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"Efecto de dos nuevos etil-carbamatos sobre diferentes estadios de *Rhipicephalus microplus* y su ecotoxicidad"

TESIS

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Dedicatoria

A mis padres Lidia y Marcos y mi hermana Nayeli

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"La ciencia no es solo una disciplina de razón, sino también de romance y pasión."
Stephen Hawking.

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ABREVIATURAS

AChE	Acetilcolinesterasa
A. mellifera	*Apis mellifera*
AIT	Adult Immersion Test
AST	Artificial Sustrate Test
CFPT	Contact Filter Paper Test
CIE_{99}	Concentración Inhibitoria de la Eclosión 99%
CL_{50}	Concentración Letal 50%
DAPI	4,6-diamidino-2-phenylindole
DMSO	Dimetil sulfóxido
E. foetida	*Eisenia foetida*
FES	Facultad de Estudios Superiores
g	Gramos
k_2	Constante de carbamilación
k_d	Constante de disociación
k_i	Constante de reacción bimolecular
K_m	Constante de Michaelis-Menten
LC_{99}	Lethal concentration 99%
LC_{50}	Lethal concentration 50%
LQM	Laboratorio de Química Medicinal
mg	Miligramos
mL	Mililitros
NOM	Norma Oficial Mexicana
OECD	Organisation for Economic Cooperation and Development
PBS	Phosphate-buffered saline
pt	Post treatment
R. microplus	*Rhipicephalus microplus*
SADER	Secretaría de Agricultura y Desarrollo Rural
V_{max}	Velocidad máxima
µg	Microgramos
µL	Microlitros

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RESUMEN

En este trabajo se evaluó el efecto de dos nuevos etil-carbamatos sobre diferentes estadios de la garrapata *Rhipicephalus microplus* y el potencial ecotóxico de estos compuestos. De los resultados obtenidos se escribieron tres artículos que se encuentran en diferentes fases de su publicación en revistas científicas internacionales especializadas en el tema.

En el primer artículo se evaluaron las alteraciones histológicas y ultraestructurales en los ovocitos de *R. microplus* y los efectos sobre la embriogénesis en huevos. Se observaron alteraciones ultraestructurales evidentes en los ovocitos de garrapatas expuestas a los etil-carbamatos, incluida la modificación de la estructura del corion, cuerpos mielínicos y vacuolas autofágicas que se asociaron con organelos degenerados (mitocondria, retículo endoplásmico y gránulos de yema), fragmentación del núcleo y aglomeración de cromatina en vesículas germinales. Ambos etil-carbamatos inhibieron la maduración de la mayoría de los ovocitos e indujeron una disminución concentración dependiente (r^2 = 0.5, p <0.05) en el porcentaje de embrionación de huevos ovipositados por garrapatas tratadas. Estos etil-carbamatos afectan el potencial reproductivo de *R. microplus* debido a sus efectos negativos sobre la ovogénesis y sus repercusiones en el desarrollo embrionario.

En el segundo artículo se evaluó la toxicidad de los dos etil-carbamatos y el propoxur en lombrices adultas *Eisenia foetida*. La mortalidad de lombrices y la pérdida de peso producida por los tres carbamatos evaluados mostraron un efecto concentración dependiente (p <0,0001) en la prueba de papel de filtro de contacto (CFPT). En la prueba de sustrato artificial (AST), la mortalidad aumentó en relación con el tiempo de exposición (p <0.0001) y la concentración (p <0.01) de los carbamatos. Sólo las lombrices de tierra expuestas en el CFPT mostraron alteraciones morfológicas. Según la LC_{50} obtenida en el CFPT, los tres carbamatos se clasificaron como muy tóxicos y, según la LC_{50} obtenida en el AST, los tres carbamatos se clasificaron como altamente tóxicos para *E. foetida*. Los valores de k_i y k_d indicaron que el LQM 919 y el LQM 996 son inhibidores débiles con menor afinidad por la acetilcolinesterasa de *E. foetida* que el propoxur. Las concentraciones en el CFPT y AST a las que se observó una

mortalidad del 100% en *E. foetida* fueron 64 y 4 veces más altas, respectivamente, que la concentración inhibitoria de la eclosión 99% reportada para las garrapatas.

En el tercer artículo se evaluaron los efectos producidos por los etil-carbamatos sobre la mortalidad y el comportamiento de abejas *Apis mellifera* mediante las pruebas de toxicidad oral aguda y contacto agudo. De acuerdo con los criterios de la OECD por vía oral, los etil-carbamatos se clasificaron como relativamente no tóxicos, mientras que el propoxur fue clasificado como altamente tóxico. En la prueba de contacto, las CL_{50} de los etil-carbamatos fueron al menos 10 veces menos letales ($p < 0.05$) que el propoxur. Los etilcarbamatos redujeron la actividad de la acetilcolinesterasa de *A. mellifera* hasta en un 30%. Los valores de k_i y k_d de ambos etil-carbamatos fueron más bajos ($p < 0.05$) que los de propoxur e indicaron que son inhibidores débiles y con baja afinidad a la acetilcolinesterasa de *A. mellifera*. Según estos resultados, los etil-carbamatos evaluados pueden considerarse un bajo riesgo ecotóxico para *A. mellifera*.

Con los resultados de esta tesis se contribuyó al entendimiento del mecanismo de acción de los etil-carbamatos y se demostró el amplio margen de seguridad y su bajo potencial ecotóxico agudo. Lo anterior señala que el uso de estos compuestos como ixodicidas es factible, no obstante, estudios sobre su estabilidad química y sobre los efectos ambientales que pudieran tener a mediano y largo plazo sobre organismos no objetivo son necesarios para su uso seguro en campo.

ABSTRACT

In this work, the effect of two new ethyl-carbamates on different stages of *Rhipicephalus microplus* tick and the ecotoxic potential of these compounds were evaluated. From the results obtained, three articles were written which are in different phases of their publication in international scientific journals specialized in the subject.

In the first article, histological and ultrastructural alterations in *R. microplus* oocytes and the effects on embryogenesis in eggs were evaluated. Ultrastructural alterations were observed in the oocytes from ticks exposed to ethyl-carbamates, including the modification of the chorion structure, myelinic bodies and autophagical vacuoles that were associated with degenerated organelles (mitochondria, endoplasmic reticulum and yolk granules), fragmentation of the nuclear chromatin and agglomeration in germinal vesicles. Both ethyl-carbamates inhibited the maturation of most oocytes and induced a concentration dependent decrease ($r2 = 0.5$, $p < 0.05$) in the embryonic percentage of eggs oviposited by treated ticks. These ethyl-carbamates affect the reproductive potential of *R. microplus* due to their negative effects on oogenesis and their repercussions on embryonic development.

In the second article the toxicity of the two ethyl-carbamates and propoxur in adult earthworms *E. foetida* was evaluated. The earthworm mortality and weight loss caused by the three carbamates showed a concentration-dependent effect ($p < 0.0001$) in the contact filter paper test (CFPT). In the artificial substrate test (AST), mortality increased in relation to the exposure time ($p < 0.0001$) and the concentration ($p < 0.01$) of carbamates. Only earthworms exposed in the CFPT showed morphological alterations. According to the LC_{50} obtained in the CFPT, the three carbamates were classified as very toxic and, according to the LC_{50} obtained in the AST, the three carbamates were classified as highly toxic to *E. foetida*. The values of k_i and k_d indicated that LQM 919 and LQM 996 are weak inhibitors with lower affinity for *E. foetida* acetylcholinesterase than propoxur. The concentrations in the CFPT and AST at which 100% mortality was observed in *E. foetida* were 64 and 4 times higher, respectively, than the 99% hatching inhibitory concentration reported for ticks.

The third article assessed the effects produced by the ethyl-carbamates on the mortality and behavior of *Apis mellifera* bees using the acute oral toxicity and acute contact tests. According to the OECD criteria orally, the ethyl-carbamates were classified as relatively non-toxic, while propoxur was classified as highly toxic. In the contact test, the LC_{50} of the ethyl-carbamates were at least 10 times less lethal ($p < 0.05$) than the propoxur. The ethylcarbamates reduced the *A. mellifera* acetylcholinesterase activity by up to 30%. The k_i and k_d values of both ethyl carbamates were lower ($p < 0.05$) than those of propoxur and indicated that they are weak inhibitors and with low affinity to *A. mellifera* acetylcholinesterase. Based on these results, the ethyl-carbamates evaluated can be considered a low ecotoxic risk for *A. mellifera*.

The results of this thesis contributed to the understanding of the mechanism of action of ethyl-carbamates and demonstrated their wide margin of safety and their low acute ecotoxic potential. The foregoing indicates that the use of these compounds as ixodicides is feasible, however, studies on their chemical stability and on the environmental effects they may have in the medium and long term on non-target organisms are necessary for their safe use in the field.

CAPÍTULO 1
Introducción

1.1 GENERALIDADES

Las garrapatas son los ectoparásitos hematófagos más importantes del ganado bovino en zonas tropicales y subtropicales del mundo (Anderson y Magnarelli, 2008). Se estima que a nivel mundial el 80% del ganado está expuesto a este ectoparásito (FAO, 1984). Las garrapatas causan severas pérdidas económicas en la ganadería bovina, ya que se estima que los costos anuales globales asociados a garrapatas y a enfermedades transmitidas por las mismas van de los 13.9 a los 18.7 billones de dólares (de Castro et al, 1997).

La infestación por estos ectoparásitos causa pérdidas económicas severas a la producción bovina, debido a que provoca anemia, disminución del consumo de alimento, disminución en la producción de carne, disminución en la producción de leche y daños a la piel de los animales. También restringe de manera importante la movilización del ganado debido a la transmisión de organismos causantes de enfermedades como la babesiosis y anaplasmosis. Adicionalmente, se incrementa el costo de producción por el empleo obligado de productos químicos y mano de obra para el control de garrapatas, y por los costos por tratamientos de las hemoparasitosis que transmiten (SADER, 2019). En México, las especies que afectan al ganado bovino son principalmente *Rhipicephalus microplus* y *Rhipicephalus annulatus* (Rodríguez-Vivas *et al*., 2006).

1.2 SITUACIÓN EN MÉXICO DE LA GARRAPATA *Rhipicephalus sp.*

En nuestro país la garrapata *Rhipicephalus* (anteriormente conocida como *Boophilus*) se encuentra ampliamente distribuida (Figura 1). La superficie libre del país comprende el 30.60% del territorio nacional. Se reconoce a los Estados de Sonora, Tlaxcala, Aguascalientes, Baja California, Chihuahua y el Norte de Baja California Sur como libres del ectoparásito. La zona en fase de erradicación abarca el 3.44% del territorio, donde se encuentran los Municipios de Los Cabos y la parte sur de La Paz en Baja California Sur; los municipios de Ahome, El Fuerte y Choix en el norte de Sinaloa; los municipios de la zona Desierto del estado de Coahuila:

Cuatrociénegas, Ocampo y Sierra Mojada. La superficie en control comprende el 65.96% del territorio nacional (SADER, 2019).

Figura 1: Clasificación del territorio nacional con relación a la presencia de *R. microplus* (SADER, 2019).

1.3 *Rhipicephalus microplus*

R. microplus es una garrapata originaria del sureste de Asia, pero se ha distribuido a través de los trópicos como Australia, Este y Sur de África, y Centro de América; se introdujo a México junto con *R. annulatus* por el sur de los Estados Unidos (George, 2000).

1.3.1 Clasificación taxonómica

R. microplus (Canestrini, 1887) es del Phylum *Arthropoda*, Clase *Arachnida*, Orden *Acarina*, Suborden *Metastigmata* y Familia *Ixodidae* (Encinas et al., 1999).

1.3.2 Morfología

El cuerpo de las garrapatas está cubierto por un exoesqueleto formado por una capa cuticular quitinosa, está formado por una porción anterior denominada gnatosoma y una porción posterior denominada idiosoma (Figura 2). Las garrapatas pertenecientes a la familia Ixodidae (garrapatas duras) poseen escudo y el gnatosoma se encuentra en posición anterior con respecto al idiosoma (Quiroz, 1984; Anderson y Magnarelli, 2008). *R. microplus* es una garrapata dura, de color café oscuro, las hembras llegan a medir hasta 1.5 cm cuando están repletas y los machos hasta 0.5 cm. El gnatosoma es corto y su escudo es de color café sin ornamentaciones (Barker et al., 2002). Los machos poseen un escudo que cubre casi todo el cuerpo, no así en las hembras en las que el escudo es pequeño y cubre aproximadamente un octavo del cuerpo, por lo que tienen la capacidad de expandir la cutícula para repletarse (Encinas et al., 1999).

El gnatosoma o capítulo está formado por los órganos bucales y forma un canal de alimentación a través del cual el alimento pasa hacia el esófago. Las estructuras que lo conforman son: base del capítulo, palpos, quelíceros e hipostoma. Los palpos poseen una función quimiosensorial y los quelíceros están adaptados para el desgarre del tejido. El hipostoma se introduce para formar el canal de alimentación, y posee una serie de prolongaciones para la fijación de la garrapata a su hospedero (Quiroz, 1984; Anderson y Magnarelli, 2008). En el caso de *R. microplus*, la base del capítulo es de forma hexagonal y los palpos no rebasan en tamaño al hipostoma (Kang et al., 1985).

El idiosoma está formado por una parte anterior o propodosoma y una posterior o histerosoma. En el propodosoma se encuentran las patas y el poro genital, mientras que en el histerosoma se encuentran los espiráculos respiratorios y el ano.

La fase larvaria es hexápoda, mientras que las ninfas y adultas son octápodas. Las patas están divididas en seis segmentos: coxa, trocánter, fémur, gena, tibia y tarso. En el tarso del primer par de patas se encuentra el órgano de Haller el cual sirve para detectar temperatura, corrientes de aire, olores y químicos (Quiroz, 1984; Anderson y Magnarelli, 2008). Los espiráculos respiratorios tienen una forma muy característica en cada género, en el caso de *R. microplus* son circulares con una mácula central y están situados posterolateralmente a la cuarta coxa (Kang et al., 1985).

Figura 2: Morfología de *R. microplus.* A. Hembra ovipositando, B. Detalle del espiráculo respiratorio, C. Detalle del capítulo y el escudo en una hembra repleta, D. Macho sobre la hembra repleta

1.3.3 Ciclo biológico

R. microplus es una garrapata de un solo hospedero (Figura 3). Su hospedero definitivo es principalmente el bovino, aunque puede encontrarse también en venados. Las garrapatas tienen cuatro estadios evolutivos en su ciclo vital: huevo, larva, ninfa y adulto.

El ciclo biológico puede durar entre 4 y 10 meses; y comprende dos fases: la fase de vida libre o fase no parásita, y la fase parásita. La fase de vida libre inicia cuando la hembra repleta se desprende del hospedero y busca un lugar para ovipositar. El periodo de preoviposición es de 2 a 39 días y el periodo de oviposición es de 4 a 44 días. Cada hembra de *R. microplus* pone entre 3500 y 4400 huevos. Después, los huevos se incuban de 14 a 146 días hasta la eclosión de las larvas (Soulsby, 1988).

En la fase de vida libre se presentan dos etapas: la etapa pasiva y la etapa de búsqueda. Durante la etapa pasiva las larvas recién nacidas adquieren la madurez para buscar al hospedero alimentándose de su vitelo. Dentro de la etapa de búsqueda, las larvas suben a las puntas de los pastos para encontrar a su hospedero, al que detectan por medio de la emisión de dióxido de carbono, la vibración y el calor corporal (Anderson y Magnarelli, 2008).

La fase parásita comienza una vez que las larvas han hallado a su hospedero, se adhieren a su pelaje, insertan en la piel sus piezas bucales y comienzan a alimentarse. Mientras se alimenta, la larva realiza la muda a ninfa y posteriormente a adulto. El macho adulto busca a la hembra para la cópula. Posteriormente la hembra fecundada se ingurgita (repleción de sangre) y finalmente se desprende del hospedero, para llevar a cabo la oviposición en el suelo (Quiroz, 1984).

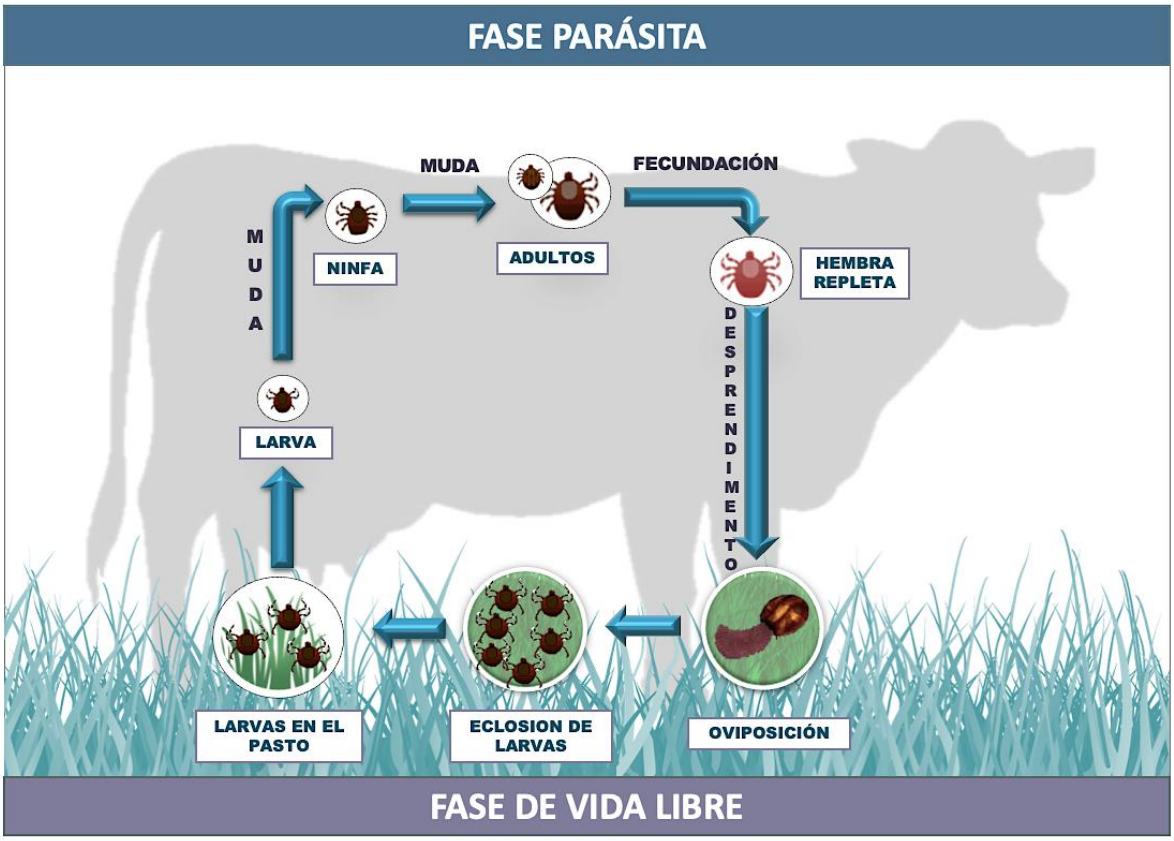

Figura 3: Ciclo biológico de *R. microplus*. Los estadios de la garrapata que ocurren sobre el hospedero son la fase parásita, y los que ocurren en el suelo pertenecen a la fase no parásita (Adaptado de Quiroz, 1984; Soulsby, 1988).

1.3.4 Patogenia

Los daños que *R. microplus* genera a su hospedero se deben a las acciones patógenas traumática, expoliatriz, inoculatriz, tóxica y antigénica. La acción traumática es causada por la perforación de la piel del hospedero con las piezas bucales de la garrapata al momento de alimentarse. La acción expoliatriz consiste en la sustracción de sangre y líquidos tisulares, cuyo principal efecto es la anemia. En este sentido, cada hembra en estado adulto es capaz de consumir entre 0.5 mL y 1.2 mL de sangre, por lo que el total de la pérdida de volumen sanguíneo está directamente relacionado al grado de infestación de los animales. Además, la anemia puede agravarse debido a hemoparasitos transmitidos que pueden tener acción lítica sobre los eritrocitos (Quiroz, 1984; Jonsson et al., 2006).

La acción inoculatriz, corresponde a la introducción de agentes patógenos causantes de otras enfermedades en el hospedero. Las garrapatas son vectores de microorganismos tales como protozoarios, ricketsias, espiroquetas y virus. *R. microplus* es el principal transmisor de *Babesia* y *Anaplasma* (Soulsby, 1988)

Las acciones tóxica y antigénica son asociadas a las secreciones salivales de la garrapata que son inyectadas por la herida y que contribuyen a prevenir la coagulación de la sangre y la reacción inflamatoria, además de que inhiben el dolor causado por la presencia de la garrapata (Quiroz, 1984).

También, se ha sugerido que las infestaciones masivas por garrapatas tienen efecto supresor del apetito en ganado de leche, basado en que los animales afectados por garrapatas consumen menor cantidad de materia seca, por lo que se observa en ellos disminución de la producción láctea y disminución de la condición corporal. Así mismo, se ha observado que los animales que no ingieren los requerimientos mínimos diarios de acuerdo con su fin zootécnico, sufren infestaciones de un grado mayor que los animales bien nutridos (Jonsson et al., 2006). En un estudio realizado en ganado Holstein-Fresian se demostró que, por cada hembra repleta, hubo una pérdida productiva de hasta 2.86 mL de leche por día y hasta 1.0 g de peso vivo en los animales infestados relacionado a un bajo consumo de materia seca (Jonsson et al., 1998; Jonsson et al., 2000).

