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**Single-stranded DNA fragments of insect-specific nuclear polyhedrosis virus act as selective DNA insecticides for gypsy moth control.**

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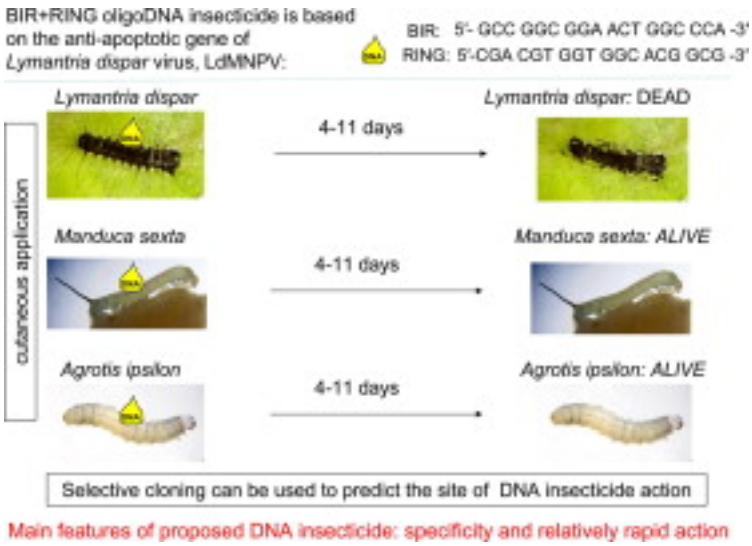
## Abstract

This paper focuses on the DNA insecticides as a novel preparation against gypsy moth (*Lymantria dispar*) based on DNA fragments of the anti-apoptotic gene of its nuclear polyhedrosis virus. It was found that the external application of a solution with two single-stranded DNA fragments from BIR and RING domains of LdMNPV (*L.dispar* multicapsid nuclear polyhedrosis virus) IAP-3 (inhibitor of apoptosis) gene induces a significantly

higher mortality of gypsy moth caterpillars in comparison with the application of the control solutions. This effect does not depend on the infection of caterpillars with LdMNPV. The results also show that DNA insecticides based on LdMNPV IAP-3 gene fragments can be selective in action, and at least are not harmful to tobacco hornworm (*Manduca sexta*) and black cutworm (*Agrotis ipsilon*). Part of the gypsy moth genome cloned with the fragments of BIR and RING domains of LdMNPV IAP-3 gene as primers, has an overlap with the corresponding part of the LdMNPV IAP-3 gene and *L.dispar* IAP-1 mRNA for an inhibitor of apoptosis protein with the high cover by query, allows assuming that we cloned a part of gypsy moth anti-apoptosis gene. This finding gives the grounding that proposed here DNA insecticides might act through the blocking of the mechanisms involved in post transcriptional expression of insect anti-apoptosis genes. The results show the insecticidal potential of the viral genome fragments that can be used to create safe and relatively fast-acting DNA insecticides to control the quantity of gypsy moth populations, important task for forestry and agriculture.

## Graphical abstract




Abbreviations:
LdMNPV
*Lymantria dispar* multicapsid nuclear polyhedrosis virus
LmMNPV
*Lymantria monacha* multicapsid nuclear polyhedrosis virus
IAP
inhibitor of apoptosis gene

BIR
baculovirus IAP repeat
RING
really interesting new gene

## Keywords

Pest control
DNA insecticides
Viral IAP genes
BIR domain
RING domain
Gypsy moth *Lymantria dispar*

## 1. Introduction

Today, during the rapid increase of human population, the regulation of pest insect quantity in agrobiocenoses and artificial plantations is very important. Regulation of pest quantity requires a safe, an effective and a cheap method. The history of insecticides evolution is very far from over, and the question of serious improvement in that field is relevant. One of the important problems is to reduce the insecticide resistance that obliges researchers to constantly search for new types of pesticides. For example, with the introduction of every new insecticide class – cyclodienes, carbamates, formamidines, organophosphates,pyrethroids, even *Bacillus thuringiensis* – cases of resistance surfaced within 2–20 years [1],[2].

There are two main kinds of insecticides, manufactured and applied today: chemical pesticides and bio-insecticides. Beyond resistance to insecticide developed in insects with the time, the main advantage of chemical pesticides such as DDT, lindane, etc. is that they are very fast in action while the main disadvantages are their unspecific action and the lengthy period of half-life that can last up to several decades. Nevertheless, many of them are still being produced and successfully used on vast territories against insects (mosquitoes, tsetse, etc.) unfortunately accompanied by the side effects on ecosystems [3]. Shortly, we may determine the action of chemical pesticides as “they save lives, paying a very high price”. The other modern insecticides, bio-insecticides, are not perfect as well and have different disadvantages according to the group they belong to. For example, viral bio-preparations based on baculoviruses have enormous potential in ecologically based microbiological control of the quantity of pests [4]. The basic problem in the use of baculoviruses is the slow infection process and delay in the death of the host [5], [6], [7]. A large field of research is devoted to improve baculoviruses by increasing the speed of their infection process through genetic modifications [8]. A possible way to improve insecticides is the development of a new preparation that will manifest the sum of

their best characteristics: fastness and cheapness of chemical pesticides and safety from baculoviral preparations.

