Caste differentiation and analyses of cuticular profile, fats and sperm production in *Vespa velutina nigrithorax* du Buysson, 1905 (Hymenoptera: Vespidae)

Vespa velutina nigrithorax du Buysson, 1905 (Hymenoptera: Vespidae) kasten diferentziazioa eta profil kutikularraren, gantzen eta espermatozoide-ekoizpenaren analisia

Izaskun Pérez de Heredia Ramírez

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Universidad del País Vasco
Euskal Herriko Unibertsitatea

Cover photo: Denis Benito

A thesis submitted by :

Izaskun Pérez de Heredia Ramírez

For the degree of Doctor of Philosophy, under the supervisión of:

Dr Juan Carlos Iturrondobeitia and **Dr Arturo Goldarazena**

Universidad del País Vasco Euskal Herriko Unibertsitatea

ZTF-FCT
Zientzia eta Teknologia Fakultatea
Facultad de Ciencia y Tecnología

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Summary

Laburpena

Resumen

Summary:

Hymenopteran alien and invasive species entail a problem for the ecosystem where they are introduced, due to the characteristics that they have such as, having spermatheca, easy dispersal, adaptation flexibility, fast development, longevity…

This is the case of *Vespa velutina* hornet, also known as yellow-legged hornet, which was introduced in 2005 by accident in Europe, specifically in Lot-et-Garonne (France). It is thought that a single inseminated queen was introduced via boat shipment from China. Since then, the species has spread throughout lots of countries in Europe.

The invasion of this species brings some damages such as: ecological damage, because is an opportunistic hornet that hunts other arthropods; economic damage, since among all arthropods it hunts the most preferred prey is the honeybee, *Apis mellifera*; and social damage, because they like urban areas to stablish their huge nests.

It is a eusocial and monogynous species in which the queen lives only for a year. The life cycle starts with a small nest built in spring that grows in size and in individuals number thorough the year. Each nest can produce up to 6000 individuals on average. Throughout autumn, the sexual individuals (future queens and males) are produced, which mate and after, the inseminated future queens hibernate while males and the rest of the colony die.

As it happens with many others insects, in this species, the chemical communication among individuals is essential. Inside this kind of communication, we can find the short distance communication in which the cuticular hydrocarbons (CHCs) are very important. Through those CHCs hornets can know the age, the caste, the physiological status… of the rest of the individuals of the same species.

This thesis has focussed mainly in two aspects of the biology of the species:

On the one hand, in the knowledge of the life cycle and in the determination of the moment in which the future queens are produced. For that, some parameters have been studied and compared with the CHCs in order to know which of them is the most suitable to distinguish future queens from workers. It was concluded that the mesoescutum width is the best one (Chapter 2).

On the other hand, it has deepened in the knowledge of a small part of the large chemical communication, analysing how different factors such as the age and the diet of emerged adult, could influence in the CHCs, quantity and quality of accumulated fats, and in sperm production (Chapters 3 and 4). At the same time, whether or not fats (quantity and quality) and sperm production present any relationship with the CHCs (Chapters 3 and 4, respectively), was studied.

The results of those two chapters show that in both cases, future queens and males, age is the variable which has a greater influence in CHCs, and also in the accumulated fats quality and in the production of sperm. However, the diet is the variable which modifies the fats quantity. There is a slight relationship between both varia-

bles, fats and sperm production, and the CHCs, although it cannot be concluded whether or not the variables directly influence the cuticle because the three are very age dependent.

Laburpena:

Himenoptero espezie exotiko inbaditzaileek arazo bat suposatzen dute sartuak diren ekosistementzako espermateka, hedapen erraza, malgutasun egokigarria, garapen azkarra, bizitza-luzera … bezalako ezaugarriak edukitzeagatik.

Hau da *Vespa velutina,* liztortzar asiarra, izeneko espeziearen kasua, Europan 2005ean ustekabeaz sartua izan zena, Lot-et- Garonne-n (Frantzia), hain zuzen ere. Intseminatutako eme bakarra Txinatik zetorren merkantzia-itsasontzi batean heldu zela uste da. Une horretatik aurrera, Europako hainbat herrialdeetan zehar sakabanatu da.

Espezie honen inbasioak kalte ekologikoak, beste artropodoak harrapatzen dituen liztortzar oportunista delako; ekonomikoak, harrapatzen dituen artropodoen artean erle arrunta, *Apis mellifera,* gustukoena duelako; eta sozialak, gune hiritarretan bere habia erraldoiak eraikitzeko joera duelako, dakartza.

Erregina urte bakar batez bizi den espezie eusozial eta moginoa da. Bizi-zikloa udaberrian sorturiko habia txiki batekin hasten da. Habia honen tamaina eta indibiduoen kopurua urtean zehar hazten doaz. Habia bakoitzak batez beste 6000 indibiduo ekoiztu dezake. Udazkenean zehar, indibiduo ugalkorrak, hurrengo urteko erreginak eta arrak, ekoiztuko dira. Hauek ugaldu eta jarraian, hurrengo urteko erreginak hibernazioan sartu eta arrak eta langileak hil egingo dira.

Beste intsektu askotan gertatzen den moduan, espezie honetan, indibiduoen arteko komunikazio kimikoa funtsezkoa da. Komunikazio mota honen barruan distantzia

txikiko komunikazioa aurki daiteke. Honetan, hidrokarburo kutikularrak (CHCak) oso garrantzitsuak dira. CHC hauen bidez, liztortzarrek espezie bereko bestelako indibiduoen adina, kasta, egoera fisiologikoa … jakin dezakete.

Tesi hau, espeziearen biologiaren bi aspektutan zentratu da:

Alde batetik, bizi-zikloa eta hurrengo urteko erreginak noiz ekoizten diren hobeto ezagutzea. Horretarako, parametro desberdinak aztertu eta CHCekin alderatu dira, langile eta hurrengo urteko erreginak hobeto zeinek bereizten dituen jakiteko. Hain zuzen ere, mesoeskuteloaren zabalera (MW) egokiena dela ikusi da (2. Kapitulua).

Beste aldetik, hain zabala den komunikazio kimikoaren zati txiki baten ezagutzan sakondu da. Emergitu osteko indibiduoen adina eta hartutako bazka bezalako faktoreek, hasteko, hurrengo urteko erreginen CHC eta metatutako gantzen kalitate eta kantitatean nola eragiten duen ikasi da (3. Kapitulua) eta jarraitzeko, faktore hauek arren CHC eta esperma-ekoizpenean zer nolako eragina duten aztertu da (4. Kapitulua). Honekin batera, bai gantzen kantitate eta kalitateak bai esperma-ekoizpenak CHCetan eraginik ote duten ikertzea izan dugu helburu (3. eta 4. Kapituluak hurrenez hurren).

Bi kapitulu hauen emaitzek, bi kasuetan adinak bai hurrengo urteko erreginen, bai arren CHCetan, metatutako gantzen kalitatean eta baita esperma-ekoizpenean ere, gehien eragiten duena dela adierazten dute. Aldiz, gantzen kantitatean, bazka da eragin handiena duena. Hau guztiaz aparte, gantzak eta esperma-produkzioa bi

aldagaien eta CHCen arteko erlazio lirain bat dagoela ondoriozta daiteke; hala ere, eragin hau modu zuzen batean gertatzen denik ezin da baieztatu, hirurak adinarekiko oso menpekoak baitira.

Resumen:

Las especies de himenópteros exóticas invasoras suponen un problema para los ecosistemas en los que son introducidas debido a las características que poseen como disponer de espermateca, fácil propagación, flexibilidad adaptativa, rápido desarrollo, longevidad…

Este es el caso de la especie de avispón *Vespa velutina*, también conocida como avispón asiático, el cual fue introducido accidentalmente en Europa, concretamente en Lot-et-Garonne (Francia), en 2005 mediante un barco de carga proveniente de China, que contenía, se cree, una única reina inseminada. A partir de este momento la especie se ha extendido por muchos países de Europa.

La invasión de esta especie trae consigo daños ecológicos ya que es un avispón oportunista que caza otros artrópodos, daños económicos debido a que entre los artrópodos que caza su presa favorita es la abeja de la miel *Apis mellifera* y, por último, daños sociales porque tiene una predilección por las zonas urbanas para crear sus enormes nidos.

Es una especie eusocial monogina en la que la reina solo vive un año. El ciclo de vida comienza con un nido pequeño creado en primavera que va aumentando de tamaño y de número de individuos a lo largo de la temporada. Cada nido puede llegar a producir hasta 6000 individuos de media. Durante el otoño se producen los individuos sexuales fértiles, futuras reinas y machos, que se aparearán, y posteriormente las futuras reinas entrarán en hibernación mientras que los machos y el resto

de la colonia muere.

Al igual que ocurre con muchos otros insectos, en esta especie, la comunicación química entre individuos es esencial. Dentro de este tipo de comunicación podemos encontrar la comunicación de corta distancia en los que los denominados hidrocarburos cuticulares (CHCs) juegan un papel fundamental. A través de estos CHCs los avispones pueden saber la edad, la casta, el estado fisiológico… del resto de individuos de la misma especie.

Esta tesis se ha centrado principalmente en dos aspectos de la biología de la especie:

Por un lado, en conocer mejor el ciclo vital y determinar en qué momento son producidas las futuras reinas. Para ello, se han estudiado distintos parámetros y comparado con los CHCs para saber cuál de ellos es el más adecuado a la hora de diferenciar las futuras reinas de las obreras. Se ha visto que la anchura del mesoescutelo (MW) es el más apropiado (Capítulo 2).

Por otro lado, se ha profundizado en el conocimiento de una pequeña parte de la extensa comunicación química, estudiando cómo afectan distintos factores, como son la edad del adulto y la dieta ingerida después de la emergencia, en los CHCs y en la cantidad y calidad de grasa acumuladas en las futuras reinas (Capítulo 3) y en los CHCs y la producción de espermatozoides en los machos (Capítulo 4). Y a su vez, se ha intentado establecer si tanto la cantidad y calidad de grasa acumulada como la producción de espermatozoides tienen alguna relación con los CHCs

(Capítulos 3 y 4 respectivamente).

Los resultados de estos dos capítulos muestran que en ambos casos la edad es la variable que más influye en los CHCs tanto de futuras reinas como de machos, en la calidad de la grasa acumulada, así como en la producción de espermatozoides. Sin embargo, es la dieta la variable que más influye en la cantidad de grasa acumulada. También se observa que existe una leve relación entre las dos variables grasa y producción de espermatozoides y los CHCs, aunque no se puede asegurar que la influencia sea de manera directa ya que los tres son muy dependientes de la edad.

Chapter 1: General introduction and aims

Due to the increase of the commercial globalization the risk to introduce an invasive alien species in new regions has been enhanced (Lockwood et al. 2005). Among those species the social Hymenoptera have well-known skills to be transported by humans and to be colonizer of new regions. Regarding the invasive skills, the most important ones would be, on one hand, having a spermatheca to store sperm of more than one male, thus, only one inseminated queen is necessary to establish a new population with a great genetic variability. On the other hand, having an efficient dispersal power which means showing high reproductive rate, longevity, wide range in the diet and habitat or effective predator defences (Moller 1996).

In the Hymenoptera order we can find the Vespidae family which includes both solitary and eusocial groups (Pickett and Carpenter 2010). Social forms include hornet wasps (Perveen and Saha 2013), embracing 23 species of *Vespa* Linnaeus, 1758 genera (Carpenter and Kojima 1997). Twenty one of these species are native to Asia, including *Vespa velutina* Lepeletier, 1836 and only 2 European (*Vespa crabro* Linnaeus, 1758 and *Vespa orientalis* Linnaeus, 1771) (Carpenter and Kojima 1997).

Invasion of the species

The natural distribution of *Vespa velutina* ranges from Afghanistan to eastern China, Indo-China and Indonesia (Villemant et al. 2011a). The subspecies *Vespa velutina nigrithorax* du Buyson 1905 (from now on simplified as *Vespa velutina*),

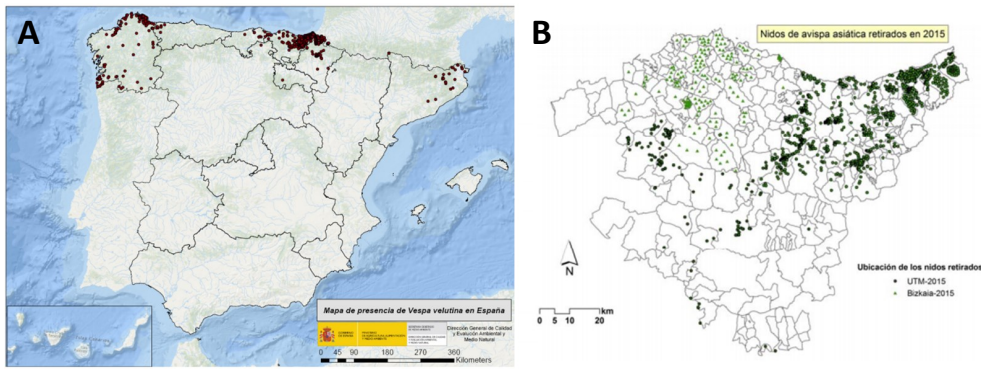
was detected in 2005 in Europe officially for the first time, more precisely in Lot-et-Garonne (France) (Haxaire et al. 2006). It is thought that only one inseminated future queen arrived to France via boat shipment from China (Arca et al. 2012). This species is also invasive in South Korea since 2003 (Kim et al. 2006) and in Japan since 2013 (Ueno 2014).

After the introduction in France, the hornet has rapidly spread to neighbouring countries such as Spain (Castro and Pagola-Carte 2010), Portugal (Grosso-Silva and Maia 2012), Belgium (Bruneau 2011), Italy (Demichelis et al. 2013), Germany (Witt 2015), England (Keeling et al. 2017) and Netherlands (Smit et al. 2018).

In Spain, the first individual found of *V. velutina* was a worker and it was observed in Amaiur (Navarra) in 2010 (Castro and Pagola-Carte 2010). Since then, the species has colonized other provinces in the north of the country and nowadays it is well-established in all Cantabrian coast (Goldarazena et al. 2015) (Figure 1A). The species is also present in provinces as Catalonia (Pujade-Villar et al. 2012) La Rioja, Burgos (MAGRAMA 2015) and Valladolid (Torres personal communication 2019). The island of Majorca in the Balearic Islands was also colonized in 2015 (Leza et al. 2017).

In the Basque Country the species can be found in the three provinces (Alava, Biscay and Guipuzcoa) (Goldarazena et al. 2015) (Figure 1B) and the number of nests removed has increased considerably from 2013 to 2017, reaching the maximum number in 2016 with 424 nests removed in Alava, 2556 in Biscay and 1718 in

Guipuzcoa. In 2017 a slight decrease was observed which probably indicates that the advance of the invasion has reached its maximum in this territory (Barandika



et al. 2018).

Figure 1: Presence of *Vespa velutina* A) in the Iberian Peninsula in 2014 (MAGRAMA 2015) and B) in the Basque Country in 2015 (Galartza 2016).

Caused damage

All Vespidae invasions imply some ecological, economic and societal impacts (Beggs et al. 2011). In the case of *V. velutina* those damages are the following ones:

Ecological damage:

V. velutina is a generalist species that preys all kind of arthropods as many hymenopterans, dipterans, arachnids… (Villemant et al. 2011b). In Asia, *V. velutina* is known as an active predator of honeybees (Abrol 2006; Tan et al. 2007). According to a study conducted by Villemant et al. (2011b) *Apis mellifera* Linnaeus, 1758

represented at least the third of the diet of the hornet. However, there is not any accurate assessment of the consequences of the predation on pollinator services (Monceau et al. 2014) on the ecosystems diversity.

Apart from the damage caused due to the predation, when an alien predator is introduced in any ecosystem, there can be a displacement or a replacement of the species that have the same ecological niche as the new arriving species (Snyder and Evans 2006). In the case of *V. velutina*, the species that could suffer these consequences is the European hornet, *Vespa crabro*, which is protected in some areas of each native range (e.g. in Germany since 1987, Federal Species Protection Ordinance-BArtSchV/Federal Nature Conservation Act-BNatSchG) (Monceau et al. 2014). The European hornet might compete with the invasive hornet for nesting sites in spring because its queens emerge from hibernation later than those of *V. velutina* which already has initiated their embryo nests (Rome et al. 2015). Apart from that, according to a study conducted by Cini et al. (2018), it appears plausible that the native hornet species might be easily outcompeted and displaced by the invasive one at foraging hotspots.

Economic damage:

As it has been previously mentioned, the predation of *V. velutina* on the domestic honeybee is supposed to be at least a third of the diet of the hornet (Villemant et al. 2011b). This predation implies economic harm for the beekeeping sector which is directly affected (Monceau et al. 2012). The damage suffered by the beekeepers

can be classified in two groups. On one side, direct damage called "homing failure": caused by the direct loss of the bees that the hornet hunts (Monceau et al. 2013a). On the other side, indirect damage called "foraging paralysis of the colony": caused due to the fact that bees do not get out to collect nectar and pollen due to the presence of hornets outside the beehive (Monceau et al. 2018). So, both the bee number and the collecting activity will decrease in the presence of *V. velutina*, which could entail the weakening and the dead of the colony (Requier et al. 2018). There is not any study which quantifies the economic loses that the introduction of *V. velutina* in Europe has supposed (Monceau et al. 2014).

Social damage:

Even though in its natural range *V. velutina* is considered as an aggressive hornet (Martin 1995), the subspecies introduced in Europe is not more aggressive than the native one when is chasing or foraging (Monceau et al. 2014). However, if the nest is disturbed, the risk to be attacked increases. In that case, the attack can be collective and virulent (Rome et al. 2011). Usually the nests of *V. velutina* are located in treetops, which reduces considerably the contact with humans (Rome et al. 2011). Nevertheless, due to the abundance in populated regions and the large size of nests, a social alarm has been created (Monceau et al. 2014). In places where hornets are very abundant the nest can be located everywhere. For that reason, the probability of been stung increases, which can be dangerous for allergic people.

Biology of the species

Life cycle:

As occurs in most *Vespa* species, *V. velutina* is a monogynous species, with colonies founded by a single queen (Spradbery 1973; Arca et al. 2012) and with annual life cycle (Figure 2) (Chauzat and Martin 2009). In spring, after spending some months in diapause, when the temperatures start raising these inseminated queens emerged from their over-wintering hibernation (Chauzat and Martin 2009).

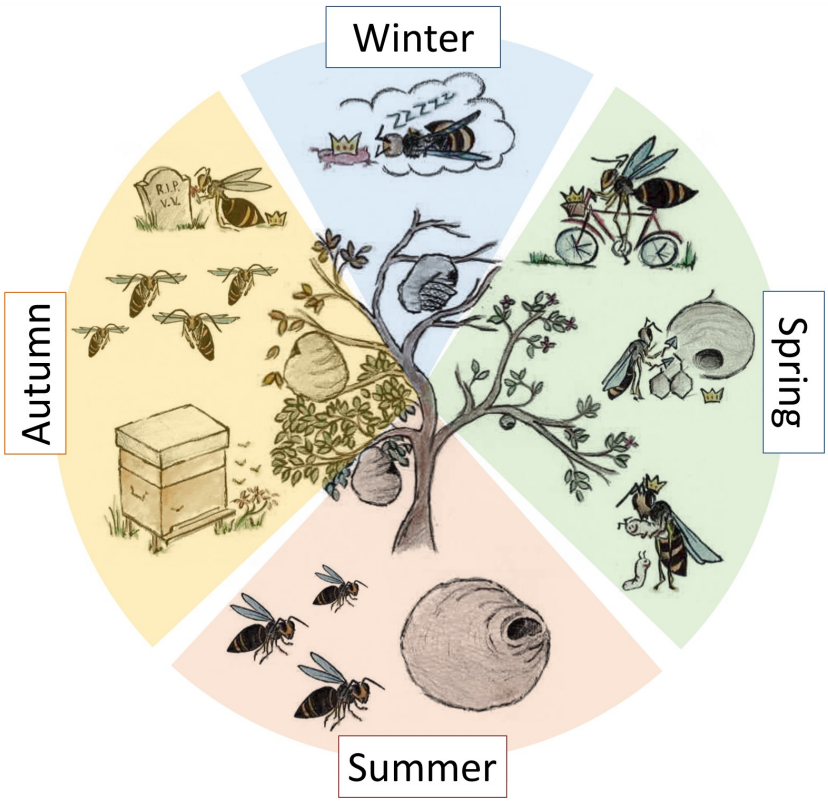


Figure 2: Scheme of the life cycle of *Vespa velutina*. (Design: Daniela Laurino).

These queens start the construction of small embryo nests (tennis ball-size one) which will be located in a safeguard place as shrubs or tree holes (Archer 2008). As in other social wasp colonies, the nests are made of coarse papery material made of masticated wood fibres (Perveen and Saha 2013). When the first comb is constructed, the queen lays the first eggs (Mastuura and Yamane 1990). These eggs will hatch after around 4-6 days (Matsuura and Yamane 1990). At this moment the queen has to start hunting insects to feed the larvae (Monceau et al. 2014). During this phase (called "the queen colony phase"), the queen is very vulnerable because she has to perform the entire tasks alone (Villemant et al. 2011b). After 48.1 days, the first cohort of workers appears (Dazhi and Yunzhen 1989). From now on, the queen´s only function is going to be laying eggs (Spradbery 1973). The rest of the tasks such as hunting preys, defending the colony, increasing the nest size… will be delegated to the workers (Spradbery 1973).

In aculeate colonies, the first cohort of workers, raised by the queen alone, is going to be formed by small size hornets because the queen reared them as quickly as possible according to the available food (Mead et al. 1994). As in the rest of the holometabolous insects, the adult size depends on the body size that the larva has when it stops feeding and therefore it stops growing (Shingleton et al. 2007). Larva size depends on the fat reserve which is linked to the food received during this stage (Strohm 2000). In several Vespidae insects, low larva / worker ratio is needed to increase the quality and/or quantity of food given to larvae and therefore, to produce workers with a higher body mass (Monceau et al. 2013b). Consequently,

the following cohorts' size will increase until the largest workers appear (Miyano 1981). There is another possible reason to explain the increment of the workers size, the cell size where are raised. The size of this cells increases while the nest gets bigger (Spradbery 1972).

Throughout the following months, the nest and the colony size will increase (Monceau et al. 2014). Until autumn, a hundred to a thousand workers will be produced by a single nest (an average of 6000 individuals) (Villemant et al. 2011b). In autumn the nest reaches its largest size (Monceau et al. 2014). Since that moment the production of new hornets will be focused on the sexual individuals, males and new queens, "*gynes*", henceforth (Monceau et al. 2014). The production of males starts about 15 days before the production of the gynes (Rome et al. 2015) and three times more males than females seem to be produced from mid-September to the end of November (Villemant et al. 2011b).

After leaving the nest, gynes will mate with males and will start to search for a sheltered place to over-winter (Matsuura and Yamane 1990). The old queen, workers and males are not going to survive the winter and the colony will die (Scwartz et al. 2012). It is estimated that a range of 90 to 99.9 % of the gynes will not survive (Bunn 1982; Matsuura 1984; Matsuura and Yamane 1990; Donovan 1991; Archer 2008, 2012). It seems that both inseminated and non-inseminated ones will be able to hibernate (Matsuura and Yamane 1990; Monceau et al. 2014). In spring, around the 98 % of the *Vespa velutina'*s new queens that survive to the winter are inseminated (personal observation).

The nest

In Vespidae family, the nets are built using plants fibres that are mixed with water and saliva. With this, they produce a mass (as a paper "*maché*") that is shaped with jaws to create new nest layers (Edwards 1980).

The construction of the nest can be separated in two phases, embryo nest and secondary nest. As previously mentioned, there is the embryo nest phase, in which the spring emerged gynes, using this sort of paper "*maché*", they have to construct a small round nest (Chauzat and Martin 2009). This nest is formed by a small comb and a cover which protects the nest completely except a hole in the lower part, the entrance (Matsuura and Yamane 1990). Those nests are usually located in sheltered places (Chauzat and Martin 2009).

When the first workers emerged, the nest will start to increase its size and in most of the cases those sheltered places are not big enough to develop the secondary nest. In those cases, nest will be relocated in more spacious and usually higher (trees branches) places (Matsuura 1984, 1991). Some workers search for an appropriate place to build the new nest. Once the scout workers have found the most suitable place, the queen will abandon the old nest and will settle in the new one. The queen never returns to the old nest. After the queen, the rest of the colony moves to the new nest. However, some workers visit the old nest to feed the larvae and newly emerged workers that are still there. When all the brood of the old nest are adult and can fly, they move to the new one and the old nest is abandoned.

(Matsuura and Yamane 1990). According to Rome et al. (2015) around 70 % of *V. velutina*'s nests are relocated.

The secondary nests of *V. velutina* find throughout the Northern hemisphere are large, round nests with many horizontal combs covered by a paper "*maché*" envelope(Perveen and Saha 2013), and they have the entrance in a lateral (Rome et al. 2011). Thanks to the envelope, the workers are able to maintain the temperature of the nest around 30 ºC, despite the ambient temperatures that may be up to 20 °C lower (Martin 1990). All nests are used only once although the same place can be used more than once (Chauzat and Martin 2009). They are usually located in the top branches of trees or in buildings although; subterranean nests have been also recorded, as occurs in other hornets (Martin 1995; Villemant et al. 2008). *V. velutina*'s nests are found in agricultural or forest habitats, but most of the French records correspond to urbanized habitats (48.53 %) (Rome et al. 2015).

Predation

The hunting behaviour of social vespids can be summarized as waiting, catching, processing the prey and bringing it to the nest (Richter 2000). This pattern is also observed in *V. velutina* that waits in a static fly in front of the hives entrance for the bees that return from pollen picking. Those bees return full of pollen and tired which make them fly awkwardly. The hornet captures the bee on fly and once the bee is caught the hornet brings it to a near tree branch where is hung with the third pair of legs. Then, using its jaws, it cuts the head and the abdomen of the bee and

chews the thorax (where the flying muscles are located) forming a mass which will be used to feed the larvae (Villemant et al. 2010). The predation in front of the hives supposed a slowdown of the foraging rate as it was explained before (Requier et al. 2018).

Nourishment

The food consumed by vespine wasps can be classified in two groups, carbohydrates and proteins (Matsuura and Yamane 1990). Carbohydrates are consumed mainly by the adults (queen, workers, gynes and males) (Richter 2000) and they are provided by flower nectar, tree sap or ripening fruit (Monceau et al. 2014). These resources are used for example, by queens newly emerged from the hibernation in spring, before the construction of the embryo nests (Monceau et al. 2014). In addition, in autumn it is usual to see males feeding in *Hedera helix* L. nectar (Monceau et al. 2014).

The proteins are obtained by hunting bees and other arthropods. Those preys are given to the brood because the larvae need proteins to develop. After the digestion of the proteins, the fifth instar of larvae produce a secretion rich in carbohydrates and amino acids. This secretion is given in exchange to the adults because they cannot digest proteins directly due to their narrow waist (Chauzat and Martin 2009). The exchange takes place by a process called "*trophallaxis*" (Wheeler 1928) in which the substances are exchanged through a mouth-mouth contact (Edwards 1980). This process can be observed between larvae and adults (queen,

workers, gynes and males) (Matsuura and Yamane 1990), but also between adults (Edwards 1980; Chapman 2013; Shukla et al. 2013). Apart from food, some chemical compounds, that take part in chemical communication of these insects, are also exchanged by trophallaxia (Chapman 2013).

Chemical communication

In many social insects, chemical communication plays an important role in the organization and maintenance of the colonies (Mitra and Gadagkar 2014).

This chemical communication is based in semiochemicals (Law and Regnier 1971). Those are classified into two categories (Nordlund and Lewis 1976) allelochemicals (Greek ***allelon*** = of one another) (Brown 1968) and pheromones (Greek ***pherein***= to transfer (carry); ***horman***= to excite) (Karlson and Butenandt 1959). The first ones are used in the communication between individuals of different species (Brown 1968) while the second ones are used by individuals of the same species (Karlson and Butenandt 1959; Karlson and Luscher 1959).

In 1963, Wilson and Bossert proposed dividing pheromones into two categories according to their mode of influence: (I) primer pheromones which induce relatively long-lasting physiological changes in receiving individuals; (2) releaser pheromones which stimulate the receiving individual to perform immediate behavioural responses.

Primer pheromones are secreted by social insects such as bees, ants, wasps and termites, for example queens (as inhibitors of ovary and queen cell construction)

or workers (as caste inhibitors) (Ali and Morgan 1990). An example of this would be the "queen substance" of the honeybee, *Apis mellifera*, the main component of which is trans-9-oxo-2-decenoic acid, from the mandibular glands (Butler et al. 1962; Slessor et al. 1990). This substance inhibits ovary development in worker bees (Jackson and Morgan 1993).

In the case of releaser pheromones they can be classified into 9 groups according to the function or the behaviour that they elicit into the receiving insect: (I) sex pheromones; (II) aggregation pheromones; (III) dispersal or spacing pheromones; (IV) alarm pheromones; (V) recruitment or trail pheromones (Birch and Haynes 1982); (VI) territorial or home range pheromones (Cammaerts et al. I977; Holldobler and Wilson I977); (VII) surface pheromones (Wilson 1971); (VIII) funeral pheromones (Wilson et al. 1958; Wilson 1963; Matthews and Matthews 1978) and (IX) invitation pheromones (Ahmadi and McClelland 1985). The assumption that all pheromones are dispersed through, and received from the air is often made. That is frequently, but not invariably. Sometimes they are dispersed through water or they are received by direct contact between individuals, or by contact with a surface (Ali and Morgan 1990). The last one is the case of surface pheromones.

Surface pheromones include recognition pheromones, releasers of grooming behaviour and secretions that stimulate food exchange (Ali and Morgan 1990). These pheromones are mainly produced and absorbed on the body surface and other insects perceive them by direct contact or over a short distance (Shorey 1973). They

are very important in social insects colonies and such pheromones allow recognition of conspecifics, nestmates, kin or even members of different castes (Jackson and Morgan 1993; Gévar et al. 2017). These pheromones are complex substances, made up by greatly varied mixtures of many compounds. Most of them are long chain hydrocarbons, located on the insects cuticle (Vander Meer et al. 1998).

Cuticular hydrocarbons

The insects cuticle is coated with a mixture of hydrocarbons (long-chain aliphatic and methyl-branched alkanes and alkenes) (Blomquist and Bagnères 2010). Those cuticular hydrocarbons (CHCs) have the primary functions of preventing desiccation and regulation of cuticular permeability (Hadley 1980), but also have semiochemical functions (Howard and Blomquist 1982) and in particular they are involved in recognition cues (Bonavita-Cougourdan et al. 1987; Vander Meer et al. 1989).

In addition, the CHCs also have a role in the sexual behaviour of insects taking part in the recognition of potential mates, the elicitation of courtship behaviour (Steiner et al. 2005, 2006, 2007; Sugeno et al. 2006; Ferveur and Cobb 2010; Ginzel 2010; Guédot et al. 2010), as well as its inhibition (Carlson and Schlein 1991).

In social insects, cuticular hydrocarbons, apart from being surface pheromones or contact sex pheromones, may be indicators of dominance and fertility (Liebig, 2010), and can contribute to the recognition of nesting sites (Espelie et al. 1990;

Steinmetz et al. 2003).

The nest surface also is covered by hydrocarbons (Espelie et al. 1990). Those hydrocarbons present a similar composition to those found on the cuticle of the wasps from that colony (Espelie and Hermann 1988, 1990; Espelie et al. 1990; Lorenzi 1992; Singer et al. 1992). In the case of the wasp *Polistes metricus* Say, 1831; newly emerged workers need to be exposed to the nest surface hydrocarbons in order to recognize their nest and nestmates (Singer and Espelie 1992; Layton and Espelie 1994).

Synthesis of hydrocarbons

There are few studies which have examined the precise site(s) where hydrocarbons are synthesized (Schal et al. 1998). However, traditionally it has been thought that hydrocarbons of cuticle arise from oenocytes, large cells that are rich in smooth endoplasmic reticulum and mitochondria and that appear only in abdominal tissues (Romer 1991). Oenocytes are often associated with fat bodies, a major source of fatty acids that could serve as precursors for hydrocarbons biosynthesis (Diehl 1975; Howard and Blomquist 2005; Juarez and Fernandez 2007; Provost et al. 2008). After the formation of those hydrocarbons, they are released into the haemolymph and transported to the cuticle by lipophorins (Van der Horst et al. 1993; Blomquist et al. 1998; Schal et al. 1998; Jurenka and Subchev 2000; Bagnères and Blomquist 2010).

Related to the nest specific odour, in 1979 Crozier and Dix proposed the model

called “Gestalt”. In this model it is proposed that, apart from the contact pheromones which are innate and genetically determined, in social hymenoptera, odours from the environment they are also used as contact pheromone. Those odours can be taken for example from the food or local odours and they are absorbed by the cuticle. So, in some cases, genetically determined pheromones are reinforced by environmental compounds.

According to this model, odour pheromones are transferred among all colony individuals by “*grooming*” and “*trophallaxis*” (Crozier and Dix 1979). But, apart from the transmission among workers it has been seen that it can be transferred from the nest surface to the individual cuticle (Crosland 1989; Espelie et al. 1990; Lorenzi et al. 2004). Like that, all workers will have a common “gestalt” odour instead of having an individualistic odour and hence the members of the same colony can recognize each other (Crozier and Dix 1979). Newly emerged individuals present a cuticular profile analogous to a “*blank slate*” because they have not acquired the nest odour (Breed et al. 2004, 2015).

Importance and aims

It was only 14 years ago that the species *Vespa velutina* was introduced to Europe and taking into account that before its introduction, very few studies about this hornet had been performed, we considered that it was necessary to try to find out more about different aspects of the life cycle. Specially identifying gynes and knowing in which moment of the life cycle they are produced, in order to apply

best control measures against the invasion.

Once we knew when sexual individuals were produced, the study was focused on the chemical communication of those individuals. The study of CHCs and especially sexual individuals' CHCs is essential for the understanding of different behavioural aspects of those social himenoptera. This is the first work where the relation between CHCs and fats and/or sperm production of *Vespa velutina* gynes and males respectively, is analysed.

Those analyses give information about how the gynes chemical profiles change according to the accumulated fats. This way, males could be able to identify and choose gynes for mating, with more probability to survive winter (i.e. those with more accumulated fats). The ability to survive winter is one of the keys of the invasion success of this species.

Another unknown and very interesting aspect of this species is the importance of the CHCs in the inbreeding avoidance. The inbreeding is one of the most harmful factors for the alien invasive species. For that reason, we consider essential to study the relation between the cuticular chemical profiles and the sperm production in males. Thus, we could know if workers could use this profile to identify sexually mature males and to expel them from the nest. Avoiding the mating between gynes and males of the same nest, which are siblings, reduces the inbreeding.

Considering all this, CHCs could be used in the future to develop selective traps to

capture sexual individuals. The trapping of those sexual individuals is fundamental for the control of the expansion of the species.

Those are the aims raised in this thesis:

Chapter 2: On the one hand, to study how the individuals' size change and how the populations vary throughout the life cycle of *Vespa velutina*. On the other hand, to distinguish new future queens, gynes, from workers and to find the easiest and most precise parameter for it, with the objective of knowing better the life cycle. This is essential to control the invasive species.

Chapter 3: To analyse on the one side, if the age and the diet can modify *Vespa velutina* gynes CHCs profile. On the other side, if those variables also have influence in their fats quantity and quality. Finally, to analyse if there is any relationship between fats (quantity and quality) and cuticular profile in gynes, to know if the cuticle could be an information source of the accumulated fats quantity and quality that could be used by males in choosing the best gyne to mate with.

Chapter 4: To analyse firstly, if the age and diet can modify *Vespa velutina* males cuticular profile. Secondly, if those variables also affect to the sperm production. Lastly, to study if sperm production and CHCs profile present any relationship in males, to know if the cuticular profile gives information on the sexual maturation stage of males that could be used by workers to know when they have to expel males of the nest.

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1. **Kapitulua:** Sarrera orokorra eta helburuak

Globalizazio komertzialaren gehitzea dela eta, lurralde batzuetan espezie inbaditzaileak sartzeko arriskua areagotu da (Lockwood et al. 2005). Espezie horien artean, himenoptero sozialek gizakien bidez garraiatuak izateko eta eskualde berriak kolonizatzeko ondo ezagutzen diren gaitasunak dituzte. Inbaditzeko gaitasun garrantzitsuenen artean, alde batetik, emeek ar baten baino gehiagoren esperma gordetzeko espermateka edukitzea dago. Hau dela eta, intseminatutako eme bakarra nahikoa da aldakortasun genetiko handiko populazio bat ezartzeko. Beste aldetik, sakabanatzeko trebetasun, ugaltze-tasa eta bizitza-luzera handia dute, habitat eta dieta tarte zabala baita harraparien aurrean defentsa eraginkorrak ere (Moller 1996).

Hymenoptera taldearen barruan, Vespidae familia aurkitzen dugu, non, bai talde bakartiak, bai eusozialak aurkitzen diren (Pickett eta Carpenter 2010). Talde sozialaren barruan liztortzarrak aurkitzen dira (Perveen eta Saha 2013). Hauek, *Vespa* Linnaeus, 1758 generoko 23 espezieetan sailkatzen dira (Carpenter eta Kojima 1997) eta espezie hauetatik 21ek *Vespa velutina* Lepeletier, 1836 barne, Asia dute jatorri naturala (Carpenter eta Kojima 1997). Beste biek aldiz, (*Vespa crabro* Linnaeus, 1758 eta *Vespa orientalis* Linnaeus, 1771) jatorri Europarra dute (Carpenter eta Kojima 1997).

Espeziearen inbasioa:

Vespa velutina-ren habitat naturala Afganistanetik Txinako ekialde, Indo-Txina eta Indonesiara zabaltzen da (Villemant et al. 2011a). Baina espezie honen subes-

pezie bat, *Vespa velutina nigrithorax* du Buyson, 1905 (hemendik aurrera *Vespa velutina* bezala laburtuta); 2005ean detektatu zen lehenengo aldiz Europan, Lot-et-Garonnen (Frantzia), hain zuzen ere (Haxaire et al. 2006). Intseminatuta zegoen eme bakar bat merkantzia-itsasontzi batean Txinatik Frantziaraino garraiatu zela uste da (Arca et al. 2012). Espezie hau Hego Korean 2003tik aurrera inbaditzailea da (Kim et al. 2006) eta baita Japonian 2013tik aurrera ere (Ueno 2014).

Frantzian sartua izan ostean, alboko herrialdeetara, hala nola Espainia (Castro eta Pagola-Carte 2010), Portugal (Grosso-Silva eta Maia 2012), Belgika (Bruneau 2011), Italia (Demichelis et al. 2013), Alemania (Witt 2015), Inglaterra (Keeling et al. 2017) eta Holandara (Smit et al. 2018) oso azkar sakabanatu da.

Espainian aurkitutako *V. velutina*-ren lehengo indibiduoa, 2010ean Amaiurren (Nafarroa) topatutako langile bat izan zen (Castro eta Pagola-Carte 2010). Hemendik aurrera, espezieak penintsularen iparraldeko bestelako probintziak kolonizatu ditu eta gaur egun Kantauri kostan ondo ezarritako espeziea kontsideratzen da (Goldarazena et al. 2015) (1A. Irudia). Katalunia (Pujade-Villar et al. 2012), Errioxa, Burgos (MAGRAMA 2015) eta Valladolid (Torres komunikazio pertsonala 2019) bezalako probintzietan ere aurkitu daiteke espezie hau. Mallorca uhartea ere, Balear uharteetan, 2015ean kolonizatua izan zen (Leza et al. 2017).

Euskal Herrian espeziea hiru probintzietan aurki daiteke (Araba, Bizkaia eta Gipuzkoan) (Goldarazena et al. 2015) (1B. Irudia) eta kendutako habien kopurua 2013tik 2017ra nabarmenki handitu da, zenbaki maximoak 2016an lortu ziren:

Araban 424, Bizkaian 2556 eta Gipuzkoan 1718 habia kenduz. 2017an murrizpen txiki bat behatu zen, inbasioa lurralde honetan bere maximora heldu dela adierazi dezakeena (Barandika et al. 2018).

