.1.8 package; Prime Group Academic Subscription No. 1865698) and specific primers described by Lord (2010)[35] (Supplement Table S1). PCR conditions after determination and optimization were described previously by Walkowiak-Nowicka et al.[31] and Walkowiak-Nowicka et al.[11]. Product

purity was checked using electrophoresis (with ethidium bromide) and confirmed by sequencing the obtained PCR product, which was made in the Sequencing and Molecular Biology Laboratory of the Faculty of Biology at the University of Adam Mickiewicz University in Poznań, Poland. The product sequence was confirmed based on sequences deposited in public databases using the Chromas Lite 2.01 and basic alignment search tool (BLAST) programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To conduct the qPCR, the suitability of the primers (Supplement Table S1), the stability of the reference gene (*syntaxin-1*), and the reaction conditions were validated and normalized a priori. The amplification of the investigated vitellogenin and *syntaxin-1* separately via qPCR was performed in a RotorGene 6000 (Corbett Research) thermocycler in the presence of SYBR-Green I fluorescent dye (Power SYBR Green Master Mix; Applied Biosystems). qPCRs of the samples as well as "no template control" and "no RT control" reactions were prepared as described by Walkowiak-Nowicka et al.[31,36]. For quantification of the products, we used the $2^{-\Delta\Delta Ct}$ method. The results are presented in a bar graph in which error bars refer to the original Ct values. Each biological sample (4 independent samples for each variant) consisted of tissues isolated from ≥ 10 females. Three technical repetitions were performed.

Impact of alloferon and its analogs on reproductive parameters

The parameters of reproduction related to offspring occurrence were determined by the method described by Walkowiak-Nowicka et al.[11] (Supplement Figure S1). Briefly, males and females were placed in the same ratio in a plastic box. On the 7th day after ecdysis, alloferon and its analogs were injected into 10 females, which were next placed with non-injected males[31]. The experiments were repeated 10 times. Each day, laid eggs were collected from the breeding medium and counted as described previously by Walkowiak-Nowicka et al.[11].

Eggs obtained on the 8th day (one day after female injection) were collected and used to assess the duration of embryonic development. To eliminate false-negative results, in the experiments, only well-developed and undamaged eggs were used. After visual assessment under a stereomicroscope, the eggs were collected, mixed with flour, and kept in an incubator (Memmert INE 400) under the conditions described by Walkowiak-Nowicka et al.[31]. The number of occurred offspring was counted daily for the next 10 days.

Impact of direct application of alloferon and its analogs on embryogenesis

Noninjected males and noninjected females were placed in plastic boxes at the same ratio. Eggs laid on the 8th day were collected and cleaned from a flour medium as described in the patent (PL No. 217868B1) and according to Walkowiak-Nowicka et al.[11] and Rosinski et al. (2011), briefly by sieving flour with eggs through a sieve with a hole diameter of 1.15 mm and then washing with a gentle stream of water at room temperature. Next, the cleaned, undamaged eggs were taken to the experiment. Twenty microliters of each tested peptide was topically applied to 10 properly developed eggs transferred to plates using a single-use plastic inoculation loop[11]. Fifteen seconds later, the excess fluid was delicately removed, and the eggs were dried on paper. In the described experiment, instead of saline, a water solution of peptides or water was used as a control. This allowed us to avoid salt crystallization on the chorion or aeropyle of the eggs. Prepared dishes with eggs were placed in an incubator under constant conditions[11]. The number of offspring was counted daily for the next 10 days. The experiments were conducted 10 times.

Analysis of concentration–inhibition of egg-laying and concentration-inhibition of hatching dependence

The inhibition parameters were calculated based on the three concentrations of compounds used. The final number of eggs laid and hatched larvae were calculated, and inhibition lines, correlation coefficients (R^2), curve equations, and IC_{50}, IC_{60}, IC_{70}, and IC_{80} values were determined using a spreadsheet calculator[37].

Statistical analysis

Statistical analysis of the data was performed with GraphPad Prism software (version 8) (Department of Animal Physiology and Developmental Biology AMU licence). Before analysis, outliers were identified, and the Shapiro−Wilk test was used to assess the normality of distributions. The homogeneity of variance was checked with Levene's test. Groups with a normal distribution and homogeneous variance were compared with ordinary one-way analysis of variance (changes in *Vg* transcript level). Nonparametric data were tested with the Kruskal−Wallis (changes in the volume, length, and width of the terminal oocytes and hatchability of larvae in two variants), and Friedman (number of eggs laid by females) tests. Two-way ANOVA was used to compare methods of administration. The data are shown as the mean value of the parameter ± SD.