1.4 CONTROL DE GARRAPATAS

Existen dos tipos de control para la ixodidosis: el control químico y el control no químico. El control químico ha sido el más utilizado y se ha basado en el empleo de productos de diferentes familias químicas. El control no químico conocido también como control biológico, se basa en el manejo de diversos métodos, como la cruza de animales para aumentar la resistencia natural determinada por la raza, el uso de inmunógenos con base en antígenos provenientes de células intestinales de las garrapatas, el uso de determinados tipos de forrajes en los potreros, el empleo de biopesticidas, el manejo de praderas de acuerdo al ciclo biológico de las garrapatas y también el control por medio de patógenos para estos ectoparásitos o el control mediante otros organismos que fungen como depredadores de fases no parásitas (Samish y Rehacek, 1999). Sin embargo, el control no químico no ha resultado del todo exitoso, debido a que los mecanismos de acción de estas medidas son muy lentos y no permiten observar un efecto inmediato; razón por la cual, se ha preferido el uso de ixodicidas químicos. En la actualidad se ha propuesto realizar un manejo integral, en el que se emplee ambos tipos de control, para retardar o evitar la aparición de resistencia a los productos químicos, así como disminuir el daño ambiental que genera el uso de estos.

1.4.1 Control químico

Hasta ahora el uso de productos químicos con efectividad contra garrapatas duras (llamados ixodicidas), ha sido el tipo de control más empleado en todo el mundo para combatirlas. Se han tratado de desarrollar ixodicidas que cumplan con una alta efectividad, sin causar daño a los animales, al humano y/o al ambiente. El avance de la investigación en la biología de las garrapatas ha dado conocimiento de su estructura, fisiología y comportamiento, lo que puede ser aplicado para diseñar fármacos con mecanismos de acción más específicos que puedan detener su reproducción, viabilidad y/o diseminación.

Los primeros intentos de aplicación de ixodicidas se realizaron en el año de 1893, utilizando como garrapaticidas aceite de semilla de algodón, aceite de pescado, petróleo crudo, keroseno, creosote, extracto de tabaco, jabón o sulfuro, que se administraban dos o tres veces por semana con ayuda de esponjas y cepillos. Posteriormente en 1895 se comenzó a sumergir al ganado en mezclas que contenían este tipo de sustancias (George, 2000; Botana et al., 2002).

El arsénico se introdujo como novedad reemplazando los remedios anteriores, y demostró ser altamente efectivo contra las garrapatas. El primer informe del uso de arsénico como acaricida, está reportado en garrapatas *R. microplus* en ganado Angus de Australia en 1896, a esta preparación se le conoció como "Queesland Dip" o "Australian Dip". Sin embargo, otras referencias indican que el arsénico se utilizó por primera vez en la República de Sudáfrica en 1893. La resistencia a este compuesto apareció alrededor de cincuenta años después de su utilización como ixodicida. Las razones más importantes para reemplazar al arsénico por insecticidas orgánicos sintéticos fueron: la cercanía de la dosis terapéutica con la concentración tóxica, la acumulación de este compuesto en los tejidos de los animales y la aparición de resistencia (George *et al*., 2004).

Después de la Segunda Guerra Mundial, se dio a conocer otro grupo de compuestos conocidos como organoclorados tales como el diclorodifeniltricloroetano, el hexaclorociclo-hexano, el hexaclorobenceno, el toxafeno y la dieldrina. Este grupo fue ampliamente utilizado en regiones resistentes al arsénico, y fueron los primeros en ser comercializados también. Su prohibición se debió a su acumulación en el tejido adiposo de los animales y su persistencia en el ambiente (George *et al*., 2004).

Posteriormente los compuestos organofosforados como el etión, coumafós, clorfenvinfós y clorpirifós reemplazaron a los organoclorados (Botana *et al.*, 2002). Los organofosforados se han utilizado ampliamente, demostrando tener una muy buena acción. Sin embargo, se ha observado que presentan una elevada toxicidad en vertebrados, además de guardar una relación con gases neurotóxicos como los gases sarín, soman y tabun (George *et al*., 2004).

Los carbamatos como el carbaryl y promacyl, son compuestos derivados del ácido carbámico que se caracterizan por tener una muy baja toxicidad. El mecanismo de acción tanto de los organofosforados como de los carbamatos, es la inhibición de la enzima acetilcolinesterasa (AChE), motivo por el que generalmente se presenta resistencia cruzada con los organofosforados (George, 2000).

Los piretroides naturales son un grupo de ixodicidas muy costosos e inestables en presencia de la luz solar, sin embargo, sirvieron como predecesores de los piretroides sintéticos que son altamente efectivos como acaricidas, incluyendo compuestos como permetrina, deltametrina, decametrina, flumetrina, cyhalotrina y cyflutrina. Su prolongado efecto residual es una

desventaja en su empleo, debido a esta característica se aumenta la presión de selección de cepas resistentes a estos ixodicidas (George *et al*., 2004).

En 1970 se sintetizaron las formamidinas y cicloamidinas como: clordimeform, clomethiuron, clenpyrin y amitraz (Botana *et al*., 2002). El clordimeform se utilizó en Australia como aditivo en los baños de inmersión para tratar a los animales con cepas resistentes a organofosforados. Sin embargo, en 1976 se retiró del mercado debido a que se comprobó su efecto carcinogénico. El amitraz es el más empleado en el mundo, debido a que es un compuesto altamente efectivo; a pesar de ser un compuesto inestable en baños de inmersión, pero si se agrega hidróxido de calcio para lograr un pH de 12 el principio activo permanece estable (George *et al*., 2004).

Las lactonas macrocíclicas son otro grupo de acaricidas, existen dos tipos; las avermectinas (ivermectina, eprinomectina y doramectina) y las milbemicinas (moxidectina) obtenidas de *Streptomyces avermitilis* y *S. higroscopicus aureolacrimosus* respectivamente. Su principal ventaja es que la dosis tóxica para las garrapatas es baja en comparación con otros productos y su aplicación puede ser por vía subcutánea, oral o epicutánea pero su alto costo limita su uso (George, 2000; George *et al*., 2004).

El fipronil es una fenilpirazolona que aplicada por vía epicutánea tiene una eficacia del 99% en animales alojados en unidades de producción intensivas, además provee de protección contra la reinfestación por larvas hasta por ocho semanas postratamiento. Sin embargo, en condiciones de producción extensiva, su vida se ve reducida, debido a las condiciones ambientales como la luz solar, que reduce su acción dos o tres semanas después de su aplicación (George *et al*., 2004).

Hacia finales del siglo XX aparecieron otros compuestos derivados de benzoil-fenil urea que actúan inhibiendo la formación de quitina, tal es el caso del lufenurón, flufenoxuron, diflubenzuron y fluazurón. Su principal efecto en garrapatas es la reducción casi total de la fertilidad y fecundidad de hembras repletas, además de producir mortalidad de fases inmaduras debido a que les impide mudar al siguiente estadio. El fluazurón puede persistir hasta por doce semanas, aunque su uso es restringido debido a que se excreta en la leche, lo cual es indeseable para la cría y para el consumo humano (George *et al*., 2004).

El metopreno es un análogo sintético de la hormona ecdisona producida por fases juveniles de la garrapata, la cual es promotora del cambio de estadio, el metopreno bloquea el desarrollo juvenil evitando que se llegue al estadio adulto. Debido a que la eccdisona es una hormona que

no poseen los mamíferos, su margen de seguridad es muy amplio, lo cual es ventajoso para su uso (Botana *et al*., 2002).

1.4.2 APLICACIÓN DE IXODICIDAS

El éxito de un ixodicida depende de la toxicidad del principio activo, la calidad y estabilidad del producto, su correcta dosificación o de la superficie alcanzada en el cuerpo del animal. Es por lo que el método de aplicación de un ixodicida dependerá de las características del producto. Actualmente diferentes métodos son empleados, los más relevantes se describen a continuación:

- *Baños de inmersión*: En este se sumerge al ganado en la solución que contiene al ixodicida, alcanzando casi toda la superficie del cuerpo del animal, por lo que es un método altamente efectivo; sin embargo, el alto costo inicial de construcción de las instalaciones y su nula portabilidad, lo hace poco práctico para las pequeñas unidades de producción (George et al., 2004).
- *Baño de aspersión*: Consiste en la aplicación del ixodicida mediante el uso de una bomba de rociado, asegurándose que el compuesto se disperse por toda la superficie corporal del animal (NOM-019-ZOO-1994). Es un método eficaz, sin embargo, la dsipersión de los pesticidas en el ambiente son una desventaja de este método.
- *Aplicación epicutánea*: También llamada "*pour-on*", consiste en la aplicación del ixodicida directamente sobre la piel del animal por la línea media dorsal, desde la cruz hasta la región coccígea. El ixodicida actúa después de dispersarse sobre la piel del animal o después de absorberse y ser ingerido por el artrópodo (NOM-019-ZOO-1994).
- *Aplicación parenteral*: Este método consiste en la inyección parenteral del ixodicida que, al absorberse y alcanzar niveles adecuados, tiene efecto sobre las garrapatas que se encuentran alimentando (NOM-019-ZOO-1994).

1.5 RESISTENCIA A LOS IXODICIDAS

La resistencia a un fármaco con acción sobre garrapatas se define como la capacidad de una fracción poblacional de estos parásitos para sobrevivir a ciertas concentraciones de productos

ixodicidas, que resultan letales o afectan la reproducción del resto de la población considerada como normal (susceptible), la cual una vez establecida es hereditaria (NOM-019-ZOO-1994).

Como describen Alonso-Díaz *et al*. (2006) y Cardozo (2007), existen tres fases en el desarrollo de la resistencia a los ixodicidas. La primera es la fase de establecimiento que ocurre cuando aparece el alelo resistente en una población, generalmente es un proceso dado por mutaciones naturales, independiente a la presión de selección. Posteriormente, se presenta la fase de desarrollo o diseminación en la que se presenta un aumento del número de individuos resistentes después del uso de ixodicidas químicos. En esta fase pueden ocurrir dos métodos de selección: la selección rápida ocurre cuando el gen de resistencia es dominante o parcialmente dominante y permite la selección de heterocigotos. La selección lenta se da cuando los alelos son recesivos. En esta fase aún no son detectables las fallas de los ixodicidas llevándose a cabo la dispersión hacia otras regiones en forma desapercibida. Finalmente en la fase emergente, el alelo resistente es lo suficientemente común en la población. En ésta la eficacia de los ixodicidas ha disminuido considerablemente debido a la alta presión de selección.

Los factores principales asociados a la evolución de la resistencia, son los genéticos, que incluyen la frecuencia de los alelos resistentes, el número de alelos, la dominancia y la expresividad de estos; los factores biológicos que son los propios de la especie, como número de generaciones, número de descendientes por generación, sobrevivencia y refugio; y operacionales del químico, lo cuales incluyen la persistencia de residuos, tipo de aplicación, formulación y umbral de aplicación y selección.

1.5.1 Mecanismos de resistencia

En las últimas décadas se realizaron numerosos estudios dirigidos a conocer los diversos procesos bioquímicos y genéticos desarrollados por las garrapatas para evitar o disminuir el efecto de los productos químicos. De acuerdo con el tipo de respuesta al producto químico, la resistencia ha sido agrupada en 4 categorías (Alonso-Díaz *et al.*, 2006):

a) Resistencia de comportamiento: El parásito modifica su conducta para evitar el químico.

b) Resistencia de la penetración: Es la modificación de algunos compuestos presentes en el exoesqueleto para impedir o retardar la penetración del producto.

c) Resistencia metabólica: Es la detoxificación del producto químico por procesos enzimáticos que radica en la modificación en las vías metabólicas del parásito.

d) Insensibilidad del sitio de acción: En esta se ve la modificación del sitio de acción del ixodicida para disminuir el efecto del producto químico.

1.5.2 Resistencia a ixodicidas en el mundo

Se ha reportado el fenómeno de resistencia desde los años 40's y 50's en Australia, Sudamérica y África, años en que aparecieron poblaciones de garrapatas resistentes a diversos compuestos organoclorados. A finales de los 50's y principios de los 60's se presentó en Australia y Sudáfrica resistencia a compuestos inhibidores de las colinesterasas como carbamatos y organofosforados. Para finales de los años 70's ya se habían reportado en Australia cepas de garrapatas resistentes a amidinas y para inicios de los 80's se tenía conocimiento de la resistencia a los piretroides en Australia (Soberanes-Céspedes et al., 2002).

1.5.3 Resistencia a ixodicidas en México

En nuestro país, se ha documentado la resistencia de *R. microplus* hacia ixodicidas organoclorados y organofosforados desde la década de los 80´s con las cepas "Tuxpan" y "Tempoal", que actualmente se distribuyen en las Huastecas Mexicanas y en el estado de Yucatán. La resistencia a piretroides se observó por primera vez en 1993. Las cepas "Coatzacoalcos", "Aldama", "La Mora" y "San Jorge", además de presentar resistencia a piretroides, también la presentaron hacia compuestos organofosforados (Alonso-Díaz et al., 2006).

Debido a la resistencia a los compuestos antes mencionados, se empleó el amitraz. Sin embargo, la alta presión de selección ejercida provocó en 2001 la aparición de cepas triple resistentes como lo es la cepa "San Alfonso", resistente a organofosforados, piretroides y amidinas (Alonso-Díaz *et al.*, 2006).

Recientemente, se ha detectado la resistencia de *R. microplus* a la ivermectina (Pérez-Cogollo et al. 2010), y también la resistencia al fipronil en cinco cepas de Tamaulipas, México (Miller et al., 2013).

1.5.4 Manejo de la resistencia a ixodicidas

Se han sugerido dos formas para controlar la resistencia en una población de garrapatas: saturación y moderación. La saturación consiste en la utilización del mismo producto hasta que

el cambio es forzoso por la dispersión de la resistencia. La concentración y frecuencia de los tratamientos se incrementan progresivamente. La moderación es un método basado en el reemplazo inmediato del compuesto al que existe la resistencia. Para lograr el reemplazo de estos compuestos se han evaluado nuevos productos con el fin de encontrar alternativas farmacéuticas para el tratamiento de la ixodidosis (Alonso-Díaz et al., 2006).

1.6 ECOTOXICIDAD

Pese a los beneficios que la mayoría de los pesticidas han traído a la humanidad como el control de plagas agrícolas y la eliminación de organismos que potencialmente transmiten enfermedades tanto a los animales como al hombre, los residuos de los diferentes compuestos, su degradación y destino final, comenzaron a ser monitoreados hasta que aparecieron efectos colaterales en organismos no objetivo como aves, peces, invertebrados e insectos (Edwards, 1973).

La ecotoxicología es la ciencia de predecir los efectos de agentes potencialmente tóxicos en los ecosistemas y en especies no objetivo (Hoffman, 2002). Una de las razones para estudiar el impacto ecotoxicológico de los pesticidas es conocer la selectividad que estos pudieran tener para proteger a los organismos no objetivo que forman parte de los ecosistemas en donde los pesticidas son aplicados. Este conocimiento es de utilidad en nuevos compuestos que se encuentran en fase de desarrollo para poder determinar si representan un riesgo potencial para el medio ambiente (Croft et al, 1998).

1.6.1 Uso de biomarcadores en estudios ecotoxicológicos

Un biomarcador es una respuesta biológica a un químico que se encuentra presente en el ambiente y que es altamente valioso para detectar posibles efectos de compuestos químicos específicos (Reinecke and Reinecke, 1994). Efectos como la letalidad, alteraciones conductuales, inhibición enzimática, entre otras, pueden ser medidas utilizando organismos que habitan comúnmente el ecosistema en donde se utiliza el compuesto a estudiar (Walker et al 2012). Las lombrices de tierra y las abejas son organismos terrestres que han sido utilizados como modelos para medir estos impactos.

1.6.1.1 Lombrices de tierra en la ecotoxicología

Las lombrices de tierra son los principales organismos del suelo ya que representan la mayor parte de su biomasa. Estos invertebrados contribuyen activamente a la formación del suelo, consumiendo materia orgánica, fragmentándola y mezclándola con las partículas de minerales para formar agregados (Ware, 1992).

Las lombrices pueden estar expuestas a los pesticidas cmo resultado de su aplicación directa, por escurrimiento, volatilización o arraste por la lluvia. El uso de pesticidas puede reducir el número o la actividad de las lombrices, lo cual puede derivar en efectos adversos sobre la fertilidad del suelo. Además, las lombrices al ser depredadas pueden generar efectos tóxicos en sus depredadores a niveles secundarios o terciarios de la cadena alimenticia (Rathore y Nollet, 2012). Por su capacidad para bioacumular o bioconcentrar pesticidas las lombrices han sido utilizadas para monitorear los efectos tóxicos de los contaminantes en el suelo.

1.6.1.2 Efectos de los pesticidas en las lombrices de tierra

Los efectos de los pesticidas en las lombrices dependen del tipo de pesticida y su tasa de aplicación. En 1990, Potter y colaboradores demostraron que una sola aplicación de diazinon, benomyl y carbaryl, causaron una disminución significativa del número de lombrices en un periodo corto. Tambien se conoce que la aplicación de fungicidas benzimidazoles y las ivermectinas causan una toxicidad extrema en estos organismos. Otros efectos como la inflamación, sangrado, enrrollamiento y contracción fueron reportadas cuando se aplicó propoxur, endosulfan y carbofuran (Rathore y Nollet, 2012).

Algunos pesticidas tienen efectos severos sobre el sistema nervioso. Los carbamatos son descritos en este aspecto como extremadamente tóxicos ya que causan inmovilidad, rigidez, enrollamiento, inflamación de los segmentos y contracciones (Roberts y Dorough, 1984). Además, los efectos sobre la reproducción como la disminución de cocones y anomalías en los espermatozoides se han observado con pesticidas como malation y dieldrina (Rathore y Nollet, 2012).

1.6.1.3 Las abejas en la ecotoxicología

Las abejas son organismos que tienen un papel ecológico muy importante ya que permiten la polinización de las plantas y elaboran productos de alto valor pecuario como la miel, el propóleo y la cera (Barganska et al, 2016).

La desaparición de abejas y otros polinizadores en todo el mundo cobró mucha importancia en las décadas recientes. Este fenómeno conocido como síndrome de colapso de la colmena se considera multifactorial, sin embargo, una de las principales causas que se ha asociado es el uso de pesticidas ya que las abejas pueden estar expuestas a los contaminantes cuando buscan alimento (vanEngelsdorp et al, 2009).

Es por lo anterior que una gran cantidad de estudios se han llevado a cabo para determinar los efectos de los pesticidas en el ambiente y en organismos polinizadores. Debido a su alta movilidad, sensibilidad y recolección de una gran cantidad de muestras de forma natural, las abejas se consideran buenos monitores biológicos (Barganska et al, 2016).

1.6.1.4 Efectos de los pesticidas en las abejas

Muchos de los pesticidas que se utilizan actualmente generan un impacto sobre las abejas. Estos efectos pueden ser letales o subletales; sin embargo, la importancia aumenta debido a que por lo general toda la colmena sufre estos impactos. Además de la mortalidad, los efectos subletales son de gran importancia ya que merman lentamente a la colmena. Los efectos subletales sobre el desarrollo son causados por reguladores del crecimiento, causando la falta de emergencia de las abejas o la disminución del número de abejas nodrizas. El fenoxicarb es conocido por causar malformaciones en las larvas y pupas; mientras que el diflubenzuron disminuye la superficie para cría y la ganancia de peso en adultas recién emergidas. Por otro lado, los efectos neurológicos como la desorientación, incapacidad de alimentarse y problemas en el aprendizaje son frecuentes en la intoxicación con piretroides como la deltametrina y lambda-cialotrina (Desneux et al, 2007).

1.7 CARBAMATOS

Los carbamatos Son compuestos moleculares sencillos derivados inicialmente de la fisostigmina, sin embargo, en la actualidad estos compuestos son de origen sintético (Gupta, 2006). Son ésteres del ácido carbámico qur estructuralmente presentan una región principal

constituida por el metilcarbamato de un fenol, con un sustituyente de carácter básico (Botana et al., 2002).

Desde los años 70's, los carbamatos se han empleado de diversas maneras, como herbicidas en la agricultura, ectoparasiticidas en veterinaria, plaguicidas en cuidados forestales e incluso en medicina humana para el tratamiento de algunas enfermedades degenerativas como Alzheimer, miastenia gravis y glaucoma, como antimicrobianos, anestésicos locales, anticonvulsivos, antiulcerosos, anticarcinogénicos, etc. (Odilón, 1993; Botana et al., 2002; Gupta, 2006).

Algunos de los carbamatos utilizados en la actualidad principalmente en la agricultura son el aldicarb, aminocarb, carbaril, carbofuran, carbosulfan, metiocarb entre otros (Gupta, 2006). En medicina veterinaria los carbamatos empleados para el control de artrópodos son el propoxur y el carbaril (Botana et al., 2002).

1.7.1 Mecanismo de acción

La mayoría de los carbamatos tienen una alta afinidad por las esterasas tales como la acetilcolinesterasa (AChE), la pseudocolinesterasa, carboxilesterasa y otras esterasas no específicas (Córdoba, 2006). La AChE actúa en las sinapsis colinérgicas terminando el impulso nervioso producido por el neurotransmisor acetilcolina. Los carbamatos son análogos de la AChE y reaccionan con la acetilcolina, prolongando la excitación nerviosa causada por el neurotransmisor acetilcolina, provocando parálisis neuromuscular y la muerte por tetanización (Sogorb y Vilanova, 2002; Tan et al., 2011). Sin embargo, los carbamatos benzimidazoles han mostrado tener un efecto en los centros de organización de los microtúbulos, especialmente con la β-tubulina de algunos protozoarios, helmintos y hongos, provocando efectos en la morfología y sobre el índice mitótico en dichos parásitos (Carvalho y Gadelha, 2007; Chávez et al., 1992; Katiyar et al., 1994). Por lo anterior, debido a que los carbamatos pueden tener mecanismos de acción diferentes, es indispensable evaluar nuevos compuestos sintetizados.

