Pheromone and population genetics analyses of *Clavigralla* species in Africa

H. Kpongbe

orcid.org 0000-0003-1629-1060

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Promoter:	Prof J Van den Berg
Co-promoter:	Prof B Torto
Assistant Promoter:	Dr F Khamis
Assistant Promoter:	Dr M Tamò

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DEDICATION

This is dedicated to Lucienne Djidago for being a wonderful and great wife, to my parents Etienne Kpongbe and Eugenie Agbeto for all the sacrifices throughout my studies.

DECLARATION BY THE CANDIDATE

I, HILAIRE KPONGBE, declare that the work presented in this PhD thesis is my own work, that it has not been submitted for any degree or examination at any other University and that all the sources I have used or cited have been acknowledged by complete reference.

Signature.......................... Date 15th May 2019............

DECLARATION AND APPROVAL BY SUPERVISORS

We declare that the work presented in this thesis was carried out by the candidate under our supervision and we approve this submission

Prof Johnnie van den Berg

Unit for Environmental Sciences and Management, North West University, Private Bag, X6001, Potchefstroom, 2520, South Africa

Signature.......................... Date 16th May 2019

Prof. Baldwyn Torto

Behavioral and Chemical Ecology Unit, International Centre of Insect Physiology and Ecology. Box 30772-00100, Nairobi Kenya

Signature.......................... Date 15th May 2019

Dr Fathiya Khamis

Department of Plant Health, International Centre of Insect Physiology and Ecology, P.O. Box 30772-00100, Nairobi Kenya

Signature.......................... Date 15/05/2019

Dr Manuele Tamò

International Institute of Tropical Agriculture (IITA), Benin

08 BP 0932Tri Postal, Cotonou, Bénin.

Signature Date...May 15, 2019.........

ABSTRACT

Cowpea (*Vigna unguiculata* (L.) Walp) and common bean (*Phaseolus vulgaris* (L.)) are major sources of protein for human and animal consumption. Production of these crops is hampered by insect pests, especially the complex of brown spiny bugs of the genus *Clavigralla* (Hemiptera: Coreidae) which causes yield loss of up to 100% in various parts of Africa. The current practice of pesticide application to control these species is not efficient and has negative impacts on human health and the environment. These species are widely distributed in Africa and has a wide range of host plants, suggesting variability in genetics and chemical profiles of this pest. Aggregation behavior is observed in *Clavigralla* spp. from the nymph to adult stages, indicating the involvement of semiochemicals. Olfactometer assays showed that the egg parasitoid, *Gryon* species (Hymenoptera: Scelionidae) could potentially be biocontrol agent for *Clavigralla* spp. *Gryon fulviventris* Crawford (Hymenoptera: Scelionidae) was attracted to the volatiles released by *C. tomentosicollis* males, suggesting involvement of semiochemicals which have not been identified yet. Additionally, this attractive compound appears to be a male pheromone of which the bio-chemical composition, and its effect on the behaviour of *Gryon* sp. have not been elucidated.

The aim of this study was to investigate the diversity of the *Clavigralla* species complex on crops in Bénin and Kenya, to elucidate aspects regarding the pheromone responsible for aggregation behavior of *Clavigralla* spp., to do a population genetics analyses of the *Clavigralla* species group. To achieve these objectives, detailed knowledge on the levels of parasitism of *Clavigralla* spp., cuticular chemistry that may influence parasitoid – pest interactions, the chemical profiles, the identity and genetic variability, and semiochemical cues mediating aggregation behavior and attraction in *Clavigralla* species and *Gryon* sp. respectively are required.

Both live and dead ethanol preserved samples of the pests as well as their eggs were collected in West Africa (Bénin) and East Africa (Kenya). Colonies were established in an insectary and egg parasitoids were recorded. Additionally, parasitism and egg cuticular chemistry were investigated. A Y-tube olfactometer was used to investigate the effect of male and female headspace volatiles of *Clavigralla* spp. on their conspecifics. Headspace volatiles of both sexes of *C. tomentosicollis*, *C. shadabi* and *C. elongata* adults were collected and analyzed. Active-components to both pest and parasitoid antennae were identified by coupled GC/electroantennographic detection (GC/EAD) and GC/MS respectively. Olfactometer assays were performed to determine the effect of male-specific compound(s) on behavior of both the pest and egg parasitoid, *Gryon* sp. The genetic diversity of the three *Clavigralla* species collected in Kenya and Bénin and their identity were established using DNA barcoding and *Cytb* primers and different molecular tools (MEGA 7, NJ, K2P, BLAST).

The parasitism assays conducted with *Gryon* sp. showed a higher incidence of parasitism of *C. tomentosicollis* eggs than that of *C. elongata*. The GC/MS analysis of

cuticular extracts obtained from *C. tomentosicollis* and *C. elongata* parasitized and unparasitized eggs identified 15 compounds of which the amount varied between the two species. Furthermore, the Y-tube olfactometer bioassays conducted with group of males and females of *C. tomentosicollis* showed that volatiles released by groups of males were strongly attractive to both sexes. Antennae of both sexes of *C. tomentosicollis* detected identical components, including a male-specific component (isopentyl butanoate) which was also detected by antennae of the egg parasitoid. Likewise, in olfactometer bioassays with the synthetic of this male-specific compound, both the pest and the egg parasitoid were significantly attracted. GC/MS analyses of headspace volatiles of the three *Clavigralla* species identified 31 components. A heat map generated from the chemistry of *Clavigralla* spp. volatiles showed separation of the three species with a higher concentration of the components in *C. tomentosicollis* volatiles compared to the other two species. A close similarity between *C. tomentosicollis* and *C. elongata* was also observed. Genetic analyses showed very low variability within the different *Clavigralla* species and populations. Great variability was observed between *C. tomentosicollis* and the other two species.

These results suggest that the alkanes present in the egg cuticula as well as isopentyl butanoate could serve as semiochemicals for *Gryon* sp., facilitating host finding and parasitism and that isopentyl butanoate is the aggregation pheromone for both sexes of *C. tomentosicollis.* These compounds are, therefore, potential candidates for future use as tools in management of these pests. Results on the genetic characteristics and distribution ranges of *Clavigralla* spp. will contribute to development of management strategies of these pests in Africa.

Key words: Aggregation pheromone, brown spiny bug, egg parasitoid, electroantennogram, genetic variability, isopentyl butanoate, kairomone, parasitism, phylogeny, semiochemical cues.

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PREFACE

This thesis follows the article format style as prescribed by the North-West University. Therefore, articles appear in published format, while manuscripts and other chapters are adjusted according to the instructions to authors of internationally accredited, scientific journals. As an additional requirement by the North-West University, Table A details the contributions of authors for each article/manuscript and provides consent for use as part of this thesis.

The following Chapters were included in this work:

Chapter 1 – Introduction, literature review, and thesis structure: **(NWU Harvard, Reference Style of the Faculty of Law and APA,** published **by the Library Services of the NWU)**

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Submitted (Chapter 3: Article 2) was adjusted according to Springer's uniform instructions to authors of which an excerpt is provided in Appendix A. Unpublished (Chapter 4: Article 3) manuscript, was adjusted according to Oxford's uniform instructions to authors of which an excerpt is provided in Appendix B. Permission was obtained from John Wiley & Sons's to present Article 1 as part of this thesis. The licence and associated terms and conditions are available in Appendix C. Also, proof of

submission of Article 2 to the Journal of Chemical Ecology is provided in Appendix D. Finally, a declaration of language editing is provided in Appendix E.

Table A: Contributions of authors and consent for use.

Author	Article	Contribution	Consent
H Kpongbe	Articles 1-3	Principal investigator: Responsible for study design, field sampling, experiments conducting, data analysis and interpretation and manuscripts and thesis writing.	[signature]
J vd Berg	Articles 1-3	As promoter, supervised the design, and execution of the study. Also provided intellectual input on data analyses and writing of articles and thesis.	[signature]
B Torto	Articles 1-3	As co- promotor, supervised the design and execution of the study. Also provided intellectual input on data analyses and writing of articles and thesis.	[signature]
F Khamis	Articles 1-3	As assistant promoter: supervised the design and execution of the study. Also provided intellectual input on data analyses and writing of articles and thesis.	[signature]
M Tamò	Articles 1-3	As assistant promoter: supervised the design and execution of the study. Also provided intellectual input on data analyses and writing of articles and thesis.	[signature]
EJ Talamas	Article 1	Gave a support in morphological identification of *Gryon* sp. He provided intellectual input.	[signature]
J Villinger	Article 3	Provided intellectual input on the experimental procedure and writing of article.	[signature]

CHAPTER 1

Introduction, literature review and thesis structure

1.1 Introduction

Cowpea. *Vigna unguiculata* (L.) Walp. is one of the most important food and forage legumes in the semi-arid tropics. This crop is cultivated in parts of Asia, Africa, Southern Europe, the Southern United States and in Central and South America (Singh 2006, Timko *et al*. 2007). Cowpea is an important source of dietary protein in areas where consumption rate of animal protein is low (Voster *et al*. 2007, Phillips *et al.* 2003). This crop significantly contributes to food security in tropical Africa where it is the most important legume (Jackai and Adalla 1997, Tamò 1991). Common bean, *Phaseolus vulgaris* (L.), is another important pulse crop grown in several countries of East Africa, particularly Burundi, Ethiopia, Kenya, Malawi, Rwanda, Tanzania and Uganda (Batureine 2009). The *per capita* consumption of common bean in Rwanda, Kenya and Uganda is approximately 50 to 60 kg year^{-1} which is considerably higher than that of Colombia and Brazil where *per capita* consumption is 4 and 17 kg year-^{1}, respectively (Beebe *et al*. 2013, Broughton *et al*. 2003).

Despite their importance, cowpea and common bean production is constrained by Hemiptera bugs that limit their production and yield (Tamò *et al*. 1997, Robin *et al*. 2010). Most pests that attack these crops cause damage from flowering until pod maturity and include flower thrips, *Megalurothrips sjostedti* Trydom (Thysanoptera: Thripidae), the pod borer, *Maruca vitrata* Fabricius (Lepidoptera: Pyralidae), the cowpea

aphid, *Aphis craccivora* Koch (Hemiptera: Aphididae) and the of complex sucking bugs that damage pods and seeds (Dreyer and Baumgartner 1994; Soyelu *et al*. 2007). The latter pest complex is dominated by the brown spiny bugs, *Clavigralla* spp. (Hemiptera: Coreidae), especially *C. tomentosicollis* Stäl (Jackai and Daoust 1986, Singh *et al*. 1990, Jackai and Adalla 1997). Damage due to *Clavigralla* spp. can vary depending on the species, the crop and the region, and yield losses of up to 100% have been observed in various parts of Africa (Singh and Allen 1980; Koona *et al.* 2001; Soyelu and Akingbohungbe 2007; Dabire-Binso *et al.* 2010; Dialoke *et al.* 2010). *Clavigralla* spp. nymphs and adults insert their rostrums through the pod walls, releasing enzyme-rich saliva which entirely digests the contents of young pods and developing seed, leaving them shriveled and of poor quality. Many farmers use chemical control to protect cowpea and common bean from pest damage, but the use of pesticides is expensive and poses health risks to both humans and the environment (Jackai and Adalla, 1997). Furthermore, current control methods such as cultural control practices, pesticide applications and resistant crop varieties used in the management of *Clavigralla* spp. are largely unsuccessful (Jackai and Adalla 1997; Adipala *et al.* 2000; Koona *et al.* 2002; Aliyu *et al.* 2007; Dzemo *et al*. 2010).

The life cycle of *C. tomentosicollis* has five nymphal instars and takes approximately 21 days to complete in an insectary (Temperature: 25 ± 3 °C, and relative humidity 34-75 %) (Dzemo and Asiwe 2010). The number of eggs laid per female ranges between 2 and 99, and eggs hatch between 7 - 10 days after oviposition. Aggregation behavior in this insect species commence during the first instar and is strongest during the second instar, after which this behavior is reduced towards the

adult stage (Egwuatu and Taylor 1976). These observations suggest that semiochemicals may be involved in the aggregation behaviour of *C. tomentosicollis*.

Generally, for group communication, stink bugs produce different semiochemicals that function as aggregation, alarm, defensive and sex pheromones (Ndo *et al.* 2007; Millar et al. 2010; Kartika *et al.* 2015). Among these are male-produced pheromones, which attract both sexes, for example the aggregation pheromone of the brown marmorated stink bug, *Halyomorpha halys* Stål (Hemiptera: Pentatomidae) (Khrimian *et al.* 2014) and that of *Nezara viridula* (L.) (Hemiptera: Pentatomidae) (Zgonik and Čokl 2014). These examples suggest that *C. tomentosicollis* volatiles could act as aggregation pheromone.

Previous studies showed that the semiochemicals produced by Coreidae play various roles in pest behaviour. These semiochemicals are defined as the chemical substances/signals that carry information between living organisms and which cause changes in their behavior (Dicke and Sabelis 1988). They are emitted by one individual and cause a response in another. These signals could have repellent or attractive effects and are subdivided into two groups: allelochemicals and pheromones. The use of semiochemicals in the host-searching and foraging behavior and parasitism by hymenopterans such as the Scelionidae has been reported by Maruthadurai *et al.* (2011) and Conti and Colazza (2012). This has been demonstrated for *N. viridula* and *Earias vittella* Fab. (Lepidoptera: Noctuidae) in its egg location by the egg parasitoid *Trichogramma brasiliensis* Ashmead (Hymenoptera: Trichogrammatidae) (Bin *et al*. 1993; Conti and Colazza 2012). The potential use of egg parasitoids *Gryon* spp. as biological control agents for pod sucking bugs in Africa was reported by Taylor (1975)

and Asante *et al*. (2000). For example, field observation showed *Gryon fulviventris* Crawford (Hymenoptera: Scelionidae) parasitism rates of 90% and higher towards the end of the cropping season (Asante *et al*. 2000). Also, the same study reported that the parasitism rate of *C. tomentosicollis* eggs by parasitoids such as *Anastatus* sp. (Hymenoptera: Eupelmidae) and *Ooencyrtus patriciae* Subba Rao (Hymenoptera: Encyrtidae) was usually lower than that for *G. fulviventris* (Asante *et al*. 2000). Moreover, an olfactometer study showed that the egg parasitoid *G. fulviventris* was attracted to the volatiles produced by *C. tomentosicollis* males (Sanou *et al*. 2019). Furthermore, *Gryon gnidus* (Nixon) and *Gryon clavigrallae* (Mineo) have been reported to parasitize brown spiny bug eggs in the field (Taylor 1975; Dreyer1996; Asante *et al*. 2000).

The Coreidae family is very diverse and includes 44 *Clavigralla* species (Dolling 1979). The same study reported that *Clavigralla horrida* Germar, (Hemiptera: Coreidae) (restricted in South Africa), was previously misidentified as *C. shadabi* in West Africa and *C. elongata* in East and Southern Africa, due to the morphological resemblance (Dolling 1979). Close morphological resemblance between *Clavigralla alpica* Bergróth (Hemiptera: Coreidae), *Acanthomia brevirostris* Stål (Hemiptera: Coreidae) and *C. tomentosicollis* has been also reported (Dolling 1979). Wide distribution of *C. shadabi*, *C. elongata*, *C. tomentosicollis* in particularly in West and East Africa have been documented (Agunbiade *et al.* 2013; Chalam *et al* 2016; Minja *et al.* 1999). Additionally, these three *Clavigralla* species are polyphagous (beans, cowpea, Hyacinth bean, chik pea pigeon pea and *Tephrosia*) (Dabre-Binso *et al.* 2005; Taylor and Omoniyi 1972).

The first genetic study of *Clavigralla* species was that by Agunbiade *et al.* (2013) on transcriptome sequence annotation which identified genes of interest for pest control and potential molecular genetic markers, and the sequencing, assembly. The second report was that which annotated the complete mitogenome of *C. tomentosicollis*, including a comparative analysis with six other currently available Coreidae mitogenomes (Steele *et al.* 2017). No recent study on genetic variability has been conducted.

Despite the economic importance of *Clavigralla* spp. in Africa especially in Bénin and Kenya, little information exists on parasitoid – pest interactions. Furthermore, no studies have investigated the influence of *Clavigralla* spp. egg-derived chemicals on *Gryon* spp. foraging behavior and parasitism. No studies have elucidated the semiochemicals used by egg parasitoids to locate this pest and variations in chemical profile as well as genetic variability between *Clavigralla* species. It is therefore important to link these aspects to improve the biological control interventions. For example, semiochemical can be used as part of a strategy to augment egg parasitoid populations in the field to attack eggs laid by early-season females instead of those produced by the first generation. Successful intervention in biological control of *Clavigralla* spp. requires knowledge of the nature and bio-chemical composition of pheromones produced by *Clavigralla* spp. as well as the effect of these compounds on congeneric species behavior and on the *Gryon sp.* activities. This knowledge will be generated through this study.

1.2 Research aims and objectives

1.2.1General aims

The aims of this thesis were to 1) determine parasitism levels of *C. tomentosicollis* and *C. elongata* eggs, and to explore the relationship between egg parasitism and egg cuticular chemistry, 2) identify the aggregation pheromone of *C. tomentosicollis* and evaluate its effect on behaviour of the egg parasitoid, *Gryon* sp., and 3) identify the chemical profiles and establish the genetic variability of *C. tomentosicollis, C. elongata* and *C. shadabi* collected in Bénin and Kenya and determine whether there is a correlation between chemical profiles and genetic variability.

1.2.2 Objectives

The specific objectives of this study were to:

I) Assess the occurrence and potential distribution of *Clavigralla* spp. and the associated egg parasitoids in Bénin and Kenya.

II) Determine the morphological and genetic identity of the key egg parasitoid recorded from *Clavigralla* spp. eggs collected in Bénin and Kenya.

III) Evaluate the levels of parasitism of *C. tomentosicollis* and *C. elongata* eggs.

IV) Determine cuticular chemistry of *C. tomentosicollis* and *C. elongata* and identify potential chemical cues used by parasitoids.

V) Evaluate the responses of adult males and females of *C. tomentosicollis* to the volatiles released by conspecifics.

VI) Determine and identify the common electrophysiological active compounds for both sexes of *C. tomentosicollis* and the egg parasitoid, *Gryon* sp., by means of GC/EAD assays and GC/MS analysis.

VII) Conduct olfactometer assays with isopentyl butanoate to assess the attractiveness of this compound to both sexes of *C. tomentosicollis* and *Gryon* sp. Females.

VIII) Analyze the volatiles of both sexes of *Clavigralla* spp. collected in Bénin and Kenya and determine the composition of chemical profiles of these species.

IX) Characterize *Clavigralla* species collected in Bénin and Kenya, establish the genetic variability and determine whether there is a correlation between their chemical profiles and genetic variability.

1.2.3 Hypotheses

The following hypotheses were considered:

I) *Clavigralla tomentosicollis* Stål and *Gryon* spp. are common and abundant species in Bénin and Kenya, and the variation in egg cuticular chemistry of *Clavigralla* species influences the level of parasitism.

II) The aggregation pheromone is produced by *C. tomentosicollis* males and this pheromone attracts the parasitoid *Gryon* sp. and may be useful in *C. tomentosicollis* management.

III) *C. tomentosicollis*, *C. shadabi* and *C. elongata* present different chemical profiles, and genetic variability exists between species and populations.

1.3 Literature review

1.3.1 Cowpea and common bean production

1.3.1.1 Overview of cowpea and common bean

Vigna unguiculata and *Phaseolus vulgaris* are the two most important food legumes grown in Africa (Singh 2006). *Vigna unguiculata* and *P. vulgaris* belong to the Order Leguminosales, Family of Fabaceae (Papilionaceae), Tribe Phaseolae, Subtribe Phaseolinae and to the Genus *Vigna* and *Phaseolus* (Singh and Rachie 1985, Debouck 1991). Cowpea is indigenous to Africa and grows throughout the continent, particularly in the semi-arid regions of West Africa (Ajeigbe *et al*. 2006, Singh and van Emden 1979). West Africa is the major center of diversity and domestication of cowpea (Ehlers and Hall 1997) whereas Southern Africa is the center of diversity of wild *Vigna* spp. (Padoulosi *et al*. 1997).

Phaseolus vulgaris was derived from independent domestication of wild common bean in the Andean and American centers (Chacon *et al*. 2005) and is grown worldwide where temperatures are moderate. Eastern and Southern Africa are the most important producers and consumers of common bean throughout the year. It is estimated that over 14 million hectares of the world's arable land is dedicated to common bean production with yield of approximately 11 million tons/year (Singh 1999). It is often grown as intercrop with cereals, plantain and bananas (Kelly 2004).

1.3.1.2 Importance of cowpea and common bean

Cowpea and common bean are widely consumed and is used both as medicinal and nutritional plants (Phillips and McWatters 1991, Hillocks *et al*. 2006). The young leaves and young pods are consumed vegetables and leaves are also used as fodder. Seeds can be eaten green or dried (Jackai and Adalla 1997; Hillocks *et al*. 2006). Cowpea and common bean also provide food for human and livestock and serve as a valuable and dependable revenue-generating commodity for farmers and grain traders (Singh 2002, Langyintuo *et al*. 2003). Countries such as Nigeria, Niger, Brazil, Burkina, Bénin, Ghana, Kenya, Uganda and Malawi are considered to be the biggest producers of cowpea in the world (Singh *et al*. 1997). Also 40% of common beans produced in Africa is marketed, but these figures tend to be lower in areas with high population densities (Wortmann *et al*. 1998).

1.3.1.3 Constraints to cowpea and common bean

Cowpea and common bean are affected by both biotic and abiotic stress factors that reduce their growth and yield. In effect, cowpea and common bean are attacked during their entire cycle from seed germination to pod maturity and during seed storage by various insect pests, pathogens and rodents (Singh and Rachie 1985). Insect pests are considered as the most important limiting factor to cowpea production (Singh and van Emden 1979, Egho 2010). During the flowering and post-flowering period, damage is caused mainly by *Megalurothrips sjostedti* (Trybom) (Thysanoptera: Thripidae), *Maruca vitrata* (Fabricius) (Lepidoptera: Crambidae) and *Clavigralla* species (Heteroptera: Coreidae). *Callosobruchus maculates* (Fabricius) (Coleoptera: Bruchidae) is an

important pest species during storage (Jackai and Daoust 1986, Singh *et al*. 1990). The pod sucking bugs (also referred to as brown spiny bugs), *Clavigralla tomentosicollis, C. shadabi, C. elongata* and *C. hystricodes* pierce the pod walls and suck the developing seeds by injecting digestive enzymes. This feeding habit leaves tiny depressions or dimples on the pod wall. The seed then rots or shrivels and loses viability. The whole pod may have a shriveled appearance (Robin *et al*. 2010).

Fig. 1. Aggregation of *C. tomentosicollis* individuals on cowpea pods.

1.3.2 The brown spiny bugs, *Clavigralla* spp.

1.3.2.1 Classification of *Clavigralla* spp.

The brown spiny bug, *C. tomentosicollis*, formerly known as *Acanthomia tomentosicollis* was originally described by Stål in 1855 from specimens collected from the Cape colony in South Africa. Stål described it as a division of Clavigrallaria, the subfamily Pseudophloeinae, the family Coreidae (Hemiptera). *Clavigralla* (*Acanthomia*) *shadabi* Dolling belongs to the genus *Clavigralla*, described by Dolling (Dolling 1978 & 1979). A list of described species of *Clavigralla* using morphological characters is provided in Table below.

Table. *Clavigralla* spp. that occur in Africa.

Species group	Species Name	Authority /Year	Geographical Distribution
Tomentosicollis	*C. tomentosicollis*	Stäl, 1855	Africa south of the Sahara except Comoro Islands
Elongata	*C. shadabi*	Dolling, 1972	West to Central Africa and, S. Sudan
	C. elongata	Signoret, 1860	Cape Verde, Central, Eastern, Southern Africa, Madagascar and Yemen.
	C. hystricodes	Stäl, 1866	Tropical Africa; from Sierra Leone to Tanzania and northern parts of South Africa

1.3.2.2 Morphological characteristics of *Clavigralla* spp.

The following brief descriptive characteristics of the *Tomentosicollis* group are adopted from the recent revisions by Dolling (1978, 1979).

The tongue of the male genital capsule is located in the anterior end of abdominal sternite VII. The pronotal disk has a pair of large, blunt, sub lateral tubercles. The membrane of the hemelytron suffused fairly evenly with brown pigments. Although some morphometric variations exist between the males and females of *C. tomentosicollis*, the adults are generally robust with lengths varying between 8.3 and 11.5 mm. The antennae and rostrum are segmented with the basal segment of the rostrum directed posteriorly at rest. The posterior femur has two major subapical spines beneath with the more distal spine which is 1.5 times longer. The posterior tibia is straight except for a slight basal curvature. Also, the female possesses a round abdomen and is bigger than the males. *C. shadabi* and *C. elongata* are narrower than

C. tomentosicollis, grey, and have a pair of elongated spines on the “shoulders”. *Clavigralla hystricodes* Stål is black and has a shorter body (Robin *et al.* 2010).

