rRNA-specific antisense DNA and dsDNA trigger rRNA biogenesis and cause potent insecticidal effect on insect pest *Coccus hesperidum* L.

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Abstract
As a completely new principle and new active substance for plant protection, unmodified DNA was shown to function as contact insecticide in 2008. CUAD (contact unmodified antisense DNA) biotechnology is built on the use of short antisense DNA oligonucleotides for insect pest control. Being a novel class of insecticides, oligonucleotide insecticides target pest rRNAs and/or pre-rRNAs and recently showed high insecticidal potential against sap-feeding insect pests, main vectors of plant DNA viruses and one of the most economically-damaging groups of herbivorous insects. In order to use all possible opportunities of CUAD biotechnology, in this article we investigated insecticidal potential of short 11-mer antisense DNA oligos in comparison with long 56-mer single-stranded and double-stranded DNA sequences for *Coccus hesperidum* control and found lower efficiency of the latter. Also in this article we show that DNA containment (DNAc) mechanism, found on sternorrhynchans, represents interesting and important for insect cell life interplay between rRNAs and different types of DNA oligos. In the course of DNAc, hypercompensation of target rRNA is triggered by all highly and all somewhat complementary DNA oligos but more pronounced later degradation of target rRNA and significant insect pest mortality is seen only in the case of perfect complementarity of oligonucleotides to target rRNA. Oligonucleotide insecticides are effective and safe control agents against sternorrhynchans and other groups of pests, easy to 'tune' to particular crops with pests, and also flexible to instantly re-create new oligonucleotide insecticides in the case of target-site resistance. Minimalist approach, short antisense DNA dissolved in water, is so potent and selective eco-friendly innovation against sternorrhynchans and other pests, and reveals entirely new dimension to plant protection – DNA-programmable insect pest control. Fundamentally important, this surprising results reveal completely new principle of regulation of rRNA expression in the cell via complementary interaction between rRNAs and unmodified antisense sequences of cell and viral DNA.

Significance Statement
Using flexible CUAD platform built on the use of oligonucleotide insecticides, any farmer, botanical garden or pest control company is capable of creating its own unique arsenal of insecticides, well-tailored for a particular population of insect pest. Most technological innovations start out very expensive and don't work well, but eventually start working well and become very cheap. CUAD biotechnology is very close to the point to be implemented at affordable price against certain groups of pests on a large scale. Results provide novel knowledge about interaction between unmodified DNA sequences and host rRNAs and sheds light on regulation of rRNA expression by complementary sequences of cell and viral DNA.

Introduction
Hemipterans, particularly sternorrhynchans, are recognized pests of many agricultural crops, being main vectors for plant DNA viruses and bacteria, and causing substantial yield losses (Huang et al. 2022; Wu et al. 2022). Hemipteran pests are mainly controlled by neonicotinoids, leading to the evolution of resistance in field populations (Yao-Fa et al. 2018; Bacci et al. 2018; Zhou et al. 2022; Elmquist et al. 2023). Also hemipterans can feed on plants and excrete honeydew contaminated with neonicotinoids at different concentrations that may cause lethal and sublethal effects on beneficial insects (Calvo-Agudo et al. 2019). Emerging genetic resistance to chemical insecticides is constantly pushing for the search of new insecticides (Siddiqui et al. 2022; Bavithra et al. 2023). History of use of chemical insecticides indicates need for a major paradigm shift in the development of insecticides in order to obtain controlling agents with multi-decade utility and has been an important goal for many decades.
In 2008, an entirely new dimension of insect pest control – DNA-programmable plant protection – was discovered when unmodified DNA was shown to possess insecticidal effect (Oberemok, 2008). Since then, the main breakthroughs in this direction were in finding the most convenient target genes (rRNA genes), in revealing mechanism of action (DNA containment) and in searching up insect pests (sternorrhynchans) with high susceptibility to this approach (Oberemok et al. 2024a). Fundamentally, for the first time we found that short unmodified antisense DNA can both up-regulate and then down-regulate expression of rRNA genes controlling 80% of cell RNA. Up-regulation and further down-regulation of expression of rRNA can be essential for rDNA

master regulation of rRNA expression, regulation of rRNA expression during replication of DNA viruses, antiviral defense against DNA viruses.

In the last few years, contact unmodified antisense DNA (CUAD) biotechnology based on oligonucleotide insecticides (briefly, olinscides or DNA insecticides) has been established as a potent and selective 'genetic zipper' method for control of many hemipteran pests, particularly sternorrhynchans: soft scale insects, armored scale insects, whiteflies, psyllids, mealybugs, and aphids, opening new horizons in green plant protection based on deoxyribonucleic acid. Recently, in Frontiers of Agronomy we published opinion article on successful list of pests targeted by oligonucleotide insecticides (Oberemok et al. 2024b), and all 13 oligonucleotide insecticides were selected from the first time according to the same algorithm and did work very efficiently; in average, the developed olinscides caused 80.04 ± 12.73% mortality for sternorrhynchans in 3–14 days after single or double treatment. Because of very efficient and easy algorithm, DNA-guided 'genetic zipper' method (CUAD biotechnology) is a unique and very potent alternative to other approaches in plant protection based on duplexes of unmodified nucleic acids and RNA-guided nucleases: RNA interference and CRISPR/Cas9 (Figure 1). While RNAi and CRISPR/Cas9 were not discovered on insect pests and initially had fundamental importance rather than practical one, CUAD biotechnology was discovered on insect pests as practical tool and recently fundamental importance of this phenomenon was revealed (Oberemok et al. 2022; Oberemok et al. 2024a; Oberemok and Gal'chinsky, 2024; Gal'chinsky et al. 2024). To date, while RNAi and CRISPR/Cas9 are excellent tools for manipulations with unmodified nucleic acids in laboratory, they do not have easy algorithms for creation of end-products for plant protection; each separate case is special and usually is sorted out using trial and error method.

Ribosomal RNAs of insect pests serve as convenient targets for oligonucleotide insecticides, since rRNA makes up 80% of all RNA in the cell; rRNAs are 'house-keeping' non-coding RNAs that have important roles in the cytoplasm and beyond, including regulation of translation, signaling and metabolism (Mattick et al. 2023). Thousands of different mRNAs make up only 5% of all RNA and use of mature rRNA and pre-rRNA for targeting substantially increases signal-to-noise ratio, ca. 10^5:1 (rRNA vs. random mRNA) (Palazzo and Lee, 2015). Thus, it is almost impossible to get substantial insecticidal effect with an oligonucleotide insecticide targeting a random mRNA, still exclusions can be found when a particular gene is strongly activated, for example, IAP genes during baculovirus infection (Oberemok et al. 2017), or when a group of genes are targeted by one antisense oligonucleotide. Oligonucleotide insecticides act through DNA containment mechanism (DNAc) (Gal'chinsky et al. 2024; Oberemok and Gal'chinsky, 2024) which turned out to be highly significant and conserved in hemipterans. DNAc is a 2-step mechanism discovered on sternorrhynchans that takes place in nucleus (Oberemok and Gal'chinsky, 2024), precisely nucleolus, where ribosome biogenesis takes place (Rawlinson et al. 2018). At first step of DNAc, target rRNA is 'arrested' and hypercompensation of target rRNA occurs (protein machinery and precise architecture of this step remains a mystery). At second step of DNAc, target rRNA degradation by RNase H takes place (Gal'chinsky et al. 2024). Formation of DNA olinscide-rRNA duplex resembles zipper mechanism. This duplex contains (or 'zips') normal target rRNA expression and leads to death of pests. Above-mentioned approach also was termed as 'genetic zipper' method (Oberemok et al. 2024c).

In our opinion, partly, oligonucleotide insecticides are efficient against sternorrhynchans due to anatomic peculiarities: they contain numerous spiracular pores, preopercular pores, simple pores, etc. (Kondo and Watson, 2022) that obviously enable deeper penetration of DNA oligos. Contact oligonucleotide insecticides, as the next-generation class of insecticides (Boukouvala et al. 2022), are supposed to have fast biodegradability, selectivity in action, and low carbon footprint (Gal'chinsky et al. 2024). Oligonucleotide insecticides for sternorrhynchans represent minimalist approach, just short antisense DNA dissolved in water, and has minimal risks for the environment. They also resolve the problem of developing target-site resistance to insecticides in insect pests (Manju et al. 2022), if unique and highly conservative sequences of rRNA genes will be used. If insecticide resistance occurs, new and efficient olinscides can be easily created displacing target site to the left or to the right from the olinscide-resistance site of target mature rRNA and/or pre-rRNA (Gal'chinsky et al. 2024; Oberemok et al. 2024c). Particularly important is necessity of complementarity of 3′-end nucleotide of olinscide to ensure maximum insecticidal

effectiveness of oligonucleotide insecticides (3'-end rule of olinscides) (Gal'chinsky et al. 2024). Importantly, non-canonical base pairing, such as A:C (C:A) and $G_{olinscide}$:U_{rRNA} (Du et al. 2005; Luige et al. 2022; Gal'chinsky et al. 2024), may occur between DNA olinscides and imperfect sites of rRNAs and should be taken into consideration during design of oligonucleotide insecticides (Gal'chinsky et al. 2024). Liquid-phase DNA synthesis based on phosphoramidite chemistry (Molina and Sanghvi, 2019; Brzezinska et al. 2023) makes it possible to significantly reduce the cost of this approach (Gal'chinsky et al. 2023), providing 0.5 USD/ha for the 'genetic zipper' method in aphid control (Oberemok et al. 2024b) and competing with current chemical insecticides. Therefore, the qualities of using unmodified nucleic acids as pesticides appear to be very promising (Kumar et al. 2022; Patil et al. 2024).