Sorturiko kalteak:

Vespidae familiako espezieen inbasio guztiek kalte ekologiko, ekonomiko eta sozialak dakartzate (Beggs et al. 2011). *V. velutina* espeziearen kasuan, kalte horiek hurrengoak dira:

Kalte ekologikoak:

V. velutina espezie jeneralista bat da, zeinek mota guztietariko artropodoak harrapatzen dituen, hala nola, himenopteroak, dipteroak, araknidoak… (Villemant et al. 2011b). Asian, liztortzar hau erleen harrapakari aktiboa izateagatik da ezaguna (Abrol 2006; Tan et al. 2007). Villeman et al-ek (2011b) egindako ikerketa batean, *Apis mellifera*-k Linnaeus, 1758 *V. velutina*-ren dietaren herena gutxienez osatzen duela ikusi zen. Hala ere, harrapakaritza honek polinizazioan eta ekosistemen dibertsitatean eragiten ari dituen kalteak zeintzuk diren jakiteko ez dago daturik (Monceau et al. 2014).

Harrapakaritzaren bidez eragindako kalteaz aparte, ekosistema batean espezie arrotz bat sartzen denean, sartutako espeziearen nitxo ekologiko berak dituen beste espezieen desplazamendua edo ordezkapena gerta daiteke (Snyder eta Evans 2006). *V. velutina* espeziaren kasuan, ondorio hauek pairatu dezakeen espeziea liztortzar Europarra (kurubiloa) da, *Vespa crabro,* zein bere jatorrizko hainbat lu-

rraldeetan babestuta dagoen (Alemanian adibidez, 1987tik aurrera. Federal Species Protection Ordinance-BArtSchV/Federal Nature Conservation Act-BNatSchG) (Monceau et al. 2014). Kurubiloak, espezie inbaditzailearekin lehiatu dezake udaberrian habia egiteko lekuengatik, Europarra Asiarra baino beranduago irteten baita hibernaziotik eta bigarren honek, jadanik hasita edukiko baitu habia primarioa (Rome et al. 2015). Honetaz aparte, Cini et al.-ek (2018) burututako ikerketaren arabera, jaioterriko liztortzarra elika puntuetan ere gainditua eta desplazatua izan daiteke inbaditzailearengatik.

Kalte ekonomikoak

Lehen aipatu izan den moduan, *A. mellifera*-k osatzen du *V. velutina*-ren dietaren herena gutxienez (Villemant et al. 2011b). Harrapakaritza honek kalte ekonomikoak suposatzen ditu batez ere erlezaintzaren sektorean (Monceau et al. 2012). Erlezainek pairatzen dituzten kalteak bi taldeetan sailkatuak izan daitezke. Alde batetik kalte zuzenak leudeke, liztortzarrek ehizatutako erleen galera zuzenetik sortutakoak ("homing failure" bezala ezagutua) (Monceau et al. 2013a) eta beste aldetik, zeharkako kalteak, erlategiaren aurrean liztortzarraren presentzia dela kausa erleek nektarra eta polena biltzera ez irteteagatik sortutakoak ("foraging paralysis of the colony" deitutakoa) (Monceau et al. 2018). Beraz, bai erleen kopurua, bai hauen biltze aktibitatea murriztuta geratuko da eta honek kolonien ahuldura eta heriotza ekar dezake (Requier et al. 2018). Oraindik ez dago ikerketarik Europan, *V. velutina*-ren inbasioak ekarri dituen galera ekonomikoak kuantifikatzeko (Monceau et al. 2014).

Kalte sozialak

Bere jatorrizko lurraldean *V. velutina* liztortzar oldarkorra kontsideratzen den arren (Martin 1995), Europan sartu den subespeziea ez da bertoko liztortzar (*V. crabro*) baino oldarkorragoa ehizatzerako orduan edo nektarrez elikatzen ari denean (Monceau et al 2014). Dena den, habia aztoratzen bada, eraso bat pairatzeko arriskua handitzen da. Kasu honetan erasoa kolektiboa eta zitala izan daiteke (Rome et al. 2011). Oro har, liztortzar asiarraren habiak zuhaitz puntetan kokatuak egoten dira eta modu honetan, gizakiarekiko kontaktua nahiko murriztuta dago (Rome et al. 2011). Alabaina, populazio handiko guneetan agertzen den liztortzar ugaritasun eta habien tamaina dela eta, alarma soziala sortu da (Monceau et al. 2014). Liztortzar asko dagoen lurralde batean habiak edonon egin ditzakete eta arriskutsua izaten da pertsona alergikoentzat, ziztada bat pairatzeko probabilitatea areagotzen delako.

Espeziearen biologia:

Bizi-zikloa:

V. velutina, beste *Vespa* espezie gehienetan gertatzen den moduan, espezie monoginoa da; hau da, erregina bakar batek sortutako duela kolonia (Spradbery 1973; Arca et al 2012) eta urtebeteko bizi-zikloa (2. Irudia) edukiko du (Chauzat eta Martin 2009). Udaberrian, diapausan hainbat hilabete pasa eta tenperaturak igotzen hasi ondoren, intseminatutako erreginak neguko hibernaziotik aterako dira (Chauzat eta Martin 2009).

Erregina hauek, habia primario txiki bat (tenis pilota baten tamainakoa) eraikitzen hasiko dira. Habia hau, babestutako lekuetan, hala nola zuhaixketan edo zirrikituetan adibidez,kokatuta egongo da (Archer 2008). Beste liztor sozial kolonietan bezala, habia, murtxikatutako egur zuntzetik lortutako paper lodi moduko material batez osatuta dago (Perveen eta Saha 2013). Habiaren lehenengo abaraska eraikita dagoenean, erreginak lehenengo arrautzak erruten ditu (Mastuura eta Yamane 1990). Arrautza hauek 4-6 egun pasa ondoren eklosionatu egingo dute (Matsuura eta Yamane 1990) eta momentu horretan erregina intsektuak ehizatzen hasi beharko da larbak elikatzeko (Monceau et al 2014). Epe hau ("the queen colony phase" deitutakoa) irauten duen bitartean, erregina oso ahula da, berak bakarrik burutu behar baititu eginbehar guztiak (Villemant et al 2011b). Arrautzak errun eta 48.1 egun beranduago agertu egingo da langileen lehenengo kohortea (Dazhi eta Yunzhen 1989). Une horretatik aurrera erreginaren funtzio bakarra arrautzak jartzea izango da (Spradbery 1973). Bestelako zereginak, hala nola, harrapakinak harrapatzea, kolonia defendatzea, habiaren tamaina handitzea… langileek burutuko dituzte (Spradbery 1973).

Akuleatuen kolonietan, erreginak bakarrik hazi dituen langileen lehengo kohortea, tamaina txikiko indibiduoez osatua egongo da. Hau, erreginak larbak elikatzeko eskuragarri dauzkan baliabide gutxiak direla medio, eta koloniak ahal duen azkarren hazteko premia duelako (indibiduo txikiak azkarrago garatuko direlako) gertatzen da (Mead et al. 1994). Bestelako intsektu holometaboloetan bezala, helduen tamaina, elikatzeari eta beraz hazteari uzten dion momentuan larbak daukan gor-

putz tamainagatik baldintzatuta egongo da (Shingleton et al. 2007). Larbaren tamaina, gantz erreserben menpekoa izango da eta azken hau larba fasean banakoak jasotako janariaren menpekoa (Strohm 2000). Hainbat Vespidae-tan, larba/langile ratio baxua beharrezkoa da larben janariaren kalitate eta/edo kantitatea handitzeko eta beraz, gorputz masa handiagoko langileak sortzeko (Monceau et al 2013b). Honen ondorioz, hurrengo kohorteen tamaina handiagotuz joango da langile handienak agertu arte (Miyano 1981). Indibiduoen tamainaren handipenerako beste azalpen posible bat dago: larbak hazten diren gelaxken tamaina. Izan ere, hauek gradualki handiagotzen doaz habia handiagotzen den heinean (Spradbery 1972).

Hurrengo hilabeteetan zehar, bai habia, bai koloniaren tamaina handituko dira (Monceau et al 2014). Udazkenera arte, habia bakar batetik ehunka langiletik milatara sortuak izango dira (6000 indibiduo batez besteko) (Villemant et al. 2011b). Habiak, udazkenean lortuko du tamaina maximoa (Monceau et al 2014). Momentu honetatik aurrera, indibiduo berrien sorrera indibiduo sexualak ekoiztera bideratuko da; hau da, arrak eta hurrengo urteko erreginak, oraindanik "*gine*" deiturikoak (Monceau et al. 2014). Arren ekoizpena gineen produkzioa baino 15 egun lehenago hasten da (Rome et al. 2015) eta hiru aldiz ar gehiago ekoizten dira gine baino erdi-irailetik azaroaren bukaerara arte (Villemant et al. 2011b).

Habia utzi ondoren, arrek gineak estaldu egingo dituzte eta ondoren gineek babestutako lekuak bilatzen hasiko dira negua pasatzeko (Matsuura eta Yamane 1990). Erregina zaharrak, langileek eta arrek ezingo diote neguari aurre egin eta hori dela eta kolonia hil egingo da (Schwartz et al. 2012). Horrez gain, gineen % 90-99.9ak

bizirauten ez duela uste da (Bunn 1982; Matsuura 1984; Matsuura eta Yamane 1990; Donovan 1991; Archer 2008, 2012), baina intseminatutako zein intseminatu gabeko gineen artean hibernatzeko gaitasunetan ezberdintasunik ez dagoela ematen du (Matsuura eta Yamane 1990; Monceau et al. 2014). Dena den, udaberrian, hibernaziotik ateratzen diren *Vespa velutina* erregina fundatzaileen intseminazio tasa % 98 ingurukoa izango da (behaketa pertsonala).

Habia

Vespidae familiako habiak, ura eta liztorren listuarekin nahastutako landare zuntzez eraikitzen dira. Modu honetan masa bat sortzen da, "*maché"* paperaren modukoa, zein barailekin forma emanez, habiaren geruza berriak sortzeko erabiltzen duten (Edwards 1980).

Bi habia fase desberdin bereiz daitezke: habia primarioa edo enbrionarioa eta habia sekundarioa. Jadanik aipatu den moduan, fase primarioan, udaberrian hibernaziotik ateratako gineek habi borobil txiki bat eraikitzen dute "*maché"* papera erabiliz (Chauzat eta Martin 2009). Abaraska zirkular bat eta material berdinarekin egindako estaldura batez osatua dago. Estaldura honek habia osoa estaltzen du, behealdean sarrera bakar bat utziz (Matsuura eta Yamane 1990). Egitura primario hau babestutako lekuetan kokatuta egoten da (Chauzat eta Martin 2009).

Lehenengo langileak jaiotzen direnean erregina lagunduko dute zeregin arruntetan. Denbora aurrera joan ahala, habiaren tamaina handiagotuz joango da eta kasu askotan hauek kokatuta dauden tokiak ez dira nahiko zabalak habia handiagoa gara-

tzeko. Kasu hauetan, habia berriz ezarri behar da leku zabalago (eta gehienetan) altuago batean (zuhaitz adarretan), habia sekundario bilakatuz (Matsuura 1984, 1991). Prozesu hau burutzeko langile batzuek leku aproposa bilatzen dute habia berria eraikitzeko. Behin langile esploratzaile hauek leku egoki bat aurkitu eta kabi berria eraikitzen dutenean, erreginak habia zaharra uzten du eta berria eraikiko den lekuan finkatuko da. Ez da inoiz habia zaharrera bueltatuko. Erreginarekin batera beste langileak ere mugituko dira habia berrira, baina erreginaren kasuan ez bezala, hainbat langile habia zaharrera bueltatzen dira geratu diren larba eta liztortzar emergitu berriak elikatzera. Habia zaharreko kumaldi guztia heldu denean eta hegan egiteko gai denean, indibiduo guztiak habia berrira mugitzen dira eta habia zaharra betirako hutsik gelditzen da (Matsuura eta Yamane 1990). Rome at al.-aren (2015) arabera, *Vespa velutina* habien % 70ean ematen da prozesu hau.

Ipar hemisferioan zehar aurkitzen diren *V. velutina*-ren habia sekundarioak, biribilak, handiak eta "*maché"* paperez egindako estalki batez inguratutako abaraska ugarikoak dira (Perveen eta Saha 2013). Habiaren sarrera alde batean kokatua dago (Rome et al. 2011). Estaldurari esker, langileak gai dira habiaren barneko tenperatura 30 ºC inguruan mantentzeko, nahiz eta kanpoko tenperaturarekiko 20 ºCko desberdintasuna egon (Martin 1990). Habia bakoitza behin bakarrik erabilia izango da, nahiz eta hau kokatua dagoen lekua behin baino gehiagotan erabilia izan daitekeen (Chauzat eta Martin 2009). Normalean habia sekundarioak zuhaitzetako goi adarretan edo eraikinetan kokatuta egoten dira; hala ere, beste liztortzar batzuekin gertatzen den moduan, batzuetan lur-azpian ere topatu izan dira

(Martin 1995; Villemant et al. 2008). Liztortzar asiarraren habiak nekazal edo baso habitatetan topatu daitezke, baina Frantzian egindako begiztapen gehienak urbanizatutako guneetan izan dira (% 48.53) (Rome et al. 2015).

Ehiza

Bespido sozialen ehiza hurrengo lau pausuetan laburbildu daiteke: itxaron, harrapatu, harrapakina prozesatu eta habiara eraman (Richter 2000). Patroi hau *V. velutina*-n ere behatu da, zeinek erlategiaren aurrealdean itxaroten duen hegaldi estatikoan, bueltatzen diren erleak harrapatzeko. Hauek polenez beteak eta nekatuta bueltatzen direnez, hegan egiteko zailtasunak dituzte. Horregatik, liztortzarrak hegaldian harrapatzen du erlea eta behin helduta duela, inguruan dagoen zuhaitz batera eramaten du, non hirugarren hanka parea erabiliz, buruz behera zintzilikatzen den adar batetik. Ondoren, barailak erabiliz, burua eta abdomena moztu eta baztertzen ditu, toraxarekin bakarrik geldituz, non hegaldirako muskulu guztiak dauden. Hau murtxikatu eta larbak elikatzeko erabiliko duen masa bat sortuko du (Villemant et al. 2010). Harrapakaritza honek lehen aipatu diren kalteak sortaraziko ditu (Requier et al. 2018).

Elikadura

Vespinae liztorrek kontsumitzen dituzten elikagaiak bi taldetan sailkatu daitezke: karbohidratoak eta proteinak (Matsuura eta Yamane 1990). Karbohidratoek helduen (erregina, langileak, gineak eta arrak) elikadura osatzen dute (Richter 2000) eta loreen nektarretik, zuhaitzen zapatik edo fruitu helduetatik lortzen dituzte, bes-

teak beste (Monceau et al. 2014). Udaberrian adibidez, habia primarioaren eraikuntza hasi baino lehen, hibernaziotik ateratako erreginek karbohidrato hauek erabiltzen dituzte (Matsuura eta Yamane 1990). Udazkenean aldiz, ohikoa da arrak *Hedera helix* L. landarearen nektarraz elikatzen ikustea (Monceau et al. 2014).

Proteinak aldiz, erle eta bestelako artropodoen ehizaren bidez lortzen dira. Harrapakin hauek kumeei ematen zaizkie, larbek, garatu ahal izateko, proteinak behar baitituzte. Bosgarren fasean dauden larbek proteina hauek digeritu eta gero, karbohidrato eta aminoazidoetan oso aberatsa den sekrezio bat ekoizten dute. Jariakin hau helduei ematen diete trukean, helduen gerri estua dela eta, ez baitira proteinak zuzenean digeritzeko gai (Chauzat eta Martin. 2009). Elkartruke hau "*trophallaxis"* deritzon prozesuaren bidez ematen da (Wheeler 1928). Hau ahozaho bidezko kontaktuaren bidez substantziak trukatzeko erabiltzen da (Edwards 1980). Prozesu hau larba eta helduen (erregina, langileak, gineak eta arrak) artean beha daiteke (Matsuura eta Yamane 1990), baina baita helduen artean ere (Edwards 1980; Chapman 2013; Shukla et al. 2013). Gainera, elikagaiez aparte, intsektuen komunikazio kimikoan parte hartzen dituzten bestelako substantziak ere elkartrukatzen dira trofalaxia honen bidez (Chapman 2013).

Komunikazio kimikoa:

Intsektu sozial ugaritan, komunikazio kimikoak kolonien antolaketa eta mantenuan rol garrantzitsu bat betetzen du (Mitra eta Gadagkar 2014).

Komunikazio kimiko hau semiokimikoetan oinarritzen da (Law eta Regnier 1971).

Semiokimiko hauek bi mailatan sailkatzen dira (Nordlund eta Lewis 1976): alelokimikoak (Grekeraz ***allelon***=bat bestearena) (Brown 1968) eta feromonak (Grekeraz ***pherein***=transferitu; ***horman***=kitzikatu) (Karlson eta Butenandt 1959). Lehenak espezie desberdineko izakien arteko komunikazioan erabiltzen dira (Brown 1968) eta bigarrenak aldiz, espezie bereko izakien artean (Karlson eta Butenandt 1959; Karlson eta Luscher 1959).

1963an, Wilson eta Bossert feromonak bi talde desberdinetan sailkatu zituzten sortzen dituzten eraginen arabera: (I) primer feromonak, zeintzuek iraupen luzeko aldaketa fisiologikoak sortarazten dituzten eta (II) feromona askatzaileak (tiratzaileak) (releaser feromonak), zeintzuek jasotzen duen indibiduoa kitzikatzen duten berehalako jarrera erantzuna izateko.

Primer feromonak erle, inurri, liztor eta termita bezalako intsektu sozialetan bai erreginek, bai langileek jariatzen dituzte. Erreginek, beste emeen obulutegien garapena eta langileek erreginen gelaxkak eraikitzea eragozteko ekoizten dituzte. Langileek aldiz, kasten inhibitzaile moduan jariatzen dituzte (Ali eta Morgan 1990). Feromona mota honetako adibide bat *Apis mellifera*-ren "erregina substantzia" deritzona da. Feromona hau erle erreginen barailan sortzen da eta bere konposatu printzipala trans-9-oxo-2-dezenoiko azidoa da (Butler et al. 1962; Slessor et al. 1990). Substantzia honek erle langileen obulutegien garapena inhibituko du (Jackson eta Morgan 1993).

Feromona askatzaileen kasuan, 9 taldeetan sailkatuak izan ahal dira hartzaileengan

sortzen dituzten portaeren edo betetzen dituzten funtzioen arabera: (I) feromona sexualak; (II) agregazio-feromonak; (III) dispertsio-feromonak; (IV) alarma-feromonak; (V) errekrutamendu- edo lorratz- feromonak (Birch eta Haynes 1982); (VI) lurralde- edo bizileku-feromonak (Cammaerts et al. 1977; Holldobler eta Wilson 1977); (VII) azalera-feromonak (Wilson 1971); (VIII) heriotz-feromonak (Wilson et al. 1958; Wilson 1963; Matthews eta Matthews 1978) eta (IX) gonbidapen-feromonak (Ahmadi eta McClelland 1985). Oro har, beti pentsatu izan da feromonak airearen bidez sakabanatu eta jasotzen direla, baina hau sarritan gertatu arren, ez da beti horrela ematen. Feromonak uraren bidez ere sakabanatu daitezke, baina ez hori bakarrik, indibiduoen arteko edo azalera batekiko kontaktu zuzenaren bidez ere jaso ahal dira (Ali eta Morgan 1990). Azken hau da azalera feromonen kasua.

Azalera feromonen artean, ezagutze-feromonak, *garbiketa* portaera (“*grooming”*) aktibatzeko feromonak eta janaria elkartrukatzeko substantzia kitzikatzaileak daude (Ali eta Morgan 1990). Beste intsektuek feromona hauek kontaktu zuzenaren bidez edo distantzia txikietara antzematen dituzte (Shorey 1973). Oso garrantzitsuak dira intsektu sozialen kolonietan espezie bereko izakiak, habia bereko izakiak, ahaideak edo baita kasta desberdineko kideak bereizteko **(**Jackson eta Morgan 1993; Gévar et al. 2017). Feromona hauek substantzia konplexuak dira, konposatu ugarien nahasketaz osatuak, baina gehienak intsektuen kutikulan aurkitzen diren kate luzeko hidrokarburoak dira (Vander Meer et al. 1998).

Hidrokarburo kutikularrak

Intsektuen kutikula hidrokarburo nahaste batez estalita dago (kate-luzezko alkeno eta alkano alifatikoak eta metilo adarkatuak) (Blomquist eta Bagnères 2010). Hidrokarburo kutikular hauek (CHCak) intsektuen lehorketa ekiditea eta kutikularen iragazkortasuna erregulatzea dute funtzio nagusi (Hadley 1980); baina, honetaz aparte, beste funtzio semiokimiko batzuk dituzte (Howard eta Blomquist 1982), bereziki ezagutze seinaleetan inplikaturik daudenak (Bonavita-Cougourdan et al. 1987; Vander Meer et al. 1989).

Baina ez hori bakarrik, CHC hauek intsektuen jokaera sexualean ere garrantzia dute bikotekide potentzialen ezagutzan parte hartuz, gorteatze jokabidea estimulatuz (Steiner et al. 2005, 2006, 2007; Sugeno et al. 2006; Ferveur eta Cobb 2010; Ginzel 2010; Guédot et al. 2010) eta inhibituz (Carlson eta Schlein 1991), besteak beste.

Gainazal edo kontaktuzko feromona sexualez aparte, intsektu sozialetan, hidrokarburo kutikularrak, dominantzia eta ugalkortasunaren indikatzaileak izan ahal dira (Liebig 2010), eta habiak egiteko guneen ezagupenean parte hartu dezakete (Espelie et al. 1990; Steinmetz et al. 2003).

Liztorren habien gainazala ere hidrokarburoz estalita dago (Espelie et al. 1990). Hidrokarburo hauek, kolonia horretako liztorren hidrokarburo kutikularren konposizio oso antzekoa aurkezten dute (Espelie eta Hermann 1988, 1990; Espelie et al. 1990; Lorenzi 1992; Singer et al. 1992). *Polistes metricus* Say, 1831 liztorraren

kasuan, langile emergitu berriak habiaren gainazaleko hidrokarburoekiko esposizioan egon behar dira habia-kideak ezagutzeko (Singer eta Espelie 1992; Layton eta Espelie 1994).

Hidrokarburoen sintesia

Ikerketa oso gutxi daude non hidrokarburoen sintesi-gune zehatzak aztertu izan diren (Schal et al. 1998). Hala eta guztiz ere, tradizionalki, kutikulan dauden hidrokarburoak oenozito deritzen zeluletan sortzen direla uste izan da (Romer 1991). Hauek abdomeneko ehunetan bakarrik agertzen diren eta erretikulu endoplasmatiko leunean, zein mitokondrietan aberatsak diren zelula handiak dira (Romer 1991). Oenozitoak askotan gantz-gorputzekin lotuak izan dira, azken hauek gantz-azidoen iturri garrantzitsua dira eta hidrokarburoen biosintesian aitzindari moduan erabili ahal dira (Diehl 1975; Howard eta Blomquist 2005; Juarez eta Fernandez 2007; Provost et al. 2008). Hidrokarburo kutikularrak, sintetizatuak izan ostean, hemolinfan askatuak dira eta hemendik lipoforinen bidez kutikularaino garraiatuak (Van der Horst et al. 1993; Blomquist et al. 1998; Schal et al. 1998; Jurenka eta Subchev 2000; Bagnères and Blomquist 2010).

Koloniaren usain espezifikoari dagokionez, Crozier eta Dix-ek, 1979ean “Gestalt” deituriko eredua proposatu zuten. Honen arabera, jaiotzetik eta genetikoki determinatutako kontaktu-feromonaz aparte, himenoptero sozialek ingurumenetik deribaturiko usaiak kontaktu-feromona modura erabiltzen dituzte. Usain hauek, besteak beste, janaria edo usain lokalak dituzte jatorri. Ingurumenetik lortutako usain

hauek, kutikulan zehar xurgatzen dira. Kasu batzuetan, genetikoki determinatutako feromona hauek ingurumeneko konposatuez indartuak izaten dira.

Eredu honen bidez, koloniaren usain feromonak "*groomming*" eta "*trophallaxis*" prozesuen bidez transferitu ahal dira koloniaren indibiduo guztien artean (Crozier eta Dix 1979). Langileen arteko transferentzia honetaz gain, habia materialetik langileen kutikulara ere pasa daitekeela ikusi da (Crosland 1989; Espelie et al. 1990; Lorenzi et al. 2004). Modu horretan, langile guztiek "gestalt usain" komun bat edukiko dute bakarkako usain bat izan beharrean eta horrela, kolonia bereko indibiduoek elkar identifika dezakete (Crozier eta Dix 1979). Indibiduo emergitu berriek "*neutro*"-a den profil kutikularra aurkeztuko dute oraindik habiaren usaia eskuratu ez dutelarik (Breed et al. 2004, 2015).

Garrantzia eta helburuak:

Bakarrik duela 14 urte Europara heldu zen espeziea izanik eta sartua izan baino lehen liztortzar honi buruz zeuden ikerketak oso eskasak direla kontuan hartuta, bere bizi-zikloari buruzko alderdi gehiago ezagutzea beharrezkoa dela uste dugu. Bereziki, gineak identifikatzea eta bizi-zikloko zer momentuan ekoiztuak diren ezagutzea, honela, inbasioaren aurkako neurri hobeak ezarri ahalko direlakoan.

Behin jakinda izaki sexualak noiz ekoizten diren, hauen komunikazio kimikoan kontzentratu ginen. CHCen eta, batez ere, izaki sexualen CHCen ezagupena himenoptero sozialen jokaeraren hainbat alderdi ezagutzeko ezinbestekoak dira. Hau da *Vespa velutina* gineen, zein arren CHCen eta gantzen eta/edo esperma-

produkzioaren arteko erlazioa analizatzen duen lehengo lana.

Analisi hauek, gineen profil kimikoak metatutako gantzen arabera nola aldatzen diren inguruko informazioa ematen dute. Honela, arrek neguan bizirauteko probabilitate handiagoa duten emeak (metatutako gantz-kantitate handiagoekin) identifikatu eta aukeratzeko gai izango lirateke, hauekin ugalduz. Neguan bizirauteko gaitasuna, espezie honen inbasio-arrakastaren gako garrantzitsuenen artean dago.

Badago ezezaguna eta oso interesgarria den beste alderdi bat: CHCen papera endogamiaren saihespenean. Endogamia faktore oso kaltegarria da espezie arrotz eta inbaditzaileentzako. Arrazoi honengatik, kutikularen profil kimikoaren eta arren esperma-produkzioaren arteko erlazioa ikertzea ezinbestekoa dela uste dugu. Modu honetan, langileek profil hau sexualki helduak diren arrak identifikatzeko erabil dezaketen jakin ahalko genuke. Ar hauek habiatik kanporatuak izango lirateke, habia berbereko gine eta arren (anai-arrebak direnak) arteko estaldura saihestuz. Hau dela medio, endogamiaren murrizketa emango litzateke.

Hau dena kontuan hartuz, etorkizunean CHCak izaki sexualak harrapatzeko tranpa selektiboak sortzeko erabiliak izan daitezke. Izan ere, indibiduo sexual hauen harrapaketa espeziaren hedapena kontrolatzeko funtsezkoa da.

Hauek dira tesi honen helburuak:

2. Kapitulua: Alde batetik, *Vespa velutina* indibiduoen tamainaren aldaketa eta populazioak bizi-zikloan zehar nola aldatzen diren ikastea. Beste aldetik, hurrengo urteko erreginak, gineak, langileetatik desberdintzea, parametro errazena eta zeha-

tzena aurkituz, bizi-zikloa hobeto ezagutzeko helburuarekin. Hau espezie inbaditzaileen kontrolerako ezinbestekoa da.

3. Kapitulua: Hasteko, adinak eta dietak *Vespa velutina* gineen CHC profilean eragin dezaketen ezagutzea. Jarraitzeko, aldagai horiek ere gantz kantitate eta kalitate alda dezaketen ikertzea. Bukatzeko, gantz (kantitatea eta kalitatea) eta profil kutikularraren arteko erlazioa aztertzea, kutikula, metatutako gantzen kantitatea eta kalitateren informazio iturri izan daitekeen jakiteko. Modu honetan, arrek informazio hau ugaltzeko eme egokiena aukeratzeko erabil lezakete.

4. Kapitulua: Alde batetik, adinak eta dietak *Vespa velutina* arren profil kutikularrean eragina duten aztertzea. Beste aldetik, aldagai horiek ere esperma-ekoizpena alda dezaketen ikertzea. Bukatzeko, esperma-ekoizpenaren eta CHC profilen arteko erlazioa zein den ezagutzea, kutikula profilak arren heldutasun sexualaren egoeraren informazio iturri izan daitezkeen jakiteko. Langileek informazio hau ar helduak habiatik noiz bota behar dituzten jakiteko erabil lezakete.

Chapter 2: Differentiating between gynes and workers in the invasive hornet *Vespa velutina* (Hymenoptera, Vespidae) in Europe

Pérez-de-Heredia I, Darrouzet E, Goldarazena A, Romón P, Iturrondobeitia JC (2017) Differentiating between gynes and workers in the invasive hornet *Vespa velutina* (Hymenoptera, Vespidae) in Europe. Journal of Hymenoptera Research.6: 119-133.

Abstract:

In the Vespinae, morphological differences of castes are generally well-marked, except for some Vespa species, where it is difficult to distinguish between future queens and workers in autumn-winter colonies. Individual weights have widely been used as a distinguishing factor but recently cuticular hydrocarbon profiles seems to be the definitive tool, although much more expensive and time-consuming. Parameters such as size (mesoscutum width), wet and dry weight were analysed, throughout several colonies, to differentiate female castes (workers and gynes) in the hornet *Vespa velutina* in Europe. These parameters were compared to cuticular hydrocarbon profiles. The results showed that in late autumn, but not earlier, populations are divided into two size groups, which, based on their CHCs profiles, can be hypothesized to correspond to workers and gynes. This differentiation mirrored a good separation by size that proves to be more accurate than weight (wet and dry). The size limit between workers and gynes is established at a mesoscutum width of 4.5 mm.

Keywords:

Caste differentiation, CHCs, chemical signature, size, weight, yellow-legged hornet

Introduction:

The Vespidae includes both solitary and eusocial groups with extensive variation among the social wasps (Cowan 1991). Caste polymorphism is one of the most widely studied point (Noll et al. 2004). Traditionally, it has been considered that Vespinae wasps (*Vespa* Linnaeus, 1858; *Provespa* Ashmead, 1903; *Dolichovespula* Rohwer, 1916 and *Vespula* Thomson, 1869) present morphological differences between female castes, with queens being larger than workers (Felippotti et al. 2009, Jeanne and Suryanarayanan 2011). However, not all species present the same degree of caste differentiation. *Dolichovespula* shows the weakest caste differentiation (Greene 1991) and *Vespula*, the highest (Spradbery 1973). In the case of *Vespa* there are species, such as *Vespa mandarinia* Smith, 1852; *V. affinis* Linnaeus, 1764; *V. crabro* Linnaeus, 1758 or *V. simillima* Smith, 1868*;* in which castes present clear size separation. By contrast, hornets like *V. tropica* Linnaeus, 1758 and *V. analis* Fabricius, 1775; show an overlap of caste sizes (Matsuura and Yamane 1990). So, in most vespine wasps, size variation among females is discontinuous, although without any clear external physical distinction between gynes and workers aside from size. It seems that *Vespa velutina* conforms to this pattern. Moreover, there are few studies of *V. velutina* on morphological differences between female castes and those use a complex wing morphometric procedure (Perrard et al. 2012).

The size difference between castes can be expressed in various ways. For example, mesoscutum width (MW) from tegula to tegula is one of the most-used parameters

to distinguish castes in some Vespidae species (Noll et al. 1997; Felippotti et al. 2009; Felippotti et al. 2010). In contrast, in some other species it is hard to find morphological features to distinguish castes; for this reason, some authors have looked into other kinds of parameters. Strassmann et al. (1984) reported differences linked to the capability of gynes to overwinter. This explained why foundresses develop multistratified fat bodies whereas workers do not (Eickwort 1969; Toth et al. 2009). For that reason, many authors have used weight to distinguish between workers and gynes (Monceau et al. 2013; Rome et al. 2015).

Apart from size and weight, cuticular hydrocarbon profiles (CHCs) can be used to differentiate between castes in a colony (Liebig 2010; Darrouzet et al. 2014), as was mentioned in Chapter 1.

In European populations of the yellow-legged hornet, *Vespa velutina*, CHCs profiles differ between individuals, depending on caste and sex (Gévar et al. 2017), as they are in several other social insects (Liebig 2010), even though there is genetic homogeneity (Arca et al. 2015) and inbreeding (Darrouzet et al. 2015). These differences are based mainly on the relative quantities of the various compounds that make up the chemical signature.

The aim of this study was (1) to study the dynamics of colony population and individual morphometric variations throughout the annual nesting cycle of *Vespa velutina* in Europe, measuring mesoscutum width, as an index of linear body size. As an alternative discriminator, (2) we tested the cuticular hydrocarbons (CHCs)

profiles of known autumn females. Finally (3), we compared the CHCs profiles with size, wet weight, and dry weight with the goal of discovering rapid, simple and useful parameters for determining castes or groups.

Material and Methods:

Sample collection

In this study, 11 nests at different developmental stages were used. These nests were collected from June to December between 2011 and 2015 at different locations in the Basque Country (Spain) and Indre-et-Loire (France) (Table 1). In both countries the species was well established (Goldarazena et al. 2015; Rome et al. 2013). The collected nests were frozen, dissected and the individuals separated by sex. Only the females were used for this study. All of the individuals were kept frozen at -20 °C until they were studied. Three types of data were analysed: size, weight, and CHCs profile of individuals.

Size and weight analyses

Size of individuals: the mesoscutum width (MW) from tegula to tegula was measured (Figure 1) in a stereomicroscope coupled to a camera system. The MW was used as an index of overall linear size (Noll and Zucchi 2002; Ohl and Thiele 2007). Size measurements are given in mm.

Weight of individuals: wet (WW) and dry weight (DW) were taken using a high precision balance (0.01mg). The wet weight was obtained after two hours of de-

frosting specimens to avoid moisture on the body surface. For dry weight, hornets were dried in an oven at 70 °C for 24h (modified from Monceau et al. 2012). Weight measurements are given in g.

Table 1: Dates and locations of collected colonies.

Colony	Date	Location
1	02/12/2011	Civray de Touraine (Tours, France)
2	22/11/2013	Tours (Tours, France)
3	02/06/2014	Ibarrangelu (Biscay, Spain)
4	22/06/2014	Loiu (Biscay, Spain)
5	23/07/2014	Mungia (Biscay, Spain)
6	26/07/2014	Gatika (Biscay, Spain)
7	28/08/2014	Lasarte (Guipuzcoa, Spain)
8	30/08/2014	Astigarraga (Guipuzcoa, Spain)
9	01/10/2014	Mungia (Biscay, Spain)
10	26/10/2014	Maruri (Biscay, Spain)
11	13/11/2015	Civray de Touraine (Tours, France)

Chemical analyses

CHCs profiles were analysed to determine the castes of individuals. CHCs were extracted by placing hornets in 1 ml of pentane and shaken for 2 minutes in a

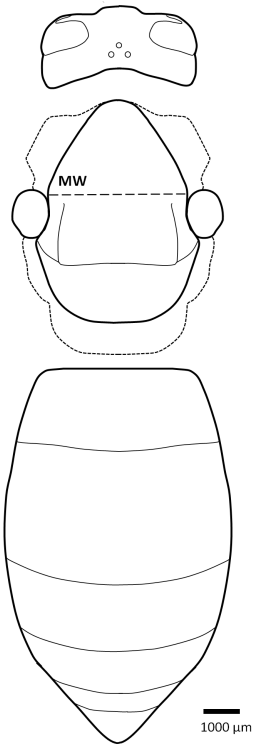

Figure 1: Dorsal view of *Vespa velutina* hornet. MW measure is indicated.

Wheaton™ V Vial™ glass. 500 µl of the extract was placed in another vial and stored at -20 °C until the samples were analysed. Ten µl of standard n-eicosane (C20) (10^{-3} g/ml) was added to each sample and, immediately afterwards, 2 µl of sample was injected into a gas chromatograph (Agilent 7820A) coupled with a flame ionisation detector (FID). The analysis was carried out with a 413HP5 (30 m × 320µm × 0.25µm) capillary column. The oven temperature programmed was from 50 °C to 200 °C (8 °C/min), from 200 °C to 315 (5 °C/min) and 315 °C for 5 min. The injection was in "*splitless"* mode and helium was used as a carrier gas

(1.7 ml/min). All data were processed with ChemStation B.04.03 software. The relative proportions of each peak were calculated as described in Bagnères et al. (1990).

Statistical analysis

MW histograms were used to see how the sizes of individuals change throughout the season. All of the females in the eight Spanish colonies, including the queens, were used.

The XLSTAT 2014 add-on for Microsoft Excel® was used to perform the Gaussian mixture model (GMM), fitted using an EM algorithm, with the MW data of 350 individuals from the four late autumn colonies pooled together to detect potential size classes between reproductive and sterile castes. Using the same individuals, identical procedure was follow to verify whether potential weight (wet and dry) classes existed.

A Principal Components Analysis (PCA) of the individual CHCs signatures of four autumn colonies was performed. The independent variables were the relative area of the most important peaks ($\geq 0.1\%$) in the chromatogram. A Cluster Analysis (Pearson correlation index and k-nearest neighbour algorithm) was performed to define the chemical groups. After that, a Discriminate Analysis with cross-validation, over those groups to test the fitness of categories separation, was performed. In order to test how the size or weight classes, got from GMMs, fit to PCA CHCs profiles, distinct representations of the PCA plots were made. The

analyses were carried out using IBM SPSS Statistics 23.

Results:

The distribution of the morphometric MW variable in the different colonies from June to October is represented in Figure 2. The frequency distribution of mesoscutum width was unimodal throughout most of the colony cycle (from early June to mid October), with a single large individual (the queen) lying outside the mode. The distribution became bimodal late in the colony cycle with the appearance of new gynes.

Apart from the modality, individual numbers and body size also changed (Figure 2). As the season went by, the number of individuals in each colony increased from N=20 in Colony 3 to N=249 in Colony 10. The same occurred with the sizes of individuals. In unimodal colonies, the MW of none of the hornets reached 4.5 mm, with the exception of the large individual which is outside the group.

However, in late-season Colony 10, which was bimodal, the size of the MW varied from 3.79 mm to 4.49 mm for the population on the left, and from 4.61 mm to 4.87 mm for the one on the right. In most of the colonies represented in Figure 2, the individual that is outside the unimodal distribution had a MW greater than 4.5 mm, except for Colony 6 where this was 4.48 mm. The MW of 4.5 mm was the threshold used to separate the two groups in the bimodal colony.

