



SERVIÇO PÚBLICO FEDERAL
UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

ASSOCIAÇÃO ENTRE CONCENTRAÇÕES DE PYRIPROXYFEN E SPINOSAD
COM BAIXA ECOTOXICIDADE COMO ESTRATÉGIA SUSTENTÁVEL PARA
PROGRAMAS DE CONTROLE DE *Aedes aegypti*

Aluno: Vanessa Santana Vieira Santos

Orientador: Prof. Dr. Boscolli Barbosa Pereira

UBERLÂNDIA - MG
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PROGRAMAS DE CONTROLE DE *Aedes aegypti***

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Tese apresentada à Universidade Federal de Uberlândia como parte dos requisitos para obtenção do Título de Doutor em Genética e Bioquímica (Área Genética)

**UBERLÂNDIA - MG
2023**



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Título do Trabalho:	Associação entre concentrações de pyriproxyfen e spinosad com baixa ecotoxicidade como estratégia sustentável para programas de controle de <i>Aedes aegypti</i> .				
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Aos vinte e três dias do mês de janeiro de dois mil e vinte e três, às 08:30 horas, reuniu-se via web conferência pela Plataforma *Microsoft Teams*, em conformidade com a Portaria nº 36, de 19 de março de 2020 da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES, Resolução de nº06/2020 e Resolução nº 19/2022 do Conselho de Pesquisa e Pós-graduação pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Genética e Bioquímica, assim composta: Dr. Dão Pedro de Carvalho Neto, Dr. Dieferson da Costa Estrela, Dr. Luis Paulo Pires, Dr. Jean Ezequiel Limongi e Dr. Boscolli Barbosa Pereira, orientador da candidata e demais convidados presentes conforme lista de presença. Iniciando os trabalhos, o presidente da mesa, Dr. Boscolli Barbosa Pereira apresentou a Comissão Examinadora e a candidata, agradeceu a presença do público e concedeu à discente a palavra para a exposição do seu trabalho. A duração da apresentação da discente e o tempo de arguição e resposta foram conforme as normas do Programa de Pós-graduação em Genética e Bioquímica. A seguir o senhor presidente concedeu a palavra, pela ordem sucessivamente, aos examinadores, que passaram a arguir a candidata. Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais. Em face do resultado obtido, a Banca Examinadora considerou a candidata:

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Esta defesa de Tese de Doutorado é parte dos requisitos necessários à obtenção do título de Doutor. O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU. Nada mais havendo a tratar foram

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Ao meu pai, Roberto, e à minha mãe,
Mariza.

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Have the courage to follow your heart and intuition. They somehow already know what you truly want to become.

(Steve Jobs)

LISTA DE TABELAS

CAPÍTULO I

Table 1. Physical and chemical properties of spinosyn A and spinosyn D.....	07
Table 2. Toxicity effect values of spinosad on different living species.....	09
Table 3. Research reports about spinosad applications for pest control.....	12

CAPÍTULO II

Table 1. Physical and chemical properties of natural larvicides used against <i>Aedes aegypti</i>	23
Table 2. Origin and mode of action of natural larvicides used against <i>Aedes aegypti</i>	23
Table 3. Toxicity effect values of natural larvicides on <i>Aedes aegypti</i> larvae....	24
Table 4. Summary of lineage strains of <i>Aedes aegypti</i> developed through genetic engineering methodologies.....	27

CAPÍTULO III

Table 1. Structural and physicochemical properties of spinosad.....	35
Table 2. Ecotoxicological parameters (95% confidence interval) and environmental risk obtained from acute, chronic and reproduction tests with <i>D. magna</i> after exposure to different concentrations of spinosad.....	38
Table 3. Reproduction parameters of <i>D. magna</i> after exposure to spinosad (5 mg/L) and control during 28 days.....	38

CAPÍTULO IV

Table 1. Technical information about larvicides tested.....	44
Table 2. <i>Daphnia magna</i> Reproduction Test parameters observed following long-term exposure (28 days) to tested mixture of low concentrations of spinosad and pyriproxyfen.....	45

Table 3. Lethality, behavior and morphological alterations in *Aedes aegypti* larvae (L3) after exposure to different dilution of the spinosad and pyriproxyfen mixture..... 45

LISTA DE FIGURAS

CAPÍTULO I

Fig 1. Structure of (a) spinosyn A and (b) spinosyn D. Spisonyn D has a methyl side group on C6.....	07
---	----

CAPÍTULO III

Fig 1. Spinosad concentrations (residual concentration) measured daily from exposed water in containers.....	37
---	----

Fig. 2. Acute toxicity of spinosad on 3rd instar <i>Aedes aegypti</i> larvae and <i>Daphnia magna</i> after a 48 h exposure.....	37
---	----

Fig. 3. Residual efficacy of spinosad on inhibition of pupal emergence of <i>Aedes aegypti</i> larvae.....	37
---	----

Fig. 4. Number (mean \pm SE) of living adults and neonates exposed (A) to low concentration of spinosad (5 mg/L) and non-exposed (control) during 21days observation period.....	38
---	----

CAPÍTULO IV

Fig 1. Inhibition of adult emergence during long-term exposure (60 days) of <i>Aedes aegypti</i> to the spinosad and pyriproxyfen mixture.....	45
---	----

Fig. 2. Daily predominance of development and behavior stages during the long-term exposure of <i>Aedes aegypti</i> to the spinosad and pyriproxyfen mixture..	45
---	----

Fig. 3. Final predominance of development stages after the long-term (60 days) exposure of <i>Aedes aegypti</i> to the spinosad and pyriproxyfen mixture.....	46
--	----

SUMÁRIO

APRESENTAÇÃO.....	01
CAPÍTULO I	
Properties, toxicity and current applications of the biolarvicide spinosad.....	04
CAPÍTULO II	
Low toxicity and high efficacy in use of novel approaches to control <i>Aedes aegypti</i>	20
CAPÍTULO III	
Evaluation of toxicity and environmental safety in use of spinosad to rationalize control strategies against <i>Aedes aegypti</i>	34
CAPÍTULO IV	
Association of low concentrations of pyriproxyfen and spinosad as an environment-friendly strategy to rationalize <i>Aedes aegypti</i> control programs....	42
CONCLUSÕES GERAIS.....	48

APRESENTAÇÃO

A dengue é uma doença infecciosa viral, cuja incidência global cresceu drasticamente nas últimas décadas. A arbovirose é caracterizada como um grave problema de saúde pública, contudo, os programas de saúde adotam como principal estratégia o uso intensivo de inseticidas para o controle dos mosquitos vetores.

Nas Américas, os vírus da dengue são transmitidos pela fêmea do mosquito *Aedes aegypti*. Apesar da relativa eficácia no controle do mosquito vetor, os pesticidas químicos são compostos recalcitrantes e poluentes, de modo que seu uso intensivo afeta não somente organismos-alvo, mas também provoca efeitos em espécies não-alvo.

Nesse sentido, este estudo teve como objetivo avaliar a viabilidade da associação entre o pesticida pyriproxyfen e o biolarvicida spinosad, ambos em concentrações testadas de baixa ecotoxicidade, como estratégia sustentável para programas de controle de *Aedes aegypti*.

A partir de extensa e crítica revisão da literatura, definimos como hipótese de estudo que a associação entre os larvicidas pyriproxyfen e spinosad tem efeito sinérgico na toxicidade específica para *A. aegypti*, de maneira que, sendo usados em baixas concentrações, não afetariam organismos não-alvo.

O texto desta tese foi desenvolvido em quatro capítulos. O Capítulo I consiste em uma revisão crítica de literatura sobre as propriedades, aplicações e toxicidade do biolarvicida spinosad, discutindo sua efetividade no controle de insetos e em intervenções de saúde pública.

O Capítulo II, também um artigo de revisão crítica, descreve e avalia abordagens seguras e ecologicamente corretas visando o combate ao *Aedes aegypti*, examinando propriedades e efeitos toxicológicos dos inseticidas, bem como discutindo tecnologias promissoras na busca por tratamentos eficazes e seguros contra o vetor.

Fundamentado pelos Capítulos I e II, no Capítulo III, avaliamos a toxicidade e a segurança ambiental do uso do spinosad, com o intuito de definir estratégias de controle do *Aedes aegypti*. Já no Capítulo IV, investigamos os efeitos da

combinação de baixas concentrações de spinosad e pyriproxyfen como alternativa sustentável de combate ao mosquito.

Os Capítulos I e II foram escritos no formato de artigo de revisão, em língua inglesa, e publicados no periódico *Journal of Toxicology and Environmental Health – Part B: Critical Reviews*. Os Capítulos III e IV foram escritos no formato de artigo científico experimental, em língua inglesa, e publicados no periódico *Chemosphere*.

CAPÍTULO I

ARTIGO DE REVISÃO

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Vanessa Santana Vieira Santos & Boscolli Barbosa Pereira

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Properties, toxicity and current applications of the biolarvicide spinosad

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ABSTRACT

Characterized as a highly valuable bioactive natural product, spinosad is a pesticide with a complex chemical structure, composed of spinosyn A and D, molecules synthesized by the actinomycete *Saccharopolyspora spinosa*. The larvicidal activity of spinosad was postulated to be a promising approach to combat crop pests and control species responsible to transmit mosquito-borne illness, including *Aedes aegypti*. Although initially deemed as relatively safe for non-target organisms and highly effective against insects and crop pests, recent studies focused on the toxicity profile detected the occurrence of side effects in different living species. Thus, the present review was undertaken to describe the properties and characteristics of spinosad. In addition to indicating potential adverse effects on living organisms, alternative uses of the biopesticide as a mixture with different compounds are provided.

KEYWORDS

Spinosad; spinosyn; toxicity; biopesticide; environmental health

Introduction

Spinosyns are metabolites generated from the aerial fermentation process of the Gram-positive filamentous bacteria *Saccharopolyspora spinosa*, an actinomycete initially isolated from a soil sample in the Caribbean Islands in 1982 (Guojun et al. 2016; Sparks et al. 1995). These molecules are defined as polyketide-macrolide compounds, composed by a single tetracyclic ring structure with two different sugars attached (Salgado 1998).

The interest in spinosyns emerged from their unique molecular structure, in addition to the potency against agriculturally relevant pests (Sparks, Crouse, and Durst 2001). In this manner, the closely related structurally spinosyn A and spinosyn D compose the natural pesticide spinosad. Spinosad is classified as an environmentally and toxicologically reduced risk compound (Williams, Valle, and Viñuela 2003). Aside from their chemical structure, spinosyns exhibit a favorable toxicological profile and also a different mode of action coupled with pesticidal efficacy (Bret et al. 1997; Salgado and Sparks 2005). It is worthwhile noting that spinosad has been suggested as a highly valuable bioactive natural product used as a pesticide

against a rich diversity of pests thus enhancing crop yields. There is an increasing interest in the use of environmentally friendly pesticides in integrated pest management procedures in order to promote the sustainability of farming systems and contributing to reducing the employment of persistent pesticides in selective control of pests (Cook 2000; Dent 2000).

Spinosad was initially commercially marketed in 1997 by Dow AgroSciences to be used as a control agent against lepidopterous pests in cotton (Salgado 1998; Thompson, Dutton, and Sparks 2000). Distinguished as an eco-friendly insecticide, it has been found to be a safe alternative owing to the low toxic actions on mammals and other non-target organisms, including insects and fish (Bacci et al. 2016; Khan 2018). Thus, the present review describes and highlights the properties, applications, and toxicity of the spinosad, emphasizing its importance for insect control in agriculture and in public health interventions.

History of spinosad

Historically, in the early 1980s, after an attempt for finding new fermentation products in non-

traditional organisms, an antibiotic screening performed at Lilly Research Laboratories (Indianapolis, USA) isolated a rare and unique actinomycete from soil collected from a sugar mill in the Virgin Islands (Mertz and Yao 1990; Salgado and Sparks 2010).

Actinomycetes are a heterogeneous group of gram-positive, high-GC content bacteria, generally anaerobic with a notable filamentous and branching growth pattern which results in an extensive colony or mycelium. Most species are prevalent in warm, aerobic soil and are harmless to higher plants and animals, while some are beneficial sources of bioactive natural products, such as antibiotics, anti-cancer compounds and immunosuppressants, including adriamycin and rapamycin, respectively (Hillel 2008; Tao et al. 2019). Consequently, the interest in the collected sample emerged due to the complex metabolites involved with an unusual activity (Mertz and Yao 1990).

Hence, taxonomic studies led to the discovery and characterization of the new species *Saccharopolyspora spinosa*, which differs from other organisms in the physiological properties and fatty acid composition attributed to the spiny spore sheath surface (Mertz and Yao 1990), and the active metabolite was termed by the generic name spinosyn, in order to correlate it with the producing microorganism (Kirst 2010; Thomson et al. 1995). Besides spinosyn A and spinosyn D, other natural spinosyns were discovered, and numerous synthetic analogs known as spinosoids synthesized (De Amicis et al. 1997; Salgado 1998).

In 1983, with the aim to explore inhibitors of mosquito larvae, an early stadium of *Aedes aegypti* larvae was incorporated in a 96-well microtiter plate assay with *Saccharopolyspora spinosa* culture broth, which enabled to record an insecticidal activity of spinosyn. Further, the screening also reported the specific activity and a strong antifeedant effect against the southern armyworm *Spodoptera eridania* coupled with mild activity on spider mites (*Tetranychus urticae*), corn rootworm (*Diabrotica undecimpunctata*) and on cotton aphid *Aphis gossypii*. In addition, the absence of antibacterial and antifungal activity also indicated that this culture possessed sensitive and selective effect (Mertz and Yao 1990; Thompson et al. 1995; Salgado and Sparks 2005; Kirst 2010).

Initially, spinosad was registered in 1997 in the United States and Korea, first formulated as a suspension concentrate for greenhouse and open-field spray application, commercialized as SpinTor Naturalyte[®]; Success Naturalyte[®] and Tracer Naturalyte[®]. Due to the potent activity against pivotal crop pests, spinosad was originally introduced by Dow AgroSciences for control lepidopterous insects in cotton crops (Salgado 1998).

Then, in 2008, spinosad was recognized as an essential insecticide for the management of key pests and as a contributor for the sustainability of the production system for crop-pest situations. As a consequence of its potent activity, the compound was incorporated in Annex II of the European Union Council Regulation 2092/91, receiving the authorization to be used in organic farming in European member states (Comission Regulation 2008).

Currently, spinosad is registered in more than 80 countries as an insecticide used to control Diptera, Lepidoptera, Coleoptera and Thysanoptera orders in agricultural, veterinary and forestry applications (Biondi, 2012). In Brazil, spinosad was also utilized as an alternative larvicide against the vector *Aedes aegypti*, hence controlling different life-threatening diseases.

Properties of spinosyns

Spinosad consists in the natural mixture of spinosyn A (C₄₁H₆₅NO₁₀) as majority and spinosyn D (C₄₂H₆₇NO₁₀) as the minor component at a typical ratio of 85:15 (A:D) in the final product (Thomson et al. 1995). Spinosyn A is the most abundant component synthesized in the fermentation of *S. spinosa*, followed by spinosyn D (Kirst 2010). Both compounds exhibit high molecular weight (731.9 and 745.9, respectively) and are characterized as relatively nonvolatile molecules (Vela et al. 2019).

Structurally, spinosyn A and spinosyn D differ only by the presence of a methyl group at C6 (Figure 1) (Waldron et al. 2000), but this impacts the physical properties, including solvent solubility values, as depicted in Table 1. In fact, spinosyns composed of a methyl group tends to be more active and less affected by alterations in the whole molecule in comparison to the

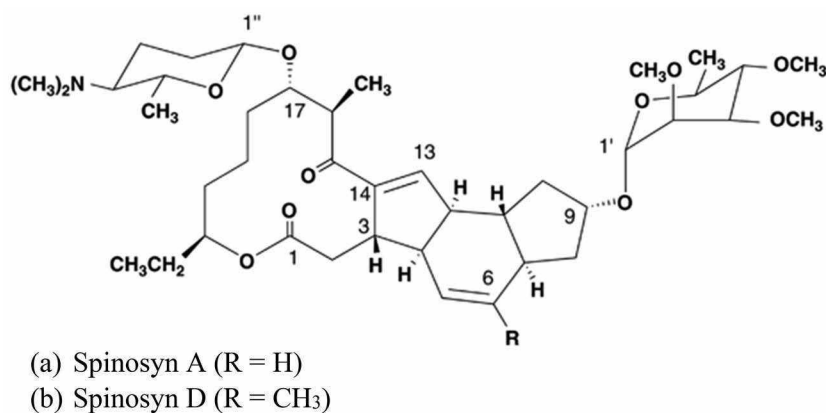


Figure 1. Structure of (a) spinosyn A and (b) spinosyn D. Spinosyn D has a methyl side group on C6.

