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Зміни репродуктивних показників *Drosophila melanogaster* при дії низьких концентрацій неонікотиноїдів та ніацину на личинковій стадії

В.А.Ковач, Н.С.Філіпоненко, Н.В.Колот, Н.Є.Волкова

Харківський національний університет імені В.Н.Каразіна (Харків, Україна)
natalia.volkova@karazin.ua

З моменту відкриття неонікотиноїди вважалися найбільш перспективними сполуками з інсектицидною активністю в силу специфічної дії на нікотинові ацетилхолінові рецептори саме комах і низьку токсичність для ссавців у використовуваних діапазонах концентрацій, а також у зв'язку з відсутністю формування стійкості до них у комах. Уведення неонікотиноїдів у сільськогосподарську практику дозволило істотно підвищити врожайність різних культур. Однак при активному повсюдному використанні даного класу інсектицидів було виявлено цілу низку негативних наслідків, зокрема для комах-запилювачів. Виявлення даних ефектів призвело до суттєвого обмеження або до заборони використання даних сполук у сільськогосподарській практиці ряду країн. Україна до переліку цих країн не входить. Виходячи з того, що препарати даного класу вільно продаються і безконтрольно використовуються, відповідні діючі речовини постійно присутні в агроценозах, та їх залишкові (не летальні) концентрації можуть впливати на життєдіяльність і репродукцію комах, причому як шкідників, так і корисних або нейтральних. Дане дослідження є експериментальним порівняльним аналізом впливу залишкових концентрацій інсектицидів неонікотиноїдного ряду і нікотинової кислоти на особливості розмноження дрозофіли. Результати експерименту показали, що нікотинова кислота чинить подібний за напрямком (пригнічення), але менш сильний (у порівнянні з використаними інсектицидами) вплив на плодючість і життєздатність генетично різних ліній дрозофіли. Виявлено генотип-залежні ефекти досліджених домішок на смертність на стадії лялечки. Встановлено також, що серед нащадків особин, які пережили вплив, частка таких, які доживають до личинкової стадії, збільшується у порівнянні з контрольною групою. Іншими словами, залежно від генотипу тривалий (в онтогенезі) вплив неонікотиноїдів і нікотинової кислоти в низьких концентраціях може зсувати добір у потомстві особин, які пережили вплив, з ранньої ембріональної стадії у бік батьківських особин (тобто впливати на гаметогенез і життєздатність гамет).

Ключові слова: *дрозофіла, інсектициди, неонікотиноїди, нікотинова кислота, залишкові концентрації, плодючість, життєздатність, смертність на стадії лялечки, ембріональна смертність, добір.*

Larval exposure to low concentrations of neonicotinoids and niacin affects *Drosophila melanogaster* indices of reproductive success

V.A.Kovach, N.S.Filiponenko, N.V.Kolot, N.Ye.Volkova

Since the discovery, neonicotinoids have been considered as the most promising compounds with insecticidal activity due to the specific effect on nicotinic acetylcholine receptors of insects and low toxicity for mammals in the applied concentration ranges, and also because of the lack of resistance formation to them in insects. Neonicotinoids introduction into agricultural practice has resulted in significant increase of different crops yields. However, with the active widespread use of this class of insecticides, a number of negative consequences have been identified, in particular for pollinating insects. The detection of these effects led to a significant restriction or ban on these compounds use in agricultural practices in a number of countries. Ukraine is not among them. Considering that insecticides of this class are freely sold and their use is uncontrolled, the corresponding active substances are constantly present in agroecosystems, and their residual (non-lethal) concentrations can affect the viability and reproduction of insects, both harmful and useful or neutral. This study is an experimental comparative analysis of the effect of residual concentrations of neonicotinoid insecticides and nicotinic acid on *Drosophila* reproduction. The results of the study have shown that nicotinic acid has a similar (inhibition), but less strong (in comparison with the insecticides used) effect on the fertility and viability of genetically different *Drosophila* stocks. The genotype-dependent effects of the studied compounds on the lethality at pupa stage have been revealed. It has also been established that among the offspring of individuals who survived under the effect of neonicotinoids and niacin, the proportion of those who survive to the larval stage increases in comparison with the control group. Therefore, depending on the genotype, the long-term (in ontogenesis) effects of neonicotinoids and nicotinic acid at low concentrations may shift the selection in the offspring of exposed individuals from the early embryonic stage towards the parents (i.e., affect gametogenesis and viability of gametes).