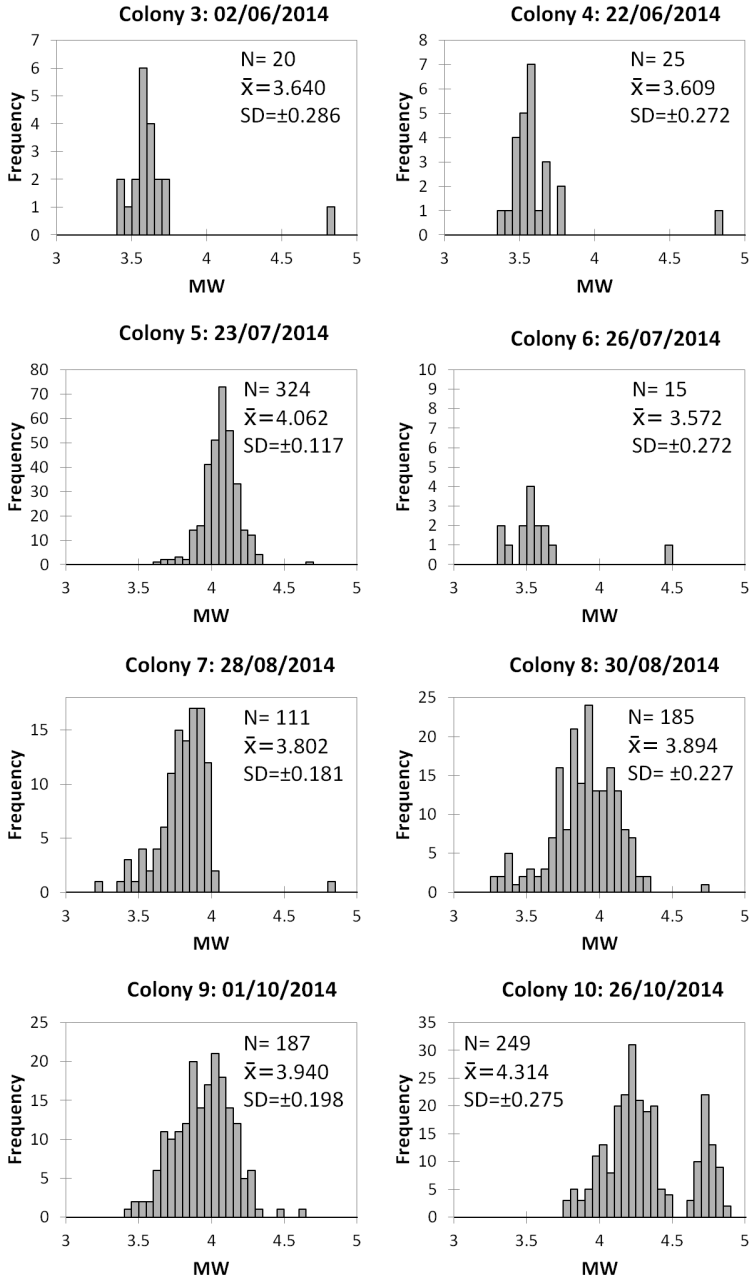

Figure 2: Histograms showing MW (mesoscutum width) from eight different colonies, sorted by collection date.

Figure 3 shows the Gaussian mixture model (GMM) of autumn colony data, performed to establish the threshold between the two populations according to size (MW) and weight (WW and DW).

The GMM analysis for MW split the distribution into two size classes, separated by a threshold or mid-point value of 4.5 mm (Figure 3A). The 5 % uncertainty level was set at 4.4 mm for workers and 4.58 mm for gynes. The same GMM analysis was performed for wet weight (WW) and dry weight (DW). In the case of WW (Figure 3B) the model did not have the same bimodal distribution as MW. Even so, the threshold calculated was 0.618 g, with the 5 % uncertainty level at 0.445 g for workers and 0.797 g for gynes. Unlike WW, the DW GMM did show a bimodal distribution (Figure 3C), with a threshold value of 0.225 g separating the two groups. The 5 % uncertainty value was 0.202 g for workers and 0.247 g for gynes.

For each of the three GMMs, the mid-point or threshold was compared to the highest values for the 5% uncertainty interval, in percentage terms, to check which of the three presented the smallest uncertainty interval. A higher percentage showed a lower uncertainty interval, resulting in a clearer separation between groups. These values were 98.25 % for MW, 77.54 % for WW, and 91.09 % for DW.

The Cluster Analysis of the CHCs profiles of the four late-season colony hornets, showed three clearly well-separated chemical groups, named as 1, 2 and 3. They are represented in the axes I and II of the PCA (Figure 4). The Discriminant Analysis showed all the hornets were chemically well classified. The group 1 hornets

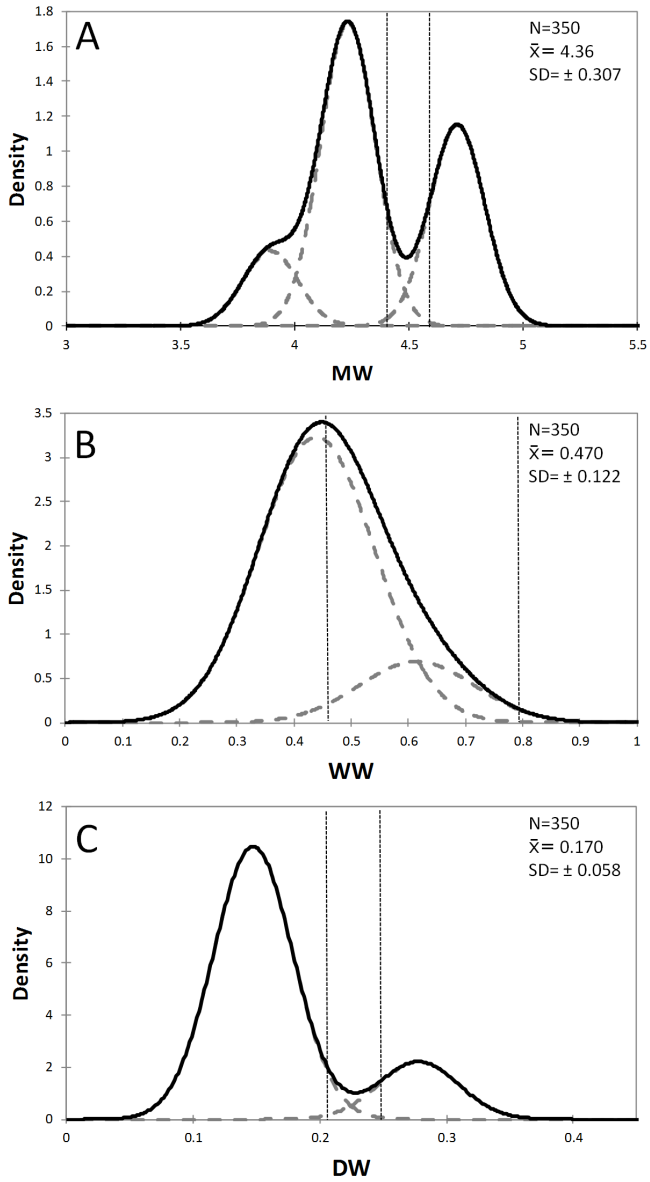

Figure 3: *Vespa velutina* size (A), wet weight (B), and dry weight C) distribution using a Gaussian Mixture Model. Two-dimensional distribution is represented by continuous line A) workers < 4.5 mm, gynes ≥4.5mm B) workers < 0.618 g, gynes ≥ 0.618 g and C) workers < 0.225 g, gynes ≥ 0.225 g. The dashed lines represent group densities. The 5% level of uncertainty is shown by dotted lines A) 4.4 mm-4.58 mm B) 0.445 g-0.797 g and C) 0.202 g-0.247 g. 4 colonies: Colony 1, N= 30; Colony 2, N= 30; Colony 10, N= 240; Colony 11, N=50.

showed to be chemically more similar to each other, since the dots cloud was more compact. The group 2 was more scattered, showing they were chemically more heterogeneous. The group 3 had very few individuals.

In the PCA of the Figure 4, ordination plots were displayed according to the size or weight class of each hornet. In the size (MW) column (Figure 4), all individuals classified as "small" belonged to the same chemical group (group 1) and the "large" to the other two (groups 2 and 3). This showed a good agreement between both PCA chemical groups and size ones. There was an exception in Colony 1, where three individuals classified as "small" appeared in the group 2.

In the column showing the PCA for wet weight (Figure 4), it can be observed that the three CHCs groups did not match well to the two WW defined groups. In Colony 1, all individuals, except one, were "light". In Colony 2 there were no hornets classified as "heavy". In Colony 10 there are four "heavy" individuals spread in the second and third CHCs groups. In the case of Colony 11 all the "heavy" hornets were in the second chemical group, most of them in the top of the group.

Lastly, in the column showing the PCA for dry weight (Figure 4), all colonies contained "heavy" individuals, which are located in the top part of the CHCs group 2.

Discussion:

Mesoscutun width (MW) seems to be one of the most common parameters used in morphometry, as it is relatively large and constant, thus minimizing errors in measurement, and can be taken easily (Noll and Zucchi 2002; Ohl and Thiele

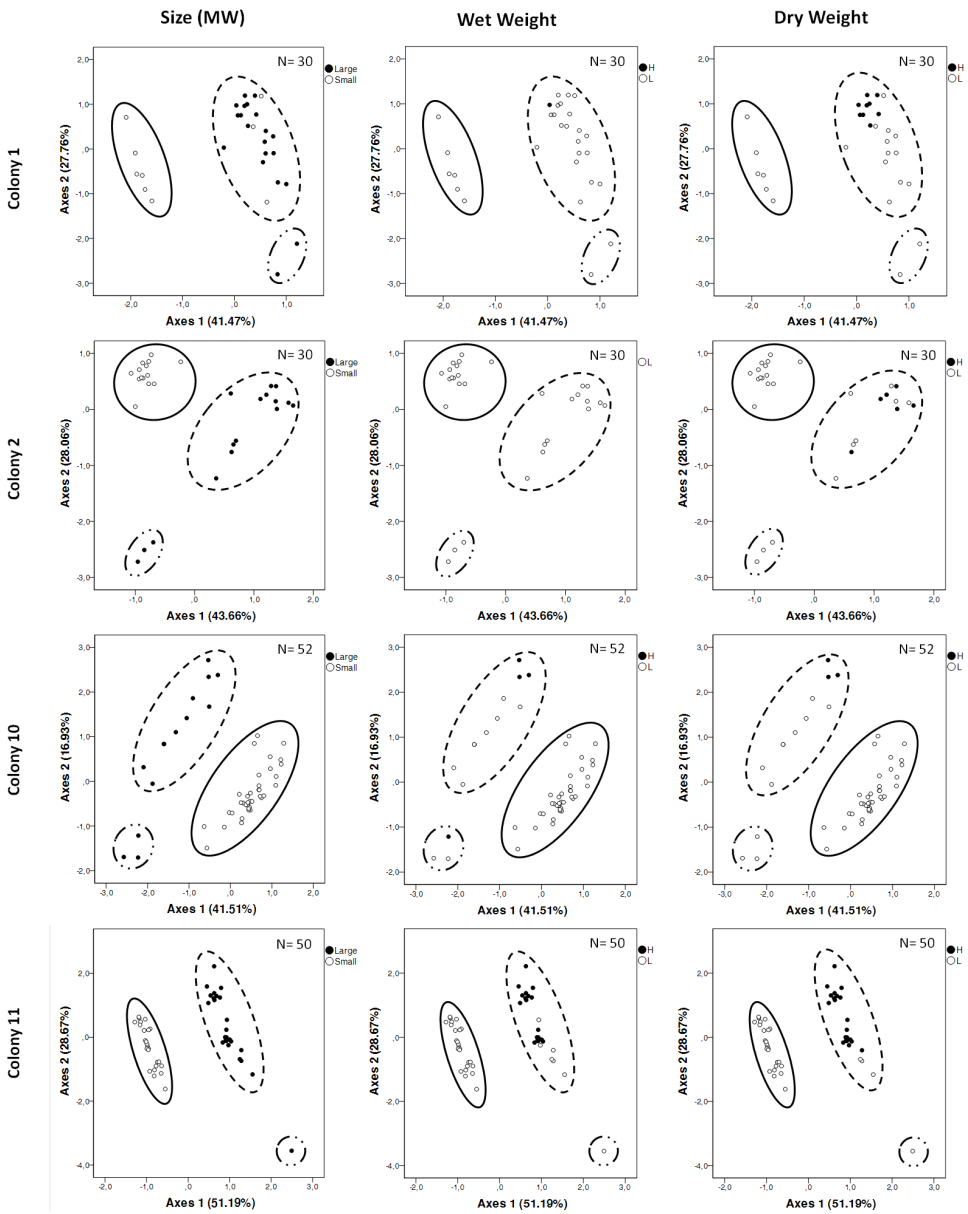

Figure 4: PCA of CHCs profiles in each of the four autumn colonies. Chemical groups are defined by continuous line: Group 1; dash line: Group 2 and dot-dash line: Group 3. PCA dots show representations according to GMMs size, wet weight and dry weight thresholds of hornets. Size, Black dots: Large females (MW ≥ 4.5 mm); White dots: Small females (MW < 4.5 mm). Wet weight, Black dots: Heavy fresh females (≥ 0.618 g); White

dots: Light fresh females (< 0.618 g). Dry weight, Black dots: Heavy dry females (≥ 0.225 g); White dots: Light dry females (< 0.225 g).

2007). As a result, this size parameter was chosen, among all the used measures, to study the dynamics of the *Vespa velutina* population as well as individual morphometric changes from June to October. The latter, had not been studied until now.

Early in the season, the number of individuals per colony was low and they were also smaller in size. However, close to the end of the colony life cycle, both individual numbers and sizes are larger and the individual size distribution changes from unimodal to bimodal. From June to early October, we observed that all of the unimodal colonies studied contained only one individual that was notably larger in size than the other females, being the queen of those colonies. Moreover, these females matched the size of individuals in the second population (MW > 4.5mm) in the autumn nests. In the other hand, females captured in early spring, which are overwintering survivor gynes, also presented MW > 4.5mm (Pérez-de-Heredia, personal observation). Therefore, it can be said that these larger autumn females will become the queens of the following year's colonies. This population dynamic is typical in aculeate colonies which are founded by a single queen. The first cohort is raised by the queen alone and comprises the smallest workers; the following cohorts increase in size until the largest workers appear. This happens together with, or is followed by, the production of gynes and males

(Wilson 1971, Miyano 1981). At the same time as gynes are being produced, female size distribution starts turning from unimodal to bimodal. This bimodality corresponds to the differentiation between castes, workers and gynes (Spradbery 1973). This size increase in females, during the annual colony cycle, is associated with the trophic advantages of having more workers in the nest to feed larvae. Another explanation for this increase in individual size is the sizes of the cells where larvae are raised, which gradually increase as the nest grows larger (Spradbery 1972). Edwards (1980) showed that, in *Vespa crabro*, the size of individuals is conditioned by the size of the cells in which they are raised. There were two size classes among males, some of which were raised in worker cells and others in gyne cells.

The bimodality of the size parameter in late autumn colonies led us to consider size as a good caste differentiator. Nevertheless, hitherto, only the weight of individuals has been used to differentiate castes in *Vespa velutina*. For that reason, we also analysed WW and DW using the GMM procedure to establish the threshold for each of them and compare the results to MW, to determine the best caste predictor.

According to the three GMMs, the MW size presents less overlap bimodality between groups, making it more accurate and reliable than either of the weights. This can be explained because once an insect emerges as an adult; its body is enclosed in a solid, non-regenerative cuticle, making body plates invariable. Unless it is damaged, no morphological changes occur in any hardened (sclerotized) body

part (O´Donnell 1998) regardless of insect age or physiological state.

The GMM for WW presents a greater overlap between groups, resulting in a unimodal distribution. This can be explained because there is great variability in the WW for individuals of the same size, influenced by differences in metabolic status, age of individuals (Hilligsøe and Holmstrup 2003) or by physiological variations, as occurs in collembolans (Verhoef 1981). By contrast, the GMM of DW presents a bimodal pattern, which means that the parameter is more constant for a given group of hornets and in consequence is more reliable.

Our study shows that the thresholds for separating the two classes or groups were 0.618 g for WW and 0.225 g for DW. These data differ a little from those observed by Rome et al. (2015), which considered that individuals with WW exceeding 0.593 g and DW exceeding 0.250 g were considered to be gynes, while those with lower weights were workers.

These discrepancies in the DW may be due to differences in methodology, such as the temperature and drying time for the individuals. Even so, the variation in the DW rank linked to 5 % uncertainty was very similar: 91.09 % in our study and 87.72 % in the data of Rome et al. (2015).

The three chemically-differentiated groups observed in the four autumn colonies, are explained as follows. Hornets of groups 2 and 3 presented sizes equal or bigger than 4.5 mm (except for three individuals in Colony 1). In addition, only hornets of the group 2 (classified as “large” hornets) presented high weights. So, following

to Rome et al. 2015, it can be hypothesized that this group belongs to the gynes. The cuticular profiles discriminate by castes, workers being in chemical group 1 and gynes in group 2. Group 3, located apart from the other two, is an undefined chemical group, different from the other two.

The aforementioned three mismatched individuals in Colony 1 have the size of workers but they have the chemical signature of gynes. It is possible that, in some nests, this type of gyne could be raised in workers' cells, resulting in small gynes. This was also observed in *Vespula germanica* (Fabricius, 1793) (Spradbery 1993), but further studies are needed to confirm that. In all cases large hornets always had gyne CHCs profiles. This can be explained because, when gynes start emerging, the production of workers is interrupted (Matsuura and Yamane 1990; Monceau et al. 2013).

Group 2, consist of both high and low weights gynes. The gynes are the only members of the colony that will survive the winter (Monceau et al. 2014). Recently-emerged gynes spend some days inside the nest before leaving it to hibernate, as long as 13-14 days in the case of *Vespa affinis* (Martin 1993). During those days, they are fed by trophallaxis with substances regurgitated by workers and larvae. Most of this food is converted into fat reserves to last the winter (Matsuura and Yamane 1990). The workers, however, have no such energy reserve, and this makes them lighter than gynes (Martin 1993). For that reason we can assume that hornets with a large MW but low weights are young gynes which have had no time to feed enough to reach high weight. All these hornets

have a similar chemical profile so, it can be concluded that PCA axis II discriminated the groups by age. Thus, the workers (group 1) are more homogeneous, because all of them have similar ages contrary to what hap pens in gynes (group 2) which have hornets with different ages. Finally, group 3 is comprised presumably by newly emerged hornets, which have not had enough time to develop and get a defined chemical profile (Lorenzi et al. 2004). Thus, it can be hypothesized that they belong to the caste of the newly emerged gynes. This is supported by the fact that there are no individuals of the chemical group 1 with a MW equal or bigger than 4.5mm.

According to the DW threshold of 0.250 g given by Rome et al. (2015), recently-emerged gynes which have no time to feed are classified in the group of light individuals, i.e. workers. The same happens with colonies collected at the end of autumn, when feeding conditions may not be ideal due to the lack of food or because there are not enough workers to feed larvae (Matsuura and Yamane 1990). Both workers and final instar larvae are feeders of recently-emerged hornets (Matsuura and Yamane 1990). So, the two castes tend to be lighter from November to December (Rome et al. 2015). The heaviest females in the chemical gynes group, which appeared close together, are probably the oldest ones. They have remained feeding in the nest for a longer time accounting for their greater amounts of reserves.

Conclusions:

Since *Vespa velutina* was introduced into Europe in 2005, a number of scientific questions have been analysed regarding this invasive species. For some of them, it is crucial to discriminate between female castes to better understand some of the biological aspects, such as when the first gynes emerge and how many gynes are produced per nest. So, considering the data set out here, *V. velutina* seems to present distinctive morphological female castes depending on their MW. Moreover, the variable rank corresponding to the 5% uncertainty level in the GMM is lower in the MW than in the weight data, with less potential for error. This is confirmed by the results from the CHCs profiles. Hornets with a MW of 4.5 mm or more are considered to be gynes, while those with a MW of less than 4.5 mm are considered to be workers. This MW size parameter is easier, faster and cheaper to measure than analysing CHCs profiles. DW worked better than WW but neither of them is as accurate as MW at least with young or not well fed gynes.

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2. Kapitulua: Europako *Vespa velutina* (Hymenoptera, Vespidae) liztortzar asiarraren gine eta langileak desberdintzatzen

Pérez-de-Heredia I, Darrouzet E, Goldarazena A, Romón P, Iturrondobeitia JC (2017) Differentiating between gynes and workers in the invasive hornet *Vespa velutina* (Hymenoptera, Vespidae) in Europe. Journal of Hymenoptera Research.6: 119-133.

Sarrera:

Vespidae familiaren barruan talde bakartiak eta eusozialak daude, liztor sozialen artean bariazio handia dagoelarik (Cowan 1991). Kasten polimorfismoa da talde sozialen artean gehien ikertu den puntuetako bat (Noll et al. 2004). Tradizionalki, Vespinae liztorrek (*Vespa* Linnaeus, 1858*; Provespa* Ashmead, 1903; *Dolichovespula* Rohwer, 1916 eta *Vespula* Thomson, 1869) eme kasten artean desberdintasunak aurkezten dituztela kontsideratu izan da, erreginak langileak baino handiagoak izanik (Felippotti et al. 2009; Jeanne eta Suryanarayanan 2011); hala ere, espezie guztiek ez dute kasten desberdintasun maila berdina aurkezten. *Dolichovespula* da kasten arteko desberdintasun txikiena aurkezten duena (Greene 1991) eta *Vespula* aldiz, handiena (Spradbery 1973). *Vespa* generoaren kasuan, kasten tamainaren banaketa oso argia aurkezten duten espezie batzuk daude; esaterako, *Vespa mandarinia* Smith, 1852; *V. affinis* Linnaeus, 1764; *V. crabro* Linnaeus, 1758 edo *V. simillima* Smith, 1868 (Matsuura eta Yamane 1990). Beste liztortzar batzuetan aldiz, *V. tropica* Linnaeus, 1758 eta *V. analis* Fabricius, 1775 bezalako espezieetan, kasten tamainan gainjarpen bat beha daiteke (Matsuura eta Yamane 1990). Beraz, Vespinae liztor gehienen artean, emeen arteko tamainaren bariazioa etena (ez-jarraitua) da, baina ez dute inolako kanpo desberdintasun fisikorik aurkezten, tamainaz aparte, gine eta langileen artean. Badirudi *Vespa velutina* mota honetakoa dela. *V. velutina*-ren eme kasten arteko desberdintasun morfologikoak aztertu dituzten oso ikerketa gutxi daude eta daudenek, hego morfometriaren oso aztertze prozesu konplexuak erabili dituzte (Perrard et al. 2012).

Kasten arteko tamainaren desberdintasuna hainbat eratan adieraz daiteke. Adibidez, Vespidae espezie batzuen artean, tegula batetik bestera dagoen mesoeskutuaren luzera da kastak desberdintzeko neurririk erabilienetarikoa (Noll et al. 1997; Felippotti et al. 2009; Felippotti et al. 2010). Aldiz, beste espezieetan kastak desberdintzeko ezaugarri morfologikoak aurkitzea zaila da; hau dela eta, hainbat autorek bestelako parametroak bilatu dituzte. Strassmann et al.-ek (1984) gineen hibernatzeko gaitasunarekin erlazionaturiko desberdintasunak aurkitu zituzten. Hibernatzeko gaitasun honek, liztortzar fundatzaileek gantz-gorputz multi-geruzatuak zergatik garatzen dituzten eta aldiz langileek ez, azaltzen du (Eickwort 1969; Toth et al. 2009). Arrazoi honengatik, hainbat autorek gorputz-pisua erabili dute langileen eta gineen artean bereizteko (Monceau et al. 2013; Rome et al. 2015).

Tamaina eta pisuaz aparte, hidrokarburo kutikularren (CHC) profilak ere erabil daitezke kolonia batean kastak desberdintzeko (Liebig 2010, Darrouzet et al. 2014), 1. Kapituluan aipatu den moduan.

Vespa velutina liztortzar asiarraren Europako populazioetan CHC profilak indibiduoen artean, kasta eta sexuaren arabera, desberdinak direla jakiten da(Gévar et al. 2017), bestelako intsektu sozialetan gertatzen den bezala (Liebig 2010), nahiz eta homogeneitate genetikoa (Arca et al. 2015) eta endogamia egon (Darrouzet et al. 2015). Desberdintasun hauek, seinale kimikoa osatzen duten hainbat konposaturen kantitate erlatiboetan oinarritzen dira, nagusiki.

Ikuspegi honetatik, ikerketa honen helburuak alde batetik, (1) Europako *Vespa*

velutina-ren populazioen dinamika eta indibiduoen aldakortasun morfometrikoak ikastea da, urteko habia-zikloan zehar, mesoeskutuaren zabalera gorputz osoaren tamainaren indizetzat erabiliz. Beste aldetik, (2) udazkeneko emeen hidrokarburo kutikularren (CHC) profilak aztertzea. Eta azkenik, (3) CHC hauek tamaina, pisu heze eta pisu lehorrarekin konparatzea, kastak edo taldeak determinatzeko CHCak baino azkarragoak, errazagoak eta baliagarriak diren beste parametro batzuk bilatzeko asmoarekin.

Material eta metodoak:

Laginen bilketa

Ikerketa honetan, garapen fase desberdinetan zeuden 11 habia erabili ziren. Habia hauek Euskal Herriko (Espainia) eta Indre-et-Loireko (Frantzia) herri desberdinetan bildu ziren ekainetik abendura, 2011tik 2015era (1. Taula). Bi herrialdeetan espeziea ondo finkatuta dago (Goldarazena et al. 2015; Rome et al. 2013). Bildutako habiak izoztu, disekzionatu eta barruan aurkitutako indibiduoak sexuaren arabera bananduak izan ziren. Ikerlan honetan, emeak bakarrik erabili ziren eta indibiduo guztiak -20 °C-tan mantendu ziren, laginak aztertuak izan arte. Hiru motatako datuak neurtu edo analizatu ziren: tamaina, pisua eta indibiduoen CHC profila.

Tamaina eta pisua

Indibiduoen tamaina: Mesoeskutuaren zabalera, tegula batetik bestera (Mesoscutum Width: MW) neurtua izan zen (1. Irudia) estereomikroskopioari ako-

platutako kamera sistema bat erabiliz eta mm-tan adierazita. MW indibiduoen tamaina linear osoaren indize gisa erabili zen (Noll eta Zucchi 2002; Ohl eta Thiele 2007).

Indibiduoen pisua: Pisu hezea (Wet Weight: WW) eta lehorra (Dry Weight: DW) neurtu ziren prezisio handiko balantza batean (0.01mg). Pisu hezea, liztortzarren desizoztetik eta 2 ordura neurtu zen, gainazalean sortutako hezetasuna ekiditeko. Pisu lehorra lortzeko, liztortzarrak labe batean lehortu ziren 24 orduz 70 °C-tara (Monceau et al. 2012tik moldatuta). Pisuen neurriak gr-tan adierazi ziren.

Analisi kimikoak

CHC profilak indibiduoen kastak zehazteko analizatuak izan ziren. CHC hauek, liztortzarrak beirazko Wheaton™ V Vial™ batean pentano mililitro batekin batera sartuz eta 2 minutuz irabiatuz erauzi ziren. Lortutako aterakinaren 500 µl beste ontzi batera pasatu eta -20 °C-tan gorde ziren laginak, analizatuak izan arte. Lagin bakoitzari n-eicosane (C20) (10^{-3} g/ml) estandarraren 10 µl gehitu eta berehala, laginaren 2 µl gar-bidezko ionizazio detektagailu (FID) bati akoplatua dagoen gas kromatografo (Agilent 7820A) batean injektatu ziren. Analisirako 413HP5 (30 m × 320 µm × 0.25 µm) kapilaritate zutabea erabili egin zen. Erabilitako labearen programa 50 °C-tik 200 °C-ra (8 °C/min), 200 °C-tik 315 °C-ra (5 °C/min) izan zen eta bukatzeko 315 °C-tan 5 minutuz mantendu zen. Injekzioa "*splitless*" erara egin eta helioa erabili zen gas eramaile gisa (1.7 ml/min). Lortutako data guztiak ChemStation B.04.03 software-arekin prozesatuak izan ziren eta piko bakoitzaren

(zeina hidrokarburo bati dagokio) proportzio erlatiboa Bagnères et al.-ek (1990) deskribatutako moduan kalkulatu zen.

Analisi estatistikoak

Urte-sasoian zeharreko indibiduoen tamainaren aldaketa ikusteko, MW-aren histogramak erabili ziren. Honetarako, Euskal Herrian bildutako 8 habietako eme guztiak, erregina barne, erabili ziren.

EM algoritmoarekin ekipatutako Eredu Misto Gaussiarrak (Gaussian Mixture Model: GMM) egiteko, Microsoft Excel®-ean instalatutako XLSTAT 2014a erabili zen. GMM hauek kasta antzuen eta ugaltzaileen artean tamaina desberdintasunik zegoen ala ez ikusteko burutu ziren. Honetaz aparte, pisuan ere (hezea eta lehorra) desberdintasunak zeuden ala ez ikusteko erabili ziren. Udazken berantiarreko lau kolonietatik lortutako 350 indibiduo, denak batera, erabili ziren GMM guztiak egiteko.

Udazkeneko lau kolonietako indibiduoen CHC datuekin Osagai Nagusien Analisia (Principal Component Analysis: PCA) burutu zen, aldagaiak kromatogrametan lortutako piko garrantzitsuenen ($\geq$ % 0.1) area erlatiboak zirelarik. Honetaz aparte, talde kimikoak zehaztu eta bereizteko, Kluster Analisia (Pearson-en korrelazio indizea eta aldameneko k-hurbilen algoritmoa erabilita) burutu zen. Honen ostean, Analisi Diskriminatzailea, zeharkako-balidazioarekin, burutu zen, aurretik lortutako CHC taldeak, erabilitako banaketa kategorietara nola egokitzen ziren ikusteko. PCAren irudikapen desberdinak egin ziren GMMetatik lortutako tamaina eta

pisu klaseak CHCen PCAei nola egokitzen zitzaizkien ikusteko. Analisi hauek IBM SPSS Statistics 23 programa erabiliz burutu ziren.

Emaitzak:

Ekainetik urrira bildutako kolonia desberdinetako MW aldagai morfometrikoaren distribuzioa erakusten da 2. Irudian. Mesoeskutuaren zabaleraren distribuzio frekuentzia unimodala da koloniaren ia ziklo osoan zehar (ekainaren hasieratik urriaren erdira arte). Distribuzio unimodala aurkezten duten kolonietan, indibiduo handi bakar bat (erregina) beha daiteke distribuziotik kanpo. Distribuzioa bimodal bihurtzen da koloniaren zikloaren bukaeran, gine berrien agerpenarekin.

Modalitateaz aparte, indibiduoen kopurua eta hauen gorputz tamaina ere aldatuz doa (2. Irudia). Sasoia aurrera doan heinean, kolonia bakoitzean dagoen liztortzar kopurua handituz doa: N=20tik 3. kolonian, N=249ra 10. kolonian. Indibiduoen tamainarekin, gauza bera gertatzen da. Izan ere, kolonia unimodaletan, ez dago MWa 4.5 mm baino handiagoa aurkezten duen liztortzarrik, taldetik kanpo gelditzen den indibiduo handi bat izan ezik. Hala ere, sasoi-amaierako 10. kolonian, bimodala dena, MW-aren tamaina 3.79 mm-tik 4.49 mm-tara doa ezkerreko populazioan, eta 4.61 mm-tik 4.87 mm-tara eskumako populazioan. 2. Irudian irudikatutako kolonietan, gehienetan distribuzio unimodaletik kanpo gelditzen diren indibiduoen MW-a 4.5 mm baino handiagoa aurkezten dute, 6. kolonian izan ezik, non 4.48 mm neurtzen duen. Beraz, 4.5 mm izan da erabilitako muga kolonia bimodaleko bi taldeak banatzeko.

3. Irudiak udazkeneko kolonien datuekin gauzatutako Eredu Misto Gausiarren (GMM) emaitzak erakusten ditu. Hauek, tamainari (MW) eta pisuei (WW eta DW) dagokienez bi populazioen arteko muga zein den finkatzeko burutu ziren.

MWrako egindako GMM analisiak, distribuzioa bi tamaina klasetan banatzen du eta banaketa hau, 4.5 mm-tako mugan ezarri da (3A. Irudia). % 5eko ziurgabetasun-maila ezarri da 4.4 mm-tan langileentzat eta 4.58 mm-tan gineentzat. GMM analisi berdina egin zen pisu heze (WW) eta lehorrarentzako (WD). WWaren kasuan (3B. Irudia), ereduak ez du distribuzio bimodalik aurkezten, MWarekin gertatzen den moduan. Dena den, 0.618 gr-tan ezarri da erdiko muga eta % 5eko ziurgabetasun-maila 0.445 gr-tan langileentzat eta 0.797 gr-tan gineentzat. WWan ez bezala, DWaren GMMak bai aurkezten du distribuzio bimodala (3C. Irudia). Kasu honetan, 0.225 gr da bi taldeak banatzen duen muga. DWrako % 5eko ziurgabetasun-maila 0.202 gr-tan ezarri da langileentzat eta 0.247 gr-tan gineentzat.

Egindako hiru GMMetako erdiko muga, kalkulatutako % 5eko ziurgabetasun-tarteko balio handienarekin alderatu zen, ehunekoetan, hiruren artean ziurgabetasun tarterik txikiena zeinek zuen egiaztatzeko. Ehuneko altuago batek ziurgabetasun tarte txikiagoa adierazten du, bi taldeen arteko banaketa argiagoa izanik. Balioak honako hauek izan ziren: % 98.25 MWrako, % 77.54 WWrako eta % 91.09 DWrako.

Sasoi-amaierako lau kolonietako liztortzarren CHC profilekin egindako Kluster Analisiak argi desberdindutako 3 talde kimiko bereizten ditu. Taldeei 1, 2 eta 3

izena eman zaie hurrenez hurren eta PCAren I. eta II. ardatzetan daude adierazita (4. Irudia). Analisi Diskriminatzaileak liztortzar guztiak ondo sailkatuak daudela adierazten du. 1. taldeko liztortzarrak kimikoki antzekoagoak dira beraien artean, puntuen hodeia elkartuagoa dagoelako 2. taldea sakabanatuago dago, kimikoki heterogeneoagoak direla adieraziz eta 3. taldeak, oso indibiduo gutxi dauzka.

PCA ordenazioan (4. Irudia), liztortzarren tamaina edo pisua adierazita ikus daiteke. Tamainaren zutabean (4. Irudia, MW), "txikiak" bezala sailkatutako indibiduoak talde kimiko berdinekoak dira (1. taldea) eta "handiak" berriz, beste bi taldeetakoak (2. eta 3. taldeak). Honek, PCAren talde kimikoak eta tamainak bat datozela erakusten du. Salbuespen bat dago 1. kolonian, non "txikiak" bezala sailkatutako 3 indibiduo, 2. taldean kokatzen diren.

Pisu hezearen PCAk agertzen diren zutabean (4. Irudia, Wet Weight), CHCaren hiru taldeak WWaren arabera definitutako taldeekin guztiz bat ez datozela ikus daiteke. 1. kolonian, indibiduo guztiak bat izan ezik "lirainak" ziren; 2. kolonian ez zegoen "astuna" bezala sailkatutako liztortzarrik; 10. kolonian lau indibiduo "astun" daude sakabanatuta 2. eta 3. taldeetan zehar; eta 11. koloniaren kasuan, liztortzar "astun" guztiak 2. talde kimikoan zeuden, gehienak gainera taldearen goiko aldean.

Bukatzeko, pisu lehorraren zutabean (4. Irudia, Dry Weight), kolonia guztietan indibiduo "astunak" beha daitezke, hauek CHC 2. taldearen goiko partean kokatuta daudelarik.

Eztabaida:

Morfometrian, mesoeskutuaren zabalera (MW) parametro erabilienetarikoa da, erlatiboki handia eta konstantea delako. Honek, neurketaren akatsak minimizatzen ditu eta gainera, modu erraz batean neur daiteke (Noll eta Zucchi 2002; Ohl eta Thiele 2007). Hau dela eta, tamaina parametro hau hautatu zen, neurtutako neurri guztien artean, *Vespa velutina*-ren populazioen dinamika ikertzeko eta ekainetik urrira indibiduoek dituzten aldaketa morfometrikoak ikertzeko ere. Azkeneko hau ez da orain arte ikertu.

Sasoiaren hasieran, kolonia bakoitzeko indibiduo kopurua txikia zen eta baita indibiduo hauen tamaina ere. Hala ere, kolonia bizi-zikloaren bukaera aldera, bai indibiduoen kopurua, bai hauen tamaina handiagoak ziren. Bestalde, banakoen tamainen distribuzioa unimodaletik bimodalera aldatzen da. Ekainetik urrira, ikertutako kolonia unimodal guztiek, bestelako emeekin alderatuz, tamaina nabarmenki handiagoko indibiduo bakar bat aurkezten zutela behatu genuen. Liztortzar hau, koloniako erregina zen. Gainera, eme handiago hauen tamaina udazkeneko habien bigarren populazioko banakoen tamainekin (MW > 4.5 mm) bat zetorren. Honetaz aparte, udaberriaren hasieran harrapatutako emeek ere, hibernazioari aurre egin diotenek, MW > 4.5 mm aurkezten dute (Pérez-de-Heredia, behaketa pertsonala). Hau dela eta, udazkenean agertzen diren eme handi horiek hurrengo urteko kolonien erreginak bihurtuko direla esan daiteke. Honelako populazioen dinamika ohikoa da erregina bakar batez fundatuak diren akuleatuen kolonietan. Lehenengo kohortea erreginak bakarrik hazten du eta langile txikienez osatua dago, baina

gero, hurrengo kohorteen langileen tamaina handiagotzen doa, indibiduo handienak agertu arte. Honako hau, arrak eta gineak ekoizten diren aldi berean edo ekoitzi baino lehen gertatzen da (Wilson 1971, Miyano 1981). Gineak ekoizten hasten diren aldi berean, emeen tamainaren distribuzioa unimodaletik bimodalera aldatzen hasten da. Bimodalitate hau kasten arteko desberdintzapenari dagokio; hau da, langileak eta gineak (Spradbery 1973). Koloniaren urte-zikloan zehar ematen den emeen tamainaren handipena, habian larbak elikatzeko langile gehiago egoteak dakartzan abantaila trofikoekin lotuta dago. Tamainaren handipena azaltzeko bestelako arrazoi bat, larbak hazten diren gelaxken tamaina izan daiteke, gradualki hazten baitira habia handitzen den heinean (Spradbery 1972). Edwards-ek (1980) erakutsi zuen *Vespa crabro* indibiduoen tamaina, hauek hazten diren gelaxken dimentsioen menpe dagoela. Arren artean bi tamaina klase zeuden, batzuk langileen gelaxketan eta beste batzuk gineen gelaxketan hazi baitziren.

Udazken berantiarreko kolonien tamaina-parametroak aurkezten duen bimodalitateak, tamaina kasten bereizle ona dela pentsarazten digu. Hala ere, orain arte banakoen pisua bakarrik erabili izan da kastak desberdintzeko *Vespa velutina*-n. Hau dela eta, WW eta DW, GMM analisiaren bidez aztertu ziren, populazioak pisuaren arabera banatzeko mugak finkatzeko eta emaitzak, MWarekin alderatuz, kasten prediktore hoberena zein den determinatzeko.

Burututako hiru GMMei dagokienez, MW tamainak bimodalitatearen gainjarpen txikiena erakusten du taldeen artean eta honek, bi pisuak baino zehatzagoa eta fidagarriagoa dela esan nahi du. Hau azaltzeko, intsektu heldu bat emergitzen de-

nean bere gorputza solido eta birsortu ezin daitekeen kutikula batean itxita dagoela ulertu behar da, gorputz-atalak aldaezinak direlarik. Hau da, kaltetua izan ezean, ez da inolako aldaketa morfologikorik emango gogortuta (esklerotizatuta) dauden gorputz ataletan (O´Donnell 1998), intsektuaren adina edo egoera fisiologikoa edozein delarik.

WWaren GMMan taldeen arteko gainjarpen handia ikusten da, distribuzioa unimodala izanik. Hau azaldu daiteke tamaina berdineko banakoen artean WWan aldakortasun handia egon daitekeelako, egoera metaboliko, adin (Hilligsøe eta Holmstrup 2003) edo aldaketa fisiologikoen eraginez, kolenboloetan gertatzen den moduan (Verhoef 1981). Alderantziz, DWaren GMMak eredu bimodala aurkezten du, liztortzar talde jakin baterako parametro konstanteagoa eta beraz, fidagarriagoa, dela adieraziz.