Table 1. Physical and chemical properties of spinosyn A and spinosyn D.

Physical property	Spinosyn A	Spinosyn D
Molecular formula	C ₄₁ H ₆₅ NO ₁₀	C ₄₂ H ₆₇ NO ₁₀
Molecular weight	731.9	745.9
Melting point	120°C	170°C
Solubility (20°C)		
Water (pH 7)	235 mg/L	0.332 mg/L
Methanol	19.0 g/mL	0.252 g/mL
Acetone	16.8 g/mL	1.01 g/mL
Vapor pressure	3.0x10 ⁻¹¹ kPa	2.0x10 ⁻¹¹ kPa
Octanol/water partition coefficient (K _{ow})	4.01	4.53

corresponding analogs with only a hydrogen at C6 (Sparks, Crouse, and Durst 2001).

As such, the structure of spinosyns were elucidated by nuclear magnetic resonance spectroscopy, X-ray and mass spectrometry, and comprises a 21-carbon polyketide-derived macrocyclic lactones to which is attached a tetracyclic core with a group of D-forosamine on the C-17 hydroxyl group as the aminosugar moiety and tri-O-methyl-L-rhamnose on the C-9 hydroxyl group as a neutral saccharide moiety (Kirst 2010; Tao et al. 2019; Waldron et al. 2000). The members of the family of spinosyns differ from each other in varying degrees on the N- or O-methylation of the deoxysugars or C-methylation on the polyketide structure (DeAmicis et al. 1997; Kirst et al. 1992; Tao et al. 2019).

Mechanism of spinosad biosynthesis

Analysis of DNA sequencing and target gene disruptions revealed the spinosyn biosynthesis

pathway is encoded by 27 genes in a 74-kb region of the *Saccharopolyspora spinosa* genome, which are involved in the synthesis, modification, and attachment of the deoxysugars and in the modification of the macrolactone (Waldron et al. 2000). Specifically, five large genes (*spn A*, *B*, *C*, *D*, and *E*) are encoding a type I polyketide synthase (PKS) and four genes (*spnF*, *spnJ*, *spnL*, *spnM*) are responsible to convert the product of PKS. The genes *spnG*, *spnH*, *spnI*, and *spnK* are related to the rhamnose attachment and methylation; *spnP*, *spnO*, *spnN*, *spnQ*, *spnR*, and *spnS*, are involved in forosamine biosynthesis; and four genes (*ORFL15*, *ORF-L16*, *ORF-R1*, and *ORF-R2*) exerted no marked effect on spinosad biosynthesis (Xue et al. 2013).

Incorporation studies established that spinosyns are assembled through a polyketide pathway responsible to create the lactone nucleus. Further, prior to incorporation of the forosamine group, the rhamnose residue needs to be added to the aglycone, that subsequently form the pseudoaglycone. The propionate originates the C-methyl groups on the polyketide and the S-adenosylmethionine is responsible for the origin of the 2 N-methyl groups of forosamine and three O-methyl groups of 2,3,4-tri-O-methylrhamnose (Kirst et al. 1992; Waldron et al. 2000).

Studies indicated that rhamnose and forosamine are derived from the common intermediate NDP-4-keto-6-deoxy-D-glucose (Tao et al. 2019). Although the two genes required for the conversion of it to NDP-rhamnose and the other two essential for the production of this intermediate molecule from

glucose-1-phosphate are not present in the spinosyn biosynthetic cluster, these play a key role in the cell wall assembly and spinosyn production (Waldron et al. 2000). Despite this, the genes related to the rhamnose methylation and transfer and other ones involved in forosamine biosynthesis are located within the spinosyn gene cluster (Tao et al. 2019).

The polyketide group of spinosyns differs from common structures of type I polyketides, including erythromycin, rapamycin and tylosin, due to the presence of three intramolecular carbon-carbon bonds (Waldron et al. 2000). This fact suggests that the actinomycete *S. spinosa* may encode different enzymes to execute these reactions, indicating interesting questions regarding the biosynthesis of the compounds (Waldron et al. 2000).

The polyketide-derived macrolides of the molecule are synthesized from common building blocks, including acetyl-CoA, methylmalonyl-CoA, malonyl-CoA, and propionyl-CoA. It is of interest that enhancing the concentration of acetyl-CoA and malonyl-CoA improves the supply of ligand sugars and may contribute to the improvement of spinosad production (Tao et al. 2019). Further, Xue et al. (2013) noted that the polyketides production might be improved through the incorporation of fatty acids during the fermentation process, hence increasing the yield of spinosad production. Thus, the synthesis of spinosyn might be elevated through increased precursor levels with upregulated biosynthesis (Tao et al. 2019).

Mode of action (MOA) of spinosad

Spinosyns are highly active macrolides and act as pesticide after ingestion by the organisms or through contact. Consequently, these chemicals are able to produce rapid death in several insect species, including caterpillars, thrips and foliage-feeding beetles, an important strategy in the management of agricultural crops (Biondi et al. 2012).

As a neurotoxic compound, the mode of action (MOA) of spinosad affects the nicotinic acetylcholine receptors (nAChRs) directly in the nervous system, precisely acting as allosteric modulator (Biondi et al. 2012). Through stimulation of nAChR and γ -aminobutyric acid (GABA) receptors, spinosad induces rapid excitation of the organism nervous system, producing paralysis and death. Several investigators showed that spinosad binds at

a different site in comparison to the neonicotinoid pesticides that act through an allosteric mechanism (Orr et al. 2009; Puinean et al. 2013). Specifically, Salgado (1998) reported that spinosad directly affects the insect central nervous system, inducing involuntary neuronal rapid excitation that consequently initiating tremors, prolonged muscle contractions, paralysis, and death.

Accordingly, the first symptoms are evident in different organisms, including houseflies and cockroaches that exhibit an elevation of the tail and lowering of the head attributed to a straightening of the hind legs. Hence, widespread fine tremors occur in all muscles of the insects and severe changes in the posture. The appendages of hard-bodied insects tremble continually and the skin appears to crawl in soft-bodied ones. Finally, the movements cease leading to the paralysis of the insects (Salgado and Sparks 2005).

Specifically, spinosad acts as an allosteric agonist of acetylcholine (Ach) by binding to nicotinic acetylcholine receptors (nAChRs), prototypical units that function as neurotransmitter ligand-gated ion channels (Salgado and Sparks 2005; Ureña et al. 2019). nAChRs $\alpha 6$ subunits are characterized by the high degree of conservation in genomic structure and amino acid identity. Normally, the subunit of nAChRs contains an extracellular N-terminal domain with 6 loops (A-F) involved in the Ach binding site. The genomes of the insects possess between 10 and 16 subunits and the target site of spinosad is the $\alpha 6$ subunit of the nicotinic Ach receptors, which is believed to result in death (Ureña et al. 2019).

Resistance to spinosad

Data suggest that different mutations in the methyl group at carbon 6 of the polyketide subunit are responsible to confer resistance to spinosad in several species (French-Constant et al. 1998; Zimmer et al. 2016). The insecticide resistance characterizes the rapid adaptive evolution and is an inherited property that involves genes in one or more insect genes. The mechanisms may occur through metabolic alterations in the levels of detoxification proteins and mutations in GABA, Ach and sodium channels receptor genes (Hemingway et al. 2004). In *Drosophila*

melanogaster, previous investigators reported that resistance of spinosad occurs due to point mutations that generate premature stop codons and amino acid alterations next to the Cys-loop motif, which result in a high level of resistance to the insecticide (Perry, McKenzie, and Batterham 2007; Watson et al. 2010; Zimmer et al. 2016). Puinean et al. (2013) found a non-synonymous point mutation of the exon 9 located in the $\alpha 6$ nAChRs in the invasive pest insect *Frankliniella occidentalis*, that occurs owing to a substitution of a glycine residue with a glutamic acid at position 275. Further, a new species was noted associated with this same replacement of amino acids residues, including the spinosad resistant species *Thrips palmi* (Bao et al. 2014) and the destructive insect pest tomato leafminer, *Tuta absoluta* (Guedes et al. 2019). In this manner, investigators examined the resistance to spinosad through the use of Crispr/Cas9 system considering this mutation. Zimmer et al. (2016) inserted the G275E mutation into *D. melanogaster* in order to demonstrate the causal role of amino acid replacements in resistance to spinosad, and the results revealed that the LC₅₀ rose from approximately 5 mg/L to 335 mg/L in the fruit flies.

Pharmacology

The pharmacokinetic and metabolism of spinosyn A and spinosyn D are similar. In fact, spinosad is rapidly absorbed and extensively metabolized. Previous studies with both components reported no significant differences in the bioavailability,

metabolism, and routes of excretion in rats as experimental models. In addition, oral administration of spinosyn A and D exhibited rapid but incomplete absorption of >70% of the dose. The excretion occurs primarily via bile in the feces (70-90%) and less than 10% is recovered in urine (US EPA 1997; WHO 2010).

Toxicological implications of spinosad

Although possessing a highly favorable environmental and toxicological profile, and considered as a potential alternative for use in integrated pest management systems (Waldron et al. 2000; Crouse et al. 2018), numerous studies focused on the toxicity of spinosad to determine the adverse effects of the compound on different living organisms. In fact, previous investigators reported that the biopesticide produced *in vivo* toxicity in mammals (Mansour, Heikal, and Mossa 2008b; Mansour, Mossa, and Heikal 2007, 2008a; Piner and Uner 2013; Stebbins et al. 2002; Yano et al. 2002). Table 2 summarizes published 50% lethal concentration (LC₅₀) values induced by spinosad.

Sub-chronic studies revealed significant signs of toxicity in Fischer rats given 0.4% spinosad (corresponding to 273.1 mg/kg/day), as evidenced by thin appearance, hypothermia and labored respiration. Further, it is worthwhile noting the alterations in activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase, and alkaline phosphatase enzymes, as well as treatment-related degenerative changes in liver,

Table 2. Toxicity effect values of spinosad on different living species.

Species	Stage	Route of administration	LC50	Reference
ICS strain			41.3	Zhang et al. 2008
Shizuoka strain			2.94	Zhang et al. 2008
<i>Poecilia reticulata</i>			>202 mg/L	Pereira et al. 2016
<i>Xiphophorus maculatus</i>			>202 mg/L	Pereira et al. 2016
<i>Podisus nigrispinus</i>			3.15 μ g L	Santos-Junior et al. 2019
<i>Daphnia magna</i>			4.1 μ g L	Santos et al. 2019
<i>Aedes aegypti</i>	III instar		60 μ g L	Antonio et al. 2009
<i>Aedes aegypti</i>	IV instar		65 μ g L	Hertlein et al. 2010
<i>Anopheles gambiae</i>	III instar		10 μ g L	Darriet and Corbel 2006
<i>Culex quinquefasciatus</i>	IV instar		100 μ g L	Liu et al. 2004
<i>Aedes albopictus</i>	IV instar		300 μ g L	Liu et al. 2004
<i>Chironomus riparius</i>			> 256 μ g L	Monteiro et al. 2019
Male rats	Adult	Oral	3738 mg/kg	Yano et al. 2002
Female rats	Adult	Oral	> 5000 mg/kg	Yano et al. 2002
Rats	Adult	Inhalation	> 5.18 mg/l air	Yano et al. 2002
Rabbits	Adult	Dermal	> 5000 mg/kg	Yano et al. 2002
Swiss mice	Adult	Oral	3500 mg/Kg	El-Naggar et al. 2017

skeletal and cardiac muscles in animals exposed to 0.1%, 0.2%, or 0.4% of spinosad (68.5, 133.5 and 273.1 mg/kg/day, respectively). Conversely, this study also showed low tumor incidence in the experimental rats, which may be explained due to the lower body weights and feed consumption, supported by the hypothesis that dietary restriction is considered a preventive mechanism and efficient inhibitor of the tumor occurrence (Chen et al. 2016; Yano et al. 2002).

Accordingly, El-Naggar et al. (2017) demonstrated that spinosad induced a significant rise in triglycerides and urea levels in male albino mice after sub-chronic exposure with 35 mg/kg. Higher concentrations also affected ALT activity and initiated a significant decrease in body weight and an increase in the relative weight of kidney and spleen. Further, these findings are in agreement with Stebbins et al. (2002) that reported a significant elevation in serum levels of ALT and AST, hence inducing hepatocellular disorder and necrosis of mice hepatocytes, and confirm that the increase in enzymatic activities is directly proportional to the extent of hepatic damage (Chinaka, Owoche, and Dozie 2011; El-Naggar et al. 2014).

The cell viability was compromised after treatment with spinosad in two mammalian cellular models, CHO-K1 and Vero, obtained from hamster ovary and monkey kidney, respectively, under three different culture conditions: serum-free, presence of 10% fetal calf serum (FCS) and 1% bovine serum albumin (BSA) supplemented media. The results were obtained through the neutral red cell cytotoxicity assay (NRU) as endpoint, and found that spinosad was highly cytotoxic for both cell lines, especially in the absence of FCS and BSA, with an estimated value of NRU_{50} of 7.55 $\mu\text{g}/\text{ml}$ for CHO-K1 cell culture and 13.46 $\mu\text{g}/\text{ml}$ for Vero cell culture after 24 hr incubation (Pérez-Pertejo et al. 2007). Conversely, the NRU_{50} value increased markedly when cells were cultured in the presence of the antioxidants glutathione (1mM), vitamin C (100 μM) and vitamin E (20 μM), indicating a significant reduction of the cytotoxic effect of spinosad. Data thus suggested an oxidative damage mediated by the biopesticide and also confirming the cytological protection pattern afforded by these agents against spinosad-mediated injury. This postulation might be supported by the lipid peroxidation observed in both

cell lines after exposure to different NRU concentrations of spinosad, a molecular mechanism directly involved in pesticide-induced toxicity (Abdollahi et al. 2004). The evidence thus indicates the involvement of free radicals in the mechanism of cell damage induced by biopesticide (Pérez-Pertejo et al. 2007).

Findings reported by Grothe, Boss, and Gries (1992) suggest that the most notable effect in the experimental animals after sub-chronic exposure is vacuolar degeneration. Data demonstrated histopathological lesions in the ovary of rats after administration of 50 ppm of spinosad in a long-term exposure, albeit vacuolar alterations in the tubular epithelial cells involving kidney were observed only when the dose reached 1200 ppm of concentration.

The sub-chronic oral administration of spinosad in male Sprague-Dawley rats, at an equivalent dose of 1/20 LD_{50} , noted marked reduction in acetylcholinesterase (AChE) activity and elevation in the concentration of malondialdehyde (MDA). In addition, the pesticide produced significant inhibition in superoxide dismutase (SOD) and glutathione S-transferase (GST) enzymes, hence altering the GSH-redox cycle, and light microscopy images revealed severe focal necrosis and degenerative alterations in the rat hepatocytes along with spinosad-mediated cytoplasmic vacuolation. These observations indicated the occurrence of DNA fragmentation, structural and numerical chromosomal aberrations followed by the induction of apoptosis in the liver of rats (Aboul-Enein et al. 2012). The study also analyzed the effect of an antioxidant mixture composed of vitamin C, vitamin E and sylimarin against the spinosad exposure, and showed a reduction in oxidative stress, less severe histological changes in hepatocytes and fewer chromosomal aberrations, indicating a potential protective effect of the antioxidants (Aboul-Enein et al. 2012).

The investigation of the acute toxicity effects of spinosad was also conducted by using different biomarkers in *Oreochromis niloticus* as a model organism. After exposure of fish to sublethal concentrations (25, 50 or 75 mg/L) of the insecticide, Piner and Uner (2013) found that spinosad-induced oxidative stress and apoptosis in the liver of the fish. The biopesticide exhibited

a significant rise in free radical production and instability of the naturally occurring antioxidant glutathione, then leading to caspase-3 activation and initiated cellular apoptosis.

Breslin et al. (2000) examined the effect of spinosad on pregnant female rats and noted a significant fall in lower body weight after oral administration of 200 mg/kg/day of spinosad on gestation day 12 (GD12) and decrease body weight gain on GD 7–10. In addition, Breslin et al. (2000) showed that New Zealand white rabbits exhibited reduced feed consumption, diminished fecal output and loss in body weight at the initial period (G7-10) at dose of 50 mg/kg.

Ecotoxicological assessment of spinosad was conducted using *Daphnia magna* as model organisms, and both acute and chronic toxicity tests demonstrated immobility effects on the microcrustaceans, in addition to a significant increase in the time of first reproduction and fall in number of neonates per female as evidenced by reproductive test (Santos et al. 2019). It is worthwhile noting that chromatographic and mass spectrometry parameters revealed that spinosad was detected at concentrations of 0.54 and 1.2 µg/L in water samples collected from different rivers near extensive agricultural practices in Spain. This might affect non-target organisms leading to damaging the ecosystem (Vega, Frenich, and Vidal 2005).