1.7.2 Degradación de los carbamatos en el ambiente

Los carbamatos son compuestos que se degradan rápidamente por agentes bioquímicos y usualmente no hay problemas de persistencia ambiental, por lo que se consideran tóxicos de riesgo a corto plazo. Algunos estudios muestran que los carbamatos más comúnmente empleados como el carbaryl, el aldicarb y el carbosulfan se degradan en el suelo en semanas por

microorganismos como los hongos *Trichoderma, Aspergillus, Fusarium, Penicillium* y *Helminthosporium*, y por bacterias como *Pseudomonas phaseolicola* (Laveglia y Dahm, 1977).

1.7.3 Nuevos etil-carbamatos sinteizados en FESC

En la Facultad de Estudios Superiores Cuautitlán se han producido una serie de nuevos carbamatos. El diseño y síntesis de estos productos se llevó a cabo por parte del grupo de investigadores del Laboratorio de Química Medicinal de la FESC (Ángeles et al., 2000).

A algunos de estos carbamatos se les ha estudiado su actividad antibiótica sobre *Escherichia coli*, *Salmonella typhi*, *Vibrio cholerae*, *Enterobacter aerogenes* y *Helicobacter pylori* demostrando tener una eficacia de más del 50% (Bernal, 2000; Reyes-González, 2007).

En cuanto a su actividad antiparasitaria, se ha demostrado que estos carbamatos tienen una actividad de entre el 50-80% sobre *Hymenolepis nana* en ratones (Bernabe-Pérez, 2007), y sobre cepas de *Giardia intestinalis* susceptibles y resistentes al albendazol (Jiménez-Cardoso *et al*., 2004); así como su actividad inhibitoria hasta en un 97% sobre el crecimiento in vitro de *Entamoeba histolytica* (Ordaz Pichardo et al., 2005).

Por otro lado, se evaluó la actividad antimicótica de estos carbamatos, en *Trichophyton mentagrophytes* y *Aspergillus fumigatus*; inhibiendo su crecimiento en más del 60% (Reyes-González, 2007).

Así mismo, nuestro grupo de investigación ha realizado pruebas con estos compuestos que han permitido demostrar su potencial ixodicida y su posible uso en animales, las cuales se presentan a continuación:

Pruebas *in vitro* para determinar el potencial ixodicida: Estas pruebas se realizaron con cepas susceptibles y resistentes de *R. microplus*, de estos ensayos se obtuvo que los etil-carbamatos identificados como LQM 919 y LQM 996 (etil-4-bromofenil carbamato y etil-4-clorofenil carbamato), inhiben la oviposición en más del 60% e inhiben la eclosión de los huevos producidos por las garrapatas tratadas con los carbamatos hasta en un 100%; además los huevos ovipositados por las garrapatas tratadas se observaron disgregados, oscuros, secos y no fueron viables (Prado-Ochoa et al., 2013; Pérez-González et al., 2013).

Pruebas sobre el mecanismo de acción: se evaluó el LQM 996 y del LQM 919 sobre la actividad de la enzima acetilcolinesterasa (AChE) estructura de los huevos y órganos reproductores de dos cepas de *R. microplus*; los resultados mostraron que los efectos de estos

carbamatos son independientes a la actividad de la AChE y que las alteraciones morfológicas de los órganos reproductores fueron debidas a la acción de estos carbamatos sobre la vitelogénesis y viabilidad de las células del ovario (Prado-Ochoa *et al*., 2014b). Un estudio mostró que éstos etil-carbamatos inducen picnosis, degeneración, vacuolización, alteración de la membrana y reducción en el depósito del corion de células ováricas de *R. microplus* tratadas *in vitro* (Escobar-Chavarría, 2014).

Pruebas toxicológicas: Se determinó la toxicidad oral aguda y dérmica aguda del LQM 996 y del LQM 919 en ratas. La dosis letal 50 oral fue de 300-2000 mg/kg y la dérmica >5000mg/kg para ambos carbamatos, aunque se presentaron algunos signos de toxicidad en la exposición oral, en la exposición dérmica no se observaron signos de toxicidad en ninguna de las dosis empleadas. Con lo anterior se demostró que los carbamatos evaluados son de baja toxicidad oral y dérmica (Prado-Ochoa *et al.,* 2014a) en mamíferos. También, se evaluó la toxicidad subcrónica en ratas. En este estudio se observaron alteraciones en algunos de los parámetros evaluados, como el hematocrito, porcentaje de reticulocitos, algunas enzimas hepáticas y creatinina en ratas expuestas a los carbamatos y se demostró la reversibilidad de estas alteraciones al suspender la exposición a los productos. Con este estudio fue posible determinar que la dosis sin efectos adversos observables (NOAEL) fue de 12.5 mg/kg/día (Prado-Ochoa *et al.,* 2014c).

En otro estudio, se evaluó por medio de la prueba de micronúcleos en sangre periférica de rata, el daño genético producido *in vivo* por los etil- carbamatos, además, se evaluó el efecto de los carbamatos *in vitro* sobre la cinética de proliferación celular en cultivos de linfocitos humanos. Los resultados de este estudio mostraron el bajo potencial genotóxico de los carbamatos estudiados en algunas dosis empleadas, así como su efecto sobre el ciclo celular (Prado-Ochoa, 2013).

Pruebas de eficacia *in vivo*: La eficacia de los etil-carbamatos sobre garrapatas parasitando bovinos fue evaluada mediante la prueba de cámaras. En este estudio se observó que estos compuestos aplicados a garrapatas *R. microplus* que se encontraban alimentandose afectaron sus parámetros reproductivos y que además afectaron el desarrollo de larvas, ninfas y adultos. La eficacia *in vivo* de estos etil-carbamatos calculada a partir de este estudio fue superior al 98% (Iturbe-Requena, 2014).

CAPÍTULO 2
Justificación

Uno de los principales ectoparásitos del ganado en zonas tropicales y subtropicales de todo el mundo es *R. microplus*. Esta garrapata es difícil de controlar en la actualidad debido a la resistencia que existe a todas las familias de ixodicidas químicos, la falta de control integral y la poca o nula aplicación de otros tipos de control como el biológico. Por lo anterior, es permanente la necesidad de crear nuevos ixodicidas para el control químico de las garrapatas. Los etil-carbamatos diseñados, sintetizados y evaluados en la FES-Cuautitlán, se han convertido en una potencial y novedosa opción para el control de las garrapatas. Debido a su alta eficacia *in vitro* e *in vivo* en cepas de garrapatas susceptibles y resistentes, su baja toxicidad aguda y subcrónica en mamíferos, se ha sugerido que pueden ser una opción para el control químico de las garrapatas en un mediano plazo.

Los estudios previos realizados por nuestro grupo sobre el mecanismo de acción permitieron conocer que el efecto de los etil-carbamatos sobre las garrapatas es principalmente a nivel reproductivo. Se conoce que estos compuestos causan daño celular a los ovarios de las hembras repletas y que los huevos ovipositados se observaron secos y disgregados, de los cuales no eclosionaron larvas. Sin embargo, se desconocía si estos efectos son debidos a falta de formación del embrión o a defectos en el desarrollo de las larvas que repercutan en su viabilidad. Por esta razón, en el primer artículo presentado se estudió el desarrollo embriogénico de *R. microplus*, desde la formación de los ovocitos dentro de las hembras repletas hasta el desarrollo de las larvas dentro de los huevos ovipositados (capítulo 6).

En la actualidad, los requerimientos para el desarrollo de nuevos ixodicidas incluyen estudios de ecotoxicidad para establecer su impacto ambiental, como afectan a organismos de vida libre y conocer si su aplicación genera modificaciones en el ecosistema. Los carbamatos forman una amplia familia de compuestos químicos con características sumamente variables, que van desde ser totalmente inertes hasta ser extremadamente tóxicos. Por lo anterior, en el segundo y tercer articulo presentado se evaluo el efecto toxico agudo de los carbamatos evaluados sobre la lombriz de tierra *E. foetida* (capítulo 7) y abejas *A. mellifera* (capítulo 8).

CAPÍTULO 3
Hipótesis

1.- La falta de eclosión de larvas a partir de huevos ovipositados por hembras repletas de *R. microplus* tratadas con los etil-carbamatos LQM 919 y LQM 996 es el resultado del daño producido por estos compuestos durante la ovogénesis.

2.- Los etil-carbamatos LQM 919 y LQM 996 no causan la muerte aguda, alteran o afectan el comportamiento de organismos de vida libre como la lombriz de tierra *E. foetida* y la abeja *A. mellifera*, por lo que son de bajo potencial ecotóxico.

CAPÍTULO 4
Objetivos

1. Conocer si el efecto de los etil-carbamatos LQM 919 y LQM 996 sobre los ovarios de *R. microplus* repercute en la embriogénesis de la garrapata.

2. Evaluar la toxicidad aguda de los etil-carbamatos LQM 919 y LQM 996 sobre lombrices de tierra adultas *E. foetida* mediante las pruebas de papel filtro y sustrato artificial, y sus efectos sobre la AChE.

3. Evaluar la toxicidad aguda de los etil-carbamatos LQM 919 y LQM 996 sobre abejas obreras *A. mellifera* mediante las pruebas oral y por contacto, y sus efectos sobre la AChE.

CAPÍTULO 5
Trabajos generados de esta tesis

La información obtenida del presente estudio generó la escritura de tres artículos de investigación. Los artículos se presentan en los siguientes capítulos, cada uno incluye resumen, introducción, material y métodos, resultados, discusión y referencias. Los artículos se encuentran en diferentes etapas para su publicación en revistas indizadas como se describe a continuación:

1. "Inhibition of *Rhipicephalus microplus* ovogenesis and embryogenesis produced by two new ethyl-carbamates." En revisión en *Ticks and Tick-borne Diseases*. Enviado, corregido por revisores de la revista, corregido por autores y en espera del resultado final.

2. "Toxic effects of new ethyl-carbamates on the morphology, mortality and acetylcholinesterase activity of *Eisenia foetida.*" Publicado en *Ecotoxicology and Environmental Safety*. 2019;176:219-25.

3. "Acute oral and contact toxicity of new ethyl-carbamates on the mortality and acetylcholinesterase activity of honey bees (*Apis mellifera*)" Enviado a *Ecotoxicology and Environmental Safety*.

CAPÍTULO 6

Oogenesis and embryogenesis inhibition induced by two new ethyl-carbamates in the cattle tick *Rhipicephalus microplus*

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Keywords: *Rhipicephalus microplus*, carbamates, oogenesis, embryogenesis, TEM

Abstract

The purpose of this work was to contribute to the understanding of the mechanism of action of two new ixodicides. The histological and ultrastructural alterations of *Rhipicephalus microplus* oocytes (San Alfonso strain) treated with two new ethyl-carbamates (ethyl-4- bromophenyl carbamate and ethyl-4-chlorophenyl carbamate) by the adult immersion test were evaluated by light microscopy and transmission electron microscopy. The carbamates' effects on embryogenesis in eggs were evaluated by fluorescence microscopy using DAPI staining. Both ethyl-carbamates inhibited the maturation of most oocytes and induced a concentration-dependent decrease ($r^2 = 0.5$, $p < 0.05$) in the embryonation percentage in the small number of eggs oviposited by treated ticks. Evident ultrastructural alterations were observed in the oocytes from ticks exposed to the ethyl-carbamates, including modification of the chorion structure,

myelinic bodies and autophagic vacuoles that were associated with degenerated organelles (mitochondria, endoplasmic reticulum and yolk granules), nucleolus fragmentation and chromatin clumping in germinal vesicles. In conclusion, these ethyl- carbamates affect the reproductive potential of *R. microplus* due to their negative effects on oogenesis and their repercussions for embryonic development.

Introduction

Rhipicephalus microplus is the most important cattle tick in tropical and subtropical areas of Mexico as well as other parts of the world. For example, annual economic losses of 3240 and 573 million dollars have been estimated in Brazil and Mexico respectively (Grisi et al., 2014; Rodríguez-Vivas et al., 2017). Infestations of cattle produce decrease in growth, anaemia and reproductive parameters, a reduction of meat and milk production, a reduction in the quality of pelts and increased transmission of diseases, such as babesiosis and anaplasmosis (de Oliveira et al., 2005; Jonsson et al., 2008).

The increasing resistance to ixodicides has generated the need to develop new molecules for tick control. It has been shown that ethyl-4-bromophenyl carbamate (LQM 919) and ethyl-4-chlorophenyl carbamate (LQM 996) reduce oviposition and inhibit the larval hatching of *Rhipicephalus microplus* strains both susceptible and resistant to organophosphates, pyrethroids and amidines (Pérez-González et al., 2014; Prado-Ochoa et al., 2013). The oral and dermic acute toxicity and oral subchronic toxicity of these ethyl-carbamates have been evaluated, and generally, their toxicity is low in mammals (Prado-Ochoa et al., 2014a; 2014b). Although cytotoxic and genotoxic effects have been reported by oral administration in rats, the concentrations that produce these effects are much higher than the concentrations proposed for their use as ixodicides (Prado-Ochoa et al., 2016).

Most commercially available ixodicides affect the nervous system of ticks (Casida and Durkin, 2013). It has been shown that the ethyl-carbamates LQM 919 and LQM 996 are weak inhibitors of acetylcholinesterase and apparently do not affect *R. microplus* larvae or adult behavior; however, these carbamates drastically alter tick reproduction (Prado-Ochoa et al., 2014c). The ovaries of engorged females treated by the adult immersion test (AIT) with these ethyl-

carbamates showed evident morphological alterations, and the produced eggs had a modified appearance, decreased size and loss of viability (Prado-Ochoa et al., 2013). However, their effects on the ultrastructural development of oocytes and their consequences on embryogenesis were not evaluated. Therefore, in this study, the morphology and development of oocytes in the ovary from females treated with ethyl carbamates were evaluated by light and electron microscopy, as well as their repercussions on the embryonic development of eggs oviposited was evaluated by fluorescence microscopy in order to contribute to the understanding of their mechanism of action.

Materials and methods

Ethyl-carbamates

The two new ethyl-carbamates evaluated were designed and synthesized in the Facultad de Estudios Superiores Cuautitlán of the National Autonomous University of Mexico (Angeles et al., 2000). The ethyl-carbamates were synthesized using halogenated derivatives of aromatic amines with ethyl chloroformate using sodium bicarbonate and acetone as the reaction medium, and they were subsequently purified by column chromatography and recrystallized. The ethyl-carbamates' structure was elucidated by spectroscopic techniques. The chemical structure, molecular weight and nomenclature of these compounds are shown in Figure 1. To prepare the dilutions, the ethyl-carbamates were previously solubilized in dimethyl sulfoxide (DMSO).

Tick strain

The San Alfonso tick strain, which is resistant to organophosphates, pyrethroids and amidines, was used (Alonso-Díaz et al., 2006; Céspedes et al., 2005; Domínguez-García et al., 2010). The *R. microplus* engorged females, which spontaneously detached from the host, were obtained from experimentally infected Holstein-Fresian cattle. Ticks were incubated at 28 oC with 80% relative humidity until they were used. The strain was provided by the National Center for Animal Health Verification Services of Mexico. This study was approved by the Internal Committee for Care of Experimental Animals of the Postgraduate Program of Animal Production and Health (UNAM, México).

Ultrastructural study of tick ovaries

Two hundred and forty engorged females distributed in 3 groups (n = 80) were used. Ticks from one group were treated with 1 mg/mL of LQM 919, ticks from other group were treated with 1 mg/mL of LQM 996, and the ticks from one last group were treated only with DMSO at 4% (negative control) using the AIT (Drummond et al., 1976). On days 1, 3, 5 and 7 post- treatment (pt), 20 ticks from each group were dissected, and the reproductive organs were separated. The ovaries from 10 ticks of each group were fixed with 4% paraformaldehyde and processed to obtain conventional histological sections stained with hematoxylin-eosin. The ovaries of the remaining 10 ticks were processed for transmission electron microscopy modifying the protocol described by de Oliveira et al. (2009). The ovaries were fixed in 2.5% glutaraldehyde (2 hours) and 1% osmium tetroxide (2 hours), contrasted with uranyl acetate (2%), dehydrated in graded ethanol series, embedded in epoxy araldite (12 hours), included in pure epon resin, ultra-sectioned and observed under a transmission electron microscope Phillips TEM®.

Embryogenesis

Eight hundred engorged females divided into 8 groups (n = 100) were used. The concentrations for each ethyl-carbamate evaluated by AIT were: 0.668, 0.066, 0.006 and 0 mg/mL (negative control). These concentrations were determined using logarithmic dilutions from the egg hatching inhibitory concentrations of 99% for *R. microplus* reported by Pérez- González et al. (2014). The eggs produced by incubated ticks during the first 48 hours were discarded, and the eggs oviposited between 48 and 72 hours were incubated at 28 °C with 80- 90% humidity to perform the experiments. The development of the embryos was evaluated by measuring the embryonation percentage and the development of embryonic structures characteristic of each stage.

Embryonation percentage

Every day, a 5 mg sample was taken from the center of the egg mass (350 to 450 eggs), and the percentage of embryonated and nonembryonated eggs was measured in an inverted microscope in triplicate. Eggs were considered embryonated when structures associated with their stage of

development (yolk, germ band, legs, idiosoma, gnathosoma, ventral furrow, among others) were observed and not embryonated when no structures were observed.

Structural development of the embryo

Each day, 100 mg of eggs from each treatment were immersed in a 5% sodium hypochlorite solution to remove the chorion from the eggs, washed with PBS (pH 7), and fixed for 20 minutes in a mixture of 5 mL of PBS with 4% paraformaldehyde and 1 mL heptane. Subsequently, the eggs were rinsed with absolute methanol and stored at -20 °C until use. Finally, the embryos were stained for 20 minutes with DAPI (4,6-diamidino-2-phenylindole, sigma®) diluted 2: 5000, washed with PBS / 0.1% Triton and mounted in glycerin for their observation under a fluorescence microscope (OLYMPUS®) at 365 nm. The microscope was coupled to a camera (Evolution MP Colors®) and the images were processed with ImagePro Premiere® software. The development of the embryos was evaluated according to the stages described by Santos et al. (2013).

Statistical analysis

The data of percentages of embryonated eggs were analyzed by lineal regression using the software Statistica for Windows 7.0. The minimum confidence used was 95%.

Results

Microscopic alterations of the ovary

The microscopic alterations observed in ovaries and oocytes were similar in ticks exposed to LQM 919 and LQM 996. On day 1 pt, most of the oocytes observed were in stage I and II (previtellogenic), and some oocytes were in stages III and IV (vitellogenic), both in the exposed ticks and in those nonexposed to ethyl-carbamates (Figures 2A and 2D). The oocytes in stage I and II from ticks exposed to the ethyl-carbamates showed degeneration of the cell membrane, vacuoles in the cytoplasm and loss of form of the germinal vesicle (Figure 2G and 2H). In oocytes III and IV, an increase in granularity and a decrease in chorion formation were observed

in relation to oocytes III and IV of untreated ticks. In addition, vacuolization of ovarian epithelial cells was observed (Figure 2I).

On day 3 pt, abundant oocytes III, IV and V, and few oocytes I and II in ticks from the control group were observed (Figure 2B). Only oocytes I and II were observed in ticks exposed to the ethyl-carbamates (Figure 2E). The alterations described on day 1 were more severe on day 3 pt, and in addition, germinal vesicle degeneration and nucleus fragmentation were observed in oocytes from exposed ticks.

On day 5 pt in exposed ticks, few IV and V oocytes were observed, which showed a large number of vacuoles in cytoplasm, shape alterations and irregular cell membranes. Only oocytes in stages IV and V were observed without apparent alterations in ticks from the control group. Most of the oocytes from ticks of the control group on day 7 pt were observed in stage V of development with their interior full of yolk granules, integral chorion and with a smooth surface (Figure 2C). In ticks exposed to the ethyl-carbamates, scarce mature oocytes were observed. The oocytes had a large number of irregular yolk vesicles and an invaginated, thin chorion (Figure 2H). The epithelium of the ovary was eroded and flattened, and the cells had pyknotic nuclei.

Ultrastructural alterations of the ovary

The alterations observed in tick oocytes were similar in groups treated with LQM 919 or LQM 996. In ticks from the control group, oocytes I and II showed a well-defined plasmatic membrane while, in oocytes IV and V, a thick chorion organized in three layers (endochorion, chorion and exochorion) and with defined channels that completely crossed the chorion was observed (Figures 3A and 3B). In all stages of oocytes from treated ticks, the chorion layers were not defined. Loss of integrity of the channels in oocytes IV and V was observed (Figure 3C and 3D).

In oocytes of all stages from the control group of ticks, the mitochondria, endoplasmic reticulum and ribosomes were observed without apparent alterations (Figure 3E). The mitochondria of oocytes III, IV and V from ticks treated with the ethyl-carbamates showed an apparent increase in number, increase in size, change in shape, and decrease in the density of the matrix and definition of the crests (Figure 3F). Degeneration of the endoplasmic reticulum was also observed.

Oocytes III, IV and V from the control group of ticks had yolk granules of variable size with a homogeneous mass of variable electrodensity and a smooth and well-defined contour (Figure 3A). In oocytes from ticks treated with the ethyl-carbamates, the yolk granules had an irregular contour with invaginations and were surrounded by myelinic bodies, vacuoles and fragmented organelles (Figures 3C, 4D and 4E).

The myelinic bodies in oocytes from the control group ticks were rounded, had homogeneously electron dense content and were dispersed in the cytoplasm of early stages oocytes or between the yolk granules in oocytes III, IV and V (Figure 3G). In oocytes from treated ticks, the myelinic bodies had irregular content (Figure 3H) and were abundantly distributed around the mitochondria, endoplasmic reticulum and yolk granules (Figures 4B, 4C and 4D). The germ vesicle of control ticks had chromatin uniformly distributed in its interior (Figure 4A). In oocytes from treated ticks, chromatin clumping, accumulation of vacuoles and myelinic bodies surrounding the germinal vesicle were observed (Figure 4B).