Gure ikerketak erakusten du bi talde edo klase banatzeko muga 0.618 gr direla WWrako eta 0.225 gr DWrako. Lortutako emaitza hauek ez datoz guztiz bat Rome et al.-ek (2015) ikusitakoarekin. Hauek, 0.593 gr-tik gorako WWa eta 0.250 gr-tik gorako DWa duten indibiduo emeak gineak kontsideratu zituzten eta balio hauek baino pisu baxuagokoak aldiz, langileak.

DWaren emaitzen desadostasunak, erabilitako metodologia ezberdinak direla eta izan daiteke, hala nola, indibiduoen lehortze-denbora eta tenperatura. Hala ere, DWaren % 5eko ziurgabetasunari lotuta dagoen tartea (portzentaietan) alderatzean, bi kasuetan oso antzekoa dela ikus daiteke, % 91.09 gure ikerketan eta

% 87.72 Rome et al.-en (2015) ikerketan.

Udazkeneko lau kolonietan behatutako hiru talde kimiko desberdinak jarraian azaltzen dira: 2. eta 3. taldeko liztortzarrek 4.5 mm-ko edo gehiagoko tamainak (MW) aurkeztu zituzten (1. kolonian agertzen diren hiru indibiduoek izan ezik). Gainera, 2. taldeko liztortzarrek bakarrik ("handiak" bezala sailkatutakoak) zuten pisu handia. Beraz, Rome et al.-en (2015) arabera, talde hau gineei dagokiela ondorioztatu daiteke. Perfil kutikularrak kasten arabera ezberdinak dira, langileak 1. talde kimikokoak eta gineak 2. talde kimikokoak izanik. 3. taldea, definitu gabeko talde kimikoa da, besteetatik desberdina eta beraz, banandua.

Lehen aipatutako 1. koloniako hiru salbuespen horiek, langileen tamaina daukate baina gineen seinale kimikoa. Habia batzuetan, gerta daiteke zenbait gine langileen gelaxketan haztea, gine txikiak sortaraziz. Fenomeno hau, *Vespula germanica*-n (Fabricius, 1793) behatu zen (Spradbery 1993), baina ikerketa gehiago beharrezkoak dira *V. velutina*-n, egiaztatua izan dadin. Kasu guztietan, liztortzar handiek beti aurkezten dituzte gineen CHC profilak. Gineak emergitzen hasten direnean langileen ekoizpena eteteak azal dezake hau (Matsuura eta Yamane 1990; Monceau et al. 2013).

2. taldean, pisu handiko zein baxuko gineak aurki daitezke. Gineak neguari aurre egingo dioten koloniako kide bakarrak dira (Monceau et al. 2014). Gine emergitu berriek habiaren barruan igaroko dituzte hainbat egun, hau utzi eta hibernazioan sartu baino lehen. *Vespa affinis*-aren kasuan 13-14 egun egongo dira barruan

(Martin 1993). Egun hauetan, langileek eta larbek berrahoratutako substantziez elikatuko dituzte gine hauek "*trofalaxia*"ren bidez. Elikagai honen gehiena erreserba-gantz bihurtzen da neguan bizirauteko (Matsuura eta Yamane 1990). Langileek aldiz, ez dauzkate honelako energia-erreserbarik eta honek gineak baino lirainagoak izatea dakar (Martin 1993). Hau dela eta, ondorioztatu dezakegu MW handia baina pisu baxua duten liztortzarrak gine gazteak direla, nahiko elikatuak izan ez direnak eta beraz, pisu handia lortzeko denbora nahiko izan ez dutenak. Mota honetako liztortzar guztiek profil kimiko antzekoa dute; beraz, PCAren II ardatzak, taldeak adinaren arabera sailkatzen dituela ondoriozta daiteke. Horrela, langileak (1. taldea), homogeneoagoak dira, denak antzeko adinak dituztelako eta gineetan (2. taldea) aldiz, kontrakoa gertatzen da liztortzarrek adin desberdinak dituztelako. Bukatzeko, 3. taldea emergitu berriko liztortzarrez osatua dagoela dirudi. Izan ere, hauek ez dute garatzeko eta profil kimiko definitu bat lortzeko denbora nahikorik izan (Lorenzi et al. 2004). Horregatik, gine emergitu berrien kastakoak direla ondorioztatu genezake. Hau, 1. talde kimikoan 4.5mm edo handiagoko MWa duen liztortzarrik ez dagoelako frogatu daiteke.

Rome et al.-ek (2015) emandako DW 0.250 gr-ko muga ontzat hartuz, emergitu berriko gineak, elikatzeko denbora nahikorik izan ez dutenak, lirainen taldean sailkatuak izango dira; hau da, langileen taldean. Gauza bera gerta daiteke udazken amaieran jasotako kolonietan, noiz elikadura baldintzak batzuetan ez diren egokiak, janari falta dela eta edo larbak elikatzeko langile kopuru nahikorik ez dagoelako (Matsuura eta Yamane 1990). Bai langileak, bai azkenengo estadioko larbak,

emergitu berriko liztortzarren elikatzaileak dira (Matsuura eta Yamane 1990). Beraz, bi kastak (langileak eta gineak) lirainagoak izaten dira urritik abendura (Rome et al. 2015). Seguru asko, gineen talde kimikoaren eme pisutsuenak, PCA-n batera agertzen direnak, dira zaharrenak . Hauek, haien erreserba kopuru altuagoaren arabera, denbora luzeagoa eman dute habian elikatzen.

Ondorioak:

Vespa velutina Europan sartu zenetik 2005ean, espezie inbaditzaile honi buruzko hainbat galdera zientifiko aztertu dira. Hauetako batzuei erantzuna emateko, guztiz erabakigarria da emeen kastak desberdintzea, hainbat aspektu biologiko ulertu ahal izateko; hala nola, lehenengo gineak noiz agertzen diren eta zenbat sortzen diren habiako. Beraz, hemen aurkeztutako datuen arabera, badirudi *V. velutina*-k emekasta morfologiko ezberdinak aurkezten dituela MWaren arabera. Gainera, GMMaren % 5eko ziurgabetasun-mailarekin kalkulatutako gainjarpena baxuagoa da MWan bi pisuetan baino, errorearen probabilitatea murriztuz. Honako hau, CHC profilen emaitzekin baieztatu daiteke: 4.5mm edo handiagoko MWa daukaten liztortzarrak gineak direla kontsideratzen da eta aldiz, 4.5mm baino gutxiagokoak langileak direla. MW tamaina-parametroa CHC profilen azterketa baino errazagoa, azkarragoa eta merkeagoa da. DWa, WWa baino hobea da, baina ez bata ez bestea ez dira MW bezain zehatzak, behintzat, gine gazte edo gaizki elikatuekin erabiltzeko.

Chapter 3: Cuticular profiles and fats in gynes of *Vespa velutina* (Hymenoptera, Vespidae)

Abstract:

Chemical communication is the oldest form of communication, spread across all forms of life. There are different factors that can influence the CHCs profiles in insects such as the age and the diet. In several insects' species, newly emerged individuals present a different cuticular profile than the oldest ones. In many species of social wasps as in *Vespa velutina,* newly emerged gynes spend several days in the nest feeding to accumulate fats to survive to the winter. Apart from that, it seems that males preferred females with more abundant fat storage. CHCs analysis and fats quantification and chemical characterization (quality) were made in gynes with different ages and diets. The CHCs are influenced especially by the age, while the fat quantity is more affected by the diet. In the case of the chemical characterization of the fats, it is the age again the most influential factor. A slight relation between CHCs and fats chemical characterization is observed. However, we cannot conclude that one factor can modify the other, because both are very age dependent.

Keywords:

CHCs, fat quantification, fats chemical characterization, *Vespa velutina* gynes.

Introduction:

Chemical communication is the oldest form of communication, spread across all forms of life (Wilson 1970). Pheromones are one of the most important signals received through the chemical sensory channel (Wyatt 2013), and they are particularly complex molecules, well-studied in insects (Howard and Blomquist 2005). These chemical signals can be volatile, and they are often used as long-distance signals, or non-volatile, which play a role in short-range communication (Gershman et al. 2014).

Among the most studied non-volatile pheromones, there can be found the cuticular hydrocarbons (CHCs), which are complex mixtures of long-chain aliphatic and methyl-branched alkanes and/or alkenes, present in the epicuticle of insects (Blomquist and Bagnères 2010). Most of the cuticular hydrocarbons studies have been focused on the Hymenoptera, one of the largest and most diverse insect orders with over 130,000 described species, in which some of them are economically and environmentally important, especially among the social bees, wasps and ants (Wilson 1971).

Even the primary function of those CHCs was to avoid insect desiccation, as it was explained in Chapter 1; they are also used by many insect species for intra- and interspecific communication (Blomquist and Bagnères 2010). Among the intraspecific communication, these CHCs are used, not only in nestmates recognition among workers of social Hymenoptera (Singer 1998), but also for the recognition

of sexual mates (Spiewok et al. 2006) and mating behaviour as well (Hora et al. 2008). Due to that, they play an important role in short-distances and male-female contact communication (Ferveur 2005).

Insects' hydrocarbons are produced in cells called oenocytes (Wicker-Thomas et al. 2009) which are located in the fat body of adult insects (Ferveur et al. 1997; Fan et al. 2003). Most of the insects synthesize CHCs *de novo* by elongation of fatty acid acyl-CoA to very long-chain fatty acids and subsequent decarboxylation to hydrocarbons (Blailock et al. 1976; Blomquist 2010; Howard and Blomquist 2005). Hence, the qualitative and quantitative blend of fatty acids may affect insects CHCs profiles (Otte et al. 2015).

Lipids are the main components of insects fat bodies, and more than 90 % of those lipids are composed by triglycerides (Bailey 1975; Canavoso et al. 2001) which have their origin in carbohydrates, fatty acids or proteins obtained from the diet (Arrese and Soulages 2010). There are several studies where it is well documented the conversion of carbohydrates, the major component of the insects diet, to lipids in the fat bodies (Bailey 1975; Briegel 1990; Hines and Smith 1963; Inagaki and Yamashita 1986; Venkatesh and Morrison 1980). The fat bodies have higher capacity for lipogenesis from glucose than for glycogen synthesis. This is the reason why the content of lipids compared to glycogen is higher in the insects fat bodies (Arrese and Soulages 2010). Both the insects' development stage and the feeding status, have influence in the amount of fatty acid incorporated by the fat bodies (Bailey 1975; Lorenz 2001; Pontes et al. 2008; Ziegler 1997).

There are different factors that can influence the CHCs profiles in insects. The first factor is the age of the individuals (Kuo et al. 2012). In several flies (Mpuru et al. 2001), parasitic wasps (Ruther et al. 2011) and in rove beetles (Peschke 1985), the newly emerged individuals have a similar CHCs phenotypes but after a week they will be sex-specific (Otte et al. 2018). In the case of the social wasp *Polistes dominulus* Christ, 1791; the proportion of CHCs changes with the age, having the young individuals more labile cuticle than the older ones which allow them to absorb environmental hydrocarbons (Lorenzi et al. 2004). The second influential factor is the diet. There are several studies which have demonstrated the influence of the diet in phenotypic plasticity of CHCs in insects such as caterpillars of different Lepidoptera species (Espelie and Bernays 1989), grasshoppers (Blomquist and Jackson 1973) and in different species of *Drosophila* Fallén, 1823 as *D. mojavensis* Patterson et al. 1940 (Etges et al. 2009; Etges and de Oliveira 2014), *D. melanogaster* Meigen, 1830 (Fedina et al. 2012) or *D. serrata* Malloch, 1927, in which this CHCs profile change can affect the mating success of the individuals (Rundle et al. 2005).

In the case of many species of social wasps, as occurs in *Vespa velutina,* the future queen after emergence spent several days in the nest feeding on larvae and workers regurgitations by trophallaxia, before leaving the nest (Rome et al. 2015). Most of this food is converted to fat reserves (Rome et al. 2015). Those fat reserves, together with fructose act as cryoprotectant to overwinter (Strassmann et al. 1984; Toth et al. 2009) and as fuel for flight and reproduction (Hill and Goldsworthy

1968; Walker et al. 1970).

Males of several species usually have preferences for matting with larger size females which are associated with a bigger likelihood to survive winter diapause, bigger capacity to found new colonies (Hunt et al. 2007, 2010; Cervo et al. 2008) and with higher fecundity (Johnson and Hubbell 1984; Harris and Beggs 1995; Gage and Barnard 1996; Ptacek and Travis 1997; Gadagakar 2001; Lüpold et al. 2011). The development of oocytes is a process that needs a large protein and fat resources (Shukla et al. 2012). Moreover, it seems likely that reproductive females could actively show their condition, while there might be a lack of male-attractive signal production by non-reproductive workers (Wen et al. 2017); thus, variability in female signal production, may explain the difference in male interest response towards the two female castes (Cappa et al. 2013). Cappa et al. (2018) showed that in the case of *V. velutina*, males present more antennation (touches with the antennae) with heavier and bigger gynes, with more abundant fat storage, and they propose that this preference is due to the CHCs profiles, as occurs in other insects (Carlson et al. 1971; Schal et al. 1990).

In this study, we wanted to see, 1) if the age and the diet of gynes may influence cuticular hydrocarbon profile , 2) if age and diet modify the fat content (quantity) and quality; and 3) if there is any relationship between CHCs profile and the fats of gynes of *Vespa velutina*. We hypothesize that both, age and diet, would influence on CHCs and fats, being the rich sugar food the most important one. It is also expected to find differences in cuticular profiles between gynes with differ-

ent fat quantity and quality.

Material and Methods:

Nest collection

During 2015 and 2017 autumn, 4 colonies of *V. velutina* were caught alive; one, C1, in Tours (France) on 13/11/2015, other 2 in different localities of Biscay (Spain), C2, in Urduliz on 18/10/2017 and C3, in Berango on 10/11/2017 and the last one, C4, in Amurrio (Alava, Spain) on 15/11/2017.

Obtaining samples

Once in the laboratory, the colonies were anaesthetised using diethyl ether. All the adults were removed and only the combs with sealed cells were place in 62 x 35 x 42 cm aerated plastic boxes (Figure 1A). Everyday the boxes were revised at the same hour and every newly emerged female was transferred to smaller plastic boxes (23 x 12.5 x 13 cm) (Figure 1B and 1C). The experiment was performed in an isolated room with 23 ±1 °C and 12 h light: 12 h dark (Poidatz et al. 2017).

Figure 1: A) Combs with sealed cells. B) Gynes in small plastic boxes. C) Gynes feeding.

The hornets of these small boxes were fed *ad libitum* (Figure 1C), half of them with a rich sugar food (R) (10 ml honey + 40 ml H_2O) while the other half were fed with a poor sugar food (P) (0.5 ml honey + 49.5 ml H_2O). The honey was Eroski® Mil flores (100 g of honey: 330 kcal, 72 g sugar, 0.6 g protein and 0.02 g salt) for human food. Hornets of both food groups were killed at 0, 3, 6 and 9 days. To kill the hornets, they were place in the freezer at -20 °C. In all boxes a piece of nest was placed as model for the synthesis of the cuticular profile (Singer and Espelie 1992; Layton and Espelie 1994; Lorenzi et al. 2004). This methodology was used for the 4 colonies.

Table 1: Number of gynes of each colony used for the different analysis.

Colony	CHCs	Fats quantification	Fats analysis
C1	80	80	75
C2	49	49	---
C3	71	71	---
C4	65	65	---
TOTAL	265	265	75

CHCs chemical analysis

For the analysis of the CHCs profile, the previously explained methodology was used (see Chapter 2, **Material and Methods**: *Chemical analysis*).

Body size estimation

For gynes' body size estimation the mesoscutum width (MW) was measured following the methodology previously explained (see Chapter 2, **Material and Methods:** *Size and weight analyses*).

Fats quantification

To quantify fats, hornets were dried in the oven during 48 h. at 80 °C and then they were weighted using a Sartorius (0.01 mg) high precision balance. Every dried hornet was place in glass tubes where they were ground. Then, 2 ml of chloroform:methanol (2:1) was added as fats solvent (Suryanaryanan et al. 2011). After shaking for a 1 min and centrifuged for 5 min, the solvent was removed. The process was repeated 2 times more adding 1.5 ml of chloroform:methanol (2:1) each time.

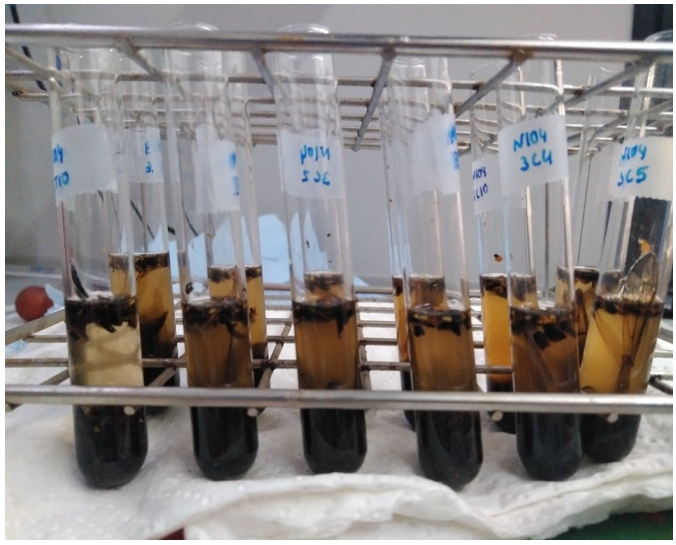

Figure 2: *Vespa velutina*'s fats extraction in chloroform:methanol.

Removed solvent of hornets of C1 was kept in the freezer at -20 °C, until it was processed (Table 1). Extracted hornets were dried during 24 h. at 80 °C and they were weighted again. The difference between the previous extracted weight and the before extracted weight was considered as the extracted fats quantity.

Fats chemical analysis

The extracted fats of 75 gynes of C1 (Table 1), were chemically analysed. After the extraction of fats, which was carried out following the methodology explained in *Fats quantification* section, the samples were evaporated under a nitrogen current flow (45 ºC, 15 psi) (TurboVap®, Zynmark). The samples were kept in the fridge (4 °C) until they were rebuilt in 1 ml hexane with 50 ppm of *n*-dotriacotane-d66 as internal standard (IS). The samples were shook during 30 seconds and filtered using a 0.45 µm filter (Whatman®). Until the moment they were injected, they were kept in the fridge (4 °C). One µl of each sample was injected in a Gas Chromatography system (6890N, Agilent) coupled with a Mass Selective Detector (5973 Single Quadrupole, Agilent) (GC-MS). The analysis was performed with a HP-5MS % Phenyl Methyl Siloxane (30 m x 250 µm x 0.25 µm) capillary column. The oven program temperature was 50 ºC (2 min), from 50ºC to 250 ºC (40 ºC/min) and from 250 ºC to 320 ºC (20 ºC/min) and 320 ºC (6 min). Injection was in "*split"* mode (1:200) and helium was used as a carrier gas (1.3 ml/min). All data were processed with MSD ChemStation E.02. 02. 1431.

Statistical analysis

For the analysis of chemical signature data, a Principal Component Analysis (PCA) of the CHCs, with supplementary variables (Age, Food and Colony), was performed. In the scatter plot of the supplementary variables, the dependent variable Fat was also represented. To estimate the % of variation explained by each variable a Redundant Variation Partitioning Analysis was performed. Both of these analyses were conducted with Canoco 5®.

In the same way, for the analysis of the chemical composition of the fats a PCA of the fats chemical components, with supplementary variables (Age and Food), was done. In this case the dependant variable Fat content (g) was also represented as a supplementary variable in the scatter plot. A Redundant Variation Partitioning Analysis was performed to estimate the % of variation explained by each variable. All these Multivariate Analysis were performed with Canoco 5®.

Gaussian Mixed effects Models (GMM) (Zuur et al. 2009; Madsen and Thyregod 2010) were used to test the effect of the Age, Food and Colony on the fat content (quantity). For this analysis the function lme() of package nlme (Pinheiro et al. 2014), with restricted maximum log-likelihood estimators (Pinheiro and Bates 2000) was performed in R (2018). Age (numeric variable) and Food (with two levels, R and P) were included in the model as fixed factors. The Colony (with four levels, C1, C2, C3 and C4) was fitted as random factor. To finish, we performed a model validation and tested whether the Fat polynomial terms (quadratic

and/or cubic terms) and special variance structures were necessary to cope with observed heteroscedasticity (Pinheiro and Bates 2000) (Appendix 1 and 2). We used "weights" argument within lme() and the varExp() functions to specify variance models with an exponential function of the Fat variance (Pinheiro and Bates 2000; Zuur et al. 2009). The function varExp() allows modelling both, increase or decrease of dispersion with the increase of the Age. For the model selection, Akaike's information criterion (AIC) and/or the log-likelihood ratio test (Zuur et al. 2009) was used in each step.

To finish, a Redundancy Analysis (RDA) was performed using Canoco 5® to analyse CHCs as response variables, and fats (chemical components) as independent variables, that were significant according to the forward selection, also performed.

Results:

Cuticular hydrocarbons (CHCs)

The 265 gynes of the 4 colonies are spatially distributed according to their cuticular profile (dependent variables) in axes I and II of the PCA (Figure 3A). This graph can be interpreted together with the supplementary independent variables Age (0, 3, 6 and 9 days), Food (R and P) and Colony (C1, C2, C3 and C4) (Figure 3B). In those graphs the main trends of CHCs variation, are shown.

First, the samples (hornets) present a great clear variation according to the age of the gynes (Figure 3B). It can be seen that there is a separated group in the right part of the ordination graph (Figure 3A) which corresponds to the newly emerged

gynes, the “0 days” group (Figure 3B). This is the age which presents more dispersion. After this group, samples of “3 days” are arranged in a middle position, also with high dispersion. The “6 days” group precede the next. To conclude, gynes with “9 days” of age form other well separated group, placed in the four quadrant of the graph, and chemically very homogenous.

Second, regarding the variables Food (R and P) and Colony (C1-C4), it can be observed that a variation according to them exists but is less important than the variation due to Age (Figure 3B), because these variables are nearer to the centre of the PCA. Colony variable present more influence than Food.

There is a clear increase of fat content linked to higher ages and in less extent, also to rich food (R). Colony 1 is the colony in which gynes present less fat content (Figure 3B).

In order to know which is the effect of the independent variables in the cuticular profile a Variation Partitioning Analysis was performed (Table 2 and Figure 4), taking into account all variables together and then each variable along without the interaction among them (Table 2).

The 3 variables together explain 70.6 % of all CHCs variability. Age is the variable which explains more % of the variability (51.9 %) (Table 2). This result is also shown in Figure 4A where it can be seen the chemical profiles according only to Age effect.

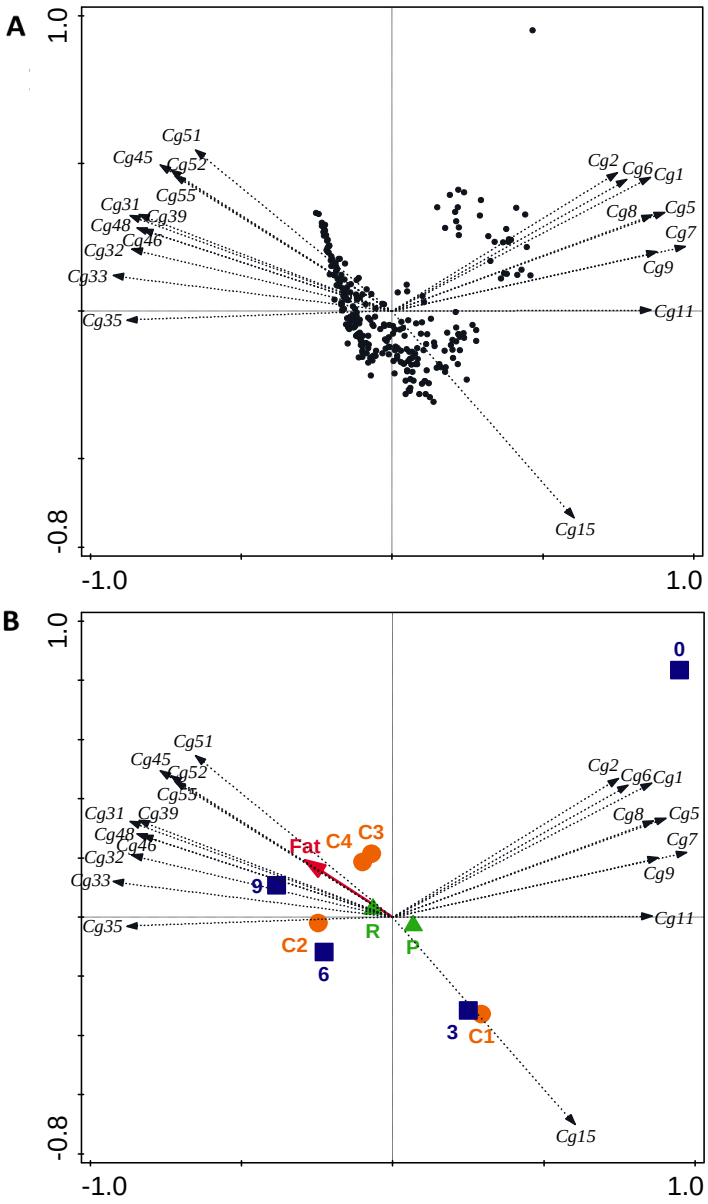

Figure 3: A) PCA of CHCs of the 265 hornets (dots). Arrows represent the 20 most important CHCs gradients. B) Scatter plot of the same PCA representing independent variables: Age (0, 3, 6 and 9 days) (blue squares), Food (R: Rich and P: Poor) (green triangles) and Colony (C1, C2, C3 and C4) (orange circles). The red arrow represents the gradient of Fat quantity.

Table 2: Variation Partitioning Results of RDA for the CHCs profiles of all gynes of 4 colonies according to the independent variables: Age (0-9 days), Food (Rich and Poor) and Colony (C1-C4).

Fraction	Variation (adj)	% of Explained	% of All	DF	Mean Square	F	p-value
Age	0.51885	73.5	51.9	3	0.17144	154	0.002
Food	0.014167	2.0	1.4	1	0.01496	13.4	0.002
Colony	0.17473	24.8	17.5	3	0.05848	52.5	0.002
Total Explained	0.70589	100.0	70.6	7	0.10196	91.5	0.002
All Variation	1	--	100.0	264	--	--	--

All gynes present the same hydrocarbons although; the proportions of those compounds are not the same in all hornets. In the groups of gynes with 0 days, the Cg2, Cg1, Cg6, Cg8 and Cg5 are the most important CHCs (Figure 4A). Those CHCs are small hydrocarbons composed of few carbons (see retention times in Appendix 3 and supplementary files). However, in gynes of 9 days, Cg35, Cg33, Cg36 and Cg39 are the principal CHCs (Figure 4A) and these hydrocarbons are bigger, due to the fact that they are composed of many carbons (see retention times in Appendix 3 and supplementary files). It seems that there are not specific CHCs related to the groups of 3 days and 6 days.

Furthermore, Food only explains 1.4 % of the total variability (Table 2). According to that and as Figure 4B shows, there are not important CHCs related to R and P food.

In the case of Colony, this variable explains 17.5 % of the CHCs profiles total variability (Table 2). There are not CHCs exclusive to any of the colonies but in Figures 3B and 4C it can be observed that C1 is chemically more different from the other colonies. In turn, C2, C3 and C4 are chemically more similar among them.

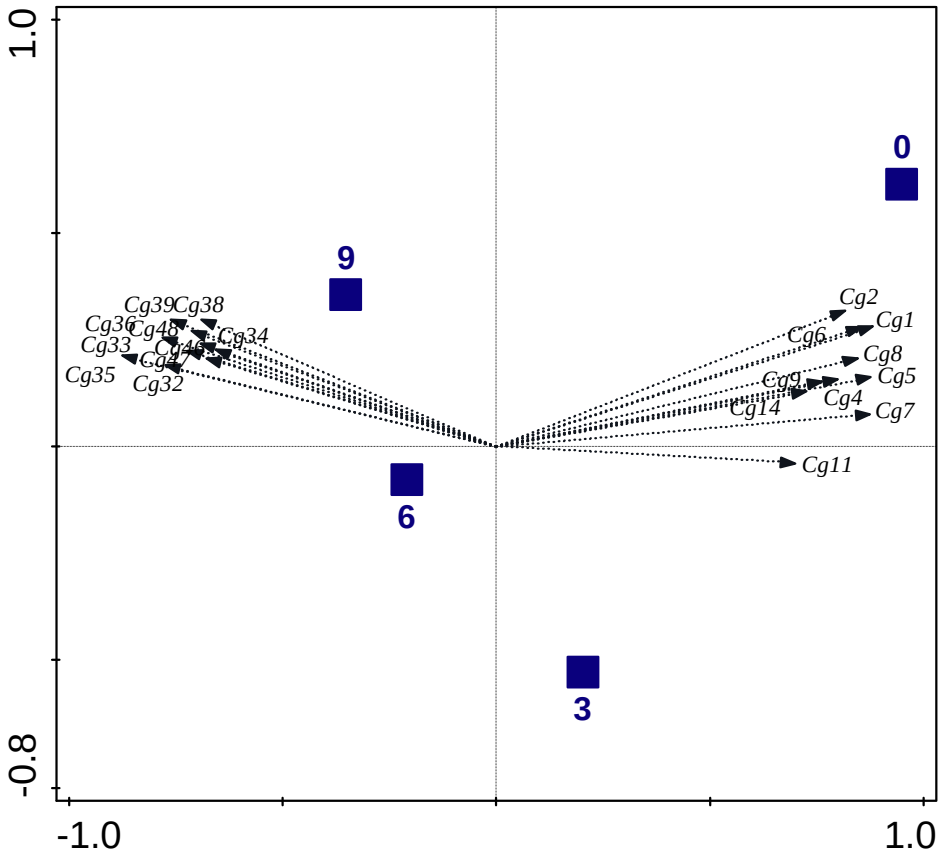

Figure 4A: PCA of the CHCs profiles according to the separated effect of Age (0, 3, 6 and 9 days). Arrows represent the 20 CHCs more important gradients.

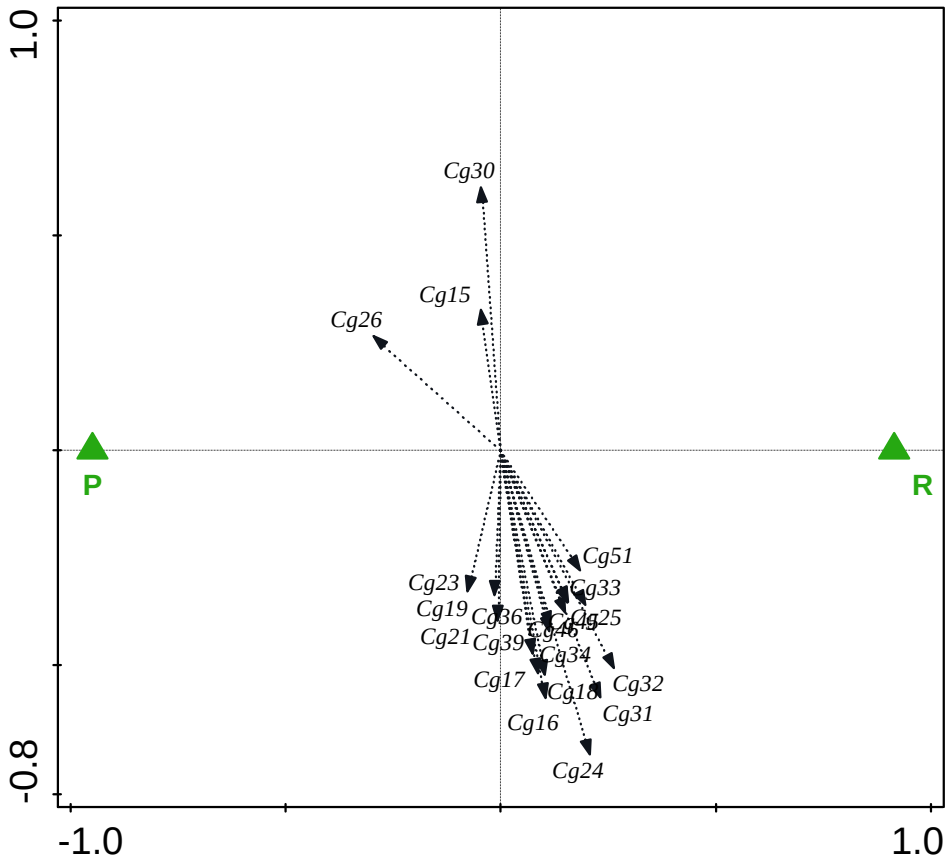

Figure 4B: PCA of the CHCs profiles according to the separated effect of Food (R: Rich and P: Poor). Arrows represent the 20 CHCs more important gradients.

Two examples of typical chemical profiles of A) a newly emerged gyne (0 days) and B) a 9 days gyne fed with rich food are represented in Figure 5. In the chemical profile of the newly emerged hornet, it has to be highlighted that in comparison to the 9 days gyne, the hydrocarbons Cg1 and Cg7 appear in mayor proportion. These correspond to *n*-C21 and *n*-C23 hydrocarbons respectively (Table 4). However, in the case of the 9 days gyne, Cg28, Cg30, Cg31 and Cg35 are

more abundant. Those CHCs are C27:1, *n*-C27, 5-MeC27 and 3-MeC27 respectively (Table 4).

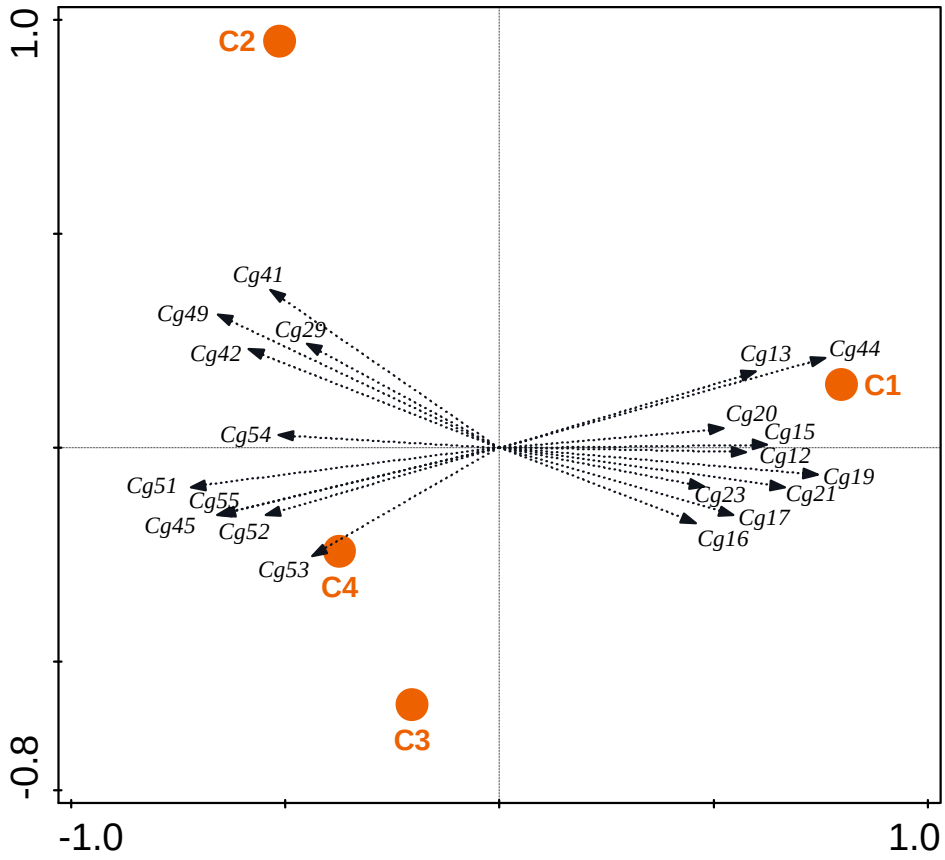

Figure 4C: PCA of the CHCs profiles according to the separated effect of Colony (C1, C2, C3 and C4). Arrows represent the 20 CHCs more important gradients.

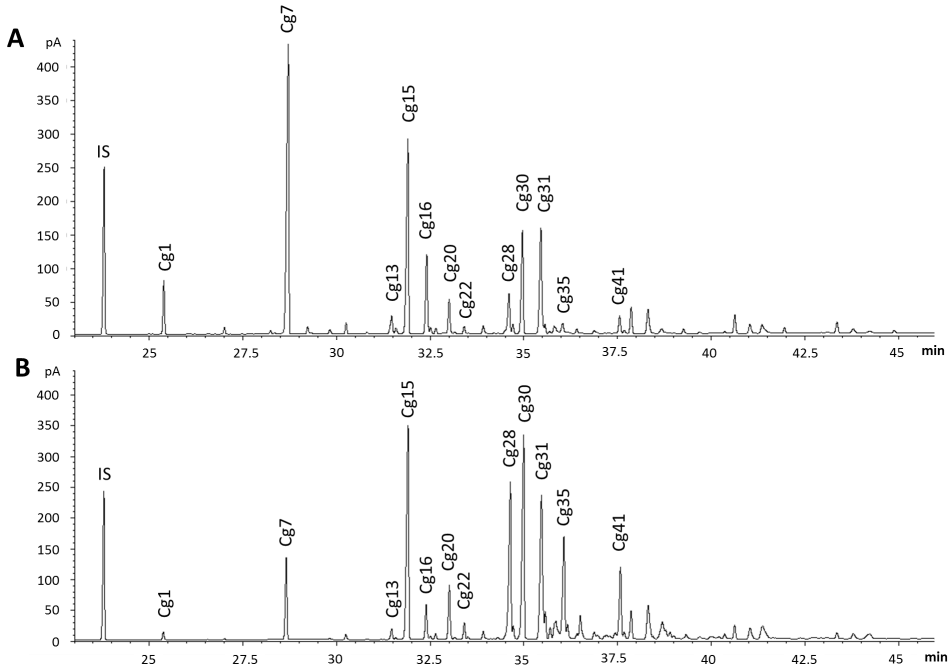

Figure 5: Typical chemical profile of A) newly emerged gyne (0 days) and B)9 days gyne fed with rich food. See Table 4 for the identification of the highlighted peaks.

Fat quantification

A regression was made to see if there was relationship between the size and the fat content of hornets. The regression with a better adjustment to the data was a polynomial regression with a R^2 value of 0.0112. It can be observed that there are small hornets with high and low fat content. The same occurs with large hornets (Figure 6). So, Fat and Size (MW) are independent.

Table 4: Identification of the main peaks of gynes profiles (Figure 5) according to Gévar et al. (2017).

Peaks	R.T. (min)	Identification
IS	23.80	*n*-eicosen
Cg1	25.43	*n*-C21
Cg7	28.71	*n*-C23
Cg13	31.47	C25:1
Cg15	31.97	*n*-C25
Cg16	32.40	13-+11-MeC25
Cg20	33.01	3-MeC25
Cg22	33.42	*n*-C26
Cg28	34.61	C27:1
Cg30	35.02	*n*-C27
Cg31	35.46	5-MeC27
Cg35	36.05	3-MeC27
Cg41	37.58	C29:1

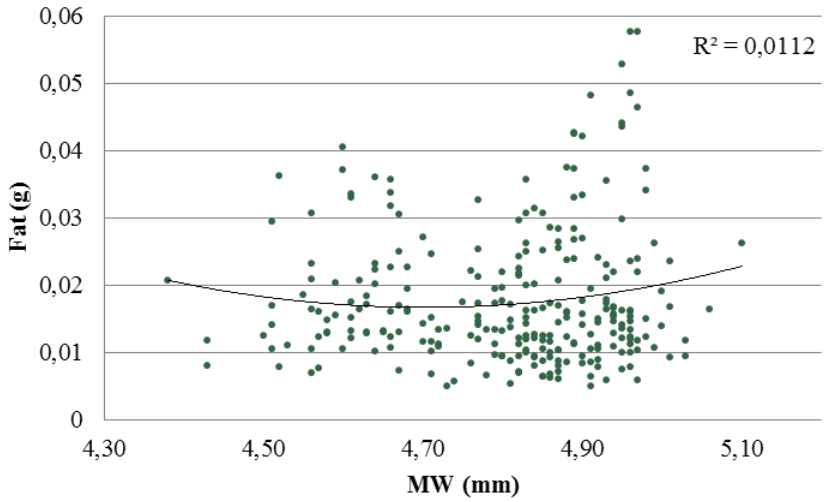

Figure 6: Polynomial regression of size (MW) (mm) and fat content (g) of 265 gynes of 4 colonies.