Use of spinosad to control *aedes aegypti*

Based on the insecticidal properties, spinosad was considered as a naturally occurring pesticide in control of several pests, including mosquitoes transmitting diseases, armyworms and other lepidopteran pests. Interestingly, the compound exhibits low toxicity to beneficial insects and demonstrates rapid degradation in the environment (DeAmicis et al. 1997; Yano et al. 2002). Although spinosad efficacious, it is relevant to highlight that certain species may differ markedly in their response according to the different stages (Williams, Valle, and Viñuela 2003). Currently, it is worthwhile noting the importance of the control of mosquito population targeting larval instars in urban areas and also management integrated procedures (PaPavela et al. 2019). *Aedes aegypti*, for instance, is the main vector of several debilitating

diseases and life-threatening arboviruses, including Zika, Chikungunya, yellow fever and dengue (Pereira et al. 2016; Santos et al. 2017). The species is an efficient vehicle by which pathogens may be spread from host to host (Attardo, Hansen, and Raikhel 2005). In fact, the eradication of these mosquito-borne illnesses remains a hurdle in regard of the use of pesticides.

Previous Fernandes et al. (2019) reported the efficacy of the treatment with spinosad against *Aedes aegypti*. Larvae were exposed to different concentrations of the biopesticide (0.025, 0.1 or 0.25 ppm) for a period of 24 h. The number of laid eggs per female decreased as spinosad concentration increased, and considering the highest concentration tested, only 30% of the eggs hatched, in opposition to 80% reached by the control group. Further, reduction in adult lifespan of the organisms was also reported. Hence, the fecundity was negatively affected by the pesticide when the individual was treated during the larval stage, and this may be attributed to malformation of the midgut during the development of the larvae (Fernandes et al. 2019). Regarding this case, it is known that adults with malformed midguts display a disability in blood digestion and nutrient absorption, which consequently may affect ovary activation, egg production, and development (Gulia-Nuss et al. 2011).

Fernandes et al. (2019) also found peroxidase-positive cells in the midgut of *Ae. aegypti* larvae and pupae after exposure of 0.250 ppm of spinosad for 24 hr, indicating the presence of oxidative stress was initiated by oxidative oxygen species (ROS) generation, which might trigger damage in the cellular structure, mitochondrial dysfunction and reduction in individual fitness and survival (Doenst, Nguyen, and Abel 2013; Fridell et al. 2005). Interestingly, Pereira et al. (2016) proposed the use of poeciliid larvivorous guppy (*Poecilia reticulata*) and platy (*Xiphophorus maculatus*) along with spinosad at concentrations lower than the lowest-observable-effect concentration LOEC (25.3 mg/L) as a safer and effective integrated approach to control *Ae. aegypti* larvae in field conditions. Efforts thus need to be directed at designing experiments that may elucidate whether low levels of spinosad are effective to control mosquito larvae for longer periods and also safe for the fish species. Kovendan et al. (2012) explored the

effectiveness of a mixture comprising methanolic leaves extract of *Carica papaya* and spinosad as a promising eco-friendly approach against *Aedes aegypti*. Kovendan et al. (2012) demonstrated effective larvicidal and pupicidal properties against I to IV instars larvae after exposure to the combined treatment.

Applications of spinosad against crop pests

Importantly, spinosad has been widely used in the agriculture field as a promising compound against resistant pests. Table 3 provides evidence for the use of the biolarvicide in the management of several species.

The Alabama house fly strain (ALHF), for example, has the ability to develop resistance to several pesticides, including permethrin, beta-cypermethrin, deltamethrin and propoxur, but it was found that the multi-resistance mechanism of this organism did not confer cross-resistance to spinosad, indicating the biopesticide as a valuable compound for integrated management of resistant house fly pests (Liu and Yue 2000). Further, although the diamondback moth *Plutella xylostella* also evolved resistance to all pesticides, it was discovered high efficacy of spinosyns for controlling the species within integrated pest management programs (Sayyed, Omar, and Wright 2004; Thomson et al 1995).

In the case of lepidopteran endoparasitoids, Schneider et al. (2003) reported that spinosad did not produce a higher mortality rate considering the treatment with parasitoid pupae and

parasitized hosts until the emergence of *Hyposoter didymator* adults. The reason may be due to a low penetration of the insecticide in pupal cocoon (Schneider et al. 2003; Williams, Valle, and Viñuela 2003). However, the natural pesticide was harmful to the adult species, affecting their life span and parasitism capacity (Schneider et al. 2000). In contrast, Medina et al. (2001) noted that spinosad did not markedly affect oogenesis when administered by ingestion or topical applications to adults of the green lacewing *Chrysoperla carnea*, a common predator of agricultural and natural habitats, and did not showed ovicidal activity. In fact, exposure of the pesticide in concentrations higher than 1 g/L failed to significantly inhibit egg hatching. The development of the emerging larvae was not affected, but spinosad exerted sublethal effects on the life span and fecundity of adults oviposition, which may be attributed to the neurotoxic activity mechanism of spinosad (Medina et al. 2001).

Analysis measured by the colorimetric (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) MTT assay in Sf9 cell line of *Spodoptera frugiperda* showed that spinosad-inhibited cell viability in a concentration and time-dependent manner. After treatment with 60 µM in 2 hr incubation, the neurotoxic compound produced cytoplasmic distension and prominent vacuolization, accompanied by a rapid increase in ROS levels. Western blot analysis also revealed that cytochrome C was released from mitochondria into the cytoplasm, mechanism directly related to cell death (Xiong et al.

Table 3. Research reports about spinosad applications for pest control.

Common name of pests	Species	Application	Reference
Diamondback moth	<i>Plutella xylostella</i>	Damage in cruciferous plants	Zhao et al. 2002
Mediterranean fruit fly	<i>Ceratitis capitata</i>	Fruit fly control	Burns et al. 2001
Caribbean fruit fly	<i>Anastrepha suspensa</i>	Fruit fly parasitoid control	Burns et al. 2001
Western flower trips	<i>Frankliniella occidentalis</i>	Damage in ornamental flowers and vector of Tospoviruses	Jones et al. 2005
Oriental fruit fly	<i>Bactrocera dorsalis</i>	Fruit fly control	Hsu and Feng 2006
Gypsy moth	<i>Lymantria dispar</i>	Damage in deciduous and evergreen trees	Wanner, Helson, Harris 2000
Spotted-wing drosophila	<i>Drosophila suzukii</i>	Damage of fruit crops	Roubos et al. 2019
Samurai wasp	<i>Trissolcus japonicus</i>		Lowenstein et al. 2019
Brown marmorated stink bug	<i>Halyomorpha halys</i>	Damage of tree fruits and hazelnuts	Lee et al. 2014
American serpentine leafminer	<i>Liriomyza trifolii</i>	Ornamental and vegetable crops	Ferguson 2004
Asian citrus psyllid	<i>Diaphorina citri</i>	Damage in citrus crops	Tofangsazi et al. 2018
Cotton leafhopper	<i>Amrasca devastans</i>	Damage of cotton crops	Saeed et al. 2018

2014), associated with upregulation and rise in p53 expression and in Bcl-2/Bax ratio due to a Bax upregulation and Bcl-2 downregulation (Yang et al. 2017).

Recently, lab bioassays revealed the influence of an aqueous suspension of spinosad against larvae of *Culex pipiens* biotype *molestus*, an important vector of the West Nile virus and filariasis. Antonios et al. (2019) reported that spinosad acted immediately after the preparation of the insecticidal solution, which is expected for the compounds that attack GABA receptors.

The assessment of different biochemical biomarkers after exposure to spinosad also indicated toxicity attributed to the biopesticide. Monteiro et al. (2019) demonstrated a significant elevation in GPx activity and lipid peroxidation in *Chironomus riparius* larvae, in addition to an increase in lactate dehydrogenase (LDH) activity following 2 µg/L treatment and in the electron transport system, determined as the most sensitive biomarker. The compound also altered the growth development, as evidenced by a delay in the evolution time of males and females (Monteiro et al. 2019).

In comparison to other dipterans, data demonstrated that a spinosad concentration of 17 µg/L induced a marked decrease of the emergence of *Polypedilum nubifer* and hence a potent lethal effect on the midge (Duchet et al. 2015). Accordingly, concentrations varying from 3.7 to 45 µg/L affected significantly *Culex pipiens* emergence (Hertlein et al. 2010), while 60 µg/L completely inhibits adult emergence of the blood-feeding mosquito (Cetin, Yanikoglu, and Cilek 2005). Further, a recent study reported cytotoxicity and histological alterations in the salivary gland cells of *Podisus nigrispinus* 30 min after exposure to spinosad (Santos-Junior et al. 2019). Over time exposure, the findings also indicated irregular epithelium, large vacuoles, an increase in cytoplasmic granules and also cell disruption (Santos-Junior et al. 2019).

Effect of spinosad as a mixture

Despite the efficacy of spinosad against specific target organisms, several investigators examined the performance of mixtures containing the compound (Darriet and Corbel 2006; Darriet et al.

2010). Various researchers investigating the efficacy of a mixture of spinosad and pyriproxyfen, a juvenile hormone analogue that blocks larval development, thus compromising adult insect characteristics and emergence (Caixeta et al. 2016; Santos et al. 2017). Darriet and Corbel (2006) found a significant synergism between both pesticides, whose mixture combined both the juvenoid activity of pyriproxyfen and the larvicidal effects of spinosad. Darriet and Corbel (2006) suggested a reduction by five and ninefold of pyriproxyfen and spinosad, respectively, to kill almost 100% mosquitoes. In agreement Darriet et al. (2010) reported that the activity of the mixture spinosad + pyriproxyfen lasted twofold longer in comparison to the use of the isolated compound. Accordingly, the use of insecticides initiated a powerful selection pressure on the target organisms, and prolonged use led to a resistance to the compound. However, the association of two pesticides with different MOA acting on different targets reduced the risk of evolution to resistance. In fact, the mixture of spinosad and pyriproxyfen combined the efficacy of the first against larvae and the second more specifically against the pupal stage, indicating a strong synergistic effect between the insecticides, hence leading to rapid mortality frequency of both larvae and pupae.

It is important to highlight that weather conditions are also able to affect the efficacy against *Ae. aegypti* larvae. Observations in this study noted that after protection from the sun and climate conditions, the combination remained active for at least 8 months against Vauclin strain, while the use of spinosad and pyriproxyfen alone lasted 3.5 and 5 months, respectively (Darriett et al. 2010). In addition, with a residual activity of 3.5 months, spinosad was suggested as a promising compound for the control of *Ae. aegypti*. The strategy of combining pesticides that work in synergy needs to be explored as an efficient approach for public health and also as an alternative to avoid the resistance of mosquitoes to conventional insecticides in critical regions.

Concluding remarks

The microbial metabolite originally synthesized by *S. spinosa* possess potent efficacy on the control of

different insect pests in several cropping systems. In this context, spinosad has been widely recommended owing to its properties as a broad-spectrum pesticide. Further, it is important to emphasize the potential performance of the bio-larvicide when combined with other compounds in the control of disease vectors and in prevention of the development of resistance.

The search for promising approaches to chemical-origin pesticides is driving the exploration of spinosad as a new alternative for the combat of several crop pests and also as a weapon against important vector of life-threatening arboviruses, such as *Aedes aegypti*. However, in order to reinforce its use and determine the maximal effectiveness, efforts require to be prioritized in areas with less effective educational networks, where farmers tend to use this pesticide in the management of crop pests repeatedly without alternating with other ones.

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CAPÍTULO II

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Low toxicity and high efficacy in use of novel approaches to control *Aedes aegypti*

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ABSTRACT

Arthropod-borne viruses are a group of etiologic agents accounting for different incapacitating diseases that progress to severe and lethal forms in animal and human targets consequently representing a significant burden on public health and global economies. Although attempts were undertaken to combat *Aedes aegypti*, the primary urban mosquito vector of several life-threatening diseases, the misuse of chemical pesticides, development of resistance, and toxicity on non-target species still need to be overcome. In this context, it is imperative for development of long-lasting, novel approaches envisioning effective control of *Aedes aegypti*, mainly in endemic regions. Thus, the present review was undertaken to describe safe and eco-friendly approaches as potential weapons against *Aedes aegypti*. Accordingly, the findings discussed indicated that biological larvicides and genetic engineering technologies constitute noteworthy alternatives of future mosquito-borne arbovirus disease control efforts.

KEYWORDS

Aedes aegypti; toxicity; biopesticide; genetic engineering

Introduction

Aedes aegypti is the primary urban vector of several human arthropod-borne diseases, including dengue, Zika, yellow fever, and chikungunya. These arboviruses are a constant threat to human and animal health worldwide, being responsible for causing acute systemic diseases and hence leading to grievous health conditions to humans (Bottino-Rojas et al. 2019; Santos, Limongi, and Pereira 2020).

Despite the efforts to control the spread of disease-carrying insects, the combat against *Aedes aegypti* remains as a challenge, especially in tropical and subtropical countries, such as Brazil, which harbor the species responsible for virus transmission. Alarming, viral surveillance practices against *Aedes aegypti* occur inadequately in endemic regions and face several issues, including underreporting of arboviruses cases and limited access to health care (Luna et al. 2020). In fact, Brazilian integrated control programs cope with the lack of a safe and effective strategy to overcome the spread of *Aedes aegypti*, when there is no licensed vaccine, specific medication, or antiviral drugs to deal with the arboviruses (Darriet et al. 2010).

In the last years, global arbovirus incidence has grown dramatically (Patterson, Sammon, and Garg 2016). Different factors driven by ecological, environmental, and socio-economic factors play key roles in the emergence of infectious diseases, such as globalization, ecology, human behavior, intensification of agriculture, deforestation and climate change, in addition to increasing mobility and geographical expansion of populations (Jones et al. 2008; Mayer, Tesh, and Vasilakis 2017).

In order to deal with factors that hamper control of mosquitoes, including high operational costs, and adverse environmental effects attributed to the use of pesticides, many efforts were undertaken to develop new control tools to eradicate mosquito-borne diseases. Emerging epidemics produced by mosquitoes represent a significant social and economic burden, markedly affecting low- and middle-income tropical regions that have unsuitable social-ecological conditions, such as access to health-care system and poor housing conditions (Heydari et al. 2017). Accordingly, the global cost associated with dengue virus infection treatment was estimated to be 8 USD.9 billion, which is higher in comparison with major infectious illness, such as Chagas and cholera (Qureshi 2020).

Various studies documented the occurrence of pesticide-resistance against natural mosquito populations globally, owing to the overuse of synthetic compounds to control young instar larvae, which has emphasized the need to develop new potential strategies for vector control (Govindarajan, Rajeswary, and Benelli 2016; Santos et al. 2017; Tao et al. 2019). Accordingly, the rapid evolution of resistance is limiting current technological designs, and efforts need to be made to cope with existing obstacles.

In spite of the efficacy to control insect pests, several chemical pesticides are recalcitrant compounds and pollutants, and kill not only vector-borne pathogens, but also favorable species and vertebrates (Bravo and Soberón 2008). In turn, natural and microbial formulations based upon *Saccharopolyspora spinosa*, *Bacillus thuringiensis*, and natural porphyrins were investigated as potent weapons against *Aedes aegypti*; and hence considered as highly valuable bioactive compounds against a rich diversity of pests (Hertlein et al. 2010; Kästel, Allgeier, and Brühl 2017; Lucantoni et al. 2011). Similarly, genetic engineering technologies were explored as potential tools to overcome hurdles of conventional vector control techniques (Figure 1) (Batool et al. 2018; Harris et al. 2011; Lucantoni et al. 2011).

In this context, beyond the intrinsic interest in *Aedes aegypti* as a vector of different life-threatening infectious diseases, the investigation of biological larvicides and application of genetic engineering techniques is a highly relevant need in order to explore safe and eco-friendly alternatives to

combat mosquitoes. Thus, the present review describes safe and eco-friendly approaches to combat *Aedes aegypti*, a species of public health interest, examining properties and toxicological effects of the compounds, as well as promising technologies in the hunt for effective, safe arboviruses treatments.

Spinosad

In 1982, a new *Saccharopolyspora* species was isolated from a soil sample collected by a vacationing scientist in the Caribbean Islands. Later, taxonomic and morphological studies confirmed the discovery of the Gram-positive filamentous actinomycete *Saccharopolyspora spinosa*, which differs from other bacteria in the physiological properties and fatty acid composition attributed to the spiny spore sheath surface (Mertz and Yao 1990).

Cultural observations found that the aerial fermentation of *Saccharopolyspora spinosa* results in a complex of natural metabolites with unusual activity, known as spinosyns (Santos and Pereira 2020). These novel polyketide-macrolides possess a distinct molecular structure, in addition to a different mode of action coupled with insecticidal efficacy (Salgado and Sparks 2005).