Biopreparations based on viral DNA fragments, termed DNA insecticides [9], [10], [11], [12], could be a safe, cheap and an effective alternative. The creation of effective biological preparations based on small DNA fragments is promising due to the important role viral DNA plays in host cells, the great variability on the one hand and specificity of sequences on the other hand, and the relatively high chemical stability. They may influence cells through mechanisms characteristic of antisense oligonucleotides [13], [14], mRNA-antisense DNAhybrids [15] and by mechanisms that resemble those both of DNA interference [16] and RNA interference [17], [18], [19]. We cannot also ignore the fact that DNA is able to interact with a large number of proteins and thus, can affect their functional activity [20].

One of the possible and practically convenient application ways of the DNA insecticides penetration into the body of a caterpillar is an external one. It is known that the presence of developed epicuticule limits the permeability of a caterpillar's covers for most insecticides. Nevertheless, chlororganic, organophosphate and other contact insecticides get through the epicuticule easily into the organism of an insect through the most permeable areas of the covers [19]. Experiments on RNA interference have shown that negatively charged double-stranded RNA (dsRNA) fragments are able to penetrate through the cuticle of the round worms [21] and insects [22] which shows that despite the extra barriers, uptake of the dsRNA by whole insect bodies is possible [22], [23]. The induction of RNA interference (RNAi) by topical application of dsRNA could be explained by passage to interior tissues via the tracheal system [19].

Our idea was to investigate the influence of fragments of a single-stranded DNA of*Lymantria dispar* MNPV IAP-3 gene on the viability of gypsy moth, a serious insect pest and a destructive defoliator with a broad host range, and one of the most recognized pests of forests and ornamental trees in the world [24]. Baculoviruses have two classes of anti-apoptosis genes – p35 and IAP genes that can block apoptosis through various mechanisms in a phylogenetically wide range of organisms [25], [26]. Most of the baculoviral IAPs with anti-apoptotic functions belong to the IAP-3 group, with certain exceptions [27]. Of note, some recent studies suggest that Ld-IAP-3 induces apoptosis of insect cells through initiatorcaspase activation [28], although the authors state that "since analysis of pro-apoptotic functions of Ld-IAP-2 and Ld-IAP-3 in LdMNPV infected Ld652Y cells is lacking in the present study, further analyses are required to find a conclusion".

Many investigations suggest an eukaryotic cellular origin for the viral IAPs [29]. Relationships between baculoviruses and their insect hosts are subject to coevolution, this should lead to long-term evolutionary effects such as the specialization of these pathogens

for their hosts [30], and the ability to affect their biochemical reactions through expression of homologous anti-apoptotic genes. All IAP genes isolated from different baculoviruses display two distinct structural features. The first of these is the presence of amino-terminal repeats of amino acid sequence termed a baculovirus IAP repeat (BIR). The second highly conserved feature of baculovirus IAP proteins is a zinc binding domain known as a RING (really interesting new gene) finger [31]. The BIR domain has been shown to be necessary for the interaction of IAP proteins with diverse pro-apoptotic factors, including invertebratedeath inducers and vertebrate and invertebrate members of the caspase family of proteases[27]. RING domains are characterized by the presence of six to seven cysteins and one or two histidines that form cross brace architecture and coordinate two zinc ions. RING domains often function as modules that confer ubiquitin protein ligase (E3) activity and, in conjunction with an ubiquitin activity enzyme (E1) and an ubiquitin conjugating enzyme (E2), catalyze the transfer of ubiquitin to target proteins [31]. For this study we chose two DNA fragments from relatively conserved BIR (sense chain) and highly conserved RING (antisense chain) domains of LdMNPV IAP-3 gene. Thus, if anti-apoptosis genes of a virus are homologous to the host anti-apoptosis genes – as it is known homologous sequences usually have the same, or very similar functions [32], then our hypothesis is the application of such fragments of viral anti-apoptosis genes is supposed to interfere with pro- and antiapoptotic pathways in the gypsy moth cells (for example, via mechanisms characteristic for the action of antisense oligonucleotides). The consequences of such application of viral IAP-3 gene fragments may be the blocking of anti-apoptosis proteins synthesis, the high level of apoptosis of affected cells and as a result, the death of an organism. Thus, viral IAP-3 gene fragments might have insecticidal effect on gypsy moth caterpillars. Besides this, every species has its own unique sequence of anti-apoptosis genes and it seems possible to create the most effective and selective DNA insecticides for certain species that will manifest the highest effect on a target insect and provide harmlessness to other members of an ecosystem.

## 2. Materials and methods

For the experiments three egg masses of gypsy moth (*L.dispar* (Lepidoptera: Erebidae) were identified and collected in the forest (Tours, France), after that they were randomized and reared under standard conditions. In average, 17 1st instar caterpillars were used per each control and experimental group for DNA insecticides treatment. The experiment was performed in 4 replicates. A water solution with single-stranded DNA fragments with a concentration of 100 pmol/μl was applied outwardly as a small drop (0.3 μl/caterpillar) in the middle of a caterpillar's body (approximately 3rd–4th setae of the abdomen) with the pipette and was absorbed by the caterpillar's body in average during 15–20 min at room temperature. Caterpillars from the control groups were treated with distilled water and either with polyA oligonucleotide 5′-AAA AAA AAA AAA AAA AAA-3′ (Eurofins