Our research group proposed the contact use of short antisense DNA in plant protection in 2008 (Oberemok, 2008), rethought DNA-programmable insect pest control in 2019 (Oberemok et al. 2024b), and continues to explore the facets of CUAD biotechnology to find the most optimal formulations. Already today, according to our estimations, 'genetic zipper' method is capable of successfully controlling 10-15% of the most serious insect pests of the world. We continue to successfully expand this approach to new groups of pests (for example, mites) (Novikov et al. 2023; Oberemok et al. 2024a; Oberemok, 2024) and also create custom-designed species-specific and interspecific mixtures of oligonucleotide insecticides against mixed insect pest populations (Gal'chinsky et al. 2024). We found that contact delivery of unmodified antisense DNAs (CUADs) is much more efficient than oral delivery of unmodified antisense DNAs (ODUADs) because of active DNases present in digestive tract of insects (Lomate and Bonning, 2016).

In this article, brown soft scale insect *Coccus hesperidum* L. (Hemiptera: Coccidae) will be used as a model object. Brown soft scale is probably one of the most polyphagous insects, having been recorded from host plants in 345 genera belonging to 121 families. *C. hesperidum* may have originated from South Africa; it has spread through trade in infested plant material. Brown soft scale feeds on phloem sap, producing sugary honeydew waste that coats nearby plant surfaces and often gives rise to sooty mould growths. The main damage is indirect, when sooty moulds coat the leaves, flowers and fruit; this blocks light and air from the leaves, impeding photosynthesis and respiration and modifying other physiological processes, resulting in loss of vigor and reduction in the size of fruit. *C. hesperidum* is not on any plant quarantine lists, because the species is so widespread already (Kondo and Watson, 2022). Brown soft scale is a common multi-crop pest in greenhouses and is well-known as the main coccid pest of citrus with an extremely wide host range such as olive, avocado, cotton, mango, cocoa, ficus, hibiscus, oleander, palm, fern and orchid (Aliakbarpour et al. 2010).

For this research, we decided to use short (11-mer) and long (56-mer) single-stranded and double-stranded DNA fragments with perfect complementarity and somewhat complementary to target rRNA sequence and evaluate their efficiency as well as obtain fundamentally important understanding of their action on insect cells. All obtained results helped to create previously unknown general scheme of interaction between unmodified DNA sequences and rRNAs of the insect cell.

Results

Mortality of C. hesperidum after treatment with single-stranded and double-stranded DNA sequences in the natural habitat

We evaluated insecticidal potential of 11-mer oligonucleotide insecticide Coccus-11 on the viability of *C. hesperidum* larvae and found significant 48.08 ± 6.56 % pest mortality ($\chi2$ = 145.79, $p < 0.001$, N = 600, df = 1) on the 2nd day compared to water-treated Control (Figure 2). Similar results, 66.33 ± 2.51% pest mortality, was obtained on the 2nd day for oligonucleotide insecticide $Coccus_{(-2)}$-11 ($\chi2$ = 249.64, $p < 0.001$, N = 600, df = 1) (Figure 2). Mortality progressively increased in Coccus-11 group up to the end of the experiment. By the 6th and 9th days of the experiment, pest mortality in Coccus-11 group reached 88.33 ± 13.42 % ($\chi2$ = 401.61, $p < 0.001$, N = 600, df = 1) and 97.66 ± 4.04 % ($\chi2$ = 463.62, $p < 0.001$, N = 600, df = 1), respectively. Similar trend was obtained for oligonucleotide insecticide $Coccus_{(-2)}$-11. By the 6th and 9th days of the experiment, pest mortality reached 91.11 ± 4.58 % ($\chi2$ = 430.49, p <

0.001, N = 600, df = 1) and 96.20 ± 2.14 % ($\chi2$ = 445.19, p < 0.001, N = 600, df = 1), respectively. Random oligonucleotide $A_2C_3G_3T_3$-11 did not show any significant insecticidal effect compared to water-treated Control, while random oligonucleotide CG-11 did show weak insecticidal trend. On the 9th day of the experiment, pest mortality rate in $A_2C_3G_3T_3$-11 and CG-11 groups were 10.33 ± 1.53 % ($\chi2$ = 0.02, p > 0.892, N = 600, df = 1) and 22.67 ± 2.52 % ($\chi2$ = 17.75, p < 0.001, N = 600, df = 1), respectively (Figure 2).
The same trend was found for long DNA sequences. In dsCoccus-56 group pest mortality increased significantly and comprised 66.33 ± 9.71 % on the 2nd day after treatment ($\chi2$ = 249.64, p < 0.001, N= 600, df = 1) compared to water-treated Control (Figure 3). In the groups treated with Coccus-$56_{antisense}$, Coccus-56_{sense}, $(ACTG)_{14}$-56 we observed pest mortality of 26.67 % ($\chi2$ = 71.51, p < 0.001, N = 600, df = 1), 31.14% ($\chi2$ = 55.43, p < 0.001, N = 600, df = 1), and 2.24% ($\chi2$ = 3.87, p > 0.05, N = 600, df = 1), respectively.
On the 6th day after treatment significant increase in pest mortality compared to water-treated Control was found in all experimental groups except $(ACGT)_{14}$-56 group (dsCoccus-56, $\chi2$ = 249.11, p < 0.001, N = 600, df = 18; Coccus-$56_{antisense}$, $\chi2$ = 96.12, p< 0.001, N = 600, df = 1; Coccus-56_{sense}, $\chi2$ = 100.27, p < 0.001, N =600, df = 1; $(ACGT)_{14}$-56, $\chi2$ = 1.53, p > 0.215, N = 600, df = 1). On average, 6.04 ± 6.62 %, 71.68 ± 8.17%, 44.33 ± 21.38 %, 40.05 ± 9.2 %, and 9.26 ± 1.43 % of insects died on the 6th day in groups of water-treated Control, dsCoccus-56, Coccus-$56_{antisense}$, Coccus-56_{sense}, and $(ACGT)_{14}$-56, respectively (Figure 3).
On the 9th day of the experiment, pest mortality was significantly higher in all experimental groups compared to water-treated Control (except $(ACGT)_{14}$-56 group) and reached 9.68 ± 3.1 % in water-treated Control, 77.09 ± 6.24 % in dsCoccus-56 group ($\chi2$ = 250.15, p < 0.001, N = 600, df= 1), 47.36 ± 19.97 % in Coccus-$56_{antisense}$ group ($\chi2$ = 96.78, p < 0.001, N = 600, df = 1), 46.42 ± 18.02 % in Coccus-56_{sense} group ($\chi2$ = 101.13, p <0.001, N = 600, df = 1), and 7.68 ± 2.51 % in $(ACGT)_{14}$-56 group (χ^2 = 0.52, p > 0.469, N = 600, df = 1) (Figure 3).
Both short (Coccus-11, $Coccus_{(-2)}$-11) and long (dsCoccus–56, Coccus-$56_{antisense}$) oligonucleotide insecticides triggered significant pest mortality. In our opinion, moderate insecticidal potential of Coccus-56_{sense} is explained by its interference with normal interaction of native 28S rRNA and ribosomal proteins, in the same manner found for antibiotic binding sites (Lin et al. 2018). Generally, short olinscides showed greater insecticidal potential in comparison with longer ones. Though olinscide dsCoccus-56 caused substantially higher pest mortality by 39.61 ± 0.91 % in comparison with Coccus-$56_{antisense}$ ($\chi2$ = 46.69, p < 0.001, N =600, df = 1) and Coccus-56_{sense} ($\chi2$ = 43.46, p < 0.001, N =600, df = 1), it was in average by 21.11 ± 0.07 % lower than for short olinscides Coccus-11 ($\chi2$ = 68.42, p < 0.001, N =600, df = 1) and $Coccus_{(-2)}$-11 ($\chi2$ = 56.43, p < 0.001, N =600, df = 1) at the end of the experiment.
The highest pest mortality was seen only for short and long olinscides and occurred between 2nd and 6th days. Of note, among all investigated short and long olinscides, dsCoccus-56 had the highest insecticidal effect (66.33 ± 9.71 % mortality) on *C. hesperidum* on the 2nd day. On the 6th day, short olinscides (Coccus-11 and $Coccus_{(-2)}$-11) significantly increased their insecticidal effect on the pest and surpassed dsCoccus-56 in efficiency (88.33 ± 13.42 % and 91.11 ± 4.58 % vs. 71.68 ± 8.17 % mortality, respectively) (p<0.05). It seems advisable to use formulations of oligonucleotide insecticides against *C. hesperidum* containing both long double-stranded and short single-stranded DNA sequences. This will allow a rapid (in 1-2 days) insecticidal effect to be achieved with double-stranded olinscides, and the remaining pest population will be reduced with short olinscides in a few days.