1.3.2.3 General distribution of *Clavigralla* spp.

Clavigralla species are widely distributed across tropical Africa and South Asia. Among them, *C. tomentosicollis* is known as a major pest throughout the African mainland (Dolling 1979). Aina (1975), in a survey on 53 farms where cowpeas were grown in mixed cropping systems with other crops in Nigeria, found 9 different species of Coreidae bugs including *C. tomentosicollis* and *C. elongata*, which infested cowpea crops. *Clavigralla tomentosicollis* was present in 42% of farms and was abundant in all four ecological zones: Rain Forest, Derived Savannah, South Guinea and North Guinea Savannah. *Clavigralla shadabi* and *C. elongata* was restricted to the derived Savannah. The same pattern of appearance was observed in the short growing season (September-December) during which the population of *C. shadabi* was lower than *C. tomentosicollis* population, the one was present in late October to November and declined in December.

1.3.2.4 Biology and Ecology of *Clavigralla* spp*.*

Clavigralla tomentosicollis is a pubescent bug, spiny, with spines on the pronotum. The *Clavigralla* spp. present a sexual dimorphism: the males are 8.3 to 9.7 mm long while females are between 9.3 to 11.5 mm long (Dolling 1979). Eggs are laid in batches (5 to 40 eggs per batch) on the pods or on the lower surfaces of leaves and take 6 to 8 days to hatch (Materu 1970). The mean fecundity of females is approximately 200 (Egwuatu and Taylor 1977). The nymphs are gregarious, and aggregations are often observed on

pods of host plants (Materu 1971). According to Dennis (2012), the total time for the development of the five nymphal instars is 18-28 days under field conditions and 16-61 days under laboratory conditions at temperatures between 18 and 30°C. The first stage takes 2 to 4 days, the second 3 to 5 days, the third 4 to 6 days, fourth 4 to 6 days and the fifth 6 to 8 days to complete. The nymphs and the adults feed on the seeds and young pods (Ali 2005). Figure 2 illustrates the morphological distinction between *C. tomentosicollis, C. hystricodes*, *C. shadabi* and *C. elongata*.

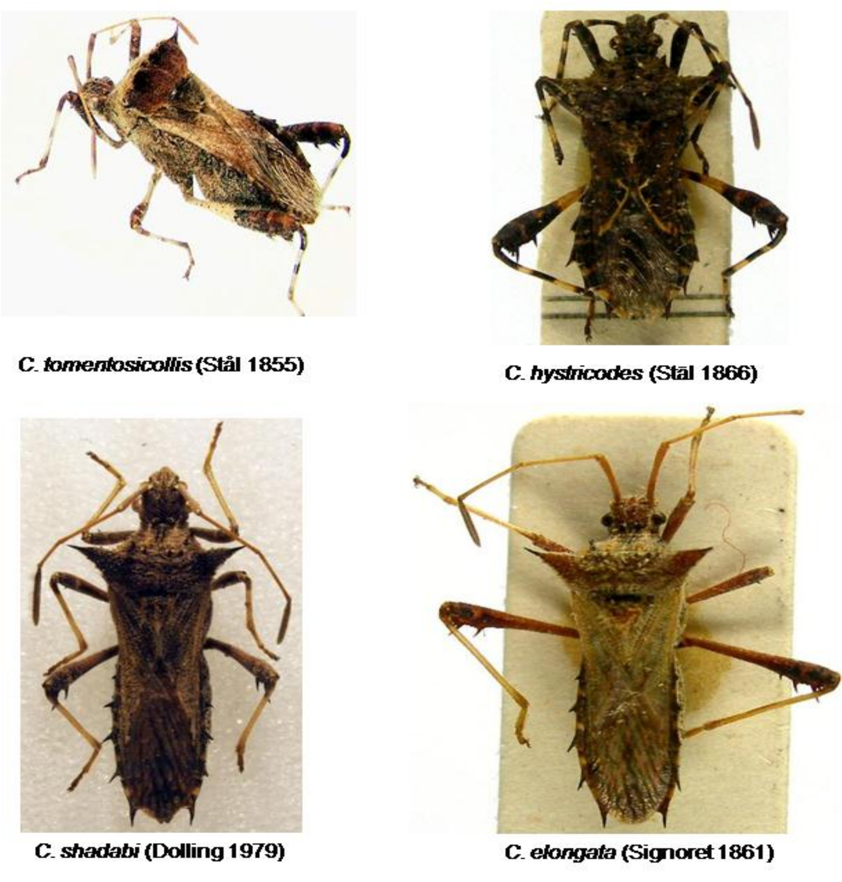

Fig. 2. Photos of *Clavigralla* spp. adults (retrieved from http://www.google.com/images?imgurl).

1.3.2.5 Host plants of *Clavigralla* spp.

Clavigralla spp. belong to the group of pod bugs that can change plant hosts during the year (Singh and Taylor 1978), with a preference for *Phaseolus vulgaris, Vigna unguiculata, Cajanus cajan, Dolichos lablab* which all belong to the order Fabales, family Fabacae. They attack other legumes and *Solanum incanum* (Dennis 2012). During the dry season, they attack also *Acanthospermum hispidium*, *Borreria raddiata, Commelina forkalaei, Lucas martinicensis* and *Tridax procumbens* (Dabiré 2005).

1.3.3 Control methods used against *Clavigralla* spp.

1.3.3.1 Chemical and varietal control

Among the several control methods used to manage the pests of cowpea and common beans, the use of the pesticides remains the most popular (Singh and Jackai 1985; Jackai and Adalla 1997). Examples of pesticides used are pyrethroids and organophosphates. Frequently used insecticides include lambda-cyhalothrin, cypermethrin, deltamethrin, and permethrin (Jackai and Adalla 1997). The management of these bugs can also be achieved via use of cowpea varieties that are resistant to *C. tomentosicollis,* for example IT86D-716, Moussa local and KVx396-4-5-2D (Dabiré *et al.* 2010).

1.3.3.2 Alternative control methods

Biological control aims at reducing the population of a given pests by using natural enemies (native and/or exotic) which are less damaging to the environment, farmers and consumers (Nomikou 2003). The biological control approach also involves the use

of entomopathogenic micro-organisms such as fungi, viruses and bacteria. For instance, high mortality of *C. tomentosicollis* adults was recorded seven days after treatment with *Metarhizium anisopliae* CPD 5 (Hypocreales: Clavicipitaceae) and *Beauveria bassiana* CPD 9 (Hypocreales: Ophiocordycipitaceae) (Ekesi 1999). Moreover, several hymenopteran parasitoids have been observed attacking *Clavigralla* spp.: *Anastatus* sp. (Hymenoptera: Eupelmidae), *Ooencyrtus patriciae* Subba Rao (Hymenoptera: Encyrtidae) and *Gryon fulviventris* Crawford (Hymenoptera: Scelionidae). Pod sucking bug management using plant extracts is limited to neem leaves and seeds extracts, which have been found to inhibit feeding and to influence the growth of nymphs (Dabiré 2001; Ostermann 1993). Furthermore, the aqueous extracts of *Boscia senegalensis* Lam. (Brassicales: Capparaceae) and *Cassia nigricans* Vahl. (Fabales: Fabaceae) have been used in *C. tomentosicollis* management (Dabiré 2001). Aliyu (2007) showed that the spray of the leaves of cowpea crops with a soap and Kerosene solution (8% concentration) in Nigeria reduced the population of *C. tomentosicollis* in the field.

1.3.3.3 Classical biological control

Biological control is the use of living organisms to suppress the population density of a specific pest organism, making it less abundant or less damaging than it would otherwise be (Eilenberg *et al*. 2001).

Four broad approaches have been distinguished in implementing biological control:

a) Conservation biological control which implies the modification of the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests (Eilenberg *et al*. 2001).

b) Seasonal inoculative biological control which implies the intentional release of a living organism as a biological control agent with the expectation that it will multiply and control the pest for an extended period but not permanently.

c) Classical biological control is the intentional introduction of an exotic, usually co-evolved, biological control agent for permanent establishment and long-term pest control (Eilenberg *et al*. 2001). It differs from seasonal inoculation in that classical biological control aims at permanent establishment of the released agent (Van Lenteren and Woets 1988).

d) Inundative biological control is the use of living organisms to control pests when control is achieved exclusively by the released organisms themselves. Effects of progeny of the released organisms are therefore not expected. Some reviewers include seasonal inoculative and inundative approaches in the augmentation strategy so that they distinguish three ways to apply biological control (Yaninek and Cock 1988; Bentley and O'Neil 1997; van Lenteren 2007). Augmentative biological control has therefore been defined as a periodic release (once or regular) of natural enemies to control pests for a short duration (Bentley and O'Neil 1997).

1.3.3 General information on the parasitoid: *Gryon fulviventris*

1.3.3.1 Classification and distribution of *G. fulviventris*

Gryon fulviventris, an egg parasitoid of *C. tomentosicollis*, belongs to Hymenoptera order, sub-order Apocrita, and to the family Scelionidae, sub-family Scelionidae (Crawford 1912). The Hymenoptera order is one of the richest orders with about 10% of terrestrial species of which 80% are parasitoids (Masner 1993). The family of Platygastroidea has two families: Scelionidae and Platygastridae, which combined have approximative 4460 species. Platygastroidea is found practically in all habitats, except the polar regions. They are especially abundant and diverse in the humid forests of tropical and subtropical regions. *Gryon fulviventris* has been reported in Africa, India and Israel (Austin *et al*. 2005). The females have a hypodermic ovipositor which they use to pierce the chorion of host eggs and lay their own eggs inside. *Gryon fulviventris* larvae that hatch consume the content of the host egg, pupates inside the egg and emerge as adult parasitoids (Masner 1993).

1.3.3.2 *Gryon fulviventris:* description, biology

The length of the female is 1-2 mm. The head and thorax are black, the abdomen ferruginous, scape testaceous and upper side medially brownish. The rest of the antennae are reddish brown with the clubs darker brown and joints of the funicle subquadrate. The male is similar to the female but has a black abdomen and brown antenna, except for the scape. The pedicel is slightly longer than the first joint of the funicle with the following joints being subquadrate. The apical joint is almost as long as

the two preceding joints combined and sculptured somewhat stronger in the male (description from Crawford 1912).

Gryon fulviventris is a solitary endoparasite, entirely developing inside host eggs. After ovipositing, the female scrapes the surface of each parasitized egg with ovipositor doing several circular lines on the oviposited part. The female has a preference of fresh host eggs (Masner 1993). Certain species of *Gryon* are found to be effective candidates for biological control. For example, *G. fulviventris* against *C. tomentosicollis* in Africa and *G. clavigrallae* against *C. gibbosa* Spinola (Hemiptera: Coreidae) and *C. scutellaris* Westwood (Hemiptera: Coreidae) in Asia (Bhagawat *et al*. 1994; Asante *et al*. 2000; Romeis *et al*. 2000). The highest parasitism rate of 73.9% have been reported for *G. fulviventris* (Asante *et al*. 2000). Additionally, Coreidae eggs are also heavily parasitized by scelionid wasps. In Japan, *Gryon pennsylvanicum* (Ashmead) (Hymenoptera: Scelionidae) is an important natural enemy of *Leptoglossus australis* (Fabricius) (Hemiptera: Coreidae) (Yasuda and Tsurumachi 1995). *Gryon pennsylvanicum* has also been considered as a potential biocontrol agent for *Leptoglossus phyllopus* (Say) and *Anasa tristis* DeGeer (Hemiptera: Coreidae) in the United States (Olson *et al*. 1996; Mitchell *et al*. 1999). Eggs of *Clavigralla* spp. in India were parasitized by both *G. clavigrallae* (Shanower *et al.* 1996) and by *G. fulviventris* in India (Singh *et al*. 1987).

G. fulviventris male *G. fulviventris* female

Fig. 3. *Gryon fulviventris* adults.

(Retrieved from: http://www.nbair.res.in/Featured_insects/images/gryon-fulviventre4.jpg on 03-02-2016).

1.3.4 Infochemicals

Vet and Dicke (1992) define infochemicals as chemicals that transmit information in an interaction between two individuals, inducing in the receiver a behavioral or physiological response.

1.3.4.1 Pheromones

The term pheromone was coined by Karlson and Lüscher (1959), for any substance secreted by an organism to the outside that causes specific reactions in the receiving organism of the same species. Pheromones are classified into several subcategories based on the type of interaction they mediate:

- Sex pheromones: chemicals that primarily affect an interaction between the sexes (e.g. sex pheromone that attracts males to females).

- Aggregation pheromones: chemicals that cause an increase in the density of the animals (usually both sexes) in the vicinity of the pheromone source.

- Trail pheromones: chemicals secreted by workers of social insects to recruit other individuals to a food source or to a new colony site.

- Alarm pheromones: chemicals that stimulate escape or defense behavior.

There are also other types of pheromones, such as dispersal pheromones and maturation pheromones.

1.3.4.2 Allelochemicals

This term was proposed by Whittaker in 1971 and is used to describe chemicals that mediate interspecific interactions. Allelochemical are classified into several subcategories:

- Allomones: chemical substances that benefit the emitter but not the receiver (e.g. venom secreted by social wasps).
- Kairomones: chemical substances that benefit the receiver but not the emitter (e.g. host location by beneficial insects).
- Synomones: chemicals that mediate mutualistic interactions; benefits both the receiver and the emitter.

1.3.5 Conclusions

Vigna unguiculata and *P. vulgaris* are used as green manure, planted to do erosion control and seeds are eaten green or dried. They provide food for man and livestock and serve as a valuable revenue-generating commodity for farmers. Their production is affected by flower and pod pests, especially the brown pod sucking bugs *C. tomentosicollis, C. shadabi, C. elongata and C. hystricodes*. These *Clavigralla* species are widely distributed in Africa and feed on a large number of host plants in the Fabacae, particularly cowpea, common bean and pigeon pea. Chemical control, cultural control and host plant resistance have been used to control *Clavigralla* spp., but these are largely not effective and do not provide sustainable management of these pests. *Gryon fulviventris*, an egg parasitoid that is widely distributed throughout Africa, and has

been reported as potential biological control agent of *Clavigralla* spp. The relationship between *Gryon* species and *Clavigralla* spp. is the topic of this thesis

1.4 Structure of thesis

This thesis is subdivided into the following chapters:

1. **Introduction, literature review, and thesis structure** provides a literature review of different aspects of *Clavigralla* species and their egg parasitoids *Gryon* sp. as well as two main group of host plants (cowpea and common beans). It presents the different control methods, definitions of chemical expressions used and presents the aims, objectives, and hypotheses. Herein, the outlines of the thesis were also presented. Emphasis is placed on the identification of the potential semiochemicals useful in the parasitism and in the location of host eggs of egg parasitoid *Gryon* sp. as well as the genetic identity and variability between the *Clavigralla* species collected in Bénin and Kenya.

   2. Article 1 presents results of a study that explored levels of egg parasitism and variation in egg cuticular chemistry of different *Clavigralla* spp. Further emphasis is placed on the occurrence of *C. tomentosicollis*, *C. shadabi* and *C. elongata* and the egg parasitoid *Gryon* sp. associated with this pest species in cowpea, French bean and pigeon pea in Bénin and Kenya. The levels of parasitism of different *Clavigralla* spp. eggs, and how the parasitism relates to host egg cuticular chemistry were investigated. This paper together with literature review provide information on distribution of *Clavigralla* species in both countries. It presents the specific cuticular components released from unparasitized eggs of *C. tomentosicollis*.

3. Article 2 reports on **isopentyl butanoate** as an aggregation pheromone of C*. tomentosicollis* and a kairomone for the egg parasitoid *Gryon* sp. The chemical profiles of *C. tomentosicollis* males and females were also identified. The results in this chapter suggest that isopentyl butanoate serves as an aggregation pheromone for both sexes of *C. tomentosicollis* and a kairomone that attracts the parasitoid *Gryon* sp.

4. Article 3 reports on identification of headspace volatile profiles of *C. tomentosicollis, C. elongata* and *C. shadabi*, establishes the genetic variability between these three *Clavigralla* species collected from Bénin and Kenya, and shows the correlation between genetic variability and volatile chemistry variation.

5. In the conclusion and recommendations section, the key findings of this study are summarized and the role of semiochemicals in the host location and parasitism by *Gryon* sp. discussed and recommendations for future studies are provided. It reports also the genetic variability between species and different populations (from Benin and Kenya) and provides recommendations for future studies.

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CHAPTER 2: ARTICLE 1

Exploring levels of egg parasitism and variation in egg cuticular chemistry in different *Clavigralla* spp.

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ORIGINAL CONTRIBUTION

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Exploring levels of egg parasitism and variation in egg cuticular chemistry in different *Clavigralla* spp.

Hilaire Kpongbe[1,2] | **Johnnie Van den Berg**[2] | **Fathiya Khamis**[1] | **Manuele Tamò**[3] | **Elijah Jacob Talamas**[4] | **Baldwyn Torto**[1]

[1]International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya

[2]Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa

[3]International Institute of Tropical Agriculture, Abomey-Calavi, Bénin

[4]Division of Plant Industry, Florida Department of Agriculture and Consumer Services, Gainesville, Florida

Correspondence
Baldwyn Torto, International Centre of Insect Physiology and Ecology (*icipe*), P.O. Box 30772-00100, Nairobi, Kenya.
Email: btorto@icipe.org

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Abstract

Clavigralla spp. (Hemiptera: Coreidae) are major pests of cowpea (*Vigna unguiculata* (L.) Walp, Fabacae), common bean (*Phaseolus vulgaris* L., Fabacae) and pigeon pea (*Cajanus cajan* L., Fabacae) in Africa. *Clavigralla* spp. egg parasitoids, *Gryon* spp. (Hymenoptera: Scelionidae), have previously been reported as potential biological control candidates. Little is known about the parasitism levels and their potential relationship with cuticular chemistry of *Clavigralla* spp. The aims of this study were to determine parasitism levels of *Clavigralla tomentosicollis* Stål (Hemiptera: Coreidae) and *C. elongata* Signoret (Hemiptera: Coreidae) eggs, and to explore the relationship between egg parasitism and egg cuticular chemistry. High parasitism levels were determined for *C. tomentosicollis* by collecting eggs from plants in mono-cropping and multi-cropping systems in farmers' fields in Bénin and Kenya between April and June 2016. Three species of *Clavigralla* were recorded: *C. tomentosicollis*, *C. shadabi* and *C. elongata*. *Clavigralla tomentosicollis* was the most common in both countries, while *C. shadabi* and *C. elongata* were only collected in Bénin and Kenya, respectively. An egg parasitoid (*Gryon* sp.) was recovered from egg batches collected from both countries. In parasitism assays using *Gryon* sp., the incidence of parasitism was higher in *C. tomentosicollis* eggs than that of *C. elongata*. Chemical analysis by coupled gas chromatography/mass spectrometry (GC/MS) of cuticular extracts obtained from *C. tomentosicollis* and *C. elongata* eggs identified fifteen compounds including ten alkanes of which the amounts varied between the two species. We speculate that *Clavigralla* spp. cuticular chemistry may serve as potential host location cues for *Gryon* sp.

KEYWORDS
egg parasitoid, occurrence, parasitism, pod sucking bug, semiochemical cues

1 | INTRODUCTION

The tribe Clavigrallini, which includes *Clavigralla* Spinola, is comprised of two genera and 44 species (Dolling, 1979). Commonly referred to as pod sucking bugs or brown spiny bugs, *Clavigralla tomentosicollis* Stål, *C. shadabi* Dolling and *C. elongata* Signoret (Hemiptera: Coreidae) all belong to the species complex that attack grain legume crops in Africa. These species occur widely in Nigeria, Burkina-Faso, Niger, Bénin, Tanzania and Kenya where they are the major pests of cowpea and French bean (Minja et al., 1999; Agunbiade et al., 2013; Chalam et al., 2016). *Clavigralla tomentosicollis* and *C. shadabi* were

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previously recorded in some regions of Bénin (Dreyer, Baumgärtner, & Tamó, 1994; Shanower, Romeis, & Minja, 1999; Egho, 2010; Agboton et al., 2014). Gethi and Khaemba (1991) reported a high prevalence of *C. tomentosicollis* and *C. shadabi* on cowpea in maize intercropping systems in Mombasa, Kenya. *Clavigralla* spp. prefer to feed on legume pods and have been reported to cause yield losses of up to 90% and reduce seed viability of up to 85% (Dreyer et al., 1994; Abate & Ampofo, 1996; Koona et al., 2001; Oparaeke, 2006a, 2006b; Soyelu & Akingbohungbe, 2007; Dabire-Binso, Ba, Sanon, Drabo, & Bi, 2010; Dialoke et al., 2010).

Cultural control practices, pesticide applications and resistant crop varieties have in the past been used in the management of *Clavigralla* spp., but these were largely unsuccessful (Jackai & Adalla, 1997; Adipala, Nampala, Karungi, & Isubikalu, 2000; Koona, Osisanya, Jackai, Tamò, & Markham, 2002; Aliyu, Ladan, Ahmed, & Abdullahi, 2007). Several studies previously reported on the potential of egg parasitoids as biological control agents for pod sucking bugs eggs in Africa (Taylor, 1975; Asante, Jackai, & Tamò, 2000). For example, in northern Nigeria, *Gryon fulviventris* (Crawford) (Hymenoptera: Scelionidae), *Ooencyrtus utetheisae* (Risbec) (Hymenoptera: Encyrtidae) and *Anastatus* sp. (Hymenoptera: Eupelmidae) were reported to parasitize eggs of *C. tomentosicollis* (Asante et al., 2000). The same study reported that *C. tomentosicollis* eggs were the most parasitized by *G. fulviventris*, which suggests that the latter species could potentially be used as a biological control agent for *Clavigralla* spp. (Asante et al., 2000). *Gryon gnidus* (Nixon) (Hymenoptera: Scelionidae), another egg parasitoid of *C. tomentosicollis*, was reported in Nigeria by Taylor (1975) while Shanower, Anitha, Bhagwat, and Dreyer (1996) reported in Kenya and Tanzania that *Gryon clavigrallae* (Mineo) (Hymenoptera: Scelionidae) also parasitized eggs of *Clavigralla* spp.

Previous chemical ecology studies have documented that chemical cues are used by egg parasitoids in host location, foraging behaviour and parasitism. This has been demonstrated for *Nezara viridula* L. (Hemiptera: Pentatomidae) (Bin, Vinson, Strand, Colazza, & Jones Jr, 1993), *Earias vittella* Fab. (Lepidoptera: Noctuidae) and *Spodoptera litura* Fab. (Lepidoptera: Noctuidae) (Maruthadurai, Gautam, & Mahesh, 2011) in their egg location by the egg parasitoid *Trichogramma brasiliensis* Ashmead (Hymenoptera: Trichogrammatidae). Likewise, *Trissolcus brochymenae* Ashmead (Hymenoptera: Platygastridae) is known to exploit *Murgantia histrionica* Hahn (Hemiptera: Pentatomidae) egg volatiles during host location and recognition (Conti, Salerno, Bin, Williams, & Vinson, 2003). Furthermore, volatiles from egg masses of *Orgyia postica* Walker (Lepidoptera: Lymantriidae) were found to attract the egg parasitoid *Telenomus euproctidis* Ratzeburg (Hymenoptera: Platygastridae) (Arakaki, Yamazawa, & Wakamura, 2011). These observations suggest that cuticular chemistry may play a role in parasitoid–pest interactions.

Despite the economic importance of *Clavigralla* spp. in Bénin and Kenya, little information exists on parasitoid–pest interactions. Furthermore, no studies have investigated the influence of *Clavigralla* spp. egg-derived chemicals in *Gryon* spp. foraging behaviour and parasitism. The aims of this study were threefold: to carry out morphological and genetic identification of the egg parasitoid recovered from *Clavigralla* spp. eggs; to determine levels of parasitism of different *Clavigralla* spp. eggs in the laboratory; and to determine whether egg cuticular chemistry differed between the different *Clavigralla* spp.

2 | MATERIALS AND METHODS

In Kenya, sampling was done during the long rains (April to May 2016) in the following six counties: Western Kenya (Kisumu), Rift Valley (Nakuru) and Eastern Kenya (Embu, Kitui, Machakos and Makueni). In Bénin, samples were collected during the long rainy season (May to June 2016) in ten villages from four regions: Southern Bénin (Abomey-Calavi), Eastern Bénin (Ketou, Pobe), Western Bénin (Klouekanme) and Central Bénin (Dassa-Zounme, Djidja). These counties and regions are considered as the main grain legume production areas in both countries (Minja et al., 1999; Gbaguidi, Dansi, Loko, Dansi, & Sanni, 2013; Ayenan, Ofori, Ahoton, & Danquah, 2017). The geographical coordinates were recorded at each site (Table 1).