MWG Operon, Germany) or random oligonucleotide 5′-GA AG GC AC TG-3′ (SibEnzyme, Russia) of concentration 100 pmol/μl (0.3 μl/caterpillar). To test the selectivity of DNA insecticides the larvae of tobacco hornworm (*Manduca sexta* (Lepidoptera: Sphingidae)) and black cutworm(*Agrotis ipsilon*, (Lepidoptera: Noctuidae)) were reared in the laboratory conditions. Second-instar tobacco hornworm and black cutworm caterpillars from different egg masses were randomized and used for the experiments. Each variant of experiment was performed in three replicates with 15 individuals of caterpillars per each variant, and the concentration of DNA fragments 100 pmol/μl (0.3 μl/caterpillar) was used to treat the caterpillars. All insects were grown on standard wheat germ-based forage at temperature 25 °C up to 11–20 days. The 11-day period for rearing of caterpillars after treatment with DNA insecticides was chosen as representative to show the dynamics of their action. Further rearing till 14 days and up to 20 days did not show additional difference in mortality versus control groups. To create DNA insecticides we designed DNA fragments according to the *LdMNPV* genome sequenced by Kuzio group [33] and found in ICTVdb (http://www.ictvonline.org). DNA fragments were synthesized by Eurofins MWG Operon (Germany) with HPSF clearance. The sequences of the applied 2 single chain DNA fragments were the following: (a) 5′-GCC GGC GGA ACT GGC CCA-3′ (134,843–134,860; sense chain; BIR domain) and (b) 5′-CGA CGT GGT GGC ACG GCG-3′ (135,159–135,142; antisense chain; RING domain).

Detection of homogeneity to the parts of BIR and RING domains of the LdMNPV IAP-3 gene fragments of gypsy moth genome was performed with polymerase chain reaction (PCR). DNA was extracted from larvae of gypsy moth. Highly pure water (Qiagen, France) was used and was exposed to a 254 nm ultraviolet lamp along with PCR tubes, $MgCl_2$ and PCR buffer to avoid contamination from the bacteria in the environment. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, France) following manufacturer's protocol. PCR reactions were performed on 2 μl (10 ng/μl) of DNA using 0.75 units of Goldstar polymerase (Eurogentec, France), 0.2 mM dNTP, 1.5 mM $MgCl_2$ and 50 pmol of each primer. DNA was initially denatured for 4 min at 95 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min hybridization at 60 °C and 1 min elongation at 72 °C, then by a final elongation step at 72 °C for 7 min. PCR products from larvae were purified using the Nucleospin Extract II Kit (Macherey–Nagel, France) and PCR sequencing was performed with a use of Big Dye Terminator v3.1 RR-100 Mix (Applied Biosystems, France). PCR reactions were performed with 5 μl of purified DNA and 0.35 μl of primers (10 pmol/μl). DNA was initially denatured for 2 min at 96 °C, followed by 30 cycles of 10 s denaturation at 96°C, 15 s hybridization at 50 °C and 4 min elongation at 60 °C. Clones were sequenced in both directions with acapillary DNA sequencer (ABI PRISM 3100, Applied Biosystems, France). DNA sequenceswere analyzed by software BLAST [34], ClustalW 2.0.3 [35] and LALIGN [36]. Specific PCR condition and 2 primers

for the viral polyhedrin gene were used for detection of possible LdMNPV contamination in gypsy moth caterpillars: 5′-GTAAAACGACGGCCAGT-3′ (forward primer), 5′-AACAGCTATGACCATG-3′ (reverse primer) [33].
We used non-parametric Pearson's chi-squared test ($\chi^2$) with Yates's Correction and Mann–Whitney test to evaluate the significance of insecticidal effect of viral IAP-3 gene fragments (Sofa Statistics 1.3.3 software).

## 3. Results

### 3.1. DNA fragments from BIR and RING domains of LdMNPV IAP-3 gene induces mortality of gypsy moth caterpillars

After the treatment with the fragments of viral IAP-3 BIR and RING domains (BIR + RING treatment) significant increase in mortality was observed already on the 4th day in comparison with both control (water treated) and polyA groups (Fig. 1). The percentage of perished caterpillars continued to rise up to 11th day of the experiment, reaching in average 24.2% of caterpillar individuals perished in the BIR + RING experimental group, 11.1% of individuals perished in the experimental group treated with polyA and 8.2% of individuals perished in the control group treated with water. Statistical analysis with $\chi^2$ test showed that the total mortality rate of caterpillars from three egg masses for 4 replicates in BIR + RING groups was significantly increased in comparison with the control ($\chi^2$ value = 6.68, corresponds to $p < 0.01$), whereas polyA oligonucleotide did not have a significant insecticidal effect on caterpillars in four replicates of the experiment (Table 1). Additionally, no significant detrimental effect on caterpillars was observed after treatment with 5′-GAAGGCACTG-3′ oligonucleotide, strengthening the evidence of specificity of BIR + RING DNA insecticides treatment.