Results

Gonado- and oocytotropic effects of alloferon and its analogs

Data from measuring the length and width of terminal oocytes were used to calculate the volume of terminal oocytes. As the data indicate (Fig. 1B and C), each increase in the volume of terminal oocytes (Fig. 1A) was an effect of both longitudinal and cross-stage oocyte growth. The greatest increase in the volume of terminal oocytes was observed after injection of alloferon and C14-alloferon at a concentration of 10^{-7} M (in the case of the control, the average oocyte volume was 21.375 μm^3, and after injection, it was greater than 90.566 μm^3) (Kruskal−Wallis $t = 74.77$, $p < 0.0001$). An increase, which was also statistically significant, was observed after injection of C8- and C16-alloferon at a concentration of 10^{-7} M and alloferon at a concentration of 10^{-3} M (in each case over 57.240 μm^3).

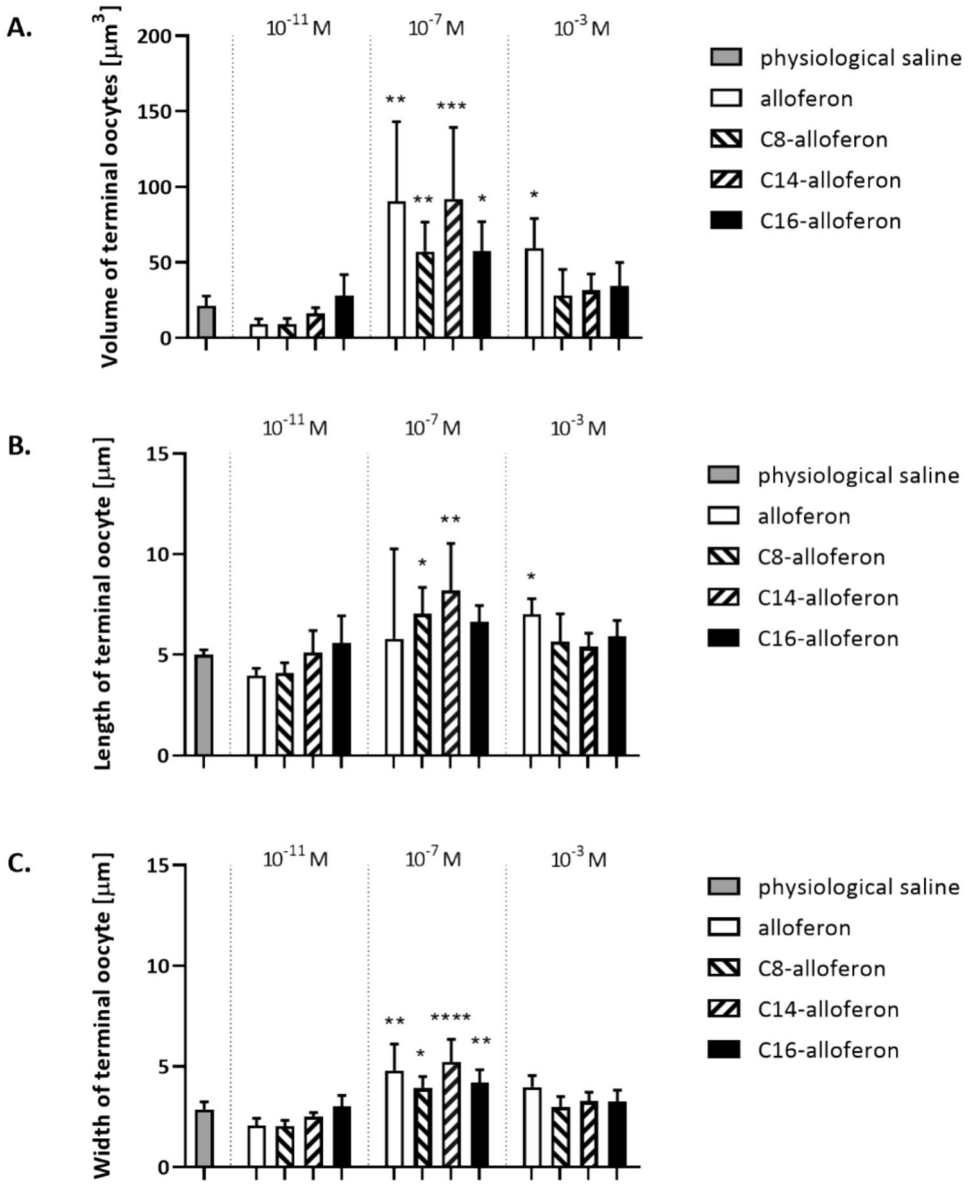

Figure 1. (**A**) Changes in the volume of the terminal oocytes of *T. molitor* females after the injection of saline (control), alloferon, C8-, C14- or C16-alloferon. The data are presented as the means ± SDs. Kruskal–Wallis test revealed significant differences among experimental groups ($t = 74.77$, $p < 0.0001$). Asterisks indicate statistically significant differences from the control values; $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). (**B**) Changes in the length of the terminal oocytes of *T. molitor* females after the injection of saline (control) and tested peptides. The data are presented as the means ± SDs. Kruskal–Wallis test revealed significant differences among experimental groups ($t = 54.52$, $p < 0.0001$). Asterisks indicate statistically significant differences from the control values; $p \leq 0.1$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). (**C**) Changes in the width of the terminal oocytes of *T. molitor* females after injection. The data are presented as the means ± SDs. Kruskal–Wallis test revealed significant differences among experimental groups ($t = 79.70$, $p < 0.0001$). Asterisks indicate statistically significant differences from the control values; $p \leq 0.1$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). In the epithelium, well-developed cells and distinct intercellular spaces between them were observed, and the development of the follicular epithelium of the oocytes was not affected (Supplement Figure S2).