Key words: *drosophila, insecticides, neonicotinoids, nicotinic acid, residual concentrations, fertility, viability, lethality at pupa stage, embryonic lethality, selection.*

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Изменения репродуктивных показателей *Drosophila melanogaster* при воздействии низких концентраций неоникотиноидов и ниацина на личиночной стадии

В.А.Ковач, Н.С.Филипоненко, Н.В.Колот, Н.Е.Волкова

С момента открытия неоникотиноиды считались наиболее перспективными соединениями с инсектицидной активностью в силу специфического действия на никотиновые ацетилхолиновые рецепторы именно насекомых и низкой токсичности для млекопитающих в используемых диапазонах концентраций, а также в связи с отсутствием формирования устойчивости к ним у насекомых. Введение неоникотиноидов в сельскохозяйственную практику позволило существенно повысить урожайность различных культур. Однако при активном повсеместном использовании данного класса инсектицидов был выявлен целый ряд негативных последствий, в частности для насекомых-опылителей. Обнаружение данных эффектов привело к существенному ограничению или к запрету использования данных соединений в сельскохозяйственной практике ряда стран. Украина в число этих стран не входит. Исходя из того, что препараты данного класса свободно продаются и бесконтрольно используются, соответствующие действующие вещества постоянно присутствуют в агроценозах, и их остаточные (не летальные) концентрации могут влиять на жизнедеятельность и репродукцию насекомых, причём как вредителей, так и полезных или нейтральных. Данное исследование представляет собой экспериментальный сравнительный анализ влияния остаточных концентраций инсектицидов неоникотиноидного ряда и никотиновой кислоты на особенности размножения дрозофилы. Результаты эксперимента показали, что никотиновая кислота оказывает сходное по направлению (угнетение), но менее сильное (по сравнению с использованными инсектицидами) действие на плодовитость и жизнеспособность генетически различных линий дрозофилы. Выявлены генотип-зависимые эффекты исследованных добавок на смертность на стадии куколки. Установлено также, что среди потомков особей, переживших воздействие, доля доживающих до личиночной стадии повышается по сравнению с контрольной группой. Другими словами, в зависимости от генотипа длительное (в онтогенезе) воздействие неоникотиноидов и никотиновой кислоты в низких концентрациях может смещать отбор в потомстве особей, переживших воздействие, с ранней эмбриональной стадии в сторону родительских особей (т.е. влиять на гаметогенез и жизнеспособность гамет).

Ключевые слова: *дрозофила, инсектициды, неоникотиноиды, никотиновая кислота, остаточные концентрации, плодовитость, жизнеспособность, смертность на стадии куколки, эмбриональная смертность, отбор.*

Introduction

Different species of phytophagous insects and other pests destroy crops, transmit plant diseases and compete for resources in various agro- and ecosystems affecting crop production that results in considerable yield losses (Tonnang et al., 2017). The search for unusual chemical structures for insect control optimization revealed neonicotinoids as a new class of insecticides (Tomizawa, Casida, 2009) with relatively low toxicity for mammals and environment. This class of systemically acting neurotoxic compounds is uptaken by plants, mainly through the roots, and is transmitted to all plant parts through xylema and phloema (Bromilow et al., 1990). This systemic property together with very high toxicity to insects enabled to propose neonicotinoids for protection of the whole plant from pest insects.

Neonicotinoids target the cholinergic system of insects' central nervous system (CNS) (Yamamoto, Casida, 1999). The nicotinic acetylcholine receptors (nAChR) playing a central role in rapid cholinergic synaptic transmission (Sattelle, 1980; Sattelle, Breer, 1990) is an important target site. Neonicotinoids act mainly agonistically on nAChRs on the postsynaptic membrane, mimicking acetyl choline (Ach) by binding with high affinity (Buckingham et al., 1997; Matsuda et al., 2005) and inducing neuronal hyper-excitation, which can lead to the insect's death within minutes (Belzunces et al., 2012; Palmer et al., 2013; Tomizawa, Casida, 2005). The vertebrate nervous system nAChR binding sites differ from those in insects, and in general they have lower numbers of such receptors with high affinity to neonicotinoids, that makes neonicotinoids to show selective toxicity for insects in comparison with vertebrates (Tomizawa, Casida, 2005; Casida, 2010; Liu et al., 2010).

Although neonicotinoids are still successful in control of many insect species, their popularity has imposed a mounting natural selection pressure for increased resistance to neonicotinoids, and in several species resistance has now reached levels that compromise the effect. Resistance to neonicotinoids (imidacloprid) can arise either through nAChR subtypes expression, detoxification mechanisms and/or structural alterations of target-site proteins (Thany, 2010). However, experimental data on this problem are limited. For example, Chao, Casida (1997), Yamamoto, Casida (1999), Sheets (2002) have

demonstrated that low doses of neonicotinoids do not cause reproductive or developmental toxicity. For target-site resistance, field-evolved mutations have only been characterized in two aphid species. So, metabolic resistance appears much more common, with the enhanced expression of one or more cytochrome P450s frequently reported in resistant strains (Bass et al., 2015).