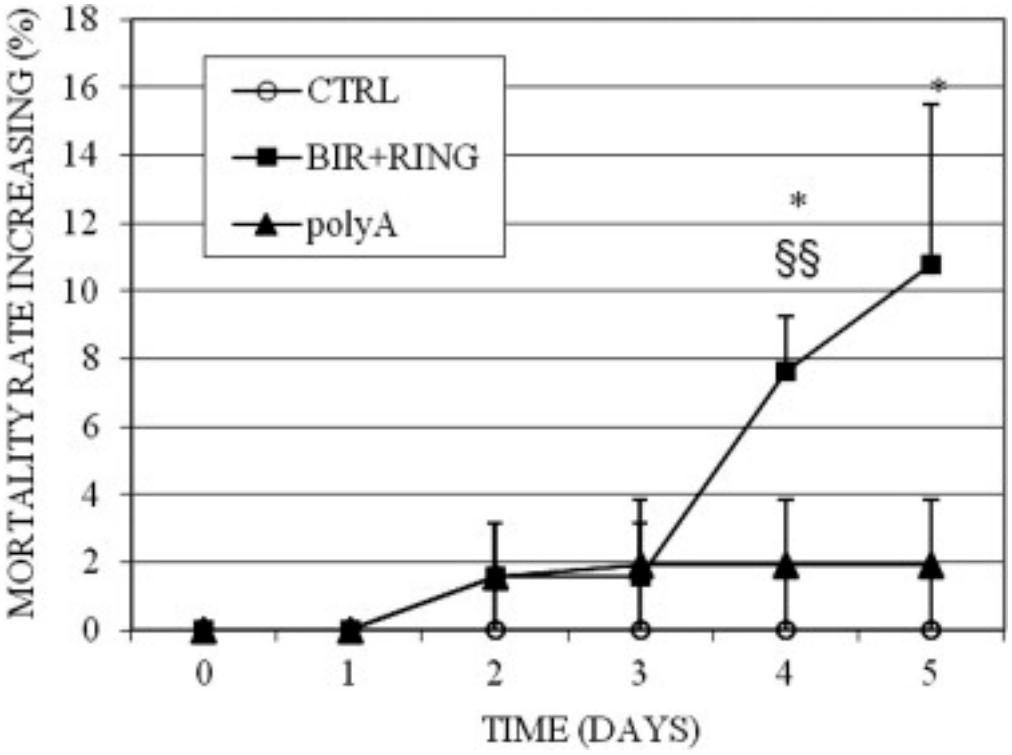



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Fig. 1. Increase in mortality in treated groups (BIR + RING and polyA) versus control group (CTRL). Means and SE are represented for 4 experimental replicates with different egg masses. Significance of difference: between BIR + RING and CTRL is indicated by * for $p < 0.05$; between BIR + RING and polyA indicated by §§ for $p = 0.06$.

Table 1. Statistical analysis of insecticidal effect of BIR + RING and polyA oligonucleotides on gypsy moth.

| Pairs | $\chi^2$ values | Number of caterpillars in a pair |
|---|---|---|
| BIR + RING/control | 6.68* | 139 |
| PolyA/control | 0.33 | 136 |

The $\chi^2$ values for experimental groups versus control group on the 11th day after the treatment. Four replicates of the experiment for 3 different egg masses are represented. Significance of difference versus control is indicated by * for $p < 0.05$.

The effect described above is dose-dependent. Ten times lower concentrations of BIR and RING fragments (10 pmol/μl) showed reduced but still significant effect for RING fragment and reduced but non-significant effect for BIR fragment. On the contrary, double concentration of DNA insecticides slightly potentiates insecticidal effect (data not shown). Thus, in our experiments concentration of 100 pmol/μl showed the best concentration-effectcorrelation.

Generally, caterpillars from BIR + RING treated group, that were going to perish, stopped to eat; they moved slower and looked more dehydrated and smaller than caterpillars from the control group what corresponds to apoptotic patterns on the cell level [37] and on the

organism level [38]. These signs possibly indicate apoptosis processes in the caterpillars that lead to further death of the insect.
Interestingly, preliminary experiments of oral administration of DNA insecticides (with oakleaves, in the same concentration as for cutaneous application) led to a significantly higher mortality of caterpillars in comparison with control but the effect was approximately 3 times lower then after cutaneous application. This indicates the advantage of cutaneous application of DNA insecticides but requires further investigation for final conclusions.

### 3.2. Sequencing of DNA fragment of gypsy moth with BIR and RING fragments as primers

In trying to understand the mechanism of action of DNA insecticides we decided to investigate if gypsy moth genome contains anti-apoptosis genes similar to viral anti-apoptosis genes. This finding would suggest that the DNA insecticides could act through the interfering with the mechanisms involved in post transcriptional expression of insect anti-apoptosis genes. Polymerase chain reaction with single-stranded fragments of BIR and RING domains of LdMNPV IAP-3 gene as primers revealed different genome parts of the gypsy moth (Fig. 2, line 1 and 2) with one prevalent band (around 200 base pairs). The idea of use of single-stranded fragments from BIR and RING domains of IAP genes as a tool for searching of anti-apoptosis genes in insects is novel and can be helpful for investigation of host anti-apoptotic genes. The most prominent fragment of insect DNA spectra (Fig. 2) was chosen for the DNA purification and further DNA sequencing.