To see if the variables Age, Food and Colony can affect the fat content of gynes a modelling was performed. Based in the results obtained in Figure 6, we decided to use the row data of fat content instead of the data corrected by the size, because there is no relation between them in our experiment. The model which adjusts the best to the data is a second-grade polynomial model:

$$y\ (T=P) = (0.0133) - (0.0012 \times Age) + (0.0002 \times Age^2) + \varepsilon_{Colony} + \varepsilon_R$$

$$y\ (T=R) = (0.0133 + 0.0075) - (0.0012 \times Age) + (0.0002 \times Age^2) + \varepsilon_{Colony} + \varepsilon_R$$

Where:

$\varepsilon_{Colony} \sim N\ (0, \sigma_{Colony}^2 = 0.0026)$

$\varepsilon_R \sim N\ (0, \sigma_R^2 = 0.0037)$

Variables, Age and Food are significant in the model (Table 5). According to the model, Age explains 8.98 % of the variability in fat content while Food explains 40.2 % of the variability. At least, Colony explains 17.01 % of the variability.

Table 5: ANOVA of the second grade polynomic model. Age: 0-9 days. Food: Rich and Poor.

	numDF	denDF	F-value	p-value
(Intercept)	1	258	147.83743	<.0001
Age	1	258	9.11159	0.0028
I(Age^2)	1	258	21.24296	<.0001
Food	1	258	119.70541	<.0001

Following the polynomial model, on the one hand, gynes fed with a poor food (red) present a decrease in their fat content during the first 3 days. However, after this moment the fat content remains constant. On the other hand, those hornets fed with a rich food (green), show an increment of the fat content thorough 9 days. An increment of the difference in fat content between both foods over time can be seen (Figure 7). There is a big variability in the data, especially in those groups of 6 and 9 days fed with rich food. In all the groups fed with poor food, data variability is very similar (Figure 7 and Table 6).

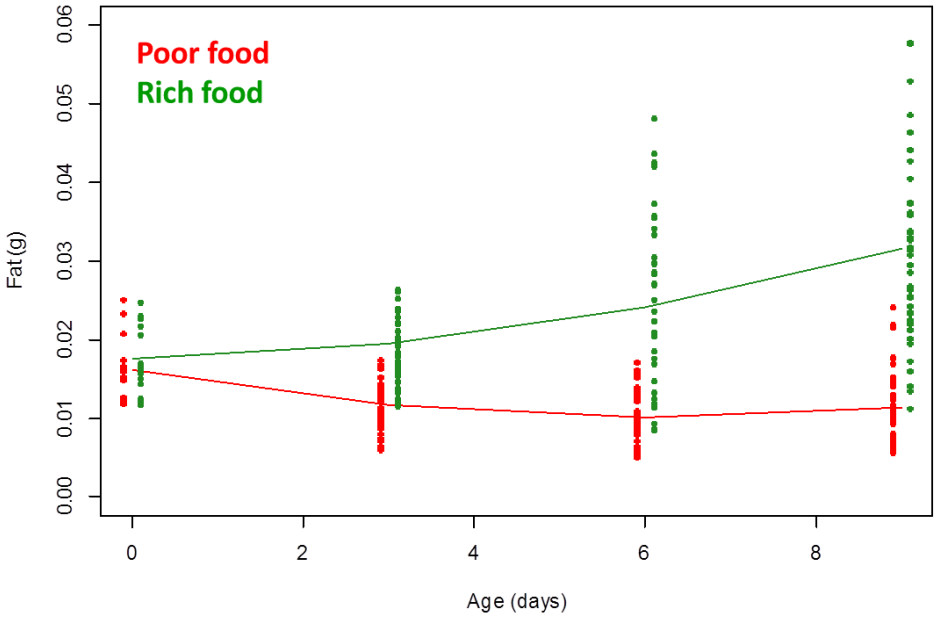

Figure 7: Effect of the Food: Rich (green) and Poor (red), in the fat content, over 9 days.

Table 6: Approximate 95 % confidence intervals for the second-grade polynomic model. Age: 0-9 days. Food: Rich food.

	Lower	Est.	Upper
(Intercept)	1.033701e-02	0.0133146576	0.0162923022
Age	-1.900768e-03	-0.0011940567	-0.0004873456
I(Age^2)	9.942135e-05	0.0001770174	0.0002546134
Food (R)	6.168490e-03	0.0075223994	0.0088763089

Fats chemical analysis

The chemical analysis of the extracted fat of gynes of Colony 1 shows that there is not such a clear relation between the variable Age and the fat chemical composition (Figure 8) as occurs with the CHCs (Figure 3). However, it seems that as occurs with the CHCs, Age explains more variability of the fat chemical composition (31.2 % of all variability) than Food (12.4 % of all variability). Both variables together explain 43.3 % of the total variability of the samples (Table 7).

Even Age has less influence in the fat chemical composition, in Figure 9A it can be seen that there are some specific fats that are clearly associated to 0, 3 and 9 days groups. In the case of gynes of 0 and 3 days, they present a very similar fat composition, been Fg26, Fg35, Fg33 and Fg21 the more characteristic. In gynes with 9 days of age; nevertheless, Fg54 and Fg1 are the most important. Hornets with 6 days of age have not a specific fat composition. Although food has less influence, there are some fats associated to the different diets, been Fg12, Fg17

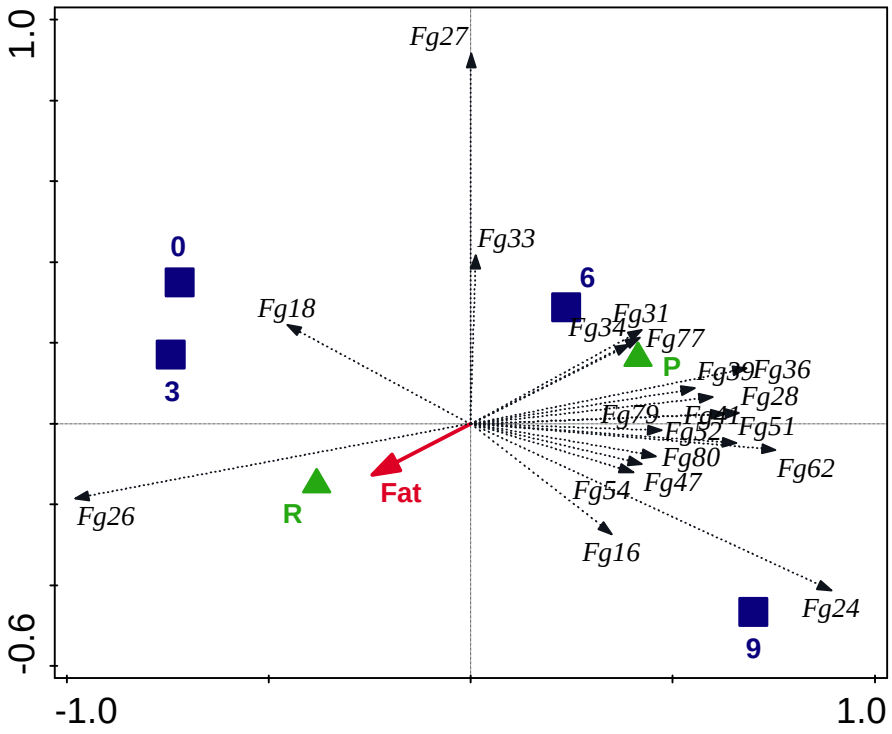

Figure 8: PCA of fats of 75 hornets of one colony (C1). Arrows represent the 20 fats more important. The independent variables: Age (0, 3, 6 and 9 days) (blue squares), Food (green triangles, R: Rich and P: Poor). Red arrow represent Fat content.

and Fg5 the most abundant fats in hornets that were fed with rich food (Figure 9B). In the other side, gynes fed with poor food have high quantities of Fg36, Fg28 and Fg31. (See Appendix 4 for the chromatograms of chemical profiles of gynes fats and Appendix 5 for the Area% and Retention Time of each fat).

Fats and CHCs

The RDA of the CHCs (response variables) and the composition of the fats peaks

Table 7: Variation Partitioning Results of RDA for the fat chemical characterization of 75 gynes of the Colony 1 according to the independent variables: Age (0-9 days) and Food (Rich and Poor).

Fraction	Variation (adj)	% of Explained	% of All	DF	Mean Square	F	p-value
Age	0.31172	72.0	31.2	3	0.11016	14.4	0.0002
Food	0.12433	28.7	12.4	1	0.12695	16.6	0.0002
Total Explained	0.43308	100.0	43.3	4	0.11593	15.1	0.0002
All Variation	1	--	100.0	74	--	--	--

(independent variables), show that there are some fats that are significantly (forward selection) related to some CHCs (Figure 10). On the one hand, the Fg33 and Fg35 fats are clearly related to Cg7, Cg5, Cg43 and Cg50. In both cases, those variables are specific of young gynes (0 and 3 days of age) (Figures 4A and 9A). On the other hand, Fg50, Fg51 and Fg55 are related to a big group of CHCs, as Cg33 or Cg35 (Figure 10), which are linked to the older gynes (6 and 9 days old) (Figures 4A and 9A).

Using the fats that have significant relevance in the RDA and that are clearly related to CHCs (Figure 10) two groups of fats can be considered for the Variation Partitioning; **Group 1:** Fg33 and Fg35, which explains the 19.2 % of the total variability and **Group 2:** Fg50, Fg51 and Fg55 which explains 31.5 % of the variability

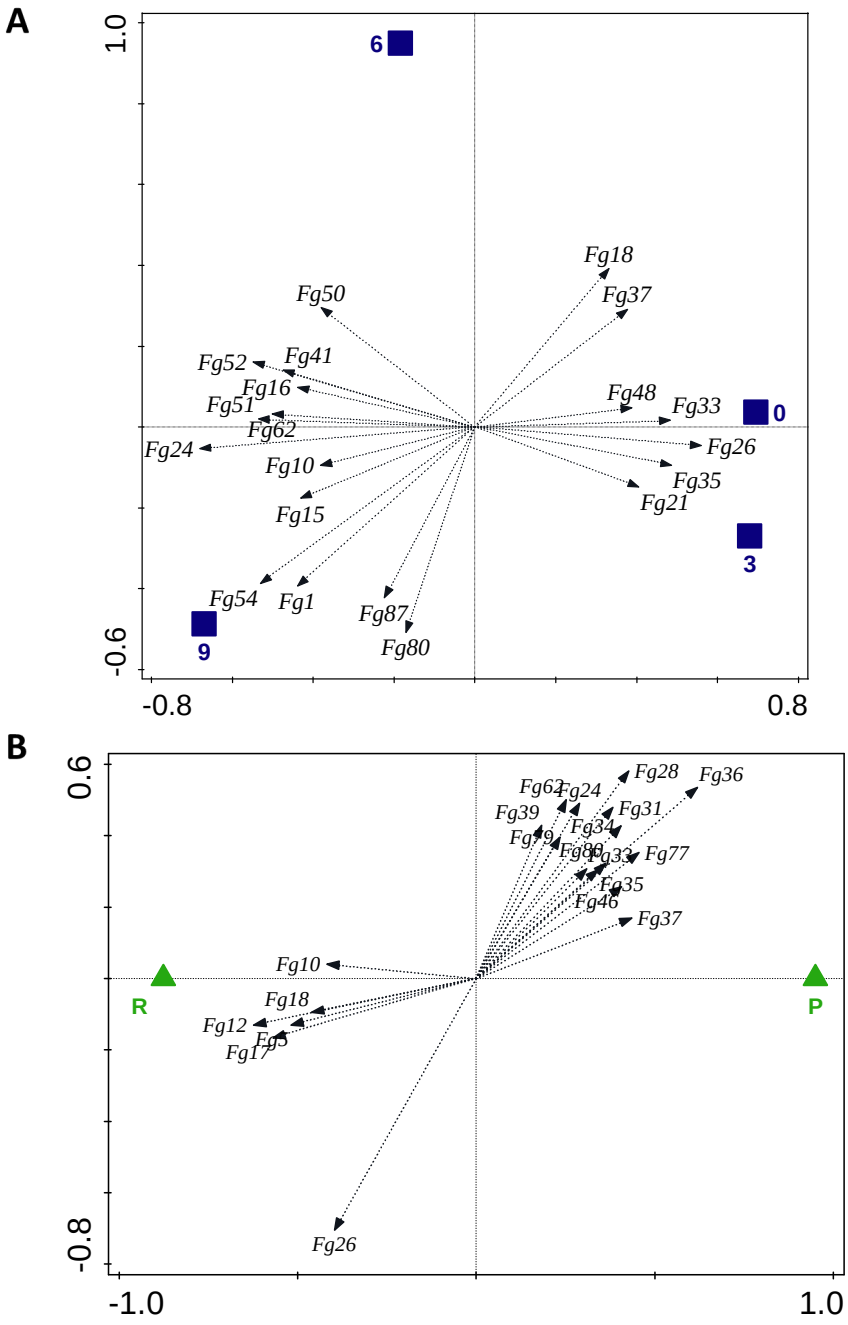

Figure 9: PCA of the fats according to the separated effect of A) Age (0, 3, 6 and 9 days) and B) Food (R: Rich and P: Poor). The plot represents the best fitted 20 fats.

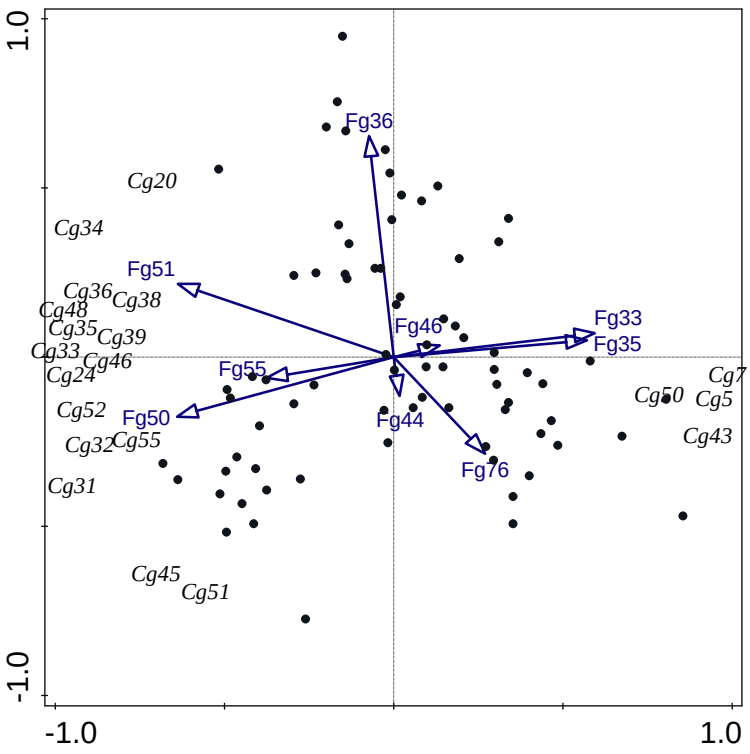

Figure 10: RDA of CHCs of 75 hornets (dots) of one colony (C1). The plot represents the best fitted 20 CHCs. The independent variables are the fats compounds (blue arrows), statistically significant in the forward selection.

(Table 8). According to this analysis both groups of fats explained the 53.2 % of all variation of CHCs of gynes of the Colony 1.

Discussion:

According to our results, the age is the variable that has bigger influence in the cuticular hydrocarbons. As it was observed in *Polistes dominulus* by Lorenzi et al. (2004), the cuticular profile of hornets changes as they get older. In the case of

Table 8: Variation Partitioning Results of the RDA of the CHCs of 75 gynes of the Colony 1 using fat components as independent variables.

Fraction	Variation (adj)	% of Explained	% of All	DF	Mean Square	F	p-value
Fg 33 and 35	0.19203	36.1	19.2	2	0.09845	15.6	0.001
Fg 50, 51 and 55	0.31485	59.2	31.5	3	0.10844	17.1	0.001
Total Explained	0.53171	100.0	53.2	5	0.11267	17.8	0.001
All Variation	1	--	100.0	74	--	--	--

V. velutina, the 0 and 9 days are extreme groups and present different proportion of CHCs which characterize each age. While, the 3 and 6 days groups seem to be intermediated stages without a clear characterization. Again, as in the research conducted by Lorenzi et al. (2004) with *P. dominulus,* the youngest hornets (newly emerged), present CHCs with lower molecular weight. Lower molecular weight hydrocarbons, are considered light and more permeable. Nevertheless, while hornets get older, the proportions of those lights hydrocarbons decrease and increase the proportion of heavy or higher molecular weight CHCs. This fact supposed that with the age, the cuticular permeability is reduced and for that, also the capacity to absorb outside compounds. Hence, hornets with 9 days present more homogeneous cuticular profile because they have acquired the definitive profile (Lorenzi et al. 2004). This fact is in accordance with the Gestalt model proposed by Crozier and Dix (1979), where they argue that passive physical interactions between the

individuals or/and the individuals and the nest material create a common chemical signature, "*gestalt odour*", that is shared by all the colony members.

In the case of the diet (Food variable), it can be seen that it has little influence in the CHCs of *V. velutina* gynes, contrary to what occurs in other insect species (Espelie and Bernays 1989; Blomquist and Jackson 1973; Etges et al. 2009, Etges and de Oliveira 2014, Rundle et al. 2005, Fedina et al. 2012). In our case, this can be explained by two possible reasons. On the one hand, because the cuticle compounds mainly will change according to its permeability and therefore in accordance with the external factors, such as the nest chemical profile, from where the hornets could absorb hydrocarbons to build the cuticle (Lorenzi et al. 2004). On the other hand, it could be that the delivered food to our hornets was not good in quality and/or quantity, as to produce changes in the chemical profile. We have fed *ad libitum* the hornets without taking into account the effect of compensatory feeding. This is, the capacity of an individual to increase its food consumption to compensate the lower food quality (Simpson and Raubenheimer 2012) making it possible to consume the same calories as in a good-quality diet (Rapkin et al. 2017). In our case, due to the difficulty to maintain the hornets alive with a poor food for long time, feeding them *ad libitum* was the only choice. However, the two given diets were good enough to see differences in fat content.

Regarding the variability due to the colony, it is seem that there is a quite important cuticle variation among hornets. The chemical profile of each nest is unique (Gévar et al. 2017) and it is created when workers rub their van der Vecht

organ against the nest surface applying a hydrocarbon layer on it (Dapporto et al. 2007; Cervo and Turillazzi 1989; Gamboa 2004). The composition of those hydrocarbons is very similar to the cuticular profile of the hornets of that colony (Dani et al. 1992, 2003). Newly emerged hornets, according to the Gestalt model of Crozier and Dix (1979), acquire the colony "odour" and thus, the rest of the individuals could recognize them as members of the colony and not attack them (Lorenzi et al. 2004; Cervo and Turillazzi 1989; Gamboa 2004). In any case, it has to be highlighted that the effect of the colony in the CHCs is far lower than the age effect. The fact that all nestmates present a common odour could be a key in the avoidance of choosing a sexual mate of the same colony, reducing the inbreeding rate.

Regarding fat content, in our study, we can see some samples in which there is no relation between the size of the hornets and their body fat content. So, the variation that we have found in the fat content data has nothing to do with hornet size, but it is related to age (Lorenz 2001), food (diet) (Bailey 1975; Ziegler 1997; Zhou et al. 2004) and/or colony variables.

The second grade polynomial model shows that in fat content data variation, the age explains less variability than the food which is a main driving factor, followed by the colony. This fact could be explained because food carbohydrates, which are one of the most important compounds in insects' diet, are transformed into fats by insects' metabolism (Arrese and Soulages 2010). In the case of *Vespa velutina*, as in other social Vespidae, gynes need to accumulate those fats to overwinter and to

found a new colony the next spring (Rome et al.2015). In the case of hornets that have been fed with a rich sugar food, the fat content increase gradually with the age. All the newly emerged gynes present similar fat content and in the case of those fed with poor sugar food, even lose fat quantity during the first three days. This phenomenon happens since the newly emerged hornets still have part of the fats accumulated when they were larvae to perform the metamorphosis (Mirth and Riddiford 2007). Over the first three days, they lose fat since they do not have enough food because they were not properly fed. From now on, they maintain their fat content more or less constant. Apart from that, data variation is much bigger in the groups of hornets that were fed with rich food. This could be explained as even they had *ad libitum* rich food to eat, we were not capable to control the quantity of food each gyne ingested. So, some of them could eat more than others which could be reflected in fat content. In the case of those fed with poor food, even if some of them could ingest more food than others, since the food has little sugar, the differences could be smaller.

As it occurs in the chemical profile of the cuticle, the composition (quality) of the extracted fats from gynes, present a bigger relation to the age than to the diet. This could be explained because, as was previously explained, the age can have influence not only in the fat quantity but also in the quality too (Anand and Lorenz 2008). The influence of the age is not as evident as in the case of the CHCs. However, there seems to be a clear similar tendency. The fats of young and old gynes are basically the same but in difference proportion. It would be very interesting to

identify them as in *Drosophila melanogaster* (Wren and Mitchel 1958) in order to try to understand the biological importance of the variation in fats proportion on *Vespa velutina*.

As it was commented before, apart from the external factors, CHCs profile could depend on internal physiological factors. When fats and CHCs are analysed together, it can be seen that there is a relationship among some of them. However, we could not be sure if the presence of some of them can influence in the presence of others, because both, age and diet have influence on CHCs and fats. For that reason either CHCs or fats, can change in parallel but as a consequence of the already cited variables (age and diet). In order to know if quantity and/or quality of fats really have any effect in CHCs profile, another kind of experiments should be done. May be modifying fats reserves in an artificial way to avoid the age effect. Apart from that, it would be very interesting to identify chemical compounds of fats, as was previously suggest, seeing if the compounds that are more related to gynes CHCs take part in the synthesis of those hydrocarbons.

In any case, the fact that a relation between the cuticular profile and the fat quantity and quality, either because one depend on the other or because both of them depend on other variables, could influence in the election of males to gynes with a cuticular profile linked to a more abundant fat storage, this was also proposed by Cappa et al. (2018). Those gynes have bigger probability to survive to winter (Hunt et al. 2007, 2010; Cervo et al. 2008) and also to be more fertile (Johnson and Hubbell 1984; Gage and Barnard 1996; Ptacek and Travis 1997; Lüpold et al.

2011). Experiments with live hornets should be performed to test this. For example, showing gynes with different fat quantity and quality to males to see which they prefer to mate with; and analysing the CHCs profiles of the chosen ones. After that, the identified hydrocarbons could be tested to be used in traps to attract males.

Conclusions:

The chemical profiles of gynes present a clear relationship to the hornets' age. The influence of the diet and the colony is less important even if the colony influences more than the diet. In our data there is no relation between the size and the fat quantity of the hornets, so fat differences are due to diet and age independent variables, been the former the most influential factor. By contrast, in the case of the fats chemical characterization, the age is the most important variable. Some fats and CHCs appear to have a clear relationship. However, as both of them depend mainly on the age, it cannot be concluded that fats can modify the CHCs. In any case, it seems possible that the cuticular profile could be used by males to identify gynes with bigger quantity and quality of fat, linked with bigger survival and fertility.

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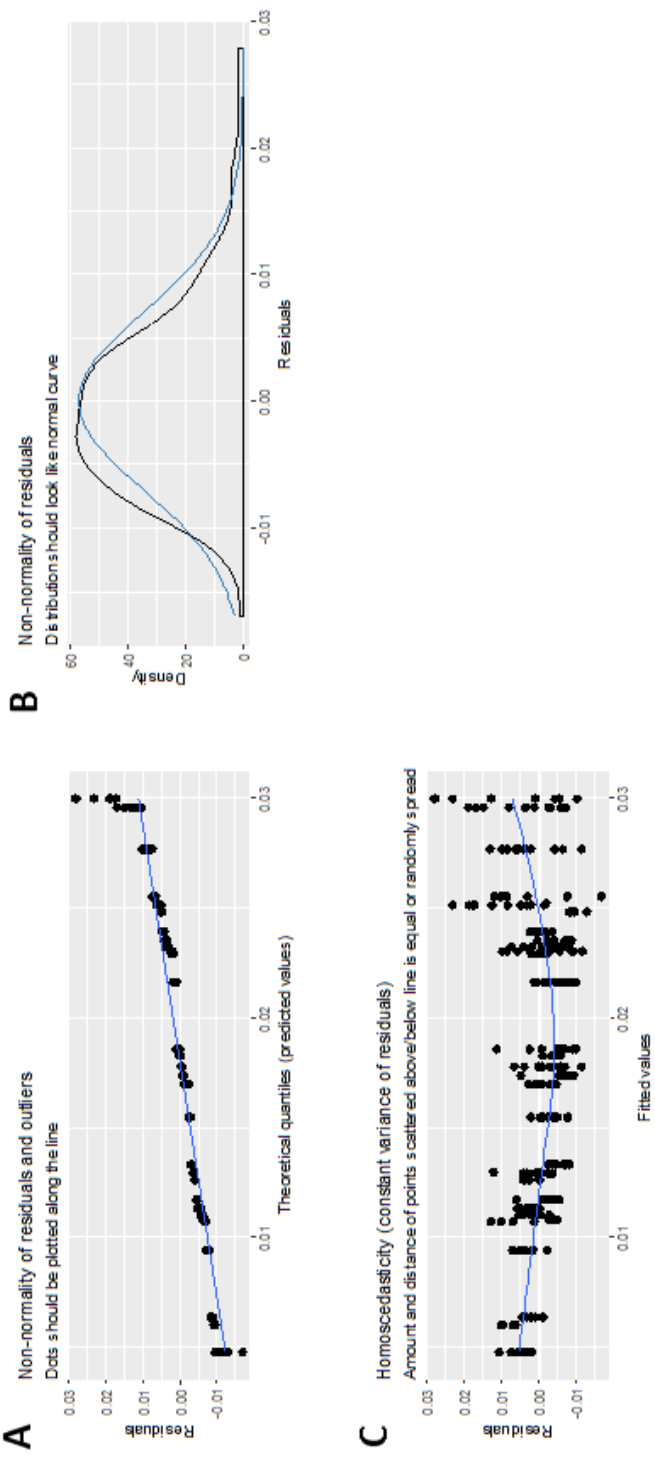
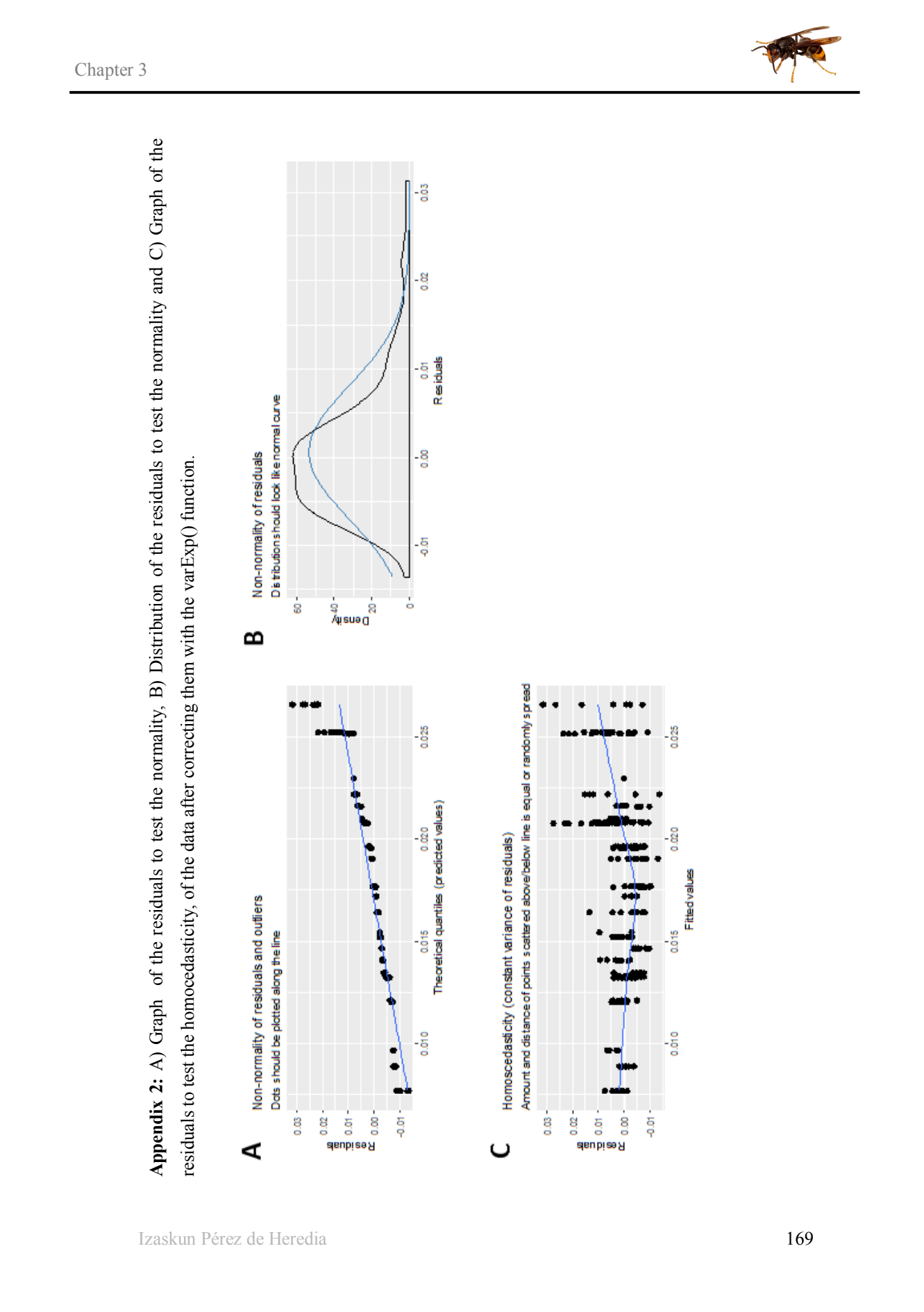


Appendix 1: A) Graph of the residuals to test the normality, B) Distribution of the residuals to test the normality and C) Graph of the residuals to test the homocedasticity, of the data before correcting them with the varExp() function.

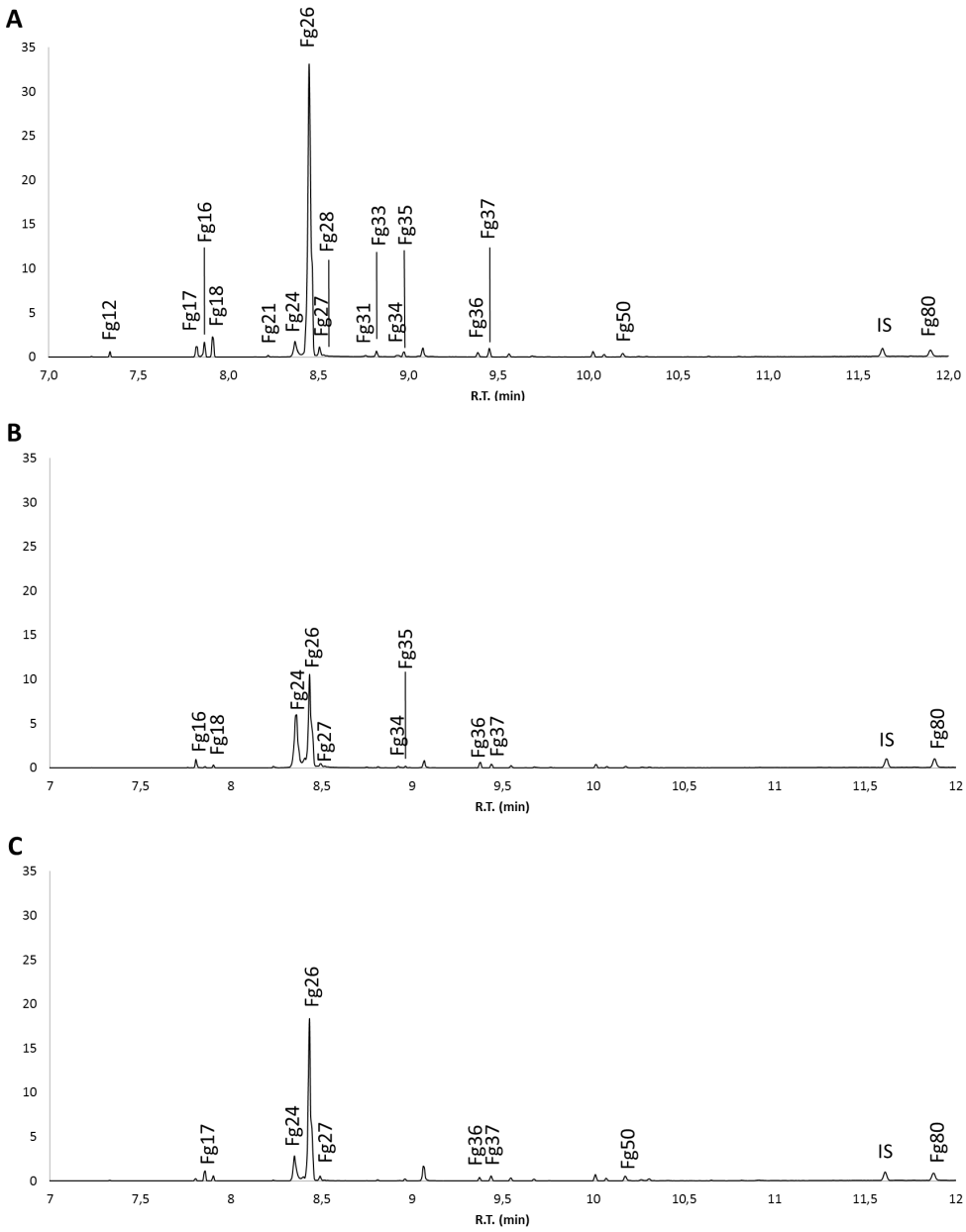


Appendix 2: A) Graph of the residuals to test the normality, B) Distribution of the residuals to test the normality and C) Graph of the residuals to test the homocedasticity, of the data after correcting them with the varExp() function.

Appendix 3: Example of the area (%) and retention time (min) of the first 18 CHCs of 18 gynes of Colony 1. Age: in days, P: poor food and R: rich food. All data available in supplementary file online: https://drive.google.com/drive/folders/1MaIFAD33uTOZtvc5UUQGaWtR3gKFn1hH

				Peaks																
				Cg1	Cg2	Cg3	Cg4	Cg5	Cg6	Cg7	Cg8	Cg9	Cg10	Cg11	Cg12	Cg13	Cg14	Cg15	Cg16	Cg17	Cg18
				Retention time (min)																
				25.43	26.12	26.50	26.57	27.03	28.26	28.71	29.24	29.83	30.06	30.27	30.82	31.47	31.65	31.97	32.40	32.60	32.65
Hornet	**Colony**	**Age**	**Food**	**Area (%)**																
1	C1	0	P	2.66	0.10	0.00	0.09	0.37	0.41	25.89	0.92	0.51	0.00	0.87	0.21	3.42	0.36	24.28	6.22	0.53	0.47
2	C1	0	P	3.05	0.07	0.00	0.06	0.46	0.24	30.79	0.88	0.34	0.00	0.89	0.16	2.02	0.26	25.84	4.36	0.45	0.24
3	C1	0	P	4.95	0.13	0.00	0.13	0.62	0.64	41.14	1.13	0.53	0.00	1.08	0.19	4.05	0.50	0.00	5.89	0.51	0.32
4	C1	0	P	3.20	0.11	0.00	0.09	0.41	0.42	28.74	0.66	0.50	0.00	0.81	0.14	3.17	0.37	23.89	4.53	0.36	0.33
5	C1	0	R	2.43	0.05	0.00	0.07	0.38	0.28	27.19	0.66	0.46	0.00	0.88	0.15	2.68	0.35	24.39	5.55	0.51	0.45
6	C1	0	R	2.63	0.07	0.00	0.08	0.38	0.37	26.80	0.69	0.52	0.00	0.86	0.17	3.17	0.39	23.79	5.38	0.49	0.42
7	C1	0	R	4.03	0.13	0.00	0.12	0.48	0.52	31.07	0.79	0.55	0.00	0.87	0.15	3.20	0.40	22.72	4.65	0.46	0.34
8	C1	0	R	3.48	0.08	0.00	0.09	0.44	0.43	29.70	0.60	0.50	0.00	0.82	0.13	3.31	0.36	23.54	4.19	0.38	0.31
9	C1	3	P	0.78	0.02	0.00	0.06	0.16	0.16	13.93	0.41	0.27	0.00	0.71	0.13	3.26	0.23	26.36	5.82	0.49	0.43
10	C1	3	P	1.30	0.03	0.00	0.03	0.21	0.13	17.09	0.31	0.26	0.00	0.58	0.08	2.22	0.25	23.18	3.50	0.30	0.31
11	C1	3	P	1.37	0.02	0.00	0.03	0.24	0.10	19.26	0.35	0.27	0.00	0.63	0.10	1.95	0.21	23.95	3.63	0.29	0.31
12	C1	3	P	1.26	0.03	0.00	0.04	0.22	0.14	17.86	0.35	0.30	0.00	0.64	0.10	2.26	0.24	23.65	3.79	0.33	0.32
13	C1	3	P	0.60	0.02	0.00	0.04	0.13	0.15	12.14	0.42	0.27	0.00	0.65	0.14	3.71	0.21	24.09	6.72	0.61	0.51
14	C1	3	P	0.79	0.03	0.00	0.04	0.17	0.15	14.94	0.34	0.29	0.00	0.71	0.11	3.59	0.23	26.74	5.46	0.47	0.43
15	C1	3	P	0.93	0.00	0.00	0.04	0.20	0.08	17.68	0.31	0.23	0.00	0.76	0.08	2.04	0.16	29.72	3.93	0.36	0.29
16	C1	3	P	0.77	0.02	0.00	0.04	0.15	0.14	14.00	0.34	0.27	0.00	0.71	0.12	3.08	0.18	27.01	5.71	0.51	0.49
17	C1	3	P	0.73	0.02	0.00	0.05	0.15	0.13	13.71	0.31	0.25	0.00	0.64	0.11	2.70	0.18	25.98	4.93	0.40	0.42
18	C1	3	P	1.12	0.02	0.00	0.02	0.20	0.11	17.17	0.28	0.23	0.00	0.58	0.08	2.19	0.23	24.05	3.61	0.26	0.29

Appendix 4: An example of chemical profiles of the Fats of a gyne A) Newly emerged B) with 9 days and fed with poor food and C) with 9 days and fed with rich food.