2.1 | Sample collection

Collection of *Clavigralla* species and their eggs were carried out during the pod filling stage of the different crops which is the preferred phenological stage for attacking the crops (Koona et al., 2001). Eggs, nymphs and adults of *Clavigralla* spp. were collected in both mono-cropping and multi-cropping systems of French bean and pigeon pea that were in some cases associated with maize in Kenya. Collections in Bénin were done in mono-cropping systems of cowpea and pigeon pea. Each site was visited once in the morning (7:30–11:00) and in the afternoon (15:00–18:00). Visual inspection of plants was conducted to search for different life stages of these pests. At each site, two fields were visited. All specimens were placed individually into small cages (13.5 cm diameter and 12.0 cm height) that contained pods of plant hosts.

Ten egg batches collected from each site were incubated separately under laboratory conditions at 25 ± 1°C and 60%–70% RH, with a photoperiod of 12:12 hr (light: dark) until nymphs hatched or parasitoids emerged. The number of the emerged *Gryon* sp. per site was recorded. This parasitoid species that emerged from eggs were morphologically identified using the taxonomic keys presented by Masner (1975, 1976) and photographed by taxonomist Dr. Elijah Jacob Talamas, Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Gainesville, FL USA. Molecular techniques were used to confirm the morphological identity.

2.2 | DNA extraction, PCR and sequencing

The identity of the *Gryon* sp. samples collected was confirmed using PCR of the D2 region of 28S rDNA (28S) in the mitochondria. Genomic DNA from 95% ethanol-preserved specimens was extracted using

TABLE 1 Localities at which *Clavigralla tomentosicollis*, *Clavigralla shadabi* and *Clavigralla elongata* were recorded in Kenya and Bénin

Localities	Villages	*Clavigralla* spp. collected			Coordinates of sampling		
		C. tom.	*C. shad.*	*C. elong.*	Latitude	Longitude	Elevation (m a.s.l.)
Kenya							
Embu	Gatirari	+	–	–	S 00°40.532'	E 037°39.187'	1,060
	Jagawneth	+	–	+	S 00°44.847'	E 037°36.151'	1,049
Kisumu	Obino	+	–	+	S 00°05.066'	E 034°52.478	1,170
Kitui	Kithinzi	+	–	+	S 01°18.155'	E 038°02.019'	1,251
Machakos	Kitimani	+	-	-	S 01°10.060'	E 037°27.287'	1,228
Makueni	Kaiani	+	–	–	S 01°52.621'	E 037°42.793'	1,113
Nakuru	Kirobon	+	–	–	S 00°18.345'	E 035°59.224'	1,930
	Wata	+	-	+	S 00°16.413'	E 036°07.172'	1,883
	Karagita	+	–	+	S 00°48.170'	E 036°26.918'	2,001
Benin							
Abomey-Calavi	IITA Station	+	+	–	N 06°25.100'	E 002°19.925'	18
Dassa-zounme	Afossogbe	+	–	–	N 07°34.382'	E 002°11.195'	137
	Ganfon	+	-	-	N 07°49.371'	E002°08.399'	128
Djidja	Oumgbega	+	–	–	N 07°17.051'	E 002°02.420'	253
	Assantoun	+	–	–	N 07°17.704'	E 002°03.109'	259
	Dridji	+	+	–	N 07°23.801'	E 022°05.048'	167
Kétou	Camp	+	–	–	N 07°18.509'	E 002°37.424'	131
	Aguidi	+	+	–	N 07°18.543'	E 002°31.583'	68
Klouékanmey	Adja-hounmey	+	–	–	N 07°02.672'	E 001°47.592'	225
Pobè	Itchagba	+	–	–	N 07°06.705'	E 002°38.722'	34
	Occurrence level	**	*	*			

Note: (+) = present; (−) = absent; (*) = present; (**) = very present.
C. tom., *Clavigralla tomentosicollis*, *C. shad.*, *Clavigralla shadabi*, *C. elong.*, *Clavigralla elongate*.

high-quality DNA extraction for RT-PCR and Sequencing kit (Qiagen) as per manufacturer's instructions. The purity and concentration of the resultant extracted DNA were determined using Nanodrop 2000/2000c Spectrophotometer (Thermo Scientific). The PCR was carried out in a total reaction volume of 20 µl containing 5X My*Taq* Reaction Buffer (5 mMdNTPs, 15 mM $MgCl_2$, stabilizers and enhancers), 10 µmole of each primer (LepD2-Fw5'AGTCGTGTTGCTTGAT AGTGCAG3' and LepD2 Rv5'TTGGTCCGTGTTTCAAGACGGG3' (Campbell, Steffen-Campbell, & Werren, 1994; Goolsby et al., 2006)), 0.5 mM $MgCl_2$, 0.25 µl My*Taq*DNA polymerase (Bioline, London, UK) and 15 ng/µl of DNA template. This reaction was set up in the Nexus Mastercycler gradient (Eppendorf). The following cycling conditions were used: initial denaturation for 2 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s annealing at 58.8°C and 1 min at 72°C, then a final elongation step of 10 min at 72°C. The target gene region was 700 base pairs. The amplified PCR products were resolved through a 1.2% agarose gel and bands analysed and documented using KETA GL imaging system trans-illuminator (Wealtec Corp, Meadowvale Way Sparks). Successfully amplified products were excised and purified using Isolate II PCR and Gel Kit (Bioline) following the manufacturer's instructions. The purified samples were shipped to Macrogen Inc. Europe Laboratory, the Netherlands, for bi-directional sequencing, and the platform used was Illumina. The successful sequences were assembled and edited using ChromasLite version 2.1.1 (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997) and Geneious version 8 (http://www.geneious.com) (Kearse et al., 2012). The sequence identities were determined using Basic Local Alignment Search Tool (BLAST) hosted by the National Center for Biotechnology Information (NCBI).

2.3 | Laboratory rearing of egg parasitoid species and *Clavigralla* species

To aid laboratory assays, the parasitoid and the *Clavigralla* species *C. tomentosicollis* and *C. elongata* were reared in the insectary at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville campus, Nairobi, Kenya. Rearing was done at 25 ± 1°C and 60%–70% RH with a photoperiod of 12:12 hr (Light: Dark). Egg batches collected from different localities in Kenya were incubated separately in sterile clear plastic cages (9.0 cm diameter x 4.5 cm height) (Foodmate 0.5 L, Kenpoly Nairobi, Kenya), with ventilated lids. Emerged parasitoids were collected by means of an aspirator

and introduced into a cage containing *C. tomentosicollis* eggs that were less than 48 hr old. Parasitoids were fed on droplets of a 90% honey solution. In Bénin, field-collected eggs were incubated in the same way as described above, after which emerged parasitoids were put into vials containing 95% ethanol for molecular/morphological identification. All rearing was done following the same conditions described above.

Clavigralla tomentosicollis and *C. elongata* were reared on young healthy pods of French bean in cylindrical clear plastic cages (18 cm diameter x 6.5 cm high) (Foodmate 2 L, Kenpoly) with a ventilated lid. The cages were lined with paper towel to absorb excess moisture and bug excretions. Each cage contained five fresh pods of French bean. Thirty to forty adults were introduced into each cage using a fine brush and an aspirator. Batches of fifty eggs that were laid on the absorbent paper were transferred to new cages of the same dimensions every 48 hr. Any dead insects were removed from the containers.

2.4 | Parasitism bioassays

Parasitism levels of *C. tomentosicollis* and *C. elongata* eggs were studied under laboratory conditions as previously described by Asante et al. (2000). Each egg batch used contained 30 eggs. The experiment consisted of two treatments for each species: (a) one unparasitized egg batch of *C. tomentosicollis* or *C. elongata* eggs (control) and (b) one parasitized egg batch of *C. tomentosicollis* or *C. elongata* eggs (test). Each treatment was replicated five times with one egg batch per replicate. Thirty fresh unparasitized eggs (1 day old) of *C. tomentosicollis* or *C. elongata* were carefully introduced into sterile clear plastic cages (9 cm diameter and 4.5 cm height) (Foodmate 0.5 L, [Foodmate 0.5 L, Kenpoly]) with ventilated lids. Five newly emerged *Gryon* sp. females selected were collected by means of an aspirator and introduced into each test cage which contained fresh unparasitized *C. tomentosicollis* or *C. elongata* eggs for a period of 12 hr (6:00 to 18:00 hr). After 12 hr, the parasitoids were removed. All egg batches were incubated for 15 days which exceeds the date of expected parasitoid emergence by 2 days. The number of parasitoids that emerged from eggs was recorded daily from 10 to 15 days after exposure to the parasitoids.

2.5 | Extraction of egg cuticular components

To obtain parasitized eggs, *C. tomentosicollis* and *C. elongata* eggs were exposed to five females of *Gryon* sp. using the methods described above. Batches of parasitized and unparasitized eggs were then used to obtain cuticular extracts following the method described by Holman, Jørgensen, Nielsen, and d'Ettorre (2010). The parasitized eggs were extracted immediately after 12 hr exposure to the parasitoids. Both, the parasitized and unparasitized egg batches of each species were placed separately into two storage vials (2 ml) containing 100 μl of pentane (HPLC Grade, Sigma-Aldrich, purity ≥ 99%.) for 12 min. The extract was collected using a microsyringe and transferred into two storage vials for GC/MS analysis. All the experiments were conducted in the insectary of the International Centre of Insect Physiology and Ecology, Duduville campus, Nairobi at 25 ± 1°C and 60%–70% RH with a photoperiod of 12:12 hr (light: dark) cycle.

2.6 | Chemical analysis

Each cuticular extract (1 μl) was analysed by coupled gas chromatography/mass spectrometry (GC/MS) on an Agilent Technologies Series A 7,890 GC coupled to a 5975C MS (inert XL/ EI/CI MSD) triple axis mass detector, equipped with an HP5 MS low bleed capillary column (30 m × 0.250 mm i.d, 0.25 μm) (J&W) in the electron impact mode at 70 eV. The GC oven temperature was 35°C for 5 min with a rise of 10°C/min to 280°C for 10.5 min, then 5°C/min to 285°C and held at this temperature for 9 min. Identification of compounds was done by comparison of mass spectral data with library data Adams2, Chemecol and NIST11. The experiments were replicated three times. Quantification was based on calibration curves (peak area vs. concentration) generated from authentic standards of identified compounds.

2.7 | Chemicals

The following synthetic standards hexadecane, hexadecanoic acid, hexadec-9-enoic acid, octadecane, nonadecane, eicosane, tricosane, tetracosane and pentacosane, hexacosane, and heptacosane were all purchased from Sigma-Aldrich, Germany (purity ≥ 97%).

2.8 | Data analysis

All statistical analyses were performed in R Development Core Team (2012) software version 3.1.2 at 5% significance level. Percentage parasitism was calculated for each treatment. Data on egg parasitism levels of the two *Clavigralla* spp. were analysed using a generalized linear model affirming quasi-binomial distribution error. Principal component analyses (PCA) were performed to compare the chemical composition of parasitized and unparasitized *C. tomentosicollis* and *C. elongata* egg cuticles.

3 | RESULTS

3.1 | *Clavigralla* spp. and parasitoid emergence from field-collected eggs

Three species of *Clavigralla* including *C. tomentosicollis*, *C. shadabi* and *C. elongata* were recorded. *Clavigralla tomentosicollis* was present at all the study sites in both countries, and it was also more abundant than *C. shadabi* and *C. elongata*. *Clavigralla shadabi* was only collected in Bénin at the following sites: Djidja (Dridji), Ketou (Aguidi) and Abomey-Calavi (IITA station). In Kenya, *C. elongata* was recorded at Embu (Jagawneth farm), Kisumu (Obino), Kitui (Kithinzi) and Nakuru (Wata and Karagita) (Table 1, Figure 1). The only egg parasitoid which emerged from the collected eggs in both countries was morphologically identified as

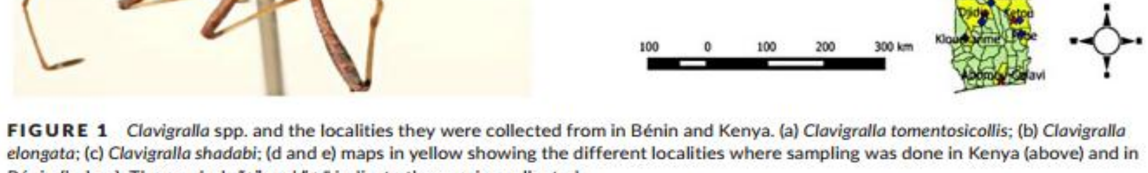


FIGURE 1 *Clavigralla* spp. and the localities they were collected from in Bénin and Kenya. (a) *Clavigralla tomentosicollis*; (b) *Clavigralla elongata*; (c) *Clavigralla shadabi*; (d and e) maps in yellow showing the different localities where sampling was done in Kenya (above) and in Bénin (below). The symbols "◆" and "★" indicate the species collected

Gryon sp. (Figure 2). In Bénin, parasitoids emerged from egg batches collected at the IITA station, Djidja (Dridji) and Ketou (Aguidi) were *Gryon* sp. Similarly, only *Gryon* sp. emerged from egg batches collected at the two sites (Machakos and Makueni) in Kenya. The numbers of emerged parasitoids recorded were, however, generally lower than those recorded in Bénin (Table 2). This preliminary data on field collection showed overall that the average number of emerged parasitoids per batch in Benin sites is two times greater than that of Kenya sites (Table 2).

3.2 | Identification of the parasitoid samples

BLAST query yielded 99% similarity of all the parasitoid sequences to *Gryon* sp. of accession number JX683193.1 (Table 3). This identity confirmed the morphological taxonomy, and which have been deposited in the GenBank with accession numbers (MK488003–MK488008). At present, the taxonomy of African *Gryon* lacks comprehensive and reliable identification tools, preventing us from attaching a species name to the species of *Gryon* reared in this study. The African species of *Gryon* are currently under revision by co-author (EJT), which includes the generation of a DNA barcode database for *Gryon* worldwide. Additional images of specimens from both the Bénin and Kenya colonies are available via the Hymenoptera Online Database (hol.osu.edu) and can be found by searching the specimen identification numbers (DPI_FSCA 00010233 and DPI_FSCA 00010182, respectively). As the revisionary work on *Gryon* progresses, identification of this species will be updated (Florida Department of Agriculture

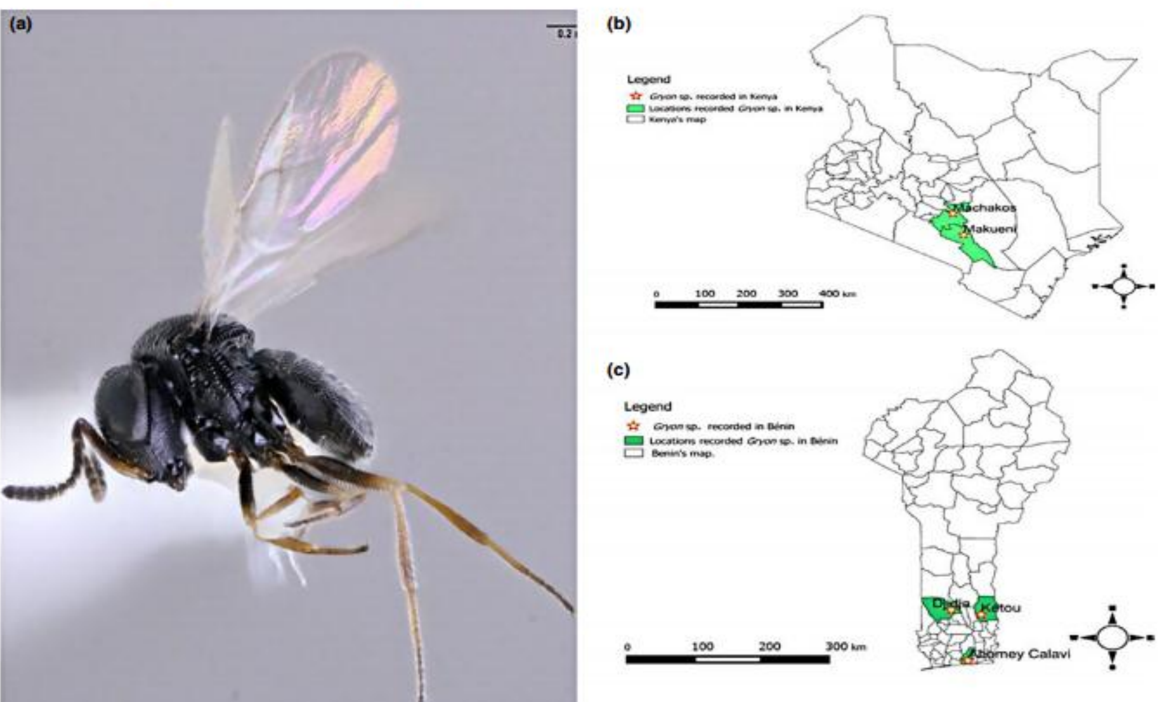


FIGURE 2 *Gryon* sp. recorded and locations they were collected from in Bénin and Kenya. (a) = *Gryon* sp. reared on *Clavigralla* spp. eggs, (b and c) = maps in green showing where *Gryon* sp. was collected in Kenya (above) and Bénin (below). Symbol "☆" indicates *Gryon* sp. recorded from *Clavigralla* spp. eggs

and Consumer Services, Division of Plant Industry, Gainesville, FL USA).

3.3 | Parasitism bioassays

There was a significant difference in the parasitism rate between *C. tomentosicollis* (mean percentage = 74.6 ± 7.2%) and *C. elongata* eggs (mean percentage = 35.3 ± 3.0%) (F = 20.01, df = 8,1; p = 0.002 (Figure 3).

3.4 | Chemical analysis

Overall, thirteen components were identified in the cuticular extracts of unparasitized and parasitized eggs of the two *Clavigralla* spp. These included two aliphatic acids, ten alkanes and one alkene whose identities were confirmed using authentic standards (Table 4; Figure 4). Four additional components were tentatively identified as phenols (two) an alkene and an alcohol based on mass spectral data only (Table 4). Differences between the cuticular chemistry of unparasitized and parasitized eggs were mainly quantitative rather than qualitative. Additionally, these differences varied within and between species and were, mainly in the dominant components, alkanes and their amounts (Table 4).

Principal component (PCA) analyses showed species separation of unparasitized and parasitized eggs (Figures 5 and 6) and were defined by seventeen cuticular components. The PCA biplot of the seventeen variables explained 77.1% (Dim 1 = 53.7% and Dim 2 = 23.4%) for unparasitized egg cuticular extract and 80.1% (Dim 1 = 57.1% and Dim 2 = 23%) for parasitized egg cuticular extract in the variance in the data set. Two groups were observed for each type of egg: group of specific cuticular extract components for the unparasitized *C. tomentosicollis* and *C. elongata* eggs and group of specific parasitized cuticular extract components for *C. tomentosicollis* and *C. elongata* eggs. Upon PCA examination, five cuticular hydrocarbons, two phenols and two aliphatic acids correlated with separation of *C. tomentosicollis* unparasitized eggs cluster from *C. elongata* unparasitized eggs (Figure 5a). The grouping of the parasitized specific cuticular extract components of *C. tomentosicollis* (heptacosane and eicosane) in the PCA separated this species from *C. elongata* (Figure 5b). Additionally, the heat map associated with the unparasitized and parasitized eggs cuticular extract components of the two species showed that the alkanes nonadecane, tricosane, tetracosane, hexacosane and the alcohols 2,4-bis(1-methyl-1-phenylethyl)- phenol, 2,4-bis(dimethylbenzyl)-6-t-butylphenol were present in high amounts in the cuticular extracts of unparasitized eggs of *C. tomentosicollis* but absent/trace from the

TABLE 2 Localities at which *Gryon* sp. were recorded from eggs of *Clavigralla tomentosicollis* in Bénin and Kenya and approximate numbers of *Gryon* sp. that emerged from field-collected eggs batches

Localities	Species collected	Coordinates		Elevation (m a.s.l.)	Number of *Gryon* sp. emerged from ten egg batches of *C. tomentosicollis* collected	Average of emerged parasitoids per batch
		Latitude	Longitude			
Kenya						
Machakos (Kitimani)	*Gryon* sp.	S 01°10.060'	E 037°27.287'	1,228	76	7.6
Makueni (Kaiani)	*Gryon* sp.	S 01°52.621'	E 037°42.793'	1,113	41	4.1
Bénin						
Abomey-Calavi	*Gryon* sp.	N 06°25.100'	E 002°19.925'	18	137	13.7
Djidja (Dridji)	*Gryon* sp.	N 07°23.801'	E 022°05.048'	167	119	11.9
Ketou (Aguidi)	*Gryon* sp.	N 07°18.543'	E 002°31.583'	68	63	6.3

cuticular extract of unparasitized eggs of *C. elongata*. Furthermore, eicosane and pentacosane were more concentrated in cuticular extract of unparasitized eggs of *C. tomentosicollis* than *C. elongata* unparasitized eggs, as well as hexadecanoic acid and octadecane which were present in high amounts in unparasitized cuticular egg extract of *C. tomentosicollis* and absent in the cuticular extract of unparasitized eggs of *C. elongata*. These compounds contributed to the separation of unparasitized eggs of the two species (Figure 6). The unidentified alkene, tricosane, tetracosane and squalene occurred in higher amounts in the cuticular extract of parasitized eggs of *C. elongata* than that of parasitized eggs of *C. tomentosicollis* and contributed largely to the separation of the two species. PCA analysis confirmed that most of the compounds were highly concentrated in the cuticular extract of unparasitized eggs than in cuticular extract of parasitized eggs of *C. tomentosicollis* but the contrary was observed for the cuticular egg extract of *C. elongata* (Figure 6).

4 | DISCUSSION

Field observations showed that *C. tomentosicollis* was the most common and abundant of the three *Clavigralla* spp. in Kenya and Bénin. *Clavigralla elongata* was recorded only in Kenya and *C. shadabi* at Djidja and Ketou in Bénin. The common and widespread occurrence of *C. tomentosicollis* in these different regions of Africa supports previous reports that these species occur in both east and west Africa (Dolling, 1979; Agunbiade et al., 2013; Chalam et al., 2016). Shanower et al. (1999) also reported *C. tomentosicollis* as a common species and *C. shadabi* as a pest of minor importance in Bénin and Nigeria. Our results support those by Dreyer and Baumgärtner (1996) which reported *C. tomentosicollis* and *C. shadabi* on cowpea in Bénin. In this study, *C. tomentosicollis* and *C. shadabi* were recorded throughout Bénin, confirming reports of its distribution across different agro-ecological zones (Dreyer et al., 1994; Agboton et al., 2014). Moreover, *C. shadabi* which was reported by Gethi and Khaemba (1991) in Mombasa, Kenya, was not recorded during this study, possibly due to geographical and seasonal differences, as well as the limited sampling that was done in Kenya. Nonetheless, our observation corroborates previous results which reported *C. tomentosicollis* and *C. elongata* on pigeon pea, hyacinth bean, gram and cowpea in Kenya (Khamala, 1978). Similar results were reported by Materu (1972) in Tanzania.

Gryon sp. was the only parasitoid species that emerged from *C. tomentosicollis* eggs in this study. We were not able to identify this egg parasitoid to species level because of the lack of reliable identification tools. Similar results were reported by Gariepy, Haye, and Zhang (2014) for some Scelionidae (*Telonomus* sp., *Trissolcus* sp.) and egg parasitoids of *Acrosternum hilare* Say (Hemiptera: Pentatomidae). Since that time, the generation of a DNA barcode database for *Trissolcus* Ashmead (Hymenoptera: Scelionidae) has enabled the development of molecular diagnostics that enable species to be identified based on the DNA barcode region.