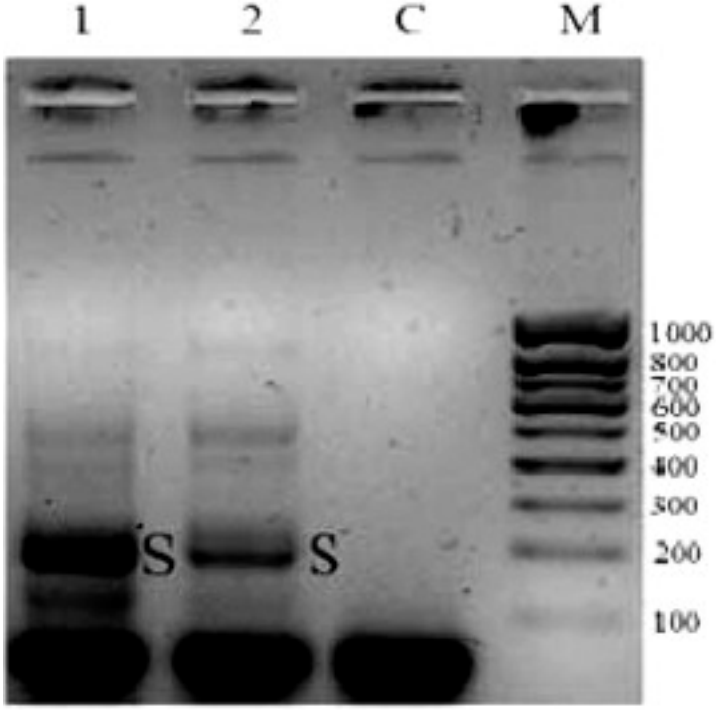


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Fig. 2. Electrophoregram of DNA amplification products from gypsy moth performed with BIR and RING IAP-3 gene DNA fragments as primers. Lines 1, 2 – representative

electrophoretic DNA spectra of two different gypsy moth individuals; C –control; M – DNA ladder from 100 to 1000 (bp with a step of 100 bp (except 900 bp); S – fragments selected for DNA sequencing.

### 3.3. Overlapping with known anti-apoptosis gene

Alignment of the fragment of LdMNPV IAP-3 gene and the sequenced genome fragment of gypsy moth performed by ClustalW 2.0.3 equipment revealed a high level of overlap between the sequences (Fig. 3). Two sequences performed 59% of identity by simple comparison and are marked with asterisk. The sequenced DNA fragment of gypsy moth genome did not match any highly similar sequences searched with BLASTN [34] in data base; because the full genome of gypsy moth has not been sequenced yet.