In the oocytes from ticks treated with the ethyl-carbamates, a large number of free vacuoles were observed in the cytoplasm, within yolk granules and in the pedicel (Figures 4B, 4D, and 4E). Some contained remains of organelles or fused to form larger vacuoles (Figure 4F).

Embryonation percentage

Figure 5 shows the percentage of embryonated eggs oviposited by ticks treated with different concentrations of ethyl-carbamates. The negative effect of both ethyl-carbamates on embryonation percentages was similar. The highest concentration of both ethyl-carbamates produced 100% embryonation inhibition, while at the medium and lowest concentrations, they produced a concentration-dependent decrease ($r^2 = 0.5$, $p < 0.05$) at all days pt.

Embryogenesis

The embryonic structures observed in eggs from the negative control group are presented in Figures 6A-D. Between days 1 and 3, the first cell divisions, which increased exponentially during development, primitive plaque (Figure 6A) and a large amount of egg yolk were observed. Between days 4 and 7, the migration of cells towards one pole was observed, and the

blastopore originated in the primitive plaque area. From the blastopore, the germ band and its segmentations were formed (Figure 6B). Between days 8 and 13, the bud and elongation of legs and pedipalps, the definition of idiosomal segments and the gnatosomal zone, and the internalization of yolk were observed (Figure 6C). Between days 14 and 21, the three pairs of legs with their defined segments, the capitulum with palps, the chelicerae and the hypostome with rows of well-defined teeth were observed. In the idiosome, the shield, anal pore and excretory sac were appreciated. Covering the body, the sensory setae were appreciated (Figure 6D). Some larvae hatched during this period. The eggs oviposited by ticks treated with the highest concentration (0.668 mg/mL) of both ethyl-carbamates showed an irregular egg shell filled with an amorphous mass and no sign of cellularization on any days pt (Figure 6 I-L). Some eggs from ticks treated with the lowest and medium concentrations (0.006 and 0.068 mg/mL respectively) showed a lack of development, as was observed in eggs from ticks treated with the highest concentration. In contrast, other eggs had embryonic development that was similar to eggs from the control group (Figure 6E-H).

Discussion

Considering that the evolutionary success of ticks is intimately related to their high prolificacy and reproductive efficiency, a product that inhibits essential stages of reproduction, such as oogenesis and embryogenesis, can be an effective and a novel control alternative. In this work, we demonstrate that the new ethyl-carbamates LQM 919 and LQM 996 produce ultrastructural alterations in the oocytes that affect the embryonic development of the eggs produced by *R. microplus* engorged females treated with these ethyl-carbamates. In general, the ovary of ticks from the Ixodidae family is formed by an epithelial wall to which oocytes are attached through cells from a structure called pedicel. According to the degree of maturation, tick oocytes have been classified from I to V. In this study, we observed that the ovaries of ticks from the control group had the same histological, ultrastructural and development characteristics as those described by other authors (de Oliveira et al., 2005; Denardi et al., 2004; Saito et al., 2005; Sanches et al., 2012). This finding indicates that the structural alterations observed in treated ticks were due to exposure to the evaluated ethyl-carbamates. It has been shown that ethyl-carbamates LQM 919 and LQM 996 alter normal ovarian development in treated ticks, decrease

the number and viability of eggs produced, and alter their morphology (Pérez-González et al., 2014; Prado- Ochoa et al., 2014c).

In this work, we observed a higher proportion of oocytes I and II than oocytes III and IV on day 1 pt, both in treated and untreated ticks. However, oocytes from treated ticks exhibited degeneration and structural alterations, suggesting that the ethyl-carbamates crossed the tick surface and reached the ovarian cells. Arnosti et al. (2011) reported alteration of the shape, vacuolation and poor chorion deposition in oocytes from *R. sanguineus* ticks treated with ricinoleic acid esters. Roma et al. (2010) observed vacuolation, irregular shape and yolk granules decrease in all stages of oocytes from *R. sanguineus* ticks treated with sublethal doses of permethrin.

It has been shown that the precursors of vitellogenin and vitellin (glycolipophosphoproteins) from the eggs of some insects and ticks, such as *Haemaphysalis longicornis*, are produced by the fatty body and transported through the hemolymph to oocytes (Raikhel and Dhadialla, 1992, Boldbaatar et al., 2010). These ethyl-carbamates, being liposoluble, could be carried by these components into the oocytes and affect their early development.

It has been reported that the chorion (the outer wall of oocytes) is formed by an outer layer called exochorion and an inner layer called endochorion. The chorion of some mites, such as *Tyrophagus perniciosus*, has pillars that separate the exochorion from the endochorion and form a locular chamber between them (Witaliñski, 1993). In addition, the chorion has pores that flow into the locular chamber (Saito, 2005, de Oliveira, 2005). In this study, we ultrastructurally observed oocytes from control ticks and determined the presence of channels that completely cross the chorion and electron-dense material that apparently circulates through them. In addition, we observed a large number of pores that probably communicate through the locular chamber with the inside and outside of the oocyte. Together, these channels and pores could form a system through which different types of nutrients from the hemolymph enter the oocyte (Figure 3B). The chorion of oocytes from treated ticks showed an evident disarray, loss of the continuity in the channels and the accumulation of electrodense material in the exochorion. Similar chorion disarrays were observed in oocytes from *R. sanguineus* ticks treated with sublethal doses of fipronil (de Oliveira et al. 2009) and dinotefuran (de Oliveira et al., 2017). The

above shows that the ethyl-carbamates and other compounds could alter the structure and probably the permeability of the chorion, which can hinder the passage of nutrients into the oocyte and lead to oocyte underdevelopment. In this work, the cytoplasmic structures of oocytes from the control group ticks were similar to those described by Saito et al. (2005), Sanches et al. (2012) and Denardi et al. (2004) in normal oocytes from different tick species; therefore, only the alterations produced by the ethyl- carbamates treatment are discussed. The oocytes from treated ticks showed clear evidence of progressive degeneration of the organelles observed, which could be a direct result of the action of ethyl-carbamates on the organelles or an indirect result of the lack of nutrients produced by the alterations of the chorion previously described. The ethyl-carbamates induced an initial increase in the number of mitochondria observed, which may be related to an increase in energy demand of the treated oocytes. Later, most of the mitochondria showed degeneration, characterized by swelling, cristolysis, structural disorganization and loss of definition. Other compounds such as deltamethrin, amitraz and fipronil also produced diminish in the number of mitochondria and mitochondrial cristolysis and swelling (de Oliveira et al., 2009; Sreelekha et al., 2017). These alterations suggest a decrease in ATP production and consequently an effect on respiratory metabolism. On the other hand, the endoplasmic reticulum also showed evident signs of degeneration, which can cause a decrease in the production of endogenous protein.

Yolk granules are storage structures for large amounts of vitelline, which is the main energy and protein reserve for embryonic development (Roma et al., 2010). In the oocytes from treated ticks, irregular and smaller yolk granules were observed, which reduces the amount of nutritional reserves. This finding probably contributes to the lack of development of the oocytes, decrease in the number of eggs produced and the lack of embryos from eggs that are oviposited. The myelinic bodies are vesicular cytoplasmic structures of the oocytes, measuring approximately 400 nm, with an electron-dense material in their interior and a well-defined, rounded contour. Although the myelinic bodies function is not well-known, they have been associated with degenerative processes in tick oocytes (Denardi et al., 2004, de Oliveira et al., 2005, Saito et al., 2005, Sanches et al., 2012). In oocytes from the control group of ticks, we observed a reduced number of well-defined myelinic bodies that were homogeneously distributed in the cytoplasm. In contrast, in the oocytes from treated ticks, there was an increased

number of myelinic bodies with irregular electrodense material and a clear association with autophagic vacuoles and degenerating organelles. In addition, we also observed an increase in the number and size of vacuoles around the degenerated organelles and within the yolk granules. We observed the fusion of several vacuoles, apparently to engulf damaged structures. In the interior of these vacuoles, remains of organelles and cellular detritus were observed. These observations suggest that myelinic bodies and autophagic vacuoles belong to a system for removing damaged structures, cell detritus and toxic compounds within the oocyte, which is stimulated directly or indirectly by the ethyl- carbamates evaluated.

The germ vesicle of the oocytes from the control ticks had a well-defined nucleolus and chromatin uniformly distributed in its interior. In the oocytes from treated ticks, we observed the following, both histologically and ultrastructurally: nucleolus fragmentation, chromatin clumping and accumulation of autophagic vacuoles and myelinic bodies surrounding the germinal vesicle, indicating damage to the genetic material. These observations are consistent with those reported by Prado-Ochoa et al. (2016), who showed that the ethyl-carbamates induce genotoxic damage characterized by an increase in the frequency of micronuclei in rat erythrocytes. In addition, nuclear damage, associated with the degeneration of mitochondria and autophagic processes was observed, pointing to apoptotic processes in the oocytes, as was suggested by Escobar-Chavarria (2014). However, cell death by other mechanisms such as necrosis and autophagy cannot be ruled out (Kroemer et al., 2009).

Recently, embryonic development in eggs of *R. microplus* and *Dermacentor andersoni* was described in detail by Santos et al. (2013) and Friesen et al. (2016), respectively. In this study, we observed that embryonic development of eggs produced by the ticks from the control group was similar to that described by these authors. Although in this work oviposition inhibition was not evaluated, a noticeable decrease in the number and size of eggs produced by ticks treated with ethyl-carbamates was observed, as was previously reported by our group (Pérez-González et al., 2014; Prado-Ochoa et al., 2014c). The eggs oviposited by ticks treated at the highest concentration of both ethyl-carbamates (0.668 mg/mL) have no evidence of embryonic development, which is probably the result of the degeneration observed in the oocytes from treated ticks. Some eggs from ticks treated with medium and lower concentrations (0.066 and 0.006 mg/mL, respectively) showed apparently normal embryonic development, while others

showed no evidence of development. No eggs were observed with incomplete or altered embryonic development. These observations suggest that at low concentrations some oocytes can resist the toxic effect of carbamates and produce an embryo. In contrast, in other oocytes, their detoxification mechanisms were not sufficient to protect embryonic development.

Recently, it has been described that some ixodicides, such as fipronil, avermectin and deltamethrin, the main site of action of which is the nervous system of ticks, secondarily and at sublethal doses affect oogenesis. de Oliveira et al., (2009), ; Friesen et al., 2003; Sreelekha et al., 2017).

The mechanism of action of these ixodicides in the reproductive system is not clear, although they probably block some of the complex interactions of the nervous system with the reproductive system.
The results of this study, together with the results of previous studies (Prado-Ochoa et al., 2014c, Escobar-Chavarría, 2017), show that ethyl-carbamates LQM 919 and LQM 996 have the ovary of ticks as their main site of action and that they are important inhibitors of reproduction. In addition, this is the first study which evaluates the effect of an ixodicide on embryonic development in *R. microplus*.

Conclusions

The ethyl-carbamates examined in this study produced important ultrastructural alterations in the oocytes of *R. microplus* ticks. The carbamates modified the structure of the chorion and produced degeneration of organelles, nuclear damage and activation of myelinic bodies and autophagic vacuoles, which was associated with the inhibition of oogenesis. These alterations probably led to the lack of embryonic development observed in the eggs of treated ticks.

Acknowledgments

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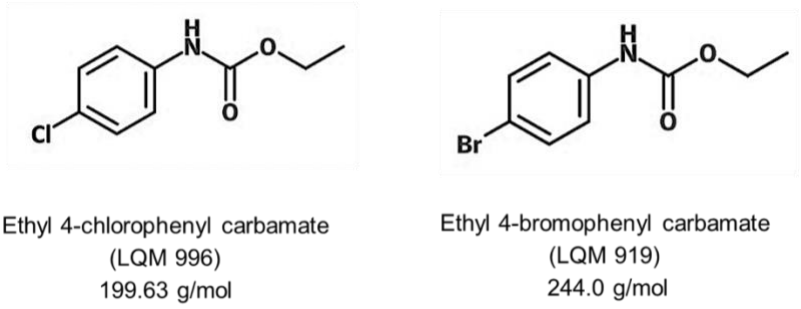
FIGURES AND TABLES



Ethyl 4-chlorophenyl carbamate
(LQM 996)
199.63 g/mol

Ethyl 4-bromophenyl carbamate
(LQM 919)
244.0 g/mol

Figure 1. Chemical structure, identification code and molecular weight of the ethyl- carbamates.

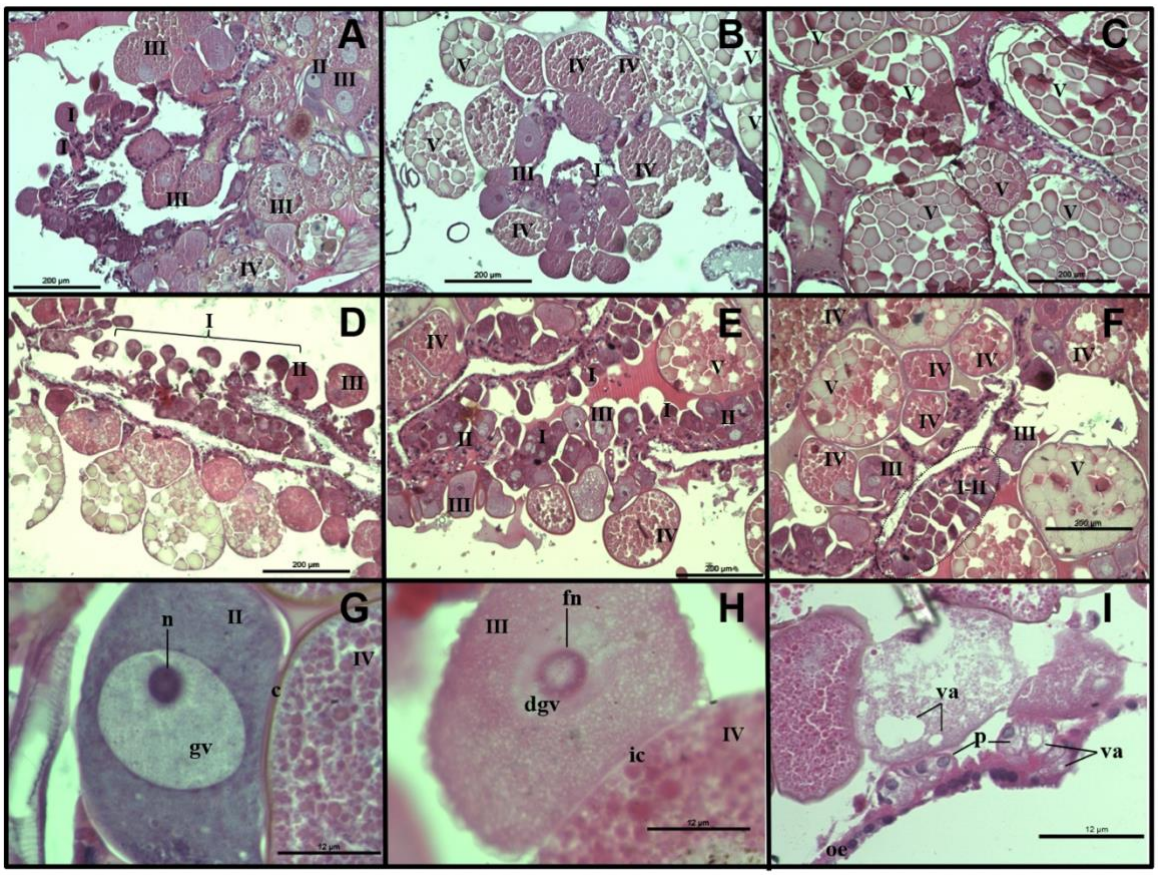


Figure 2. Histological sections (HE-stained) of ovaries from *Rhipicephalus microplus* ticks treated by the Adult Immersion Test. Ovaries from control ticks at 1 (A), 3 (B) and 7 (C) days post-treatment with dimethylsulfoxide (vehicle). Oocytes from ticks at 1 (D), 3 (E) and 7 (F) days post-treatment with 1 mg/ml of LQM 919. G, Detail of oocytes from control tick. H, Detail of oocytes from ethyl-carbamate exposed tick. I, Detail of ovarian epithelium and pedicel from ethyl-carbamate exposed tick. I-V = oocyte I-V; n = nucleolus; fn = fragmented nucleolus; gv = germinal vesicle; dgv = degenerated germinal vesicle; va = vacuole; oe = ovarian epithelium; c = chorion; ic = incompletely deposited chorion; p = pedicel.

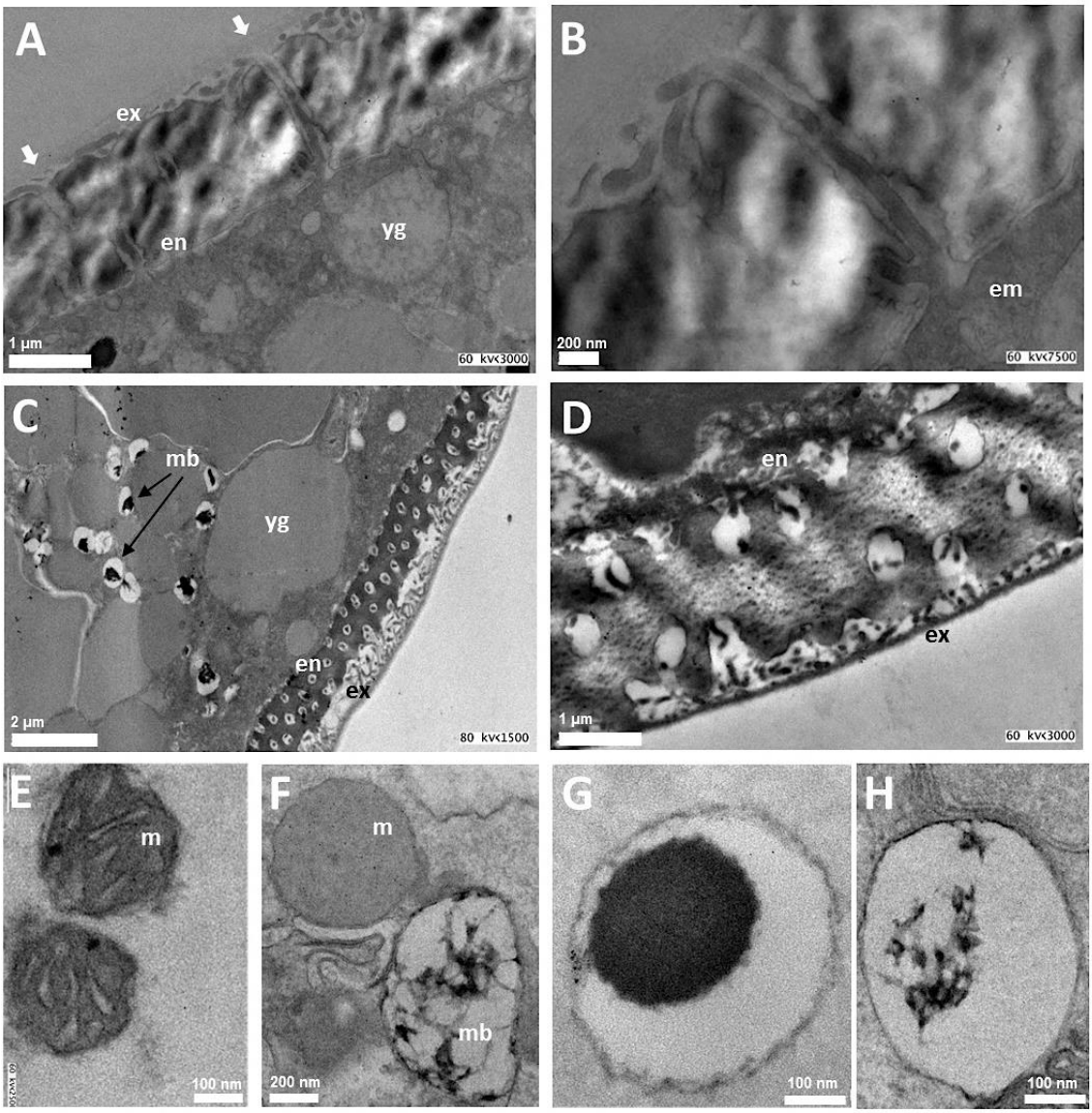


Figure 3. TEM micrographs of oocytes from *Rhipicephalus microplus* ticks exposed or nonexposed (control) to LQM 919 or LQM 996 ethyl-carbamates. A, Chorion structure of an oocyte from a control tick: ex = exochorion, en = endochorion, yg = yolk granule, arrows show the chorion channels. B, Detail of a chorion channel; note the electrodense material (em) across to channel. C, Chorion structure of an oocyte from an exposed-tick; note the disarray of the exochorion and the presence of multiple myelinic bodies (mb). D, Detail of the chorion of an exposed-tick oocyte; note the absence of channels. E, Mitochondria (m) from control tick oocyte. F, Degenerated mitochondria next to a myelinic body in an exposed-tick oocyte. G, Appearance of a myelinic body in control tick oocytes. H, Appearance of a myelinic body in an exposed-tick oocyte.