TABLE 3 Results of *Gryon* sp. sequences data analyses

Sample name	ID from GenBank	Accession no.	Query %	E value	ID %
GRB-1-D2	*Gryon* sp. CT-2012b voucher OSUC 266,775 28S ribosomal RNA gene, partial sequence	JX683193.1	76	0	99
GRB-3-D2	*Gryon* sp. CT-2012b voucher OSUC 266,775 28S ribosomal RNA gene, partial sequence	JX683193.1	76	0	99
GRB-5-D2	*Gryon* sp. CT-2012b voucher OSUC 266,775 28S ribosomal RNA gene, partial sequence	JX683193.1	76	0	99
GRK-8-D2	*Gryon* sp. CT-2012b voucher OSUC 266,775 28S ribosomal RNA gene, partial sequence	JX683193.1	76	0	99
GRK-9-D2	*Gryon* sp. CT-2012b voucher OSUC 266,775 28S ribosomal RNA gene, partial sequence	JX683193.1	76	0	99
GRK-10-D2	*Gryon* sp. CT-2012b voucher OSUC 266,775 28S ribosomal RNA gene, partial sequence	JX683193.1	76	0	99

Note: Abbreviations: GRB, *Gryon* from Bénin, GRK, *Gryon* from Kenya

This study showed that *Gryon* sp. seemed to be common in both Bénin and Kenya. This observation is consistent with previous studies, which showed that *G. fulviventris* was the most abundant egg parasitoid of *Clavigralla* spp. in northern Nigeria (Asante et al., 2000) compared to *O. utetheisae* and *Anastatus* sp. which were not recorded during this study. Previously, *G. gnidus* and *G. clavigrallae* have been reported as *Clavigralla* spp. egg parasitoids in Nigeria, Asia and India, respectively, and showed lower parasitism rates than that of *G. fulviventris* (Taylor, 1975; Shanower et al., 1996; Romeis, Shanower, & Madhuri, 2000). As reported from previous studies on *Gryon* spp., field parasitism rates can range from 69% to 74% (Taylor, 1975; Shanower et al., 1996; Asante et al., 2000). For example, the egg masses collected on cowpea in Nigeria showed that *G. fulviventris* parasitized up to 74% *C. tomentosicollis* eggs (Asante et al., 2000). Similar results have been reported for *G. gnidus* and *G. clavigrallae* showing that field parasitism rates of egg masses can reach 69% (Taylor, 1975; Shanower et al., 1996). Thus, although these observations suggest that *Gryon* sp. may be the principal and most common egg parasitoid of *Clavigralla* spp. in West Africa, future studies need to determine whether field parasitism rates of *Gryon* sp. in Benin and Kenya agree with this range (69%–74%).

Our results also showed that *Gryon* sp. parasitized higher numbers of *C. tomentosicollis* eggs than *C. elongata* eggs under laboratory conditions. This result confirms not only our field observations but also the parasitism rate of *G. fulviventris* reported in Nigeria on *C. tomentosicollis* eggs (Asante et al., 2000), and that of *G. clavigrallae* on *Clavigralla scutellaris* Spinola, and *C. gibbosa* (Westwood) (both Hemiptera: Coreidae) eggs in India (Romeis et al., 2000). This finding suggests that *C. tomentosicollis* eggs may contain contact chemical cues for parasitism by the parasitoid.

Chemical analysis showed significant quantitative differences in the profiles of unparasitized and parasitized eggs dominated by alkanes, which varied within and between the two species. Whereas these alkanes were upregulated in the cuticular profile

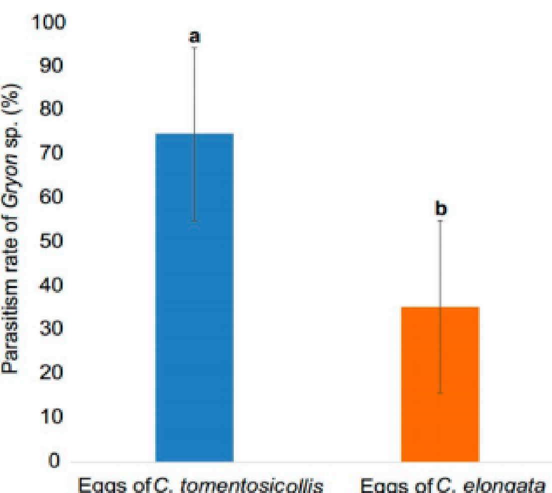


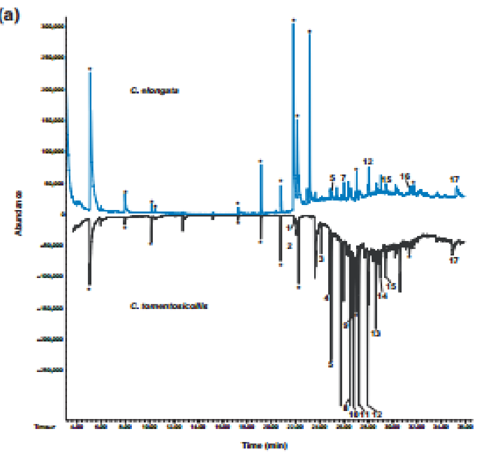
FIGURE 3 Comparison of parasitism rate of *Gryon* sp. ±SE (%) between *Clavigralla tomentosicollis* eggs and *Clavigralla elongata* eggs. Mean percentage was calculated for the parasitized eggs in five replications. Different letters "a" and "b" indicate significant differences

of unparasitized eggs of *C. tomentosicollis*, they were downregulated in that of the eggs of *C. elongata*. The opposite pattern was observed in the parasitized eggs which may account for the higher parasitism of *Gryon* sp. of *C. tomentosicollis* eggs than those of *C. elongata*. Additional research will be needed across a wide range of samples collected from different localities and seasons in Kenya and Benin to confirm this suggestion and to investigate the possible contribution of background egg volatiles in parasitoid attraction. A previous study showed that the high concentration of hydrocarbons extracted from *E. vittella* larvae influenced the foraging behaviour and parasitism of *T. brasiliensis* (Maruthadurai

TABLE 4 Compounds identified in pentane extracts of parasitized and unparasitized eggs of *C. tomentosicollis* and *C. elongata*

Peak #	t_R(min)	Compound	Category	Retention Index	Mean amount detected (ng/egg/min ± SE)			
					C. elong. unparasitized eggs	*C. tom.* unparasitized eggs	*C. elong.* parasitized eggs	*C. tom.* parasitized eggs
1	22.82	Hexadec-9-enoic acid	Fatty acid	1848	tr	0.27 ± 0.09	-	-
2	22.99	Hexadecanoic acid	Fatty acid	1864	tr	0.28 ± 0.00	-	-
3	25.15	Octadecane	Alkane	2091	tr	0.06 ± 0.02	0.06 ± 0.02	tr
4	26.01	Nonadecane	Alkane	2185	tr	0.08 ± 0.00	-	-
5	26.05	Eicosane	Alkane	2189	0.06 ± 0.02	0.07 ± 0.03	tr	0.06 ± 0.00
6	27.47	Unidentified alkene[a]	Alkene	2357	-	-	0.08 ± 0.02	0.06 ± 0.00
7	27.49	Unidentified alcohol[a]	Alcohol	2359	0.27 ± 0.00	tr	-	-
8	27.63	Tricosane	Alkane	2376	tr	0.09 ± 0.03	0.07 ± 0.02	0.06 ± 0.00
9	27.79	2,4-bis(1-methyl-1-phenylethyl)- phenol[a]	Phenol	2395	tr	0.28 ± 0.00	-	-
10	27.93	2,4-bis(dimethylbenzyl)-6-t-butylphenol[a]	Phenol	2413	tr	0.29 ± 0.09	-	-
11	28.39	Tetracosane	Alkane	2470	tr	0.09 ± 0.03	0.07 ± 0.02	0.06 ± 0.02
12	29.18	Pentacosane	Alkane	2572	0.06 ± 0.02	0.07 ± 0.02	0.06 ± 0.02	0.06 ± 0.02
13	29.86	Hexacosane	Alkane	2659	tr	0.08 ± 0.00	0.07 ± 0.02	0.07 ± 0.00
14	30.14	Squalene	Alkene	2694	tr	0.06 ± 0.02	0.08 ± 0.01	0.07 ± 0.00
15	30.72	Heptacosane	Alkane	2860	0.06 ± 0.02	0.06 ± 0.02	tr	0.07 ± 0.02
16	32.82	Triacontane[a]	Alkane	2952	0.06 ± 0.02	tr	0.06 ± 0.02	0.06 ± 0.02
17	36.85	Hentriacontane[a]	Alkane	3832	0.06 ± 0.02	tr	0.07 ± 0.03	0.07 ± 0.02

Note: Identification based on comparison of retention time (RT) with mass spectral library data and retention index.
Quantification was based on calibration curves (peak area vs. concentration) generated from authentic standards of identified compounds;
(−), Not detected, tr, trace, *C. tom.*, *Clavigralla tomentosicollis*, *C. shad.*, *Clavigralla shadabi*, *C. elong.*, *Clavigralla elongata*
[a]compounds identified based on mass spectral data only.



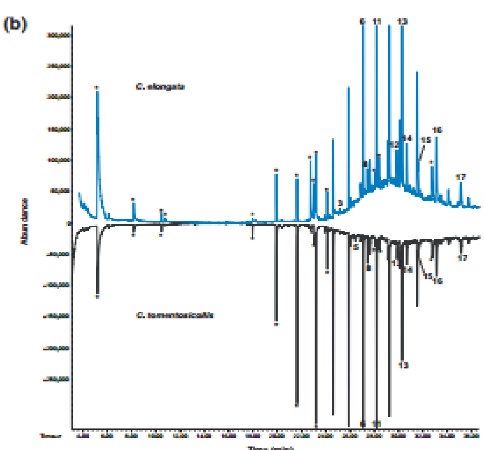


FIGURE 4 GC/MS profiles of cuticular extracts of unparasitized and parasitized eggs of *Clavigralla tomentosicollis* and *Clavigralla elongata*. (a) profiles of unparasitized eggs (above) and (b) profiles of parasitized eggs (below). Peak numbers indicate the identified compounds (Table 4); (*) means impurity

et al., 2011). Cuticular components extracted from pupa of *Lucilia sericata* Meigen (Diptera: Calliphoridae) and *Myrmecia gulosa* Fabricius (Hymenoptera: Formicidae) were found to be used for prey recognition (Dietemann, Peeters, Liebig, Thivet, & Hölldobler, 2003; Moore, Pechal, Benbow, & Drijfhout, 2017). Additionally, cuticular components have been implicated as important chemical cues used by trichogrammatids for location of their egg hosts (Paul, Srivastava, Dureja, & Singh, 2008). In the current study, hexadecenoic acid was identified as a specific compound in the cuticular extract of *C. tomentosicollis* unparasitized eggs. This compound was also reported by Michereff et al. (2016) to occur amongst the volatiles emitted by egg clusters of the stink bug *Euschistus heros* Fabricius (Heteroptera: Pentatomidae) as an attractant of the egg parasitoid *Telenomus podisi* Ashmead (Hymenoptera: Platygastridae). Our results suggest that cuticular chemistry of eggs of *Clavigralla* species may play a role in host location in parasitism of *Gryon sp*. Therefore, future study should explore the use of egg cuticular extracts to enhance *Gryon* sp. parasitism activity and management of *Clavigralla* spp.

Morphological and molecular tools were not able to identify this parasitoid (*Gryon* sp.) to species level. We identified cuticular chemicals compounds in the unparasitized eggs of *C. tomentosicollis*, which may play important roles in the host finding behaviour of *Gryon* spp. The importance of egg parasitoids in the management of *Clavigralla* species was previously highlighted in Nigeria and Tanzania using *Gryon gnidus*, *Ooencyrtus patriciae* Subba Rao (Hymenoptera: Encyrtidae) and *Ooencyrtus kuvanae* (How) (Hymenoptera: Encyrtidae) because of the high levels of combined egg parasitism (62%) observed (Matteson, 1981). Our results suggest that the parasitoid *Gryon* sp. could potentially be used as a biological control agent in the integrated management of *Clavigralla* spp. in Kenya and Bénin. It will also be important to evaluate the effect of the identified specific compounds associated with unparasitized eggs of *C. tomentosicollis* in the host location of *Gryon* sp. and to study the genetic diversity and identity of *C. tomentosicollis* from different regions of Africa.

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CONFLICT OF INTERESTS

Authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

HK, JVB, FK, MT and BT conceived and designed research. HK conducted experiments in and analysed data. HK, JVB, FK, MT and BT

FIGURE 5 Plots of proportion of seventeen cuticular components of *Clavigralla tomentosicollis* and *Clavigralla elongata* eggs using principal component (PCA) analysis. Solid lines indicate specific component present in cuticular extract of unparasitized and parasitized eggs of *Clavigralla tomentosicollis* and *Clavigralla elongata*. (a) unparasitized eggs and (b) parasitized eggs

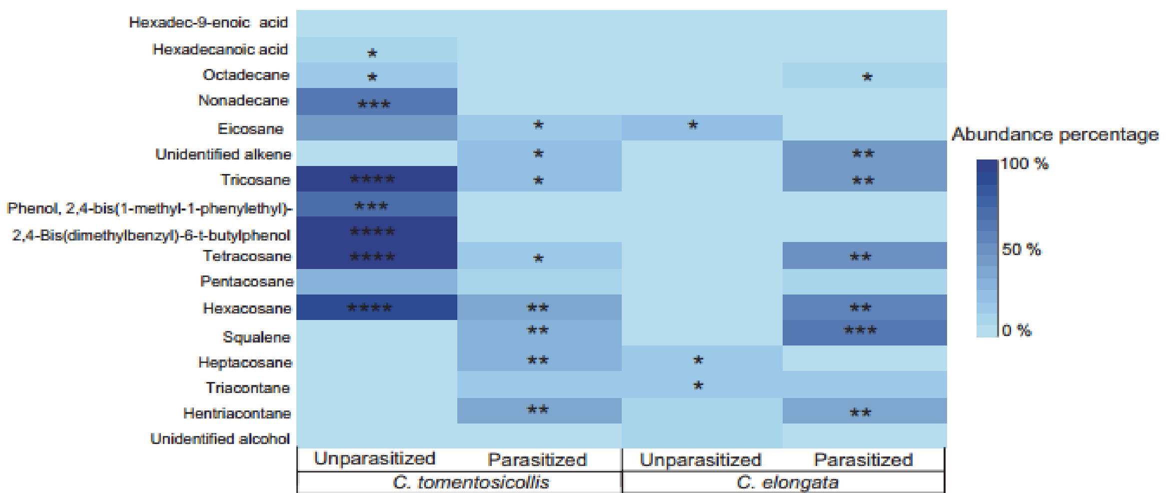

FIGURE 6 Heat map showing compounds detected in cuticular extracts of unparasitized and parasitized eggs of *Clavigralla tomentosicollis* and *Clavigralla elongata*. The number of asterisk (*) indicates the level of the compound content. The letters A, B, C and D indicate the different types of *Clavigralla tomentosicollis* and *Clavigralla elongata* eggs used

wrote the manuscript. EJT gave us support morphological identification of *Gryon* sp. All authors edited the manuscript and approved the final version.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

DATA ACCESSIBILITY

Gryon sp. could be found in hol.osu.edu (deposit number: DPI_FSCA 00010233 and DPI_FSCA 00010182). Sequences data are available in GenBank with accession numbers (MK488003–MK488008). Raw chemical data are available in the Data S1 and S2.

ORCID

Hilaire Kpongbe https://orcid.org/0000-0003-1629-1060

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SUPPORTING INFORMATION

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CHAPTER 3: ARTICLE 2

ISOPENTYL BUTANOATE: AGGREGATION PHEROMONE OF THE BROWN SPINY BUG, *Clavigralla tomentosicollis* (HEMIPTERA: COREIDAE), AND KAIROMONE FOR THE EGG PARASITOID *Gryon* sp. (HYMENOPTERA: SCELIONIDAE)

Submitted for review to the **Journal of Chemical Ecology**

HILAIRE KPONGBE[1, 2], JOHNNIE VAN DEN BERG[2], FATHIYA KHAMIS[1], MANUELE TAMÒ[3] AND BALDWYN TORTO*[,1]

[1]*International Centre of Insect Physiology and Ecology (icipe), P.O.BOX 30772-00100, Nairobi, Kenya*

[2]*IPM-Program, Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa*

[3]*International Institute of Tropical Agriculture (IITA), 08 BP 0932 Tri Postal, Cotonou, Bénin*

*Corresponding author:

P.O. Box 30772-00100, Nairobi-Kenya; (Tel: +254 20 863 2000. Fax: +254 20 863 2001. Email: btorto@icipe.org, ORCID: 0000-0002-5080-9903)

3.1 ABSTRACT - The brown spiny bug, *Clavigralla tomentosicollis* Stål (Hemiptera: Coreidae) is a key pest of leguminous crops in many countries in Africa, causing significant yield losses especially in cowpea, pigeon pea and common beans. Although *C. tomentosicollis* uses olfaction to aggregate, little is known about the identity of the aggregation pheromone. This study aimed to identify the aggregation pheromone of *C. tomentosicollis* and to test its potential role in the behavior of its egg parasitoid, *Gryon* sp. In Y-tube olfactometer bioassays, only male volatiles strongly attracted both sexes of *C. tomentosicollis*. Coupled gas chromatography/electroantennographic detection (GC/EAD) and GC/mass spectrometry were used to identify antennally-active compounds from male volatiles. Antennae of both sexes detected identical components including a male-specific component, identified as isopentyl butanoate, which was also detected by antenna of the egg parasitoid. In olfactometer bioassays, both sexes of *C. tomentosicollis* and the egg parasitoid responded to isopentyl butanoate. These results suggest that isopentyl butanoate serves as an aggregation pheromone for both sexes of *C. tomentosicollis* and a kairomone to attract the parasitoid in the management of *C. tomentosicollis*.

Key Words-Aggregation pheromone, biological control, *Clavigralla tomentosicollis*, *Gryon* sp., kairomone.

3.2 INTRODUCTION

The brown spiny bugs (*Clavigralla* spp.) are key pests of leguminous crops including cowpea, pigeon pea and common bean in Africa, causing significant damage and yield losses of between 44 - 100% in these crops (Dabire et al. 2005; Aliyu et al. 2007; Dialoke et al. 2010). The high level of damage caused by a key species, *C. tomentosicollis,* is largely attributed to its aggregation behavior on host crops (Dabire et al. 2005). Both nymphs and adults of *C. tomentosicollis* aggregate, with aggregation indices varying between 1.61 and 2.30 depending upon the insect stage (Egwuatu and Taylor 1976, 1977; Dzemo and Asiwe 2010). This suggests that olfaction may play a role in the aggregation of this bug, which if identified may lead to its semiochemical management. Like most spiny bugs, the life cycle of *C. tomentosicollis* involves eggs (2 to 99, laid on pods or leaves), which hatch (7-10 days) into nymphs (five nymphal instars), with a total developmental time of 20-21 days before emerging into adults. In the laboratory, adults can live up to 161 days (Dzemo and Asiwe 2010).

Previous studies have shown that *C. tomentosicollis* can be managed using the egg parasitoids *Gryon gnidus* (Nixon), *Gryon clavigrallae* (Mineo), and *Gryon fulviventris* Crawford (all Hymenoptera: Scelionidae) (Taylor 1975; Dreyer1996; Asante et al. 2000). Additionally, it has been demonstrated in olfactometer assays that the male-produced pheromone of *C. tomentosicollis* attracts *G. fulviventris* (Sanou et al. 2019). However, in this study the male volatiles eliciting attraction in the parasitoid were not identified. These observations suggest that semiochemicals may also be

involved in parasitoid location of prey, which, if identified, could potentially be exploited to enhance parasitoid performance in the management of *C. tomentosicollis*.

The aim of this study was to investigate the olfactory basis of aggregation in *C. tomentosicollis* and to identify the aggregation pheromone. Additionally, we evaluated the responses of the egg parasitoid, *Gryon* sp. to the aggregation pheromone. To achieve this, we used behavioral, electrophysiological and chemical analyses.

3.3 MATERIALS AND METHODS

3.3.1 Insects. Eggs, nymphs and adults, of *C. tomentosicollis* were collected on French beans and pigeon pea in six counties in Kenya: Makueni (01°52.621" S, 037°42.793" E), Machakos (01°10.060" S, 037°27.287" E), Embu (00°40.532" S, 037°39.187" E; 00°44.847" S, 037°36.151" E), Kitui (01°18.155" S, 038°02.019" E), Nakuru (00°18.345" S, 035°59.224" E; 00°16.413" S, 036°07.172" E) and Kisumu (00°05.066" S, 034°52.478" E) in separate containers and then transferred to the laboratories of the International Centre of Insect Physiology and Ecology (*icipe*), Duduville campus, Nairobi, Kenya (01°63 13' 25.3" S, 36° 53' 49.2" E; 1600 m a.s.l.). They were reared on young healthy pods of French bean (*Phaseolus vulgaris*, Fabaceae) in cylindrical clear plastic cages (18 cm diameter x 6.5 cm high) (Foodmate 2 l, Kenpoly, Nairobi, Kenya) with ventilated lids. The cages were lined with paper towel to absorb excess moisture as well as bug excretions. Each cage contained five healthy fresh pods of French bean. Thirty to forty adults were introduced into cages using a fine brush and an aspirator. Batches of fifty eggs that were laid on the absorbent paper were transferred to new cages of the same dimensions every 48 hr, whereas any dead insects were discarded.

Egg batches collected from different localities in Kenya were incubated separately in sterile clear plastic cages (9.0 cm diameter x 4.5 cm height) (Foodmate 0.5 l, Kenpoly, Nairobi, Kenya) with ventilated lids. Emerged parasitoids, identified by molecular techniques to genus level as *Gryon* sp. (Khamis, pers. comm) were collected by means of an aspirator and introduced into a cage containing *C. tomentosicollis* eggs that were less than 48 hr old. Parasitoids were fed on droplets of a 10% honey solution. All the rearing was conducted at 25 ± 1 °C and 60 - 70% RH with a photoperiod of 12:12 hr (Light: Dark).

3.3.2 Olfactometer Assays. A Y-tube olfactometer (stem, 10 cm; arms, 23 cm each at 60° angle; internal diameter, 2.3 cm) was used to investigate the responses of adult males and females to volatiles released by conspecifics in a laboratory maintained at 25 ± 1 °C and 60 - 70% RH with a photoperiod of 12:12 hr (Light: Dark). A battery powered portable vacuum pump (assembled at the USDA/ARS-CMAVE, Gainesville, FL), was used to draw charcoal-purified clean air over odor sources before it entered the Y-tube. Airflow through each of the olfactometer arms was set at 174 ml min^{-1} (combined flow 348 ml min^{-1}).

The following odor treatment combinations were tested: (1) blank vs. blank, (2) male vs. blank, (3) female vs. blank, and (4) male vs. female. To account for the possible effects that volatile sources could have on insect response, all tests were conducted using a group of 5, 10 and 20 adult individuals of the same sex, respectively as odor sources. Thirty 7-8-day old adults (27-28 days old after hatching) (30 replicates) of each sex were tested and the odor source was changed after 10 replicates. The position of the arms containing treatment and control odors was

changed after every five tested individuals to avoid any positional bias. The Y-tube was cleaned with liquid soap and water, rinsed with acetone and then with distilled water and dried in an oven at 100 °C for 3 hr after every five replicates.

One male or female was introduced into the Y-tube via the entrance of the stem, and the choice made by the individual recorded after 10 min. When an individual entered one of the arms, its response was recorded. When the insect moved further than 5 cm into one of the arms within a period of 10 min and spent at least 30 sec in the arm of the olfactometer, it was considered a valid choice. However, if the insect exited the arm before moving 5 cm into the selected arm and then moved to the other arm and spent at least 30 sec there, the latter was considered as its choice. All the experiments were conducted between 09:00 and 16:00 which correspond to the period when adults are most active in the field (Dreyer et al.1994).

3.3.3 Collection of Volatiles. The headspace volatile collection system used was the same as that described by Njihia et al. (2017) for collecting volatiles from mature green berries. Three quick-fit glass jars (250 ml each) (Sigma Scientific, Gainesville, FL, USA)), containing either sexually mature males (20; 7-8 days old), females of similar age or no insects (control) were used. No food was provided inside the containers. Charcoal-purified air at a flow rate of 260 ml min^{-1} was passed over the jar containing the insects and through a previously cleaned Porapak Q filter (30 mg, mesh size 80–100, Supelco, Bellefonte, PA), for 24 hr. Volatiles adsorbed on the Porapak Q filters were each eluted with 200 μl dichloromethane (Analytical grade, Sigma Aldrich, St. Louis, MO) and then stored at -80 °C until use.

3.3.4 Chemical Analyses. Samples were analyzed by coupled gas chromatography/electroantennographic detection (GC/EAD) analysis using antennae of *C. tomentosicollis* males and females (7-8 days old adult) as well as those of *Gryon* sp. The GC/EAD used a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with a HP-5 MSI column (30 m x 0.25 mm diameter x 0.25 μm film thickness, (Agilent, Technologies, Inc., California, USA), with nitrogen as the carrier gas at a flow rate of 1.2 ml min^{-1}. Volatiles were analysed in the splitless mode at an injector temperature of 280 °C and a split valve delay of 3 min. The oven temperature was held at 35 °C for 3 min, programmed at 10 °C min^{-1} to 280 °C and maintained at this temperature for 10 min. The column effluent was split 1:1 for simultaneous detection by a flame ionization detector (FID) and EAD. The antennal preparation was made by filling in two sharpened glass capillaries with Ringer saline solution (Kugel 1977). One of the capillaries was inserted into the excised head/pro-thorax. The distal end of the antenna was then placed in a saline filled electrode. The antennal signal was detected through an amplifier (Syntech, Hilversum, The Netherlands), which was acquired and processed by IDAC-4 data acquisition controller (Syntech, Hilversum, The Netherlands) and later analysed with an EAG 2000, GC/EAD software (Syntech) to generate simultaneous FID and EAD signals on a computer. Aliquots (3 μl) of volatile samples and commercially-purchased synthetic EAD-active compounds dissolved in dichloromethane were analysed. EAD responses were considered positive when three or more positive responses to the same sample were recorded.