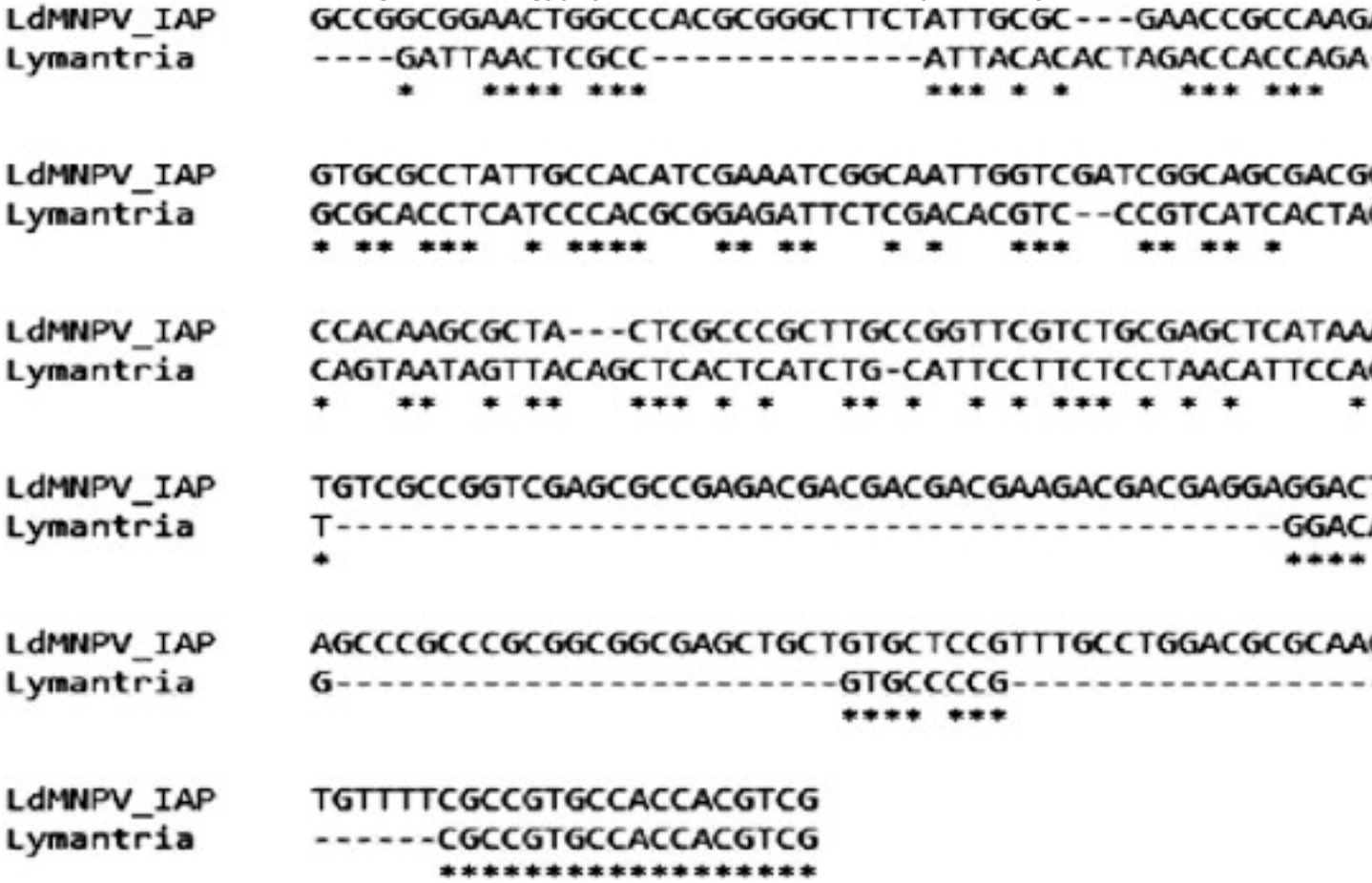


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Fig. 3. Alignment of the sequenced DNA fragments of gypsy moth (*Lymantria dispar*) and part of LdMNPV IAP-3 gene. Dashes are missing nucleotides in database, asterisks are overlapping nucleotides. Last 18 nucleotides are the part of RING sequence.

A search with the TBLASTX [34] in the nucleotide collection of genome fragments of gypsy moth revealed *L.dispar* IAP-1 mRNA (described by Sugiyama Y. group in 2012 and available in http://www.ncbi.nlm.nih.gov/nuccore/409924403) for an inhibitor of apoptosis protein that has 20% cover by query with the sequenced DNA fragment. This

sequence according to the significance of alignment became second behind only *L.dispar* actin mRNA that has 51% cover by query with the sequenced DNA fragment. Since *L.dispar* IAP-1 mRNA for inhibitor of apoptosis protein does not contain introns that the sequenced fragment of gypsy moth genome might contain, two sequences did not perform highly similar alignment. Thus, obtained results of sequence and overlapping allow to assume that we have found a fragment of an anti-apoptosis gene of gypsy moth. The results of DNA sequencing show that the reverse primer (fragment of a RING domain of LdMNPV IAP-3 gene) performed perfect complementary base pairing on the fragment of gypsy moth genome sequence (lower part of the Fig. 3). LALIGN program [36], that finds the best local alignments between two sequences, revealed 100.0% identity in 18 bp overlap (185–202:300–317) between two sequences. On the contrary, BIR domain fragment of LdMNPV IAP3 gene used as a forward primer did not perform a perfect complementary base pairing on the genome sequences of the investigated insects (Fig. 4).

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Lymantria       AATTTTTAAATTCCGCCCGCAC
LdMNPV_IAP      --TGGGCCAGTTCCGCCGGC--
                       ******* **
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Fig. 4. Alignment of the part of the sequenced DNA fragment of gypsy moth (*Lymantria dispar*) andcomplementary DNA strand for the forward primer (corresponded to BIR domain fragment of LdMNPV IAP gene). Dashes are missing nucleotides in database, asterisks are overlapping nucleotides.

### 3.4. LdMNPV contamination exclusion

To understand whether the sensitivity of gypsy moth caterpillars to DNA insecticides is associated with the virus infection, eggs from the same egg masses used for BIR + RING treatment were checked for *LdMNPV* contamination using the primers detecting the part of the viral polyhedrin gene (Fig. 5; line V, 1–4) since transovarial transmission of the virus occurs in gypsy moth populations [39]. Investigated egg masses, the source of all experimental caterpillars, were free of virus (Fig. 5, a part of investigated samples are present) as shown the absence of DNA amplicons in lines 1–4.

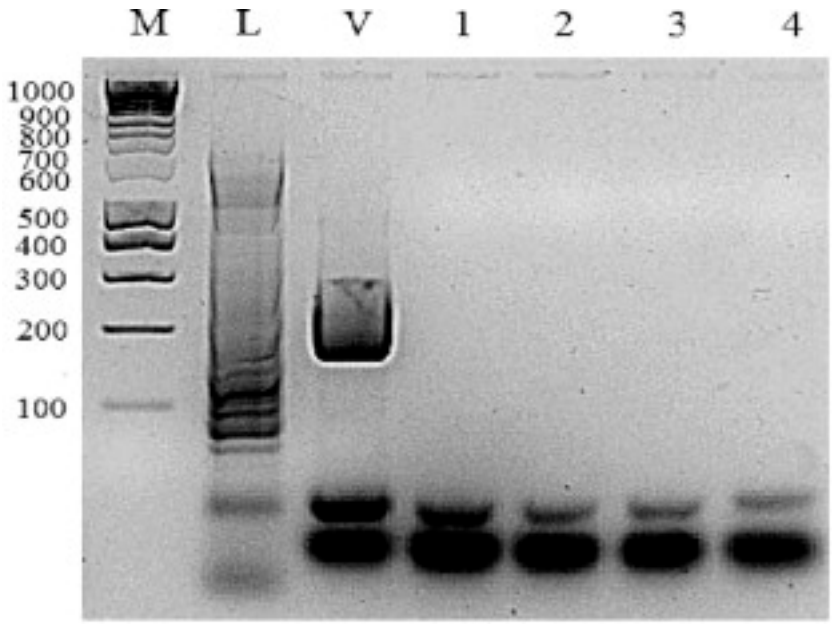


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Fig. 5. Electrophoregram of DNA amplification products of gypsy moth. M – DNA ladder from 100 to 1000 bp with a step of 100 bp (from bottom to top); 1–4 – electrophoretic DNA spectra of different individuals from three egg masses + polyhedrin primers; V (positive control) – *Lymantria dispar*multicapsid nuclear polyhedrosis virus + polyhedrin primers; L – *Lymantria monacha* multicapsid nuclear polyhedrosis virus + IAP-3 gene primers.