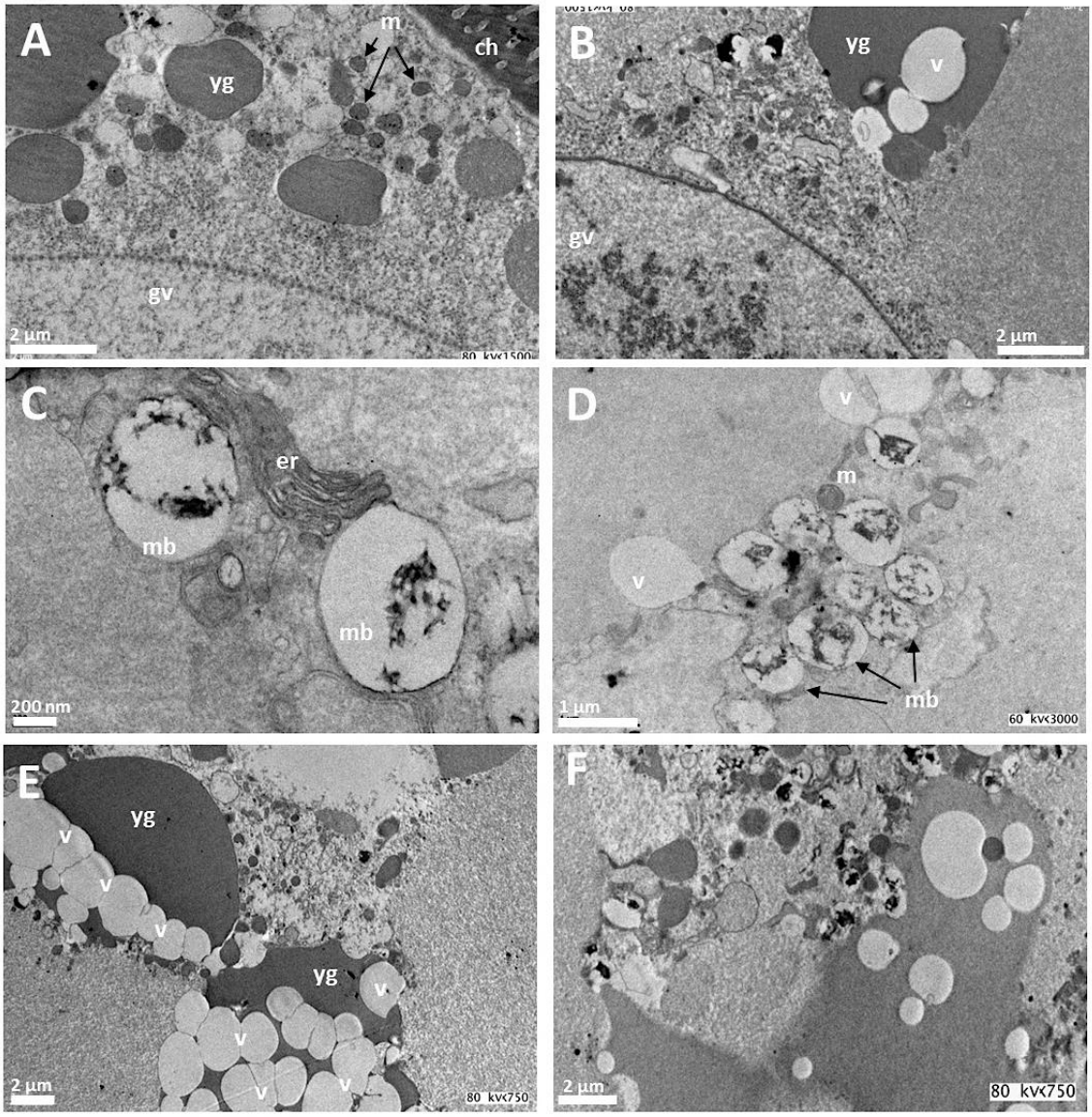


Figure 4. TEM micrographs of oocytes from *Rhipicephalus microplus* ticks exposed or nonexposed (control) to LQM 919 or LQM 996 ethyl-carbamates. A, Germinal vesicle (gv), yolk granule (yg) and cytoplasmic structures from a control tick oocyte. B, Germinal vesicle and cytoplasmic structures from exposed-tick oocyte; note the chromatin clumping, the presence of vacuoles and the disarray of cytoplasmic structures. C, Myelinic bodies (mb) next to the endoplasmic reticulum (er) from exposed-tick oocyte. D, Myelinic bodies next to mitochondria (m), vacuoles (v) and cytoplasmic detritus from exposed-tick oocyte. E, Multivacuolated yolk granules from an exposed-tick oocyte in an advanced degeneration stage. F, Myelinic bodies, vacuoles and degenerated organelles from an exposed-tick oocyte in an advanced degeneration stage.

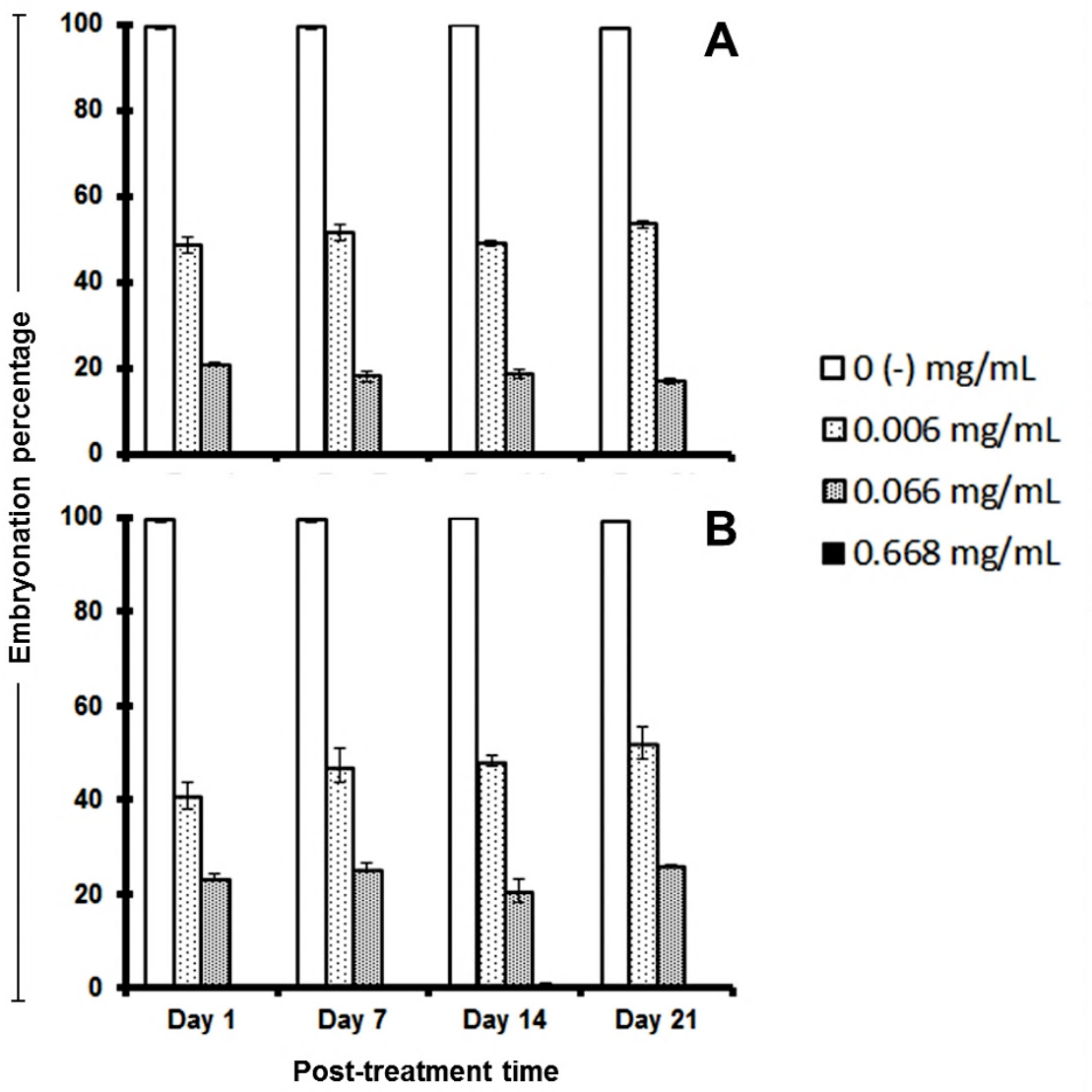


Figure 5. Embryonation percentage values (mean±SE) of eggs from *Rhipicephalus microplus* ticks treated with different concentrations of; A. ethyl-4-bromophenyl carbamate (LQM 919) and B. ethyl-4-chlorophenyl carbamates (LQM 996).

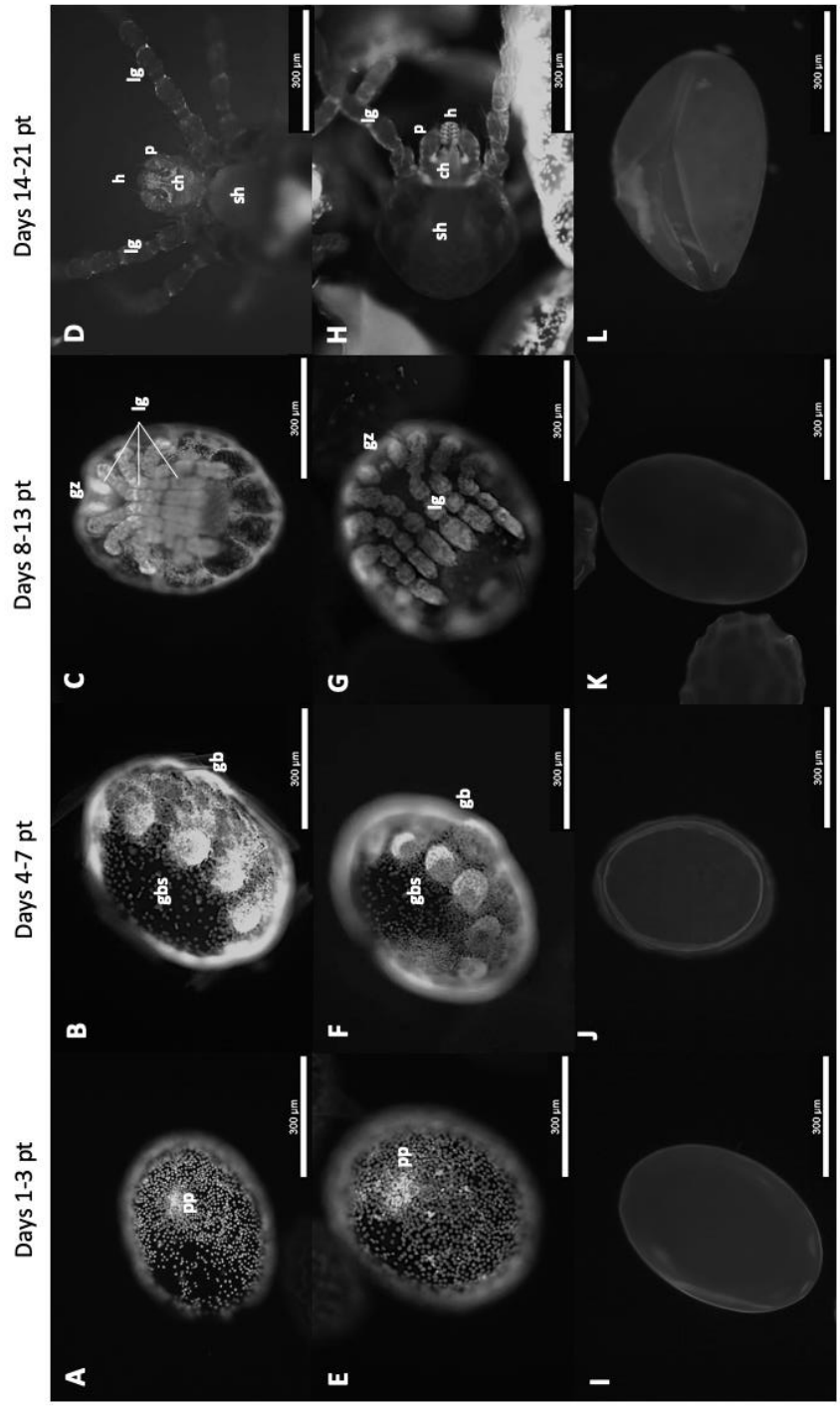


Figure 6. Microphotographs of DAPI-stained eggs from *Rhipicephalus microplus* ticks at different days post-treatment with ethyl-4-bromophenyl carbamates (LQM 919). A-D Eggs from negative control group ticks. E-H Eggs from ticks treated with 0.066 mg/mL (medium concentration). I-L Eggs from ticks treated with 0.668 mg/mL (highest concentration). ch= chelicerae; gb= germ band; gz= gnatosomal zone; h= hypostome; lg= legs; p= palps; pp= primitive plaque; gbs= germ band segments; sh=shield.

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CAPÍTULO 7

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Toxic effects of new ethyl-carbamates on the morphology, mortality and acetylcholinesterase activity of *Eisenia foetida*

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ABSTRACT

The toxicity of the ixodicidal carbamates ethyl-4-bromophenyl carbamate (LQM 919), ethyl-4-chlorophenyl carbamate (LQM 996) and propoxur on *Eisenia foetida* adults was evaluated to estimate their ecotoxic potential. The earthworm mortality and weight loss produced by the three evaluated carbamates showed a concentration-dependent effect ($p < 0.0001$) in the contact filter paper test (CFPT). In the artificial soil test (AST), mortality increased in relation to the exposure time ($p < 0.0001$) and the concentration ($p < 0.01$) of the carbamates. Only the earthworms exposed in the CFPT showed morphological alterations. According to the LC_{50} obtained in the CFPT, the three carbamates were classified as very toxic and, according to the LC_{50} obtained in the AST, the three carbamates were classified as highly toxic for *E. foetida*. The values of k_i and k_d indicated that LQM 919 and LQM 996 are weak inhibitors with lower affinity for the acetylcholinesterase of *E. foetida* than that of propoxur. The concentrations in the CFPT and AST at which 100% mortality was observed in *E. foetida* were 64- and 4-fold higher, respectively, than the egg hatching inhibitory concentration 99% reported for ticks.

1. Introduction

Carbamates are a broad group of chemical compounds derived from carbamic acid. Their toxicity is variable, with some being so toxic that they were used as poisonous gases during the First World War (Gupta, 2006). In contrast, some benzimidazole-carbamates (albendazole, mebendazole, fenbendazole and oxfendazole, among others) have such low toxicities that they are used as antiparasitic drugs in humans and domestic animals (Botana López et al., 2002). Considering these attributes, it is not correct to extrapolate the toxicological data from one carbamate to others, even if they are from the same family. Therefore, when a new carbamate is synthesized, it is necessary to study its toxicity individually.

Some carbamates such as propoxur and carbaryl have been used as pesticides for arthropod control in the agriculture industry (Mwanza et al., 2008). Ticks are ectoparasites with a great impact in livestock production in tropical and subtropical areas in worldwide (George, 2006). The widespread use of ixodicides has generated the emergence of tick strains resistant to most commercial products. Our group designed and synthesized two new ethyl carbamates with high efficacy toward strains of ticks of the species *Rhipicephalus microplus*, which are multiresistant to commercial ixodicides (Pérez-González et al., 2014; Prado-Ochoa et al., 2013). The oral and dermic acute toxicity, oral subchronic toxicity, cytotoxicity, genotoxicity and mutagenicity of these ethyl-carbamates have been evaluated, and generally, their toxicity is low in mammals (Prado-Ochoa et al., 2014a, 2014b; 2016; Strassburger Madrigal, 2017). Commonly, the ixodicides are applied to livestock by immersion or aspersion baths, and due to this practice, the products are dispersed into the environment, and potentially, other nontarget organisms may be exposed to the effects they cause. Therefore, it is necessary to study the ecotoxic effects of these ethyl-carbamates before they are used in the field.

Earthworms play an essential role in terrestrial ecosystems. They represent the majority of the total invertebrate biomass in the soil, contributing to the degradation of organic matter, recycling inorganic compounds (mainly nitrogen) and conducting the formation of soil (Rodríguez-Castellanos and Sanchez-Hernandez, 2007). Due to its ease of cultivation, low cost and high sensitivity to environmental contaminants, *Eisenia foetida* has been recommended by the OECD (Organization for Economic Cooperation and Development) as a biomarker for toxicity studies involving soil organisms (Li et al., 2017; OECD, 1984; Xing et al., 2017). For the above reasons, in this study, the

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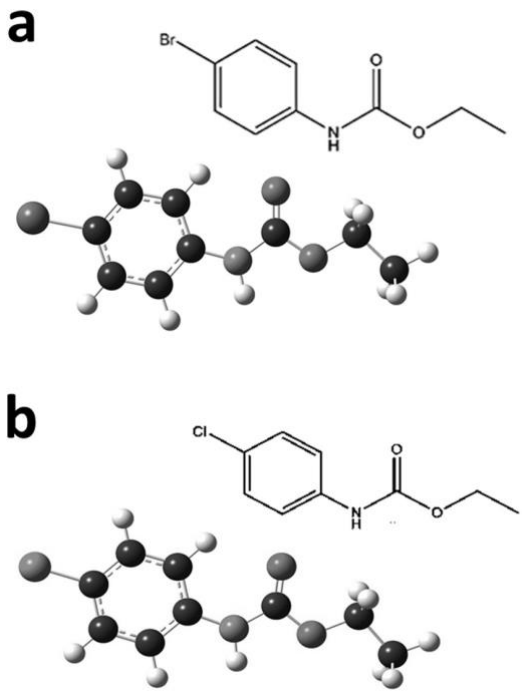

Fig. 1. Chemical structure of: a, ethyl-4-bromophenyl carbamate (LQM 919) and b, ethyl-4-chlorophenyl carbamate (LQM 996).

toxicity of the new ixodicidal ethyl-carbamates toward *E. foetida* adults was evaluated to estimate the ecotoxic potential of the compounds.

2. Materials and methods

2.1. Carbamates

Ethyl-4-bromophenyl carbamate (LQM 919) and ethyl-4-chlorophenyl carbamate (LQM 996) were designed and synthesized at the Universidad Nacional Autónoma de México using a benzimidazole molecule as the structural base, as described by Angeles et al. (2000). The identification codes, nomenclature, chemical structures and molecular weights of the ethyl-carbamates are shown in Fig. 1.

2.2. Earthworm maintenance

E. foetida earthworms were maintained according to the 207 OECD guide recommendations (1984). The earthworms were kept in plastic boxes at 18–20 °C in a substrate of dirt and dried leaves, with 80–90% humidity. Weekly, they were fed fermented fruits and vegetables. The development of a complete life cycle was verified by the observation of cocoons, juvenile and adult earthworms. In all experiments, sexually mature earthworms with weights of 250–550 mg were used.

2.3. Contact filter paper test (CFPT)

The test was conducted according to the description in the 207 OECD guide (1984). Six hundred and twenty-five earthworms were used, and they were dieted for 3 h prior to the experiments. Groups of 5 earthworms were individually exposed to filter paper impregnated with different concentrations of ethyl-carbamates. The concentrations used for ethyl-carbamates (LQM 919 and LQM 996) were 2.6, 5.3, 10.5, 21, 42, 84, 168 and 336 μg/cm^2 on the filter papers. Propoxur® (2-2 (1-methylethoxyl) phenyl methylcarbamate, 99% pure, Sigma-Aldrich 45,644) was used as a positive control at the same concentrations, and negative control filter papers were impregnated with trichloroethylene 99%. The exposed earthworms were maintained at room temperature and in darkness for 48 h. The weight of each earthworm was measured individually at the beginning and at the end of the test. Subsequently, the mortality percentage, lost weight percentage (initial weight - final weight ÷ initial weight X 100) and lethal concentrations (LC_{50} and LC_{99}) were calculated. Morphophysiological data were recorded (movement, displacement and general appearance). This experiment was repeated 5 times.

The concentrations of each ethyl-carbamate were selected based on a previous test in which we used the 99% egg hatching inhibitory concentration ($EHIC_{99}$) in ticks (Pérez-González et al., 2014), which was equivalent to 5.3 μg/cm^2 in the CFPT, and then increased the concentration twice by a serial method until a mortality of 100% was produced in the earthworms (336 μg/cm^2).

2.4. Artificial soil test (AST)

The test was conducted according to the description in the 207 OECD guide (1984). One thousand and four hundred forty earthworms previously adapted to the artificial substrate (70% industrial sand, 20% kaolin and 10% sphagnum peat) at room temperature for 24 h were used and were distributed into 144 groups (n = 10). Earthworms from each group were exposed over 14 days to the different treatments in flasks containing 750 g of the substrate, the moisture content was adjusted to 35% of the final weight. Earthworms from eight groups were exposed to each concentration of LQM 919, LQM 996 (2.09, 4.18, 8.14, 16.73 and 33.46 mg/kg of substrate), and propoxur (3.33, 6.66, 13.32, 26.64 and 53.28 mg/kg of substrate). As negative controls, three groups of earthworms exposed to absolute ethanol (13.3 mL/kg of substrate) were used. The earthworm mortality and morphophysiological data were recorded at days 1, 2, 7 and 14 of exposure. Lethal concentrations (LC_{50} and LC_{99}) from each treatment were calculated.

The range of concentrations for LQM 919 and LQM 996 was selected by taking the mean of the $EHIC_{99}$ in ticks (Pérez-González et al., 2014), which is equivalent to 8.14 mg/kg of substrate in the artificial soil test. From the $EHIC_{99}$, two major concentrations and two minor double concentrations were prepared.

2.5. Enzyme extraction

Acetylcholinesterase enzymes (AChE) were extracted from earthworms following the protocol described by Rao and Kavitha (2004). The anterior six segments of each earthworm were macerated in 0.1 M phosphate buffer with a pH of 7.5. The homogenates were centrifuged (12,000 *g* at 4 °C), and the supernatant was also centrifuged. The protein concentration was determined by the technique described by Bradford (1976) and adjusted to 1 μg/μL.

2.6. Determination of AChE kinetics

The activity of AChE in extracts of *E. foetida* was measured by the method of Ellman et al. (1961), as modified by Prado-Ochoa et al. (2014c). Seven absorbance readings were performed at 2-min intervals in an Ascent ELISA plate reader (Labsystems) at 405 nm and 30 °C. The kinetic constants K_m (Michaelis–Menten constant) and V_{max} (maximum velocity) were calculated by nonlinear regression, using least-squares algorithms to fit the Michaelis–Menten function using Graph Pad Prism® software.