Male, female and control volatiles (1 μl each), were analyzed by coupled gas chromatography/mass spectrometry (GC/MS) on an Agilent Technologies Inc. Series

A 7890 GC coupled to a 5977A MS (inert XL/EI/CI MSD) triple axis mass detector, equipped with a HP-5 low bleed capillary column (30 m × 0.250 mm i.d., 0.25 μm) (J&W, Folsom, CA, USA) in the electron impact mode at 70 eV. The GC oven temperature was 35 °C for 5 min with a rise of 10 °C min^{-1} to 280 °C for 10.5 min, then 5 °C min^{-1} to 285 °C and held at this temperature for 9 min. Identification of compounds was done by comparison of mass spectral data with library data; Adams2, Chemecol and NIST11. Additionally, the identities of several compounds were confirmed using mass spectral data and retention times of synthetic compounds where available. Quantification was based on calibration curves (peak area vs. concentration) generated from authentic standards of identified compounds. The experiments were replicated three times.

3.3.5 Olfactometer Assays with Male-specific Compounds. Y-tube olfactometer assays described earlier were used to evaluate EAD-active compounds. The responses of both sexes of *C. tomentosicollis* and the parasitoid *Gryon* sp. were evaluated in the assays. In the bioassays with the parasitoid, airflow through each of the olfactometer arms was maintained at 85 ml min^{-1}. The female parasitoids used in bioassays were 1-2 days old. The tests conducted were: (1) solvent vs. solvent, (2) isopentyl butanoate vs. solvent. The naturally-occurring dose of isopentyl butanoate, 152 ng μl^{-1}, was tested after which higher and lower concentrations of 304 ng μl^{-1} (obtained by doubling the natural dose) and 76 ng μl^{-1} (half the natural dose) were used to test the response of females and males of *C. tomentosicollis*, and that of *Gryon* sp. The natural release rate of isopentyl butanoate by one insect in an hour is 63.3 ng h^{-1}. Ten microliters (10 μl) of sample equivalent to each concentration were applied on to 2 cm

x 2 cm pieces of filter paper (Whatman filter Nº1) by means of a syringe and air dried for 30 sec. Controls consisting of dichloromethane (10 µl) were prepared similarly. Control and treated filter papers were then placed separately into the olfactometer arms. The treated filter paper was changed after every five replicates.

3.3.6 Chemicals. 2-methylbutanoic acid, isopentyl butanoate, 2-methylbutyl 3-methylbutanoate, 2-methylbutyl 2- methylbutanoate were all purchased from Sigma Aldrich, Germany, with purity ≥ 98%. Dichloromethane, 2-methylpropanoic acid, 6-methyl-5-hepten-2-one, limonene, acetophenone were all purchased from Sigma Aldrich, Germany (purity ≥ 95%).

3.3.7 Data Analyses. All statistical analyses were performed in R software version 3.1.2 (R Core Team 2012) at 5% significance level. Chi-square (χ^2) analyses were used to determine (1) the significant difference of *C. tomentosicollis* male and female choices for conspecific volatiles against the respective controls and between volatiles of the different sexes, (2) the comparative response of male and female of *C. tomentosicollis* and egg parasitoid *Gryon* sp. to the three different doses of isopentyl butanoate against the respective controls. The total number of replicates used in these bioassays was 30 per experiment but only the respondents (n) were considered in the analysis.

3.4 RESULTS

3.4.1 Olfactometer Assays. The responses of males and females to the control treatments in the Y-tube were not significantly different ($P > 0.05$). Both males and females were significantly attracted to volatiles of the group of twenty males compared

to the control ($P < 0.05$) (Fig. 1C). In paired assays, both sexes were more attracted to odors released from the group of twenty males than to the group of twenty females ($P < 0.05$) (Fig. 1C). There were no significant differences between male and female responses when tested against odors from the group of 20 females and the control ($P > 0.05$). Likewise, no preference was observed in these experiments (except a response of female) when males and females were provided with a choice between the odors from a group of five or ten males or females ($P > 0.05$) (Figs.1A &B). Nonetheless, females were strongly attracted to the volatiles from the group of ten males compared to the control treatment ($P < 0.05$) (Table 1).

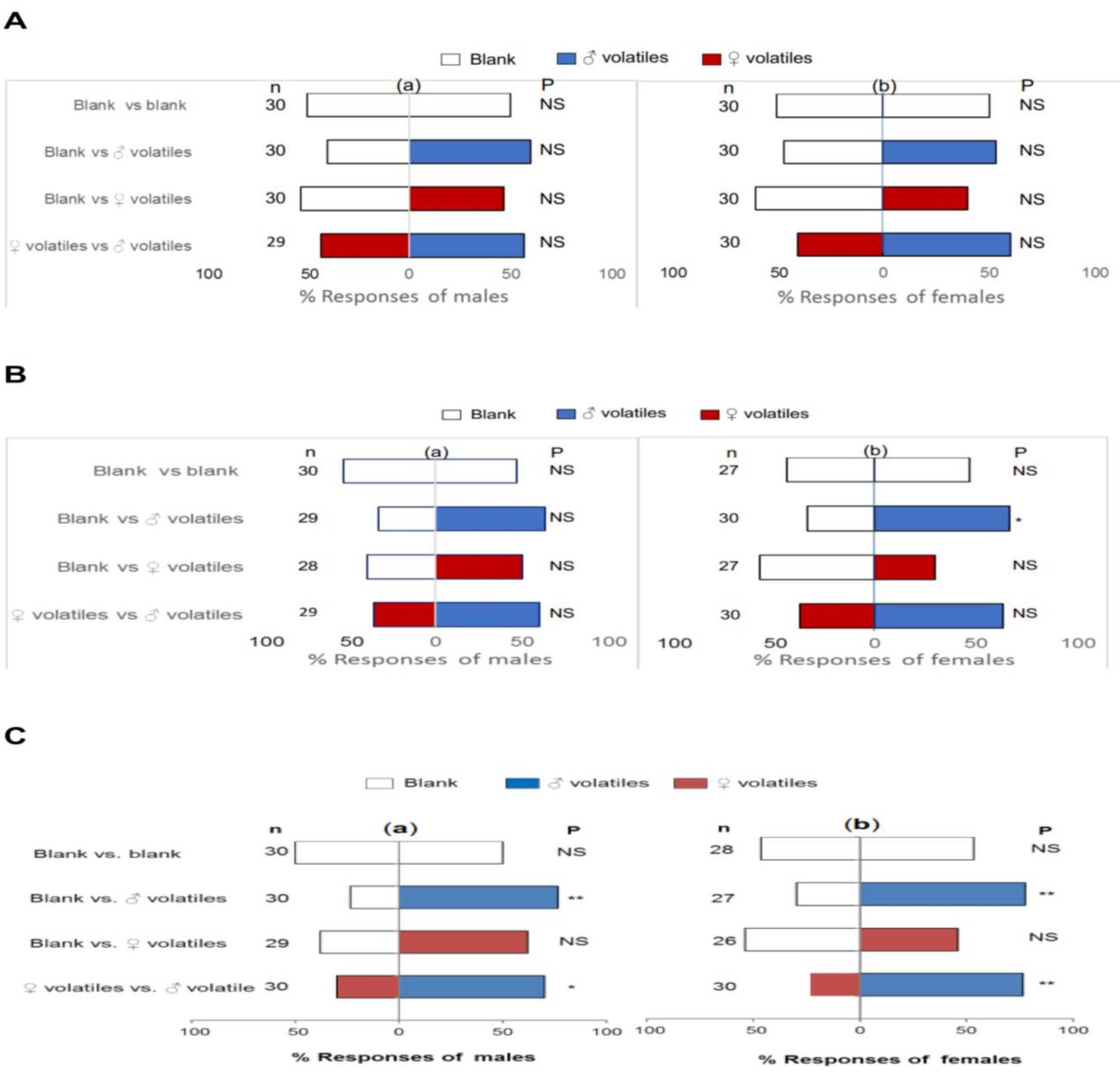

Fig. 1 Olfactometer responses of *C. tomentosicollis* males and females to their conspecific volatiles. A = group of 5 individuals, B = group of 10 individuals, C = group of 20 individuals. (**a**) = responses of males, (**b**) = responses of females. Thirty adults' males/females (7-8 days old) were tested individually for choice between blank and odors from a group of twenty conspecific males or females. Asterisks indicate significant difference levels: *$P < 0.05$, **$P < 0.01$. n = number of choices, P = probability, NS = non-significant.

TABLE 1 BEHAVIORAL RESPONSES OF *Clavigralla tomentosicollis* MALES AND FEMALES SUBJECTED TO CONSPECIFIC VOLATILES OF THREE GROUPS OF DIFFERENT SIZES

		Male choice			**Female choice**		
Different groups	Tests	χ^2	*df*	*P*-value	χ^2	*df*	*P*-value
	Blank vs. Blank	0	1	1	0.1333	1	0.715
Group of 5 individuals	Males vs. Blank	1.6897	1	0.193	0.0344	1	0.852
	Female vs. Blank	0.3103	1	0.577	1.2	1	0.273
	Male vs. Female	0.5333	1	0.465	0.5714	1	0.449
Group of 10 individuals	Male vs. Blank	1.96	1	0.161	3.8462	1	0.049
	Female vs. Blank	0.1666	1	0.683	2.4615	1	0.116
	Male vs. Female	0.6666	1	0.414	1.8148	1	0.177
Group of 20 individuals	Male vs. Blank	8.5333	1	0.003	4.4815	1	0.034
	Female vs. Blank	1.6897	1	0.193	0.1538	1	0.694
	Male vs. Female	4.8	1	0.028	8.5333	1	0.003

χ^2 = Chi-square, df = Degrees of freedom

3.4.2 Chemical Analyses. Chemical analysis of the headspace volatiles of *C. tomentosicollis* males and females showed similar profiles (Fig. 2). However, differences were both quantitative and qualitative, with the identification of a male-specific component identified as isopentyl butanoate and confirmed with an authentic standard (Fig. 2). Common components identified in male and female volatiles are indicated in Table 2. The identities of seven of these components; 6-methyl-5-hepten-2-

one, acetophenone, limonene, isopentyl butanoate, 2-methylbutyl 2-methylbutanote, 2-methylbutyl 3-methylbutanoate, 2-methylbutanoic acid, were confirmed using authentic standards, with the others by mass spectral library data only. The retention times and mass spectra of compounds identified, and authentic standards used for confirmation were exact matches.

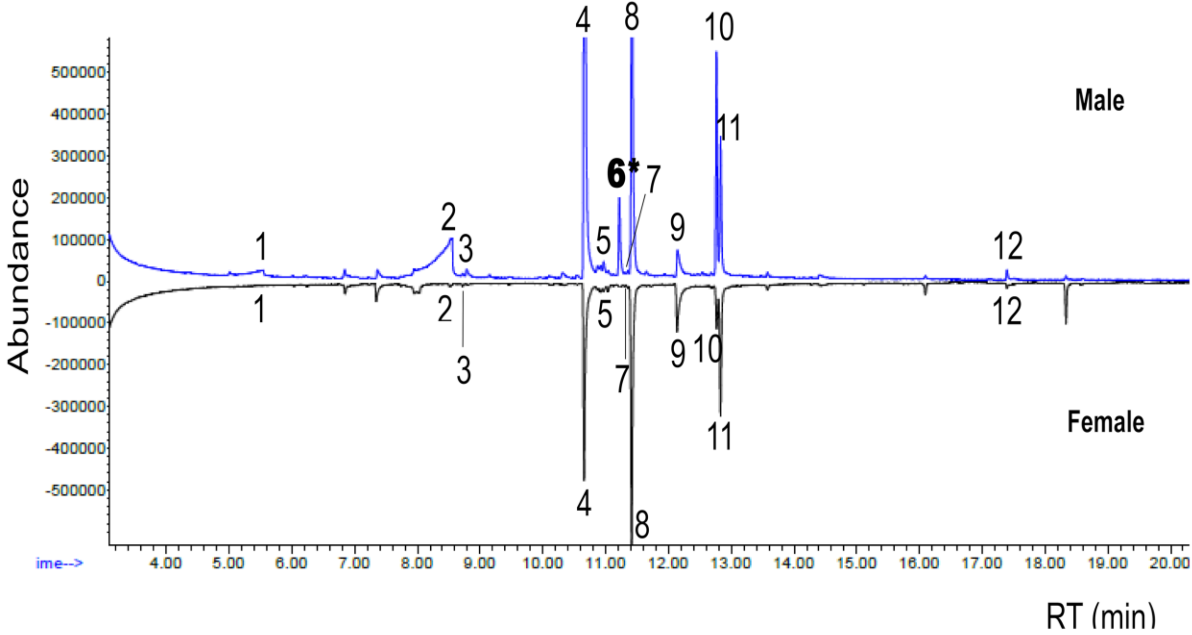

Fig. 2 Chemical profiles of *C. tomentosicollis* male and female volatiles showing male-specific compound, **isopentyl butanoate*** in bold, RT = retention time

TABLE 2 COMPOUNDS DETECTED BY MEANS OF GC-MS ANALYSIS OF HEADSPACE VOLATILES OF *Clavigralla tomentosicollis* MALES AND FEMALES

Peak #	Retention time (min)	Compounds	Category	Retention index	Male	Female
1	5.52	2-Methyl propanoic acid	Aliphatic acid	768	+	trace
2	8.41	2-Methyl butanoic acid	Aliphatic acid	882	+	+
3	8.86	Nonane	Hydrocarbon	900	+	+
4	10.66	6-Methyl-5-Hepten-2-one	Ketone	980	+	+
5	10.97	Isobutyl 2-methylbutanoate	Ester	993	+	trace
6*	**11.22**	**Isopentyl butanoate***	**Ester**	**1012**	**+**	**-**
7	11.35	*p*-Cymene	Monoterpene	1013	+	trace
8	11.42	Limonene	Monoterpene	1017	+	+
9	12.14	Acetophenone	Ketone	1057	+	+
10	12.76	2-Methyl butyl 2-methylbutanoate	Ester	1090	+	+
11	12.83	2-Methyl butyl 3-methylbutanoate	Ester	1097	+	+
12	17.40	2-Epi-alpha-funebrene	Monoterpene	1409	+	+

A group of 20 C. tomentosicollis males/females (7 to 8 days old adults) was used for volatile collection.

*(+) = present = (-) absent, (**6***) = male-specific compound identified, and which elicited electrophysiological responses in males and females of C. tomentosicollis and Gryon sp.*

In GC/EAD assays, the male-specific compound and its authentic standard, isopentyl butanoate, elicited antennal detection in both sexes of *C. tomentosicollis* and females of the parasitoid *Gryon* sp. (Fig. 3).

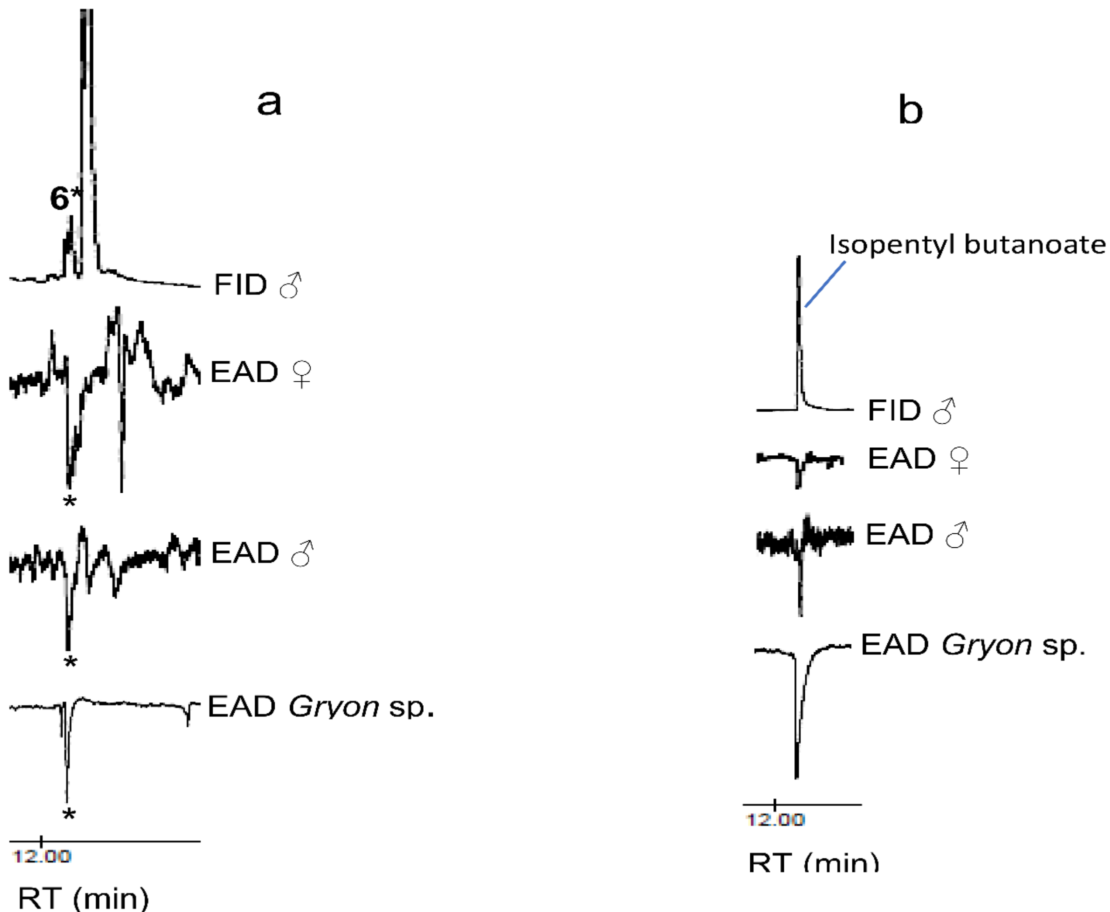

Fig. 3 Electroantennograms showing the male-specific compound, isopentyl butanoate (**6***) detected by *C. tomentosicollis* male and female, and parasitoid in the male crude volatiles. (**a**) and synthetic standard identified from male adult volatiles (**b**). RT = retention time.

3.4.3 Olfactometer Assays with Male-specific Compound. The olfactory responses of *C. tomentosicollis* males and females and the parasitoid *Gryon* sp. to the solvent controls

were not significantly different (P > 0.05) (Fig. 4 a, 4b and 4c). Dose-response assays using the male-specific compound isopentyl butanoate showed that males and females were significantly attracted to similar concentrations (P <0.05) (Fig. 4a & 4b). Furthermore, only females and the parasitoid were significantly attracted to isopentyl butanoate at the lower dose of 76 ng μl^{-1} (equivalent to half the naturally-occurring concentration) (P < 0.05) (Fig. 4 b). The parasitoid *Gryon* sp. was attracted to the naturally-occurring concentration of isopentyl butanoate (Fig. 4 c), but not to the higher concentration (304 ng μl^{-1}) (P > 0.05) (Table 3).

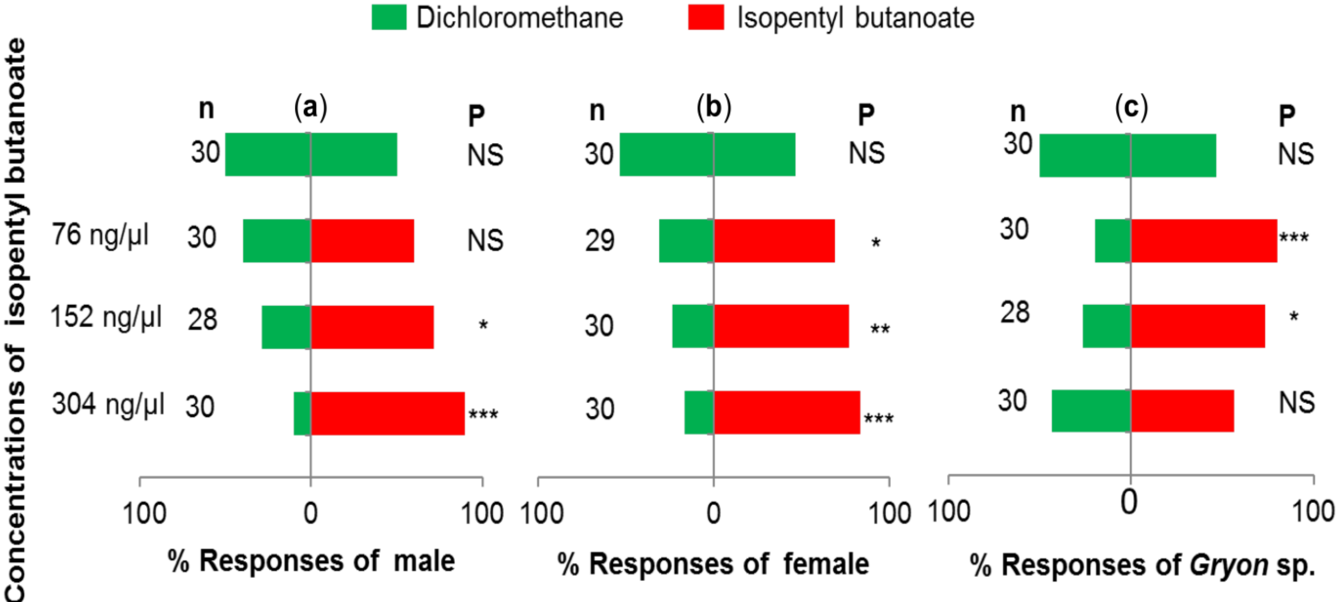

Fig. 4 Olfactometer responses of male and female *C. tomentosicollis* and *Gryon* sp. to different doses of the synthetic compound isopentyl butanoate. (**a**) = responses of males, (**b**) = responses of females and (**c**) = responses of *Gryon* sp. Thirty *C. tomentosicollis* adults' males/females (7-8 days old) and thirty females *Gryon* sp. (1-3 days old) were tested individually for choice between three doses of isopentyl butanoate solution and dichloromethane, Asterisks means significant difference levels: *P < 0.05, **P < 0.01, ***P < 0.001. n = number of choices, P = probability, NS = non-significant.

TABLE 3 *Gryon* sp. AND *Clavigralla tomentosicollis* MALE AND FEMALE BEHAVIORAL RESPONSES TO DIFFERENT DOSES OF ISOPENTYL BUTANOATE IN OLFACTOMETER BIOASSAYS

	Male choice			Female choice			*Gryon* sp. choice		
Treatments	χ^2	*df*	*P*-value	χ^2	*df*	*P*-value	χ^2	*df*	*P*-value
Dichloromethane vs. Dichloromethane	0.133	1	0.715	0	1	1	0.034	1	0.852
Concentration 1 vs. Dichloromethane	1.2	1	0.273	4.172	1	0.041	10.8	1	0.001
Concentration 2 vs. Dichloromethane	5.142	1	0.023	8.533	1	0.003	6.533	1	0.010
Concentration 3 vs. Dichloromethane	19.2	1	< 0.001	13.33	1	< 0.001	0.533	1	0.465

χ^2= Chi-square, df = Degree of freedom. Concentration 1, 2 and 3 are the different concentrations used (concentration 1 = 76 ng μl^{-1}*, concentration 2 =* 152 ng μl^{-1}*, concentration 3 =* 304 ng μl^{-1}*)*

3.5 DISCUSSION

Our results showed that *C. tomentosicollis* males and females were strongly attracted to the volatiles released by males, indicating that males release olfactory cues that act as an aggregation pheromone for both sexes. This result agrees with previous studies on certain Hemipterans (e.g. *Plautia stali*, *Triatoma infestans*) which showed differential attraction of both sexes to male odors (Guerenstein and Guerin 2004; Jang et al. 2011). Moreover, a study on *Pristhesancus plagipennis* Walker (Hemiptera: Reduviidae) showed that both sexes were attracted to volatiles released from the male dorsal

abdominal glands (James et al. 1994). Although it is less common in hemipterans for females to produce the aggregation pheromone, in *Neomegalotomus parvus* Westwood (Hemiptera: Coreidae), females were found to produce an aggregation pheromone which attracted both sexes (Laumann et al. 2012). These results indicate that the production and release of aggregation pheromones in hemipterans is both species- and sex-dependent.