Structurally, the natural mixture of spinosyn A and spinosyn D composes the biological larvicide spinosad, formulated at a typical ratio of 85:15, respectively (Thompson et al. 1995). Both spinosyns are nonvolatile molecules with high molecular weight and differ only by the presence of a methyl group at C6 (Table 1) (Waldron et al. 2000).

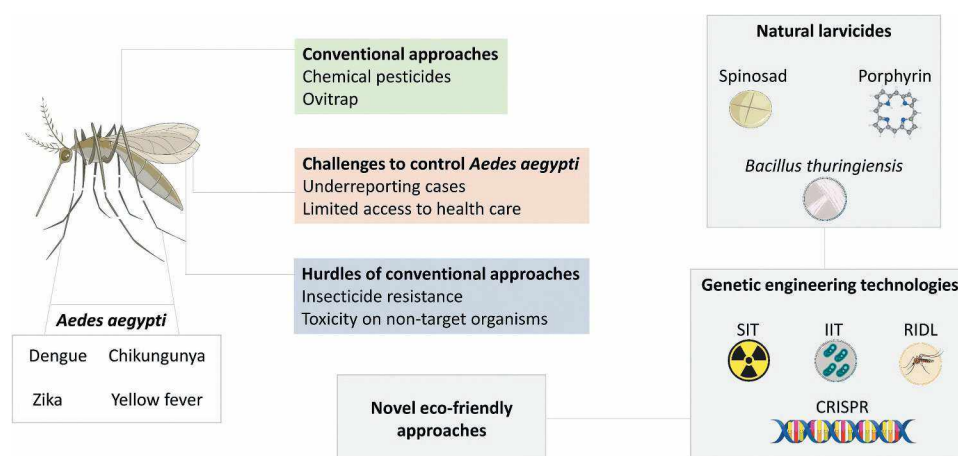


Figure 1. Overview of the current scenario, remaining challenges and novel approaches to control *Aedes aegypti*.

Table 1. Physical and chemical properties of natural larvicides used against *Aedes aegypti*.

Physical property	Spinosyn A	Spinosyn D	Porphyrin
Molecular formula	C ₄₁ H ₆₅ NO ₁₀	C ₄₂ H ₆₇ NO ₁₀	C ₂₀ H ₁₄ N ₄
Molecular weight	731.9 g/mol	745.9 g/mol	310.4 g/mol
Melting point	120°C	170°C	360°C
Solubility (20°C)			
Water (pH 7.0)	235 mg/L	0.332 mg/L	0.07847 mg/L
Methanol	19.0 g/mL	0.252 g/mL	
Acetone	16.8 g/mL	1.01 g/mL	
Vapor pressure	3.0x10 ⁻¹¹ kPa	2.0x10 ⁻¹¹ kPa	6.96x10 ⁻⁶ kPa
Octanol/water partition coefficient (K _{ow})	4.01	4.53	6.01

Originally, spinosad was commercially marketed by Dow AgroSciences as a biological control agent of lepidopterous pests in cotton crops (Salgado 1998). Currently, the compound is recognized as an eco-friendly pesticide used to protect against different pests, and it has been extensively used in the management of agricultural crops to increase crop yields, in animal health applications and also to control head lice in humans (Puinean et al. 2013).

The mode of action of spinosyns occurs after ingestion or contact by the organisms. Specifically, spinosad acts by binding on the nicotinic acetylcholine receptors (nAChRs) directly in the nervous system as an allosteric agonist of acetylcholine (Salgado and Sparks 2005). Evidence also suggests a minor impact on the γ -amino butyric acid (r) receptor neurotransmitter and on GABA-gated chloride channels (Table 2) (Biondi et al. 2012; El-Naggar et al. 2017). Thus, through stimulation of nAChRs and GABA receptors, exposure to spinosad produces involuntary neuronal excitation of the insect central nervous system (CNS), hence rapidly leading to tremors, prolonged muscle contractions, absence of feeding activity, paralysis, and death (Orr et al. 2009; Salgado 1998).

In addition to favorable toxicological profile and low toxic actions on mammals and other non-target organisms (Bacci et al. 2016), the need to overcome development of resistance to synthetic pesticides is driving the exploration of spinosad as a potential weapon to combat important vector of life-threatening infectious diseases, such as *Aedes aegypti* (Santos and Pereira 2020).

In fact, spinosad exerts a variety of sublethal effects on natural enemies and is able to markedly affect demographic traits in parasitoids and predators (Köhler and Triebkorn 2013). Lab studies reported relative susceptibility of *Aedes aegypti* larvae and toxicity effects to different spinosad formulations, as summarized in Table 3. The findings clearly demonstrate that mortality of *Aedes aegypti* after exposure to the natural larvicide accumulates steadily over a period of 72 hr, and established that spinosad resulted in significant and irreversible larval mortality (Hertlein et al. 2010). However, although deemed as a highly valuable bioactive molecule, the side effects of acute and chronic pesticide-mediated toxicity on different living organisms have raised concerns.

Some investigators reported *in vivo* toxicity in mammals and non-vertebrate model organisms (Mansour, Mossa, and Heikal 2008; Piner and Üner 2013). Sub-chronic and chronic dietary studies in Fischer rats demonstrated significant signs of toxicity after administration of spinosad, including hypothermia, thin appearance, and deep and labored respiration (Yano et al. 2002). Light microscopic images noted phospholipidosis in animal models, which is in accordance to other cationic amphiphilic drugs (Reasor, Hastings, and Ulrich 2006). Clinical tests demonstrated altered levels in serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase, and alkaline phosphatase (AP) activities, following by a degenerative change in liver, cardiac, and skeletal muscles after administration of 0.1%, 0.2%, and 0.4% of spinosad

Table 2. Origin and mode of action of natural larvicides used against *Aedes aegypti*.

Compound	Origin	Mode of action	Reference
Spinosad	<i>Saccharopolyspora spinosa</i>	Stimulation of nAChRs and GABA receptors	Biondi et al. 2012, El-Naggar et al. 2017
Bti	<i>Bacillus thuringiensis</i> svar. <i>israelensis</i>	Ingestion of crystal inclusions with release of protoxins	Batool et al. 2018
Porphyrin	Natural or synthetic organic heterocyclic molecules	Photoexcitation activity causing tissue damage	Lucantoni et al. 2011

Table 3. Toxicity effect values of natural larvicides on *Aedes aegypti* larvae.

Strain	Stage	LC50	Exposure duration	Reference
Bti (wild type)	IV instar	19.8 ng mL	24 h	Juárez-Hernández et al. 2015
Bti-pEHchiA74Δsp	IV instar	8.02 ng mL	24 h	Juárez-Hernández et al. 2015
Bti-pEBchiA74Δsp	IV instar	9.6 ng mL	24 h	Juárez-Hernández et al. 2015
Bti	III instar	54.0 ng mL	24 h	Ignoffo et al. 1981
Bti	IV instar	0.33 mg L	24 h	Lopes, Arantes, and Cenci 2010
Spinosad	III instar	60 ng mL	1 h	Antonio et al. 2009
Spinosad Tech AI	IV instar	65 ng mL	24 h	Hertlein et al. 2010
Spinosad 120 SC	III instar	12 ng mL	48 h	Hertlein et al. 2010
Spinosad 120 SC	IV instar	28 ng mL	48 h	Hertlein et al. 2010
Spinosad 480 SC	III instar	7 ng mL	48 h	Hertlein et al. 2010
C14 Porphyrin	III instar	0.77 mg L	1 h	Lucantoni et al. 2011
C14 Porphyrin	III instar	0.15 mg L	12 h	Lucantoni et al. 2011

(68.5, 133.5, and 273.1 mg/kg/day, respectively (Yano et al. 2002). Inflammatory lesions were observed in multiple tissues and pathologic necrosis seen in thyroid glands and lungs of female rats (Yano et al. 2002).

Sub-chronic studies were performed after exposure of male albino mice to low and high concentrations of spinosad (35 and 350 mg/kg, respectively) (El-Naggar et al. 2017). Accordingly, measurement was undertaken of several biochemical biomarkers, including enzymatic activities of serum ALT and AST, total triglycerides, urea levels, total protein, and albumin levels, in addition to the histological and immunohistochemical alterations in liver, kidney, and cerebellum tissues of mice. Treatment with 35 mg/kg detected a significant increase in triglycerides and urea levels, and nevertheless, the higher dose produced neurodegenerative alteration in Purkinje neurons, elevation in relative weight of kidney and spleen and severe necrosis in hepatocytes (El-Naggar et al. 2017). The results are in accordance with previous observations, which confirmed hepatocellular disorder and necrosis of mice hepatocytes associated with rise in serum ALT and AST enzyme activities (Stebbins et al. 2002), in addition to ROS generation in liver cells, led to cell activity dysfunction, in particular nuclear pyknosis and DNA damage (Aboul-Enein et al. 2012). Interestingly, these findings are not in agreement with WHO (2010) report, which mentioned no evidence of neurotoxicity in rats and no marked effect on the CNS of either human or animals after exposure to spinosad. It is noteworthy that long-term tests in mice and rats revealed histological alterations (WHO 2010). In mice, doses above the no-observed adverse-effect level NOEL (11 mg/kg body weight per day) produced chronic inflammation, hyperplasia, and hyperkeratosis of the stomach, skeletal myopathy,

inflammatory changes, and vacuolation of pancreas, ovaries, parathyroid, and epididymal epithelial cells as a consequence of cytoplasmic lamellar inclusion bodies and lysosomal storage disorder (WHO 2010).

Aquatic toxicology assessment was performed using *Daphnia magna* as model system to evaluate spinosad on non-target species (Santos et al. 2019). After exposure to different concentrations in both acute and chronic toxicity tests, data showed immobility effects on microcrustacean, and reproductive tests reported a significant increase in the time of first reproduction of *D. magna* and decrease in number of neonates per female. Remarkably, evidences of this study also indicated that a 100-fold lower concentration of the biological compound might be used against mosquito larvae as a rational dosage to avoid toxicity risks for beneficial species (Santos et al. 2019).

Despite spinosad is deemed as an environmentally friendly effective alternative against different larvae and insect pests, data indicate that the pesticide also affects several physiological functions such as the activity of antioxidant enzymes, that are beneficial for arthropods and mammals. Thus, far, there is no natural compound that targets just insect pests and leaves non-target insects unaffected. Efforts need to be directed at designing novel integrated and innovative approaches that may elucidate whether low levels of spinosad are effective to control *Aedes aegypti* for longer periods and safely without damage to other species in field situations (Pereira et al. 2016).

Porphyrin

Porphyryns are a large group of natural or synthetic organic heterocyclic compounds with reliable light-harvesting ability (Liu et al. 2020). Characterized as

amphipathic molecules, porphyrins (1) interact with membranes, (2) may be photosensitized and (2) contain a metal atom in the center of the tetrapyrrolic structure (Neris et al. 2018). Allied with their derivatives, porphyrins have been explored as new tools for nanotechnology and medical applications, especially in skin treatments and photodynamic therapy, recognized as a promising technique in the treatment of certain types of cancer (Silva et al. 2010).

Importantly, porphyrins were found to be highly effective against mosquito larvae, and proposed as a novel and eco-friendly alternative to synthetic chemical insecticides for vector control (Lucantoni et al. 2011; Neris et al. 2018). In order to examine this possibility, Lucantoni et al. (2011) demonstrated a potent photosensitizing activity of an analogue of the C¹² porphyrin against *Aedes aegypti* larvae. Fluorescence microscopy analysis detected the presence of the C¹⁴ porphyrin in the intestine, suggesting that larval death occurred after cecal and midgut epithelial damage initiated by photoexcitation properties of the porphyrin (Table 2).

Souza et al. (2014) reported that the mechanism for introducing the photosensitizer into *Aedes aegypti* larvae is predominantly through digestion. Under photodynamic reaction of porphyrin, the mortality rate was enhanced which has emerged as a promising strategy for control of urban vectors.

It is noteworthy that amphiphilic porphyrin demonstrates potential photo-killing activities against insect pests owing to the ability to cross cell membranes and distribute in relatively large amounts within the insect tissues due to sufficient water solubility (Lucantoni et al. 2011). In contrast, hydrophilic compounds are not able to cross the lipid bilayer, hindering the distribution of porphyrin into the insect target tissues and, consequently, are inefficient photo-insecticidal agents (Ben Amor et al. 1998), while hydrophobic molecules may be transformed into photo-biologically inactive dimers or oligomers in the aqueous medium, which is a challenge when targeting aquatic pesticide organisms (Karunaratne et al. 2005).

Further, C¹² porphyrin effectively showed photo-inactivation of the malaria vector *Anopheles gambiae* and *Anopheles arabiensis* under lab conditions. Fabris et al. (2012a) demonstrated associated porphyrin with two different carriers, the anionic co-polymer

Eudragit® S 100 (EU-C12) and a fraction of cat food pellets (CF-C12) composed of 80% proteins, 10% fat, 10% carbohydrates, minerals, and vitamins. Data showed that EU-C12 porphyrin has a greater efficacy in comparison to CF-C12, probably because Eudragit® is an oral drug vehicle that unfolds at pH values >8 and releases C¹² porphyrin more readily in larval gastric ceca. CF-C12 displays a higher stability in water, which should avoid release of the sunlight-activated larvicide and its binding with non-target organisms, hence reducing the risk of deleterious effects in the aqueous environment (Fabris et al. 2012a). Thus, studies were conducted to explore optimization and validation tests for assessing porphyrin formulations as potential photo-activated molecules for controlling mosquito larvae. The aim was to develop a cost-effective product with a low impact on the environment (Fabris et al. 2012a, 2012b).

Specifically, Neris et al. (2018) noted that porphyrins exhibit a potential broad-spectrum effectiveness in the treatment of different enveloped viruses, including Zika and Chikungunya viral particles. Lab bioassays revealed viral envelope protein loss induced by porphyrin, which consequently affected viral morphology, adsorption and entry into target cells (Neris et al. 2018). In addition, porphyrins exerted modulatory effects on heme oxygenase activity and the ability to generate reactive oxygen species (ROS) (Neris et al. 2018). Previously, Assunção-Miranda et al. (2016) found that heme Co-protoporphyrin IX (CoPPIX) and Sn-protoporphyrin IX (SnPPIX) were directly inactivated both dengue virus and yellow fever virus replication in a dose-dependent manner, demonstrating that the molecular structure of porphyrin might be used to design new broad spectrum antiviral compounds with enhanced activity.

However, porphyrin faces challenges in regard to ecotoxicological studies. Indeed, toxicity assessment of C¹² porphyrin was performed on ciliated protozoa *Colpoda inflata* and *Tetrahymena termophila*, and branchiopod crustaceans *Artemia franciscana* and *Daphnia magna* (Fabris et al. 2012b). The study reported a significant affinity of porphyrin for *C. inflata* and *T. termophila*, but revealed a high sensitivity of *Daphnia magna* to the compound. In acute toxicity tests with this microcrustacean (OECD 2004), there was increased rate of motility and high

level of photosensitivity (Fabris et al. 2012b). Microscopic images indicated porphyrin accumulation in the digestive tract at 1–10 mM doses and, for higher amounts, it was also associated with the exoskeleton of *Daphnia magna* (Fabris et al. 2012b). *Artemia franciscana* nauplii were markedly more resistant associated with no significant differences in motility rate when compared to controls (Fabris et al. 2012b). Regarding the protozoan *Colpoda inflata*, significant adverse effects were induced at doses greater than 1 mM, followed by inactivation and elevated rate of cysts at 10.0 mM porphyrin (Fabris et al. 2012a, 2012b).

Certainly, porphyrin derivatives should remain to be explored through genetic engineering mechanisms or formulations with different carriers in order to establish an effective and safe approach against life-threatening disease vectors, such as *Aedes aegypti*, and achieve a low toxicological impact on non-target species.

Bacillus thuringiensis* svar. *israelensis

Bacillus thuringiensis (Bt) is a rod-shaped, Gram-positive entomopathogenic bacterium abundant in soil and plants, and has been widely used as biological agent against natural enemies, displaying an important role in insect pest control management and public health (Lajmanovich et al. 2015; Palma et al. 2014). The pathogenic mechanism of action underlying *Bacillus thuringiensis* is attributed to an insect-Bt interaction process which involves insect immune responses and Bt toxic proteins (Contreras et al. 2015).

Initially, conventional Bt compounds were developed and targeted primarily to control lepidopteran pests of agricultural and forestry crops (WHO 2007). In 1990's after careful screening and consideration of the effectiveness of *Bacillus thuringiensis* svar. *israelensis* (Bti) in public health programmes, more than 200 tons of this biolarvicide was applied annually in global mosquito campaigns (Becker 1998). To date, owing to the short life cycle, high specificity to mosquito larvae and minimal side effects to non-target species, Bti is considered the most environmental friendly alternative to chemical compounds available for controlling dipteran vectors of viral and parasitic diseases, including *Aedes aegypti* (Kästel, Allgeier, and Brühl 2017).