From the other hand, with the active widespread use of this class of insecticides, a number of negative consequences have been identified, in particular for pollinating insects (Moffat et al., 2016; Tomizawa, Yamamoto, 1992; Barbara et al., 2008; Shi et al., 2017; Di Prisco et al., 2013; Henry et al., 2012). The detection of these effects led to a significant restriction or ban on these compounds use in agricultural practices in a number of countries. Ukraine is not among them. Considering that insecticides of this class are freely sold and their use is uncontrolled, the corresponding active substances are constantly present in agroecosystems, and their residual (non-lethal) concentrations can affect the viability and reproduction of insects, both harmful and useful or neutral. This study is an experimental comparative analysis of the effect of residual concentrations of neonicotinoid insecticides and nicotinic acid on *Drosophila* reproduction.

Materials and methods

Two *Drosophila melanogaster* stocks were used to carry out the experiment: the wild type stock *Canton-S* (*C-S*) and the mutant one – *knirps* (*kni*^{ri}) from the Collection of drosophila stocks of Genetics and Cytology Department of V.N.Karazin Kharkiv National University that is among objects that constitute National Heritage of Ukraine. The gene *knirps* [*radius incompletus*] (*kni*^{ri}) occupies the 3-47.0 locus (http://flybase.org/reports/FBgn0001320). The normal allele of this gene ensures the formation of a complete radial wing vein, and *kni*^{ri} mutation interrupts the vein, dividing it into two fragments: proximal and distal (Vasilyeva, 2005). The mutation is characterized by 100% penetrance and highly variable expressivity, which, apparently, is controlled by several systems of modifier genes (Vasilyeva, 1984). The *kni*^{ri} stock used in the study was synthesized by N.S.Filiponenko from the original *st ri* stock using a system of crosses with the balancer stock *CyO/Pin; Ly/TM3*. The stock used here is characterized by stable phenotypic manifestation: the proximal part of radial wing vein presence only. The symbol *ri* will be used further to mark this stock through the text.

Insecticides thiamethoxam (MW 291.71) (3-[(2-Chloro-1,3-thiazol-5-yl) methyl]-5-methyl-N-nitro-1,3,5- oxadiazinan-4-imine) – ACTARA® (dry powder) – and acetamiprid (MW 222.676) (N-[(6-chloro-3-pyridyl)methyl]-N'-cyano-N-methylacetamidine) – MOSPILAN (dry powder), as well as NICOTINIC ACID – pyridine-3-carboxylic acid (MW 123.111) (in a solution) were used for the experiment. Actara is an insecticide used for cultivated plants protection from a complex of sucking and leaf-eating pests. Hazard category: according to FAO classification thiamethoxam consider to be moderately hazardous to humans (WHO class III). Route of entry: contact and oral. Manufacturer: Syngenta TM. Packing: bag 0,004 kg. Mospilan is a systemic insecticide of a broad-spectrum of action. Hazard category: according to US EPA acetamiprid has been classified as an "unlikely" human carcinogen, causing generalized, nonspecific toxicity in mammals, and did not appear to have specific target organ toxicity (https://www3.epa.gov/pesticides/chem_search/reg_actions/registration/fs_PC-099050_15-Mar-02.pdf). Route of entry: contact and oral. Manufacturer: Nippon Soda Co., Ltd., Japan. Packing: soluble powder, available in foil bags of 2.5 g. Nicotinic acid (niacin) is water-soluble vitamin of the B complex required for the formation of coenzymes NAD and NADP (The vitamins …, 2007). Manufacturer: PrJSC "Pharmaceutical Firm "Darnitsa". Packing: solution for injection in vacuum sealed ampoules (10 mg/ml).

Neonicotinoids (Actara, Mospilan) and niacin were added to the culture medium (fed to the larvae), so that the final concentrations of supplements in the medium were 0.005 mg/ml and 0.01 mg/ml respectively. The need to use different concentrations is caused by the lethal effect of higher concentrations of neonicotinoids.

For each stock, the experiment was carried out according to the following scheme: from the collection, the parental pairs (P) of each stock were randomly selected for each studied group: control – offspring developed on a standard medium; experimental – the offspring developed on a medium with the addition of "Actara" (actara), "Mospilan" (mospilan) or "Nicotinic acid" (niacin). Parental individuals were placed in tubes with medium supplied with appropriate tested compounds (5 ml). In the offspring (F1) the number of individuals surviving to the stage of the pupa (fertility; number of individuals), the death rate during metamorphosis (pupa lethality; %), and the number of individuals surviving to the stage of adults (viability; number of individuals) were recorded. For this virgin females and males of each experimental

group (in the age of 3 days) were placed in test tubes with medium in the amount of 2♀ and 2 ♂. The egg laying period was 7 days. Then the parents were removed. For each experimental group we analyzed 5–10 tubes in parallel, the data on which were averaged. The analysis of changes occurred in gametes of individuals survived in the presence of neonicoticotinoids or niacin in the medium was carried out according to their progeny (F2). That is imagoes F1 were used as parents to assess embryonic mortality. The offspring F2 in all cases developed on a standard (minimal) medium.