Target rRNA expression of C. hesperidum during DNAc

During DNA containment mechanism, hypercompensation of target rRNA was triggered by all DNA oligos (Figures 4-5, 7). Hypercompensation of 28S rRNA progressively increased from the 2nd to the 6th day (peak for all experimental groups, except Coccus-11 that had peak on the 2nd day) and then decreased by the 9th day. Generally, short oligos triggered lower level of rRNA hypercompensation than long oligos. Short and long random oligos took an intermediate position in relation to level of rRNA hypercompensation (Figures 4-5, c). As a trend, short oligonucleotide insecticides (Coccus-11 and $Coccus_{(-2)}$-11) triggered the lowest level of rRNA hypercompensation (Figure 4, b) while long olinscides ($Coccus56_{antisense}$ and dsCoccus56) triggered the highest

(Figure 5, b). That is why for short olinscides we detect in average 3.75 times more decreased concentration of target rRNA compared to short random DNA oligos on 6th day (peak of rRNA hypercompensation) (Figure 4, b, c). In turn, for long olinscides we see 4.25 times more substantial decrease in target rRNA concentration on 9th day (after pronounced peak of rRNA hypercompensation on 6th day) (Figure 5, b, c). For both short and long olinscides substantial decrease in rRNA concentration after rRNA hypercompensation is explained by the work of RNase H. For long random DNA oligos concentration of rRNA decreased by 2.04 times and for short random DNA oligos concentration of rRNA decreased by 3.02 times between 6th and 9th days, respectively, and this corresponds to normal half-life of rRNAs that lasts 3-5 days and is degraded by ribonucleases (Yi et al. 1999).

Coccus-11 and Coccus$_{(-2)}$-11 caused almost similar mortality but dynamics of rRNA expression in these two groups substantially differed. Only Coccus-11 down-regulated expression of target rRNA gene by 1.63 times on the 6th day. It can be explained by myriad binding partners of rRNA that limit its accessibility to antisense oligonucleotides and RNase H recruitment (Bachellerie et al. 2002; Will and Luhrmann, 2001).

To date, it is not clear why long olinscides trigger more pronounced rRNA hypercompensation than long random DNA oligos, before substantial decrease of target rRNA by RNase H occurs. Precise architecture of rRNA hypercompensation remains a mystery. We hypothesize that special proteins (rRNA sensing proteins) could monitor the proper functioning of rRNA by interacting with it. As previously mentioned, myriad binding partners of rRNA exist (Bachellerie et al. 2002; Will and Luhrmann, 2001) and some of them can perform this function. When highly complementary DNA-rRNA duplex is formed, these rRNA sensing proteins break off and transmit a signal to start transcription on rDNA. The more proteins break off and transmit the signal, the stronger rRNA hypercompensation is. Based on the experimentally found proportion in the rRNA hypercompensation peaks between 56 nucleotides long olinscides and water-treated Control on the 6th day (ca. 9:1) (Figures 4,5), each such specific protein may interact with 6-7 nucleotides in rRNA. Concept of rRNA sensing proteins could explain why somewhat complementary DNA oligos trigger rRNA hypercompensation. Even complementarity of 6-7 nucleotides in a row will be enough to trigger rRNA hypercompensation. All tested here DNA oligos have this threshold level of complementarity to *C. hesperidum* 28S rRNA in GenBank for rRNA of *C. hesperidum* (found by BLAST program). rRNA looks like a sensor for highly and somewhat complementary sequences of DNA oligos and rRNA hypercompensation is one of the first cell reactions on them. Apparently, that is why Coccus56$_{sense}$ does not trigger pronounced rRNA hypercompensation but causes significant pest mortality. It does both somewhat complementary interaction with rRNA and proper interaction with rRNA sensing proteins, thus, it interferes with normal interaction of native 28S rRNA and ribosomal proteins. Interestingly, short olinscides caused 5.76 times lower hypercompensation of target 28S rRNA in comparison with long olinscides. Short olinscides are 5.1 times shorter than long olinscides and obtained data corresponds to the hypothesis of existence of RNA sensing proteins. The longer is olinscide, the higher hypercompensation of target rRNA is occurred.

Of note, long olinscides can serve as models (or prototypes) that simulate interplay between host rRNAs and DNA viruses that normally infect hemipterans (Guo et al. 2022). As a trend, antisense sequences Coccus56$_{antisense}$ and dsCoccus56 triggered 2.36 times stronger hypercompensation of 28S rRNA on the 6th day (peak of rRNA hypercompensation for all experimental groups, except Coccus-11) than Coccus56$_{sense}$ and (ACGT)$_{14}$-56. This phenomenon could be used by DNA viruses. It is obvious that DNA viruses can also take advantage of hypercompensation of rRNAs observed during the DNA containment mechanism in order to increase the number of rRNAs for extra ribosomes necessary for their replication (Miller et al. 2021). Bioinformatics studies indicate that hijacking of host genes by viruses is common (Cerio et al. 2010). Indeed, during coevolution, DNA viruses could not miss the opportunity to hijack rRNA genes via lateral transfer of the nucleic acid from the host organism (Evseev et al. 2021) as key players in metabolism, since rRNA makes up four fifths of all RNA in a cell (Deng et al. 2022), and more than 60% of all energy is spent on the production and maintenance of ribosomes (Szaflarski et al. 2022). Most DNA viruses replicate in nucleus and most viruses interact with the nucleolus that plays a major role in virus life and assembly of ribosomes (Salvetti and Greco, 2014). The canonical function of

nucleoli is ribosome biogenesis, where they support rRNA synthesis, processing, and assembly into pre-ribosomal subunits (Rawlinson et al. 2018). It is not difficult to imagine that a complementary fragment of DNA of virus genome can regulate host rRNA synthesis according to the ON/OFF principle, using this trigger to influence the cell (to turn on this mechanism and turn it off when required). This mechanism, deemed rRNA synthesis switchboard, can help virus initiate rRNA synthesis and the formation of new ribosomes with minimal energy consumption (Oberemok and Gal'chisnky, 2024), using the complementary interaction of specific sequences of viral DNA and host rRNA.
Fundamentally very important, found here potent effect of antisense unmodified DNA on rRNA shows potential of cell DNA in regulation rRNA expression. Thus, cell DNA may serve not only as the matrix for rRNA synthesis but as regulatory molecule of its expression. These findings significantly change our understanding of regulation of rRNA biogenesis.

Histological studies
We also performed histological studies (Figure 6, a, b) to detect hypercompensation of rRNA in insect cells caused by DNA oligos. We chose the 2nd day of the experiment with short DNA oligos where substantial rRNA hypercompensation was detected in Coccus-11 group compared to water-treated Control. Upon histological examination with hematoxylin and eosin, we found that in the Control group, the apical parts of the insect cells have outgrowths, in the recesses between which vesicles of the Golgi apparatus emerge with polysaccharides, forming the cuticle layer by layer. In insects of the control group, the network of such outgrowths is well defined, and the layering of the cuticle is also noticeable. From the basement of membrane side, the epithelium is washed by hemolymph (Figure 6, (b) – Control). Interestingly, in the $A_2C_3G_3T_3$-11 group, the color saturation of the cuticle was similar compared to water-treated Control group, and the thicknesses of cuticle layer was similar as well (Figure 6, (b) – $A_2C_3G_3T_3$-11). Insects from the $A_2C_3G_3T_3$-11 group show slight thinning of the cuticle layer and loss of layering.
In the Coccus-11 group, with a sufficiently high epithelium and preservation of the apical processes, the thickness of the cuticle is less than in the control (Figure 6, (b)–Coccus-11). The cytoplasm of epithelial cells appears denser, with a large number of dark-stained hematoxylin granules. It is known that hematoxylin stains nucleic acids (DNA and RNA): the cell nucleus, ribosomes and RNA-rich areas of the cytoplasm (Ortiz-Hidalgo and Pina-Oviedo, 2019). Thus, such intensive staining of insect cells in the Coccus-11 group proves rRNA hypercompensation and increase level of rRNA biogenesis found by RT-PCR in comparison with water-treated Control and $A_2C_3G_3T_3$-11 groups on the 2nd day after the treatment with DNA oligos (Figure 4, a).