Structurally, Bti is composed of crystalline protein inclusions containing prototoxins which are part of the 3-domain Cry toxin family and the cytolytic toxin. Prototoxins are crystalline and cytolytic proteins whose individual components display low toxicity for certain insects and other invertebrates, such as protozoa, mites, flatworms, and nematodes (Baek et al. 2019). However, the molecules act in synergy, and this complex composition of Bti crystals exhibits a higher larvicidal activity.

The mode of action of Bti involves ingestion of crystal inclusions by larvae and their solubilization in the midgut by gut proteases; hence, leading to release of prototoxins which are proteolytically cleaved and converted into activated toxins (Table 2). Then, the toxins interact with the midgut receptors, producing pore formation in the membrane, triggering cell permeability and osmotic lyses that damage the epithelium, resulting in the death of larvae within a few hours (Batool et al. 2018; Bravo, Gill, and Soberón 2007).

Previously Smouse and Nishiura (1997) found that the δ -endotoxins synthesized by Bti during the sporulation phase of the strain-induced apoptosis in larval midgut cells of *Culex pipiens* mosquitoes, accounting for part of the insecticidal mechanism of the toxin. Further, it was found that several agricultural pests including *Spodoptera frugiperda* and *Plutella xylostella* and arthropod vector of human life-threatening diseases such as *Anopheles*, *Culex* and *Aedes* species are also susceptible to these toxins (Martínez-Zavala et al. 2020).

Despite the efficacy of Bti formulations as natural larvicides, chronic exposure treatments for *Aedes aegypti* control in tropical regions and physiological and molecular mechanisms that larvae might display in response to Bti require further studies in order to confirm it as a safe and effective innovative approach (Carvalho et al. 2018).

Notably, the water-dispersable granule formulation VectoBac WDG, synthesized from Bti (strain AM 65–52) is already recognized as a public health pesticide and specifications are published by World Health Organization (WHO World Health Organization 2007). The formulation was employed in broad acreage treatments and exhibited over 90% residual efficacy in container habitats. Accordingly, the application rate recommended for

mosquito control is 8 mg/L of Bti VectoBac WDG (Farajollahi et al. 2013).

Nevertheless, although considered environmentally safe, ecotoxicological data of microbial pesticides are still limited on non-target aquatic invertebrates and mammals. It is worthwhile noting that the environmental risk assessment of *Bacillus thuringiensis* var. *israelensis* on freshwater macroinvertebrate *Chironomus riparius* indicated delay in adult emergence (Bordalo et al. 2020). Lethal and sublethal effects occurred at doses lower than those recommended for field application in pest control programs (Bordalo et al. 2020). In addition, ecotoxicological studies of Bti formulation Introban® performed using the common frog *Leptodactylus latrans* showed enzymatic impairment of glutathione S-transferase and catalase activities and histopathological changes in the intestine, as well as formation of micronuclei and erythrocyte nuclear abnormalities after 48 hr exposure (Lajmanovich et al. 2015).

Genetic engineering was also utilized to improve the toxicity of Bti against vector diseases. Juárez-Hernández et al. (2015) reported that Bti engineered to produce stable inclusions of chitinase enhanced the toxicological effect against *Aedes aegypti* larvae by at least 2-fold, as depicted in Table 3, suggesting the possibility to generate even more potent recombinant strains with increased toxicity to *Aedes aegypti* species. In this context, the extensive worldwide use of Bt formulations in public health programs of mosquito-borne diseases emphasizes the importance of the investigation in relation to the effectiveness to combat potential arbovirus vectors along with a safe toxicological profile on non-target species. Undoubtedly, intensive studies need to be performed aiming to optimize and validate effectiveness and safety of biolarvicides using *Aedes aegypti* vector species. The outcome of such investigations

and the assessment of potential alternatives might be discovered with novel technologies of genetic engineering.

Genetic engineering technologies

Conventional vector control approaches have largely been unsuccessful to prevent outbreaks of infectious arbovirus diseases. Thus, advances in genetic management strategies were undertaken to identify and characterize potential tools to control *Aedes aegypti* mosquitoes as presented in Table 4 (Olson and Franz 2015).

Basically, the most known methods involving genetic engineering encompass population suppression or population replacement (Williams et al. 2020). The sterile insect technique (SIT), for example, has historically been applied to control insect pest populations dating back to the mid-1930 s. This proven technology to suppress wild populations involves periodic mass production followed by release of irradiated sterile male mosquitoes in target areas to outnumber the fertile wild-type mating partners (Bushland, Lindquist, and Knipling 1955; Olson and Franz 2015). Currently, nuclear radiation is also widely used in agriculture pest management both in mass-produced operations of natural enemies and in the SIT (Cai et al. 2018). In Switzerland, the approach has been applied to control cherry fruit fly, revealing positive results. The development of SIT is ongoing as an attempt to achieve a less costly, more robust, and more broadly applicable technique (Ware 2019).

However, even deemed very effective in controlling selected insect pests, the method has never been successfully applied in large-scale mosquito control strategies. The operational difficulty of irradiation in field conditions, loss of fitness, and shortened

Table 4. Summary of lineage strains of *Aedes aegypti* developed through genetic engineering methodologies.

Strain	Genetic-based technique	Reference
OX513A	RIDL	Harris et al. 2011
OX3604 C	RIDL	Wise de Valdez et al. 2011
WB2-BRA	SIT/IIT	Carvalho et al. 2020
WB2-MEX	SIT/IIT	Carvalho et al. 2020
WB2	<i>Wolbachia</i> intervention	Carvalho et al. 2020
wMelPop	<i>Wolbachia</i> intervention	McMeniman et al. 2008
AAEL010097-Cas9	CRISPR/Cas9	Li et al. 2017
AAEL000582-Cas9	CRISPR/Cas9	Kistler, VossHall, and Matthews 2015

lifespan of mosquitoes are among the most prominent concerns of the effectiveness of SIT programs (Marrelli et al. 2006; Qsim et al. 2017).

The *Wolbachia*-based incompatible insect technique (IIT) is a notable microbe-mediated infertility technique which emerged as an alternative to overcome obstacles of traditional methods, such as reduction of overall fitness and mating competitiveness of released males, and has been used both in lab and field experiments in different countries (Panagiotis and Bourtzis 2007). The Gram negative bacteria *Wolbachia* establishes symbiotic relationships with a wide range of species, and produces reproductive alterations in the hosts, such as parthenogenesis, feminization, male killing and cytoplasmic incompatibility. IIT consists of sustained release of *Wolbachia*-infected incompatible males in order to sterilize the targeted female population (Papathanos et al. 2018). It is noteworthy that cytoplasmic incompatibility is a form of male sterility was effectively employed as a pest population suppression tool and underlying mechanism against *Aedes aegypti* (Moretti et al. 2018).

Transgenic lineages of *Aedes aegypti* were examined according to the “release of insects carrying a dominant lethal” (RIDL) strategy, known as a potential improvement on SIT and representing another genetic engineering modification method (Olson and Franz 2015). RIDL is defined as an insect sterile approach, deemed to be specific and ecologically friendly, and is also based upon male release with the aim to introduce sterility or lethality in the target population by utilizing a strain that carries dominant, sex-specific lethal genetic system (Qsim et al. 2017; Thomas et al. 2000).

The *Wolbachia*-based intervention, SIT and RIDL face the major obstacle of sex separation. Despite the effectiveness and ability to reduce the reproductive potential of mosquitoes, currently, there is no robust strategy to promote an efficient separation of males from females or genetic sexing strains during mass rearing of several arthropod species, including *Aedes aegypti*. Indeed, the rudimentary methods available are not sufficient for mass-release programs, and the high cost of needed improvements to scale-up is a major disadvantage (Papathanos et al. 2018).

Thus, future research needs to be designed to develop technologies and primary genetic components, along with highly efficient sex separation methods for mosquito control strategies. Further, the effect of releasing genetically modified mosquitoes on the population dynamics of non-target vectors needs to be investigated in order to assess the impact on the dynamics of disease transmission and the cost-benefit needs to be explored, due to its critical importance in the integrated vector management framework (Wilke, Beier, and Benelli 2018).

Recently, CRISPR-based genome editing has revolutionized the ability for precise genome manipulations and brought new perspectives to genetically modified mosquitoes research. In fact, CRISPR has emerged as an innovative technology to provide global solutions to protect crop yields and control vector-borne diseases and invasive pest species (Kandul et al. 2019). As depicted in Table 4, transgenic *Aedes aegypti* strains expressing Cas9 in the germline were developed, and the genetic manipulation resulted in a quite effective CRISPR/Cas9 transgenic system. Li et al. (2017) provided improvements in the consistency and specificity of genome modifications using this approach. These findings thereby created new lines for exploration of novel population control technologies targeting *Aedes aegypti* populations.

Concluding remarks

The need to exploit new strategies and control agents arise from the spread of *Aedes aegypti* worldwide and due to the sustained permanence of this species in already occupied territories, resulting in different life-threatening infectious illnesses. Owing to the intensive proliferation of mosquitoes throughout the year, the tropical endemic countries require higher frequency of treatments and, therefore, an increased selection pressure to enhance the use of larvicides.

It is to be assumed, herein, that the pesticide resistance is an increasingly urgent global issue regarding agricultural systems and control of vector-borne diseases. In this sense, the search for promising strategies to chemical-origin pesticides is driving the exploration of novel biolarvicides as a weapon against important vector of several debilitating arboviruses, including dengue, yellow fever,

Chikungunya, and Zika virus. Further, developments of efficient and environmentally friendly genetic engineering technologies for *Aedes aegypti* population control are required.

Thus, although progress achieved during the last decades concerning the knowledge and use of natural larvicides and genetic engineering-based tools, scientific and surveillance efforts need to focus on the development of long-lasting, effective mosquito control strategies, with a safe toxicological profile and ability to overcome potential hurdles for large-scale implementation.

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CAPÍTULO III

ARTIGO CIENTÍFICO EXPERIMENTAL

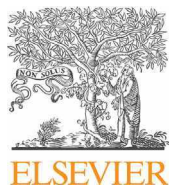
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Evaluation of toxicity and environmental safety in use of spinosad to rationalize control strategies against *Aedes aegypti*



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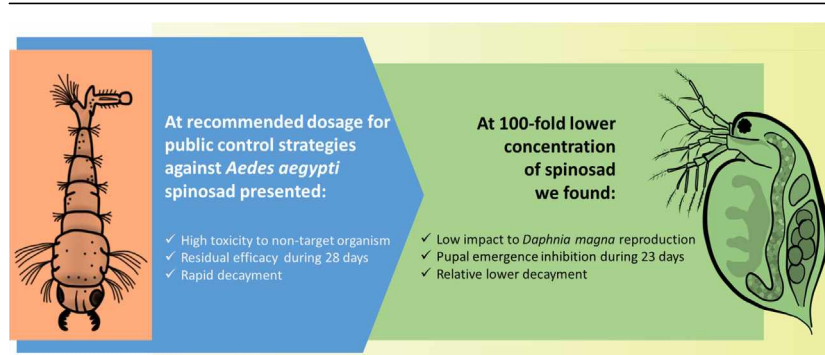
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HIGHLIGHTS

- Ecotoxicological parameters of spinosad were evaluated on *Daphnia magna*.
- Acute and chronic toxicity tests revealed a high toxicity risk of spinosad.
- Reproductive parameters were not markedly affected due to the rapid decay of spinosad.
- A 100-fold lower concentration showed satisfactory performance to control *Aedes aegypti*.

GRAPHICAL ABSTRACT



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ABSTRACT

Spinosad is a naturally-occurring insecticide used for the management of *Ae. aegypti* larvae. The assessment of ecotoxicological parameters of spinosad is required for verifying the environmentally-friendly behavior of the compound and for evaluating toxicity values on non-target species. Thus, the aim of the study was to conduct toxicity tests using *Daphnia magna* as model organism after exposure to different concentrations of spinosad. Immobility effects were observed in both acute and chronic toxicity tests at the concentration of 2.5 µg/L, and *D. magna* exhibited an EC_{50-48 h} of 4.1 µg/L and EC_{50-7d} of 9.3 µg/L. Also, the reproductive test showed a significant increase in the time of first reproduction and decrease in the number of neonates per female. However, due to the rapid decay of spinosad, other reproductive parameters were not markedly affected. Thereby, considering the satisfactory control performance against *Aedes aegypti*, a 100-fold lower concentration of spinosad can be used against the larvae, and owing to the residual efficacy observed, the application of the pesticide in the field may be rationalized while offering environmental safety.

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1. Introduction

Aedes aegypti is a well-known vector of several debilitating and life-threatening arboviruses, including Zika fever, Chikungunya, yellow fever and dengue (Black et al., 2002; Pereira et al., 2016). In an attempt to get the proteins necessary to develop their eggs,

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female mosquitoes feed on human blood and hence disease-causing viral pathogens are passed to it (Brown et al., 1994; Bhatt et al., 2013; Duvall et al., 2019).

Historically, as a native species of Africa, *Ae. aegypti* was introduced to South America in the 16th century with the advent of slave trade (Powell and Tabachnick, 2013). Importantly, the need of public health policies related to basic sanitation and infrastructure favored the dissemination of this species, which became highly domesticated, adapted to humans and currently is the main vector of critical tropical emerging infectious diseases (Gubler, 2011).

Therefore, hurdles remain mainly in tropical and subtropical countries regarding the efforts to overcome the spread of *Ae. aegypti* and, thus, public programs need to develop strategies to combat it (Pereira et al., 2016). Remarkably, one challenge to the Brazilian public programs to control *Ae. aegypti* is the selection of a bioraricide that will effectively exterminate mosquitoes without causing deleterious effects on non-target organisms.

Spinosad is a naturally derived biorational pesticide obtained through the aerobic fermentation of the actinomycete *Saccharopolyspora spinosa* (Bond et al., 2004; Kirst, 2010). Structurally composed of two active metabolites, spinosyn A and spinosyn D, containing a polyketide-derived tetracyclic macrolide with two saccharides, this insecticide has been gaining attention owing to its potential for use in integrated pest management systems (Williams et al., 2003; Huan et al., 2015), acting to control several insects, including caterpillars, flies, thrips, drywood termites and, as recently discovered, *Ctenocephalis felis* (Thompson and Sparks, 2002; Orr et al., 2009; Huan et al., 2015).

Spinosad present a unique mechanism of action which involves the disruption of nicotinic acetylcholine receptors and GABA-gated ion channels of the nervous system of insects (Kirst, 2010; Huan et al., 2015). Findings also affirm that this mode of action is not restricted to insects, and propose a different nicotinic receptor subunit that acts as an underlying molecular alternative site for spinosyns (Orr et al., 2009; Pereira et al., 2016).

Classified as an environmentally and toxicologically reduced risk compound by the United States Environmental Protection Agency, spinosad established a new standard for low environmental and human risk and hence is considered an effective tool in providing more final products while also protecting the environment (Thompson and Sparks, 2002). In fact, besides the chemically unique structure, spinosad reveals insecticidal efficacy and favorable toxicological profile (Salgado and Sparks, 2005).

Although it is known as an ecologically friendly insecticide (Del Rio-Galvan et al., 2016), documented cases of resistance of spinosad

are still limited (Zhao et al., 2002), and assessment of the ecotoxicological risk of this compound in organisms derived from aquatic ecosystems is crucial in order to achieve a suitable balance between the amount of the pesticide applied to control disease-carrying insects and the rational environmental management (Santos et al., 2017).

Daphnids are defined as aquatic microcrustaceans predominantly filter-feeding which play a crucial role in the aquatic ecosystem dynamics due to their significant trophic level in the food chain as primary consumers (Santos et al., 2017). Besides that, the species is extensively used as model organism in ecotoxicological tests owing to the high sensitivity for pollutants, easy culture and short life cycle (Lv et al., 2018; Santos et al., 2019).

In this sense, the objectives of the study were: (i) to determine the acute toxicity (LC50) of spinosad in *Ae. aegypti* larvae; (ii) to assess the potential for emergency inhibition of *Ae. aegypti* pupae using low spinosad concentration and (iii) to analyze the environmental safety of spinosad at low concentration by ecotoxicity assays using *D. magna*.

2. Material and methods

2.1. Larvicide

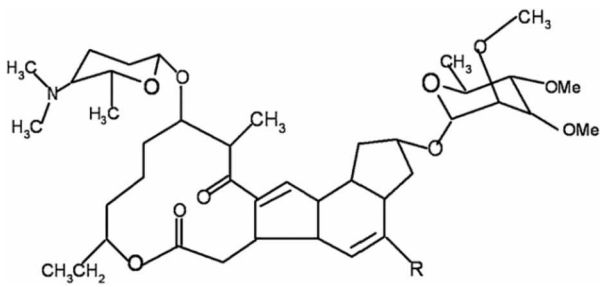
The Spinosad larvicide (Trade name: Natular DT; Chemical Abstract Service [CAS] Registry Number: 131929-60-7 - Spinosyn, used by WHO Acute list) used in the study with active ingredient 1.25 g/kg was obtained from the Center for Zoonosis Control of Uberlândia, Brazil. The structure and chemical composition are shown in Table 1.