As a criterion for changes occurring in gametes of imagoes, the frequency of dominant lethal mutations (DLM) at early stages of embryogenesis was used (Tikhomirova, 1990).The frequency of DLM was defined as the percentage of eggs that stopped their development at a certain stage to the total number of eggs laid. To perform the experiment, virgin imagoes F1 were separated according to their sex within the 1st day after eclosion and were kept separately until sexually mature age (three days) in vials with temporary culture medium. Then males and females were put together for 12 hours for mating. After that inseminated females were placed in Petri dishes (d=10 cm) with temporary medium in an amount of 10 individuals per dish for 8 hours to obtain eggs. After the time, egg production was counted. Accounting was carried out with stereoscopic microscope (Delta Optical NTX-3C). Then the eggs were placed in a thermostat (t=23ºC) for 48 hours. After the time, the DLM level was recorded according to the following parameters: white eggs – early embryonic lethality (the first 6–9 hours of embryonic development) – eDML; yellow and brown – late embryonic lethality – lDLM. For each variant of the experiment, 5–15 measurements were performed. The term of egg development arrest was confirmed by comparison with the standard photos of different stages of *D. melanogaster* embryonic development (Bownes, 1975). For this purpose, from each Petri dish, separately white and yellow eggs were collected on the single concave microscope slides in a 3% solution of sodium hypochlorite (NaOCl), which provides rapid dechorionization of the embryo. The analysis of embryos was carried out using the microscope "Konus" (at magnification of ×400) (Kostenko et al., 2015). The time of egg development arrest was confirmed by microscopic studies for all undeveloped embryos.

For fertility and viability indicators the arithmetic means, standard deviations, and standard errors were calculated. The effect of neonicotinoids and niacin on these indicators of studied stocks was established using a quantitative analysis of variance (ANOVA). The effects of the genotype and the presence of supplements in the culture medium on the levels of pupa, early and late embryonic mortality were established by two-way analysis of variance for qualitative characteristics (Plokhinsky, 1969). Calculations were carried out using Microsoft Excel and Statistica 6.0 software.

Results and discussion

The results of the experiment show that fewer individuals of both used drosophila stocks survived till pupa stage (Fig. 1) under the influence of supplements to the medium studied in concentrations under investigation. Anyway, initial differences in fertility between stocks (higher fertility in *C-S*; lower – in *ri* – Fig. 1, control) are observed in all experimental variants, even they become more distinct. The most pronounced reduction in fertility is observed in the case of larval development in culture medium supplied with actara (0.005 mg/ml). Mospilan (0.005 mg/ml) seems to be less effective. And in the case of *C-S* stock its effect does not differ from niacin one. On the contrary, in the case of *ri* stock it is almost as effective as actara. The effect of niacin is the weakest among supplements studied in spite of concentration being the highest (0.01 mg/ml).

The results of ANOVA corroborate significant genotype dependent ($F=75.33$; $p<0.001$) effect of supplement presence in culture medium ($F=29.05$; $p<0.001$), and reveal the interaction of two factors ($F=3.22$; $p<0.05$).

Surely, actively feeding larvae are affected by various chemical compounds used to protect plants, especially those of contact mechanism of action and due to oral consumption. So, the most active selection might take place within individuals at this stage; especially while the first contact with the agent. Still, applying various insecticides one should take into account different susceptibility (survivability) of individuals of different genotypes.

On the contrary to fertility index, the initial level of pupa lethality does not differ significantly between *D. melanogaster* stocks used when larvae develop in standard culture medium (Fig. 2). Both analyzed neonicotinoids demonstrate similar pronounced effect – rise of pupa lethality index in *C-S* stock, but not in *ri* stock. Niacin induces pupa lethality in *ri* stock but not in *C-S* stock. These results show that neonicotinoids-mediated effects at larva-pupa period was more pronounced in *ri* stock.