### 3.5. PCR extension of BIR and RING fragments on LmMNPVgenome

Additionally, we observed that the use of IAP-3 gene fragments as primers revealed in genome of *Lymantria monacha* multicapsid nuclear polyhedrosis virus (LmMNPV) several bands (Fig. 5; line L). It means that fragments of BIR and RING domains of LdMNPV IAP-3 gene are able to initiate PCR not only in gypsy moth but also in phylogenetically close LmMNPV. Thus, used IAP-3 gene fragments may be also the useful searching tool for analysis of sequences close to viral anti-apoptosis genes in insects (Fig. 3.) and viruses for their further investigation.

### 3.6. Safety for tobacco hornworm and black cutworm caterpillars

Our next results show that single-stranded fragments of LdMNPV IAP-3 gene (BIR + RING) did not have a significant effect (low $\chi^2$ values) on the viability of tobacco hornworm andblack cutworm second instar caterpillars after 11 days of experiment in comparison with control and polyA group emphasizing the selectivity of DNA insecticides (Table 2). During the 11-day period on average 8.3% of individuals of tobacco hornworm perished in the experimental group treated with fragments of viral IAP-3 BIR and RING domains, 21.7% of individuals perished in the experimental group treated with polyA and 18.3% of individuals perished in the control group treated with distilled water. In the experiments with black cutworm during 11-day period on average 26.7% of individuals of

black cutworm perished in the experimental group treated with fragments of viral IAP-3 BIR and RING domains, 26.7% of individuals perished in the experimental group treated with polyA and 30% of individuals perished in the control group treated with distilled water.

Table 2. Statistical analysis of insecticidal effect of BIR + RING and polyA oligonucleotides on tobacco hornworm and black cutworm.

| Pairs | $\chi^2$ values | Number of caterpillars in a pair ($N$) |
|---|---|---|
| *Tobacco hornworm* | | |
| BIR + RING/control | 2.6 | 120 |
| PolyA/control | 0.2 | 120 |
| *Black cutworm* | | |
| BIR + RING/control | 0.08 | 60 |
| PolyA/control | 0 | 60 |

The $\chi^2$ values for experimental groups versus control group on the 11th day after the treatment. Three replicates of the experiment for different egg masses are represented.

This implies that DNA insecticides based on fragments of BIR and RING domains of LdMNPV IAP-3 gene have a significant margin of safety in action and harmlessness to non-target insects such as tobacco hornworm and black cutworm.

## 4. Discussion

The found effect of the viral DNA fragments on its host can be used for the creation of selective fast-acting insecticides to protect plants from the gypsy moth and other phyllophagous insects [9]. Such phenomenon seems to be adaptively important for a system host-virus and may be one of natural pathways of interaction between the gypsy moth and*L.dispar* multicapsid nuclear polyhedrosis virus and requires further investigation. Viral DNA fragments acted as inducers (for death) in the host cells of the gypsy mothcaterpillars. Generally caterpillars from the experimental group that were going to perish stopped eating; they moved slower, insects looked more dehydrated and smaller than caterpillars from the control group. These signs look like manifestation of series of apoptotic processes that made progress in caterpillars. The fragment of the RING domain might play mainly the insecticidal role in a pair BIR + RING, probably acting as an antisense molecule. Since the fragment of BIR belongs to a sense strand, the role of BIR domain fragment in action of DNA insecticides is not clear for the moment and can be counted as auxiliary one.

The results of DNA sequencing show that RING domain fragment used as a reverse primer entirely coincides with such sequence of LdMNPV IAP-3 gene. This indicates that this antisense single-stranded DNA fragment may be used as DNA insecticides if it belongs to an anti-apoptosis gene or a gene that is vitally important for the functioning of a

cell. Part of the gypsy moth genome cloned with the fragments of BIR and RING domains of LdMNPV IAP-3 gene as primers, has high overlap with the part of LdMNPV IAP-3 gene and *L.dispar*IAP mRNA for inhibitor of apoptosis protein, and allows assuming that we cloned a part of the gypsy moth anti-apoptosis gene. This finding gives the grounding that the DNA insecticides might act through the blocking of the mechanisms connected with the expression of anti-apoptosis genes. The action of RING domain fragment might not be limited only by apoptosis/anti-apoptosis scenario since IAPs are also involved in mitoticchromosome segregation, cellular morphogenesis, copper homeostasis and intracellular signaling [40]. Notable, while overall significant gypsy moth mortality was observed after DNA insecticide treatment in comparison with control, a few egg masses of gypsy moth showed resistance to the DNA insecticides. Further studies will show whether or not some specific factors could enhance or weaken the action of DNA insecticides. We suppose the additional effect from the factors able to operate with apoptosis–anti-apoptosis system of a host insect.

Caterpillars from the studied egg masses were free of LdMNPV and this means that viral DNA fragments could interfere with reactions that were wholly under the control of the genetic apparatus of the insect. Thus, the effect of DNA insecticides observed in this study does not depend on the infection of caterpillars with the virus. Of interest, the effect of DNA insecticides can be faster compared with the direct virus effect because during experimental infection of insects with nucleopolyhedroviruses, the latent period of illness lasts from 3–5 up to 10–12 days [7], [41] similar to period observed in this study.