Appendix 5: Example of the area (%) and retention time (min) of the first 18 fats of 18 gynes of Colony 1. Age: in days, P: poor food and R: rich food. All data available in supplementary file online: https://drive.google.com/drive/folders/1MaIFAD33uTOZtvc5UUQGaWtR3gKFn1hH

			Peaks																
			Fg1	Fg2	Fg3	Fg4	Fg5	Fg6	Fg7	Fg8	Fg9	Fg10	Fg11	Fg12	Fg13	Fg14	Fg15	Fg16	Fg17	Fg18
			Retention time (min)																
			5.22	5.71	6.58	6.65	6.77	6.89	6.90	7.00	7.06	7.23	7.31	7.33	7.48	7.72	7.77	7.82	7.86	7.91
Hornet	Age	Food	Area (%)																
1	0	P	0.00	0.00	0.00	0.00	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.86	0.00	0.00	0.00	1.43	2.84	3.41
2	0	P	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.08	0.00	0.61	0.00	0.00	0.00	2.00	2.37	3.34
3	0	R	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.07	0.00	0.50	0.00	0.00	0.00	1.65	2.10	2.55
4	0	R	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.59	0.00	0.00	0.00	1.45	2.28	2.92
5	3	P	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.62	0.00	0.00	0.00	0.00	5.22	2.78
6	3	P	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.41	0.00	0.00	0.00	0.90	4.00	3.08
7	3	P	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.31	0.00	0.00	0.00	0.39	2.66	2.32
8	3	P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.28	1.14
9	3	P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.43	0.00	0.00	0.00	0.58	3.52	2.37
10	3	P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.23	0.00	0.00	0.00	0.64	1.72	2.60
11	3	P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.76	1.53	2.23
12	3	P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.00	0.40	1.22	2.22
13	3	P	0.00	0.00	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.32	0.00	0.00	0.00	0.26	2.04	2.41
14	3	P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33	1.05	1.88
15	3	P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.49	0.48	1.70
16	3	P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.30	0.43	1.18
17	3	R	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.11	0.00	0.47	0.00	0.00	0.13	2.12	3.35	2.39
18	3	R	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.80	0.00	0.00	0.00	0.73	5.11	2.62

3. **Kapitulua:** *Vespa velutina* (Hymenoptera, Vespidae) gineen profil kutikularrak eta gantzak

Sarrera:

Komunikazio kimikoa, bizi-forma guztien artean zabalduriko komunikazio mota zaharrena da (Wilson 1970). Feromonak dira sentsore-kimikoen kanalen bitartez jasotzen diren seinale garrantzitsuenetarikoak (Wyatt 2013) eta intsektuetan, partikularki konplexuak eta ondo aztertutako molekulak dira (Howard eta Blomquist 2005). Seinale kimiko hauek lurrunkorrak direnean, distantzia handiko seinale bezala erabiltzen dira eta ez-lurrunkorrak direnean, distantzia txikiko komunikazioan parte hartzen dute (Gershman et al. 2014).

Gehien ikertutako feromona ez-lurrunkorren artean, hidrokarburo kutikularrak (CHCak) aurki daitezke (Blomquist eta Bagnères 2010). Hauek, kate-luzeko alkeno eta/edo alkano alifatiko eta metilo adarkatuen nahasketa konplexuak dira, intsektuen epikutikulan aurkitzen direnak (Blomquist eta Bagnères 2010). Hidrokarburo kutikularrei buruzko ikerketa gehienak deskribaturiko 130.000 espezie baino gehiago dituen Hymenoptera taldean ardaztu dira (Wilson 1971). Orden honen barruan bai ekonomikoki, bai ekologikoki garrantzitsuak diren espezieak daude, batez ere, erle sozial, liztor eta inurrien artean (Wilson 1971).

Nahiz eta, 1. Kapituluan azaldu den moduan, CHCen funtzio primarioa intsektuen lehortzea ekiditea izan, hauek ere komunikazio intra- eta interespezifikoan erabiltzen dira, hainbat intsektu espezieetan (Blomquist eta Bagnères 2010). Himenoptero sozialen arteko komunikazio intraespezifikoan, CHCak ez dira bakarrik habia bereko kideak ezagutzeko baliagarriak (Singer 1998), kide sexualak ezagutzeko

(Spiewok et al. 2006) eta baita estaltze portaerarako (Hora et al. 2008) ere erabiltzen dira. Hau dela eta, garrantzi handia dute distantzia txikiko eta ar-eme arteko kontaktu komunikazioan (Ferveur 2005).

Intsektuen hidrokarburoak oenozito deitzen diren zeluletan sortzen dira (Wicker-Thomas et al. 2009). Zelula hauek, intsektu helduen gantz-gorputzetan aurkitzen dira (Ferveur et al. 1997; Fan et al. 2003). Intsektu gehienek, *de novo* sintetizatzen dituzte CHCak, acyl-CoA gantz-azidoen elongazioaren bidez, kate luzeko gantz-azido bat sortuz eta hauen deskarboxilazioaren bitartez, hidrokarburoak ekoiztuz (Blailock et al. 1976; Blomquist 2010; Howard eta Blomquist 2005). Modu honetan, gantz-azidoen ezaugarri kualitatibo eta kuantitatiboek intsektuen CHCen profiletan eragina dute (Otte et al. 2015).

Lipidoak dira intsektuen gantz-gorputzen konposatu nagusia, lipido horien % 90a baino gehiago triglizeridoak direlarik (Bailey 1975; Canavoso et al. 2001). Triglizerido hauek, dietatik lortutako karbohidratoak, gantz-azidoak edo proteinak dituzte jatorri (Arrese eta Soulages 2010). Hainbat ikerketa daude non gantz-gorputzetan lipidoak sortzen direla erakusten den, intsektuen dietaren konposatu nagusiak diren karbohidratoak jatorriz hartuz, (Bailey 1975, Briegel 1990; Hines eta Smith 1963; Inagaki eta Yamashita 1986; Venkatesh eta Morrison 1980). Gantz-gorputzak glukosatik abiatuz glukogeno sintesi baino lipogenesi gaitasun handiagoa du. Hau dela eta, intsektuen gantz-gorputzetan lipido kantitatea handiagoa da glukogeno kantitatearekin alderatuta (Arrese eta Soulages 2010). Intsektuen garapen eta elikatze egoerek, gantz-gorputzen bidez sortutako gantz-azidoen

edukieran eragina dute (Bailey 1975; Ziegler 1997; Lorenz 2001; Pontes et al 2008).

Intsektuen CHC profiletan eragina izan dezaketen faktore ezberdinak daude. Lehena, izakien adina litzateke (Kuo et al 2012). Euli batzuetan (Mpuru et al. 2001), liztor parasitikoetan (Ruther et al. 2011) eta hainbat kakalardotan (Peschke 1985), izaki emergitu berriek CHCen fenotipo oso antzekoa aurkezten dute, baina aste bat pasa ondoren, hidrokarburo kutikular hauek sexuarekiko espezifikoak bilakatzen dira (Otte et al. 2018). *Polistes dominulus* Christ, 1791 liztor sozialaren kasuan, CHC proportzioak izakien adinarekin aldatzen dira. Kasu honetan, indibiduo gazteek helduek baino kutikula iragazkorragoak dituzte, ingurumeneko hidrokarburoak xurgatu ahal izateko (Lorenzi et al. 2004).

Bigarrena, dieta litzateke. Hainbat ikerketatan, dietak CHC fenotipoaren plastikotasunean duen eragina frogatu da. Adibidez, Lepidoptera espezie desberdinen beldarretan (Espelie eta Bernays 1989), matxinsaltoetan (Blomquist eta Jackson 1973) eta *Drosophila* Fallén, 1823 generoaren espezie desberdinetan; hala nola, *D. mojavensis* Patterson et al. 1940 (Etges et al 2009; Etges eta de Oliveira 2014), *D. melanogaster* Meigen, 1830 (Fedina et al. 2012) edo *D. serrata* Malloch, 1927. Azken honetan, CHC profilaren aldaketek, indibiduoen ugaltze-arrakastan eragina izan dezakete (Rundle et al. 2005).

Liztor sozialen espezie ugaritan, *Vespa velutina*-n gertatzen den moduan, hurrengo urteko erreginak (gineak) emergitu ondoren, zenbait egun pasatzen dituzte habian,

hau utzi baino lehen, larba eta langileen berrahoratzez elikatuz, trofalaxiaren bidez (Rome et al. 2015). Janari honen gehiena gantz-erreserbetan bihurtzen dira (Rome et al. 2015). Gantz-erreserba hauek, fruktosarekin batera, gineak neguan bizirauteko kriobabesle bezala (Strassmann et al. 1984; Toth et al. 2009) eta hegan egiteko, zein ugaltzeko fuel bezala (Hill eta Goldsworthy 1968; Walker et al. 1970) erabiltzen dute.

Espezie ezberdinetako arrek tamaina handiko emeekin estaltzea nahiago dute, hauek neguko diapausari bizirauteko probabilitate, kolonia berriak sortzeko gaitasun (Hunt et al. 2007, 2010; Cervo et al. 2008) eta ugalkortasun handiagoak dituztelako (Johnson eta Hubbell 1984; Harris eta Beggs 1995; Gage eta Barnard 1996; Ptacek eta Travis 1997; Gadagakar 2001; Lüpold et al. 2011). Oozitoen garapena, proteina eta gantz-erreserba ugari behar dituen prozesua da (Shukla et al. 2012). Gainera, eme ugalkorrek ugalkortasun hau modu aktibo batean erakustea oso probablea da, langile ez-ugalkorrek arrek erakartzeko seinalerik sortzen ez duten bitartean (Wen et al. 2017). Honek azaldu dezake zergatik arrek interes desberdina aurkezten duten bi eme kasten aurrean (Cappa et al. 2013).

Cappa et al.-ek (2018), *V. velutina*-ren kasuan, arrek gine pisutsuago eta handiagoekin antenazio (antenekin egindako ukipenak) gehiago burutzen zituztela ikusi zuten, hauek gantz-erreserba handiagoa zutelarik. Hau dela eta, lehentasun hau CHC profila dela eta izan zitekeela proposatu zuten (Cappa et al. 2018), beste intsektuetan suertatzen den moduan (Carlson et al. 1971; Schal et al. 1990).

Ikerketa honetan, hauek dira aztertu nahi izan diren alderdiak: 1) Ea adinak eta dietak liztortzarren profil kutikularretan eragina daukaten, 2) ea emergitu osteko gineen adinak eta dietak metatutako gantzen edukiera (kantitatea) eta kalitatea alda dezaketen eta 3) ea erlaziorik dagoen *Vespa velutina* gineen CHC profil eta gantzen artean. Gure hipotesiak hurrengoak dira:, bai adinak eta bai dietak, profil kutikularrean eta baita gantzetan eragina edukiko dutela, bazka aberatsa garrantzitsuena izanik. Gantz kantitate eta kalitate desberdineko gineen artean profil kutikularren desberdintasunak aurkitzea espero da.

Material eta metodoak:

Habien bilketa

2015eko eta 2017ko udazkenetan, *V. velutina*-ren 4 kolonia bildu ziren, bizirik. Lehena, C1, Tours-en (Frantzia) 2015/11/13an, beste bi Bizkaiko (Espainia) herri desberdinetan, C2, Urdulizen 2017/10/18an eta C3, Berangon 2017/11/10an. Azkena, C4, Amurrion (Araba, Espainia) 2017/11/15an.

Laginen bilketa

Behin laborategian, koloniak dietil eterra erabiliz anestesiatu ziren. Heldu guztiak kendu eta bakarrik gelaxka operkulatuak zituzten abaraskak utzi ziren. Hauek aireztatutako plastikozko kutxetan (62 x 35 x 42 cm) (1B eta 1C. Irudiak) sartu ziren. Egunero, kutxak ordu berdinean berraztertzen ziren eta eme emergitu berriak plastikozko kutxa txikiagoetara (23 x 12.5 x 13 cm) pasatzen ziren. Esperimentua 23 ±1 °C-ko tenperaturan eta 12 h argi:12 h iluntasuneko zikloak (Poidatz et al.

2017) zituen isolatutako gela batean burutu zen.

Kutxa txikiko liztortzarrek *ad libitum* elikatuak izan ziren, erdia azukrean aberatsa zen janari batekin (Rich: R) (10 ml eztia + 40 ml H_2O) eta beste erdia aldiz, azukrean pobrea zen janari batekin (Poor: P) (0.5 ml eztia + 49.5 ml H_2O). Erabilitako eztia, gizakiaren kontsumorako Eroski® Mil flores (100 g eztia: 330 kcal, 72 g azukre, 0.6 g proteina eta 0.02 g gatza) izan zen. Bi taldeetako liztortzarrak 0, 3, 6 eta 9 egunetan hil ziren. Hiltzeko, laginak -20 °C-tan izoztuak izan ziren. Kutxa guztietan habia zati bat sartu zen, liztortzarrek profil kutikularra sortzeko eredu bezala erabili ahal izateko (Singer eta Espelie, 1992; Layton eta Espelie, 1994; Lorenzi et al 2004). 4 koloniekin metodologia berdina erabili zen.

CHCen analisi kimikoa

CHC profilen analisia burutzeko erabilitako metodologia 2. Kapituluan **Material eta Metodo**etan (*Analisi kimikoak*) azaltzen den metodologia bera da.

Tamainaren estimazioa

Gineen tamaina estimatzeko mesoeskutuaren zabalera (MW) neurtu zen jadanik azaldutako metodologia jarraituz (ikusi 2. Kapituluko **Material eta Metodoak**: *Tamaina eta pisua*).

Gantzen kuantifikazioa

Gantzak kuantifikatzeko, liztortzarrak 48 orduz 80 °C-tan lehortuak izan ziren eta ondoren, prezisio handiko Sartorius (0.01 mg) balantza erabiliz pisatu ziren. Le-

hortutako liztortzar guztiak beirazko tutuetan sartu eta birrindu ziren. Gero, kloroformo:metanol (2:1) 2 ml gehitu zen gantzak disolbatzeko (Suryanaryanan et al. 2011). Minutu batez irabiatu ostean, laginak zentrifugatu (5 min) eta disolbatzailea kendu zen. Prozesu hau beste bi aldiz errepikatu zen 1.5 ml kloroformo:metanol (2:1) gehituz aldi bakoitzean. C1eko laginetatik kendutako disolbatzailea izozkailuan gorde zen -20 °C-tan beranduago analizatua izateko (1. Taula). Erauzitako liztortzarrak 24 orduz lehortuak izan ziren 80 °C-tan eta berriro pisatuak. Erauziak izan baino lehen eta erauziak izan osteko pisuen arteko desberdintasuna, erauzitako gantzen edukiera zela kontsideratu zen.

Gantzen analisi kimikoak

Erauzitako C1-eko 75 gineen gantzak (1. Taula) kimikoki analizatuak izan ziren. *Gantzen kuantifikazioa* atalean azaldutako metodologia jarraitu eta gero lortutako disolbatzaileen laginak nitrogenozko fluxu baten bidez (45 C°, 15 psi) (TurboVap®, Zynmark) lurrundu ziren. Laginak hozkailuan mantendu ziren (4 °C-tan), bakoitza 50 ppm-ko *n*-dotriacotane-d66 (barneko estandarra: IS) zuen 1ml hexanoan berrezekitu arte. Honen ostean, laginak 30 segundoz irabiatu, eta ondoren, 0.45 µm-ko filtroa (Whatman®) erabiliz iragazi ziren. Lagin bakoitzeko 1 µl, Masa-Aukeratzaile detektagailu (5973 Single Quadrupole, Agilent) bati akoplatutako Gas-Kromatografo sistema (6890N, Agilent) (GC-MS) batean injektatu zen. Analisirako HP-5MS % Phenyl Methyl Siloxane (30 m x 250 µm x 0.25 µm) kapilaritate zutabea erabili egin zen. Erabilitako labearen programa 50 ºC (2 min), 50 ° C-tik 250 °C-ra (40 °C/min), 250 °C-tik 320 °C-ra (20°C/min) eta 320 °C-tan 6

minutu mantendu zen. Injekzioa "*split*" moduan (1:200) egin eta helioa erabili zen gas eramaile moduan (1.3 ml/min). Lortutako data guztiak MSD ChemStation E.02. 02. 1431 software-arekin prozesatu ziren.

Analisi estatistikoak

Seinale kimikoen datuak aztertzeko, CHCen Osagai Nagusien Analisia (Principal Component Analysis: PCA) Adina, Bazka eta Kolonia (Age, Food eta Colony) aldagai osagarriekin burutu zen. Aldagai osagarrien dispertsio grafikoan, Gantz edukiera (Fat content) menpeko aldagaia ere irudikatu zen. Bi analisi hauek Canoco 5® programa erabiliz burutu ziren.

Modu berean, gantzen konposatu kimikoen PCA bat burutu zen, aldagai osagarriekin (Age eta Food), gantzen konposizio kimikoa aztertzeko. Kasu honetan ere, menpeko aldagaia Fat content (g) adierazi zen dispertsio grafikoan. Age eta Food aldagaiak azaltzen zuten aldakortasunaren % zein den estimatzeko Erredundantzien Aldakuntza-Banaketaren Analisia (Redundant Variation Partitioning Analysis) egin zen. Analisi hauek Canoco 5®-aren bidez egin ziren.

Efektu Misto Gaussiar Ereduak (Gaussian Mixed Effects Models, GMM) (Zuur et al. 2009; Madsen eta Thyregod 2010) erabili ziren Age, Food eta Colony aldagaiek gantz edukieran zer nolako eragina zeukaten ikusteko. Horretarako, R (2018) programako nlme paketeko (Pinheiro et al. 2014), lme() funtzioa erabili zen log-likelihood estimatzaile mugatuarekin (Pinheiro eta Bates 2000).

Age (aldagai numerikoa) eta Food (bi mailekin, R eta P) faktore finko bezala ge-

hitu ziren ereduan. Colony aldagaia (lau mailekin, C1, C2, C3 eta C4) aldiz, zorizko faktore bezala gehitua izan zen. Bukatzeko, ereduaren balioespena egin eta Fat content kantitatearen termino polinomialak (termino koadratikoak eta/edo kubikoak) eta aldakortasunerako egitura bereziak, behatutako heterozedastizitateari aurre egiteko beharrezkoak ote ziren, aztertu zen (Pinheiro eta Bates 2000) (1. eta 2. Apendizeak). "Weights" argudioak erabili ziren lme() eta VarExp() funtzioen barruan bariantzen ereduak Fat bariantzaren funtzio esponentzialarekin zehazteko (Pinheiro eta Bates 2000; Zuur et al. 2009). varExp() funtzioak, dispertsioaren handipena edo murrizpenaren eredua egitea ahalbidetzen du, Age-aren handipenarekin. Eredua aukeratzeko, Akaike informazio kriterioa (AIC) eta/edo log-likelihood ratioaren testa (Zuur et al. 2009) erabili ziren pausu bakoitzean.

Amaitzeko, Analisi Erredundantea (RDA) egin zen Canoco 5® erabiliz CHCak erantzun aldagai bezala eta gantzak (konposatu kimikoak) aldagai independente modura aztertzeko. Aldagai independente modura aukeratze-progresibo-aren arabera esanguratsuak ziren Fat konposatu kimikoak erabili ziren.

Emaitzak

Hidrokarburo kutikularrak (CHCak)

Lau kolonietako 265 gineak espazialki sakabanatuta daude profil kutikularraren arabera (menpeko aldagaiak) PCAren I. eta II. ardatzetan zehar (3A. Irudia). Grafiko hau, aldagai independenteekin batera, Age (0, 3, 6 eta 9 egun), Food (R eta P) eta Colony (C1, C2, C3 eta C4), interpretatu daiteke (3B. Irudia). Grafiko hauetan,

CHCen aldakortasunaren joera ikus daiteke.

Hasteko, laginek (liztortzarrek) aldakortasun argia aurkezten dute gineen adinaren arabera (3B. Irudia). Ordenazio grafikoaren eskuin aldean, bananduta ikus daitekeen taldea (3A. Irudia), gine emergitu berriei dagokien "0 egun" taldea da (3B. Irudia). Adin honek aurkezten du aldakortasun handiena. Talde honen ostean, "3 egun"eko laginak grafikoaren erdiko zonaldean kokatuak daude, hauek ere sakabanapen handiarekin. Hurrengoa, "6 egun"eko taldea da eta bukatzeko, "9 egun" adinekoa. Hauek, grafikoaren laugarren koadrantean kokatzen dira eta ondo bereiztutako talde bat osatzen dute, kimikoki oso homogeneoa.

Jarraitzeko, badago bariazio bat Food (R eta P) eta Colony (C1-C4) aldagaiei lotuta, baina txikiagoa da adinari dagokion aldakortasunarekin konparatuta. Hau argi ikusten da 3B. Irudian, bai Food, bai Colony aldagaiak PCAren erdialdetik hurbil daudelako. Colony aldagaiak Food aldagaiak baino eragin handiagoa dauka.

Adinari lotutako gantz-edukieraren handipen argi bat dago, baita bazka aberatsarekin ere, nahiz eta honen eragina txikiagoa izan. Colony aldagaiari dagokionez, C1eko gineak dira gantz kopuru gutxien erakusten dutenak (3B. Irudia).

Aldakortasunaren Zatiketa Erredundantearen Analisia burutu zen profil kutikularrean aldagai independenteek zer nolako eragina duten ezagutzeko (2. Taula eta 4. Irudia). Alde batetik, aldagai guztiak batera hartu ziren kontuan eta bestetik, aldagai bakoitza besteen eragina kontuan hartu gabe (2. Taula).

Hiru aldagaiek batera CHCen % 70.6ko aldakortasuna azaltzen dute. Age da alda-

kortasunaren ehuneko altuena (% 51.9) azaltzen duen aldagaia (2. Taula). Honako emaitza hau ere 4A. Irudian erakusten da, non profil kutikularretan Age aldagaiaren eragina bakarrik ikus daitekeen.

Gine guztiek hidrokarburo berdinak erakusten dituzte, nahiz eta konposatu hauen proportzioak berdinak ez izan liztortzar guztietan. 0 egun adineko gineetan, Cg2, Cg1, Cg6, Cg8 eta Cg5 dira CHC garrantzitsuenak (4A. Irudia). Hauek, hidrokarburo txikiak dira, karbono gutxi batzuez osatuak (ikusi atxikipen denborak 3. Apendizean eta fitxategi osagarrian). Hala ere, 9 egun adineko gineetan Cg35, Cg33, Cg36 eta Cg39 dira CHC nagusiak (4A. Irudia). Hauek, hidrokarburo handiagoak dira, hainbat karbonoz osatuak (ikusi atxikipen denborak 3. Apendizean eta fitxategi osagarrian). Badirudi ez daudela 3 egun eta 6 egun adineko taldeei modu espezifiko batean lotutako CHCak.

Horretaz gain, Food aldagaiak aldakortasun totalaren % 1.4a bakarrik azaltzen du (2. Taula). Hau bat dator 4B. Irudian ikusitakoarekin; hau da, R eta P bazkak ez daudela garrantzi handiko CHCei lotuta.

Colony aldagaiari dagokionez, CHCen profilen aldakortasun totalaren % 17.5a azaltzen du (2. Taula). Ez dago CHCrik kolonia baterako esklusiboak denik. Hala ere, 3B eta 4C. Irudietan ikus daiteke C1 kolonia beste koloniekin alderatuz kimikoki desberdinagoa dela. C2, C3 eta C4 aldiz, kimikoki antzekoagoak dira.

Profil kimikoen bi eredu erakusten dira 5. Irudian: A) gine emergitu berri batena (0 egun) eta B) azukrean aberatsa den bazka batekin elikatutako 9 eguneko gine

batena. Gine emergitu berriaren kasuan, 9 eguneko ginearen profilarekin konparatuta, Cg1 eta Cg7 hidrokarburoei dagozkien pikoak, proportzio handiago batean agertzen direla azpimarratu behar da. Piko hauek *n*-C21 eta *n*-C23 hidrokarburoei dagozkie hurrenez hurren. Hala ere, 9 eguneko ginearen kasuan, Cg28, Cg30, Cg31 eta Cg35 dira ugariagoak. CHC hauek, C27:1, *n*-C27, 5-MeC27 eta 3-MeC27 dira, hurrenez hurren (4. Taula).

Gantz-edukiera

Erregresio bat burutu zen liztortzarren tamaina eta gantz-edukieraren arteko erlazioa zein zen ikusteko. Datuei hobeto egokitzen zen erregresioa, polinomikoa zen. Honek 0.0112 balioko R^2 aurkeztu zuen. Gantz-edukiera handi zein txikiko liztortzar txikiak daudela beha daiteke. Gauza bera gertatzen da tamaina handiko indibiduoekin (6. Irudia). Beraz, gantz-edukiera (Fat) eta tamaina (MW) independenteak dira.

Age, Food eta Colony aldagaiak gantz-edukieran zer nolako eragina duten ikusteko eredu bat egin zen. 6. Irudian ikus daitezkeen emaitzetan oinarrituz, gantz-edukieraren datu gordinak erabiltzea, eta tamainarekin ez zuzentzea erabaki zen, gure esperimentuan ez baitzegoen inolako erlaziorik bi aldagai hauen artean. Datuekin bat zetorren eredua bigarren-graduko eredu polinomikoa da:

$$y\,(T=P) = (0.0133) - (0.0012 \times Age) + (0.0002 \times Age^2) + \varepsilon_{Colony} + \varepsilon_R$$

$$y\,(T=R) = (0.0133 + 0.0075) - (0.0012 \times Age) + (0.0002 \times Age^2) + \varepsilon_{Colony} + \varepsilon_R$$

Non:

$\varepsilon_{Colony} \sim N\ (0, \sigma_{Colony}^2 = 0.0026)$

$\varepsilon_R \sim N\ (0, \sigma_R^2 = 0.0037)$

Bai Age eta bai Food aldagaiak esangarriak dira ereduan (5. Taula). Izan ere, honen arabera, Age-k gantz-edukieraren aldakortasunaren % 8.98a azaltzen du eta Food-ek aldiz, % 40.2a. Bukatzeko, Colony aldagaiak aldakortasunaren % 17.01a azaltzen du.

Eredu polinomikoa jarraituz, alde batetik, azukre gutxiko bazkarekin elikatutako gineek (gorriak) lehenengo 3 egunetan gantz-edukieraren galera pairatzen dute. Baina momentu honetatik aurrera, gantz-edukiera konstante mantentzen da. Beste aldetik, azukrean aberatsa den bazkarekin elikatutako liztortzarrek (berdeak), 9 egunetan zehar gantz-edukieraren igoera aurkezten dute. Denbora joan ahala bi bazken arteko gantz-edukieraren desberdintasunak handiagoak egiten dira (7. Irudia). Datuek aldakortasun handia aurkezten dute, batez ere R bazkarekin elikatutako 6 eta 9 egun-eko taldeetan. P bazkarekin elikatutako talde guztietan aldagarritasuna oso antzekoa da (7. Irudia eta 6. Taula).

Gantzen analisi kimikoa

C1-eko gineen gantz-estraktuen analisi kimikoek erakusten dute ez dagoela hain erlazio argia hauen konposizio kimiko eta Age aldagaiaren artean (8. Irudia), CHCekin ez bezala (3. Irudia). Hala ere, CHCekin gertatzen den moduan, Age aldagaiak gantzen konposizio kimikoaren aldakortasun gehiago azaltzen du

(aldakortasun totalaren % 31.2) Food aldagaiak baino (aldakortasun totalaren % 12.4). Bi aldagaiek batera % 43.3a azaltzen dute (7. Taula).

Lehen aipatu den moduan, Age aldagaiak eragin gutxiago dauka gantzen konposizio kimikoan, CHCan duenarekin konparatuta; hala ere, 9A. Irudian 0, 3 eta 9 eguneko taldeekin lotuta dauden gantzak daudela ikus daiteke. 0 eta 3 eguneko gineek gantz-konposizio kimiko oso antzekoa erakusten dute, konposatu bereizgarrienak Fg26, Fg35, Fg33 eta Fg21 direlarik. 9 eguneko gineetan aldiz, Fg54 eta Fg1 dira garrantzitsuenak. 6 eguneko liztortzarrek ez dute gantz konposizio berezirik. Nahiz eta bazkak eragin gutxi izan, badaude hainbat gantz dieta desberdinei lotuta daudenak. Alde batetik, Fg12, Fg17 eta Fg5 gantzak dira garrantzitsuenak R bazka izan duten liztortzarretan (9B. Irudia). Beste aldetik, P bazka izan dutenetan, Fg36, Fg28 eta Fg31 dira garrantzitsuenak. (Ikus 4. Apendizean gineen gantzen profil kimikoen kromatogramak eta 5. Apendizean gantz bakoitzaren % Area eta atxikipen denbora).

Gantzak eta CHCak

CHCen (erantzun aldagaiak) eta gantz konposatu kimikoen (aldagai independenteak) RDAk, gantz batzuek (aukeratze progresiboen bidez aukeratutakoak) CHC batzuekin erlazio esanguratsua dutela (10. Irudia) erakusten du. Alde batetik, Fg33 eta Fg35 gantzak Cg7, Cg5, Cg43 eta Cg50 hidrokarburoekin erlazionatuta daude. Bi kasuetan, aldagai horiek gine gazteekiko espezifikoak dira (0 eta 3 egunekoak) (4A eta 9A. Irudiak). Beste aldetik, Fg50, Fg51 eta Fg55 gantzak CHCen talde

handi batekin daude lotuta hala nola, Cg33 edo Cg35 (10. Irudia). Hauek, gine zaharretan (6 eta 9 egunekoak) agertzen dira (4A eta 9A. Irudietan).

Gantzen bi talde erabili dira Aldakortasunaren Zatiketa egiteko. Bi talde hauek osatzeko, RDAn garrantzi esanguratsua eta CHCekin erlazio argia zeukaten gantzak erabili ziren. **1. Taldea:** Fg33 eta Fg35. Talde honek CHCen aldakortasun totalaren % 19.2a azaltzen du. **2. Taldea:** Fg50, Fg51 eta Fg55 % 31.5a azaltzen duena (8. Taula). Analisi honen arabera, bi taldeek batera C1eko gineen CHCen aldakortasunaren % 53.2a azaltzen dute.

Eztabaida

Gure emaitzei dagokienez, adina da hidrokarburo kutikularretan (CHCak) eragin handiena duen aldagaia. Lorenzi et al.-ek (2004) *Polistes dominulus* (Christ, 1791) espeziean ikusi zuten bezala, liztortzarrak helduak egiten diren heinean, hauen kutikulen profilak aldatuz doaz. *V. velutina*-ren kasuan, 0 eta 9 eguneko taldeak muturreko taldeak dira eta adin bakoitzeko espezifikoa diren CHCen proportzio desberdinak aurkezten dituzte. 3 eta 6 eguneko taldeak aldiz, tarteko egoeran daude eta ez daukate karakterizazio argirik. Berriz, Lorenzi et al. (2004)ek *P. dominulus*-ekin burututako ikerketan ikusi zuten bezala, liztortzar emergitu berriek pisu molekular txikiko CHCak aurkezten dituzte. Pisu molekular txikiko hidrokarburoak lirainagoak eta iragazkorragoak dira. Alabaina, liztortzarrak helduagoak egiten diren heinean, hidrokarburo lirain horien proportzioa gutxitu eta pisu molekular handiko hidrokarburo kutikularren (astunak) proportzioa handitzen da. Honek,

adinarekin, kutikularen iragazkortasunaren murrizketa eta hau dela eta, baita kanpotik konposatuak xurgatzeko gaitasunaren galera ere suposatzen du. Horregatik, 9 eguneko indibiduoek profil kutikular homogeneoagoa aurkezten dute, behin betiko profila lortu baitute (Lorenzi et al. 2004). Hau bat dator Crozier eta Dix-ek (1979) proposatutako Gestalt ereduarekin. Honetan, jadanik azaldu den moduan, indibiduoen arteko eta/edo indibiduo eta habia-materialen arteko interakzio fisiko pasiboen bidez, koloniaren kide guztien artean partekatutako "*gestalt usain*" deituriko seinale kimiko komuna sortzen da.

Dietaren kasuan (Food aldagaia), *V. velutina* gineen CHCetan eragin gutxi duela ikus daiteke, beste intsektu espezieetan gertatzen ez den bezala (Espelie eta Bernays 1989; Blomquist eta Jackson 1973; Etges et al. 2009, Etges eta de Oliveira 2014, Rundle et al. 2005, Fedina et al. 2012). Gure kasuan, bi arrazoi posible daude hau azaltzeko. Alde batetik, kutikularen konposatuak batez ere kutikularen iragazkortasunaren arabera aldatuko direlako; hau da, kanpo faktoreen arabera. Faktore hauen adibide bat, habiaren profil kimikoa izan daiteke. Izan ere, honetatik liztortzarrek kutikularen profila sortzeko hidrokarburoak xurgatzen dituzte (Lorenzi et al. 2004). Beste aldetik, gerta liteke eskainitako bazkaren kalitatea eta/edo kantitatea ez zela aproposa izan, profil kimikoan aldaketak sortzeko. Liztortzar guztiak *ad libitum* elikatuak izan ziren, konpentsazio elikadura kontuan hartu gabe; hau da, izaki baten gaitasuna bazka kontsumoa handitzeko eta modu horretan, kalitate txikiagoko bazka konpentsatzeko (Simpson eta Raubenheimer 2012). Modu horretan, kalitate oneko dieta batean kontsumituko ziren kaloria berdinak

lor daitezke (Rapkin et al. 2017). Gure kasuan, azukrean pobrea zen bazkarekin liztortzarrak luzaroan bizirik mantentzeko, *ad libitum* elikatzea zen aukera bakarra. Hala ere, eskainitako bi bazkak egokiak izan ziren gantz-edukieran aldaketak sortzeko.

Koloniaren aldakortasunari dagokionez, badago aldakortasun garrantzitsu bat liztortzarren artean. Habia bakoitzak bere profil kimikoa du (Gévar et al. 2017), langileek beraien van der Vecht organoa habiaren gainazalaren kontra igurtziz hidrokarburo geruza bat uzten dutenean sortzen dena (Cervo eta Turillazzi 1989; Gamboa 2004; Dapporto et al. 2007). Hidrokarburo hauen konposizioa, habia horretako liztortzarren profil kutikularrean agertzen diren hidrokarburoen konposizioaren oso antzekoa da (Dani et al. 1992, 2003). Crozier eta Dix-ek (1979) proposatutako Gestalt ereduaren arabera, emergitu berriko gineek koloniaren "usaina" eskuratuko dute, modu honetan koloniako beste indibiduoek kolonia-kide bezala errekonozituko dituzte eta ez dituzte erasoko (Lorenzi et al 2004; Cervo eta Turillazzi 1989; Gamboa 2004). Dena den, koloniaren eragina CHCetan, adinarena baino askoz txikiagoa dela aipatu beharra dago. Habia berdineko indibiduo guztiek usain komun bat edukitzeak, ugaltzeko kolonia berdineko liztortzar bat aukeratzeko saihespenean garrantzi handia izan dezake. Honela endogamia murriztuko litzateke.

Gantzen edukierari dagokionez, gure ikerketan ez zen inolako erlaziorik behatu liztortzarren tamaina eta gantz-edukieraren artean. Beraz, ikus daitekeen gantz-edukieraren aldakortasunak ez dauka zer ikusirik tamainarekin eta bai aldiz, adina (Lorenz 2001), bazka (dieta) (Bailey 1975; Ziegler 1997; Zhou et al 2004) eta/edo

kolonia aldagaiekin.

Bigarren mailako eredu polinomikoak, adinak, bazkak eta koloniak baino gantz-edukieraren aldakortasun gutxiago azaltzen duela erakusten du. Bazkaren kasuan, intsektuen dietan karbohidratoak konposatu garrantzitsuenetarikoak direla eta hauek intsektuen metabolismoaren bidez gantzetan bihurtzen direla esan beharra dago (Arrese eta Soulages 2010). *Vespa velutina*-n, bestelako Vespidae sozialetan bezala, gineek gantzak metatu behar dituzte neguari aurre egiteko eta hurrengo udaberrian kolonia berriak sortzeko (Rome et al.2015). Azukrean aberatsa den janariarekin elikatutako liztortzarretan, gantz-edukiera adinarekin gradualki handituko da. Gine emergitu berri guztiek antzeko gantz-edukiera aurkezten dute eta azukre gutxiko bazkarekin elikatuen kasuan, lehenengo hiru egunetan zehar galduko da. Gertakari hau liztortzar emergitu berriak larba zirenean metamorfosia burutzeko metatutako gantzen parte bat oraindik mantentzen dutelako azal daiteke (Mirth eta Riddiford 2007). Lehenengo hiru egunetan zehar, gantza galtzen dute dieta egoki bat ez edukitzeagatik, baina hemendik aurrera, gantz-edukiera konstante bat mantenduko dute gutxi gora behera. Honetaz aparte, azukrean aberatsa den bazkarekin elikatutakoen taldeetan datuen aldakortasuna handiagoa da. Hau, bazka aberatsarekin *ad libitum* elikatu zirelako eta indibiduo bakoitzak hartutako kantitatea kontrolatzeko gai izan ez ginelako azal daiteke. Beraz, batzuek beste batzuek baino gehiago jan zezaketen, gantz-edukieran islatuz. Bazka pobre batekin elikatutakoen kasuan, nahiz eta batzuek beste batzuek baino gehiago jan, janariak azukre gutxiago zuenez, gerta liteke desberdintasunak txikiagoak izatea.

Kutikularen profil kimikoan gertatzen zen moduan, gineetatik erauzitako gantzen konposizio kimikoak (kalitatea) erlazio handiagoa erakusten du adinarekin dietarekin baino. Hau azaldu daiteke, lehen aipatu den moduan, adinak eragina eduki dezakeelako ez bakarrik gantz-edukieran (kantitatea), baizik eta hauen kalitatean ere (Anand eta Lorenz 2008). Adinaren eragina ez da CHCetan bezain argia; hala ere, joera berdina ikusten da. Gine gazteen eta helduen gantzak berdinak dira baina proportzio desberdinetan. Oso interesgarria izango litzateke gantz hauek identifikatzea *Drosophila mellanogaster*-aren kasuan bezala (Wren eta Mitchel 1958), horrela, *Vespa velutina*-n gantz hauen proportzioen aldaketek zer nolako garrantzi biologikoa duten ulertu ahal izateko.

Aurretik komentatu den bezala, kanpo faktoreez aparte, CHCen profila ere barne faktore fisiologikoen menpe egon daiteke. CHCak eta gantzak elkarrekin aztertu zirenean, hainbaten arteko erlazioa beha zitekeen. Hala ere, ezin gara ziur egon baten presentziak bestearen presentzian eragin zuzenik eduki ote dezakeen, zeren biak adinaren eta dietaren menpekoak baitira. Arrazoi hori dela eta, bai CHCak bai gantzak modu paralelo batean alda daitezke, baina adinaren eta dietaren eraginarengatik izatea gerta daiteke. Gantzen kantitate eta/edo kalitateak CHCen profiletan nolako eragina duen benetan jakiteko, beste motatako esperimentuak egin beharko ziren. Agian, gantz-metaketak modu artifizial batean aldatzen, adinaren eragina saihesteko. Honetaz aparte, gineen CHC-ekin erlazio handiena duten gantzak hidrokarburo horien sintesian parte hartzen duten ikusteko, gantzen konposatu kimikoak identifikatzea oso interesgarria dela uste dugu.

Bai kutikularen profila, bai gantzen kantitate eta/edo kalitatearen erlazioa bata bestearen menpekoak diren edo beste aldagaien menpekoak diren ezin jakin arren, gineen kutikularen profilak ugaltzeko emea aukeratzeko orduan, arren lehentasunean eragina izan dezakeela ikusi da. Arrek gantz metaketa ugarienak dituzten emeak aukeratuko lituzkete beraien kutikularen arabera Cappa et al. (2018)ek proposatzen duten moduan. Izan ere, eme hauek neguari aurre egiteko (Hunt et al. 2007, 2010; Cervo et al. 2008) eta emankorragoak izateko (Johnson eta Hubbell 1984; Gage eta Barnard 1996; Ptacek eta Travis 1997; Lüpold et al. 2011) probabilitate handiagoa baitute. Hau frogatzeko hainbat esperimentu buru beharko litzateke izaki biziekin. Adibidez, arrei gantz-kantitate eta kalitate desberdineko gineak eskainiz, ar hauek ugaltzeko zein eme nahiago duten aztertzeko. Honen ostean, aukeratutako eme hauen CHC profilak analizatuz, hidrokarburo horiek arrak harrapatzeko tranpetan erabili ahalko lirateke.

Ondorioak:

Gineen profil kimikoek liztortzarren adinaren araberako sakabanatze argi bat erakusten dute. Bazka eta koloniaren eragina ez da hain garrantzitsua, nahiz eta bigarrena handiagoa izan. Ez dago gineen tamaina eta gantz-edukiaren arteko inolako erlaziorik gure datuetan; beraz, ondoriozta daiteke gantz-edukiaren desberdintasunak aldagai independenteak direla kausa ematen direla, bazka izanik gehien eragiten duena. Gantzen karakterizazio kimikoari dagokionez, berriz ere adina da garrantzi handiena duena. Hainbat CHCk eta gantzek erlazio arin bat erakusten dute. Hala ere, biak adinaren menpekoak dira batez ere, eta hori dela eta, ezin dugu on-

dorioztatu batak bestea aldatzen duenik. Edozein kasutan ere, posible ematen du arrek gineen profil kutikularra gantz-kantitate eta kalitate hobeko emeak identifikatzeko erabili dezaketela, eme hauek biziraupen eta emankortasun handiagoa dutelarik.