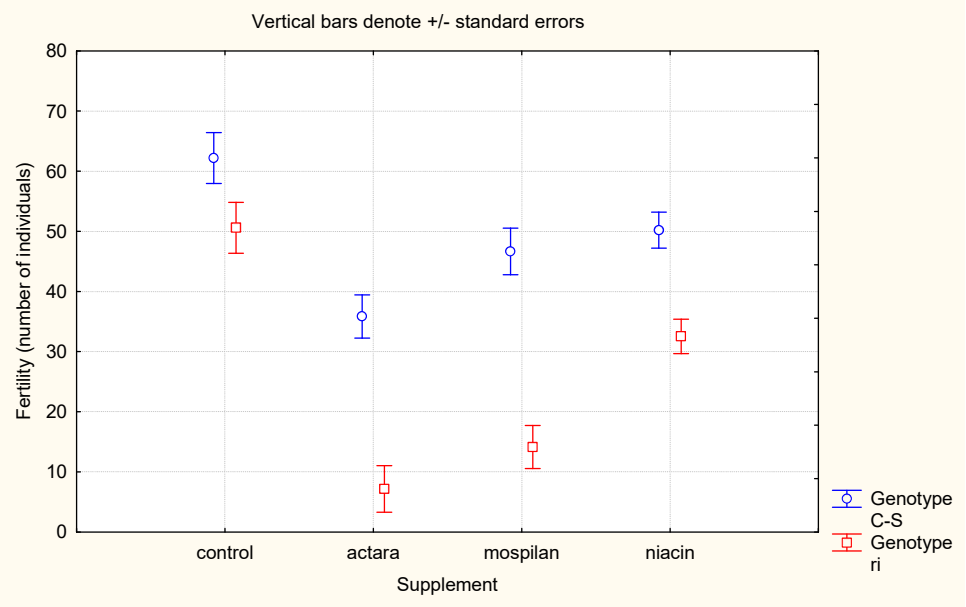

Fig. 1. The genotype dependent effects of larval exposure to low doses of neonicotinoids and niacin on *Drosophila melanogaster* fertility (blue, circles – *C-S*; red, squares – *ri*)

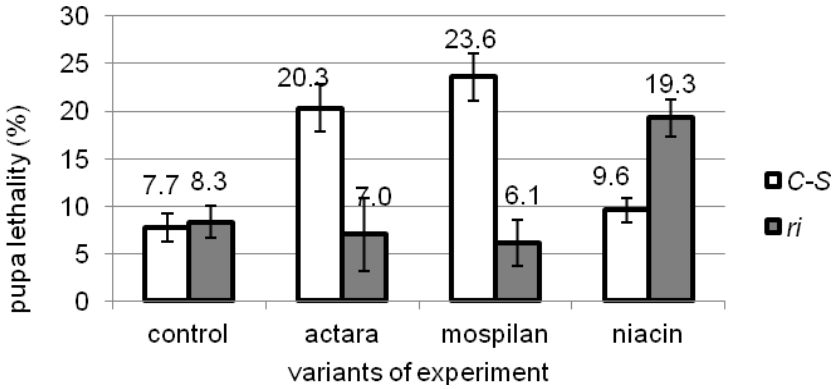

Fig. 2. The genotype dependent effects of larval exposure to low doses of neonicotinoids and niacin on *Drosophila melanogaster* pupa lethality

The results of two-way ANOVA for qualitative characteristics of *Drosophila melanogaster* pupa lethality index under larval exposure to low doses of neonicotinoids and niacin prove that existing variability of the index depends on the genotype ($F=16.35$; $p<0.001$), on the presence of supplement in culture medium ($F=6.26$; $p<0.001$), as well as on these two factors interaction ($F=24.27$; $p<0.001$).

The trend we observe for fertility index (Fig. 1) is kept for the viability of stocks studied (Fig. 3–5), although the analysis of sex-specific viability reveals (compare Fig. 3 and 4) that there are strong genotype-dependent differences in females' viability (the index for *C-S* stock is higher than for *ri*) but not in males' one when larvae develop in the standard culture medium. Under the influence of supplements used these differences become more pronounced, especially in females. We also can say that drosophila males of both stocks used tend to be less viable if being a larva they were subjected to low doses of neonicotinoids and niacin.