Oligonucleotide insecticides are dominantly contact insecticides
During the experiment we noticed that *C. hesperidum* occurred mainly on the leaf undersides of *P. tobira* in the investigated population. It was interesting to check whether olinscides have characteristics of systemic insecticides that move through plants (Tudi et al. 2021). To do this, leaf uppersides without pest were treated with olinscide Coccus-11. As a control, leaf undersides containing pest also were treated. On the leaf undersides, olinscide Coccus-11 caused substantial mortality of the pest (85.3 ± 8.9 %) compared to water-treated Control group ($\chi 2$ = 76.4, $p < 0.001$, N = 140, df = 1), while application of olinscide Coccus-11 on the leaf uppersides caused moderate mortality of pest on the leaf undersides and comprised 31.8 ± 7.1 % ($\chi 2$ = 11.6, $p < 0.001$, N = 133, df = 1) (Table 1).
Random (somewhat complementary) oligonucleotide $A_2C_3G_3T_3$-11 did not show significant insecticidal effect compared to water-treated Control on either surface of leaves on the 4th day. Thus, oligonucleotide insecticides based on unmodified antisense DNA do not possess pronounced characteristics of systemic insecticides and direct contact of oligonucleotide insecticides with insect integument is required for substantial insecticidal effect.

Discussion
In our studies on sternorrhynchans, particularly *C. hesperidum*, we found that DNA containment mechanism consists of 2 steps and represents fundamental and previously unknown interplay between different types of DNA oligos (olinscides and random oligos) and rRNAs (Figure 7).

Ribosomal RNA plays role of a potent sensor for penetrated DNA oligos and comprises 80% of the cell RNA performing important functions during protein biosynthesis, apoptosis, DNA damage repair, etc. Thus, regulation of synthesis of rRNA is of immense value for cell life (Wang et al. 2015; Pecoraro et al. 2021). Obviously, DNA containment mechanism can serve as a module in DNA repair, virus-host relationship, action of extracellular DNA, regulation of rRNA expression by cell, etc. and is evolutionary conserved regulatory process of insects. At the same time, DNAc is the tip of the iceberg of the processes that take place in response to DNA oligos, especially in the light of the protein machinery involved in these processes.

Sequences of DNA oligos highly complementary to rRNA (olinscides)

At first step of DNAc, an antisense DNA oligonucleotide (olinscide) complementarily interacts with target mature rRNA (in other words, blocks target rRNA) and/or 47S pre-rRNA, thus, interferes with normal functioning of mature ribosomes and processing of 90S pre-ribosomes; this process is accompanied with substantial insect pest mortality. Target rRNA hypercompensation by the DNA-dependent RNA polymerase in insect cells occurs in response to proper 'arrest' of target rRNA by antisense DNA oligonucleotide. Both 'old' blocked target rRNA in ribosomes (ribosomes apparently do not function properly in this situation) and 'newly' synthesized target rRNA and polycistronic rRNA transcripts (47S pre-rRNA) can be found by RT-PCR and, thus, we detect target rRNA hypercompensation. Precise architecture of this step remains a mystery. At second step of DNAc, RNase H cleaves target rRNA and substantial decrease in its concentration occurs; this process is also accompanied with substantial insect pest mortality. Both short and long olinscides, either constantly keep the target rRNA concentration low (Coccus-11 and $Coccus_{(-2)}$-11 in comparison with short random DNA oligos), or they dramatically reduce it after the peak of rRNA hypercompensation ($Coccus56_{antisense}$ and dsCoccus56 in comparison with long random DNA oligos), which in both cases is explained by the work of RNase H. Obviously, RNase H protects its unmodified DNA guide from degradation by DNases.

DNA containment mechanism (STEP 1+, hypercompensation of rRNA) can play crucial role in regulation of rRNA expression by complementary cell DNA (direct rDNA master regulation) and viral DNA (rRNA switchboard mechanism) (Oberemok and Gal'chinsky, 2024) as well as can be recruited in innate immunity system (Gal'chinsky et al. 2024) against ssDNA viruses for which hemipteran insects serve as major vectors (Wang and Blanc, 2021 ; Wu et al. 2022) and also against DNA viruses that normally infect them (Guo et al. 2022). Insect nucleases can cleave specific DNA sequences of the invader producing ssDNA oligonucleotides and subsequently initiate degradation of target viral RNAs (Gal'chinsky et al. 2024) (Figure 8). Obtained here results with unmodified DNAs (single-stranded and double-stranded) is a profound model for the described process and entirely new dimension to regulation of rRNA genes controlling 80% of cell RNA.

Sequences of DNA oligos somewhat complementary to rRNA (random oligos)

At first step of DNAc, a random (somewhat complementary) DNA sequence in imperfect manner interacts and not properly blocks target mature rRNA and/or 47S pre-rRNA, as a result, weakly interferes with normal functioning of mature ribosomes and processing of 90S pre-ribosomes.

Obviously, in this case, target rRNA hypercompensation by the DNA-dependent RNA polymerase is the reaction on DNA oligo as possible damage of cell DNA. Insect cells mobilize their strengths through elevation of protein biosynthesis (for example, it is known that pre-ribosomal RNA reorganizes DNA damage repair factors in nucleus during meiotic prophase and DNA damage response) (Gai et al. 2022). Both weakly arrested 'old' RNA in ribosomes and 'newly' synthesized target rRNA and polycistronic rRNA transcripts (47S pre-rRNA) can be found by RT-PCR and, thus, we detect target rRNA hypercompensation. At second step of DNAc, ribonucleases cleave excess of rRNAs and deoxyribonucleases cleave penetrated DNA oligos. rRNAs have 3-5 days long cellular half-lives (Yi et al. 1999) and we see substantial 2-3 times decrease in concentration of rRNA between 6th and 9th days without significant pest mortality for groups with random DNA oligos. Obviously, DNA oligos without perfect complementarity to target rRNAs can't recruit RNase H efficiently and insect cells normalize their life degrading random

DNA oligos. Generally, random oligos trigger cell proliferation and increase in cell volume, cells do not die massively, and substantial mortality of insects does not occur (Oberemok, 2019).
Thus, short and long single-stranded DNA oligonucleotides having weak interaction with target rRNA also trigger its hypercompensation. Non-specific interactions become inevitable with the increase of participating biomolecules and cell requires very clear signal – perfect or almost perfect complementarity of a DNA oligo to target rRNA – of whether to die or not. All other weak signals which are caused by random DNA oligos are subject to homeostasis. Subsequently, significant pest mortality is caused only by olinscides with perfect or almost perfect complementarity to target rRNA (Gal'chinsky et al. 2024). Obtained results open up vast opportunities for potent and selective insect pest control as well as reveals completely new principle of biogenesis of the most abundant RNA in the cell – rRNA.
Importantly, previously found extracellular DNA (exDNA) of *P. tobira* having high insecticidal potential against *C. hesperidum* (Oberemok et al. 2022), was shown in our experiments to comprise of both double-stranded and single-stranded 50-100 nucleotides long fragments of *P. tobira* DNA (under publication). Thus, under natural conditions, plant DNA can be partially biodegraded and appear in the form of a nanolayer of DNA insecticides on the surface of plant leaves and can be an example of plant adaptation to sap-feeding insects having sedentary lifestyle. Of note, length of *P. tobira* exDNA is shorter in summer than in winter and can be explained by stronger UV radiation that degrades DNA. As natural DNA insecticide, found exDNA acts by DNA containment mechanism and can comprehensively affect the work of many genes inside insect pest cells since insect pests and host plants possess similarities in genome sequences (Gilbert and Maumus, 2023), including rDNA sequences. It is amazing that 'genetic zipper' method, elaborated by scientists in the lab to benefit of mankind, has its previously unknown and strikingly similar analogue in nature.
Recently, it was shown that the expression of piwi-interacting RNAs (piRNAs), a group of small non-coding RNAs (sncRNAs) ranging from 26 to 31 nt in length, was depleted with unmodified antisense deoxyribonucleotides during *Blatella germanica* embryogenesis (Gonzalvo et al. 2024). Gonzalvo et al. (2024). Their research work also demonstrated great potential of unmodified antisense oligonucleotides that have barely been used to regulate insect gene expression (Oberemok et al. 2018), though their enormous potential in insect pest control as 'genetic zipper' method (Oberemok et al. 2024b; Oberemok et al. 2024c) and assistance in understanding of fundamental cell events (direct rDNA and viral DNA master regulation of rRNA expression) have been shown (Oberemok, 2024; Obermok and Gal'chinsky, 2024; Gal'chinsky et al. 2024; Oberemok et al. 2024a). Unfortunately, for a long time it was believed that unmodified oligonucleotides are toxic for cells and are very quickly degraded in all eukaryotic cells under the action of nucleases (Egli and Manoharan 2023), including insects (Peng et al. 2018). In historical retrospective, the mode of action of unmodified antisense DNA and its potential application as insecticide have not been investigated on insect pests. No attempt had been made to test this hypothesis until 2008 (Oberemok, 2008) and later DNA insecticides successfully transformed into the potent and selective DNA-programmable tool of insect pest control based on CUAD biotechnology.
Obtained results with antisense and double-stranded DNA fragments helped to create general architecture of interplay between rRNA and DNA based on DNA containment mechanism, important for both practical application (DNA-programmable insect pest control) and fundamental understanding of functioning of the cell (regulation of rRNA synthesis). Tested here unmodified DNAs serve as a simple and robust models of investigation of complementary interactions between cell DNA and rRNAs as well as between viral DNA and rRNAs unraveling new facets of crucial and previously unknown roles played by nucleic acids (Figure 8). Using complementary DNA sequences, both cell DNA and viral DNA are capable of directly causing rRNA hypercompensation in the cell. It is not difficult to imagine that cell DNA or viral DNA can regulate rRNA synthesis according to the ON/OFF principle, using this trigger to influence the cell (to turn on this mechanism and turn it off when required). Direct rDNA and direct viral DNA master regulation initiate rRNA synthesis and the formation of new ribosomes with minimal energy consumption, using the complementary interaction between specific sequences of DNA, either cell or viral, and rRNAs (Oberemok and Gal'chinsky, 2024). Also cell DNA and viral DNA can