2.7. Inhibition of AChE activity in vitro and in vivo

The *in vitro* AChE inhibition kinetics produced in *E. foetida* due to carbamates were determined according to the method of Chen et al. (2001). The AChE inhibition rate was measured in quadruplicate in 20 μL of the homogenate (1 μg of protein/μL) containing untreated earthworms in the presence of 1.2×10^{-4} M of acetylthiocholine with

DTNB (5-5 dithiobis-2 nitrobenzoate) and different concentrations of LQM 919, LQM 996 (0, 195, 390, 780, 1560, 3125 and 6250 µM) or propoxur (0, 0.04, 0.2, 1, 5, 25 and 125 µM). The inhibition kinetics were monitored at 405 nm and 30 °C. Seven absorbance readings were collected at 2-min intervals (Pruett, 2002). The dissociation constant (k_d), bimolecular reaction constant (k_i), carbamylation constant (k_2) and 50% inhibitory concentrations (IC_{50}) were calculated (Chen et al., 2001; Pruett, 2002; Rao et al., 2003).

The *in vivo* inhibition of AChE activity was evaluated as described for the *in vitro* test. Extracts of earthworms exposed over 48 h to different concentrations of LQM 919 (22.5, 45 and 90 µg/cm^2), LQM 996 (17.5, 35 and 70 µg/cm^2) and propoxur (17.5, 35 and 70 µg/cm^2) in the CFPT were used. The concentrations used correspond to the LC_{50} obtained in the CFPT and two minor double concentrations. To calculate the rate of activity reduction, the following equation was used:

$$\%\text{AChE activity reduction} = 100 - \left(\frac{V_{[I]}}{V} \times 100 \right)$$

where:

V = AChE activity without the inhibitor. The slope of the reaction rate is created by an increase in absorbance over time.
$V_{[I]}$ = AChE activity for each inhibitor concentration. The slope of the reaction rate is created by an increase in absorbance over time.

2.8. Statistical analysis

The effects of the carbamate concentrations on the mortality and weight loss of earthworms in the CFPT, as well as the effects of time and concentration in each period, on the mortality of earthworms in the AST were analyzed by linear regression.

The mortality and weight loss data from the earthworms in the CFPT were analyzed by one-way ANOVA. The mortality data of earthworms in the AST obtained during different periods were analyzed by ANOVA for repeated samples, using the Statistica for Windows® software. All differences between means were established using Tukey's test. The lethal concentrations of each carbamate were calculated by Probit analysis, conducted with the Polo-Plus program (LeOra Software®). The minimum confidence used in all tests was 95%.

3. Results

3.1. Contact toxicity

The linear regression showed a concentration-dependent effect on the mortality of *E. foetida* in the three carbamates evaluated ($r^2 \geq 0.73$; $p < 0.0001$). The lowest concentrations that produced mortality were 42, 10.5 and 21 µg/cm^2 for LQM 919, LQM 996 and propoxur, respectively.

The initial average weight of the earthworms subjected to the treatments (n = 468) was 369.68 ± 70 mg. For all three products, the weight loss had a positive correlation with the concentration ($p < 0.001$). The final percentages of weight loss produced by LQM 919, LQM 996 and propoxur were 26.7 ± 3.5, 25.5 ± 3.7 and 20.2 ± 4.4, respectively. Due to the high mortality, there were not enough weight loss data for the concentrations of 168 and 336 µg/cm^2.

The most common morphological alterations observed in the earthworms exposed to different concentrations of the evaluated carbamates are shown in Fig. 2, and the percentages of earthworms that presented these alterations are shown in Table 1. In all the earthworms exposed to the carbamates, the extrusion of coelomic fluid was observed, as well as less-frequent (≤6%) swelling and vesicle formation. The earthworms of the negative control group showed no apparent alterations. The majority of dead earthworms exposed to the ethyl-carbamates presented liquefaction.

3.2. Soil toxicity

The earthworm mortalities after exposure to the different concentrations of carbamates in AST are presented in Fig. 3. For the three carbamates, earthworm mortality increased in relation to the time of exposure ($p < 0.0001$) and to the concentration ($p < 0.01$). None of the earthworms exposed to carbamates showed apparent alterations.

3.3. Lethal concentrations

The LC_{50} and LC_{99} values obtained in the CFPT and AST are presented in Table 2. In accordance with the classification system of Roberts and Wyman Dorough (1984) and according to the LC_{50} values obtained in the CFPT, the three carbamates used were classified as very toxic toward *E. foetida*. In accordance with the classification system of the OECD (2003) and according to the LC_{50} values obtained in the AST, the three carbamates were classified as highly toxic toward *E. foetida*.

3.4. AChE inhibition

The basal parameters of the AChE of *E. foetida* were $V_{max} = 0.021 \pm 0.001$ M/min/L and $K_m = 1.85 \times 10^{-4} \pm 1.09 \times 10^{-5}$ M. The kinetic constants—the bimolecular reaction constant (k_i), dissociation constant (k_d) and carbamylation constant (k_2)—and the 50% inhibitory concentrations (IC_{50}) obtained in the *in vitro* test are presented in Table 3. The k_i values of LQM919 and LQM 996 toward AChE were lower ($p < 0.05$) than those of propoxur. The k_d was higher ($p < 0.05$) for the carbamates LQM 919 and LQM 996 than for propoxur. The k_2 values of LQM 919, LQM 996 and propoxur were not significantly different ($p > 0.05$). All propoxur concentrations evaluated produced *in vitro* reduction in the AChE activity of close to 100%, so it was not possible to establish the IC_{50} of propoxur. The carbamates LQM 919 ($r^2 = 0.69$) and LQM 996 ($r^2 = 0.62$) produced a concentration-dependent reduction of AChE activity ($p < 0.001$).

The IC_{50} values obtained in the *in vivo* tests were 50.07 (C.I. 21.3–117.2), 41 (C.I. 36.09–46.5) and 79 (C.I. 38–165) µg/cm^2 for LQM 919, LQM 996 and propoxur, respectively. The activity reduction was concentration-dependent ($p < 0.05$). The carbamates LQM 919 (r2 = 0.91), LQM 996 ($r^2 = 0.93$) and propoxur ($r^2 = 0.82$) produced a concentration-dependent reduction of AChE activity ($p < 0.001$).

4. Discussion

The use of ixodicides in livestock has favored an increase in food production in tropical and subtropical areas of the world through the control of different species of ticks (Willadsen, 2006). Due to the morphological, metabolic and physiological similarities between ticks and other organisms, ixodicides can affect both target and nontarget organisms (Stepić et al., 2013). Many pesticide ecotoxicity studies have been conducted after pesticide commercialization (Boxall et al., 2003). Currently, the development of new ixodicides should include environmental impact studies to estimate the potential risks of their use. In this study, the toxicity of the new ethyl carbamates LQM 919 and LQM 996 toward earthworms (*E. foetida*), which have been used as biomarkers to evaluate the impact of pesticides on soil, was evaluated. Our results show that these ethyl-carbamates are capable of inducing mortality and morphological alterations in *E. foetida* at concentrations higher than those effective against ticks.

The CFPT has been used as a screening test to achieve an approximation of the toxicity degree of a chemical toward earthworms (OECD, 1984; Luo et al., 1999; Wang et al., 2012). This test is relatively simple and reproducible and allows easy observation of morphological alterations in earthworms. However, it does not represent what happens during natural exposure in a terrestrial ecosystem (Miyazaki et al., 2002; Wang et al., 2012). We used propoxur as a positive control because its toxic potential and LC_{50} are well-known (Roberts and Wyman

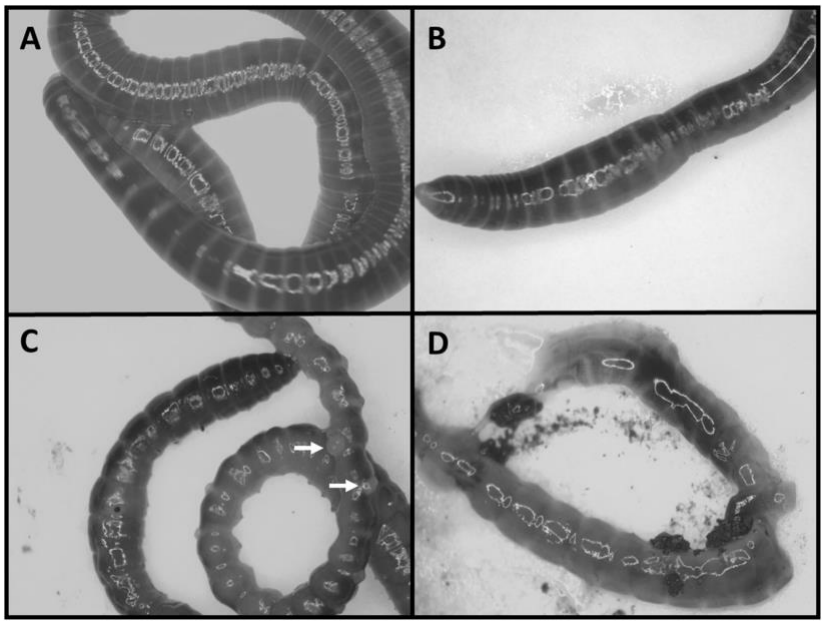

Fig. 2. Effects of ethyl-carbamates on the morphology of *E. foetida* earthworms after 48 h of exposure. A) Nonexposed earthworm (control). B) Metameres swelling and coelomic fluid extrusion. C) Metameres segmentation and production of vesicles (arrows). D) Liquefaction of dead earthworm.

Table 1
Percentages of morphological and behavioral alterations observed in *Eisenia foetida* earthworms exposed to different concentrations of ethyl-carbamates and propoxur in the contact filter paper test.

Carbamate code	Concentration ($\mu g/cm^2$)	Metameric segmentation (%)	Random contractions (%)	Coiling inability (%)
LQM 996	84	100	50	80
	42	33	27	80
	21	21	37	58
	10.5	36	60	64
	5.25	4	48	56
	2.63	8	20	52
LQM 919	84	82	67	8
	42	43	57	57
	21	20	50	60
	10.5	0	20	48
	5.25	8	16	44
	2.63	0	0	36
PROPOXUR	84	40	64	0
	42	60	90	0
	21	64	100	0
	10.5	40	96	0
	5.25	4	32	0
	2.63	0	0	0

Dorough, 1984; Ware, 1980). In this work, we observed that LQM 919 and LQM 996 produced concentration-dependent mortality, which allowed us to estimate the corresponding LC_{50} and LC_{99} values. Based on the confidence intervals and the proximity of the LCs, we consider that the acute toxicities of both ethyl carbamates and that of propoxur are similar (Table 1).

To compare the acute toxicities of different chemicals toward *E. foetida*, Roberts and Wyman Dorough (1984) proposed the following classification system based on CFPT: LC50% < 1 $\mu g/cm^2$, supertoxic; LC_{50} 1–10 $\mu g/cm^2$, extremely toxic; LC_{50} 10–100 $\mu g/cm^2$, very toxic; LC_{50} 100–1000 $\mu g/cm^2$, moderately toxic and LC_{50} > 1000 $\mu g/cm^2$, relatively nontoxic. The acute toxicities of pesticides for commercial use are highly variable. Some are supertoxic, such as carbofuran and neonicotinoids (acetamiprid, imidacloprid, among others), and others are extremely toxic such as ivermectin, pyridaphenthion, dicrotophos, fonophos and most carbamates. Considering the results obtained in this study, LQM 919 and LQM 996 (Table 2) should be classified as very toxic in the CFPT. Therefore, we consider that these ethyl-carbamates should be grouped with other pesticides with lower environmental impacts such as malathion, parathion, chlorpyrifos, and cypermethrin, among others (Roberts and Wyman Dorough, 1984; Wang et al., 2012; Chen et al., 2014).

Morphological changes in *E. foetida* are one of the most adequate indicators by which to monitor the toxicity of chemicals in the CFPT. Weight loss is a good indicator of chemical stress (Shi et al., 2007). In this study, the weight loss was concentration-dependent for all the three carbamates. The extrusion of coelomic fluid observed in most earthworms exposed to carbamates and contributed to the observed weight loss, and it is probably part of the earthworm strategy for reducing the chemical stress and eliminating the pesticide. In the CFPT the cuticle of the earthworms is directly exposed to the chemical, which favors its absorption, arrival to the coelomic fluid and transport throughout the body (Jager et al., 2003). Macroscopic alterations occurred in the whole body of earthworms exposed to carbamates, and the most frequent lesions were inflammation in some metameres, segmentation and liquefaction. These alterations are similar to those produced by profenofos, chlorpyrifos and carbofuran (Gilman and Vardanis, 1974; Rao et al., 2003; Reddy and Rao, 2008). These types of alterations have been associated histologically with the disintegration of the cuticular layer, glandular epithelium, intestinal cells and muscle cells (Reddy and Rao, 2008; Wang et al., 2015; Li et al., 2017).

In general, in artificial soil tests, earthworms are exposed to chemicals under similar conditions to those of exposure when chemicals are poured into the soil. Hence, absorption occurs mainly through the intestine (OECD, 1984; Ware, 1980; Wang et al., 2012). In the AST, we observed that LQM 919 and LQM996 produced concentration- and time-dependent mortality. The highest mortality was observed at 14 days of exposure for the three evaluated carbamates, regardless of the concentration in the artificial soil. No apparent morphological alterations were observed in earthworms, even in the dead ones. The above findings suggest that the absorption of carbamates is slower through the intestinal route than through the cuticle and that the observed toxicity is probably due to the accumulation of carbamates after several days of exposure.

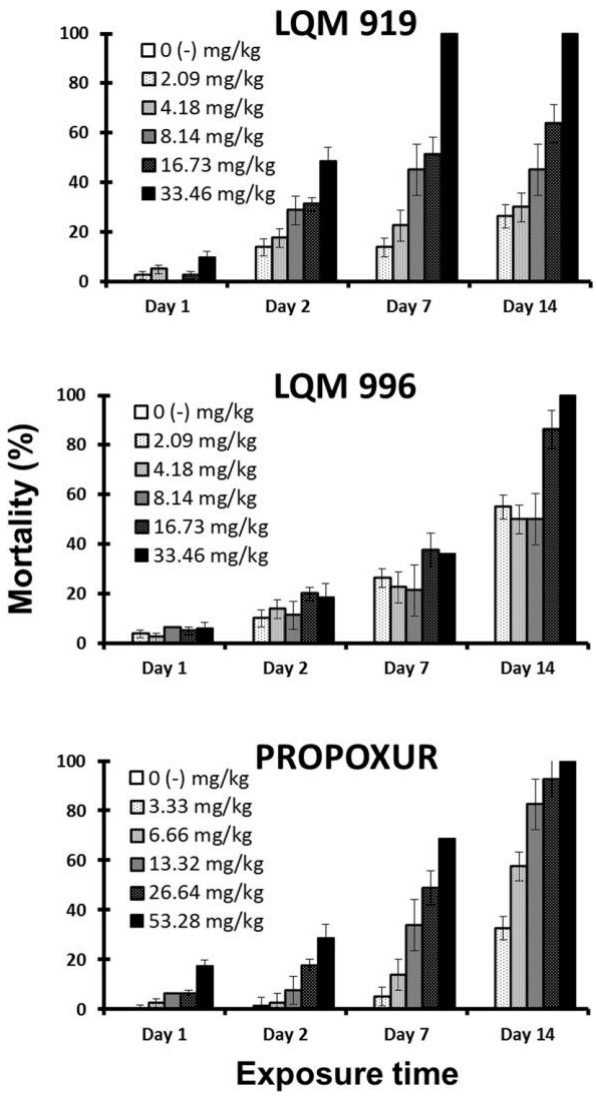

Fig. 3. Mean (± SE) mortality percentages of *E. foetida* earthworms exposed to different concentrations of two ethyl-carbamates and propoxur in the artificial soil test at different days of exposure.

Additionally, we observed that the surviving earthworms continued with their normal life cycle when they were returned to an artificial substrate without carbamates (data not shown). This suggests that the mechanisms of detoxification of earthworms allow them to eliminate the toxic effects produced by these carbamates.

The LC_{50} of any pesticide is a mathematical prediction obtained from data generated using a range of concentrations that can be adjusted for Probit analysis, so this range of data must be selected in a particular way for each pesticide. In the present study, the concentrations used in the AST allowed us to calculate the LC_{50} of each carbamate for *E. foetida*, which allows comparison between them. Considering the LC_{50} confidence limits of the three carbamates, no differences were observed among them. According to the OECD (2003) classification, the two ethyl-carbamates, together with propoxur, can be classified as very toxic in the AST.

Earthworms exposed to sublethal concentrations of carbamates in the CFPT presented behavioral alterations such as coiling inability and random contractions that suggested nervous system effects. The evaluation of AChE activity is the fastest and most effective way to determine when chemicals modify the neurological functions of an organism.

The most reliable criterion by which to evaluate the inhibitory power of a chemical on AChE is the k_i. In this study, the k_i values of LQM 919 and LQM 996 were lower ($p < 0.05$) than the k_i of propoxur, which means that LQM 919 and LQM 996 have lower inhibitory powers than does propoxur. The k_d is directly proportional to the carbamate concentration required to inhibit AChE, so an increase in the value of this constant reflects a reduced affinity of the chemical for AChE (Yi et al., 2006). In the present study, the k_d values of the ethyl-carbamates were approximately 10,000-fold greater than that of propoxur, which indicates that they have a lower affinity to the AChE of *E. foetida*. The k_2 reflects the rate of formation of the AChE-inhibitor complex. The study showed no differences ($p > 0.05$) between the values for ethyl-carbamate and propoxur, so the corresponding rates of inhibition of the complex are similar. Although higher concentrations of the carbamates LQM 919 and LQM 996 reduced the AChE activity by close to 100%, their values of k_i and k_d indicate that they are weak inhibitors and of lower affinity to the AChE of *E. foetida* than a potent inhibitor such as propoxur.

The ethyl-carbamates LQM 919 and LQM 996 have been reported as effective inhibitors of the development and reproduction of the *R. microplus* tick. Both are able to drastically inhibit oviposition and larval hatching in the adult immersion test (Prado-Ochoa et al., 2013; Pérez-González et al., 2014). Some of the parameters used to measure this activity are the percentage of egg hatching (% EH) and the 99% egg hatching inhibitory concentration ($EHIC_{99}$) derived from the activity of carbamates. In the present study, the average $EHIC_{99}$ value of the carbamates (0.628 mg/mL) for *R. microplus*, which was equivalent to 5.3 μg/cm^2, was taken as a reference for the selection of the concentration ranges of the carbamates for *E. foetida* exposure in the CFPT. The concentration that was able to inhibit 99% of the hatching of *R. microplus* larvae did not produce mortality in earthworms in the CPFT. However, it did produce some reversible alterations such as random contractions and inability to coil.

On the other hand, in the AST, the concentration equivalent to the $EHIC_{99}$ for *R. microplus* was 8.14 mg/kg. This concentration produced less than 29% earthworm mortality at 2 days of exposure, less than 45% at 7 days of exposure and 50% at 14 days of exposure. The concentrations at which 100% mortality of *E. foetida* was observed were 64- and 4-fold greater than the $EHIC_{99}$ in the CFPT and AST, respectively. The above results indicate that both carbamates are less toxic toward *E. foetida* than toward *R. microplus*.

Additionally, to estimate the real ethyl-carbamates toxicity risk, their chemical stability, rate of degradation, kinetics of persistence and other soil factors that could diminish their toxic activity should be considered. Moreover, earthworm avoidance behavior toward the toxic chemicals could reduce their exposure level in the environment. Finally, to complement the ecotoxicity studies, and a toxic effect evaluation of these ethyl-carbamates is required using other organisms,

Table 2
Lethal concentrations produced by ethyl-carbamates LQM 919, LQM 996 and propoxur in *Eisenia foetida* in two different tests.

Carbamate	Filter paper test (μg/cm^2)				Artificial soil test (mg/kg)			
	LC_{50}	95% limits	LC_{99}	95% limits	LC_{50}	95% limits	LC_{99}	95% limits
LQM 919	88.06	72.94–106.16	313.06	224.36–563.68	5.8	4.3–7.9	118.6	55.1–510.1
LQM 996	67.98	55.38–83.58	316.4	217.77–234.37	2.2	1.3–3.1	115.3	48.3–657.5
PROPOXUR	66.94	53.60–84.22	302.56	201.54–620.64	4	3.2–4.7	41.4	28.8–71.0

Table 3
Kinetics constants for the inhibition (media ± DS) of *Eisenia foetida* AChE by ethyl-carbamates LQM 919, LQM 996 and propoxur (k_2 = carbamylation constant, k_d = dissociation, and k_i = bimolecular reaction constant, IC50% = Inhibitory concentration 50%, CI = Confidence intervals).

Carbamate	IC_{50} (μM)	CI (95%)	k_i (M^{-1})	k_d (M)	k_2 ($\times 10^{-3}$ min^{-1})
LQM 919	3046	1442–6433	0.70 ± 0.23 (x10^4)^a	1.2 ± 0.5 ($\times 10^{-5}$)^a	3.6 ± 0.8 ($\times 10^{-2}$)^a
LQM 996	5546	2584–11,900	0.59 ± 0.09 (x10^4)^a	0.51 ± 0.1 ($\times 10^{-5}$)^a	2.4 ± 0.3 ($\times 10^{-2}$)^a
PROPOXUR	< 0.04*	–	137 ± 65.9 (x10^4)^b	9 ± 5 ($\times 10^{-8}$)^b	2.3 ± 0.2 ($\times 10^{-2}$)^a

* Lowest concentration tested. Different letters indicate significant differences between the means ($p < 0.05$).

such as honey bees, dung beetles, water fleas, fresh water worms and fishes, to determine the real limitations of their use.

5. Conclusions

The ethyl-carbamates evaluated in *E. foetida* produced concentration-dependent mortality, morphologic alterations, behavioral changes and weak AChE inhibition. These effects were produced at higher concentrations than the effective concentration reported for ticks (target organism). In general, the toxic effects of LQM 919 and LQM 996 in *E. foetida* were minor compared to those produced by propoxur and other reported carbamates.