Vg expression level

Injection of alloferon and its analogs caused changes in the *Vg* transcript level (ordinary one-way ANOVA with Dunnett's multiple comparisons test $F = 3.328$, $p = 0.0173$) (Fig. 2). Twenty-four hours after the injection of C8-alloferon ($p = 0.0072$) and C14-alloferon ($p = 0.0287$), the expression level of *Vg* increased statistically significantly in comparison to that in the control.

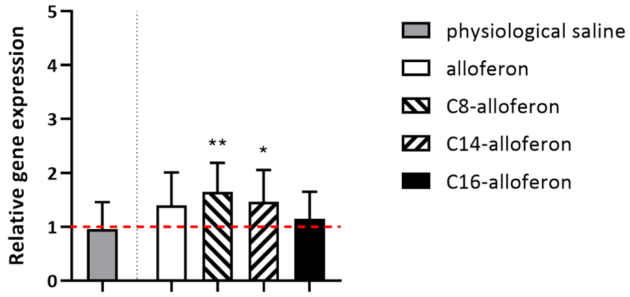

Figure 2. The *Vg* transcript levels in *T. molitor* female fat body tissue 24 h after injection of PS (control), alloferon, C8-, C14- or C16-alloferon at a concentration of 10^{-7} M were calculated via the relative quantification method. The data are presented as the means ± SDs, $n > 190$. Statistical significance was tested by one-way ANOVA with Dunnett's multiple comparisons test ($F = 3.328$, $p = 0.0173$). Asterisks indicate statistically significant differences from the control values; $p \leq 0.05$ (*), $p \leq 0.01$ (**). Red line – expression level in the control.

Parameters of reproduction after injection

Injection of alloferon and its analogs at concentrations of 10^{-11}, 10^{-7}, and 10^{-3} M impacted the number of eggs laid by females (Fig. 3); however, the changes were not statistically significant (Friedmann's test with Dunn's multiple comparisons test). The tested peptides did not change the time at which oviposition started—in each variant, females started laying eggs between the 4th and 5th days after ecdysis (Fig. 3A, B, C). The control females injected with PS for 10 days in the experiment duration laid 114 eggs (calculated per one female). C8-alloferon in concentrations of 10^{-11} and C14-alloferon at concentrations of 10^{-3} M reduced the number of eggs to 49. After injection of C14-alloferon at a concentration of 10^{-7} M the opposite effect was caused, so the increase in the number of eggs laid to 126 in ten days. The correlation between the concentration of the tested substances and the inhibition of egg laying was very high (> 0.9) for alloferon, C8-alloferon, and C14-alloferon but low for C16-alloferon (< 0.5). The concentration-inhibition equations were $y = 0.1455x + 5.743$, $y = -0.0221x + 4.473$, $y = 0.0221x + 5.2873$ and $y = -0.1007x + 3.3321$, respectively. As the equations show, the bias of the line is very low, and the inhibition increases very slowly.