directly cause rRNA degradation (for example, discarding incorrectly synthesized rRNA or reducing pre-rRNA and rRNA concentration) recruiting DNA-guided RNase H. RNase H is one of the ubiquitous enzymes with the highest concentration in the cell nucleus and plays a key role in the interactions between DNA and RNA cleaving RNA in RNA/DNA duplex (Cerritelli and Crouch, 2009).

Conclusion

Fundamentally, oligonucleotide insecticides act on *C. hesperidum* through DNA containment mechanism and cause substantial pest mortality. Ribosomal 28S rRNA has been proven to be a good target for both short and long olinscides. Surprisingly, short antisense olinscides are more effective against *C. hesperidum* than long single-stranded and long double-stranded olinscides. We hypothesize that closely related representatives of superorder Hemipteroidea (Paraneoptera) as well as groups of insect pests that are infected by DNA viruses and actively involved in the transmission of plant DNA viruses will be the most susceptible to oligonucleotide insecticides dissolved in water (without additional substances enhancing insecticidal effect). This is because they apparently have a natural DNA containment defense mechanism against DNA viruses that helps them track and degrade viral mRNAs (Gal'chinsky et al. 2024). Vice versa, DNA viruses also can use DNA containment mechanism (hypercompensation of rRNA) in order to increase the number of rRNAs for extra ribosomes in the cell necessary for their replication. The rapidly accumulating amount of genomic information indicates that a noticeable number of viral genomes contain sequences related to ribosomal RNA (Evseev et al. 2021). Also complementary sequences of cell DNA could regulate rRNA expression through formation of DNA-rRNA duplexes and initiation of DNA containment mechanism. It is important to note that cell DNA and viral DNA do not take any risk to be degraded when they recruit RNase H, while rRNA can be either up-regulated or down-regulated by complementary cell DNA and viral DNA as direct master regulator of rRNA biogenesis. Our discovery made with the help of unmodified DNAs reveals previously unknown dimension to gene regulation of rRNA genes controlling four fifths of all RNA in the cell.

Practically, the introduction of 'genetic zipper' method into plant protection techniques will help create a flexible system for adaptation of oligonucleotide insecticides to the constantly changing genetics of pests during microevolution. Moreover, agriculture substantially shifted to monoculture state and favors outbreaks from insect pests, thus, flexible methods should be invented to counteract them. Fundamental goal of short oligonucleotide insecticides – healthy crops without losing yield – helps to easily adapt to microevolution of pests and, to our latest projections, affordability of the approach makes it very perspective to be implemented in agriculture on a large scale. One of the most alluring features of DNA-guided 'genetic zipper' method based on CUAD biotechnology is that oligonucleotide insecticides target single species while non-target species stay unharmed. Moreover, we are now able to predict the effectiveness of oligonucleotide insecticides on various insect pests based on their effectiveness in closely related species (Oberemok et al. 2024c). We are on the cusp of insecticide development turning into use of construction set composed of nitrogenous bases and genomic sequences of insect pests as instructions for assembling effective and safe oligonucleotide insecticides against sternorrhynchans and other groups of pests. Interestingly, the researchers and collaborators in this field call olinscides as "intelligent insecticides" because they act selectively and effectively with good results (Kumar et al. 2024). Oligonucleotide insecticides are fulfilling a 90-year-old dream of creation of potent and selective chemical insecticides with highly adaptable structure to microevolution of insects.

Materials and Methods

Origin of C. hesperidum L.

We identified *C. hesperidum* larvae (Kondo and Watson, 2022) on *Pittosporum tobira* Thunb. (Apiales: Pittosporaceae) in the Nikita Botanical Garden (Yalta, Crimea) (situated at 44°30′41.9″N latitude and 34°13′57.3″E longitude) and used them for the experiment. During 8 replicates of the experiment, around 10400 larvae were treated, which were included in the statistical calculations to assess the survival rate. Insects were counted for each replicate of each variant of the experiment on 20 leaves of *P. tobira*. Mortality was calculated on the 2nd, 6th, and 9th days; the

number of dead individuals was divided by the total number of individuals on the leaf and multiplied by 100%.

Applied short (Coccus-11; Coccus$_{(-2)}$-11) and long (dsCoccus-56; Coccus-56$_{antisense}$) olinscides
We used GenBank database (*C. hesperidum* isolate S6A395 large subunit 28S ribosomal RNA gene, partial sequence; https://www.ncbi.nlm.nih.gov/nuccore/MT317022.1, accessed on 27 March 2024) to design short (Coccus-11, 5′–CCA–TCT–TTC–GG–3′; Coccus$_{(-2)}$-11, 5′–CAC–CAT–CTT–TC–3′, it is situated 2 nt to the right from Coccus-11 antisense sequence) and long (Coccus-56$_{antisense}$, 5′–CCA–TCT–TTC–GGG–TAC–CAG–CGT–GCA–CGC–TGT–AGG-TGC–GCC–CCA–GTT–CGT–CGA–CGG–TC–3′ and its corresponding double-stranded copy, dsCoccus-56) sequences as contact oligonucleotide insecticides. We also performed DNA sequencing to check if oligonucleotide insecticides complementarily match with 28S rRNA sequences in investigated population of *C. hesperidum*. We checked and it matched (data not shown). Olinscides were dissolved in nuclease-free water (100 ng/μL) and applied using a hand sprayer to *P. tobira* leaves (mg of olinscides per m^2 of leaves), 10 mL of the solution was used per m^2. All sequences were synthesized using ASM 800E DNA synthesizer (BIOSSET, Novosibirsk, Russia).

Applied short ($A_2C_3G_3T_3$-11; CG-11) and long (Coccus-56$_{sense}$; (ACGT)$_{14}$-56) random oligos
As a control, we used short ($A_2C_3G_3T_3$-11, 5′–AAC–CCG–GGT–TT–3′; CG-11, 5′–CGC–CGC–CGC–CG–3′) and long (Coccus-56$_{sense}$, 5′–GAC-CGT-CGA-CGA-ACT-GGG-GCG-CAC-CTA-CAG-CGT-GCA-CGC-TGG-TAC-CCG-AAA-GAT-GG–3’; (ACGT)$_{14}$-56, 5′–ACG-TAC-GTA-CGT-ACG-TAC-GTA-CGT-ACG-TAC-GTA-CGT-ACG-TAC-GTA-CGT-ACG-TAC-GT–3′) sequences. They were also dissolved in nuclease-free water (100 ng/μL) and applied using a hand sprayer to *P. tobira* leaves (mg of olinscides per m^2 of leaves), 10 mL of solution was used per m^2. All sequences were synthesized using ASM 800E DNA synthesizer (BIOSSET, Novosibirsk, Russia).
Evaluation of 28S rRNA expression of C. hesperidum
C. hesperidum larvae were ground using a pestle in 1.5 mL tube to prepare them for RNA extraction by ExtractRNA kit (Evrogen, Moscow, Russia) according to the manufacturer’s instructions. Three independent extractions were carried out to produce the replicates for each treatment. For each extraction, 10 larvae were used from each group (Control, Coccus-11, Coccus$_{(-2)}$-11, $A_2C_3G_3T_3$-11, CG-11, dsCoccus-56, Coccus-56$_{antisense}$, Coccus-56$_{sense}$, (ATCG)$_{14}$-56. The quality and concentration of the extracted total RNA were assessed using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). 1.5% agarose gel was used to run electrophoresis in TBE (Tris-borate-EDTA) buffer (10 V/cm) for 30 min, and 5 μL of the eluted RNA volume was loaded for each group. Among all experimental groups, the quantity, intensity, and pattern of the RNA bands were equal.
For reverse transcription, the total RNA (50 ng) was annealed with Coccus-R primer (5′–ACG–TCA–GAA–TCG–CTG–C–3′) and analyzed using FastStart Essential DNA Green Master kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions. The reaction was conducted at 40 °C for 60 min in a LightCycler® 96 (Roche, Basel, Switzerland). cDNA (2 μl) was added to the mixture with FastStart SYBR Green MasterMix kit (Roche, Basel, Switzerland). Primers, forward 5′–ACC–GTC–GAC–GAA–CTG–G–3′ and reverse 5′–ACG–TCA–GAA–TCG–CTG–C–3′, were used for quantitative real-time PCR studies to calculate the concentration of *C. hesperidum* 28S rRNA. The qPCRmix-HS SYBR (Evrogen, Moscow, Russia) master mix was used according to the manufacturer’s instructions. The following procedure of 10 min initial denaturation at 95 °C, followed by 30 cycles with 10 s denaturation at 95 °C, 15 s annealing at 62 °C, and 14 s elongation at 72 °C was used for amplification on a LightCycler® 96 instrument (Roche, Basel, Switzerland). PCR was repeated in triplicate for each condition. Finally, to estimate the specificity of amplification and the presence of additional products, all the PCR products were melted.