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CAPÍTULO 8

Acute oral and contact toxicity of new ethyl-carbamates on the mortality and acetylcholinesterase activity of honey bee (*Apis mellifera*)

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ABSTRACT

The effects produced by the ethyl-carbamates: ethyl-4-bromophenyl carbamate (LQM 919) and ethyl-4-chlorophenyl carbamate (LQM 996) on the mortality and behavior of *Apis mellifera* were evaluated by the acute oral toxicity test and the acute contact toxicity test. The oral lethal dose, 50% of the ethyl-carbamates was >145.24 μg per bee, and the oral lethal dose, 50% of propoxur was 0.072 μg per bee. Therefore, according to the OECD criteria, the ethyl-carbamates were classified as relatively nontoxic orally; meanwhile, propoxur was classified as highly toxic orally. In the contact test, lethal concentrations, 50% of the ethyl-carbamates were 4.83 and 2.23 μg/cm^2 for LQM 919 and LQM 996, respectively; therefore, they were at least 10-fold less lethal ($p < 0.05$) than propoxur (0.22 μg/cm^2). The ethyl-carbamates reduced the activity of *A. mellifera* acetylcholinesterase by up to 30%. The k_i and k_d values of both ethyl-carbamates were lower ($p < 0.05$) than those of propoxur and indicated that they are weak inhibitors and with low affinity to

A. mellifera acetylcholinesterase, which along with the absence of behavioral alterations suggests that the mortality caused by ethyl carbamates is not related to damage to the nervous system. According to these results, the evaluated ethyl-carbamates can be considered a low ecotoxic risk for *A. mellifera*.

Key Words: ethyl-carbamates, ecotoxicity, acetylcholinesterase , *Apis mellifera*

1. INTRODUCTION

Bees play an essential role in the ecological balance of ecosystems; they are important pollinators of crops and promote genetic variation of plants, and also, they are the main producers of honey, propolis, and wax (Mizrahi and Lensky, 2013). In recent decades, there has been a significant decrease in the number of bee colonies in Europe, the United States, and other regions of the world (vanEngelsdorp et al., 2009, vanEngelsdorp and Meixner, 2010; Potts et al., 2016). Several factors such as modification of ecological niches, infectious diseases, and the indiscriminate use of pesticides have contributed to the decrease of bee colonies (vanEngelsdorp et al., 2010). Pesticides used in agriculture for the control of arthropods, such as DDT, chlorpyrifos, dichlorvos, malathion, coumaphos, tau-fluvalinate, flumethrin, imidacloprid, and thiamethoxam, have lethal and sublethal effects on bees (Johnson et al., 2010; Henry et al. al., 2012). In some countries, the phenomenon called Colony Collapse Disorder has been detected, which is characterized by a rapid loss of adult bees, but not the queen or brood, from a hive (Faucon et al., 2002, Biesmeijer et al., 2006). One of the causes associated with this disorder is the exposure of bees to pesticides used in agriculture (Desneux et al., 2007, Henry et al., 2012). Owing to their high susceptibility and constant exposure to contaminants during the search for food, bees have been used as biomarkers in ecotoxicity studies to measure the environmental impact of pesticides (Bargańska et al., 2015).

The ethyl-4-bromophenyl carbamate (LQM 919) and the ethyl-4-chlorophenyl carbamate (LQM 996) are new ethyl-carbamates developed at the National Autonomous University of Mexico, which have shown high efficacy toward strains of cattle ticks *Rhipicephalus microplus* multiresistant to commercial ixodicides (Prado-Ochoa et al., 2013; Pérez-González et al., 2014). Several studies including acute and subchronic toxicity, genotoxicity, and cytotoxicity have

shown that these ethyl carbamates are of low toxicity in mammals (Prado-Ochoa et al., 2014a, 2014b, 2016) and of low mutagenicity in bacteria (Strassburger-Madrigal et al. al., 2017). Recently, it was reported that these ethyl carbamates produced concentration-dependent mortality, morphological alterations, behavioral changes, and weak acetylcholinesterase inhibition in the nontarget organism *Eisenia foetida* (Iturbe-Requena et al., 2019); therefore, it is probably that these molecules also have negative effects on other nontarget organisms that may potentially be exposed. In this study, the acute toxic effects of the new ethyl-carbamates on the mortality and acetylcholinesterase activity of *Apis mellifera* were evaluated.

2. MATERIALS AND METHODS

2.1. Bees (*Apis mellifera*)

Foraging adult workers were collected from the top of a hive maintained in the municipality of Cuautitlán Izcalli, State of Mexico (subhumid temperate climate, 2250 m above sea level). The collection was made during the spring-summer season.

2.2 Carbamates

The design and synthesis of ethyl-4-bromophenyl carbamate (LQM 919) and ethyl-4-chlorophenyl carbamate (LQM 996) were made according to Angeles et al. (2000). The identification codes, nomenclature, and toxicity in different organisms of the ethyl-carbamates are shown in Table 1. Propoxur (2,2-(1-methylethoxyl) phenyl methylcarbamate, 99% pure, Sigma-Aldrich 45644) was used as a positive control.

2.3 Acute oral toxicity test (AOTT)

The assay was performed according to the guide 213 of the OECD (1998). The collected bees were kept in plastic containers in groups of ≈20 bees; they were dieted for 2 hours prior to the experiments. Subsequently, to each group of bees, 400 µL of a treatment diet with different concentrations of LQM 919 or LQM 996 (0, 0.63, 1.25, 2.5, 5, 10, and 20 µg/µL), or different concentrations of propoxur (0.03, 0.06, 0.12, 0.25, 0.5, and 1 µg/µL) solubilized in 4% dimethyl sulfoxide (DMSO) and 50% sucrose, was offered during 4 hours. After carbamates exposure, bees were kept in darkness, at 25 ± 2ºC, at relative humidity of 65-75%, and with a maintenance

diet (50% sucrose) *ad libitum* for the rest of the experiment. To calculate the ingested dose per bee, the amount of rejected diet at 4 hours was subtracted from the offered treatment diet, and then, the amount was divided by the number of bees in each group to obtain ingested microliters per bee, and finally, the microliters were multiplied by the offered solution concentration. Mortality percentage and behavioral alterations (circle turns, inability to tip over, inability to fly, and inability to feed) were recorded at 4, 24, and 48 hours postexposure. All the tests were performed in triplicate.

2.4 Acute contact toxicity test (ACTT).

The test was carried out modifying the guide 214 of the OECD (1998). The entire internal surface of polypropylene containers was homogenously sprayed with a solution of different concentrations of LQM 919 or LQM 996 (0, 0.9, 1.8, 3.6, 7.2, and 14.4 μg/cm^2), or different concentrations of propoxur (0, 0.9, 1.8, 3.6, and 7.2 μg/cm^2) diluted in absolute ethanol, and dried at room temperature for 24 hours prior to the test to ensure total evaporation of the solvent. Groups of ≈20 newly collected bees were placed in each container and kept in this way with continuous exposure to carbamates, in darkness, at 25 ± 2ºC, at relative humidity of 65-75%, and with a maintenance diet (sucrose at 50%) *ad libitum* for 48 hours. Mortality percentage and behavioral alterations (circle turns, inability to tip over, inability to fly, and inability to feed) were recorded at 4, 24, and 48 hours of exposure. All the tests were performed in triplicate.

2.5 *In vitro* acetylcholinesterase (AChE) inhibition

Newly collected bees were euthanized by freezing at -20°C, and the heads were separated. All procedures were performed at 0-4 ºC. Four heads were homogenized in PBS (pH 7.5) with a micropestle. The homogenate was centrifuged at 12,000 *g*, and the supernatant was again centrifuged under the same conditions. The protein concentration of supernatants was determined by the technique of Bradford (1976) and adjusted to 1 μg/μL. The activity of AChE in extracts of *A. mellifera* was measured by the method of Ellman et al. (1961) and modified by Prado-Ochoa et al. (2014c). The kinetic constants of Michaelis-Menten (K_m) and the maximum velocity (V_{max}) were obtained. The *in vitro* AChE inhibition kinetics produced in *A. mellifera* because of carbamates were determined according to the method described by Iturbe-Requena et al. (2019). Different concentrations of LQM 919, LQM 996 (0, 0.19, 0.39, 0.78, 1.56, 3.12, and 6.25 mM),

or propoxur (0.04, 0.2, 1, 5, 25, and 125 μM) were used. The dissociation constant (k_d), bimolecular reaction constant (k_i), carbamylation constant (k_2), and 50% inhibitory concentrations (IC_{50}) were obtained (Chen et al., 2001, Pruett, 2002).

2.6 Statistical analysis

The effects of the carbamate concentrations and exposure time on the mortality of *A. mellifera* in the ACTT and AOTT were analyzed by linear regression. The averages of the kinetic constants were analyzed by one-way ANOVA, and the difference between means was established by the Tukey test using Statistica for Windows 7.0. The LC_{50} of each carbamate was calculated by Probit analysis, conducted with the Polo-Plus program (LeOra Software®). The minimum confidence used in all tests was 95%.

3. RESULTS

3.1. Acute oral toxicity

Figure 1 shows the mortality of bees in oral exposure to LQM 919, LQM 996, and propoxur during different postexposure periods (4, 24, and 48 hours). None of the negative control groups had mortality ≥10%. No differences were observed ($p > 0.05$) between the treated groups and the negative control (0 mg/mL) at any of the LQM 919 concentrations evaluated or at the different postexposure times (Figure 1A). In bees orally exposed to LQM 996, there was an increase in concentration-dependent mortality ($p < 0.05$) at 4, 24, and 48 hours postexposure ($r^2 = 0.24$, $r^2 = 0.27$, and $r^2 = 0.29$, respectively); the highest mortality achieved was 27%, with the highest concentration (20 μg/μL) at 48 hours (Figure 1B). In bees orally exposed to propoxur, there was an increase in concentration-dependent mortality ($p < 0.02$) at 4, 24, and 48 hours postexposure ($r^2 = 0.24$, $r^2 = 0.27$, and $r^2 = 0.28$, respectively). The highest mortality observed was 100% (1 μg/μL) at 24 and 48 hours postexposure (Figure 1C). The LD_{50} of propoxur orally was 0.072 μg per bee (C.I. 0.012-0.164). The mortality data of LQM 919 and LQM 996 did not adjust for the LD_{50} calculated in the Probit model; however, on the basis of the highest concentration evaluated, LD_{50} was >145.24 μg per bee. No apparent behavioral alterations were observed in bees exposed to any carbamates or concentrations used.

3.2 Acute contact toxicity

Figure 2 shows the mortality of bees produced by exposure to the carbamates evaluated in the ACTT during different periods of exposure (4, 24, and 48 hours). The bees exposed by contact to LQM 919, LQM 996, and propoxur showed an increase in concentration- dependent mortality ($r^2 \geq 0.73$, $r^2 \geq 0.69$, and $r^2 \geq 0.35$, respectively; $p < 0.001$) and time-dependent mortality ($r^2 \geq 0.38$, $r^2 = 0.36$, and $r^2 \geq 0.77$, respectively; $p < 0.005$). The LC_{50} of LQM 919, LQM 996, and propoxur were 4.83 (C.I. 3.98-5.97), 2.23 (C.I. 2.01-2.46), and 0.23 (C.I. 0.03-0.44) μg/cm^2, respectively. No apparent behavioral alterations were observed in bees exposed to any carbamates or concentrations used.

3.3 AChE inhibition

The basal parameters of the AChE of *A. mellifera* were $K_m = 4.9 \times 10^{-5} \pm 1.9 \times 10^{-5}$ M and $V_{max} = 0.027 \times 10^{-4} \pm 4 \times 10^{-4}$ M/min/L. The kinetic constants (k_i, k_d, and k_2) and the IC_{50} obtained are presented in Table 2. The k_i values of LQM 919 and LQM 996 toward *A. mellifera* AChE were lower ($p < 0.05$) than those of propoxur. The k_d was higher ($p < 0.05$) for the carbamates LQM 919 and LQM 996 than for propoxur. The k_2 values of LQM 919, LQM 996, and propoxur were not different ($p > 0.05$). Only the data obtained from propoxur adjusted to the Probit model; the IC_{50} was 16.25×10^{-4} M (C.I. 6.7×10^{-4}-39×10^{-4}).

The effects of LQM 919, LQM 996, and propoxur on the reduction of AChE activity in *A. mellifera* are shown in Figure 3. The results show that carbamates evaluated reduce AChE activity in *A. mellifera* ($p < 0.05$) with regard to negative controls. The LQM 919 and LQM 996 reduced AChE activity by <30%, while propoxur reduced AChE activity by 99%.

4. DISCUSSION

One of the most serious problems with the use of pesticides is their environmental impact; nowadays, the current international regulations and social tendencies demand that products which are used for the control of plagues be kind to the environment (Correa, 2011). The development of new products requires evaluating the effect on both target and nontarget organisms and their possible environmental impact. The most internationally accepted tests to evaluate the environmental impact of a pesticide are the AOTT and ACTT in bees, which are regulated by guidelines 213 and 214 of the OECD. The results of this study showed that

according to the OECD criteria, the new ethyl carbamates are classified as relatively nontoxic orally for *A. mellifera* and showed to be at least 10-fold less lethal in contact exposure than the propoxur.

The proposed administration route for these ethyl-carbamates for tick control is by spraying the product on the cattle skin, which brings on the dispersion of the ethyl-carbamates in the environment and the contamination of the flowers where the bees are feeding. The AOTT is designed as a first step to assess the acute risks of pesticides on worker bees (OECD 1998a). According to the OECD (2003), pesticides with an LD_{50} oral higher than 10.9 μg per bee are classified as relatively nontoxic; therefore, these ethyl-carbamates fall into this classification. In comparison, the LD_{50} of propoxur was 0.072 μg per bee, which places it according to the OECD as highly toxic. Other chemicals such as chlorpyrifos (0.25 μg), fipronil (0.004 μg), imidacloprid (0.003 μg), and malathion (0.38 μg) have low oral LD_{50} and therefore are of very high toxicity to bees, despite which they are used commercially as ixodicides (Stoner and Eitzer, 2013).

One of the ways in which bees come into contact with pesticides is when they land on contaminated surfaces. Several studies have shown the danger of some pesticides for bees in indirect contact tests (Marletto et al., 2003, Bailey et al., 2005, Laurino et al., 2011). On the basis of tests that better simulate the conditions of exposure to pesticides in the field, we modified the conventional technique of the guide 214 of the OECD (1998b) to assess the exposure of bees when they land on a surface contaminated with the pesticide. In this study, the LC_{50} observed were 4.83, 2.23, and 0.23 μg/cm^2 for LQM 919, LQM 996, and propoxur, respectively. The results obtained by this form of exposure are difficult to compare with the topical contact toxicity test; however, LC_{50} obtained in this study show that the ethyl-carbamates evaluated are between 10- and 20-fold less toxic than propoxur. In addition, both the concentration-dependent and the time-dependent effects observed with the propoxur showed the efficacy and sensitivity of the test.

Many of the carbamates such as propoxur, carbofuran, and carbaryl, among others, are strong inhibitors of AChE, and their inhibition grade is directly related to their toxicity (Johnson, 2015). AChE is responsible for the rapid hydrolysis of acetylcholine in cholinergic synapses and allows precise control of nerve transmission modulation. The k_i is considered a reliable parameter with which to evaluate the inhibitory power of some substances over AChE (Main, 1964). In the

present study, we observed that the ethyl-carbamates, although they reduced the *A. mellifera* AChE activity by as much as 30%, had lower k_i values ($p < 0.001$) than those of propoxur, a potent AChE inhibitor (approximately 1500-fold less). The aforementioned shows that the ethyl-carbamates LQM 919 and LQM 996 are weak inhibitors of AChE.

The affinity of a compound to AChE can be measured by the rate at which the compound inhibits its activity, the rate of inhibitor-AChE complex formation, and enzyme carbamylation (Pruett and Pound, 2006). The k_d values are directly proportional to the ethyl carbamate concentration required to inhibit AChE; therefore, an increase in k_d values indicates a reduced affinity of carbamate to AChE (Yi et al., 2006). The k_2 indicates the rate of inhibitor-AChE complex formation; consequently, changes in the k_2 denote decreases or increases in the carbamylation velocity. In this study, although the k_i values for the ethyl-carbamates were similar to those for propoxur, the k_2 values calculated for ethyl-carbamates were greater ($p < 0.01$) than those for the propoxur. These results indicate poor affinity of the ethyl-carbamates evaluated to *A. mellifera* AChE and therefore are weak inhibitors. Previous studies have shown that these ethyl-carbamates are also weak and low-affinity inhibitors to *R. microplus* AChE (Prado-Ochoa et al., 2014c). On the other hand, it has been shown that these compounds are also of low affinity to *E. foetida* AChE; however, they can reduce their activity by up to 100% (Iturbe-Requena et al., 2019).

The concentration of both ethyl carbamates that reduces oviposition and inhibits 99% of the larvae hatching of *R. microplus* is about 0.66 mg/mL (Pérez-González et al., 2014). In studies carried out by our group (data not shown), we established spray baths with a solution of LQM 919 or LQM 996 at 1 mg/mL for the control of *R. microplus* in bovine. This proposed concentration is 8- and 20-fold lower than the concentrations that produced the maximum mortality of bees in the ACTT and AOTT, respectively. The aforementioned suggests that there is a wide safety margin for bees when using the proposed dose for tick control.

Many pesticides currently being used have effects on the nervous system of bees or on their larval development (Pham-Delegue et al., 2002; Zhu et al., 2014). In this study, behavioral alterations in the bees were not observed in the two acute toxicity tests used. This observation, together with the low affinity of both carbamates to *A. mellifera* AChE, suggests that the

mechanism that induces the death of bees is independent of the involvement of their nervous system. However, the effects of ethyl-carbamates in the medium and long term are unknown. Testing sublethal doses to assess the effects of ethyl-carbamates on learning, memory, and larval development of *A. mellifera* is necessary.

5. CONCLUSIONS

According to the OECD criteria, the ethyl-carbamates evaluated are relatively nontoxic orally for *A. mellifera*, while propoxur is classified as highly toxic. In the contact test, the ethyl-carbamates were at least 10-fold less lethal than propoxur. The ethyl-carbamates evaluated are of low affinity to and weak inhibitors of *A. mellifera* AChE, which together with the absence of behavioral alterations suggests that bees' death is not related to the involvement of the nervous system.

FIGURES AND TABLES

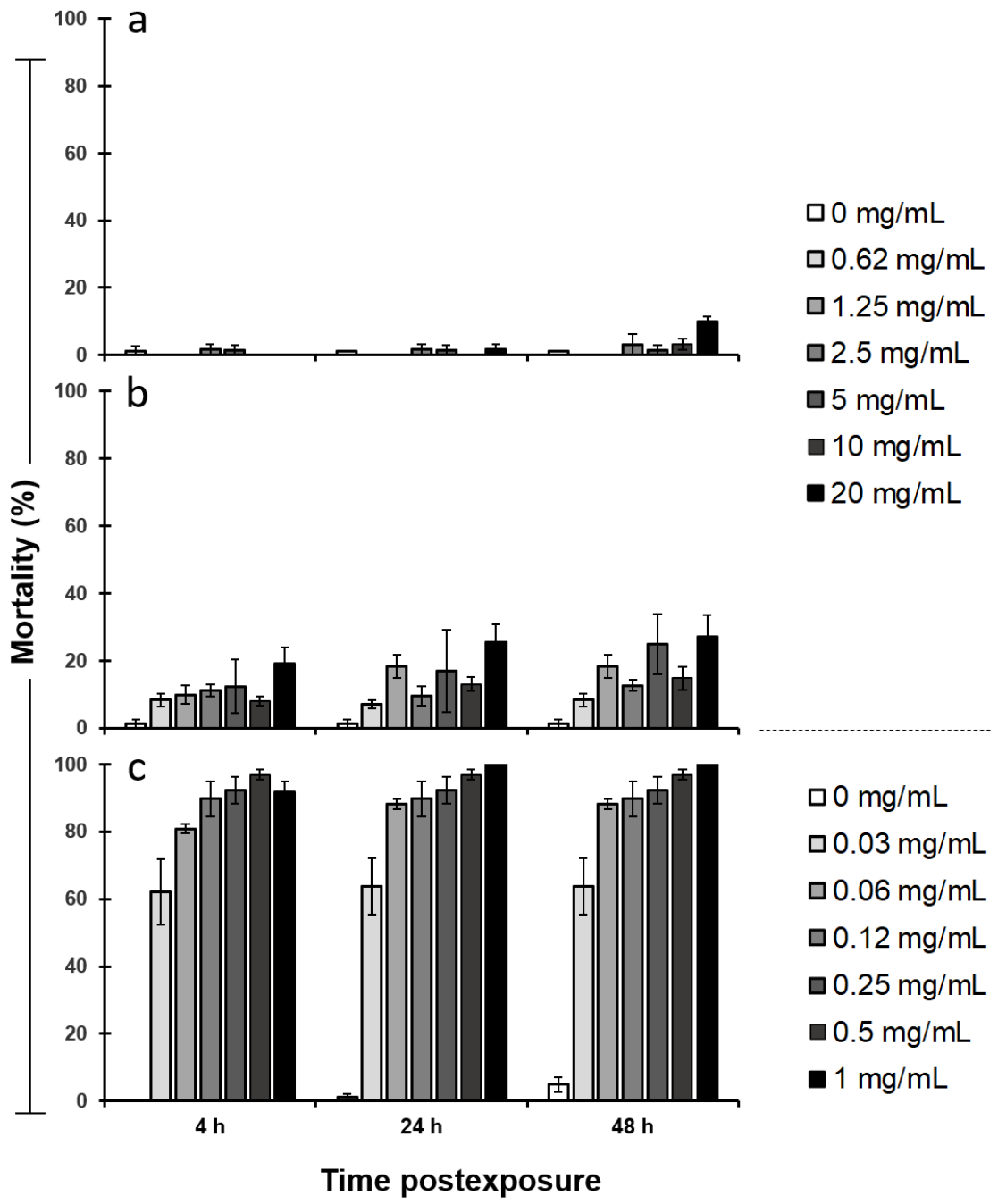

Figure 1. Mean (±SE) of mortality percentages of Apis mellifera bees exposed to different concentrations of a) LQM 919, b) LQM 996 and c) propoxur in the acute oral test at different days of exposure.