Chapter 4: Cuticular profiles and sperm production in males of *Vespa velutina* (Hymenoptera, Vespidae)

Abstract:

CHCs are essential components in insects' communication. They play an important role in short-distances and male-female communication. It is well-known that different factors such as age or diet could have influence in the cuticular chemical profile. However, few studies show the influence of the sperm production on pheromones. CHCs profile and sperm production were analysed in males of *Vespa velutina* with different ages and diets in order to see if the modification of the sperm production could change the chemical profile. Both CHCs profile and sperm production present a great dependence on the age of the individuals, more than on the diet provided. It is possible to distinguish young males from older ones according to their CHCs. We cannot conclude that the sperm production can modify the cuticular profile of *Vespa velutina*'s males because both are very age dependent. However, it seems possible that workers could use males CHCs profile to know if they are sexually mature being the oldest males the ones which present more sperm quantity.

Keywords:

CHCs, sperm production, *Vespa velutina* males

Introduction:

The cuticular hydrocarbons (CHCs) of social hymenopterans serve as protective device (Blomquist and Bagnères 2010) and they play an important role against the desiccation (Gibbs and Rajpurohit 2010). But not only that, they are also essential components in communication (Mitra and Gadagkar. 2014) both inter- and intra-specific (Howard and Blomquist 2005; Blomquist and Bagnères 2010), as well. In the intraspecific communication, they play an important role in short-distances and male-female contact communication (Ferveur, 2005), giving information about the age (Lorenzi et al. 2004; Kuo et al. 2012), nutritional quality taken (Espelie and Bernays 1989; Blomquist and Jackson 1973; Etges et al. 2009; Etges and Oliveira 2014; Fedina et al. 2012; Rundle et al. 2005), fertility rate (Liebig 2010) or relat-edness rate (Howard and Blomquist 2005) of each individual.

There are several studies that show the relationship between both, the age and the diet to the CHCs profiles in insects (Ferdina et al. 2012; Etges and Oliveira 2014). In the same way, the CHCs profiles of gynes of the yellow-legged hornet *Vespa velutina,* are influenced by the age and the diet as it was discussed before (see Chapter 3). In this species, as occurs in other Vespidae (Lorenzi et al. 2004) the newly emerged individuals have a different cuticular profile than the already ma-ture ones. Apart from the age, the diet that each gyne takes after the emergence is also affecting, although the diet effect on CHCs profile is lower (see Chapter 3).

Even though, there are few studies dealing with relationship between sperm and

pheromones production, Blaul and Ruther (2011) showed that in the parasitic wasp *Nasonia vitripennis* (Walker, 1836), the biosynthesis of male sex pheromone is directly linked with spermathogenesis.

In insects, the males germ cell development occurs in compartments called cysts which are found inside the testicular follicles (Baccetti and Bairati 1964). Inside each cyst, during the spermatogenesis, the spermatogonia go through mitotic divisions generating a constant number of cells. These, after undergoing two meiotic divisions, become spermatids (Lino-Neto et al. 2008). The males of the invasive yellow-legged hornet *Vespa velutina* present an average of 201 follicles, which involve a high investment in sperm production (Poidatz et al. 2017). Likewise, in other Vespidae, the spermatogenesis of this species is a synchronous event with only one wave of sperm production which begins at the pupa stage and ends in the adult stage, approximately 10 days after the emergence (Poidatz et al. 2017).

The sperm production is a costly process contrary to what it has been thought (Bunning et al. 2015) and it can depend on several variables as the age and diet. In the case of the age, there are some research showing a positive effect of the age in the sperm production (Mahmood and Reisen 1982; Ponlawat et al. 2007). However, the few studies performed to know the relationship between diet and sperm production in insects have not obtained clear conclusions (Bunning et al 2015). For example, Queensland fruitflies, *Bactrocera tryoni* (Froggatt, 1897), (Perez-Staples et al. 2008); *Drosophila melanogaster* Meigen, 1830 (McGraw et al. 2008); red flour beetles, *Tribolium castaneum* (Herbst, 1797), (Fedina and Lewis

2006); Indian-meal moth, *Plodia interpunctella* Hübner, 1813, (Gage and Cook 1994) and the cockroach *Nauphoeta cinerea* (Olivier, 1789) (Bunning et al. 2015) show a positive influence of the diet quality in sperm production. Meanwhile, ladybirds *Adalia bipunctata* (Linnaeus, 1758) (Perry and Rowe 2010) and Mediterranean fruit flies *Ceratitis capitata* (Wiedemann, 1824) (Blay and Yuval 1997) present opposite results.

In vespine wasps of temperate zones, new queens (gynes) and males are produced when the number of workers reaches a maximum peak (Matsuura and Yamane 1990). In the case of *V. velutina* the production of those reproductive individuals occurs in autumn (Monceau et al. 2014). Before this period, males can also be present, but they are non-fertile diploid ones (Darrouzet et al. 2015). The production of autumn males occurs a little before the emergence of gynes and generally continues even after it (Matsuura and Yamane 1990). After the emergence, the reproductive individuals remain inside the nest for 1-2 weeks (Matsuura and Yamane 1990), 8 days for males in *V. simillima* (Martin 1991) and 8-11 days for males in *V. affinis* (Martin 1993). During those days, they are fed by trophallaxis with substances regurgitated by workers and larvae (Matsuura and Yamane 1990). This phenomenon was also observed in *V. velutina* (Personal observation). During this resting period, *V. velutina* males could reach sexual maturation as it is expected to occur in other *Vespa* species (Poidatz et al. 2017).

After this period, both, gynes and males, leave the nest, and they never return back to it. Workers continue their labour, showing no interest in the departing individu-

als (Matsuura and Yamane 1990). In *V. simillima*, however, if some males do not fly away and stay walking in the nest surface, they are bitten by workers with the mandibles, forcing them to leave the nest (Matsuura unpublished). This phenomenon has also been observed in captivity-maintained *V. velutina* (Monceau et al. 2013). This behaviour may be a strategy to avoid the inbreeding (Tabadkani et al. 2012).

The hymenopterans are hoplodiplod with a single locus complementary sex-determination (sl-CSD) (Whiting 1943). It is well-known that inbreeding is especially harmful in species of this group because it leads to homozygosity at the sex determination locus, producing diploid males (Cook and Crozier 1995). That diploid male presence implies particularly high fitness costs to the colony since they do not make any work and are usually sterile. Even they were not sterile, they could produce diploid sperm and in consequence sterile triploid female progeny (Heimpel and de Boer 2008). As it is mentioned before, diploid males are observed in spring nests of *V. velutina*, suggesting that the species can be experiencing a genetic bottleneck (Darrouzet et al. 2015).

So, taking into account all previously explained aspects, a study with *V. velutina*'s males was performed to know, 1) if the age and the diet have influence in the CHCs profile configuration as it occurs in gynes of the same species, 2) if the age and the diet affect the sperm production and 3) if there is any relationship between the production of sperm and cuticular profile in males. We hypothesized that both, age and diet, will have influence in CHCs profiles and in sperm production, being

the rich sugar food the most important one. On the other hand, it is also expected to find differences in cuticular profiles between males with high and low sperm quantity.

Material and Methods:

Nest collection

In autumn, three mature colonies of *V. velutina* were captured in the province of Biscay (Spain). Colony 1 and 2 were collected in the locality of Galdakao on 25/10/2016 and on 03/11/2016 respectively. Colony 3 was collected in Urduliz on 22/10/2017.

Obtaining samples

Once the colonies were in the laboratory, they were anaesthetised using diethyl ether and all the adult hornets were removed. The sealed cells combs were placed in aerated big plastic boxes (62 x 35 x 42 cm) (Figure 1A). Every day at the same time, the boxes were checked and new emerged hornets were removed to be used in the experiment. For this study only males were used. The experiment was performed in an isolated room with 23 ±1 °C, 12 h light: 12 h dark (Poidatz et al. 2017).

Emerged males were placed in smaller plastic boxes (23 x 12.5 x 13 cm) (Figure 1B and 1C) and fed *ad libitum,* half of them with rich sugar food (R) (10 ml honey + 40 ml H_2O) and the other half with a poor sugar food (P) (0.5 ml honey + 49.5

ml H_2O). The honey was Eroski® Mil flores (100 g of honey: 330 kcal, 72 g sugar, 0.6 g protein and 0.02 g salt) for human food.

Figure 1: A) Sealed cells combs in aerated plastic boxes. B) and C) Small plastic boxes with males inside.

Colony	CHCs analysis	Sperm counting
C1	90	90
C2	90	90
C3	90	90
Total	270	270

Table 1: Number of males used of each colony for the different analyses performed.

Five hornets of each group were killed by freezing the same day they were born and other 10 of each group were killed by freezing at 3, 6, 9 and 12 days of age (N = 90 individuals) (Table 1). In each box a piece of nest was also introduced so as to be used by newly emerged hornets as hydrocarbon profile example which is specific for each nest (Singer and Espelie 1992; Layton and Espelie 1994; Butts et al. 1995). This procedure was performed in the three colonies (Table 1).

CHCs analysis

For the CHCs analysis, each hornet was introduced in a test tube (5 ml Ø 12 x 75 mm) with 1ml of pentane and n-eicosene (C20) (10^{-3} g/ml), internal standard (IS); shaken for 2 minutes and then extracted the supernatant liquid. The samples were stored in other vials at -20 °C until they were analysed. One µl of sample was injected into a Gas Chromatograph (GC) coupled to a Mass Spectrometer (MS) (Agilent Technologies 7890A GC System and Agilent Technologies 5975C inert XL MSD with Triple-Axis Detector). Analysis was carried out with an HP-5MS Phenyl Methyl (30 m x 250 µm x 0.25 µm) capillary column. The oven temperature program was 50 °C (1 min), from 50 °C to 200 °C (8 °C/min), from 200 °C to 315 (5 °C/min) and 315 °C (5 min). The injection was in "*splitless"* mode and helium was used as a carrier gas (1.5 ml/min). All data were processed with MSD ChemStation E.02.02.1431 software. The relative proportion of each peak was calculated as described in Bagnères et al. (1990).

Sperm counting

Males were dissected using stereomicroscope and dissecting forceps. The two seminal vesicles (Figure 2A), where the sperm is stored, were extracted and after, ground in a 1 ml Eppendorf®, each one with 500 ml of Ringer solution. Ten µl DAPI (1.5 µg/µl) were added to each sample. The spermatozoa of each seminal vesicle were counted using a Neubauer® chamber under a fluorescence microscope (Figure 2B and 2C). After that, the total spermatozoa were calculated adding

the spermatozoa of both seminal vesicle of each male.

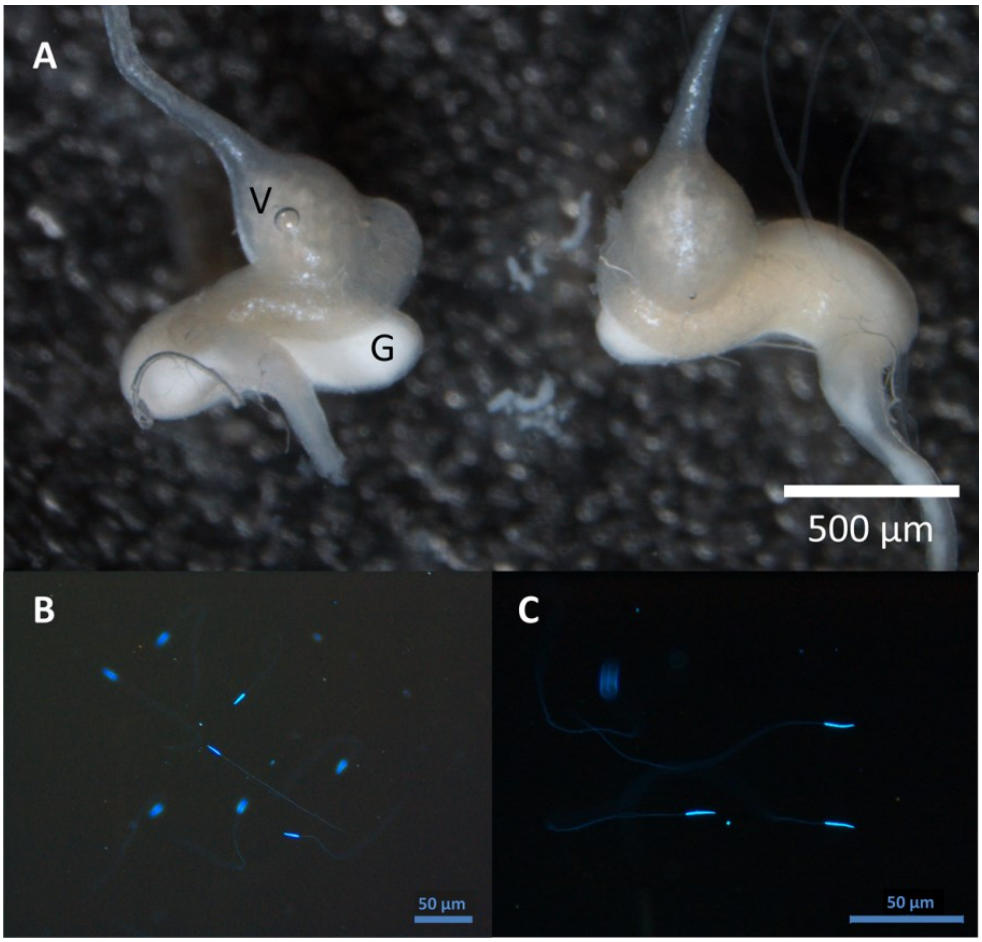


Figure 2: A) Seminal vesicles (V) and seminal glands (G) of *Vespa velutina*'s male. B) and C) *Vespa velutina*'s spermatozoa under fluorescence microscope.

Statistical Analysis

For the analysis of chemical signatures data (CHCs) a PCA with Age, Food and Colony supplementary variables, was performed. In the scatter plot, the sperm

quantity was also represented as a supplementary variable. In order to estimate the % of variation explained by each variable a Redundant Variation Partitioning Analysis was performed. Both of these multivariate analyses were conducted with Canoco 5®.

To test the effect of the Age, Food and Colony on the Sperm production of males, the Gaussian mixed effects models (Zuur et al. 2009; Madsen and Thyregod, 2010) were used. For this analysis the function lme() of package nlme (Pinheiro et al. 2014), with restricted maximum log-likelihood estimators (Pinheiro and Bates 2000) was used in R (2018).

Age (numeric variable) and Food (with two levels, R and P) were included in the model as fixed factors. Colony (with three levels, C1, C2 and C3) was fitted as random factor. To finish, a model validation was performed, and it tested whether the Sperm quantity polynomial terms (quadratic and/or cubic terms) and special variance structures were necessary to deal with observed heteroscedasticity (Pinheiro and Bates 2000) (Appendix 1 and 2). We used "weights" argument within lme() and the varExp() functions to specify variance models with an exponential function of the Sperm variance (Pinheiro and Bates 2000; Zuur et al. 2009). The function varExp() allows modelling both, increase or decrease of dispersion with the increase of the Age. For the model selection, Akaike's information criterion (AIC) and/or the log-likelihood ratio test (Zuur et al. 2009) was used in each step.

Results:

Cuticular hydrocarbons (CHCs)

The spatial distribution in axes I and II of the PCA of the 3 colonies' male hornets (samples) according to their CHCs (dependent variables) is presented in the Figure 3A. This PCA can be represented together with the supplementary independent variables (Figure 3B) where the main trends of CHCs variation can be interpreted. There is a clear great variation of the samples according to the age of males (Figure 3A) and, in a less extent, to food taken and colony to which they belong. It can be seen that in the right part of this ordination graph is located the group of hornets corresponding to newly emerged, "0 days", clearly showing a different cuticular profile (Figure 3B). This is the age class which presents the highest dispersion.

Regarding the rest of the hornets, it has to be highlighted that those corresponding to the ages "3" and "6 days" are more disperse than the "9" and "12 days" ones (Figure 3B). The last two groups (9 and 12 days) present a similar profile among them. The cuticular profile goes changing from the newly emerged ones to the oldest hornets. On the other hand, food and colony type also affects the cuticular profile but less than age because these variables are closer to the centre of the graph (Figure 3B).

The sperm arrow indicates that the number of spermatozoa strongly increases with the age and much less with the food or the colony. Comparing these later two,

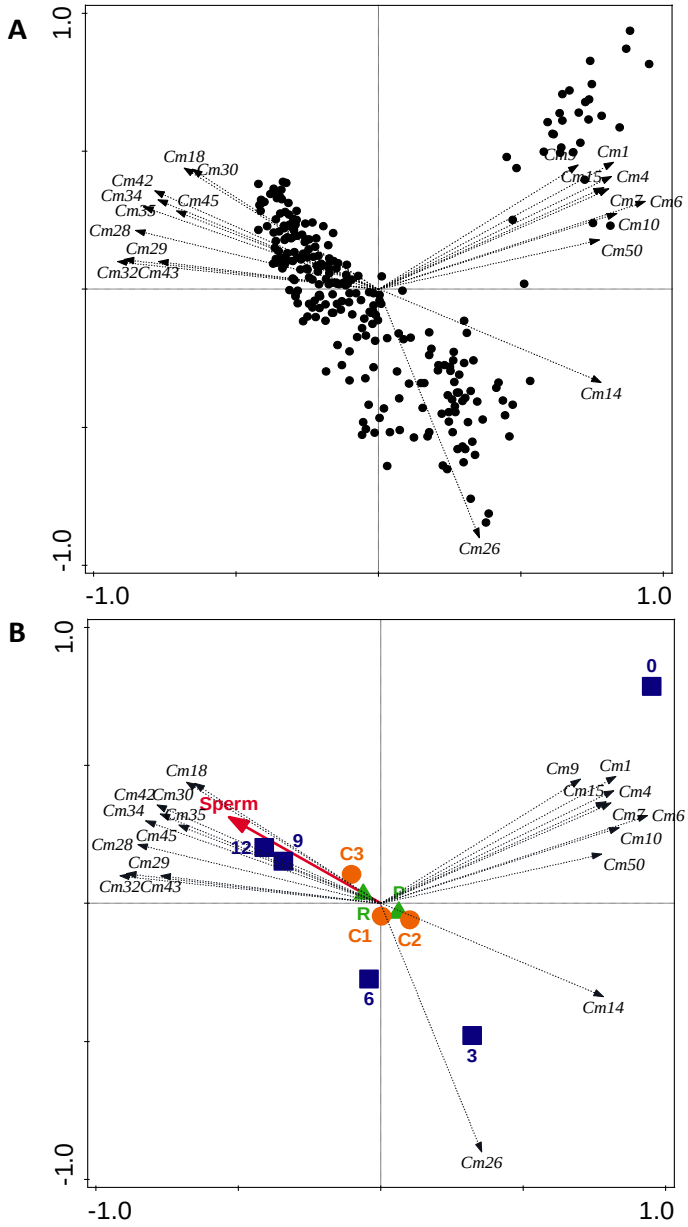


Figure 3: A) PCA of CHCs of 270 hornets (dots) of 3 colonies, with different ages and food. Arrows represent the 20 most important CHCs gradients. B) Scatter plot of the same PCA showing the supplementary variables (independents): Age (0, 3, 6, 9 and 12 days) (blue squares), Food (R: Rich and P: poor) (green triangles) and Colony (C1, C2, and C3) (orange circles). The red arrow represents the gradient of the Sperm quantity.

sperm amount is slightly higher in the well-fed hornets and in those belonging to Colony 3.

The variation partitioning analysis (Table 2 and Figure 4) shows the chemical profiles variability explained by each variable, Age, Food and Colony, removing the effect of the others and the variability explained by the 3 variables together, which reaches the 75.4 %.

Table 2: Variation Partitioning Results of RDA for the CHCs profile of all males of 3 colonies according to the independent variables: Age (0-12 days), Food (R: rich and P: poor) and Colony (C1-C3).

Fraction	Variation (adj)	% of Explained	% of All	DF	Mean Square	F	p-value
Age	0.7001	92.8	70.0	4	0.17399	191	0.001
Food	0.015125	2.0	1.5	1	0.01570	17.2	0.001
Colony	0.048247	6.4	4.8	2	0.02459	26.9	0.001
Total Explained	0.75443	100.0	75.4	7	0.10869	119	0.001
All Variation	1	--	100.0	269	--	--	--

Age, again, is the variable which explains bigger variability, 70 % of all variability (Table 2). It can be seen in Figure 4A that the CHCs present a clear spatial distribution according to the Age, as is shown in the PCA (Figure 3).

Even all male hornets have most of the CHCs (see below for exceptions), the proportions are different. It has to be highlighted that in males with 0 days, the five CHCs more important are Cm1, Cm4, Cm6, Cm9 and Cm7 (Figure 4A). Those CHCs are small hydrocarbons (composed of few carbons) (see retention times in Table 3, Appendix 3 and supplementary files). However, in the case of males with 12 days, Cm45, Cm34, Cm35, Cm18 and Cm42 are the five more important (Figure 4A). Those CHCs, except Cm18, are big hydrocarbons (composed of many carbons) (see retention times in Table 3, Appendix 3 and supplementary files). Males with 3 and 6 days have not CHCs that are specific for those ages (Figure 4A).

Contrary to variable Age, Food only explains the 1.5 % of all variability (Table 2). Figure 4B shows that there are a very few CHCs related to Food variable. Even though, in the case of R there are few more than in the case of P.

By its own, the variable Colony explains the 4.8 % of all variability (Table 2). This also is represented in Figure 4C, where there can be seen some CHCs that are more specific for each colony. Cm31 is very specific of the Colony 3. This CHC mainly appears in this colony (See Appendix 3 and supplementary files). In the case of Cm22, even it only appears in some males of the Colony 2 (See Appendix 3 and supplementary files) it has not so much importance.

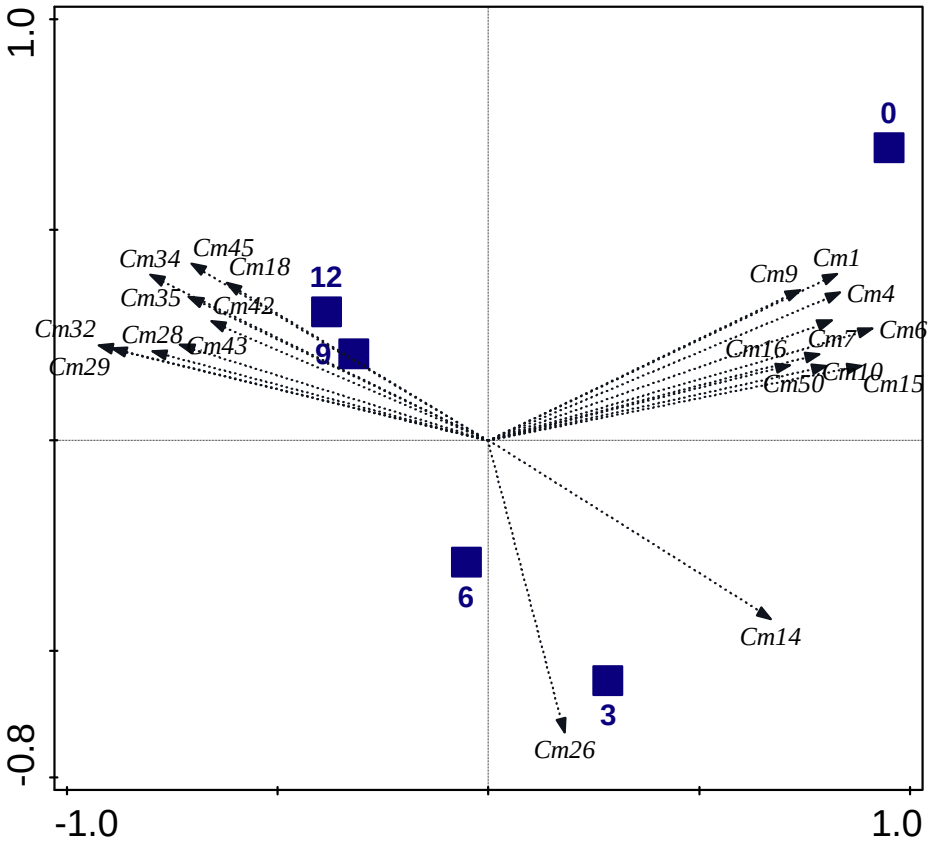


Figure 4A: PCA of the CHCs profiles according to the separated effect of Age (0, 3, 6, 9 and 12 days). Arrows represent the 20 CHCs more important gradients.

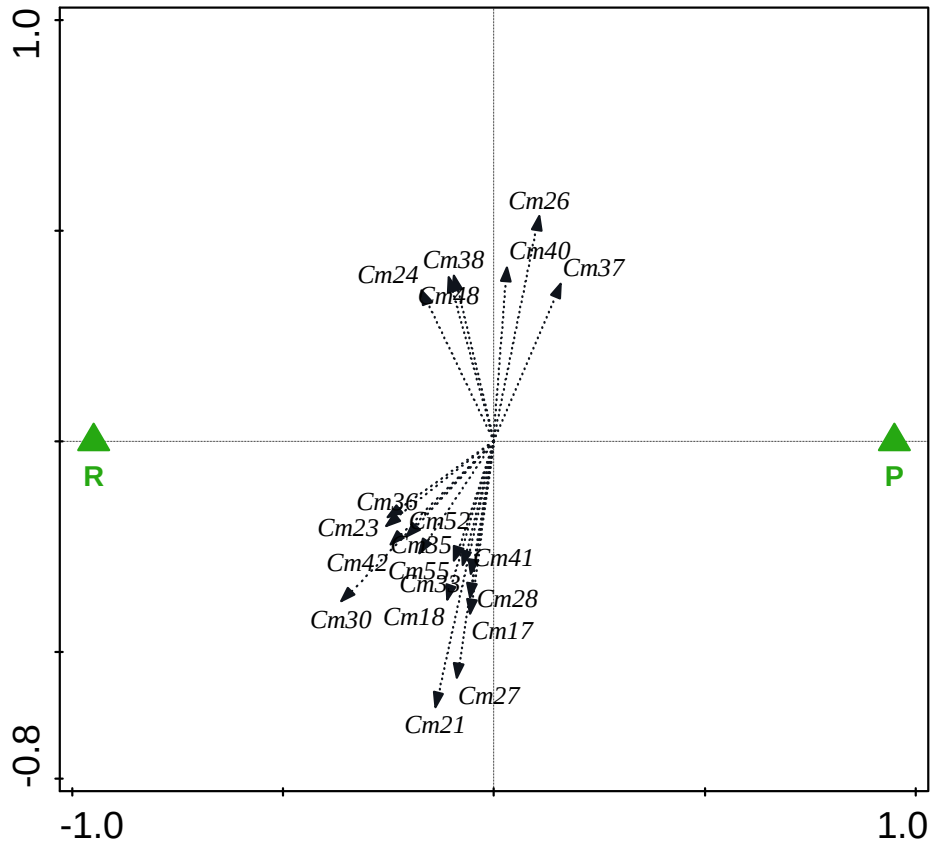


Figure 4B: PCA of the CHCs profiles according to the separated effect of Food (R: rich and P: poor). Arrows represent the 20 CHCs more important gradients.

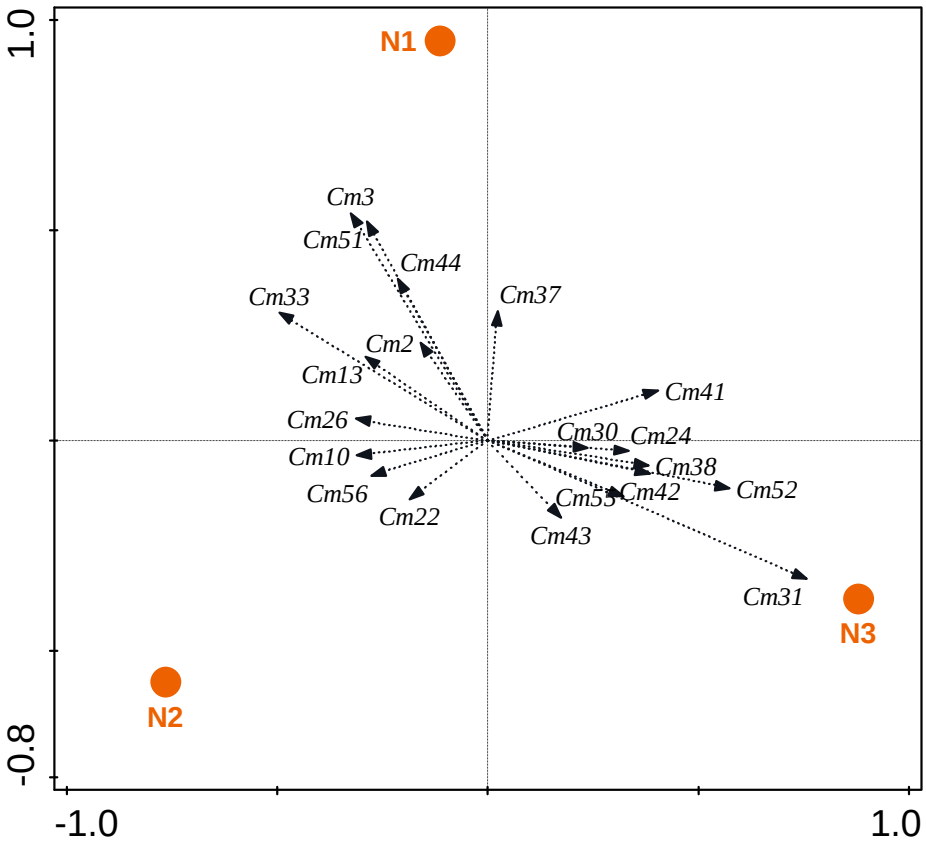


Figure 4C: PCA of the CHCs profiles according to the separated effect of Colony (C1, C2 and C3). Arrows represent the 20 CHCs more important gradients.

Table 3: Retention times of hydrocarbons of Kovacs pattern.

R. T. (min)	Hydrocarbon
22.16	C20 (IS)
28.01	C24
33.70	C28
38.82	C32
43.40	C36

In Figure 5 it can be observed the typical chemical profiles of newly emerged male and 12 days male which have been fed with rich food. It can be seen that in the newly emerged males, the peaks of Cm1 and Cm6 hydrocarbons are higher than in the 12 days males, what means that they appear in higher proportion. These hydrocarbons are *n*-C21 and *n*-C23 hydrocarbon respectively (Table 4). However, Cm24, Cm26, Cm27 and Cm30 hydrocarbons are more abundant in the case of 12 days males. Those CHCs are C27:1, *n*-C27, 5MeC27 and 3-MeC27 respectively (Table 4).

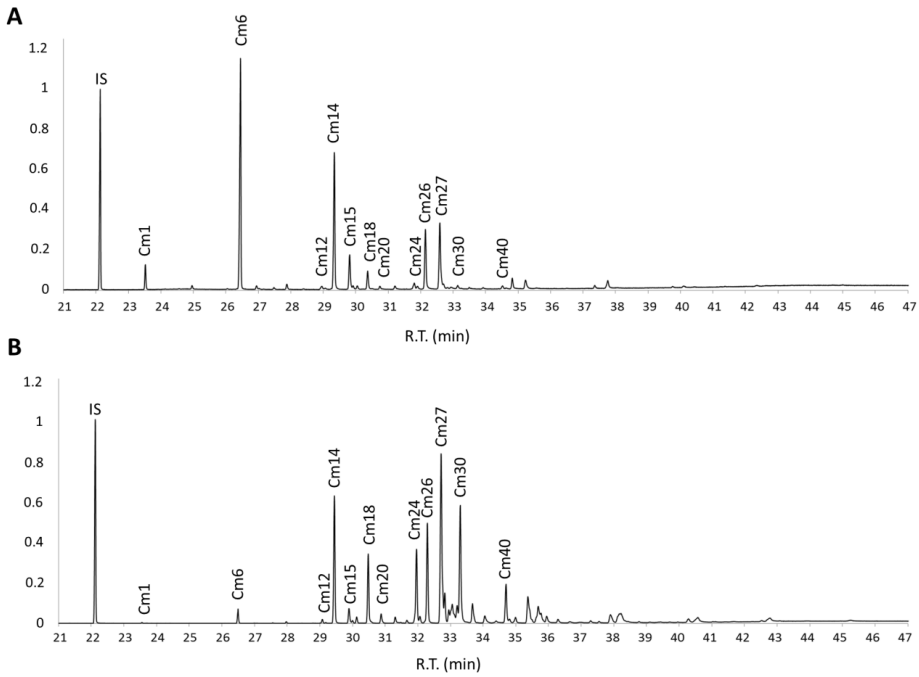


Figure 5: Typical CHCs profile of A) a newly emerged male (0 days) and, B) a male with 12 days fed with rich food. In both cases each peak was corrected with Internal Standard (IS). See Table 4 for the identification of the mentioned peaks.

Table 4: Exact identification of the mayor peaks of males' profiles (Figure 5) according to Gévar et al. (2017).

Peaks	R.T. (min)	Identification
IS	22.12	*n*-eicosen
Cm1	23.61	*n*-C21
Cm6	26.44	*n*-C23
Cm12	29.06	C25:1
Cm14	29.4	*n*-C25
Cm15	29.9	13-+11-MeC25
Cm18	30.47	3-MeC25
Cm20	30.85	*n*-C26
Cm24	31.9	C27:1
Cm26	32.2	*n*-C27
Cm27	32.7	5-MeC27
Cm30	33.047	3-MeC27
Cm40	34.93	C29:1

Sperm production

To analyse the relationship between sperm production and Age, Food and Colony variables, a modelling was performed. The model that better adjusts to the data is a second-grade polynomial model:

$$y\,(T=P) = (-58437.4) - (40994.3 \times Age) + (23284.2 \times Age^2) + \varepsilon_{Colony} + \varepsilon_R$$

$$y\,(T=R) = (-58437.4 + 128446.5) - (40994.3 \times Age) + (23284.2 \times Age^2) + \varepsilon_{Colony} + \varepsilon_R$$

Where:

$\varepsilon_{Colony} \sim N\,(0, \sigma_{Colony}^2 = 93297.91)$

$\varepsilon_R \sim N\,(0, \sigma_R^2 = 151259.91)$

Variables, Age and Food are significant in the model although Age is the most important (Table 5). According to this model, Age explains an 89.2 % of the variability in Sperm production while Food only explains an 8.1 % and Colony a poor 0.8 % of the total variability.

Table 5: ANOVA for the model.

	d.f	d.f (residual)	F-value	p-value
(Intercept)	1	264	2.48	0.1168
Age	1	264	102.66	<.0001
$I(Age^2)$	1	264	51.75	<.0001
Food	1	264	8.25	0.0044

In Figure 6 it can be observed that males fed with a poor food (red) show a small increase in the production of sperm over 12 days. The first spermatozoa appear at

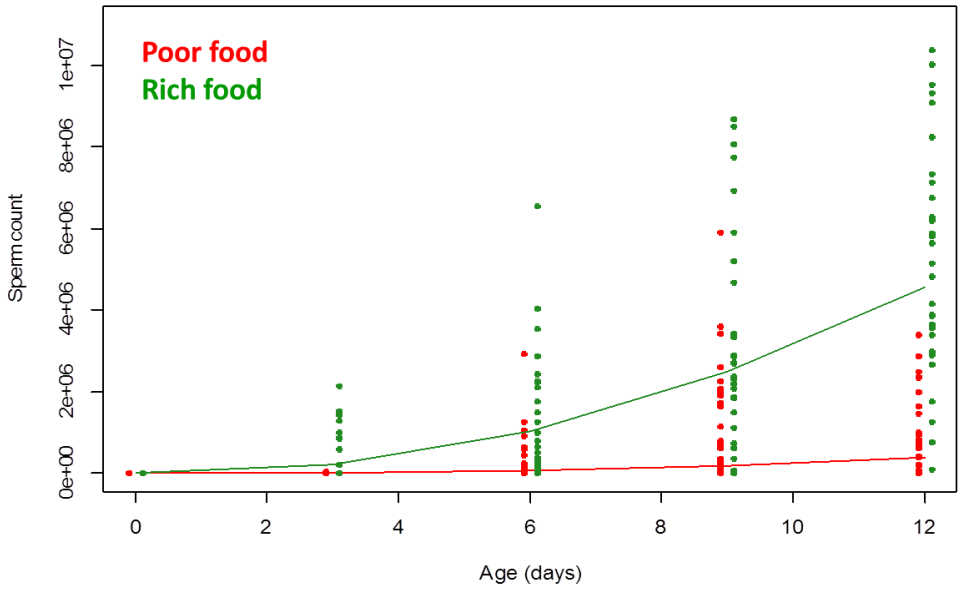


Figure 6: Effect of Food: Rich (green) and Poor (red), in the production of Sperm, over the variable Age (days).

the age of 6 days. However, males fed with rich food (green) have bigger increase thorough 12 days, appearing first spermatozoa at the age of 3 days. During the first 6 days the food type has not much effect but while the time goes on, this effect becomes bigger. There is a big variability among the data especially among males fed with rich food (R) (Figure 6 and Table 6).

Discussion:

According to the model of Gestalt, proposed by Crozier and Dix in 1979, and as was explained in Chapters 1 and 3, the newly emerged individuals will present a "blank slate" cuticular profile that, while the individuals get older, changes and

Table 6: Approximate 95 % confidence intervals for the model.

	lower	est.	upper
(Intercept)	-185356.43	-58437.39	68481.65
Age	-87179.11	-40994.34	5190.43
I(Age^2)	16911.33	23284.23	29657.13
Food R	40376.27	128446.50	216516.72

gets the odour of the nest which allow nestmates to identify each other as members of the same colony. This is the case of *Vespa velutina*'s males because, similarly as it was seen in gynes (See Chapter 3), the cuticular profiles of males present a clear variation according to the age of the hornets, as was also observed in *Polistes dominulus* wasp (Lorenzi et al. 2004). The newly emerged (0 days) and the oldest (12 days) males form two groups with clear differences between them in terms of CHCs profile. Inside the group of newly emerged individuals, the cuticular profiles are more heterogeneous comparing to the 12 days age group hornets which present much more similar CHCs. These results were also observed for gynes (see Chapter 3). This observation could be explained due to the fact that the youngest hornets have cuticular profiles with lower molecular weights hydrocarbons, making the cuticle more permeable and at the same time "neutral". In oldest individuals however, those low molecular weight compounds are replaced by higher molecular ones. For that reason, the cuticle permeability decreases as the definitive

profile is obtained (Lorenzi et al. 2004). That fact could also explains the similarity between the CHCs of the 9 and 12 days males because at this age the cuticular profile will change very little since 9 and 12 days males already present the nest odour. Those ages, 9-12 days, seem to correspond with the days that males of other species spend inside the nest feeding before leaving it, around 8 days in *V. simillima* (Martin 1991) and 8-11 days in *V. affinis* (Martin 1993).

In the case of the food, as it also occurs for gynes (see Chapter 3), it hardly influences males cuticular profile in contrast to what it was observed in other insects species (Espelie and Bernays 1989; Blomquist and Jackson 1973; Etges et al. 2009, Etges and de Oliveira 2014, Rundle et al. 2005; Fedina et al. 2012). Otte et al. (2014) proved that fatty acid composition of the diet, instead of the quantity of its components, produced differences in the CHCs profile of *Phaedon cochleariae* (Fabricius, 1792). So, it may be that the difference between the two types of diets in our experiment, different concentration of honey, was not enough to cause changes in CHCs.

In the case of males, the variable Colony has low influence in the cuticular profile variation compared to observed in gynes, but, in any case, greater than the one caused by the diet (food). This is explained since all the colonies used for males' experiments were got in the Basque Country (north Spain), so may be chemically more homogeneous, while among the colonies used for gynes' experiments, one of them was caught in the region of Tours (France) and the other 3 in the Basque Country. This explanation is supported by the observations made by Gévar et al.