Alloferon and its peptidomimetics caused changes in the number of occurring offspring (Kruskal−Wallis test, $t = 52.10$, $p < 0.0001$). After the injection of peptides at each concentration (except for C16-alloferon at a concentration of 10^{-3} M), a decreasing trend in the number of hatching larvae was observed (Fig. 4). Injection of C8-alloferon at a concentration of 10^{-11} M caused the most significant reduction, and only 20.3% of the larvae hatched after its application, whereas in the control, 99.3% of the larvae hatched ($p = 0.0029$). Hatchability decreased to 55.0% after the injection of C14- and C16-alloferon at a concentration of 10^{-11} M ($p = 0.0368$, $p = 0.0368$) and to 54.1% after the application of C8-alloferon at a concentration of 10^{-7} M ($p = 0.0064$). No changes in embryogenesis duration were observed (Supplement Figure S3). For each variant, embryogenesis lasted a minimum of 6 days. All the compounds inhibited hatchability in a concentration-dependent manner. The correlation was greater than 0.8 in all cases. However, as in the case of egg laying, the cure grows very slowly.

Parameters of reproduction after topical application

The application of alloferon and its peptidomimetics to the egg surface caused changes in the number of hatched larvae (Kruskal–Wallis test, $t = 34.91$, $p < 0.0005$), as indicated by a trend toward a reduced number of hatched larvae (Fig. 5). The application of C16-alloferon at a concentration of 10^{-4} M caused the most significant reduction, from 88.04% in the case of the control to 46.67% ($p = 0.003$). Hatchability decreased to 50.3% after the application of alloferon at the same concentration ($p = 0.0012$) and to 53% after the application of C14- and C16-alloferon at a concentration of 10^{-8} M ($p = 0.0036$, $p = 0.0047$). No changes in the duration of embryogenesis duration were detected for any of the embryogenesis variants, which lasted for a minimum of 6 days (Supplement Figure S4). On the 6th day, 76.7% of the 'control' larvae emerged. In turn, the application of peptides reduced larval emergence from 23.3% to 36.6% range in the case of 10^{-12} M concentration, from 26.7% to 47% range in the case of concentration 10^{-8} M, and from 16.7% to 30.0% range in the case of concentration 10^{-4} M.

Two methods of administration, injection, and topical application, were also compared. The analysis confirmed significant differences between the methods of application of the compounds (two-way ANOVA, $p < 0.0001$, $F = 16.44$).

Discussion

In this work, for the first time, the gonadotropic and oocytotropic properties and effects of alloferon, C8-, C14-, and C16-alloferon on the embryogenesis and larval hatchability of *T. molitor* beetles were examined. The results of the present study indicate the spectrum of physiological effects of alloferon and its analogs, constituting the basis for the knowledge needed for further research. The physiological effects demonstrated on *T. molitor* beetles indicated the potential of using alloferon as an active bioinsecticide. However, these studies were mainly aimed at understanding the physiological activity of the peptide, about which little is known and can potentially be used, for example, in the pharmaceutical industry [24,38]. Nevertheless, an attempt to administer the compounds