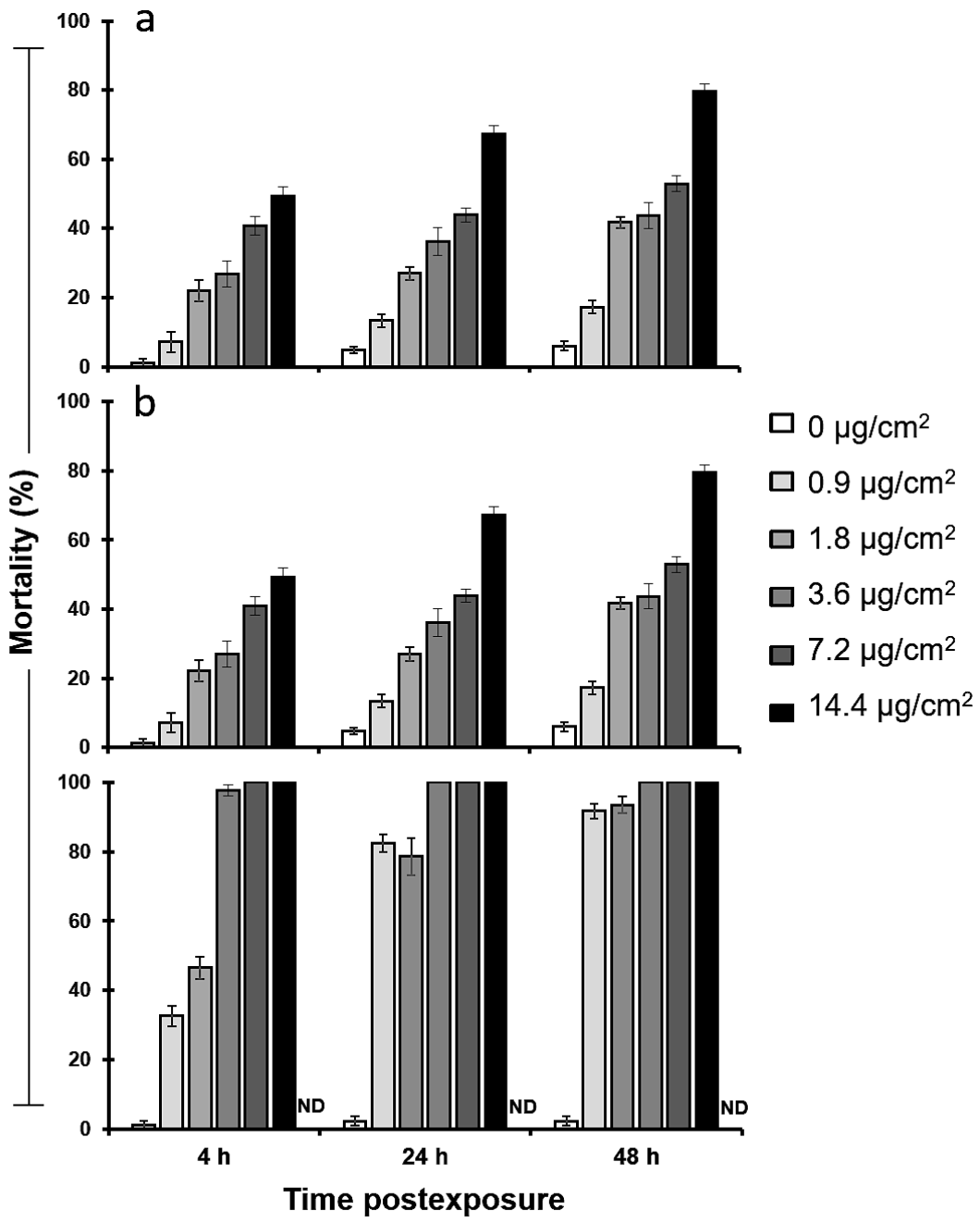

Figure 2. Mean (±SE) of mortality percentages of Apis mellifera bees exposed to different concentrations of a) LQM 919, b) LQM 996 and c) propoxur in the acute contact test at different days of exposure.

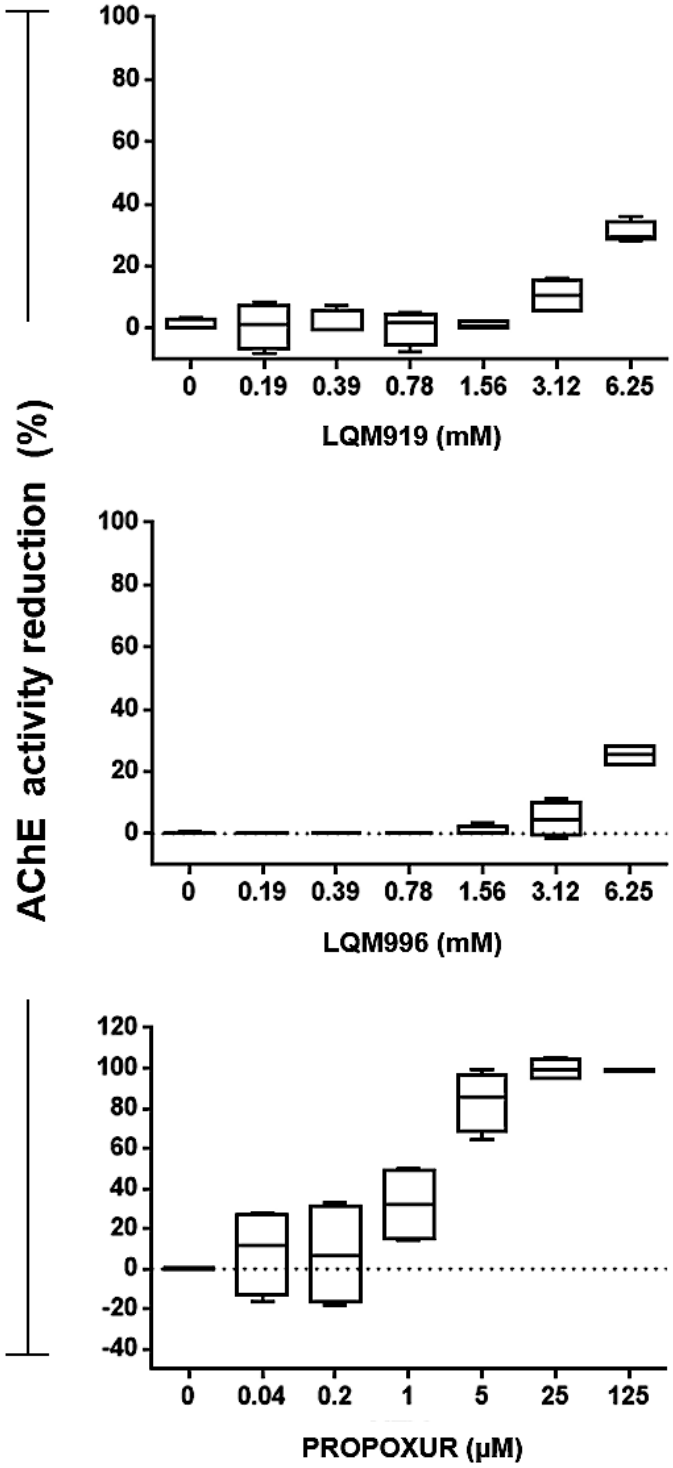

Figure 3. Effects of the ethyl carbamates and propoxur on AChE activity reduction (as a percentage of the control group activity) in Apis mellifera extracts.

Table 1.- Name, identification code, efficacy and toxicity of the evaluated ethyl-carbamates in different organisms.

Carbamate	Identification code	Ticks[1]	Rats[2]			Earthworms[3]	
		Efficacy vs. *Rhipicephalus microplus* ($EHIC_{99}$)	Acute oral toxicity (LD_{50})	Acute dermal toxicity (LD_{50})	Subchronic oral toxicity (NOAEL)	Filter paper test (LC_{50})	Artificial soil test (LC_{50})
Ethyl-4-bromophenyl carbamate	LQM 919	0.668 mg/mL (C.I. 0.485–1.033)	300 - 2000 mg/kg low hazard	>5000 mg/kg low acute toxicity	12.5 mg/kg	88.06 mg/cm^2 (C.I. 72.94–106.16)	5.8 mg/kg (C.I. 4.3–7.9)
Ethyl-4-chlorophenyl carbamate	LQM 996	0.589 mg/mL (C.I. 0.490–0.739)	300 - 2000 mg/kg low hazard	>5000 mg/kg low acute toxicity	12.5 mg/kg	67.98 mg/cm^2 (C.I. 55.38–83.58)	2.2 mg/kg (C.I. 1.3–3.1)

$EHIC_{99}$ = Egg hatching inhibitory concentration 99%; LD_{50} = Letal dose 50%; NOAEL = nonobserved adverse effect level; LC_{50} = Letal concentration 50%.

[1] Prado-Ochoa et al., (2013); Pérez-González et al., (2014)

[2] Prado-Ochoa et al., (2014a,b)

[3] Iturbe-Requena et al., (2019)

Table 2.- Inhibitory concentrations and kinetics constants (mean ± SE) for the *in vitro* inhibition of *Apis mellifera* AChE by different carbamates (k_2 = carbamylation constant, k_d = dissociation, and k_i = bimolecular reaction constant).

Carbamate	IC50% (μM)	C.I. (95%)	k_i (M^{-1})	k_d (M)	k_2 ($X10^{-3}$ min^{-1})
LQM919	---	---	1.2 ±0.17 ($x10^2$)	2.4 ± 2.6 ($x10^{-4}$)^a	2.8 ± 2.6 ($x10^{-2}$)^a
LQM996	---	---	1.7 ±0.97 ($x10^2$)	1.63 ± 1.15 ($x10^{-4}$)^a	2.1 ± 1.1 ($x10^{-2}$)^a
Propoxur	1.6 $x10^{-3}$	0.6-3.9 ($x10^{-3}$)	2.5 ±4.5 ($x10^5$)	1.34 ± 1.64 ($x10^{-6}$)^b	5.0 ± 4.6 ($x10^{-2}$)^a

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CAPÍTULO 9
Discusión

Las garrapatas *R. microplus* son uno de los principales problemas de la ganadería bovina que causan grandes pérdidas económicas en las zonas tropicales y subtropicales de México y el mundo. Aunque la aplicación de productos químicos ha sido la forma de control más utilizada, sin embargo, debido a su uso indiscriminado han aparecido cepas de *R. microplus* que han desarrollado diferentes grados de resistencia a todas las clases de ixodicidas disponibles en el mercado (Rodríguez Vivas et al., 2012). El desarrollo de nuevas moléculas con efectivas contra garrapatas que no presente resistencia cruzada con otros ixodicidas es una opción para el control de las garrapatas en la actualidad. Sin embargo, antes de que un ixodicida pueda ser comercializado debe ser evaluado en diferentes aspectos. En la FES- Cuautitlán se diseñaron y sintetizaron una serie de nuevos carbamatos, de los cuales se seleccionaron dos etil-carbamatos con eficacia ixodicida (Ángeles et al., 2000, Prado-Ochoa et al., 2013). Algunas de las pruebas que se han realizado mostraron que estos dos compuestos son de baja toxicidad en mamíferos, poseen una alta eficacia *in vitro e in vivo* sobre cepas susceptibles y resistentes de *R. microplus* (Prado-Ochoa et al., 2013; Pérez-González et al., 2013; Iturbe-Requena, 2014) y que su mecanismo de acción en garrapatas es independiente a la inhibición de la enzima AChE (Prado-Ochoa et al, 2014b; Escobar-Chavarría, 2014). Con estos estudios se demostró que los etil-carbamatos LQM 919 y LQM 996 son una novedosa opción en el control de garrapatas, sin embargo, es indispensable antes de su comercialización estudios más profundo de su mecanismo de acción y sobre todo evaluar su impacto ambiental. Por lo anterior, en este trabajo doctoral se realizaron experimentos para profundizar en el conocimiento del mecanismo de acción de los etil-carbamatos LQM 919 y LQM 996 y en sus efectos ecotóxicos en organismos no objetivo, los resultados se muestran en los tres artículos presentados anteriormente.

En trabajos previos realizados por Prado-Ochoa et al. (2014b) y Escobar-Chavarría (2017), se mostró que los etil-carbamatos LQM 919 y LQM 996, impactan negativamente en la viabilidad y maduración de los ovocitos de las garrapatas *R. microplus*. Estos compuestos generan apoptosis en las células del pedicelo y en los ovocitos en desarrollo (Escobar-Chavarría, 2017). Por otro lado, los huevos ovipositados por las hembras tratadas con estos compuestos

presentaron huevos de menor tamaño, opacos y de apariencia más oscura, la masa de huevos se observó poco hidratada por lo que se disgregaba con facilidad; además no se presentaba eclosión de larvas de estos huevos (Prado-Ochoa et al, 2013). Sin embargo, se desconocía si estas alteraciones impedían el desarrollo del embrión de la garrapata, por lo que en el primer artículo presentado se estudió la embriogénesis de las garrapatas cuando las hembras repletas son tratadas con los etil-carbamatos (capítulo 6). Los resultados obtenidos en este estudio permitieron corroborar y profundizar en la observación de las alteraciones a los ovocitos a nivel histológico y ultraestructural, demostrando que los etil-carbamatos afectan el número y desarrollo de los ovocitos, y que generan daños en estructuras como la vesícula germinal, los gránulos de yema y el corion, además de la degeneración de organelos como las mitocondrias y cuerpos mielínicos. Por otro lado, se observó en los estudios de embriogénesis utilizando la tinción de fluorescencia de DAPI que en los huevos de las hembras repletas expuestas a la CIE_{99} de los etil-carbamatos no existe celularización desde las etapas tempranas de la embriogénesis, lo que repercute en el desarrollo del embrión. Lo anterior demuestra que el mecanismo de acción de estos compuestos está relacionado directamente con la ovogénesis en las hembras repletas, las alteraciones del corion observadas en los ovocitos probablemente modifican el paso de elementos hacia el interior y evita que haya suficientes nutrientes dentro del ovocito para que se desarrolle el embrión de la larva de *R. microplus*, lo que provoca la ausencia de eclosión de los huevos producidos por hembras que han sido tratadas con los etil-carbamatos. Este mecanismo de acción es distinto al de los carbamatos conocidos, por lo que un producto cuyo efecto es directo sobre la reproducción de la garrapata sería una alternativa novedosa y a la que difícilmente pueda existir algún tipo de resistencia cruzada con otros ixodicidas.

Debido a la alta eficacia de los etil-carbamatos sobre garrapatas y a su baja toxicidad en mamíferos, se ha propuesto se puedan utilizar para el control de las garrapatas en un mediano plazo. Sin embargo, se debe considerar que la forma propuesta de aplicación de soluciones de los etil-carbamatos mediante baños de aspersión en bovinos, lo que provocaría escurrimiento de estas soluciones al ambiente y la exposición a estos compuestos a organismos no objetivos. En el segundo y tercer artículo (Capitulos 7 y 8) se evaluó el efecto que los etil-carbamatos sobre organismos no objetivo que habitan en ecosistemas terrestres.

Las lombrices de tierra juegan un papel crucial en la descomposición de la materia orgánica y su integración en el suelo (Edwards, 1994 capítulo). Es mediante el escurrimiento de los pesticidas y su filtración en el suelo que las lombrices de tierra quedan expuestas a ellos, por lo que las lombrices *E. foetida* han servido como modelo para monitorear los efectos de los pesticidas en el suelo (Walker et al., 2012). En el segundo artículo utilizando las pruebas de papel filtro (CFPT, por sus siglas en inglés) y sustrato atificial (AST por sus siglas en inglés) recomendadas por la OECD (Guia 207, 1984) se estudió los efectos tóxicos agudos que producen los etil-carbamatos a las lombrices (Capítulo 7). Los resultados obtenidos mostraron que los etil-carbamatos aplicados a las concentraciones recomendadas para su uso en bovinos (0.6 mg/mL) no produjo mortalidad de lombrices en la CFPT y un máximo de 50% de mortalidad de lombrices después de 14 días de exposición en la AST. Sin embargo, son capaces de producir mortalidad hasta en un 100% en la CFPT cuando se usa 64 veces la concentración recomendada en bovinos y en la AST cuando se usa 4 veces la concentración recomendada. Por otro lado, la evaluación del efecto de los etil-carbamatos sobre la actividad de la AChE demostró que son débiles inhibidores y de baja afinidad a esta enzima en *E. foetida*. Si bien, de acuerdo con la clasificación de Roberts y Dorough (1984), estos etil carbamatos se consideran muy tóxicos para las lombrices de tierra, su toxicidad es menor a la de algunos carbamatos como el carbaril, carbosulfan y propoxur que actualmente se comercializan en México y el mundo, los cuales son considerados extremadamente tóxicos.

Las abejas son organismos polinizadores de gran importancia, ya que además de elaborar productos de alto valor pecuario (miel propoleo, cera, entre otros), facilitan la variación genética de las plantas, por lo que la desaparición de las colmenas alrededor del mundo ha cobrado importancia en las últimas décadas (Bradbear, 2009). Una de las causas que se ha asociado al colapso de las colmenas es el uso extensivo de los pesticidas, ya que las abejas entran en contacto con estos cuando se posan sobre partes de las plantas contaminadas o cuando se alimentan de néctar que contiene los pesticidas (Johnson, 2014). Se ha observado que los pesticidas inducen en las abejas efectos letales y subletales como la pérdida de memoria, desorientación e incluso incapacidad de alimentarse (Desneux et al., 2006). La aplicación de ixodicidas por baños de aspersión provoca la volatilización de los compuestos dispersión en el ambiente. En el tercer artículo se estudiaron los efectos tóxicos agudos que producen los etil-carbamatos sobre abejas *A. mellifera* (Capítulo 8). Los resultados obtenidos en abejas mostraron

que los etil-carbamatos aplicados por vía oral a las concentraciones recomendadas para su uso en bovinos (0.6 mg/mL) produjo una mortalidad de 10% y del 50% en la exposición por contacto. La máxima mortalidad observada en la exposición oral fue del 30% cuando se usa 33 veces la concentración recomendada en bovinos y del 80% en la exposición por contacto cuando se usa 2.8 veces la concentración recomendada en bovinos. Esto muestra la diferencia que hace la vía de exposición, mientras que en la prueba oral las abejas tienen la posibilidad de rechazar el alimento contaminado por palatabilidad, en la prueba por contacto quedan inherentemente expuestas al pesticida y a sus efectos. Adicionalmente los estudios sobre la AChE mostraron que los etil-carbamatos tienen una baja afinidad y que son débiles inhibidores de esta enzima en *A. mellifera*. Por la cantidad de producto que se requiere para producir mortalidad en abejas, de acuerdo con la clasificación de OECD (2003), estos etil-carbamatos se consideran relativamente no toxicos. En comparación, otros ixodicidas que se utilizan comercialmente como el clorpirifos, fipronil e imidacloprid son considerados de muy alta toxicidad en abejas.

Perspectivas

Los presentes estudios contribuyen a mejorar el entendimiento del mecanismo de acción a nivel reproductivo de los etil-carbamatos. En futuros estudios se debe abordar los mecanismos moleculares que permitan conocer los eventos por los cuales se produce la muerte celular de los ovocitos, así como los mecanismos por los cuales se inhibe el desarrollo de las fases parásitas de la garrapata *R. microplus*.

Además, se realizaron los primeros estudios del efecto de estos etil-carbamato sobre organismos no objetivo. Se observó que tienen un potencial ecotóxico bajo y un amplio margen de seguridad si se utilizan a las dosis efectivas contra garrapatas. Estudios que evalúen otros efectos subletales, estabilidad química y las vías de degradación y el destino final de los etil-carbamatos complementarían esta línea de investigación y garantizarían la seguridad para su uso comercial.

En conclusión, estos etil-carbamatos son una buena alternativa para el control de *R. microplus* a mediano plazo, sin embargo, aún es necesario realizar pruebas de campo y de formulación para poder ser utilizados en el sector agropecuario.

CAPÍTULO 10
Conclusiones

- Los etil-carbamatos LQM 919 y LQM 996 actúan sobre las células del ovario de las garrapatas inhibiendo la vitelogénesis y el desarrollo de los ovocitos, siendo este efecto el que evita el desarrollo embrionario de las larvas.

- Los etil-carbamatos LQM 919 y LQM 996 se clasifican como *altamente tóxicos para E. foetida* en las pruebas de papel filtro y de sustrato artificial, sin embargo, son menos tóxicos que el propoxur, sin embargo, producen el 100% mortalidad en la CFPT cuando se usa 64 veces la concentración recomendada en bovinos y en la AST cuando se usa 4 veces la concentración recomendada.

- A pesar de causar la mortalidad de lombrices *E. foetida,* los etil-carbamatos son inhibidores débiles y con poca afinidad a su AChE.

- Los etil-carbamatos LQM 919 y LQM 996 se clasifican como relativamente no toxicos para *A. mellifera* en las pruebas oral y por contacto. La máxima mortalidad observada en la exposición oral fue del 30% cuando se usa 33 veces la concentración recomendada en bovinos y del 80% en la exposición por contacto cuando se usa 2.8 veces la concentración recomendada en bovinos.

- Los etil-carbamatos LQM 919 y LQM 996 son inhibidores débiles y con poca afinidad a la AChE de *Apis mellifera*.

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