Histochemical Assay
Dehydration and paraffin impregnation were carried out in a Logos microwave histoprocessor (Milestone, Sorisole, Italy). Tissue blocks were sectioned into slices 4 μm thick and stained with

hematoxylin and eosin. The following reagents for fixation, dehydration, wiring, and staining were used: formalin 10%, isopropyl alcohol, paraffin, o-xylene, and a set of dyes of hematoxylin and eosin (Biovitrum, St. Petersburg, Russia).

Statistical Analyses

The standard error of the mean (SE) was determined and analyzed using the Student's t-test to evaluate the significance of the difference in 28S rRNA concentration between control and experimental groups on the 2nd, 6th, and 9th days. The non-parametric Pearson's chi-squared test ($\chi 2$) with Yates's correction was performed to evaluate the significance of the difference in mortality between control and experimental groups on the 2nd, 6th, and 9th days. All above-mentioned calculations were preformed using Prism 9 software (GraphPad Software Inc., Boston, USA).

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Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Figures and Tables

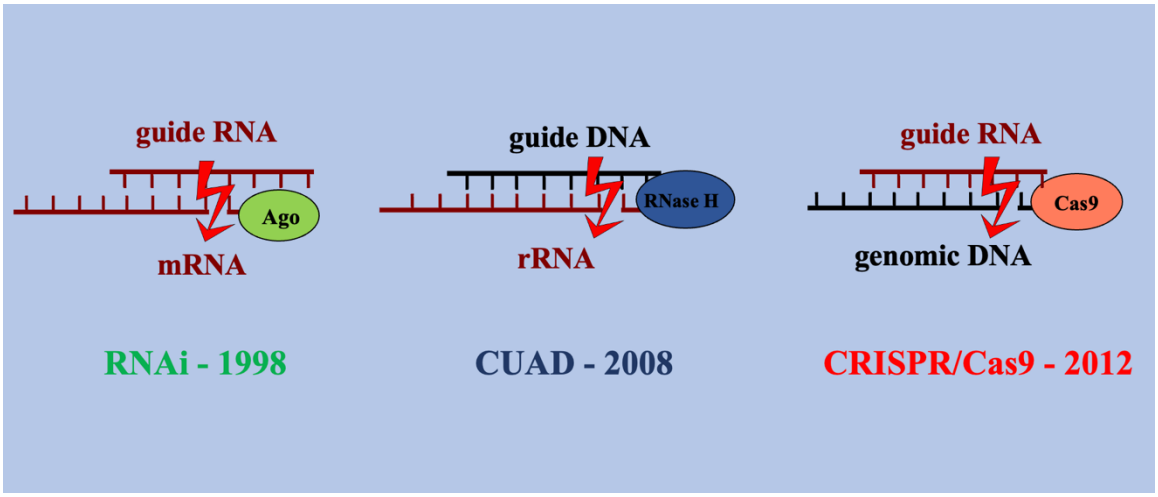

Figure 1. Plant protection technologies (RNAi, CUAD, CRISPR/Cas9) based on formation of duplexes of unmodified nucleic acids (RNAi: guide RNA-mRNA; CUAD: guide DNA-rRNA; CRISPR/Cas9: guide RNA-genomic DNA) and action of nucleic acid-guided nucleases (Argonaute, briefly Ago; RNase H; CRISPR associated protein 9, briefly Cas9).

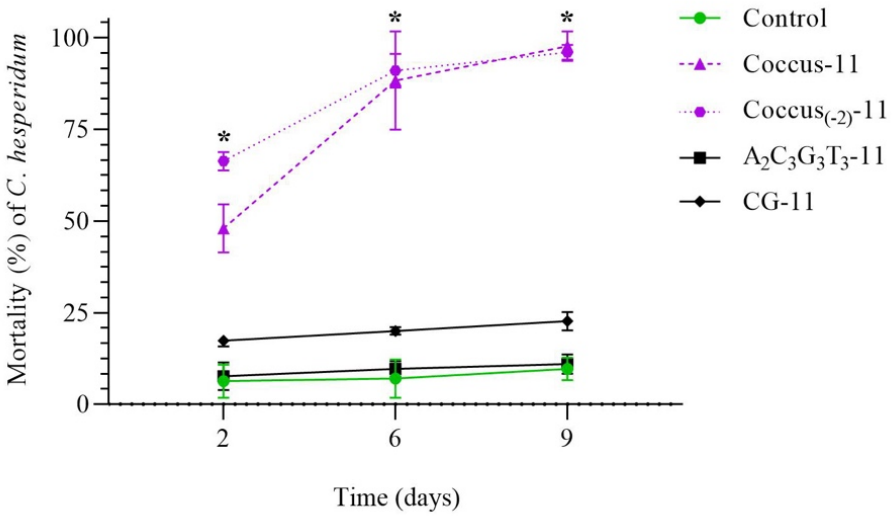

Figure 2. Dynamics of mortality of *C. hesperidum* after contact treatment with water, Coccus-11, Coccus$_{(-2)}$-11, $A_2C_3G_3T_3$-11, and CG-11. The significance of differences in the groups of oligonucleotide insecticides (Coccus-11 and Coccus$_{(-2)}$-11) compared to water-treated Control is indicated by * at $p < 0.05$.

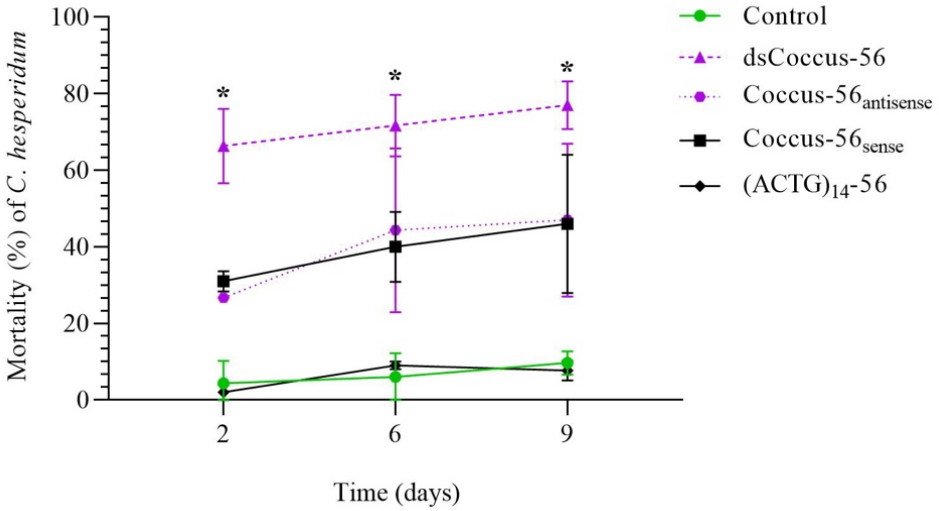

Figure 3. Dynamics of mortality of *C. hesperidum* after contact treatment with water, dsCoccus-56, Coccus-$56_{antisense}$, Coccus-56_{sense}, and $(ACTG)_{14}$-56. The significance of differences in the groups of the experiment (dsCoccus-56, Coccus-$56_{antisense}$, Coccus-56_{sense}) compared to the water-treated Control is indicated by * at $p < 0.05$.