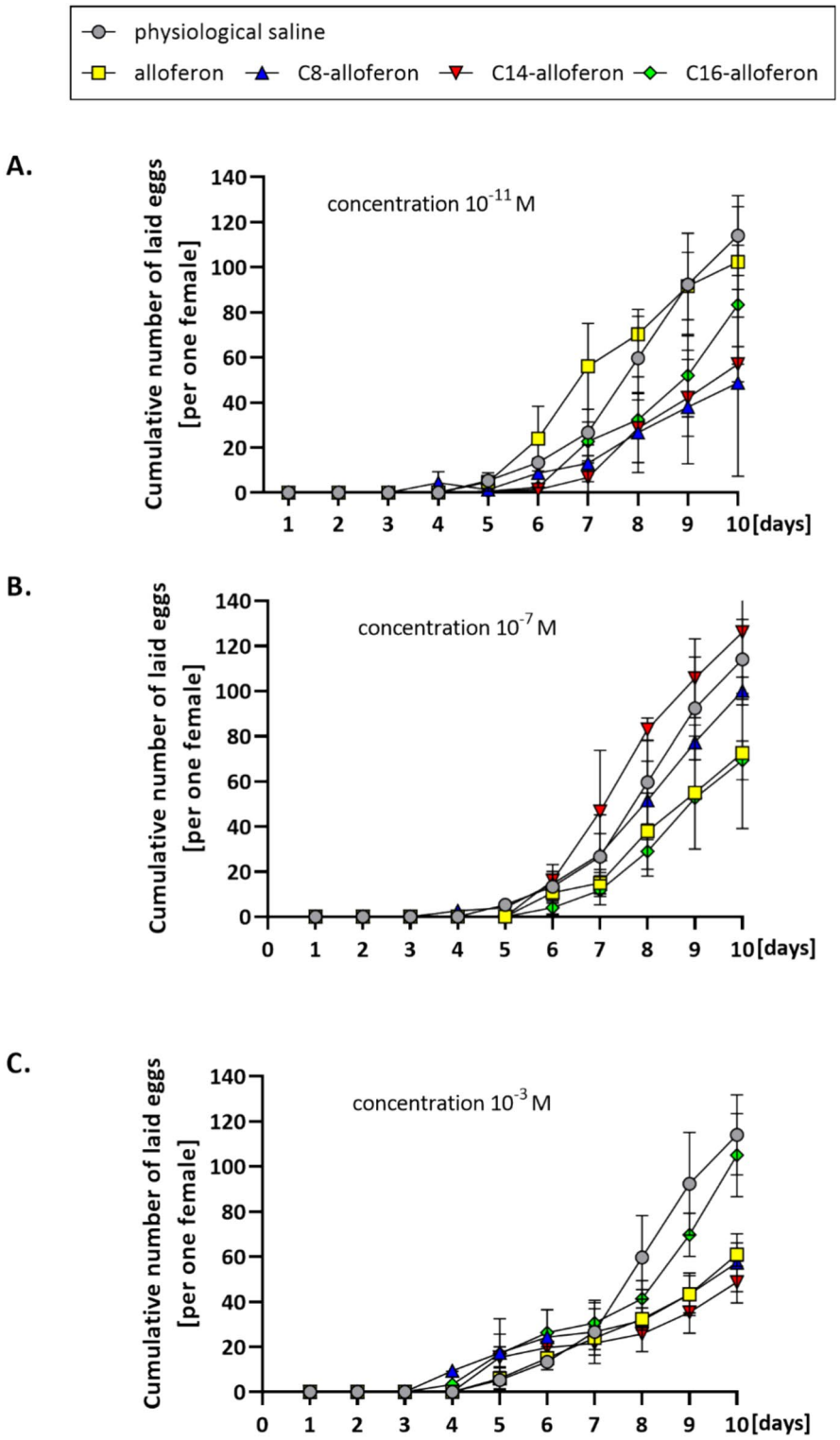

Figure 3. Cumulative number of eggs laid by one female after the application of PS (control), alloferon, and the C8, C14, and C16-alloferon. On the graph mean values ± SDs are presented, $n > 60$. No significant changes were observed (Friedmann's test with Dunn's multiple comparisons test).

topically and thus obtain statistically significant results indicates possible application for pest control, although the research should be extended with additional concentrations and experiments related to, for example, the passage of the compounds through the cuticle—the assessment of the obtained activity will be possible thanks