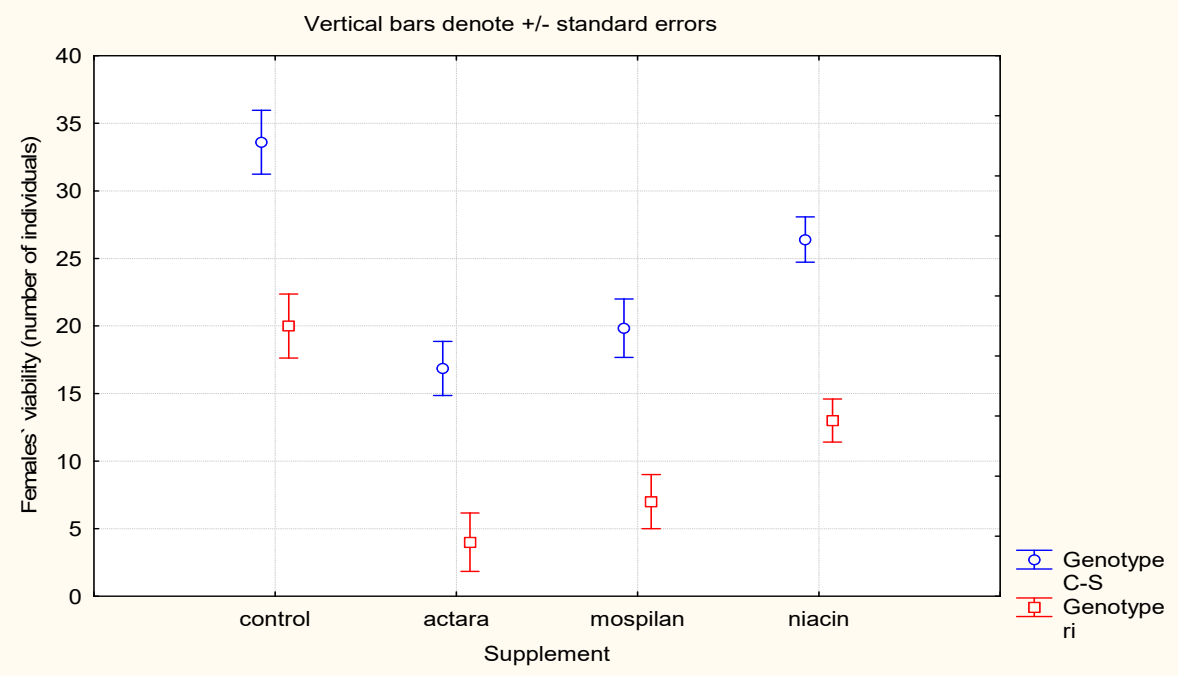

Fig. 3. The genotype dependent effects of larval exposure to low doses of neonicotinoids and niacin on *Drosophila melanogaster* females' viability (circles – *C-S*; squares – *ri*)

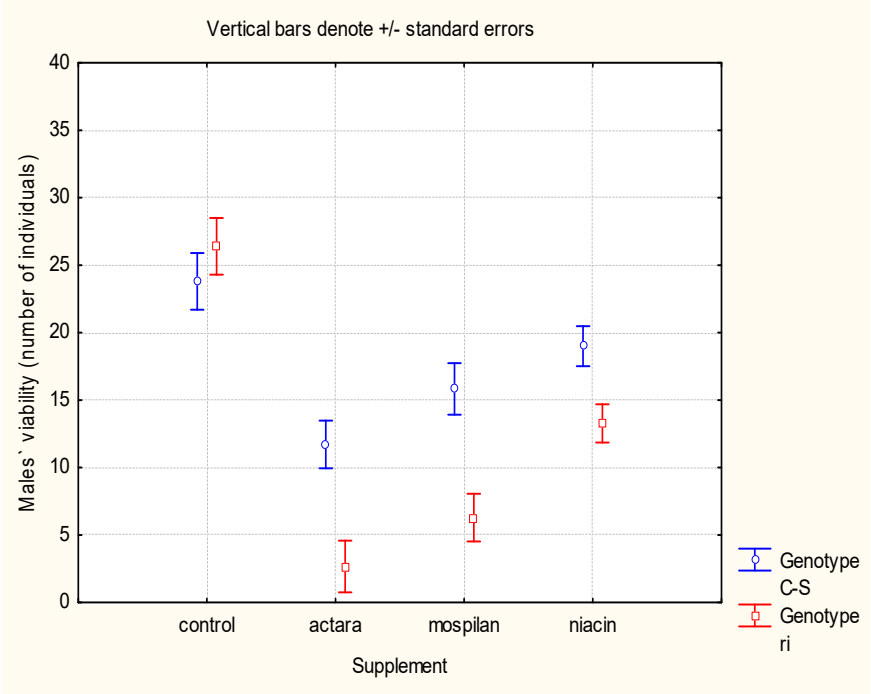

Fig. 4. The genotype dependent effects of larval exposure to low doses of neonicotinoids and niacin on *Drosophila melanogaster* males' viability (circles – *C-S*; squares – *ri*)

The results of ANOVA corroborate significant genotype dependent ($F=81.99$; $p<0.001$) effect of supplement presence in culture medium ($F=21.77$; $p<0.001$) on females' viability, as well as on males'

one (the genotype effect – F=17.74; p<0.001; the supplement presence effect – F=30.68; p<0.001). We also observe the effect of two factors interaction (F=3.88; p<0.05) on males' viability.

The changes of general index of viability (Fig. 5) completely reflect fertility index changes with the significant effect of genotype (F=59.19; p<0.001) and supplement presence in the culture medium (F=33.08; p<0.001).