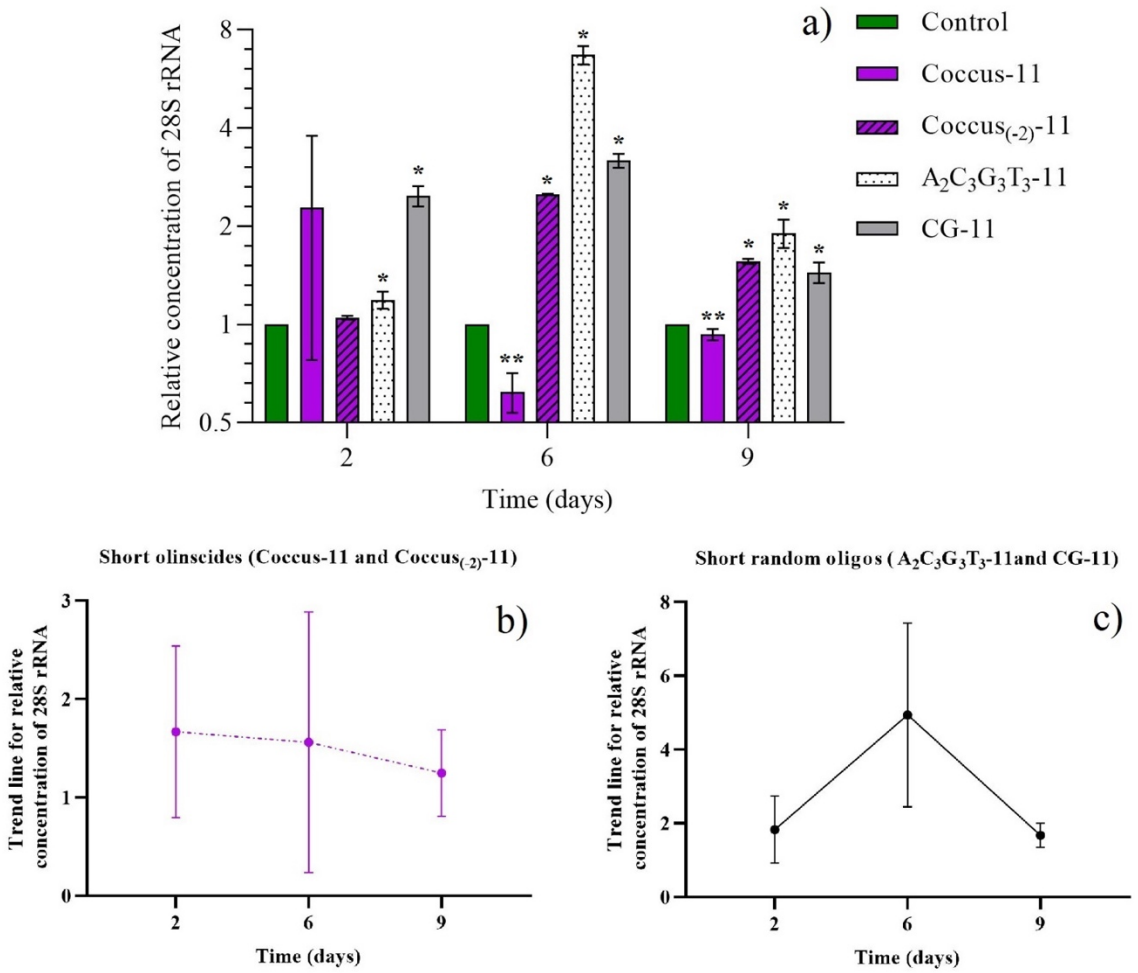

Figure 4. Dynamics of the relative concentration of 28S ribosomal RNA (average concentration) for short olinscides and short random oligos; * is marked when concentration of target 28S rRNA is significantly higher compared to water-treated Control ($p < 0.05$); ** is marked when concentration of 28S rRNA is significantly lower compared to water-treated Control ($p < 0.05$).

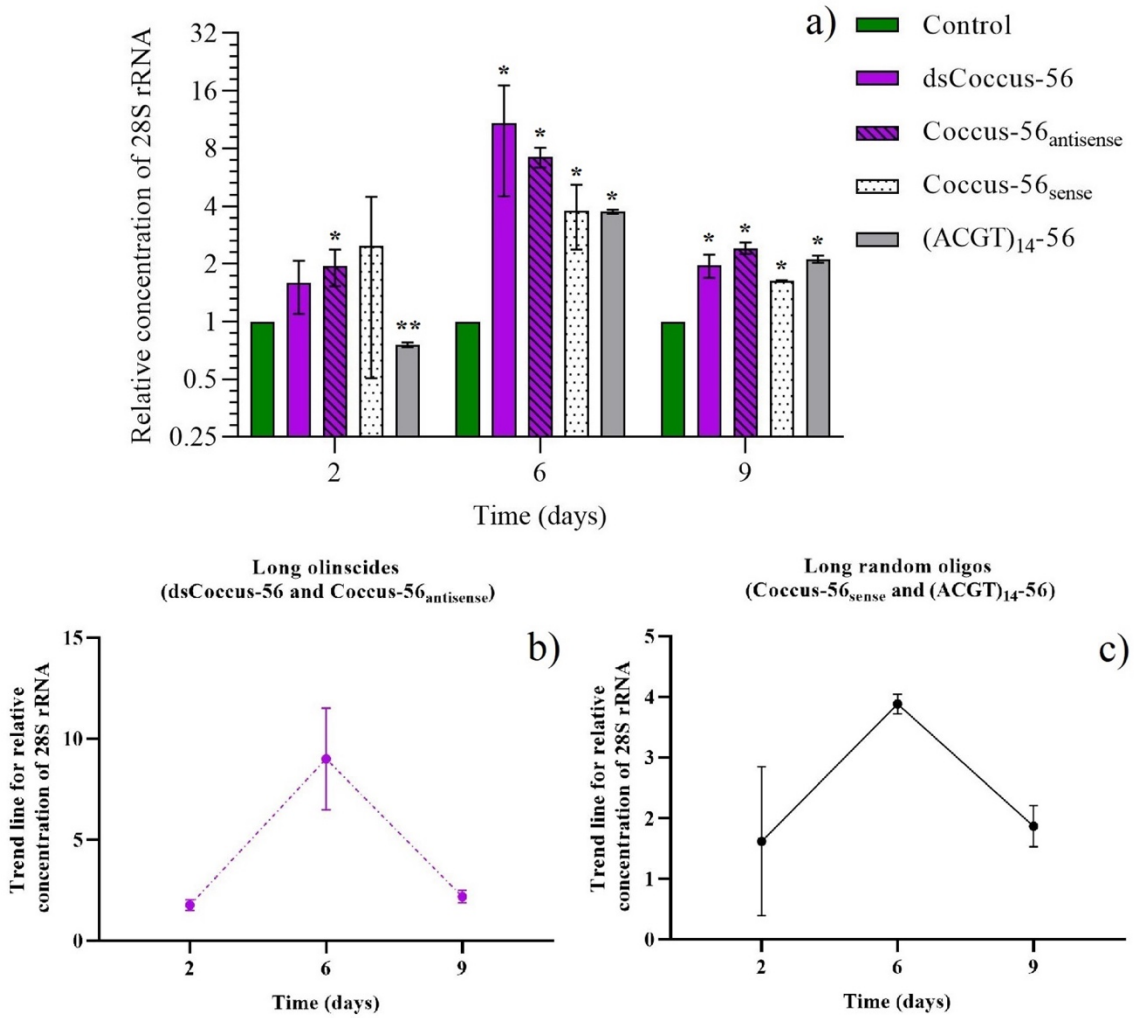

Figure 5. Dynamics of the relative concentration of 28S ribosomal RNA (average concentration) for long olinscides and long random oligos; * is marked when concentration of target 28S rRNA is significantly higher compared to water-treated Control ($p < 0.05$); ** is marked when concentration of 28S rRNA is significantly lower compared to water-treated Control ($p < 0.05$).

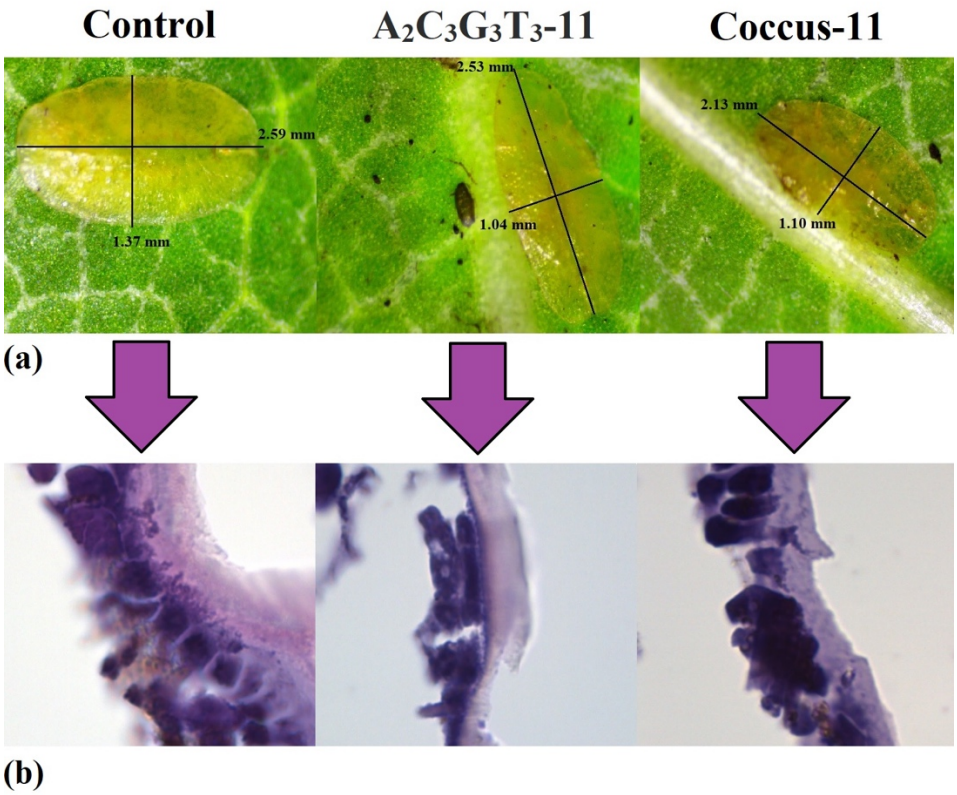

Figure 6. The effect of water (Control), random oligo ($A_2C_3G_3T_3$-11), and oligonucleotide insecticide (Coccus-11) on *C. hesperidum* larvae on the 2nd day of the experiment; (a) insect morphology was investigated with light microscopy; (b) the integument of *C. hesperidum* is represented by a cylindrical single-layer epithelium from different groups of the experiment stained with hematoxylin and eosin.