Spinosad concentrations in the treatments were determined daily over 28 days by sampling of 100 mL from each container. Spinosad was extracted from samples with 40 mL dichloromethane, dried at 30 °C and resuspended in 1 mL acetonitrile. Then, samples (50 µL) were measured in a HPLC device and read by UV detector at 243 nm.

2.2. Organisms and culturing

D. magna Straus 1820 (Cladocera, Crustacea) were obtained from continuous cultures maintained in the Laboratory of Ecotoxicology, Federal University of Uberlândia, Brazil, according to Guideline 202 (OECD, 2004) established by Organization for Economic Cooperation and Development. The organisms were cultivated in synthetic media Elendt M4, pH 7.8 ± 0.2, total hardness 47 ± 5 mg/L in CaCO₃,

Table 1
Structural and physicochemical properties of spinosad.

Chemical structure	Physicochemical properties	
 <p>C₈₃H₁₃₂N₂O₂₀ MW: 1477.9 g/mol</p>	Microbial origin	Mixture
	<i>Saccharopolyspora spinosa</i>	85% spinosyn A 15% spinosyn D

Note. MW = Molecular Weight.

which was renewed three times per week. The water fleas were fed daily with *Arthrospira platensis* algal suspension on a basis of 150 µg/organism/day. *D. magna* culture was maintained in acclimated aquaria with dilution water (pH 7.2 ± 0.1 , total hardness of 246 ± 7 mg/L in CaCO₃) at 20 ± 1 °C, under a 16: 8 h light/dark photoperiod for 24 h before starting the tests. The dilution water used for acclimatization of the organisms in the aquaria was considered as negative control and also for preparation of all test solutions.

Mosquitoes were collected in different neighborhoods from urban perimeter in Uberlândia, representing areas of highest level of infestation of *Aedes aegypti*. Eggs of *Ae. aegypti* were obtained using ovitraps according methodology proposed by Silva and Limongi (2018). At laboratory conditions, eggs were hatched in plastic containers with 1 L of tap water (dechlorinated) to obtain the larvae.

2.3. Tests setup with *Aedes aegypti* larvae

Acute toxicity test with *Ae. aegypti* larvae was performed based on the World Health Organization protocol (World Health Organization, 1981). According this recommendation, batches of mosquito larvae must be exposed to different concentrations of larvicide in four replicate tests at each concentration. Third instar larvae of *Ae. aegypti* were exposed to six different concentrations of Spinosad active ingredient (500; 250; 125; 62.5; 31.25 and 15.63 µg/L). For each concentration, 200 L3 larvae (quadruplicate with 50 larvae) were exposed in plastic bowls containing 100 mL of solution. Mortality was recorded after 48 h of exposure.

Test of residual efficacy of Spinosad were conducted on sixteen polyethylene water containers (with capacity of 25 L) filled with 20 L of dechlorinated tap water (twelve were treated with three different concentrations of Spinosad and four were maintained with water as control). The tests were performed in quadruplicate, using the field recommend concentration of Spinosad (500 µg/L) and two lower concentrations (50 µg/L and 5 µg/L) to estimate the potential of inhibition of pupae emergence. The plastic containers used in the tests were covered with mosquito nets to prevent oviposition by external insects. Each container was colonized with 50 L3 larvae and daily monitored for checking mortality records during 28 days.

2.4. Environmental safety tests

Acute toxicity tests with *D. magna* were carried out according to the Guideline 202 of Organization for Economic Cooperation Development (OECD, 2004).

The acute toxicity tests were performed in quintuplicate, in a static system, using five neonate daphnids per each concentration of Spinosad (20 mL glass beakers containing 10 mL of six different concentrations of pesticide diluted in reconstituted water, ranging from 1.25 to 80 µg/L Spinosad active ingredient with a 0.5 dilution factor) and to a negative control (reconstituted water only) for 48 h (with medium renovation after 24 h). Immobilization endpoint was recorded after exposure period by observing the organisms under a stereomicroscope.

Chronic toxicity tests for lethality assessment of *D. magna* during 7 days of exposure were performed according to OECD Guideline 211 (OECD, 2012). Tests were conducted in a semi-static system, and culture media was renewed every 48 h. For exposure to the insecticide, 10 animals were individually held at each test concentration (the same used in acute test) performed in 100 mL glass beakers containing 50 mL of test solution.

2.5. *Daphnia magna* reproduction test

Based on the test of residual efficacy of spinosad, daphnids were exposed to the lowest tested concentration (5 µg/L and control) for 21 days. The young female daphnids (parent animals) with age less than 24 h at the start of the experiments were derived from a healthy parthenogenetic stock culture.

Ten parent animals were maintained individually, one per test beaker, containing 50 mL of test solution and control. Both the time to the first reproduction and reproduction rates (brood size, number of broods per female, number of neonates per female and total number of living offspring per female) of *D. magna* were monitored for each individual organism and recorded every 24 h. The offspring resulted from each parent animal was counted daily and removed.

2.6. Statistical analysis

Data were tested for normality using the Shapiro–Wilk test before all analysis. For tests with a normal distribution, Bartlett's test for homogeneity was used. Lethal concentrations (LC_{50–48h}) for L3 *Ae. aegypti* larvae and confidence interval was estimated using probit analysis. For *D. magna* tests, the median immobility concentration (EC_{50–48h}) was calculated according to OECD Guideline 171 (OECD, 2012). To analysis of chronic exposure and reproduction tests with *D. magna*, the estimation of the CL_{50–7d} (lethality on 50% of daphnids after 7 days of chronic test) and EC_{50–21d} (50% reduction in reproductive output of parental daphnids after 21 d of exposure) were calculated according to OECD Guideline 211 (OECD, 2012).

Additionally, to assessment of the spinosad environmental safety for *D. magna*, a risk quotient (RQ) was calculated by dividing the value of recommended and lowest tested (5 µg/L) concentrations of use for the pesticide by the EC_{50–48h} value calculated in the acute toxicity tests. For all analysis, p values < 0.05 were considered statistically significant.

3. Results

Spinosad concentrations (residual concentration) were analyzed daily and results showed rapid decrease of initial exposure levels (500; 50 and 5 µg/L). After 48 h of exposure, residue concentrations were 70.1 ± 10.2 ; 13.3 ± 7.5 and 2.2 ± 0.3 µg/L, respectively. Residue concentrations were at limit of quantification (0.2 µg/L) after 27, 21 and 17 days, respectively, after the start of test (Fig. 1).

Acute toxicity test with *Ae. aegypti* third instar larvae (L3) showed a lethal concentration of 85.3 µg/L (CI: 50.1–145.1 µg/L), which is 5.86-fold lower than the recommended dosage (500 µg/L) for field interventions against the insect development (Fig. 2).

The results related to the assessment of residual efficacy of spinosad are shown in Fig. 3. The treatments performed with the three concentrations tested did not differ until the 22nd day, guaranteeing pupal emergence inhibition in at least 92% of the exposed containers. The lowest concentration tested maintained residual efficacy greater than 80% inhibition until the last day of the test. Inhibition of emergence of pupae at all concentrations tested was significantly different from control.

Table 2 shows the results of the ecotoxicological evaluation of spinosad in *D. magna*. From the 2.5 µg/L concentration of spinosad, immobility effects were observed in the organisms exposed in both acute and chronic exposure tests. As can be observed, the toxicity calculated for the acute test (EC_{50,48h}) was higher than the chronic toxicity (EC_{50,7d}), considering that there was a renewal of the medium after 24 h in the first one, whereas in the chronic one, there was a continuous decrease of the concentration of spinosad in the middle.

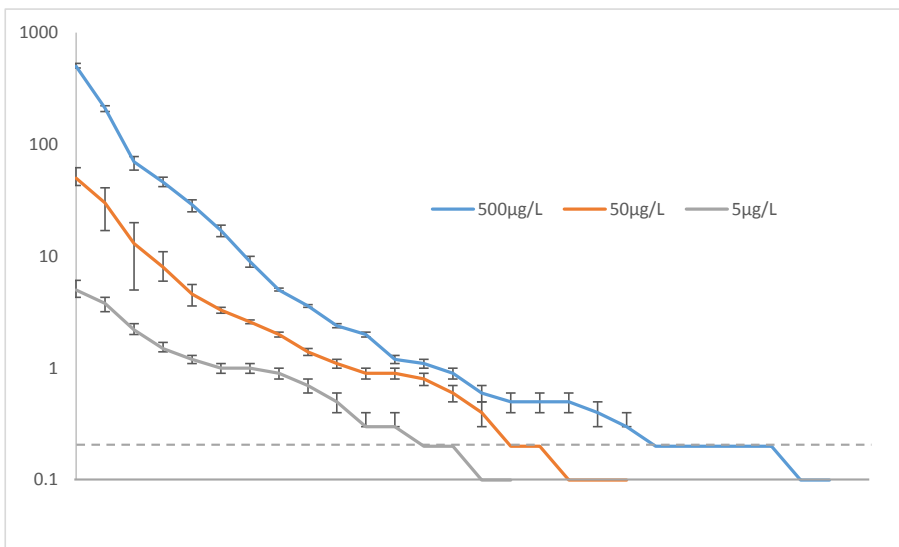


Fig. 1. Spinosad concentrations (residual concentration) measured daily from exposed water in containers.

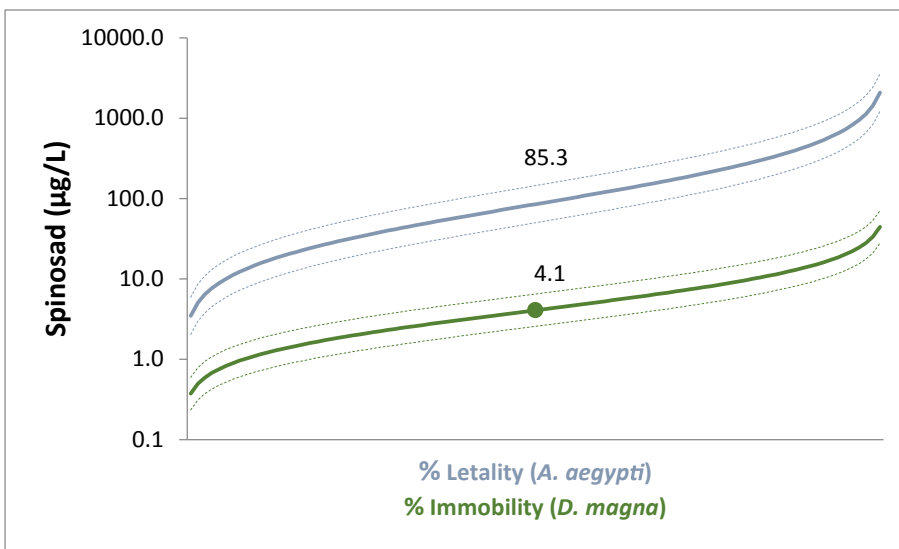


Fig. 2. Acute toxicity of spinosad on 3rd instar *Aedes aegypti* larvae and *Daphnia magna* after a 48 h exposure.

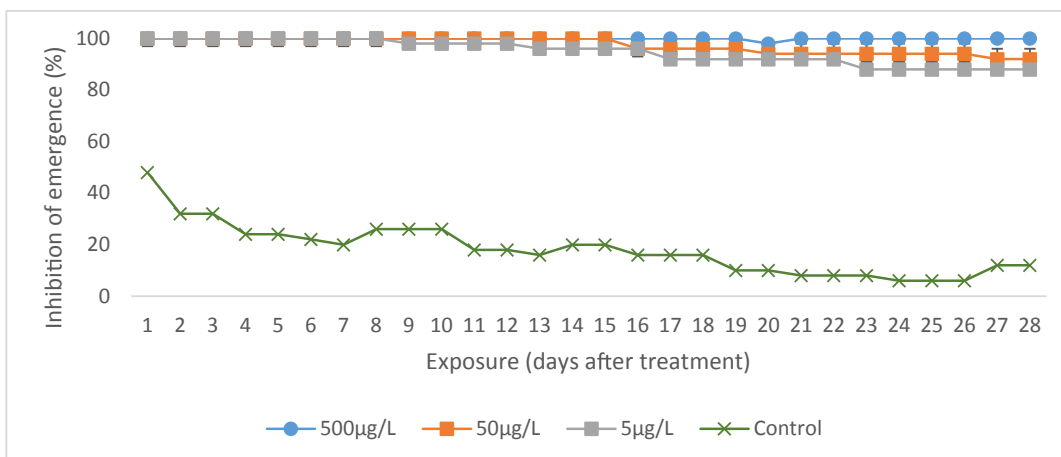


Fig. 3. Residual efficacy of spinosad on inhibition of pupal emergence of *Aedes aegypti* larvae.

Table 2

Ecotoxicological parameters (95% confidence interval) and environmental risk obtained from acute, chronic and reproduction tests with *D. magna* after exposure to different concentrations of spinosad.

Ecotoxicological parameters of exposure		($\mu\text{g/L}$)	CI95%
Acute exposure (48 h)	NOEC	1.25	0.8–1.9
	LOEC	2.5	1.5–3.9
	EC50	4.1	2.6–6.5
Chronic exposure (7d)	NOEC	1.25	0.8–2.0
	LOEC	2.5	1.4–4.1
	EC50	9.3	5.9–14.9
RQ in usual concentration (500 $\mu\text{g/L}$)	121.9	High Risk	

Note. The Risk Quotient (RQ) categories used were: high risk ($\text{RQ} > 0.5$), medium risk ($0.05 < \text{RQ} < 0.5$) and low risk ($\text{RQ} < 0.05$).

Table 3

Reproduction parameters of *D. magna* after exposure to spinosad (5 $\mu\text{g/L}$) and control during 28 days.

Reproduction parameters	Spinosad (5 $\mu\text{g/L}$)	Control
Time to production of first brood (days) ^a	8.4 \pm 0.5*	7.8 \pm 0.4
Number of broods per female ^b	5.1 \pm 0.4	5.4 \pm 0.8
Brood size ^c	22.0 \pm 1.4	24.2 \pm 2.7
Number of neonates per female ^d	104 \pm 13.8	130.7 \pm 12.9*
Number of living offspring per female ^e	81.3 \pm 18.8	101.5 \pm 15.6*

Note. Student *t*-Test.

^a $p = 0.04$.

^b $p = 0.31$.

^c $p = 0.19$.

^d $p = 0.03$.

^e $p = 0.02$.

The use of high concentrations in the field during actions to combat larvae of *Ae. aegypti* mosquito associated to the high toxicity evaluated in the acute exposure reveals a high risk of environmental toxicity (ETR) of spinosad for *D. magna*.

Fig. 2 allows to compare the susceptibility of *Ae. aegypti* larvae to *D. magna*, showing that the non-target organism is significantly more susceptible to spinosad.

The reproductive parameters of *D. magna* after chronic exposure of 28d to the initial test concentration of spinosad 5 $\mu\text{g/L}$ are presented in Table 3. The exposure to the larvicide increased the time to production of the first brood and reduced the number of neonates and living offspring per female in comparison to control.

As previously shown, ecotoxicological parameters revealed that acute exposure to spinosad caused high toxicity to *D. magna* (Table 2; Fig. 2). However, reproductive parameters (number of broods per female and brood size) were not significantly altered by chronic exposure to larvicide at the diagnostic dose tested (5 $\mu\text{g/L}$).

According to Fig. 4, it is possible to observe that the rapid decay of the tested concentration of spinosad (Fig. 4A) allowed that the reproduction of *D. magna* was not much affected in relation to the control group (Fig. 4B), although the population decreased on the first day of exposure.

4. Discussion

Aedes aegypti is the main vector of acute systemic diseases of worldwide occurrence. Urban and epidemic transmission cycles have been causing outbreaks throughout tropical and subtropical regions (Batth et al., 2013).