Our results also show that aggregation pheromones are only effective when a critical number of individuals are present. In the present study, we found that the volatiles released by 20 males acted as an aggregation pheromone for both sexes. This result corroborates a previous study which showed that the aggregation response of both sexes of the southern chinch bug, *Blissus insuhris* Barber (Hemiptera: Lygaeidae) to conspecific volatiles increased with increasing number of the bugs (Addesso et al. 2012).

On the other hand, we found that females responded more strongly to male volatiles when provided with a choice of ten males and control. This suggests that male odors may also serve as a potential sex pheromone for females. A previous finding showed that females of *Pellaea stictica* Dallas (Hemiptera: Pentatomidae) were attracted to male volatiles (Fávaro et al. 2015). Likewise, females of *Edessa meditabunda* Fabricius (Hemiptera: Pentatomidae) are attracted to the male volatiles (Zarbin et al 2012).

Chemical analysis showed both quantitative and qualitative differences in the emission profiles of male and female volatiles. The major compounds identified included ketones, monoterpenes, esters and aliphatic acids. These classes of compounds are

commonly associated with many insect Orders including the Hemiptera (Aldrich 1975; James et al. 1994; Guerenstein and Guerin 2004; Audino et al. 2007; Yusufoglu et al. 2018). A previous study reported the presence of isopentyl butanoate in the volatiles of male *Triatoma infestans* Klug (Hemiptera: Reduviidae), but its role in the behavior in this insect was not established (Audino et al. 2007). In our GC/EAD analysis of male volatiles, antennae of both sexes of *C. tomentosicollis* detected similar components, including the male-specific compound isopentyl butanoate. Interestingly, isopentyl butanoate was also detected by the egg parasitoid *Gryon* sp. The fact that both sexes of *C. tomentosicollis* and the egg parasitoid detected isopentyl butanoate suggests that it may play a role in the aggregation/sex communication observed with the living insects and host location by the parasitoid. Other components detected in the male volatiles may play a role, for example, in enhancing intra-and inter-specific communication when combined with or without isopentyl butanoate, which would require additional research.

In the olfactometer studies with isopentyl butanoate, as found with the odors of living insects, male and female responses were dose dependent, with both sexes responding to the naturally-occurring concentration of the compound released by males in their emission profile. The fact that only females responded to half the naturally-occurring concentration of isopentyl butanoate agrees with our previous results with odors of living insects and confirms the dual role that male volatiles play in the behavior of both sexes of *C. tomentosicollis*; as an aggregation pheromone for both sexes and sex pheromone for females depending upon the concentration. It is also noteworthy that at higher emission profiles with living insects and concentration of isopentyl butanoate, both sexes of *C. tomentosicollis* were attracted, which suggests that in the behavioral

context, isopentyl butanoate could be used as an attractant/aggregation cue in both sexes and mating recognition for females of this insect. Additionally, isopentyl butanoate has also been identified as a component of some plant volatiles. For example, it has been demonstrated that mashed banana volatiles containing isopentyl butanoate attracted both males and females of the scarab beetle *Pachnoda interrupta* Olivier (Coleoptera: Scarabaeidae) (Wolde-Hawariat 2008). Future studies should explore whether host plants of *C. tomentosicollis* also contain isopentyl butanoate.

In our study, the parasitoid *Gryon* sp. responded to isopentyl butanoate in a dose-dependent manner; responding strongly to the naturally-occurring concentration and half of the concentration of isopentyl butanoate, but not at higher concentrations, as observed for females of the host *C. tomentosicollis*. These results suggest that responses shown by both the parasitoid and females of the host are ecologically relevant. At low doses, whereas females may utilize this compound for sex attraction, the parasitoid on the other hand may associate detection of the compound with both the presence of females and their eggs. It is possible that egg-associated chemicals (volatiles and cuticular components) may contribute to parasitoid location of eggs, which would require further studies. Nonetheless, our results also suggest that the olfactory systems of females of *C. tomentosicollis* and the parasitoid are fine-tuned to detect isopentyl butanoate. Indeed, a recent study showed that the volatiles from a group of *C. tomentosicollis* males were attractive to *G. fulviventris* females when they were given a choice between male volatiles and control/host plant (cowpea) volatiles (Sanou et al. 2019). Our results indicate that isopentyl butanoate could be the male-produced aggregation pheromone eliciting attraction in *G. fulviventris* females. It would be

interesting to investigate whether different species of *Gryon* utilize the same or different pheromones to locate *C. tomentosicollis* and other related hemipterans. This finding lends support to previous studies which reports the use of prey volatiles by hymenopterans to locate their hosts (Yasuda 1998; Aldrich and Zhang 2002; Fatouros et al. 2008; Maruthadurai 2011). For example, in field evaluation studies of male and female volatiles of *Leptoglossus australis* Fabricius (Heteroptera: Coreidae), only traps baited with male volatiles attracted the parasitoid *Gryon pennsylvanicum* Ashmead (Hymenoptera: Scelionidae) (Yasuda 1998). In similar field studies, the parasitoid *Telenomus calvus* Johnson (Hymenoptera: Platygastridae) showed a clear preference for male volatiles of *Podisus maculiventris* Say (Hemiptera: Pentatomidae) (Fatouros et al. 2008). Furthermore, the egg parasitoid *Trissolcus brochymenae* Ashmead (Hymenoptera: Platygastridae) was found to exploit male volatiles of *Murgantia histrionica* Hahn (Hemiptera: Pentatomidae) as an attractant to locate females and to find their eggs (Conti et al. 2003).

In summary, we showed that olfaction plays a key role in the aggregation of *C. tomentosicollis*. We identified several classes of compounds in both sexes of *C. tomentosicollis*, with the ester isopentyl butanoate as specific to male volatiles. Furthermore, we identified isopentyl butanoate as an aggregation pheromone for both sexes of *C. tomentosicollis* and a kairomone for the egg parasitoid *Gryon* sp. These results, therefore, provide the fundamental baseline information required for field evaluations of isopentyl butanoate in the management of *C. tomentosicollis*.

3.6 AUTHOR CONTRIBUTIONS

HK, JVB, FK, MT, and BT conceived and designed research. HK conducted experiments in and analyzed data. HK, JVB, FK, MT, and BT wrote the manuscript. EJT gave us support morphological identification of *Gryon* sp. All authors edited the manuscript and approved the final version.

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3.8 *Compliance with ethical standards*. Authors declare no conflict of interest.

3.9 Ethical approval. This article does not contain any studies with human participants or animals performed by any of the authors.

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CHAPTER 4: ARTICLE 3

Chemistry of headspace volatiles and genetic variability of *Clavigralla* species (Hemiptera: Coreidae) in Kenya and Bénin

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Hilaire Kpongbe[1,2*], Fathiya Khamis[1], Villinger Jandouwe[1], Johnnie Van den Berg[2], Manuele Tamò[3] and Baldwyn Torto[1]

[1]International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya (hkpongbe@icipe.org; fkhamis@icipe.org; jandouwe@icipe.org; btorto@icipe.org),

[2]Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa (johnnie.vandenberg@nwu.ac.za),

[3]International Institute of Tropical Agriculture, Abomey-Calavi, Bénin (m.tamo@cgiar.org).

[1*]Corresponding author

P.O. Box 30772-00100, Nairobi-Kenya; (Tel: +254 720901973. Email: hkpongbe@icipe.org, ORCID: **0000-0003-1629-1060**)

4.1 Abstract

The production of leguminous crops continues to decline in West and East Africa particularly in Bénin and Kenya. This decline is partly ascribed to damage by the complex of pod-sucking bugs, *Clavigralla* spp. (Hemiptera: Coreidae). Apart from the feeding damage, *Clavigralla* species are also a major contributor in the loss of seed viability. The *Clavigralla* complex is distributed throughout Africa. They have a wide host range, especially the Leguminosae (Fabacae), including cowpea and common beans. This wide host range diversity could induce genetic variability and chemical profile variation that make *Clavigralla* spp. effective as herbivores of these plant species. This study aimed to identify the chemical profiles and establish the genetic variability of the three most important *Clavigralla* species collected in Bénin and Kenya. The *Clavigralla* spp. sequences generated from the populations used in the study showed very low variability (0.02), which is within acceptable ranges for population and species. However, the *Clavigralla tomentosicollis* Stål (Hemiptera: Coreidae) clustered in a distinct clade from *Clavigralla shadabi* Dolling and *Clavigralla elongata* Signoret (all Hemiptera: Coreidae), which were monophyletic. Coupled gas chromatography/mass spectrometry (GC/MS) analysis identified thirty-one components including common and specific components from headspace volatiles collected from these three species. A heat map generated from the chemistry of volatiles of the three *Clavigralla* spp. showed that the components were more abundant in *C. tomentosicollis* volatiles than *C. shadabi* and *C. elongata* with a close similarity between *C. tomentosicollis* and *C. elongata.*

Key words: Blast query, chemical profile, genetic variability, genetic distance

4.2 Introduction

Cowpea (*Vigna unguiculata* L. Walp), common bean (*Phaseolus vulgaris* L.) and pigeon pea (*Cajanus cajan* L. Millsp.) (all Fabales: Fabacae) are the most important leguminous crops cultivated and consumed in Africa (Tamò 1997, Batureine 2009). Annual cowpea production in West Africa is estimated at 5,833,904 tonnes while in Kenya production is estimated at 146,807 tonnes (FAOSTAT 2016). In Kenya cowpea production generates approximately USD 687.6/tonne annually however no static is available for the West African cowpea production (FAOSTAT 2016). Furthermore, 101,821 tonnes and 728,160 tonnes of beans dry production were reported in Bénin and Kenya respectively (FAOSTAT 2016) with an annual income of USD 794.7/tonne in Bénin (FAOSTAT 2011) and USD 744.2/tonne in Kenya (FAOSTAT 2016).

Yield losses of these legumes are often attributed to pest infestations, specifically by the legume pod borer *Maruca vitrata* Fabricius (Lepidoptera: Crambidae), the cowpea aphid *Aphis craccivora* Koch (Hemiptera: Aphididae), the flower thrips *Megalurothrips sjostedti* Trybom (Thysanoptera: Thripidae), the cowpea weevil *Callosobruchus maculatus* Fabricius (Coleoptera: Chrysomelidae) (a storage pest), and a complex of pod-sucking insects including the brown pod-sucking bugs *C. tomentosicollis*, *C. shadabi,* and *C. elongata* (Dreyer and Baumgartner 1994, Koona et al. 2004, Soyelu et al. 2007). Both the nymphal and adult stages of *Clavigralla* species suck sap from these legumes' pods, inducing yield losses of up to 100% (Egwuatu and Taylor 1976, Dialoke et al. 2010).

The Coreidae family of the order Hemiptera is very diverse and includes 44 *Clavigralla* species (Dolling 1979). Due to close morphological resemblance of the

Clavigralla species, some species have been misidentified. For example, *Clavigralla horrida* Germar (restricted in South Africa), has been misidentified as *C. shadabi* in West Africa and *C. elongata* in East and Southern Africa (Dolling 1979). The close morphological relationship between *C. horrida* and *Clavigralla alpica* Bergróth was reported as well as *C. tomentosicollis* which is morphologically very similar to *Coreus scutellaris* Westwood and *Acanthomia brevirostris* Stål (Dolling 1979). *Clavigralla shadabi*, *C. elongata* and *C. tomentosicollis* are distributed in Africa, particularly in West and East Africa (Agunbiade et al. 2013, Chalam et al. 2016, Minja et al. 1999), hence stringent identification of these *Clavigralla* species is needed to develop efficient control measures. In addition, a wide host range (beans, cowpea, Hyacinth bean, chickpea, pigeon pea and *Tephrosia*) has been documented for these three species (Dabire-Binso et al. 2005, Taylor and Omoniyi 1972), which might influence the insects' chemical profiles.

Currently, mitochondrial cytochrome c oxidase subunit 1 (*cox1*) is commonly used as a molecular marker for molecular phylogenetics, population genetics and species identification in animals (Hebert et al. 2003, Simon et al. 2006, Nelson et al. 2012). Previous studies have confirmed the effectiveness and suitability of the *cox1* as a suitable DNA barcoding marker for most insect groups though with limited ability to identify some groups of closely related crop pest species (Khamis et al. 2012, Lee et al. 2013). However, the mitochondrial cytochrome b (*cyt b*) gene in identification has also been found to be effective for differentiating between Hemiptera species (Giordano et al. 2005, Piffaretti et al. 2013, Steele et al. 2017). For example, the mitochondrial *cyt b* gene was used in the *Triatoma infestans* Klug (Hemiptera: Reduviidae) identification

(Giordano et al. 2005) and to generate a DNA barcode library for *C. tomentosicollis* (Steele et al. 2017).

In this study, we identified the chemical profiles and confirmed the identities of three *Clavigralla* species (*C. elongata*, *C. shadabi*, *C. tomentosicollis*) collected from different localities and host plants in Bénin and Kenya. Furthermore, the genetic variability within and between species was explored to ascertain correlation between the chemistry of their headspace volatiles and genetic variability within populations of the pest.

4.3 Materials and methods

4.3.1 Insect collection

Live and ethanol preserved (95%) specimens of nymphs and adults of *Clavigralla* spp. were collected on French beans (*P. vulgaris*) and pigeon pea (*C. cajan*) from six counties in Kenya (Makueni, Machakos, Embu, Kitui, Nakuru and Kisumu). In Bénin, the same samples were collected on cowpeas (*V. unguiculata*) and pigeon pea (*C. cajan*) in ten villages from four regions: Southern Bénin (Abomey-Calavi), Eastern Bénin (Ketou, Pobe), Western Bénin (Klouekanme) and Central Bénin (Dassa-Zounme, Djidja). For each sample population, the geographical coordinates were recorded using a GPS device (Garmin, etrex(R) 30, Garmin international Inc, USA) (Table 1 & Fig. 1). Young healthy pods of French bean (*P. vulgaris*) were used to maintain the live insects for volatile collection.

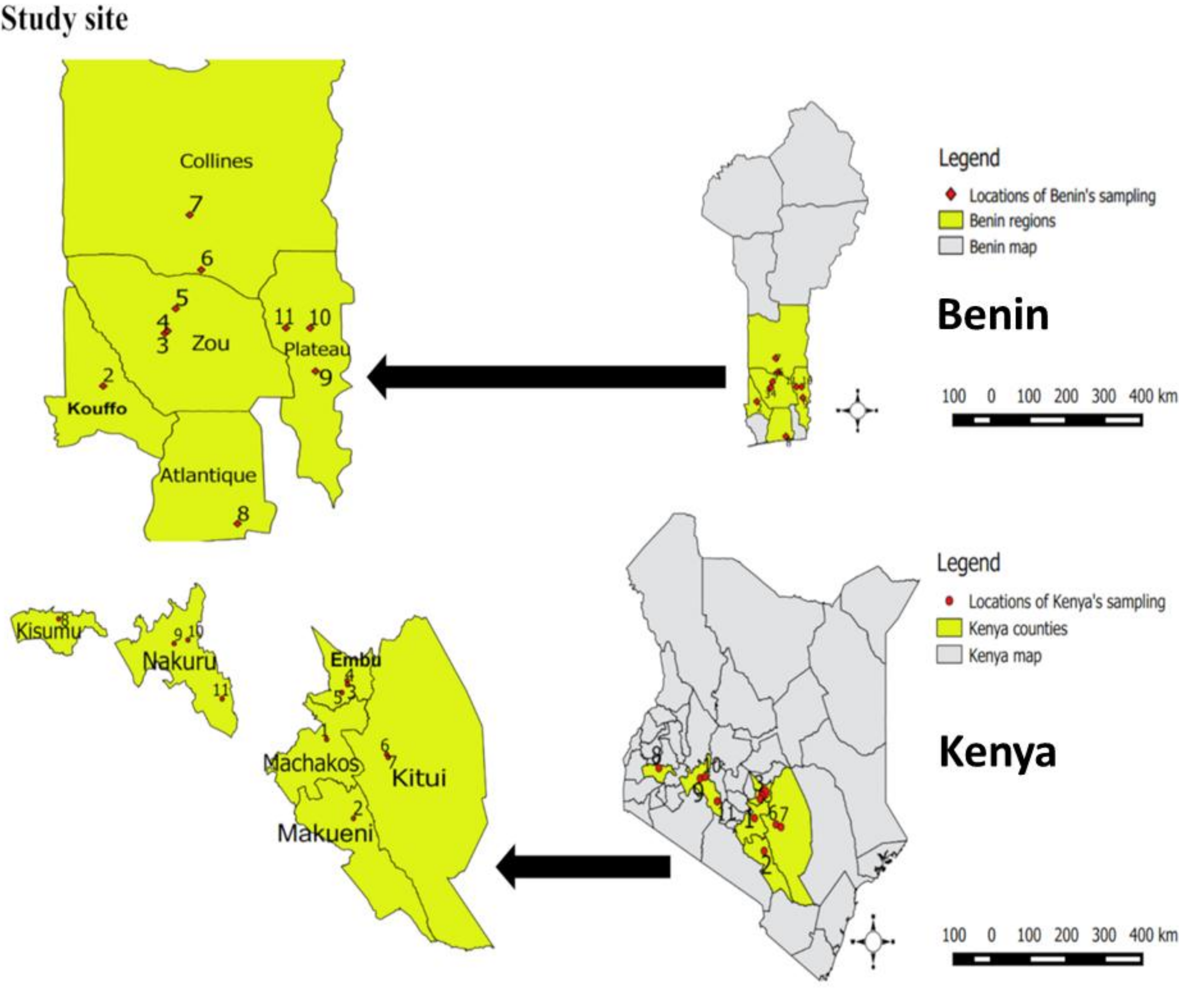

Fig. 1. Maps showing the regions where *C. tomentosicollis*, *C. shadabi* and *C. elongata* were collected in Bénin (above) and Kenya (below).

Table 1. References of *Clavigralla* spp. collection sites in Benin and Kenya.

Species ID	Host plants	Country	Localities	Villages	GPS co-ordinates	Elevation (m)
C. tom	Pegeon pea	Kenya	Machakos	Kitimani	S 01°10.060' E 037°27.287'	1228
C. tom	Pegeon pea	Kenya	Makueni	Kaiani	S 01°52.621' E 037°42.793'	1113
C. tom	Pegeon pea	Kenya	Embu (1)	Gatirari	S 00°40.532' E 037°39.187'	1060
C. tom	Pegeon pea	Kenya	Embu (2)	Gatirari	S 00°40.465' E 037°39.353'	1156
C. tom & C. elong	French beans	Kenya	Embu (3)	Jagawneth farm	S 00°44.847' E 037°36.151'	1049
C. tom & C. elong	Cowpeas	Kenya	Kitui (1)	Kithinzi	S 01°18.155' E 038°02.019'	1251
C. tom	Pegeon pea	Kenya	Kitui (2)	Kithinzi	S 01°19.770' E 038°02.864'	1253
C. tom & C. elong	French beans	Kenya	Kisumu	Obino	S 00°05.066' E 034°52.478'	1170
C. tom	Pegeon pea	Kenya	Nakuru (1)	Kirobon	S 00°18.345' E 035°59.224'	1930
C. tom & C. elong	Cowpeas	Kenya	Nakuru (2)	Wata	S 00°16.413' E 036°07.172'	1883
C. tom & C. elong	French beans	Kenya	Karagita	Nga'rama farm	S 00°48.170' E 036°26.918'	2001
C. tom	Pegeon pea	Benin	Toviklin	Houedogli	S 00°48.168' E 036°26.932'	114
C. tom	Pegeon pea	Benin	Adja-Honmey	Lycée Agricole Farm	N 07°02.672' E 001°47.592'	225
C. tom	Cowpeas	Benin	Djidja	Oungbega	N 07°17.051' E 002°02.420'	253
C. tom	Cowpeas	Benin	Djidja	Assantoun	N 07°17.704' E 002°03.109'	259
C. tom & C. shad	Cowpeas	Benin	Djidja	Dridji	N 07°23.801' E 022°05.048'	167
C. tom	Cowpeas	Benin	Dassa	Afossogbe	N 07°34.382' E 002°11.195'	137
C. tom	Cowpeas	Benin	Dassa	Ganfon	N 07°49.371' E002°08.399'	128
C. tom & C. shad	Cowpeas	Benin	Abomey Calavi	IITA Station	N 06°25.100' E 002°19.925'	18
C. tom	Cowpeas	Benin	Pobè	Itchagba	N 07°06.705' E 002°38.722'	34
C. tom	Cowpeas	Benin	Ketou	Camp	N 07°18.509' E 002°37.424'	131
C. tom & C. shad	Cowpeas	Benin	Ketou	Aguidi	N 07°18.543' E 002°31.583'	68

C. elong = *Clavigralla elongata*, C. shad = *Clavigralla shadabi*, and C. tom = *Clavigralla tomentosicollis*

4.3.2 Collection of headspace volatiles

The headspace volatile collection system used in this study is as described by Njihia et al. (2017). Volatiles were collected from *C. tomentosicollis*, *C. elongata*, and *C. shadabi*. Three glass jars (250 ml each) (Sigma Scientific, Gainesville, FL, USA), containing either sexually mature males (n = 20; 7-8 days old), females of similar age of each species, or no insects (control) were used. No food was provided inside the containers. Charcoal-purified air at a flow rate of 260 ml min^{-1} was passed over the jar containing the insects and through a previously cleaned Porapak Q filter (30 mg, mesh size 80–100, Supelco, Bellefonte, PA), for 24 h. Volatiles adsorbed on the Porapak Q filters were each eluted with 200 µl dichloromethane (Analytical grade, Sigma Aldrich, St. Louis, MO) and then stored at -80 °C until used.

4.3.3 Chemical analysis

Male and female of *C. tomentosicollis*, *C. elongata* and *C. shadabi* and control volatiles (1 µl each), were analyzed by coupled gas chromatography-mass spectrometry (GC/MS) on an Agilent Technologies Inc. Series A 7890 GC coupled to a 5977A MS (inert XL/EI/CI MSD) triple axis mass detector, equipped with a HP-5 MSI low bleed capillary column (30 m × 0.250 mm i.d., 0.25 µm) (J&W, Folsom, CA, USA) in the electron impact mode at 70 eV. The GC oven temperature was set at 35 °C for 5 min with a rise of 10 °C/min to 280 °C for 10.5 min, then 5 °C/min to 285 °C and held at this temperature for 9 min. Volatile compounds were identified by comparison of mass spectral data with library data Adams2, Chemecol and NIST11. In addition, structural assignments of several compounds were confirmed using synthetic compounds where

available. Quantification was based on calibration curves (peak area vs. concentration) generated from authentic standards of identified compounds. The experiments were replicated three times.

4.3.4 Chemicals

The synthetic standards used include: toluene, 2-methyl propanoic acid, octane, 2-methyl butanoic acid, alpha pinene, 6-methyl-5-hepten-2-one, decane <n->, isopentyl butanoate, undecane<n>, limonene, 2-methylbutyl 2-methylbutyrate, 2-methylbutyl isovalerate, dodecane, decanal, methyl eugenol, pentadecane, Valencene (Sigma Aldrich, Germany) (purity ≥ 97%).

4.3.5 PCR, sequencing and data analysis

Genomic DNA from 95% ethanol-preserved specimens was extracted from individual hind legs using the Isolate II Genomic DNA Kit (Bioline, London, United Kingdom), following the manufacturer's instructions. The resultant DNA was eluted in a final 50 µl volume then quality and quantity checks done using the Nanodrop 2000/2000c Spectrophotometer (Thermo Fischer Scientific, Wilmington, USA). Mitochondrial *cyt b* gene sequences were amplified by polymerase chain reaction (PCR) using the *cytb*-J-1-933 (5'-TCTTTTTGAGGAGCWACWGTWATTAC-3') and *cytb*-N-11367 (5'-AATTGAACGTAAAATWGTRTAAGCAA-3') primes (Belshaw and Quicke 1997). The PCRs were carried out in a total reaction volume of 20 µl containing 5x My Taq Reaction Buffer (5 mM dNTPs, 15 mM MgCl2, stabilizers and enhancers) (Bioline, London, UK), 0.5 pmol/µl of each primer, 0.5 mM MgCl2, 0.0625 U/µl My Taq DNA polymerase (Bioline, London, UK) and 15 ng/µl of DNA template. These reactions were

set up in the Nexus Mastercycler gradient (Eppendorf, Germany) using the following cycling conditions: initial denaturation for 2 min at 95 °C, followed by 40 cycles of 30 sec at 95 °C, 40 sec annealing at 45.5 °C and 1 min at 72 °C, then a final elongation step of 10 min at 72 °C. The amplified PCR products were resolved through 1.2% agarose gels. DNA bands on the gel were analyzed and documented using KETA GL imaging system trans-illuminator (Wealtec Corp, Meadowvale Way Sparks, Nevada, USA). Successively amplified products were excised and purified using Isolate II PCR and Gel Kit (Bioline, London UK) following the manufacturer's instructions. The purified samples were shipped to Macrogen Inc. Europe Laboratory (Netherlands) for bi-directional sequencing.