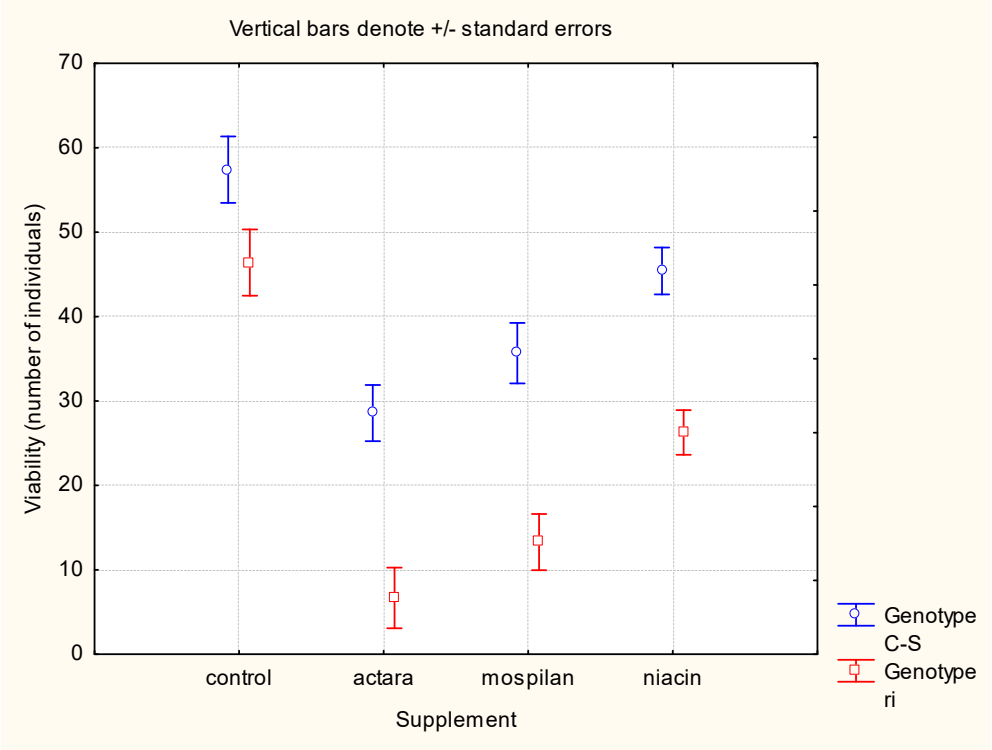

Fig. 5. The genotype dependent effects of larval exposure to low doses of neonicotinoids and niacin on *Drosophila melanogaster* imagoes viability (circles – *C-S*; squares – *ri*)

It should be said that both fertility and viability reduction is a typical nonspecific reaction of laboratory *Drosophila* stocks on diet changes, especially on the excess of the supplement (see, for example, Volkova et al., 2010, 2013). Still, among various compounds examined by our research group the effect of neonicotinoids is the most pronounced.

Survivability of F2 progeny from individuals developed in the presence of neonicoticotinoids or niacin in the medium was analyzed according to the effectiveness of larva hatching from eggs (embryonic lethality indexes) (Fig. 6, 7). According to the results obtained there is a strong effect of genotype both on early (F=27.55; p<0.001) and late embryonic lethality (F=909.21.55; p<0.001). Initially *C-S* and *ri* stocks are contrast in these indexes: *C-S* stock is characterized by rather low level of eDLM (Fig. 6) but relatively high level of lDLM (Fig. 7); on contrary, *ri* stock is characterized by high level of eDLM (Fig. 6) and low level of lDLM (Fig. 7). Parents' development in the culture mediums supplied with neonicotinoids or niacin raised slightly the level of eDLM among *C-S* offspring but reduced significantly the level of eDLM among *ri* offspring (Fig. 6). The effect on lDLM depends on supplement type (Fig. 7). For example under actara supplement we observe the reduction of index in both stocks that is more pronounced in *C-S* one. Mospilan appears to have no effect on this index in concentration used. While parents' development in the culture medium supplied with niacin tends to result in index reduction among *C-S* offspring but rises it significantly among *ri* offspring. The two-way ANOVA for qualitative characteristics proved the effect of supplement as well as the combined effect of both controlled factors on early and late embryonic lethality indexes. Therefore, depending on the genotype, the long-term (in ontogenesis) effects of neonicotinoids and nicotinic acid at low concentrations may shift the selection in the offspring of exposed individuals from the early embryonic stage towards the parents (i.e., affect gametogenesis and viability of gametes).