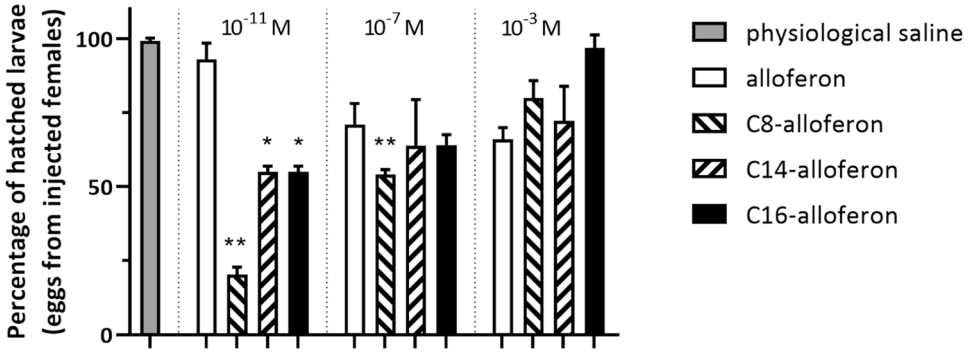

Figure 4. Percent hatchability of larvae from eggs obtained from females injected with PS (control), alloferon, or C8-, C14- or C16-mimetics 10 days after injection. The data are presented as the means ± SDs, $n > 60$. Kruskal–Wallis test revealed significant differences among experimental groups ($t = 52.10$, $p = 0.0001$). Asterisks indicate statistically significant differences from the control values; $p \leq 0.05$ (*), $p \leq 0.01$ (**).

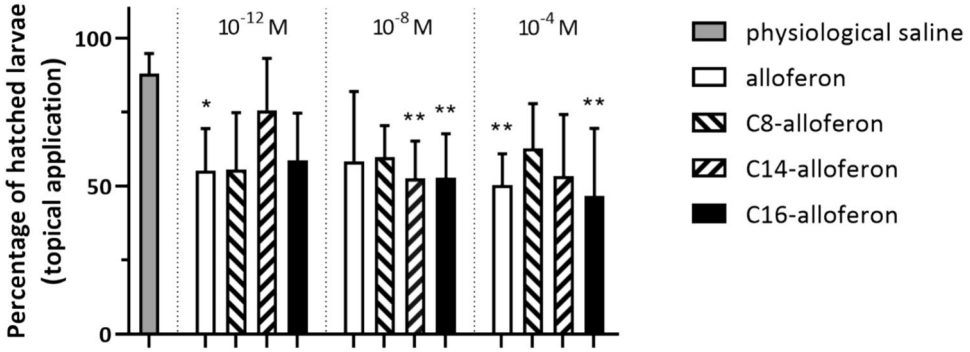

Figure 5. Hatchability of larvae observed after topical application of water solutions of alloferon, C8-, C14-, and C16-alloferon on eggs obtained from noninjected females, expressed as a percentage. Water was used as a control. The graph presents the mean values ± SD, $n > 60$. Kruskal–Wallis test revealed significant differences among experimental groups ($t = 34.91$, $p < 0.0005$). Asterisks indicate statistically significant differences from the control values; $p \leq 0.05$ (*), $p \leq 0.01$ (**).

to our research in which the compounds were directly administered into the insect's body. In our research, we used an approach involving the attachment of a fatty acid residue to the native molecule of alloferon. To date, synthesized peptidomimetics of neuropeptides and hormones, in which similar modifications were used, showed increased hydrophobic properties, as well as increased ability to penetrate across the cuticle, prolonged half-time of the active form or increased resistance to proteolytic factors [39–41]. Our research showed that the attachment of caprylic, myristic, and palmitic fatty acids to the native molecule of alloferon changed the bioinsecticidal potential of the compound. However, the effect was not observed in each experiment and concentration, so further studies are necessary. These compounds influence the functioning of the female reproductive system. Injection of peptides at a concentration of 10^{-7} M resulted in the stimulation of ovarian development, as indicated by an increase in the length of the ovarioles, and the acceleration of terminal oocyte development, as indicated by a significant increase in volume. The peptides show the greatest effect at an intermediate concentration, which may be adequate to fully saturate the receptor. The lack of effect after the injection of peptides at a higher concentration may be the result of compensation for the action of the compounds—lower or even lack of effect may be the result of activation of different signalling pathways or other mechanisms that in some way limit the action of the applied compounds. It might also be the effect of hormesis—i.e. an effect in which a compound is beneficial in low concentrations, but harmful in high concentrations. The amount of compound provided may also be appropriate to maximize the proteolytic effect of the compounds found in the haemolymph. In turn, low concentrations may not be sufficient to produce the maximum effect. It should also be emphasized that time may also be an important factor; changing this parameter could indicate that peptides at lower concentrations are also effective [39]. The observed changes in the ovarian growth rate, morphology, and oocyte maturation may be due to the effect of the injected peptides on the levels of hormones, e.g., ecdysone, which acts through the ecdysteroid receptor (EcR) and has a direct effect on the development of ovarioles, vitellogenesis and oocytes[42]. Studies conducted on ovaries isolated from the fruit fly *Drosophila melanogaster* (Meigen) have shown that changes in the transcript levels of genes responsible for both the regulation of maturation and the degeneration of oocytes may be the result of increased levels of ecdysone, resulting in the initiation of oocyte apoptosis[43].