4.3.6 Sequence data analysis

The successful sequences were assembled and edited using Chromas Lite Version 2.1.1 (Thompson et al. 1997) and Geneious Version 8 (http://www.geneious.com) (Kearse et al. 2012). The sequence identities were determined using Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) hosted by the National Center for Biotechnology Information (NCBI). Multiple alignments of the assembled, trimmed sequences were done using Clustal X software (version 2.1) (Thompson et al. 1997). The evolutionary history was inferred by using the maximum likelihood phylogenies based on Kimura 2-parameter (K2P) distance model (Kimura 1980). Evolutionary analyses were conducted in MEGAX (Kumar et al. 2018). The reliability of the clustering pattern in the tree was evaluated using a bootstrap analysis with 1,000 replicates. The analysis involved 79 nucleotide sequences for all the samples and representative samples, respectively. All positions containing gaps and missing data were eliminated.

There was a total of 299 positions in the final dataset. The estimates of evolutionary divergence between sequences were calculated using Kimura 2-parameter (K2P) distance model (Kimura 1980) and the principal component plot then developed from the genetic distance matrix by GenAlEx 6.5 (Peakall and Smouse 2006, 2012). The correlation within species and between species was plotted using Principal Component Analysis (PCA) to generate 1) the genetic correlation within *C. tomentosicollis* populations from Bénin and Kenya and 2) the genetic relationship between *C. elongata*, *C. tomentosicollis, C. shadabi. Anoplocnemis curvipes* F. (Coreidae) and *Nysius thymi* (Wolff) (Lygaeidae) were used as out groups.

4.4 Results

4.4.1 Chemical analysis

Overall, thirty-one components were identified in the headspace volatiles of male and female *C. tomentosicollis*, *C. shadabi*, and *C. elongata*. These included two aliphatic acids, five hydrocarbons, one ketone, four esters, one alcohol, three monoterpenes, one benzenoid and two aldehydes whose identities were confirmed using authentic standards (Table 2). Eleven additional components were tentatively identified based on mass spectral data only as: two monoterpenes, two alcohols, three esters, one ketone, two benzenoids and another (Table 2). Variation within/between the headspace volatiles chemistry of each species were reported and seems to be quantitative rather than qualitative. Additionally, these differences varied within and between species and were, mainly in the dominant components; esters and monoterpenes. Interestingly, the heat map associated with the headspace volatiles

components of the three species showed that out of the 31 components identified, 12 showed a significant quantitative difference between the three species. This result showed that each species has specific components. Indeed, beta gurjunene, undecane, valencene, methyl eugenol, 1,2,4-trimethyl benzene, decane <n>, 2-methylbutyl 2-methylbutyrate, decanal and menthol were present in high amounts in the *C. tomentosicollis* volatiles but absent/trace from the volatiles of *C. shadabi* and *C. elongata*. Moreover, 2-methylbuthyl isovalerate, beta gurjunene, limonene and isopentyl butanoate were more concentrated in volatiles of *C. shadabi* than *C. elongata* volatiles. Also, only 2-methylbuthyl isovalerate and isopentyl butanoate were present in high amounts in the volatiles of *C. shadabi* but in trace amounts in *C. tomentosicollis* volatiles. In *C. shadabi* volatiles, 2-methylbuthyl isovalerate was more concentrated compared to *C. tomentosicollis* volatiles but the contrary was observed in *C. shadabi* volatiles (Fig. 2). The distribution of these compounds contributed to the separation of the three species. The dendrogram generated from clustering of the species based on volatiles trapped in this study, showed that *C. tomentosicollis* and *C. elongata* volatiles clustered the two species together while the *C. shadabi* clustered separately from the two species (Fig. 2).

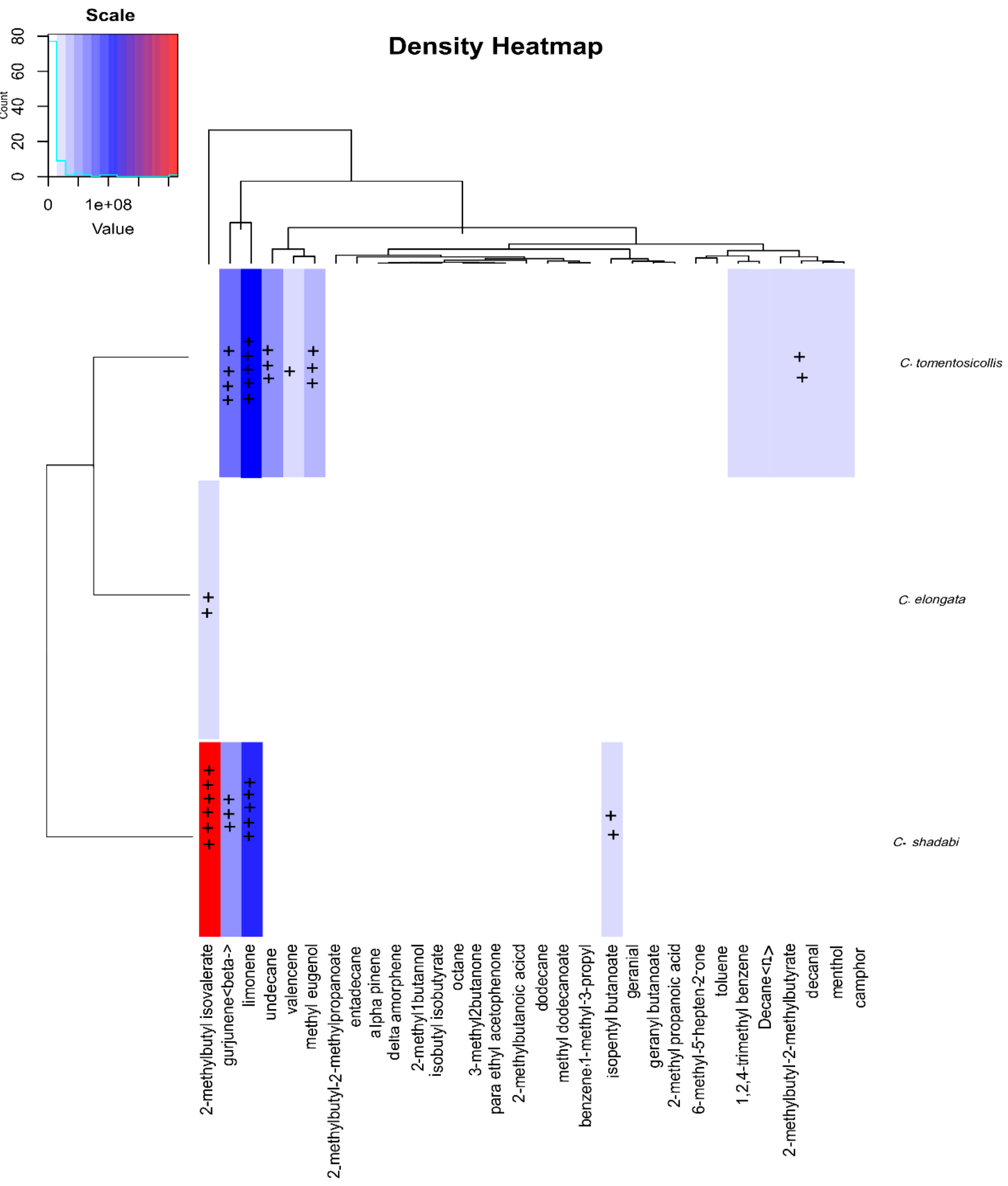

Fig. 2. Heat map showing the correlation between headspace volatile chemistry of *C. tomentosicollis*, *C. elongata* and *C. shadabi.* The number of plus (+) indicates the level of the compound content.

Table 2. GC/MS analysis of the volatiles emitted by *C. tomentosicollis*, *C. shadabi* and *C. elongata*. The compounds identified and confirmed with standards are in bold.

					C. tomentosicollis		***C. shadabi***		***C. elongata***	
N°	RT (min)	Compound name	Category	Q (%)	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
1	4.29	2-Methyl-1-butanol	Alcohol	72	-	-	-	-	+	-
2	5.05	**Toluene**	Benzenoid	93	+	+	+	+	-	-
3	6.41	**2-Methyl propanoic acid**	Aliphatic acid	72	+	-	-	-	-	-
4	8.8	**Octane**	Hydrocarbon	81	-	+	-	-	-	-
5	9.15	Isobutyl isobutyrate	Ester	74	-	-	-	-	+	+
6	9.37	**2-Methyl butanoic acid**	Aliphatic acid	90	+	-	+	+	+	+
7	9.54	**Alpha pinene**	Monoterpene	91	+	-	-	-	-	-
8	10.73	**6-Methyl-5-hepten-one**	Ketone	93	-	+	+	-	-	-
9	10.82	1,2,4-Trimethyl benzene	Benzenoid	96	+	+	+	+	-	-
10	10.94	**Decane<n>**	Hydrocarbon	94	-	+	+	+	-	-
11	11.08	Isobutyl 3-methylbutanoate	Ester	90	-	-	+	-	+	+
12	11.29	2-Methylbutyl 2- methyl propanoate	Ester	90	+	+	-	-	+	+
13	11.31	**Isopentyl butanoate**	Ester	72	+	-	+	+	-	-
14	11.49	**Limonene**	Monoterpene	96	+	+	+	+	+	+
15	11.93	1-Methyl-3-propyl-benzene	Benzenoid	94	-	+	-	-	-	-
16	12.75	**Undecane<n>**	Hydrocarbon	97	+	-	-	-	-	-
17	12.83	**2-Methyl butyl 2-methylbutyrate**	Ester	91	+	+	+	+	+	+
18	12.90	**2-Methylbutyl isovalerate**	Ester	90	+	+	+	+	+	+
19	13.55	Camphor	Other	94	+	-	+	+	-	-
20	13.99	Menthol	Alcohol	63	-	+	-	-	-	-
21	14.34	**Dodecane**	Hydrocarbon	97	-	-	-	+	-	-
22	14.44	**Decanal**	Aldehydes	62	+	-	+	+	-	-
23	15.34	Para-ethyl acetophenone	Ketone	94	+	+	-	-	-	-
24	15.41	Geranial	Aldehydes	95	-	-	+	-	-	-
25	15.42	**Pentadecane**	Hydrocarbon	93	-	-	+	+	-	-
26	16.95	Delta amorphene	Monoterpene	90	+	-	-	-	-	-
27	17.26	**Methyl eugenol**	Alcohol	97	-	+	+	+	-	-
28	17.77	Beta-gurjunene	Monoterpene	99	-	+	+	+	-	-
29	18.50	**Valencene**	Monoterpene	99	-	+	+	-	-	-
30	18.61	Geranyl butanoate	Ester	96	-	-	+	-	-	-
31	18.72	Methyl dodecanoate	Ester	97	+	-	-	-	-	-

(+) = present, (-) = absent. Q = quality

4.4.2 Sequence data analysis

The *cyt b* PCRs consistently gave excellent amplifications with strong amplicons of between 360- 375 bp. All the sequencing reads were of high quality (≥ 96%) with almost no miss amplifications. Most of BLAST queries yielded ≥ 97% similarity of all the *Clavigralla* spp. sequences to *C. tomentosicollis* of GenBank accession number KY274846 (Table 3). This identity confirmed the morphological taxonomy of *C. tomentosicollis* but not of the other two *Clavigralla* specimens. Maximum likelihood phylogenetic trees with the highest log likelihood (-1946.74) are shown in Figures 3a and b.

Despite the presence of *A. curvipes* and *N. thymi* as out groups, the phylogenetic tree was paraphyletic to *C. tomentosicollis*, *C. elongata* and *C. shadabi* with two distinct branches. All *C. tomentosicollis* clustered within one clade together the *C. tomentosicollis* reference sequence (GenBank accession KY274846.1) with 33 clusters without significant distances between them. The phylogenetic tree resulting from this study was not influenced by host-plants (*V. unguiculata*, *P. vulgaris* and *C. cajan*) and sampling regions (Kenya or Bénin) (Figs. 3a and b). The second branch consisted of the other *Clavigralla* specimens (*C. elongata* and *C. shadabi*), without significant distances between them (0.01).

Estimates of evolutionary divergence over sequence pairs between groups are detailed in Table 4. Genetic distance between *C. tomentosicollis* samples collected from Bénin and Kenya was 0.02. This distance between *C. tomentosicollis* from GenBank (KY274846.1) and those from Kenya and Bénin were 0.02 and 0.01 respectively. Moreover, the genetic distance which separated *C. elongata* to *C.*

tomentosicollis from this study and that from GenBank was 0.14 and that of *C. shadabi* was 0.13. The relationship between *C. elongata* samples collected from Kenya and *C. shadabi* from Bénin is very close, with genetic distance 0.01 (Table 4). All *Clavigralla* species separated from *N. thymi* (KP310647.1) with a genetic distance greater than 0.80, confirming the phylogenetic tree analysis. Furthermore, *C. elongata* and *C. shadabi* exhibited a very distant relationship with *A. curvipes* (KY906099.1) at 1.00. The principal component analysis (PCA), revealed the existence of mainly two distinct clusters, i.e. a cluster of *C. tomentosicollis* samples from both countries and that from GenBank, and a cluster of the two other *Clavigralla* species. The two outgroups *N. thymi* and *A. curvipes* were isolated separately. Furthermore, *C. tomentosicollis*, *C. elongata* and *C. shadabi* were closely associated on the same axis (Coord. 2) and separated from the other two species picked from GenBank. These results are congruent with phylogenetic tree and genetic distance (Fig. 4).

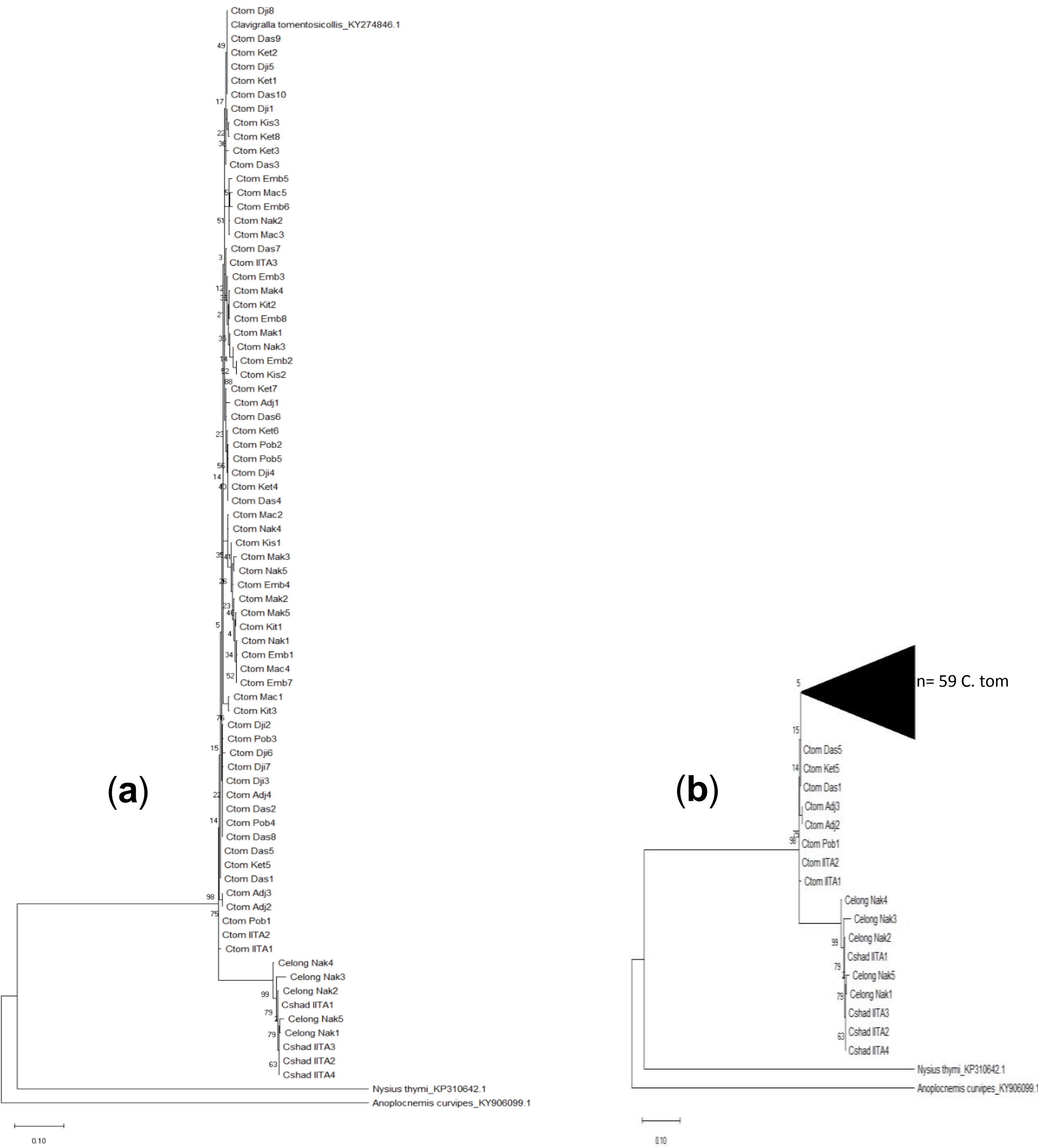

Fig. 3. Maximum likelihood tree showing evolutionary relationships between *C. tomentosicollis*, *C. elongata* and *C. shadabi* samples generated by MEGA X (Kumar et al. 2018). **a** = full tree, **b** = simplified tree.

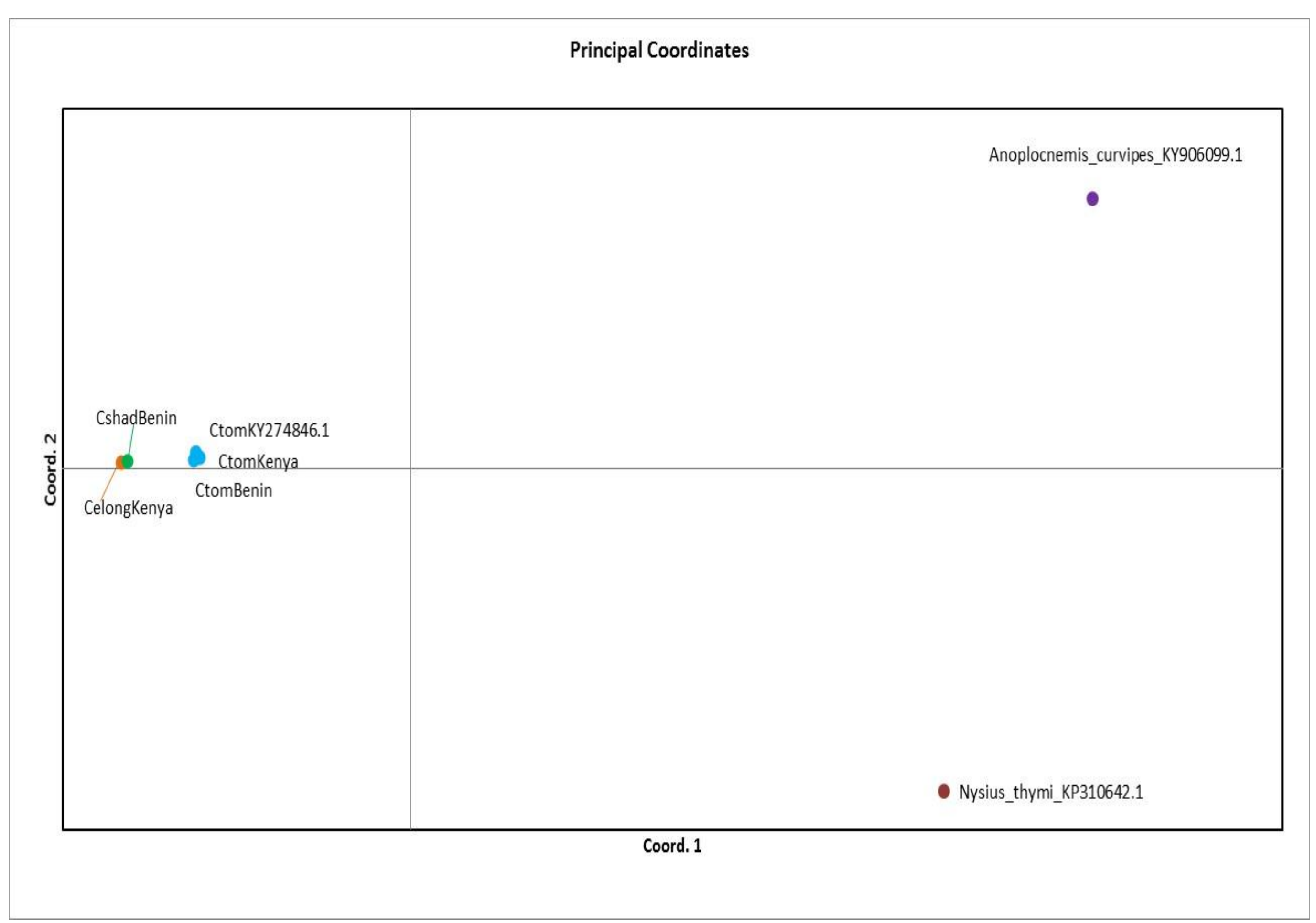

Fig. 4. Principal component analysis (PCA) plot generated from the genetic distance matrix of *C. tomentosicollis*, *C. shadabi* and *C. elongata* samples collected in Kenya and Bénin, using GenalEx.

Table 3. Results of *Clavigralla spp.* sequences data analyses.

Code name	Localities	ID from GenBank	Accession no.	Query %	E. value %	ID %
CTmk-K	Machakos-Kitimani	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	96%	0	99%
CTmk-K	Machakos-Kitimani	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	96%	0	99%
CTmka-K	Makueni-kaiani	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	96%	0	99%
CTmka-K	Makueni-kaiani	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	97%	0	98%
CTeg-K	Embu-Katirari	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	96%	0	99%
CTeg-K	Embu-Katirari	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	96%	0	100%
CTej-K	Embu-Jagawneth	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	96%	0	99%
CTej-K	Embu-Jagawneth	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	97%	0	98%
CTkki-K	Kitui-Kitinzi	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	97%	0	100%
CTkki-K	Kitui-Kitinzi	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	96%	0	99%
CTkio-K	Kisumu-Obino	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	96%	0	98%
CTkio-K	Kisumu-Obino	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	97%	0	97%
CTnk-K	Nakuru-Kirobon	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	96%	0	97%
CTnk-K	Nakuru-Kirobon	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	96%	0	99%
CTnka-K	Karagita Naivasha	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	96%	0	99%
CTnka-K	Karagita Naivasha	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	96%	0	97%
CEnk-K	Nakuru-Kirobon	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	86%	5.00E-130	99%
CEnk-K	Nakuru-Kirobon	*Clavigralla tomentosicollis* mitochondrion, complete genome	LT221849.1	86%	4.00E-126	99%
CTkah-B	Klouekanmey-Adja	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	97%	2.00E-85	100%
CTkah-B	Klouekanmey-Adja	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	97%	4.00E-180	100%
CTdja-B	Djidja-Oungbega	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	98%	0	99%
CTdja-B	Djidja-Oungbega	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	96%	0	99%
CTdjd-B	Djidja-Dridji	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	98%	0	96%
CTdjd-B	Djidja-Dridji	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	99%	0	99%
CTda-B	Dassa-Affossogbe	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	97%	0	98%
CTda-B	Dassa-Affossogbe	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	97%	0	99%
CTdg-B	Dassa-Ganfon	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	97%	0	97%
CTdg-B	Dassa-Ganfon	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	98%	0	96%
Ctai-B	Abomey-Calavi (IITA)	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	96%	0	99%
Ctai-B	Abomey-Calavi (IITA)	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	96%	0	99%
Ctai-B	Abomey-Calavi (IITA)	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	98%	0	95%
Cshai-B	Abomey-Calavi (IITA)	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	88%	3.00E-142	98%
Cshai-B	Abomey-Calavi (IITA)	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	86%	1.00E-125	99%
CTpi-B	Pobe-Itchagba	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	96%	0	99%
CTpi-B	Pobe-Itchagba	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	97%	0	98%
CTkc-B	Ketou-Camp	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	98%	0	98%
CTkc-B	Ketou-Camp	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	98%	0	99%
CTka-B	Ketou-Aguidi	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	97%	0	100%
CTka-B	Ketou-Aguidi	*Clavigralla tomentosicollis* mitochondrion, complete genome	KY274846.1	97%	0	97%

CE = *Clavigralla elongata* from Kenya, Csh = *Clavigralla shadabi* from Bénin and CT = *Clavigralla tomentosicollis* from Bénin and Kenya.