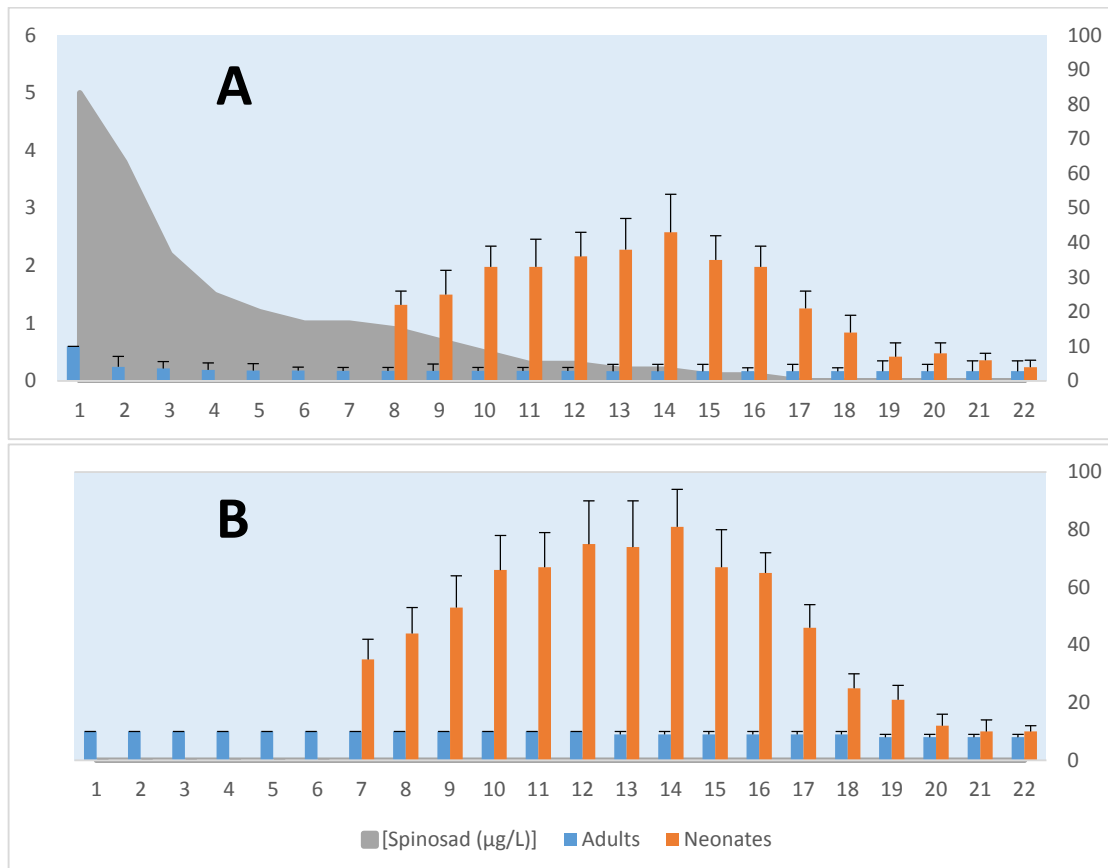


Fig. 4. Number (mean \pm SE) of living adults and neonates exposed (A) to low concentration of spinosad (5 $\mu\text{g/L}$) and non-exposed (control) during 21days observation period.

As already pointed out, the combination of both spinosyn A and spinosyn D constitutes the naturally occurring compound spinosad, obtained from the fermentation broth of the soil actinomycete *S. polyspora* (Kirst, 2010). Accordingly, spinosad has been used as insecticidal agent in the management of a wide range of insect pests and, due to the natural origin, the compound is often referred to an environmentally-friendly behavior (Huan et al., 2015). In fact, owing to the tendency to degrade faster in the environment, natural larvicides are expected to show less toxicity to non-target organisms in comparison to synthetic compounds (Anogwih et al., 2015). However, despite widespread application of spinosad, researches on the environmental safety and ecotoxicity of this pesticide are still scarce.

The present study was based on the impacts of spinosad on non-target organism. Although the insecticide is effective for pest control, we confirmed that spinosad showed a low safety behavior considering *D. magna* as model organism. Notwithstanding that, we presumed that the use of concentrations lower than the recommended dosage used in the field constitute an effective and safe control of *Ae. aegypti* larvae. Notably, while the recommended dosage to control the insect development is 500 µg/L, our findings revealed that the lowest concentration (5 µg/L) maintained the residual efficacy of spinosad by inhibiting the pupal emergence of the mosquito in 80%. In fact, pesticide evaluations on natural enemies should include not only acute toxicity assessment, but also residual toxicity (Desneux et al., 2007). Previous studies showed that concentrations of 50 µg/L were able to control over 95% of *Culex* spp. larvae, which is 10-fold lower than the recommended dosage. Also, it was reported a LC_{50–24h} of spinosad of 25 µg/L and 24 µg/L against 3rd and 4th instars of *Ae. aegypti* and *Anopheles albimanus*, respectively (Jiang and Mulla, 2009).

Regarding the rapid decrease of spinosad in the culture media, this is confirmed in this study since, at 5 µg/L, *D. magna* reproduction was not significantly affected after chronic exposure. In fact, insecticides undergo several dissipation processes after application in the field, including photolysis, hydrolysis, leaching, volatilization and microbial degradation (Adak and Mukherjee, 2016). This occurs primarily due to the loss of the forosamine sugar of the larvicide structure and reduction of the 13, 14-bond on the macrolide ring (Cleveland et al., 2002). In agreement with our data, Adak and Mukherjee (2016) also demonstrated that after 30 days, 92.3% of spinosad dissipated in soil conditions.

Besides that, the environmental risk assessment is essential in predicting the safety of the chemicals, and depending on the value, this might limit their application in the field (Abe et al., 2014). Our findings indicated that spinosad is highly toxic to *D. magna* even at low concentrations, showing a high environmental toxicity risk to daphnids. Further, it is also important to highlight that microcrustaceans have been widely applied in several evaluations of environmental safety, since they are influenced by physico-chemical alterations of the water and interactions with larvicides may cause deleterious impacts on the ecosystem (Simões et al., 2011).

In conclusion, spinosad revealed high toxicity risk to non-target species at the recommended dosage in field applications. Alternatively, a 100-fold lower concentration showed satisfactory control performance against *Ae. aegypti* larvae, which is also environmentally safe, evidencing that excessive and unnecessary dosage of spinosad to control mosquitoes need to be avoided. Furthermore, it is also interesting to considerer different strategies to combat *Ae. aegypti*, for example, the use of spinosad in a rational dosage or integrated biological approaches to control the mosquito larvae for longer periods.

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CAPÍTULO IV

ARTIGO CIENTÍFICO EXPERIMENTAL

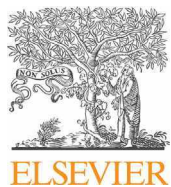
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Association of low concentrations of pyriproxyfen and spinosad as an environment-friendly strategy to rationalize *Aedes aegypti* control programs



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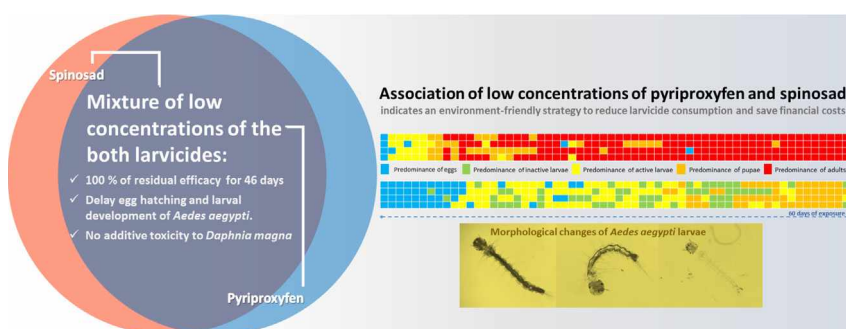
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HIGHLIGHTS

- An environment-friendly strategy to control *Aedes aegypti* was purposed.
- Association of low concentrations of larvicides was tested against *Aedes aegypti*.
- Ecotoxicological parameters of the mixture was performed on *Daphnia magna*.
- The tested mixture only altered the behavior and development of *Aedes aegypti*.
- Results present an alternative way for public control programs to be more efficient.

GRAPHICAL ABSTRACT



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ABSTRACT

The association of low concentrations of pyriproxyfen and Spinosad, a naturally-occurring insecticide, was evaluated as an environment-friendly strategy to rationalize *Aedes aegypti* control programs by reducing larvicide consumption, saving financial costs and increasing residual effect against mosquitoes development. Firstly, the ecotoxicological parameters of the mixture was performed on non-target species *Daphnia magna* and the results confirmed that the low concentrations used in this larvicide mixture were not able to alter the reproductive parameters of chronically exposed microcrustaceans. In contrast, the mixture altered the behavior and development of *Aedes aegypti*, effectively inhibiting the emergence of adult insects for a long period. The results confirm the hypothesis that even at very low concentrations, the combination of the Spinosad and Pyriproxyfen larvicides offers an opportunity for *Aedes aegypti* public control programs to be more efficient.

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1. Introduction

Aedes aegypti is the main vector of important acute systemic diseases, such as dengue and yellow fever, and has also been associated with the large-scale emergence of viruses Chikungunya

and Zika (Halstead, 2015; Musso et al., 2015; Bottino-Rojas et al., 2019). In fact, the spread of emerging arboviruses still represents a global public health concern, mainly in tropical and subtropical regions which harbour the mosquito species responsible for virus transmission (Silva et al., 2019). Thus, in these areas, the species is the target of considerable public health efforts and the most common strategies to combat the vector are based upon chemical larvicides or insecticidal tools which affect *Aedes aegypti* by different modes of action, affecting larvae and adults, respectively (Devine et al., 2009; Santos et al., 2017).

Remarkably, *Aedes aegypti* viral surveillance occurs inadequately in endemic countries, and it is still a challenge to the Brazilian integrated control programs to overcome the spread of disease-carrying insects with an effective and economically viable larvicide without causing deleterious effects on non-target organisms (Silva et al., 2019). Furthermore, special attention should be given to the improvement in health surveillance systems to control the mosquito vector in order to prevent outbreaks of diseases, as there is no licensed vaccines, effective antiviral agents or specific medication available to deal with the arboviruses (Darriet et al., 2010).

Dengue control has been primarily conducted by combating the vector. Thus, insecticides have been widely applied in the mosquito breeding sites in order to target mosquito larvae. However, despite the pivotal role in the control of *Aedes* vectors, the irrational and intensive use of pesticides can cause environmental and health impacts, hence leading to negative effects, such as selection of resistance populations of pests and reduction of biodiversity of insects and soil microorganisms (Vontas et al., 2012; Wagan et al., 2014).

The use of conventional pesticides, as the organophosphates and pyrethroids (World Health Organization, 2016), is declining owing to the resistance developed by *Aedes aegypti*, and hence the exploration of new compounds to control the vector should be considered. In this sense, currently, different biotechnological approaches have been explored in order to curb the transmission focus of systemic diseases (Bhatt et al., 2013). The first strategy is the use of the biolarvicide known as Spinosad, a naturally occurring neurotoxic pesticide synthesized through the fermentation of the soil actinomycete *Saccharopolyspora spinosa* (Sparks et al., 1995; Guojun et al., 2016). Also, in a previous study, Pereira et al. (2016) propose the integration of pest management systems using larvivorous fish with low concentrations of spinosad as a different vector control method. In addition, natural alternatives have been tested, including β -cyclodextrin complexed with *Lippia gracilis* essential oil, which is a new promising reference due to the high efficacy and strong larvicidal activity against *Aedes aegypti* larvae (Galvão et al., 2019).

However, although is derived from a naturally occurring species, recent studies have been highlighted the widespread use of biological compounds, such as Spinosad, to control vectors and pests of economic and public health importance. As a consequence of the intensive use, this approach has also been causing potential adverse effects against non-target organisms, including arthropods, which can be natural predators of *Aedes aegypti* (Biondi et al., 2012).

Thus, in order to succeed dealing with this, the challenge is to rationalize the control of *Aedes aegypti* by reducing the amount of larvicide used simultaneously by improving the efficacy of Brazilian public programs, hence reducing the costs and the environmental impact. In this sense, research into new approaches aimed at reducing the use of larvicides or limiting the development of resistance in *Aedes aegypti* has been conducted towards the mixture of two compounds, each one showing a different mechanism of action.

Importantly, World Health Organization recommends alternative strategies in resistance management programs, as mixtures,

rotations or mosaics, by using pesticides sharing different modes of action (WHO, 2003; Ahmad et al., 2009). The employment of mixtures is favoured when the pesticide effectiveness is high and exposure is low (Levick et al., 2017).

Recently, the association between Spinosad and Pyriproxyfen has been investigated as an interesting strategy against mosquitoes. Characterized as a juvenile hormone analog, Pyriproxyfen is a potential insect growth regulator and it has been used to control a wide range of arthropods and vectors of diseases. Field and laboratory findings have found good residual activity of the compound against *Aedes aegypti*, whose treatment from it results in death typically at the pupal stage (World Health Organization, 2000; Hustedt et al., 2017). Also, Spinosad does not show cross-resistance with conventional pesticides, and reveals high potential to combat mosquitoes (Darriet et al., 2010).

However, the assessment of the applicability of these insecticide intercropping strategies rarely considers environmental safety and ecotoxicological parameters for non-target organisms. Accordingly, the negative effects on the environmental and public health caused by the irrational use of larvicides can be measured based on ecotoxicological assays using model organisms, such as *Daphnia magna* (Pino-Otín et al., 2019; Santos et al., 2019).

In this sense, the present study aimed to investigate an association of low concentrations of Pyriproxyfen and Spinosad, following ecotoxicological assays with *Daphnia magna*, in order to propose an environment-friendly strategy to rationalize *Aedes aegypti* control programs.

2. Material and methods

2.1. Larvicides

The larvicides used in the study were obtained from the Center for Zoonosis Control of Uberlândia, Brazil. Technical information about the compounds are presented in Table 1.

Based on results previously obtained by the authors for acute toxicity tests of the larvicides studied in *D. magna* (Santos et al., 2017, 2019), the initial concentration of Spinosad and Pyriproxyfen used in the mixture tested in the present investigation was established using the 'No Observed Effect Concentration' (NOEC) of Pyriproxyfen and Spinosad (0.63 and 1.25 $\mu\text{g/L}$, respectively).

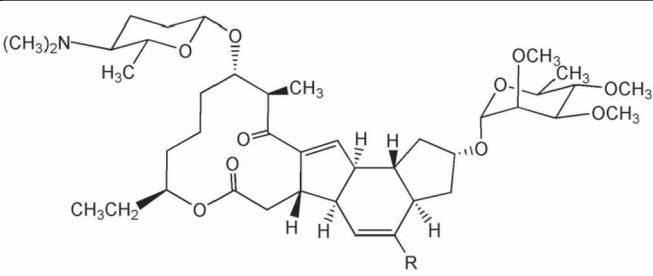
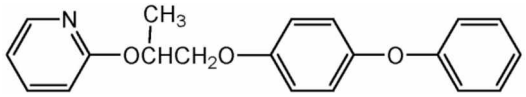
2.2. Biological material

Aedes aegypti eggs were collected using ovitraps by agents from the Center for Zoonosis Control of Uberlândia, Brazil, in urban areas of highest level of infestation of the vector (Silva and Limongi, 2018).

Daphnia magna Straus 1820 (Cladocera, Crustacea) were obtained from continuous cultures maintained in the Laboratory of Ecotoxicology, Federal University of Uberlândia, Brazil, following recommendations of Organization for Economic Cooperation and Development Guideline 202 (Organization for Economic Cooperation and Development, 2004). Daphnids were fed daily with *Arthrospira platensis* algal suspension on a basis of 150 $\mu\text{g/organism/day}$, and the culture medium synthetic media Elendt M4 (pH 7.8 ± 0.2 , total hardness $47 \pm 5 \text{ mg/L}$ in CaCO_3) was renewed three times per week.

D. magna culture was maintained in acclimated aquaria under specific conditions of dilution water (pH 7.2 ± 0.1 , total hardness of $246 \pm 7 \text{ mg/L}$ in CaCO_3) in a temperature of $20 \pm 1 \text{ }^\circ\text{C}$, 16: 8 h light/dark photoperiod for 24 h before starting the tests. The negative control was the dilution water used for acclimatization of the organisms in the aquaria, which was also considered for preparation of all test solutions.

Table 1
Technical information about larvicides tested.

Larvicide	Trade name	CAS	Presentation	AI (%)	FRC (µg/L)	Chemical structure
Spinosad	Natular DT	131929-60-7	Tablets	7.48	500	
Pyriproxyfen	Sumilarv	95737-68-1	Granular formulation	0.5	10	

Note. CAS: Chemical Abstract Service; AI: Active Ingredient; FRC: Filed Recommended Concentration.

2.3. Ecotoxicological tests

To test environmental safety of the proposed Pyriproxyfen/Spinosad mixture, reproduction parameters of *D. magna* were evaluated by the comparison between long-term exposure (28 days) to the mixture and control.

The *Daphnia magna* reproduction test was performed in a semi-static system (with media renovation every 48 h), using 10 young female daphnids for mixture and control tests. The test was conducted by individual exposure of each parent female in beakers (capacity of 100 mL) containing 50 mL of Pyriproxyfen/Spinosad mixture (exposed group) or dilution water used for acclimatization (control groups).