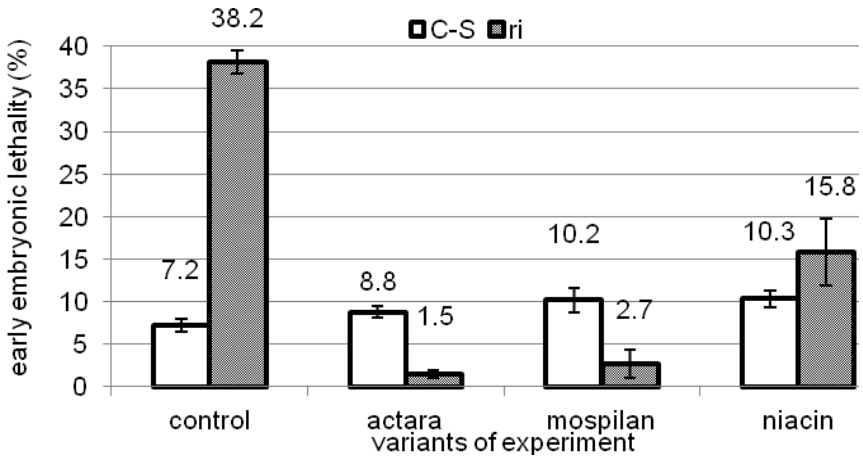

Fig. 6. The genotype dependent effects of larval exposure to low doses of neonicotinoids and niacin on *Drosophila melanogaster* early embryonic lethality

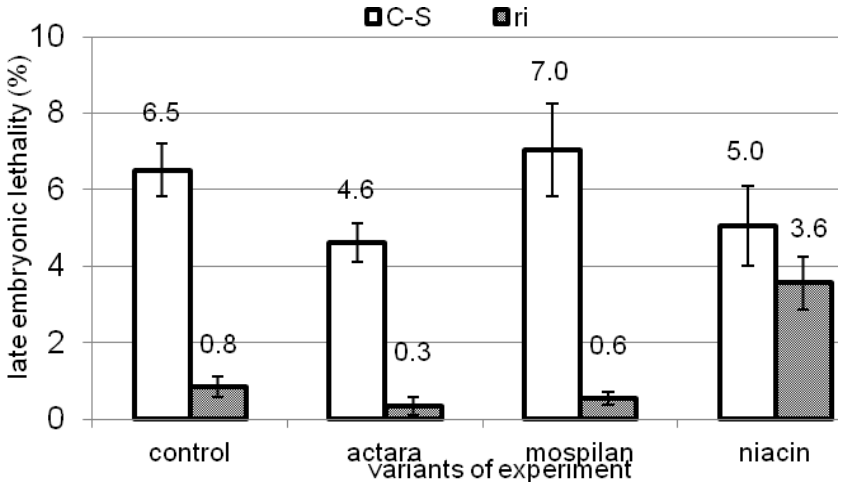

Fig. 7. The effects of larval exposure to low doses of neonicotinoids and niacin on *Drosophila melanogaster* late embryonic lethality

The genotype-determined differences can be explained from the point of view of nucleotides substitutions in nAChR subunit genes that can affect receptor-ligand affinity. Genome studies have identified 11 nAChR subunit genes in the honeybee (Jones et al., 2007, 2006), compared with 10 each in *D. melanogaster* (Jones et al., 2007). There are core nAChR subunits conserved between various insect species with over 60% of amino acid sequences homology (Jones et al., 2007; Sattelle, 2009). However, at least one divergent subunit in the fruit fly, mosquito and honeybee has less than 20% homology (Sattelle, 2009). In *D. melanogaster* genes encoding the ligand-binding α-type (Dα1, Dα2, Dα3, Dα4, Dα7) subunits and for the structural *β*-type subunits (Dβ1 and Dβ2) have overlapping distributions in various regions of the nervous system. However, expression of homomeric receptors with either the D*α*1 (ALS) or D*α*2 (SAD) alone does not generate a functional receptor and *Drosophila β*-type (ARD and SBD) subunits do not contribute to functional receptor expression. Coexpression of the *Drosophila α*- and *β*-type subunits in various combinations does not produce any electrophysiological or biochemical response. At present, the functional receptor with ion channel property and/or ligand binding activity can be generated only when any of the three *α*-type subunits is coexpressed with the vertebrate (chick or rat) *β*-type subunit. These results strongly suggest the importance of the *β*- or non-*α*-type subunit and the

heterooligomeric status of the native *Drosophila* nAChR with possible involvement of unidentified subunit(s) (Lansdell, Millar, 2000).

Conclusions

The results of the experiment showed that niacin has a similar effect (reduction of indexes) on the fertility and viability of the genetically different *Drosophila* stocks in comparison with the neonicotinoids used, but the effect of neonicotinoids is more pronounced. The genotype-dependent effects of the studied supplements on pupa mortality were also established. Depending on the genotype, low concentrations of neonicotinoids and niacin may enhance selection processes at feeding (larva) and metamorphosis (pupa) stages of insect development that will result in better survivability of embryos formed by imagoes survived under the pressure of such factor. Therefore, the formation of insects' resistance to neonicotinoids is possible in agroecosystems.

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Представлено: Т.О.Єлецька / Presented by: T.O.Yeletska
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