Table 4. Estimates of evolutionary divergence of mitochondrial Cytob gene region over sequence pairs between groups as determined using Kimura 2-parameter model in MEGA X (Kimura et al. 1980).

	Ctom Kenya	**Celong Kenya**	**Cshad Benin**	**Ctom Benin**	**CtomKY274846.1**	**Nysius_thymi_KP310642.1**	**Anoplocnemis_curvipes_KY906099.1**
Ctom Kenya	0.0000						
Celong Kenya	0.1420	0.0000					
Cshad Benin	0.1385	0.0128	0.0000				
Ctom Benin	0.0224	0.1414	0.1371	0.0000			
CtomKY 274846.1	0.0213	0.1483	0.1437	0.0103	0.0000		
Nysius_thymi_KP310642.1	0.8197	0.9158	0.9098	0.8158	0.8319	0.0000	
Anoplocnemis_curvipes_KY906099.1	0.8960	1.0089	0.9957	0.8786	0.8815	1.0797	0.0000

Ctom = *Clavigralla tomentosicollis*, Celong = *Clavigralla elongata*, Cshad = *Clavigralla shadabi.*

4.5 Discussion

The consistence of the components 2-methylbutyl 2-methylbutyrate, 2-methylbutyl isovalerate, limonene and 2-methyl butanoic acid in all samples of *C. tomentosicollis*, *C. elongata* and *C. shadabi* volatiles suggest that these components are specific to *Clavigralla* spp. and can be used to distinguish them from the other groups. This finding agrees with a previous study which detected the consistence of tridecane and (E)-2-decanal in the volatiles released by diapausing and non-agitated adults of the brown marmorated stink bug, *Halyomorpha halys* Stål (Hemiptera: Pentatomidae) as specific components which can be used to differentiate the diapausitive state of this species (Vl et al. 2014, Nixon et al. 2018). The variation of the headspace volatiles chemistry of these three *Clavigralla* species suggests that these components may be involved in the differentiation of these species. *C. tomentosicollis*, *C. shadabi* collected on cowpea exhibited more similarity in chemical profiles compared to *C. elongata* collected on French bean. Despite this similarity, differences in chemical profiles of the three species were observed. These differences most likely resulted from the host plants from which they were collected. The influence of host plant on the variation in the chemistry of volatiles has been demonstrated in *Pyrrhocoris apterus* L., *Pyrrhocoris aegyptius* L., and *Pyrrhocoris tibialis* Stål (all Heteroptera: Pyrrhocoridae) which feed on different host plants (*Tilia cordata*, *Alcea rosea*, and *Hibiscus rosa-sinensis*) (Krajicek et al. 2016). In addition, the variation in their chemical profiles could also be explained by the fact that the sample collection was done randomly regardless of age, which requires further investigation. That was reported by Fávaro et al. (2011) who demonstrated that the variation in insect age influences the chemistry of *Pallantia macunaima* Grazia

(Hemiptera: Pentatomidae) volatiles. Interestingly, the occurrence of species-specific components reported in the current study explains the distinction of the three species shown in Fig. 2. Furthermore, the common compound (2-methylbutyl 2-methylpropanoate) shared by *C. tomentosicollis* and *C. elongata* justifies the close phylogenetic relationship between these two species as demonstrated Krajicek et al. (2016). Moreover, the separated branch of *C. shadabi* suggests that the quantity of the component is an important factor in the separation of these species.

The 5' end of the mitochondrial gene region is known to be used in the identification of insect species and other organisms, restoration of the origin of introduced species, uncovering of presence of cryptic species, and in the examination of genetic variability (Hebert et al. 2004, Park et al. 2011). Furthermore, the evolution of morphological and advances in molecular methodologies provide powerful tools for species identification and facilitate characterization and species delineation. Currently, in most hemipterans' genetic identification, DNA barcoding is used as a molecular tool (Barman et al. 2017, Park et al. 2011). Armstrong and Ball (2005) documented DNA barcoding as a tool that can expedite species identification in the absence of taxonomic expertise, interception of immature stages or damaged specimens with reduced morphological features. In the current study, we firstly tested the DNA barcoding primers (Folmer et al. 1994) to recover the barcode segment of the mitochondrion genome of *C. tomentosicollis, C. elongata* and *C. shadabi* since this primer pair was previously used for similar studies (Barman et al. 2017, Kaur and Sharma 2017, Khamis et al. 2012). However, these primers did not reliably amplify the gene region of interest, hence alternative primers targeting *cyt b* gene were used. The *cyt b* provided

satisfactory amplification of the target mitochondrion genome. All the sequences generated in the study linked to a barcode of *C. tomentosicollis* (KY274846.1) that was recently uploaded (Steele et al. 2017). The higher similarity (97%) of all the *Clavigralla* spp. sequences to *C. tomentosicollis* confirms the identity of our samples. These results confirm the finding of Steele et al. (2017) that *cyt b* can be used for *C. tomentosicollis* identification. Species identification based on *cyt b* was reported for other Hemiptera species also. For example, the mitochondrial *cyt b* gene (750 bp) was used in to identify of *Brachycaudus* species (Hemiptera: Aphididae) (Piffaretti et al. 2013) and *T. infestans* (Giordano et al. 2005). These reports consolidate our results in the choice of the marker (*cyt b*) in the insect identification. Additionally, the results in the current study is confirms previous studies which highlighted the effectiveness of DNA barcoding in identification of the hemipteran pest species (Barman et al. 2017, Kaur and Sharma 2017, Park et al. 2011, Jung et al. 2011).

The Maximum Likelihood model-based phylogenetic analysis results grouped all *C. tomentosicollis* samples collected from different localities and different host plants in Bénin and Kenya on the same branch with a mixture of the colony within in the clusters. Low genetic distance (0.02) was observed between and within colonies of all *C. tomentosicollis* samples, indicating that there is no a genetic diversity within the *C. tomentosicollis* species. Likewise, *C. elongata* and *C. shadabi* were clustered on the same branch with very low genetic distance (0.01) between them. This result suggests that these two species are genetically linked and requires supplementary study. These results are similar to those of a previous study on *Chlorochroa* spp. (Hemiptera: Pentatomidae) which presented similar genetic distance between species (0.04) and

within species (0.01) (Barman et al. 2017). A similar genetic distance (≥ 0.02) to our results was reported for *Nezara viridula* (L.) (Hemiptera: Pentatomidae) (Tembe et al. 2014) and other Pentatomidae (Hemiptera) from India (Kaur and Sharma 2017), consolidating our finding. The lower genetic distance observed between species in this study (0.02) is similar to that reported in different vertebrate species at *cyt b* primers (Avise and Walker 1999). In contrast, *C. elongata* and *C. shadabi* were branched separately to all *C. tomentosicollis* samples collected from both countries and presented a genetic distance ranged between 13 to 0.14, indicating genetic variability between the two species and *C. tomentosicollis*. These results show that *C. elongata* and *C. shadabi* are different species from *C. tomentosicollis*. This finding concurs with the study of 39 species of the family Anthocoridae (Hemiptera) who's interspecific genetic distances ranged between 0.12 to 0.19 (Jung et al. 2011). The genetic variability between species recorded in the current study conforms with a previous study which reported an interspecific divergence of 0.10 and 0.19 within the same and different genera respectively of 380 species of true bugs (Hemiptera) (Park et al. 2011). Moreover, the genetic diversities observed between *C. elongata* and all *C. tomentosicollis* samples (0.14) and then between *C. shadabi*. and all *C. tomentosicollis* samples (0.13) were comparable to the genetic diversity reported for a brown marmorated stink bug (H. halys) population from Canada and New Zealand (0.13 and 0.18, respectively) (Cesari et al. 2017). Additionally, the different clustering of the same species from the same location with low genetic variation could be a misidentification, cryptic taxa, ancestral polymorphisms, or introgression. For example, males and females of *Homaemus aeneifrons* extensus (Hemiptera: Scutelleridae) specimens collected from western

Canada were classified as distinct sibling species because of distinct male genitalic characters (Walley 1929). Previous studies have shown many of these cases involve cryptic species, which could be the case in the current study. Our result supports the hypothesis which stipulates that the separation of *Plagiognathus obscurus* Uhler (Hemiptera: Miridae) specimens into two groups with a low genetic distance of 0.04 are due to the existence of cryptic species (Park et al. 2011), consolidating our results.

The three *Clavigralla* species exhibited variable chemical profiles. We conclude that this variation seems quantitative rather than qualitative and that it depends on the host plants that the specimens were collected from. Moreover, the blast query on our samples identified three different species: *C. tomentosicollis*, *C. elongata* and *C. shadabi.* Our study reports for the first time the genetic identity of the two latter species which have been deposited in the GenBank with accession numbers (MK945668-MK945672 and MK945673- MK945676). Our study showed a close genetic relationship within species but a variability between *C. tomentosicollis* and others two species. Very close genetic relationship between *C. elongata* and *C. shadabi* was also documented and the genetic variability observed between species was independent from host plants and sample collections areas. Our finding showed that the variation in chemical profiles does not in general depend on genetic variability. However, a correlation between the chemical profiles and genetic variability is reported for *C. tomentosicollis* and *C. shadabi*. This study will facilitate the development of biological control strategies for *Clavigralla* spp. management in both countries. The assemblage of individuals in the clustering within species could be due to cryptic taxa and requires further investigations

with more stringent markers. Another consideration is to evaluate effect of host plants and insect ages on variation in chemical profile.

4.6 Data Availability Statement:

DNA sequences were deposited to GenBank with accession numbers: *C. elongata* (MK945668- MK945672), *C. shadabi* (MK945673- MK945676), *C. tomentosicollis* (MK945677- MK945743).

4.7 Author contributions

HK, JVB, FK, MT, and BT conceived and designed research. HK conducted experiments in and analyzed data. HK, JVB, FK, MT, and BT wrote the manuscript. EJT gave us support morphological identification of *Gryon* sp. All authors edited the manuscript and approved the final version.

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4.9 Conflict of Interest Statement:

Authors declare no conflict of interest.

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CHAPTER 5

Conclusion and future trends

5.1 Testing of hypotheses

This study aimed to: 1) determine parasitism levels of *Clavigralla tomentosicollis* Stål (Hemiptera: Coreidae) and *Clavigralla elongata* Signoret (Hemiptera: Coreidae) eggs, and to explore the relationship between egg parasitism and egg cuticular chemistry variation, 2) identify the aggregation pheromone of *C. tomentosicollis* and test its effect in the egg parasitoid, *Gryon* sp. behavior, and 3) identify the chemical profiles and establish the genetic variability of these three *Clavigralla* species collected in Bénin and Kenya and determine whether there is correlation between chemical profiles variation and genetic variability. This study was successful in achieving the aims and completing the associated objectives. The outcome of this study is summarized per hypothesis:

II) *Clavigralla tomentosicollis* Stål and *Gryon* spp. are the most common and abundant species in Bénin and Kenya, and the variation in egg cuticular chemistry of key *Clavigralla* species influences the level of parasitism of this egg parasitoid.

Base on the literature (chapter 1) and results (Kpongbe *et al*. 2019) (Chapter 2: Article 1) reported in this study, we concluded that *Clavigralla* species and its associated egg parasitoid *Gryon* sp. are widely distributed in Bénin and Kenya. Our study recorded *C. tomentosicollis* and *Gryon* sp. as common and widespread in Bénin and Kenya, while *C. elongata* and *C. shadabi* were specific to Kenya and Bénin respectively. In addition, the egg parasitoid recorded from *Clavigralla* spp. eggs was identified as *Gryon* sp., which

was also previously reported as common and a potential candidate of *Clavigralla* spp. in Africa (Asante *et al*. 2000). The high parasitism rate recorded in laboratory assays for *C. tomentosicollis*, confirms this aspect. Furthermore, the differences in cuticular chemistry of unparasitized eggs of *C. tomentosicollis* and *C. elongata* could explain the differences observed in the parasitism of eggs of the different species. These volatiles could influence parasitoid attraction and the stated hypothesis is accepted.

III) The aggregation pheromone is produced by *C. tomentosicollis* males and it could be an attract of the parasitoid *Gryon* sp., and useful in *C. tomentosicollis* management.

In Chapter 3 (Article 2) we used behavioral, chemical and electrophysiological assays to identify the aggregation pheromone compounds and their effects on the egg parasitoid *Gryon* sp. behavior. Result from Y-tube assays using groups of *C. tomentosicollis* males and females separately showed that volatiles released by groups of males were strongly attractive to both sexes. GC/EAD analysis and GC/MS analysis showed that the male-specific compound, isopentyl butanoate, elicited positive antennal and behavioral responses in male and female *C. tomentosicollis* and *Gryon* sp. Isopentyl butanoate served as aggregation pheromone for both sexes of *C. tomentosicollis* and kairomone for *Gryon* sp. and can be used in the host location by the parasitoid. Therefore, the stated hypothesis is accepted.

IV) *C. tomentosicollis*, *C. shadabi* and *C. elongata* present different chemical profiles, and there is genetic variability between species and populations. The chemistry variation correlates with genetic variability.

The variation in chemical profiles, genetic identity and genetic variability between species were described in this section (Chapter 4: Article 3). The correlation between chemical profiles and genetic variability was also established. Results from the chemical analysis presented a variation in chemical profiles of the three *Clavigralla* species and showed that this variation was related to host plant and species. Our results also showed that the amount of the different compounds contained in the volatile profiles can contribute to the distinguishing of species. Genetic variability between species was observed but also within species and population. This variability was independent of host plant and locations. Furthermore, despite the lack of correlation between variation in chemical profiles and genetic variability of *C. tomentosicollis* and *C. elongata*, a positive relationship between the variation of chemical profiles and genetic variability was recorded for *C. tomentosicollis* and *C. shadabi*. From results obtained in this study, the stated hypothesis is accepted.

5.2 Conclusion

This study investigated *Clavigralla* spp. and its associated egg parasitoid occurrence in Bénin and Kenya, the levels of parasitism of *C. tomentosicollis* and *C. elongata* eggs, the egg cuticular chemistry and the chemical cues that influence the pest-parasitoid relationship, the variation in chemical profiles and genetics of *C. tomentosicollis, C. shadabi* and *C. elongata.* It also highlighted the role of an aggregation pheromone and its role in *Gryon* sp. attraction, indicating that it may have potential for use in pest management. Specifically, the ecology and behavior of *Clavigralla* species and *Gryon* sp. and semiochemicals that could play a role in host location egg parasitism were

investigated. The absence of an adequate biological control strategy and knowledge of *Gryon* spp. in the control of *Clavigralla* spp. hampers the effective control of this pest and the profitability of cowpea and common bean production. Although different control methods were developed to protect these crops, efficient control of *Clavigralla* spp. remains a challenge (Jackai and Oghiakhe 1989, Jackai and Adalla 1997).

We conclude that the focus of future research should be on 1) using the specific egg cuticular chemistry that serves as semiochemical cues for parasitoids to increase their activities, 2) employing the aggregation pheromone as semiochemical in the recruitment of the egg parasitoid, *Gryon* sp., and 3) development of a biological control strategy for *Clavigralla* spp., based on their distribution patterns and genetic variability between species.

5.3 Future trends

More comprehensive research should be undertaken on both *Clavigralla* spp. and their parasitoids in East and West Africa. Our results and the high parasitism rates by *G. fulviventris* reported in Nigeria on *C. tomentosicollis* eggs (Asante *et al*. 2000) and that of *G. clavigrallae* on *Clavigralla scutellaris* Spinola and *C. gibbosa* (Westwood) (both Hemiptera: Coreidae) eggs in India (Romeis *et al.* 2000) highlighted the importance of parasitism of *C. tomentosicollis* eggs compared to the other species. Since cuticular compounds have been shown to be important chemical cues used by parasitoids for locating the egg of hosts, their attraction and the prey recognition (Dietemann *et al*. 2003, Paul *et al*. 2008, Moore *et al*. 2017), these aspects need further investigation. In the current study, hexadecenoic acid was identified as a specific compound in the

cuticular extract of unparasitized eggs of *C. tomentosicollis*. This compound was also reported by Michereff *et al*. (2016) to occur amongst the volatiles emitted by egg clusters of the stink bug *Euschistus heros* Fabricius (Heteroptera: Pentatomidae) as an attractant of the egg parasitoid *Telenomus podisi* Ashmead (Hymenoptera: Platygastridae). Therefore, future studies have to address the attractiveness of volatiles of egg masses of the different *Clavigralla* species to *Gryon* sp., which could potentially also play a role in the parasitism. Furthermore, it will also be important to evaluate the effect of the identified specific compounds associated with unparasitized eggs of *C. tomentosicollis* in the host location by *Gryon* sp.

The production of aggregation pheromone in the metathoracic scent gland of male stink bugs as well as nymphs was previously reported (Aldrich 1975, James *et al*. 1994, Aldrich *et al.* 2000, Fávaro *et al*. 2011, Kartika *et al*. 2015,). In addition, the use of insect-infested host-plant volatiles in the location of hosts by parasitic hymenopterans has been previously reported (Meiners 1997, Powell *et al*. 1998, Calatayud *et al*. 2001). Future research needs to investigate the source of these compounds, its presence in different instar nymphs as well as other *Clavigralla* species. Another consideration should be to evaluate the role of *Clavigralla*-infested host-plant volatiles alone, and in combination with isopentyl butanoate in the host-searching of parasitoids, since this could lead to development of provide *Clavigralla* spp. Management strategies.

Future study of the effect of host plant and insect stages on variation in chemical profile is needed.

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APPENDIX A

Instructions to authors (excerpt) Springer

Article structure Chemical Ecology

Title Page. The title should be centered and in capital letters (no bold); genus and species should be in upper- and lower-case italics. Author names should be centered and in capital letters (no bold). The corresponding author should be designated with an asterisk. The address(es) should be centered and in italics under the author names. Multiple authored manuscripts with multiple addresses should identify authors with their corresponding institutions by using consecutive numerical superscripts. The address(es) should be centered and in italics under the authors' names. Abstract (150 to 250 words) and Key Words (6 keyworks). These headers should be left justified, in bold, and followed by a dash. The text of the abstract should begin immediately after the dash in regular font (i.e., no bold). "Key Words" is two words, each with the first letter capitalized and in bold. The key words immediately follow the dash. The first letter of the first key word is always capitalized. Others are in lower case unless the word is a proper name. Key words are separated by commas, and the list ends with a period. All the headers are upper case (no bold or italic) and centered. Subheading phrases, where appropriate, should be written in italics with the first letter of each word capitalized with the exception of prepositions, conjunctions, articles etc. and end with a period. The subheading phrase begins a paragraph. The text follows on the same line, immediately after the subheading.

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The title page should include the name(s) of the author(s); a concise and informative title; the affiliation(s) and address(es) of the author(s); the e-mail address, and telephone number(s) of the corresponding author. If available, the 16-digit ORCID of the author(s)

REFERENCES

Citation

Cite references in the text by name and year in parentheses. Some examples: Negotiation research spans many disciplines (Thompson 1990). This result was later contradicted by Becker

and Seligman (1996). This effect has been widely studied (Abbott 1991; Barakat et al. 1995a, b; Kelso and Smith 1998; Medvec et al. 1999, 2000).

Reference list:

Reference list entries should be alphabetized by the last names of the first author of each work. Order multi-author publications of the same first author alphabetically with respect to second, third, etc. author. Publications of exactly the same author(s) must be ordered chronologically. Ideally, the names of all authors should be provided, but the usage of "et al" in long author lists will also be accepted:

Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. https://doi.org/10.1007/s00421-008-0955-8

Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086

Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb.

http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

APPENDIX B

Instructions to authors (excerpt)_Oxford

Article structure

Research article: No limit (under 7500 words recommended). The manuscript should be divided in the following parts: abstract, introduction, materials and methods, results and discussion. Make sure your paper has the following items: continuous line numbers; double-spaced lines; a title page and abstract in the main document. A main document and tables in a doc, docx…. Figure and table legends in the main document. Provided no more than 250 words in the abstract and five (5) keywords. References listed in alphabetical order, cited by author and year in the text (not numbered). Figures and tables at the end of the main document after the references or uploaded as separate files. Figure legends should be included at the end of the main text after the references, and table legends should be next to their corresponding tables. Text is single-column. Place the acknowledgments after the text. Following the Acknowledgments, you may include a statement of author contribution outlining the specific contributions of each author to the article.

Title Page

The title page should include: (1) the full name, mailing address, telephone number, and email address of corresponding author; (2) a title which should be as short as possible. Only include common names that are listed in the ESA Common Names of Insects & Related Organisms. Do not include authors of scientific names. Insert "([Order]: [Family])" immediately after the name of the organism; (3) list of all the authors in the order the names should be published and (4) the full affiliation addresses of all authors. If there are multiple affiliations, designate through numbered footnotes.

Body

The main text includes an introduction, the materials and methods, the results, a discussion

References

EndNote style is "Environmental Entomology," and Reference Manager style is "Journal of Medical Entomology". Only cite published or formally accepted (in press) articles, not submitted articles. References should be in alphabetical order. If multiple references from the same author are cited, those references should be in chronological order. Abbreviate journal titles according to the most recent issue of BIOSIS Serial Sources. For non-English titled journals that are cited

in the references, the title of the journal should be spelled out. Systematics-related articles may specify that all serial titles be spelled out for final publication.

Sample reference styles

Journal Articles

Evans, M. A. 2000. Article title: subtitle (begin with lowercase after colon or dash unless first word is a proper noun). J. Abbr. 00:000–000.

Evans, M. A., R. Burns, and A. A. Dunn. 2001. Article title. J. Abbr. 00: 000–000.

In Press

Evans, M. A. 2002. Article title. J. Econ. Entomol. (in press).

Books

Burns, R. 2001. Title (initial cap only): subtitle (no initial cap after colon). Publisher, city, state abbreviation or country.

Article/Chapter in Book

Tyler, A., R.S.T. Smith, and H. Brown. 2001. Onion thrips control, pp. 178–195. In R. S. Green and P. W. White (eds.), Book title, vol. 13. Entomological Society of America, Lanham, MD.

No Author Given

(USDA) U.S. Department of Agriculture. 2001. Title. USDA, Beltsville, MD

(IRRI) International Rice Research Institute. 2001. Title. IRRI, City, State or Country.

Proceedings

Martin, P. D., J. Kuhlman, and S. Moore. 2001. Yield effects of European corn borer (Lepidoptera: Pyralidae) feeding, pp. 345–356. In Proceedings, 19th Illinois Cooperative Extension Service Spray School, 24–27 June 1985, Chicago, IL. Publisher, City, State.

Theses/Dissertations

James, H. 2001. Thesis or dissertation title. M.S. thesis or Ph.D. dissertation, University of Pennsylvania, Philadelphia.

Software

SAS Institute. 2001. PROC user's manual, version 6th ed. SAS Institute, Cary, NC.

Online Citations

Reisen, W. 2001. Title. Complete URL (protocol://host.name/path/file.name) and/or DOI (Digital Object Identifier)

APPENDIX C

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APPENDIX D

Proof of submission

Re: "ISOPENTYL BUTANOATE: AGGREGATION PHEROMONE OF THE BROWN SPINY BUG, *Clavigralla tomentosicollis* (HEMIPTERA: COREIDAE), AND KAIROMONE FOR THE EGG PARASITOID *Gryon* sp. (HYMENOPTERA: SCELIONIDAE)"

Full author list: HILAIRE KPONGBE; JOHNNIE VAN DEN BERG; FATHIYA KHAMIS; MANUELE TAMÒ; BALDWYN TORTO

Dear Mr. Hilaire Kpongbe,

We have received the submission entitled: "ISOPENTYL BUTANOATE: AGGREGATION PHEROMONE OF THE BROWN SPINY BUG, *Clavigralla tomentosicollis* (HEMIPTERA: COREIDAE), AND KAIROMONE FOR THE EGG PARASITOID *Gryon* sp. (HYMENOPTERA: SCELIONIDAE)" for possible publication in Journal of Chemical Ecology, and you are listed as one of the co-authors.

The manuscript has been submitted to the journal by Dr. Dr. Baldwyn Torto who will be able to track the status of the paper through his/her login.

If you have any objections, please contact the editorial office as soon as possible. If we do not hear back from you, we will assume you agree with your co-authorship.

Thank you very much.

With kind regards,

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APPENDIX E

Declaration of language editing

Language editing statement

To whom this may concern,

I, Prof. Johnnie Van den Berg, hereby declare that the thesis titled: “Pheromone and population genetics analyses of *Clavigralla* species in Africa” by Hilaire Kpongbe has been edited for language correctness and spelling by some of the supervisors. No changes were made to the academic content or structure of this work.

J van den Berg

16 May 2019

Prof. Johnnie Van den Berg Date