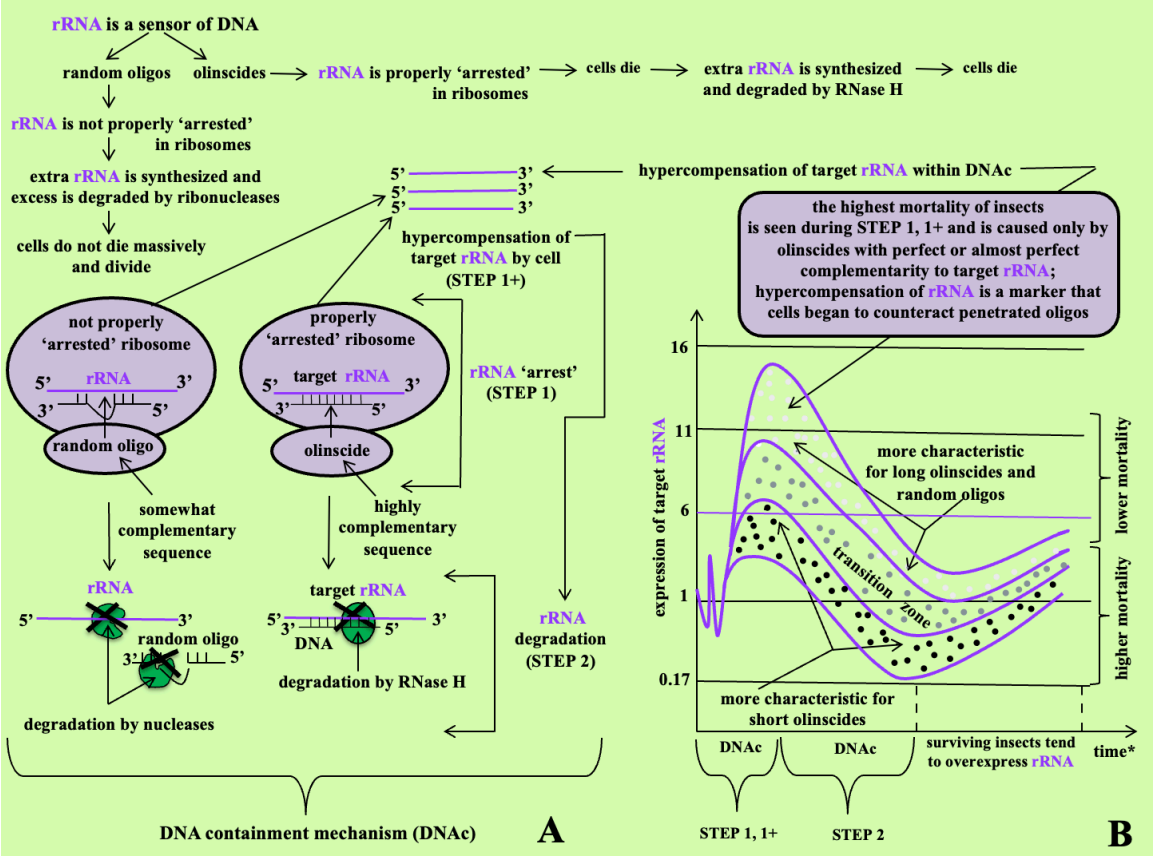

Figure 7. DNA containment mechanism (DNAc) of Sternorrhyncha representatives (A) and trends of target rRNA expression triggered by olinscides and random oligos during DNAc (B); * — time varies depending on distinct species and used oligos, usually 2 steps (full cycle) of DNAc occurs within 2 weeks; transition zone — can be detected for short olinscides, long olinscides and random oligos.

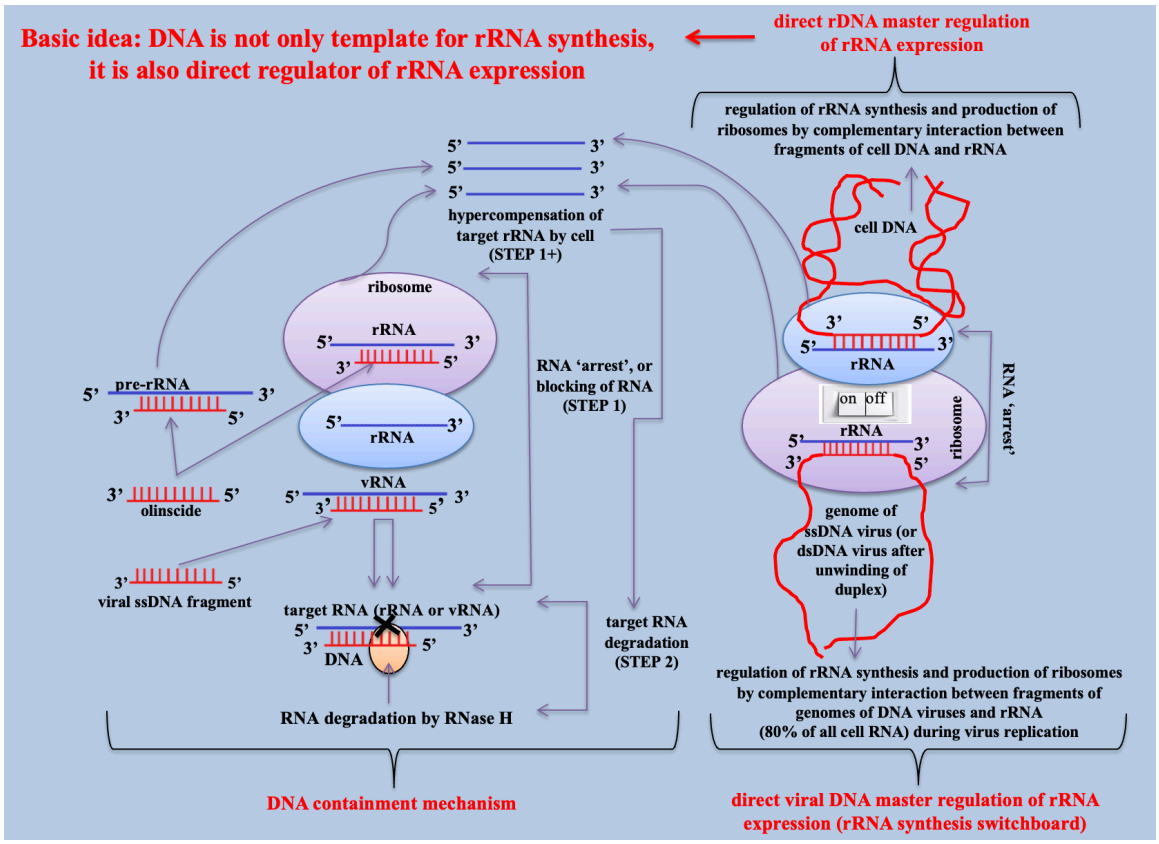

Figure 8. Mode of action of oligonucleotide insecticides, viral single-stranded DNA fragments, regulation of rRNA synthesis via direct rDNA and direct viral DNA master regulation based on DNA containment mechanism

Table 1. Mortality of *C. hesperidum* after application of water, random oligo $A_2C_3G_3T_3$-11, and olinscide Coccus-11 on the undersides and uppersides of *P. tobira* leaves on the 4th day;
* – significance of the difference compared to water-treated Control, $p<0.05$

Leaf uppersides			Leaf undersides		
Control	$A_2C_3G_3T_3$-11	Coccus-11	Control	$A_2C_3G_3T_3$-11	Coccus-11
7.2 ± 1.9 %	7.9 ± 5.5 %	31.8 ± 7.1 %*	7.5 ± 2.4 %	16.6 ± 6.8 %	85.3± 8.9 %*