The time to the first reproduction and the parameters of reproduction rates (brood size, number of broods per female, number of neonates per female and total number of living offspring per female) of *Daphnia magna* were controlled for each individual organism and recorded every 24 h. The offspring resulted from each parent animal was calculated daily and removed.

2.4. Hatching and development/behavior monitoring

In order to evaluate the toxicological effects of the mixture, groups of 50 *Aedes aegypti* eggs were exposed in quadruplicate (200 eggs per test) to different concentrations of the mixture (100%, 50%, 25% and 12.5%) and to dechlorinated water without larvicide (control group). For exposure, 200 mL beakers containing 100 mL of the tested solution were used. In order to avoid oviposition of insects in the environment, the containers were covered with mosquito nets. The temperature was maintained between 25 and 28 °C, the relative humidity ranged from 50 to 60% and the photoperiod was restricted to 12 h of light: 12 h of darkness.

Initially, lethality, behavioural aspects and predominant morphological alterations of L3 stage larvae exposed to different dilutions of the mixture were monitored at 3 time intervals (10 min, 24 h and 48 h).

Besides that, in order to evaluate the residual effect of the larvicide mixture, four experimental groups (quadruplicate) containing 100% of the tested combination of Spinosad and Pyriproxyfen were kept under evaluation until 60 days of exposure, with the addition of 50 eggs per day and removal of dead organisms. Based on the daily observation records of the exposed groups, it was

possible to determine the inhibition of adult emergence and patterns of predominance of the developmental phase and insect behavior, considering the following categories: predominance of eggs; predominance of inactive larvae; predominance of active larvae; predominance of pupae and predominance of adults.

2.5. Statistical analysis

Data were evaluated for normality using the Shapiro–Wilk test before all analysis. Also, Bartlett's test for homogeneity was used for tests with a normal distribution. Predominance of development and behavioural stage was recorded on basis of absolute frequency of events. To compare reproduction parameters with *D. magna* (reduction in reproductive output of parental daphnids after 28 d of exposure) between control and exposed groups, a Student-t test was performed. P values < 0.05 were considered significant for all statistical analysis.

3. Results and discussion

Aedes aegypti is the main vector of life-threatening diseases of worldwide occurrence, currently considered one of the greatest health threats. Veritably, urban and epidemic transmission cycles have been causing several outbreaks throughout tropical and subtropical regions. In this sense, studies have been explored the potential of different compounds to control larvae and mosquitoes of the species (Bhatt et al., 2013).

In the current work, based on the preliminary results (Santos et al., 2017, 2019), NOEC values obtained in ecotoxicological tests which considered Spinosad and Pyriproxyfen concentrations much lower than those recommended for use in *Aedes aegypti* control programs (Table 1) were combined. Findings revealed that this association did not cause chronic effects on *D. magna*, as shown by the reproductive parameters evaluated (Table 2). Thus, these results are consistent with the effect observed when *D. magna* populations were submitted to each larvicide in isolation. This means that the use of the mixture maintains the environmental safety standard for the evaluated non-target species *Daphnia magna*. Therefore, at the combined concentrations, Spinosad and Pyriproxyfen showed no additive ecotoxicity effect for the reproductive parameters evaluated in *D. magna* during 28 days of exposure.

The effects on larvae of *Aedes aegypti* are depicted in Table 3. As

Table 2
Daphnia magna Reproduction Test parameters observed following long-term exposure (28 days) to tested mixture of low concentrations of Spinosad and Pyriproxyfen.

Reproduction parameters	Mixture	Control
Time to production of first brood (days) ^a	8.2 ± 0.4	7.9 ± 0.3
Number of broods per female ^b	5.2 ± 0.5	5.1 ± 0.6
Brood size ^c	19.1 ± 3.7	23.7 ± 3.5
Number of neonates per female ^d	99.2 ± 20.1	109.5 ± 21.4
Number of living offspring per female ^e	90.0 ± 21.2	99.3 ± 22.2

Note. Student *t*-Test; ^a*p* = 0.15; ^b*p* = 0.48; ^c*p* = 0.28; ^d*p* = 0.21; ^e*p* = 0.19.

can be observed, the mixture caused lethality, behavioural and morphological changes in L3 at 10min, 24 h and 48 h exposure.

Also, the mixture inhibited pupae formation and adult emergence, and induced mortality in a dose-dependent manner. The pesticides caused significant alterations in swimming behavior of *Aedes aegypti* larvae, including displacement and speed, which is a strong evidence of the sublethal effect of the mixture against the species (Table 3).

Fig. 1 shows that the mixture inhibited the emergence of viable adults by 100% for 46 days, and at the end of 60 days of exposure,

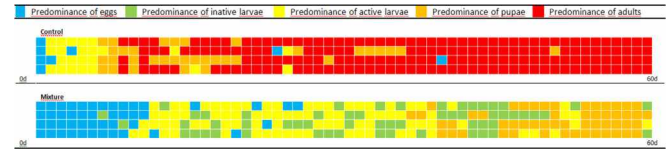


Fig. 2. Daily predominance of development and behavior stages during the long-term exposure of *Aedes aegypti*. To the Spinosad and Pyriproxyfen mixture.

the inhibition rates were still above 85%. In the control group, adult emergency inhibition ranged from 4 to 44%.

Fig. 2 reveals that the mixture acted by delaying egg hatching and larval development of *Aedes aegypti*. From day 45 of exposure to the mixture, the larval development was predominant, but most of them did not survive, hence reducing the production of adult mosquitoes. In the control group, from the 9 day of exposure, adult mosquito production was prevalent.

Under the effect of exposure to the mixture, as shown in Fig. 3, the larval and pupal phases were predominant in the evaluated period, which is consistent with the insecticidal properties of SPN and Pyr, respectively.

Spinosad has a unique mechanism of action which directly

Table 3
Lethality, behavior and morphological alterations in *Aedes aegypti* larvae (L3) after exposure to different dilution of the Spinosad and Pyriproxyfen mixture.

10 min			24 h			48 h		
Lethality	Behavior change	Morphological alteration	Lethality	Behavior change	Morphological alteration	Lethality	Behavior change	Morphological alteration
Control 0	—	—	0	—	—	0	—	—
12.5%	0	Low excitation	25%	Moderate excitation	Slightly altered abdominal segments	100%	Paralysis	Slightly altered abdominal segments
25%	0	Low excitation	33%	Moderate excitation	Slightly altered abdominal segments	100%	Paralysis	Moderately altered abdominal segments
50%	0	Low excitation	40%	Moderate excitation	Abdominal segments poorly defined	100%	Paralysis	Abdominal segments poorly defined
100%	0	Moderate excitation	75%	Strong excitation	Abdominal segments poorly defined	100%	Paralysis	Abdominal segments poorly defined

N = 50 larvae per test (quintuplicate).

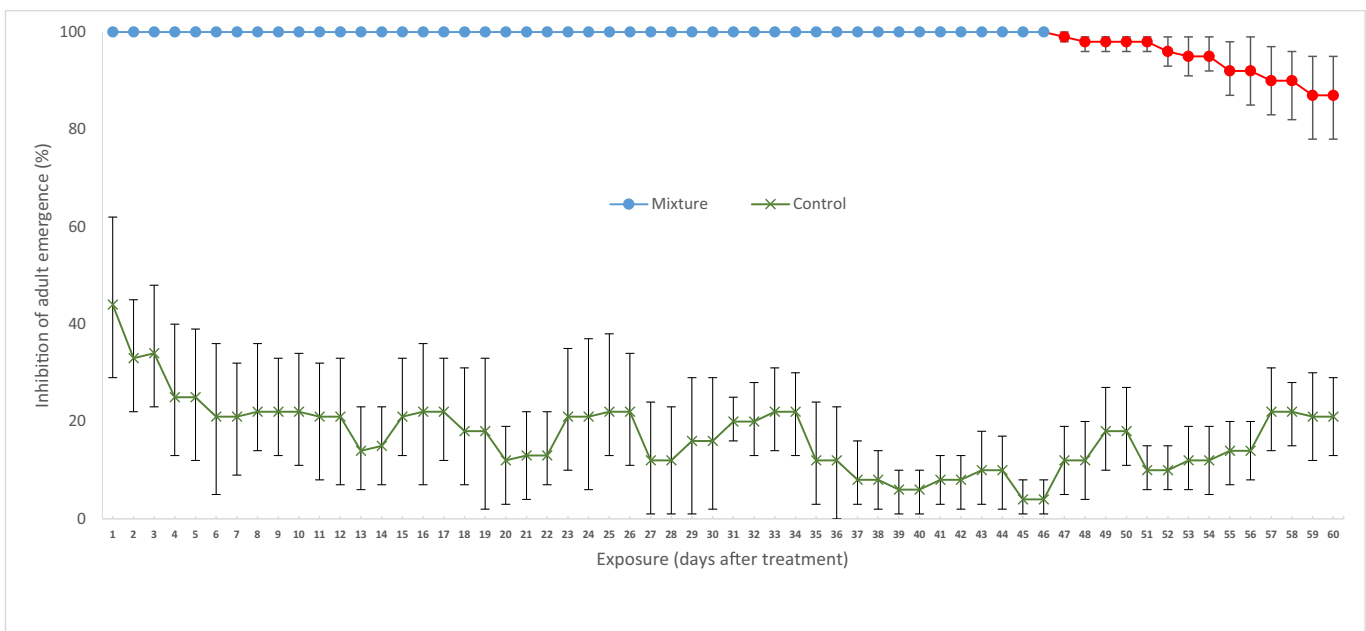


Fig. 1. Inhibition of adult emergence during long-term exposure (60 days) of *Aedes aegypti* to the Spinosad and Pyriproxyfen mixture.

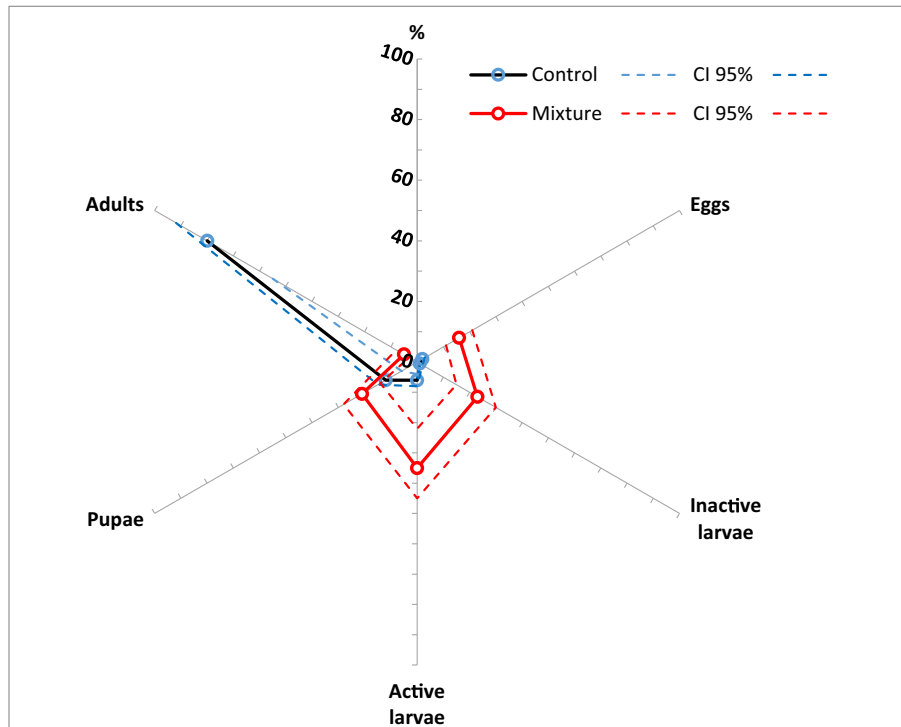


Fig. 3. Final predominance of development stages after the long-term (60 days) exposure of *Aedes aegypti* to the Spinosad and Pyriproxyfen mixture.

affects the insect central nervous system, hence leading to involuntary neuronal excitation that produces tremors, muscle contraction, paralysis and death (Salgado, 1998; Ureña et al., 2019). The biochemical interaction with the biolarvicide implicates the disruption of nicotinic acetylcholine receptors and GABA-gated ion channels of the nervous system of insect species (Kirst, 2010; Z. Huan et al., 2015).

On the other hand, Pyriproxyfen is classified as a juvenile hormone analog, acting as an insect growth regulator (Dzieciolowska et al., 2017; Alves et al., 2019). The chemical regulates several post-embryonic development processes and insect reproduction, exerting disturbance of larval development and effective inhibition of adult characteristics (Wang et al., 2013; Caixeta et al., 2016). Hence, while Spinosad has a strong effect on the early larval stages of *Aedes aegypti*, Pyriproxyfen acts on the late stages of the species.

Despite the efficacy of isolated Spinosad and Pyriproxyfen against specific target organisms for pest control, few studies have been addressed to investigate the performance of mixtures composed by both compounds specifically on the development of *Aedes aegypti* (Darriet and Corbel, 2006; Darriet et al., 2010). Therefore, in this study, we aimed to evaluate the efficacy of the mixture of Spinosad and Pyriproxyfen as a potential strategy to rationalize control programs by reducing the pesticide consumption and, consequently, saving financial costs.

Notably, our findings revealed that the mixture has no additive toxicity to *Daphnia magna*, showing a favorable toxicological profile on the non-target organism, but acts synergistically in inhibiting the development of adult mosquitoes, enabling the prolongation of vector control campaigns. In fact, the association of pesticides which work in synergy and that share different mechanisms of action is a promising tool for the needs of public health.

Notwithstanding that, the residual efficacy of the mixture was able to prevent the adult emergence of *Aedes aegypti* in 100% during 45 days of exposure. It altered the behavior and development of larvae, effectively inhibiting the emergence of adult insects for a long period. The results confirm the hypothesis that even at very low concentrations, the combination of the Spinosad and Pyriproxyfen larvicides offers an opportunity for *Aedes aegypti* public control programs to be more efficient.

Although mixtures of pesticides have been used in agricultural practices for more than 30 years, this strategy has not yet been properly explored in relation to the public health. But remarkably, we presumed that the use of the association between Pyriproxyfen and Spinosad constitute an effective and safe control of *Aedes aegypti*. This novel approach represents an efficient alternative against the species, notably in specific habitats that provide suitable conditions and environmental resources for the mosquito survival (Wilke et al., 2019). Thus, it is essential to highlight the use of the mixture as a sustainable management practice to control urban vector of diseases and to overcome the development of resistance in field populations, evidencing that new strategies can substitute excessive and unnecessary dosage use of pesticides to combat mosquitoes and also may help to guide improvements in global public health programs.

CRedit authorship contribution statement

Vanessa Santana Vieira Santos: Data curation, Formal analysis, Methodology, Writing - review & editing. **Jean Ezequiel Limongi:** Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing - review & editing.

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CONCLUSÕES GERAIS

As intervenções tradicionais de controle do *Aedes aegypti* priorizam o uso intensivo de inseticidas, provocando a seleção de populações de mosquitos resistentes, dificultando o controle do vetor e contribuindo com o aumento do número de casos da doença. Além disso, por apresentar ausência de especificidade ao organismo-alvo, estas ações de controle vetorial apresentam significativo impacto ecológico. Dessa forma, torna-se essencial o desenvolvimento de estratégias eficientes e ecologicamente corretas para o combate do mosquito.

Nesse sentido, no primeiro estudo experimental, em que realizamos ensaios de toxicidade utilizando diferentes concentrações do biolarvicida spinosad para determinar seu efeito sobre as larvas de *Aedes aegypti* e sua segurança ambiental - empregando a espécie não-alvo *Daphnia magna* - os achados revelaram que o uso da concentração recomendada de spinosad pelos programas públicos de combate ao *Ae. aegypti* apresentou elevada toxicidade à *D. magna*. Ainda, identificamos que uma concentração 100 vezes menor do biolarvicida demonstrou eficácia no controle das larvas do mosquito e baixo impacto na reprodução de *D. magna*.

No segundo estudo experimental, por meio do qual investigamos o efeito da associação de baixas concentrações do biolarvicida spinosad com o pesticida pyriproxyfen em larvas de *Ae. Aegypti*, também avaliamos os parâmetros ecotoxicológicos da exposição conjunta dos químicos em *D. magna*. Os resultados demonstraram que esta combinação de larvicidas não apresentou toxicidade aos microcrustáceos, mas atuou sinergicamente na inibição do desenvolvimento de mosquitos *Aedes aegypti* adultos, confirmando a hipótese testada.

Estes resultados podem subsidiar campanhas de controle do vetor *Aedes aegypti*. Nessa direção, concluímos que o uso da associação entre spinosad e pyriproxyfen representa uma solução mais racional, eficaz e sustentável às necessidades dos programas de saúde pública voltados ao controle vetorial.