This could suggest that alloferon, C8-, C14-, and C16-alloferon caused a decrease in the level of ecdysone, thus accelerating oocyte maturation.

Juvenile hormone (JH) is another regulator of ovarian development and function, particularly during the process of vitellogenesis and oocyte maturation in insects[44,45]. The effect of this hormone can be altered by the influence of alloferon and its peptidomimetics on the function of the *corpora allata* glands that produce and release JH[46]. These compounds did not affect the growth or maturation of follicular cells or intercellular space formation, so the increase in the size of mature oocytes may be the result of changes in the metabolic activity of JH[44], as this hormone stimulates the synthesis and release of yolk proteins from cells of the fat body into the haemolymph and increases their uptake by the oocyte. This suggests that the increased dimensions of oocytes may result from the stimulating effect of compounds on the synthesis of vitellogenins and more efficient delivery of this protein during the process of vitellogenesis to oocytes, which in turn is possible due to the accelerated growth of the ovarioles and the correct formation of the follicular epithelium[47].

Alloferon, C8-, C14- and C16-alloferon not only presented gonado- and oocytotropic effects but also had an impact on the process of oviposition; the injection of females with alloferon and its analogs reduced the number of emerged offspring. This suggests that despite their accelerating effect on the development of oocytes, peptides do not accelerate the laying of eggs. Further experiments showed that the effects of alloferon and its analogs on *T. molitor* reproduction are more complex because of the processes of oviposition and embryogenesis as well as larval hatching. After injection of the tested compounds, the females laid eggs from which fewer larvae hatched. Presumably, this may be the result of laying unfertilized eggs, perhaps as an effect of the compounds on the functioning of the complex *corpora cardiaca/corpora allata* and stimulating them to increase the release of JH[48]. Soares et al.[48] confirmed on the stinkbug *Podisus nigrispinus* Dallas that an increased level of JH may result in the laying of unfertilized eggs[48]. Sugime et al.[49] indicated that in *D. melanogaster,* supplying JH with food resulted in the laying of an increased number of 'virgin' eggs. Similar relationships between the titer of the juvenile hormone in the haemolymph and the production of unfertilized eggs by *Aedes aegypti* L. mosquitoes were detected by Van Ekert et al.[50]. Another potential reason may be the disturbing activity of compounds on the spermatheca, which releases sperm, causing asynchrony with the release of oocytes from ovaries[51].

In the analysis of the effects of alloferon and its analogs on reproductive processes in *T. molitor* during ovulation and/or fertilization, it was necessary to investigate whether these compounds exhibited embryotoxic activity. For this purpose, tests were carried out on eggs laid by noninjected females.

The performed experiments proved that the application of alloferon and its peptidomimetics at each of the concentrations resulted in a reduced number of hatching larvae. The results indicated that the tested compounds applied directly on the fertilized egg surface penetrated the chorion layer or passed through to the egg via the micropyle, causing an embryotoxic effect. Here, the most effective analog was analog with palmitic fatty acid, which resulted in a significant reduction in the number of emerged larvae. These observations can be explained by studies that correlate the mechanism of migration of compounds across the cuticle and the degree of toxicity with both the size of the molecule and the hydrophobic properties resulting from the structure[52].

In summary, analysis of the structure-activity relationships of alloferon and its mimetics revealed that they can be considered potential bioinsecticides by combining a long list of peptides and neuropeptides, such as allatostatins, pyrokinins, myosuppressins or trypsin-modulating oostatic factor (TMOF), of insect origin. Each of the compounds described in this paper has potential insecticidal activity and can be used for further research on obtaining active bioinsecticide molecules[53,54]. The obtained results also fill the gap in knowledge about the physiological activity of the compound in insects and indicate further properties that can be used in the pharmaceutical industry, for example, when using the compound or its analogs as adjuvants. However, to obtain a full picture of the function and activity of alloferon, further studies are needed.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

KWN, SCh, and GR contributed to the study conception and design. Material preparation, data collection, and analysis were performed by KWN, SCh, ZA, and JPB—synthesis of compounds by MK. KWN wrote the first draft of the manuscript, and all the authors commented on previous versions of the manuscript. All the authors have read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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