The selectivity and safety of pesticides is probably the main characteristic that has to be checked before a pesticide will be applied to avoid harm to balance of an ecosystem. This research work showed that DNA insecticides can be selective in action and are not harmful to tobacco hornworm and black cutworm. Since every species has its own unique DNA sequence it might be possible to create the most effective and selective DNA insecticides on the base of highly conserved sequences of distinct species (such as RING domain of the viral IAPs) that will manifest the highest effect on a target pest insect and provide harmlessness to other members of an ecosystem. Worth noting that BLAST programme does not find any sequences in human genome that are similar with the sequences from applied here DNA insecticides, thus they look safe for the humans.

Generally, the influence of single-stranded DNA fragments on apoptosis or cellular cycle is an innovative branch of biology. It suggests that DNA could have an ability to coordinate particular cellular pathways, not only as a part of a genome but also as fragments of it. Idea described in this article is novel, and there is no available data in scientific literature about research testing the influence of very short viral single-stranded DNA fragments of anti-apoptosis genes that could work as DNA insecticides. Some authors are close to this

idea but they are mostly concentrated on the phenomenon of RNA interference [18], [19], [22],[42].
In particular, in [42] the authors suggest that RNA interference itself could be envisaged as an insect control tool through targeting vital genes, although efficient systems of double-stranded RNA formulation and delivery must be developed. Also the approach of administering double-stranded RNAs by feeding, as seen in *Epiphyaspostvittana*[43] and*Rhodniusprolixus*[44], paves the way in this field, and the possibility of delivering the dsRNA by soaking, as in *Caenorhabditiselegans*[45], should also be considered, for example, in particularly permeable stages of aquatic insects [42]. Direct spray of aqueous solution of dsRNA on newly hatched *Ostrinia* furnalalis larvae was performed [18], resulted in considerable mortalities, and this effect correlated with the down regulation of the target gene expression as verified by qPCR. This easy and applicable delivery method demonstrated the possibility of dsRNAs penetrating the insect integument and triggering RNAi. RNAi-based pest control can be facilitated by high-throughput dsRNA delivery methods reviewed in [22].
While the idea of DNA insecticides may resemble mechanisms characteristic of antisenseoligonucleotides [13], [14], mRNA-antisense DNA hybrids [15] and mechanisms that resemble those of DNA interference [16] and RNA interference [19], it has its own peculiar features such as its external application, small size of oligonucleotides, single-stranded DNA molecule and the concept of using viral anti-apoptosis genes. Although presently we do not have complete genomic sequence of gypsy moth in data bases, LdMNPV has already been sequenced [33]. In this situation, viral DNA can provide valuable information on some vitally important genes that have cellular origin, such as anti-apoptosis genes [29], that can be used for pest insect control. The perspective of such approach in practice is seen clearly because it provides the same effect with less effort. For example, instead of a baculovirus preparation, we could use small parts of a viral genome and get the same effect.
One of the closest ideas to our concept of DNA insecticides on the base of viral IAPs is the idea described in [23]. In this study, expression of an inhibitor of apoptosis protein 1 gene (AaeIAP1) was suppressed by applying of relatively long dsRNA diluted in acetone to the dorsal thorax of adult mosquito females what led to a significant mortality. Our research work also shows the high potential of use of IAP gene fragments as a perspective means of pest insect regulation.
An important issue is to show that proposed here DNA insecticides will be safe for the environment. In the literature is under discussion the potential hazards posed by RNA interference (RNAi)-based pesticides and genetically modified crops to non-target organismsinclude off-target gene silencing, silencing the target gene in unintended organisms, immune stimulation, and saturation of the RNAi machinery [46], [47]. Currently,

there is no much evidence regarding possible side effects of DNA insecticides on the environment. We recently evaluated the possible side effects of the DNA insecticides on wheat (*Triticum aestivum*). We did not find negative effect of DNA insecticides on the plant investigating some important biochemical parameters (concentration of glucose and activity of alkaline phosphatase) and biomass of wheat sprouts [48].
Important further elaboration in the creation of DNA insecticides we see in the better understanding of mechanism of their action, additional reduction of the concentration of DNA fragments in the end-product and increasing of their effectiveness through DNA carriers using.

## 5. Conclusions

In this article we show the insecticidal potential of the viral DNA fragments that can be used to create safe, relatively inexpensive and fast-acting DNA insecticides to control the quantity of gypsy moth populations, a serious pest of agriculture and forestry. For the used egg masses we found that effect of DNA insecticides does not depend on the infection ofcaterpillars with LdMNPV. PCR and DNA sequencing techniques helped us to find that proposed here DNA insecticides might act through the interfering/blocking of the mechanisms involved in post transcriptional expression of insect anti-apoptosis genes. The results show that DNA insecticides based on LdMNPV IAP-3 DNA fragments can be selective in action, and at least are not harmful for tobacco hornworm (*M.sexta*) and black cutworm (*A.ipsilon*). Thus, use of insect-specific short single-stranded DNA fragments as DNA insecticides paves the way in the field of creation of “intellectual” insecticides that “think” before they act.

## Author contributions

Volodymyr V. Oberemok designed and performed experiments, analyzed data, and wrote the paper. Oleksii A. Skorokhod analyzed data and wrote the paper.

## Conflict of interest disclosure

The authors declare no conflict of interest and no competing financial interests.

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