(2017), who observed that the chemical profile of every nest in *V. velutina* is unique. This nest unique chemical profile seems to have high importance in configuring the cuticular chemical profile (Singer and Espelie 1992; Layton and Espelie 1994; Lorenzi et al. 2004) and thus serve in recognition of nestmates (Gamboa et al. 1996; Espelie et al. 1990; Lorenzi et al. 2004) as it was previously explained.

As it occurs with gynes' size and fat quantity (see Chapter 3), the size of autumn males has not influence in the number of spermatozoa produced (Poidatz et al. 2017). For that reason, the observed variations in the spermatozoa production are mainly due to the variable Age, followed by Food and lastly by Colony. As in our case, there are several studies where the spermatozoa number increases with the males' age (Mahmood and Reisen 1982; Ponlawat et al. 2007). According to Poidatz et al. (2017) *V. velutina* males, need 10 days to produce spermatozoa. During this time, males would stay inside the nest feeding as it occurs in other hornets' species (Martin 1991; Martin 1993). Nevertheless, under our experimentation conditions, the production of spermatozoa is gradual from the 3rd day, in some males fed with rich sugar food, or from the 6th day, in some males fed with a poor sugar food. This is also observed in the stingless bee *Melipona beecheii* Illiger, 1806; in which better fed larvae, it produced sperm earlier than the not well-fed ones (Pecht -May et al. 2012).

In other insect species, the diet has positive influence in sperm production (Gage and Cook 1994; Fedina and Lewis 2006; Perez-Staples et al. 2008; McGraw et al.

2008; Bunning et al. 2015). Nevertheless, our results are contrary; showing that diet in *V. velutina* has a very small effect on spermatozoa production. This could happen because the food provided by workers during the larvae phase is very important in the sperm production (Pech-May et al. 2012), may be even more than the food intake after the emergence. In the same way, this could explain the effect of the colony; even if it is small, because there may be different resources in the surrounding area available for rearing the larvae. In any case, there is an interaction between the age and the diet; since, the difference in spermatozoa between the two diet groups is much higher in hornets of the latest ages than in early ones.

Again, as it occurs with gynes' fats, for a given age, males fed with rich sugar food present a higher spermatozoa amount and more variability in data compared to those fed with a poor sugar food. It can be said that the food ingested by the insects cannot be controlled by the observer and, even all hornets have food available *ad libitum*, the eaten quantity could be different. This could explain the high differences found in intra group sperm production.

Taking into account our results, we cannot ensure that cuticular profile is directly affected by the sperm quantity, as both are strongly age dependent. However, it can be hypothesized that the chemical profile could be used by workers to know the age of males and to know indirectly if they are sexually mature or not. In this way they could have the possibility to expel them from the nest (Monceau et al. 2013). This fact would prevent the possibility of mating inside the nest and so, endogamy (Tabadkani et al. 2012). More experiments are necessary to know

whether sperm quantity controls the chemical profile in males, comparing males CHCs with spermatogenesis stopped to non-stopped ones (i.e. via hormones or high temperature) and testing, with hornets *in vivo,* if those males induce aggressiveness in workers. The question remains open.

Conclusions:

It is evident that in the case of males there is a clear distribution of the cuticular profile according to the age of the hornets. However, the diet provided and the colony to which they belong, have less influence in this profile, having the colony more influence than the diet. In the case of the sperm production, it is again the age of the individuals which has greater influence, followed by the diet and by the colony. There is an interaction between the age and the diet on the sperm production, producing more sperm the oldest males fed with rich food. According to our results, we hypothesized that the cuticular profile could be used by workers to know when males are sexually mature. Nevertheless, we could not be sure if it is the sperm production directly which influence the cuticular profile since both are age dependent.

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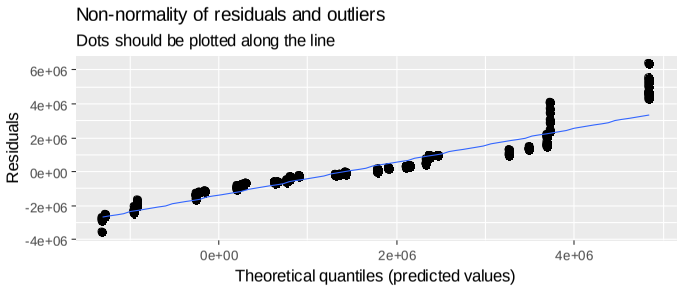
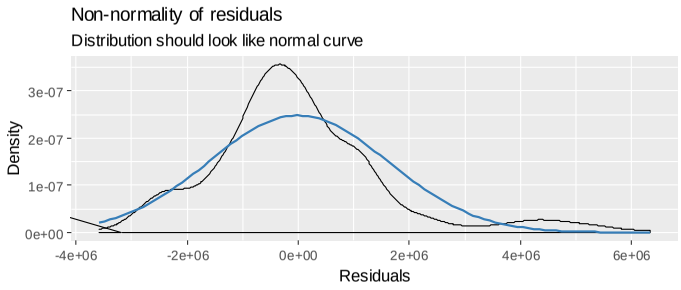
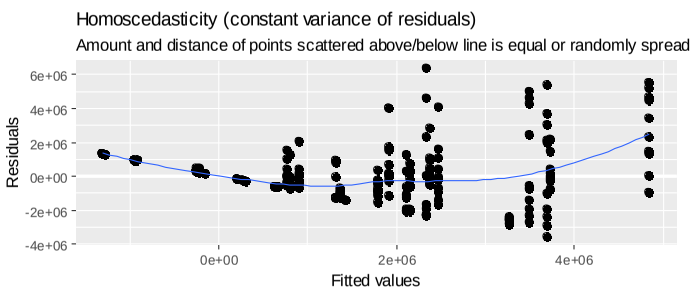
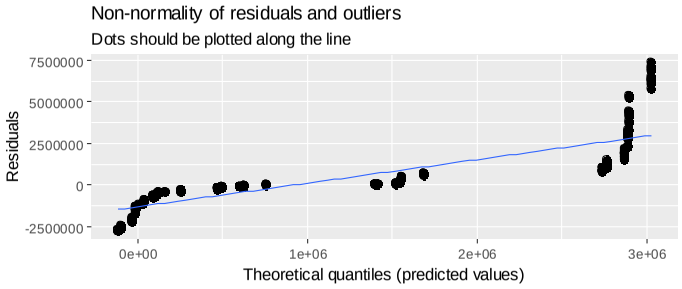
Appendix 1: A) Graph of the residuals to test the normality, B) Distribution of the residuals to test the normality and C) Graph of the residuals to test the homocedasticity, of the data before correcting them with the varExp() function.

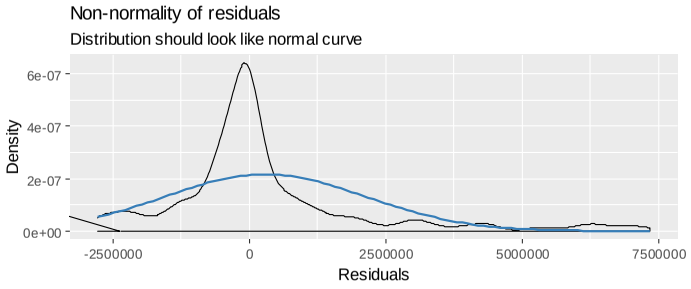


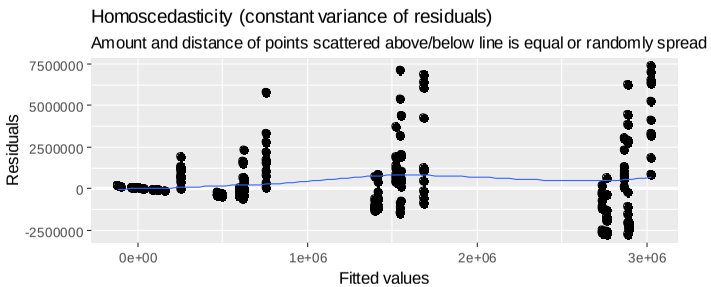




Appendix 2: A) Graph of the residuals to test the normality, B) Distribution of the residuals to test the normality and C) Graph of the residuals to test the homocedasticity, of the data after correcting them with the varExp() function.







Appendix 3: Example of the area (%) and retention time (min) of the first 18 CHCs of 18 males of Colony 1. Age: in days, P: poor food and R: rich food. All data available in supplementary file online: https://drive.google.com/drive/folders/1MaIFAD33uTOZtvc5UUQGaWtR3gKFn1hH

				Peaks																
				Cm1	Cm2	Cm3	Cm4	Cm5	Cm6	Cm7	Cm8	Cm9	Cm10	Cm11	Cm12	Cm13	Cm14	Cm15	Cm16	Cm17	Cm18
				Retention time (min)																
				23.61	24.5	24.64	25.05	26.1	26.44	27.04	27.13	27.5	27.9	28.49	29.06	29.18	29.4	29.9	30.03	30.15	30.47
Hornet	Colony	Age	Food	Area (%)																
1	C1	0	P	2.27	0.00	0.00	0.33	0.14	24.00	0.39	0.09	0.16	0.66	0.00	0.83	0.25	19.42	6.86	0.53	0.47	2.74
2	C1	0	P	2.54	0.00	0.00	0.40	0.20	23.47	0.43	0.00	0.21	0.63	0.00	1.01	0.27	17.63	6.84	0.61	0.39	2.65
3	C1	0	P	3.26	0.00	0.19	0.56	0.22	27.43	0.42	0.10	0.20	0.77	0.14	1.20	0.38	18.14	6.46	0.43	0.42	2.38
4	C1	0	P	1.77	0.00	0.12	0.34	0.17	18.21	0.31	0.00	0.18	0.60	0.00	0.86	0.27	17.97	5.81	0.52	0.39	2.75
5	C1	0	P	1.91	0.00	0.11	0.38	0.22	15.96	0.42	0.10	0.15	0.63	0.09	0.92	0.25	17.87	6.22	0.41	0.37	2.24
6	C1	0	R	2.72	0.00	0.00	0.40	0.24	25.64	0.29	0.00	0.18	0.67	0.07	1.10	0.32	20.29	5.45	0.35	0.32	2.18
7	C1	0	R	2.42	0.00	0.00	0.40	0.22	23.51	0.27	0.00	0.16	0.66	0.00	0.88	0.30	18.95	5.45	0.40	0.31	2.30
8	C1	0	R	1.76	0.00	0.18	0.31	0.21	17.04	0.38	0.00	0.16	0.62	0.00	0.84	0.33	18.75	7.73	0.53	0.41	2.38
9	C1	0	R	2.36	0.00	0.06	0.36	0.11	22.05	0.42	0.09	0.27	0.72	0.10	0.86	0.29	18.45	5.92	0.56	0.45	2.86
10	C1	0	R	2.30	0.00	0.08	0.35	0.20	20.69	0.38	0.10	0.20	0.76	0.07	0.99	0.29	18.22	6.00	0.48	0.36	2.86
11	C1	3	P	0.77	0.69	1.40	0.15	0.09	7.67	0.14	0.00	0.06	0.32	0.00	0.58	0.16	19.36	2.58	0.18	0.20	1.70
12	C1	3	P	0.67	0.45	0.96	0.33	0.08	9.08	0.18	0.00	0.09	0.34	0.00	0.97	0.17	19.50	3.45	0.23	0.22	1.85
13	C1	3	P	0.38	0.27	0.45	0.10	0.00	7.03	0.08	0.00	0.03	0.29	0.00	0.44	0.12	20.72	2.32	0.20	0.21	1.65
14	C1	3	P	0.42	0.58	0.91	0.10	0.00	8.01	0.11	0.00	0.06	0.34	0.00	0.54	0.15	19.83	3.10	0.22	0.22	1.97
15	C1	3	P	0.37	0.56	0.68	0.08	0.07	7.50	0.13	0.00	0.10	0.34	0.00	0.63	0.14	21.94	3.16	0.25	0.28	2.14
16	C1	3	P	0.33	0.22	0.54	0.15	0.00	6.80	0.13	0.00	0.09	0.32	0.00	0.43	0.08	20.89	3.37	0.26	0.26	2.26
17	C1	3	P	0.36	0.28	0.54	0.11	0.00	7.62	0.11	0.00	0.07	0.34	0.00	0.38	0.12	21.40	3.41	0.24	0.28	2.30
18	C1	3	P	0.48	1.26	1.35	0.22	0.08	9.04	0.14	0.00	0.07	0.37	0.00	0.71	0.14	19.99	3.07	0.25	0.21	1.91

4. Kapitulua: *Vespa velutina* (Hymenoptera, Vespidae) arren profil kutikularrak eta esperma-ekoizpena

Sarrera:

Himenoptero sozialetan, hidrokarburo kutikularrak (CHCak) babes tresna bat dira (Blomquist eta Bagnères 2010) eta lehortzearen aurrean eginkizun garrantzitsu bat betetzen dute (Gibbs eta Rajpurohit 2010). Baina ez hori bakarrik, konposatu ezin-bestekoak ere dira komunikazioan (Mitra eta Gadagkar 2014), bai inter- zein intra-espezifikoan (Howard eta Blomquist 2005; Blomquist eta Bagnères 2010). Komunikazio intraespezifikoan distantzia txikiko eta ar-eme kontaktuen arteko komunikazioan garrantzi handia dute, indibiduo bakoitzaren (Ferveur, 2005) adinari (Lorenzi et al. 2004; Kuo et al. 2012), hartutako bazkaren kalitateari (Espelie eta Bernays 1989; Blomquist eta Jackson 1973; Etges et al. 2009; Etges eta de Oliveira 2014; Fedina et al. 2012; Rundle et al. 2005), ugalkortasun-tasari (Liebig 2010) edo kidetasun motari buruzko informazioa emanez (Howard eta Blomquist 2005).

Hainbat ikerketa daude, non adina eta bazka (dieta) intsektuen CHC profilekin erlazionatzen diren (Ferdina et al. 2012; Etges eta Oliveira 2014). *Vespa velutina* liztortzarraren kasuan ere, lehen eztabaidatu den moduan (ikusi 3. Kapitulua), adinak eta bazkak CHC profilean eragina edukiko dute. Espezie honetan, beste Vespidae batzuetan gertatzen den bezala (Lorenzi et al. 2004) emergitu berri diren indibiduoen profil kutikularrak, helduak direnen profiletatik desberdinak dira. Adinaz aparte, gine bakoitzak emergitu ostean hartzen duen bazkak ere eragina edukiko du, nahiz eta honen influentzia txikiagoa da (ikus 3. Kapitulua).

Honetaz aparte, bestelako ikerketak daude, non esperma eta feromona produkzioaren arteko erlazioa ikasten den. Blaul eta Ruther-ek (2011) *Nasonia vitripennis* (Walker, 1836) liztor parasitikoan, arraren sexu-feromonaren biosintesia eta espermatogenesia zuzenean lotuta daudela erakutsi zuten.

Intsektuetan, testikulu folikuluetan kokatuta dauden konpartimentuetan, kiste deiturikoak, ar-zelula germinalak garatzen dira (Baccetti eta Bairati 1964). Espermatogenesian, kiste hauen barruan, espermatogoniak zelula kopuru konstante bat sortzen du zatitze mitotikoen bidez. Zelula hauek, bi meiosi pairatu ostean, espermatidetan bihurtzen dira (Lino-Neto et al. 2008). *Vespa velutina*-ren arrek 201 folikulu dituzte batez beste eta honek esperma-produkzioan inbertsio handia suposatzen du (Poidatz et al. 2017). Beste Vespidae espezieko liztorretan bezala, espermatogenesia gertaera sinkronikoa da, esperma-produkzio olatu bakarrean eman eta pupa estadioan hazi eta heldu estadioan bukatzen dena, emergitu eta 10 egun beranduago gutxi gora behera (Poidatz et al. 2017).

Urteetan zehar pentsatu izan denaren kontra, esperma-produkzioa prozesu garestia da (BunningBunning et al 2015) eta aldagai desberdinen menpe dago; hala nola, adina eta bazka. Adinaren kasuan, esperma-produkzioan efektu positiboa erakusten duten hainbat ikerketa daude (Mahmood eta Reisen 1982; Ponlawat eta Harrington 2007). Hala ere, bazkaren kasuan egin diren ikerketetan ez dira ondorio argiak lortu (Bunning et al 2015). Adibidez, *Bactrocera tryoni* (Froggatt, 1897) Queensland-eko frutaren eulia (Perez-Staples et al 2008), *Drosophila melanogaster* Meigen, 1830 eulia (McGraw et al 2008), *Tribolium castaneum* (Herbst, 1797)

irinako kakalardo gorria (Fedina eta Lewis 2006), *Plodia interpunctella* Hübner, 1813 Indiako-irinaren sitsa (Gage eta Cook 1994) eta *Nauphoeta cinerea* (Olivier, 1789) labezomorroaren (Bunning et al 2015) kasuetan, bazka kalitatearen eragin positiboa behatu zen esperma-produkzioan. Bitartean, *Adalia bipunctata* (Linnaeus, 1758) marigorringoek (Perry eta Rowe 2010) eta *Ceratitis capitata* (Wiedemann, 1824) Mediterraneoko fruta-euliek (Blay eta Yuval 1997), kontrako emaitzak aurkezten dituzte.

Zona epeleko bespino liztorretan, erregina berriak (gineak) eta arrak, langileen kopuru maximo bat lortzen denean produzitzen dira (Matsuura eta Yamane 1990). *Vespa velutina*-ren kasuan, indibiduo ugalkorren produkzioa udazkenean ematen da (Monceau et al. 2014). Momentu hau baino lehen arrak ere aurkitu daitezke, baina ar diploide ez-ugalkorrak izango dira (Darrouzet et al. 2015). Udazkeneko arren ekoizpena gineen agerpena baino pixka bat lehenago ematen da eta normalean gine guztiak emergitu arte mantentzen da (Matsuura eta Yamane 1990). Emergitu ostean, indibiduoak 1-2 aste bitartean egoten dira habia barruan (Matsuura eta Yamane 1990), 8 egun *V. simillima* arren kasuan (Martin 1991), eta 8-11 egun *V. affinis* arrentzako (Martin 1993). Denbora honetan zehar, larbak eta langileak berahoratutako substantziekin elikatuak dira trofalaxiaren bidez (Matsuura eta Yamane 1990). Fenomeno hau *V. velutina*-ren kasuan ere ikusi izan da (behaketa pertsonala). Atseden denbora honen bitartean, beste *Vespa* espezieetan gertatzen den moduan, arrak sexualki helduko direla uste da (Poidatz et al. 2017).

Denboraldi hau eta gero, bai gineek, bai arrek habia utzi eta ez dira inoiz bueltatuko. Langileek beraien eginkizunekin jarraituko dute, habia uzten duten izakiei kasurik egin gabe (Matsuura eta Yamane 1990). Hala ere, *V. simillima*-n, arren bat habiatik ez badoa hegan egiten eta honen gainazalean ibiltzen gelditzen bada, langileek eraso egingo diote eta koskatzen saiatuko dira barailak erabiliz, habiatik alde egitera behartuz (Matsuura publikatu gabea). Honelako jarrera ere ikusi izan da laborategian mantendutako liztortzar asiarrean (Monceau et al. 2013). Jokaera hau endogamia saihesteko izan daiteke (Tabadkani et al. 2012).

Himenopteroak izaki haplodiploideak dira, sexua determinatzeko lokus osagarri bakar batekin (sl-CSD) (Whiting 1943). Oso ezaguna da honelako espezieetan zein kaltegarria den endogamia, sexua determinatzeko lokus-ean homozigosia sortzen baitu, ar diploideak emanez (Cook eta Crozier 1995). Ar hauen presentziak, koloniari koste handia suposatzen dio, hauek kolonian inolako lanik betetzen ez dutelako eta gainera antzuak izaten direlako. Antzuak ez direnek, esperma diploidea sortzen dute eta beraz, antzuak diren eme triploideak ekoiztuko dira (Heimpel eta de Boer 2008). Lehen aipatu den moduan, ar diploideak behatu izan dira udaberriko *V. velutina*-ren habietan, espezie honek botila-lepoa pairatzen ari duela aditzera emanez (Darrouzet et al. 2015).

Hau guztia kontuan hartuz, hainbat esperimentu burutu ziren *V. velutina*-ren arrekin 1) espezie berdineko gineetan gertatzen den moduan, adinak eta bazkak CHC profilen konfigurazioan zer efektu duten, 2) adinak eta bazkak esperma-produkzioan zer nolako eragina duten eta 3) esperma-ekoizpena eta profil kutiku-

larrean artean ze erlazio dagoen jakiteko. Gure hipotesiak honako hauek dira: bai adinak bai bazkak, CHC profilean zein esperma-ekoizpenean eragina edukiko dute, azukrean aberatsa den bazka eraginkorrena izanik. Honetaz gain, esperma-kopuru altua eta baxua duten arren artean, profil kutikularren desberdintasunak ikustea espero da.

Material eta Metodoak:

Habien bilketa

Udazkenean, *V. velutina*-ren 3 kolonia heldu bildu ziren Bizkaiko probintziaren gune desberdinetan (Espainia). 1. eta 2. koloniak, Galdakao lurraldean 2016/10/25ean eta 2016/11/03ean hurrenez hurren eta 3. kolonia, Urdulizen harrapatu zen 2017/10/22ean.

Laginen bilketa

Behin koloniak laborategian zeudelarik, heldu guztiak dietil eterra erabiliz lokartu eta kendu ziren. Gero, operkulatutako gelaxkak zituzten abaraskak plastikozko kutxa handietan kokatu ziren (62 x 35 x 42 cm) (1A. Irudia). Egunero ordu berdinean, kutxak aztertu eta indibiduo emergitu berriak kentzen ziren esperimentuetan erabiliak izateko. Ikerketa honetarako, arrak bakarrik erabili ziren. Esperimentuak 23 ±1 °C eta 12 h argi: 12 h iluntasun zuen gela isolatu batean egin ziren (Poidatz et al. 2017).

Emergitutako arrak plastikozko kutxa txikiagoetan kokatu (23 x 12.5 x 13 cm)

(1B eta 1C. Irudiak) eta *ad libitum* elikatu ziren, erdia azukrean aberatsa zen bazka batekin (R) (10 ml eztia + 40 ml H_2O) eta beste erdia azukrean pobrea zen bazka batekin (P) (0.5 ml eztia + 49.5 ml H_2O). Erabilitako eztia, giza elikadurarako Eroski®Mil flores (100 g eztia: 330 kcal, 72 g azukre, 0.6 g proteina eta 0.02 g gatza) izan zen.

Bazka-talde bakoitzeko 5 liztortzar izoztuta hil egin ziren jaio ziren egun berdinean eta bazka talde bakoitzeko 10 indibiduo 3, 6, 9 eta 12 eguneko adinarekin (N = 90 indibiduo) (1. Taula). Kutxa bakoitzean habia zati bat sartu zen liztortzar emergitu berriek, habiarekiko espezifikoa den hidrokarburo profil eredu bezala erabil zezaten (Singer eta Espelie 1992; Layton eta Espelie 1994; Butts et al. 1995). Prozedura hau hiru koloniekin jarraitu zen (1. Taula).

CHCen analisia

CHCen analisientzako, liztortzar bakoitza 1ml pentano eta *n*-eicosene (C20) (10^{-3} g/ml) barne estandarra (IS) zuen kristalezko saiodi batean (5 ml Ø 12 x 75 mm) sartu zen. Bi minutuz irabiatu eta gero, gelditzen zen likidoa beste hodi batera pasa zen eta -20 °Cetan gorde zen analizatua izan arte. 1 µl lagin Masa Espektrometro (MS) bati akoplatua dagoen Gas Kromatografo (GC) (Agilent Technologies 7890A GC System eta Agilent Technologies 5975C inert XL MSD Ardatz Hirukoitzeko Detektagailuarekin) batean injektatu zen eta analisirako HP-5MS Phenyl Methyl (30 m x 250 µm x 0.25 µm) kapilaritate zutabea erabili egin zen. Labearen programa 50 °C (1 min), 50 °C-tik 200 °C-ra (8 °C/min), 200 °C-tik

315 °C-ra (5 °C/min) eta 315 °C-tan 5 minutuz mantenduz izan zen. Injekzioa "*splitless*" moduan egin zen eta helioa erabili zen gas eramaile moduan (1.5 ml/ min). Lortutako data guztiak ChemStation E.02.02.1431 software-arekin prozesatuak izan ziren. Lortutako kromatogramen piko bakoitzaren proportzio erlatiboa Bagnères et al.-ek (1990) deskribatutako modura kalkulatu zen.

Espermatozoideen zenbaketa

Arrak, estereomikroskopioa eta disekzio matxardak erabiliz disekzionatu ziren. Bakoitzari bi semen-besikulak (2A. Irudia), non esperma gordetzen den, erauzi zitzaien eta gero 500 ml Ringer soluzioa zuen 1 ml-ko Eppendorf® batean birrindu egin ziren. Ondoren, lagin bakoitzari 10 µl DAPI (1.5 µg/µl) gehitu zitzaizkion. Semen-besikula bakoitzaren espermatozoide kopurua Neubauer® ganbara bat erabiliz zenbatu zen fluoreszentziazko mikroskopioan (2B. eta 2C. Irudiak) eta espermatozoide kopuru totala ar bakoitzaren bi semen-besikulen espermatozoideak gehituz kalkulatu zen.

Analisi estatistikoak

Profil kimikoen analisiak egiteko, Age (adina), Food (bazka) eta Colony (kolonia) aldagai osagarri bezala zuen Osagai Nagusien Analisia (ONA, PCA) egin zen. Dispertsio grafikoan esperma-kopurua (Sperm) ere irudikatu zen aldagai osagarri bezala. Aldagai bakoitzak azaltzen duen aldagarritasun % zein den jakiteko Erredundantzien Aldakuntza-Banaketaren Analisia (Redundant Variation Partitioning Analysis) burutu zen. Aldagai anitzeko bi analisi hauek Canoco 5® programa era-

biliz gauzatu ziren.

Efektu Misto Gaussiar Ereduak (Gaussian Mixed Effects Models, GMM) (Zuur et al. 2009; Madsen eta Thyregod 2010) erabili ziren Age, Food eta Colony aldagaiek esperma-ekoizpenean zer eragin zuten aztertzeko. R (2018) programaren nlme paketearen lme() funtzioa (Pinheiro et al. 2014), log-likelihood estimatzaile mugatuarekin (Pinheiro eta Bates 2000) erabili zen analisi hau burutzeko.

Age (aldagai numerikoa) eta Food (bi mailekin R eta P), faktore finko bezala sartu ziren ereduan eta Colony (3 mailekin, C1, C2 eta C3), faktore aleatorio bezala. Bukatzeko, ereduaren balidazio bat egin zen ea Sperm kantitatearen termino polinomialak (termino koadratikoak eta/edo kubikoak) eta aldakortasunerako estruktura bereziak, behatutako heterozedastizitateari aurre egiteko beharrezkoak ziren aztertzeko (Pinheiro eta Bates 2000) (1. eta 2. Apendizeak). lme() eta varExp() funtzioetan, “weights” izeneko argumentua erabili zen Sperm aldagarritasunaren funtzio esponentzialarekin, aldagarritasun eredu bat zehazteko (Pinheiro eta Bates 2000; Zuur et al. 2009). varExp() funtzioak, datuen dispertsioaren eredua egitea ahalbidetu zuen, bai handitzen, bai txikitzen bada ere, Age aldagaiaren handiagotzearekin. Eredua aukeratzeko orduan, Akaike informazio kriterioa (AIC) eta/edo log-likelihood ratio testa (Zuur et al. 2009) erabili ziren pausu bakoitzean.

Emaitzak:

Hidrokarburo kutikularrak (CHCak)

Hiru kolonietako arren (laginak) CHCekin (menpeko aldagaiak) gauzatutako

PCAren I. eta II. ardatzen distribuzio espaziala irudikatu da 3A. Irudian. PCA hau aldagai osagarri independenteekin batera aurkeztu daiteke (3B. Irudia), non CH-Cen aldakortasun joera nagusiak uler daitezkeen. Adinaren araberako aldaketa handia erakusten dute laginek, baita bazka eta koloniarekiko ere, baina neurri txikiagoan. Ordenazio grafikoaren eskumako partean ar emergitu berrien "0 egun" taldea kokatzen dela argi ikus daiteke (3B. Irudia). Talde honek profil kutikular bereiztua erakusten du (3A. Irudia). Adin klase hau da sakabanatze handiena erakusten duena. Beste arrei arreta jarriz, esan beharra dago "3 egun" eta "6 egun"eko taldekoak "9 egun" eta "12 egun"eko taldekoak baino sakabanatuagoak agertzen direla. Azken bi taldeek (9 eta 12 egun) beraien artean profil antzekoak aurkezten dituzte (3A. Irudia). Profil kutikularra liztortzar emergitu berrietatik helduenetara aldatuz doa. Bestalde, bazkak eta koloniak ere eragina dute, baina txikia adinarekin konparatuta. Hau ondorioztatu daiteke aldagai hauek grafikoaren erdian kokatuta daudelako (3B. Irudia).

Esperma irudikatzen duen geziak, adinarekin espermatozoide kopurua nabarmenki handitzen dela eta aldiz, bazka eta koloniarekin askoz gutxiago adierazten du. Azken biak bakarrik kontuan hartuz, esperma-kopurua pixka bat altuagoa da ondo elikatutako liztortzarretan eta baita 3. koloniakoetan (3B. Irudia).

Erredundantzien Aldakuntza-Banaketaren Analisia (Redundant Variation Partitioning Analysis) (2. Taula eta 4. Irudia), bai aldagai bakoitzak (Age, Food eta Colony) besteen eragina kenduta, bai hirurak batera, profil kimikoen aldagarritasunaren zer % azaltzen duten adierazten du. Hiru aldagaiak batera kontuan hartuz, al-

dagarritasunaren % 75.4a azaltzen da.

Age berriz, aldakortasun gehien azaltzen duen aldagaia da, aldakortasun totalaren % 70a (2. Taula) hain zuzen ere. 3. Irudiko PCAn ere ikus daitekeen moduan, 4A. Irudian, Age aldagaiaren araberako CHCen distribuzio espazial argia adierazten da.

Nahiz eta ar guztiek CHC gehienak eduki (ikusi aurrerago salbuespenak), hauen proportzioak desberdinak dira. 0 eguneko arretan, Cm1, Cm4, Cm6, Cm9 eta Cm7 (4A. Irudia) dira bost hidrokarburo garrantzitsuenak. CHC hauek hidrokarburo txikiak dira (karbono gutxikoak) erretentzio denbora baxuak aurkezten dituztelarik (ikusi 3. Taula, 3. Apendizea eta fitxategi osagarria). Hala ere, 12 eguneko arretan, Cm45, Cm34, Cm35, Cm18 eta Cm42 dira (4A. Irudia) nagusiak. Hauek, Cm18 kenduta, hidrokarburo handiak dira, erretentzio denbora altukoak eta beraz, karbono ugarikoak (ikusi 3. Taula, 3. Apendizea eta fitxategi osagarria). 3 eta 6 eguneko arrek ez dute adin horietako espezifikoak diren CHCrik aurkezten (4A. Irudia).

Age aldagaiarekin gertatzen zen kontrara, Food-ek aldakortasun totalaren % 1.5 bakarrik azaltzen du (2. Taula). 4B. Irudiak bazkari loturiko oso CHC gutxi daudela erakusten du. Hala ere, R bazka izandakoetan, P bazka izandakoetan baino gehiago agertzen dira, gutxi izan arren.

Colony aldagaiak berak bakarrik, CHCen aldakortasunaren % 4.8a azaltzen du (2. Taula). Gauza bera ikus daiteke 4C. Irudian, non CHC batzuk koloniekiko espezi-

fikoak direla beha daitekeen. Cm31, 3. koloniarekiko oso espezifikoa da. Honako CHC hau batez ere kolonia honetan agertzen da (ikus 3. Apendizea eta fitxategi osagarria). Bestalde, nahiz eta Cm22 hidrokarburoa 2. koloniako ar batzuetan bakarrik agertu (ikus 3. Apendizea eta fitxategi osagarria), ez dauka garrantzi handirik.

Ar emergitu berri baten eta 12 eguneko eta R bazkarekin elikatutako beste ar baten profil kimiko tipikoak beha daitezke 5. Irudian. Liztortzar gaztean, Cm1 eta Cm6-en pikoak helduan baino altuagoak dira, proportzio handiagoan agertzen direla adieraziz. Piko hauek, *n*-C21 eta *n*-C23 hidrokarburoei dagozkie hurrenez hurren (4. Taula). Beste aldetik, Cm24, Cm26, Cm27 eta Cm30, 12 eguneko liztortzarretan dira oparoagoak. Hauek C27:1, *n*-C27, 5MeC27 eta 3-MeC27 dira hurrenez hurren (4. Taula).

Esperma-ekoizpena

Age, Food eta Colony aldagaien eta esperma-ekoizpenaren arteko erlazioa aztertzeko, eredu bat egin zen. Datuei hobeto egokitzen zitzaion eredua bigarren mailako eredu polinomikoa zen:

$$y\,(T=P) = (-58437.4) - (40994.3 \times Age) + (23284.2 \times Age^2) + \varepsilon_{Colony} + \varepsilon_R$$

$$y\,(T=R) = (-58437.4 + 128446.5) - (40994.3 \times Age) + (23284.2 \times Age^2) + \varepsilon_{Colony} + \varepsilon_R$$

Non:

$\varepsilon_{Colony} \sim N\,(0, \sigma_{Colony}^2 = 93297.91)$

$\varepsilon_R \sim N\,(0, \sigma_R^2 = 151259.91)$

Age eta Food aldagaiak dira esangarriak ereduan, nahiz eta Age izan garrantzitsuena (5. Taula). Eredu honen arabera, Age-k esperma-kopuruan dagoen aldakortasunaren % 89.2a azaltzen du eta Food eta Colony aldagaiek aldiz, % 8.1a eta % 0.8a, hurrenez hurren.

Bazka pobre batekin elikatutako arretan espermaren ekoizpena oso gutxi handitzen da 12 egunetan zehar (6. Irudia, gorriz), lehenengo espermatozoideak 6. egunean agertzen direlarik. Bazka aberats batekin elikatutako arrek aldiz, handipen handiagoa erakusten dute 12 egun hauetan zehar (berdez). Kasu honetan, 3. egunean behatuko dira lehenengo espermatozoideak. Lehenengo 6 egunetan, bazkak ez du efektu handia erakusten, baina denbora aurrera joan ahala, efektu hau handiagotuz joango da. Aldakortasun handia dago datuetan, batez ere bazka aberatsarekin elikatutakoetan (6. Irudia eta 6. Taula).

Eztabaida:

Crozier eta Dix-ek 1979ean proposatutako Gestalt ereduaren arabera, eta jadanik 1. eta 3. Kapituluetan azaldu den bezala, indibiduo emergitu berriek profil kutikular “neutroa” aurkeztuko dute. Liztortzarrak helduak egiten diren heinean, hau aldatuz joango da eta habiaren usaina lortuko dute. Honi esker, habia kideek errekonozituko dituzte. Honako hau da *Vespa velutina* arren kasua, gineetan behatu den moduan (ikusi 3. Kapitulua), arren profil kutikularrak aldaketa argia pairatzen duelako adinarekin, *Polistes dominulus* liztorrean gertatzen den moduan (Lorenzi et al. 2004). Ar emergitu berriek (0 egun) eta helduenek, CHC profilari dagokionez,

bi talde oso desberdin osatzen dituzte. Lehenengoen taldearen barruan, profilak heterogeneoagoak dira 12 egunekoekin konparatuz, CHC profil antzekoagoak dituztenak beraien artean. Honako emaitza hauek gineetan ere behatu ziren (ikusi 3. Kapitulua).

Behaketa hau, indibiduo gazteen kutikula pisu molekular txikiko hidrokarburoz osatua dagoelako azaldu daiteke. Honela, iragazkorragoa eta aldi berean "neutroa" da. Liztortzar zaharragoetan aldiz, pisu molekular txikiko konposatu hauek, pisu molekular handiko hidrokarburoz ordezkatuak dira. Arrazoi honengatik, kutikularen iragazkortasuna murrizten da behin betiko profila lortuz (Lorenzi et al. 2004). Gertakari honek 9. eta 12. egunetan behatzen diren CHCen antzekotasuna ere azal dezake. Adin honetan profilak oso aldaketa gutxi pairatzea gerta liteke, adin honetako liztortzarrek jada habiaren usaina lortu dutelako. Adin hauek, 9-12 egun, bat datoz beste liztortzar espezie batzuetako arrek habia utzi baino lehen honen barruan elikatzen pasatzen dituzten egun kopuruarekin, 8 egun gutxi gora behera *V. simillima* (Martin 1991) eta 8-11 egun *V. affinis*-en (Martin 1993) kasuetan.

Bazkak, gineetan gertatzen zen bezala (ikusi 3. Kapitulua) eta beste intsektu espezieetan behatu denarekin konparatuta (Blomquist eta Jackson 1973; Espelie eta Bernays 1989; Rundle et al. 2005; Etges et al. 2009; Fedina et al. 2012; Etges eta de Oliveira 2014), oso eragin txikia du arren kutikulan. Agian, bi bazken arteko desberdintasunak, hau da, eztia kontzentrazioa, ez zen nahikoa CHCetan aldaketak sortzeko, Otte et al.-ek (2014) erakutsi zuten moduan. Autore hauek, konposatu bakoitzaren kantitatearen ordez, *Phaedon cochleariae*-en (Fabricius, 1792) CHCe-

tan aldaketak sortzen zituena dietaren gantz-azidoen konposizioa zela ikusi zuten.

Arren kasuan, Colony aldagaiak profil kutikularraren aldaketan eragin txikia du gineetan gertatzen zenarekin konparatuta. Hala ere, eragin hau bazkak eragiten duena baino handiagoa da. Agian, arren esperimentuetarako erabilitako kolonia guztiak Euskal Herrikoak izateak, beraz agian homogeneoagoak, eta gineen kasuan erabilitako kolonia bat Tours-ekoa (Frantzia) eta beste hiruak Euskal Herrikoak izateak azal dezake fenomeno hau. Honako azalpen hau bat dator Gévar et al. -ekin (2017), zeintzuek *V. velutina*-ren habia bakoitzak profil kimiko berezi bat duela antzeman zuten. Profil kimiko honek, habiaren barruan bizi diren intsektuen kutikularen konfigurazio kimikoan garrantzi handia du (Singer eta Espelie 1992; Layton eta Espelie 1994; Lorenzi et al 2004) eta honek aldi berean, lehen aipatu den moduan, habia bereko kideak ezagutzeko baliagarria da (Gamboa et al. 1996; Espelie et al. 1990; Lorenzi et al 2004).

Gineen tamaina eta gantz kantitatearekin gertatzen den moduan (ikusi 3. Kapitulua), udazkeneko arren tamaina ez dauka eraginik esperma-ekoizpenean (Poidatz et al 2017). Arrazoi honengatik, esperma-ekoizpenean behatutako aldaketak Age aldagaiak, Food eta Colony aldagaiez jarraitua, eragindakoak dira. Gure kasuan bezala, beste hainbat ikerketa daude, non espermatozoide kopurua adinarekin handitzen doan (Mahmood eta Reisen 1982; Ponlawat eta Harrington 2007). Poidatz et al.-en (2017) arabera, *V. velutina*-ren arrek 10 egun behar dituzte espermatozoideak ekoizteko. Denbora honetan zehar, arrak habiaren barruan mantentzen dira elikatzen, beste liztortzar espezie batzuetan gertatzen den moduan (Martin 1991;

Martin 1993). Hala ere, gure esperimentazio baldintzetan, espermatozoideen ekoizpena graduala izan da 3. egunetik aurrera azukrean aberatsa den bazkarekin elikatutako arretan, edo 6. egunetik aurrera, azukrean pobrea den bazkarekin elikatutakoen kasuan. Hau ere eztenik gabeko *Melipona beecheii* Illiger, 1806 erlean behatu izan da, zeinetan hobeto elikatutako larbek besteek baino goizago ekoizten dituzten espermatozoideak (Pecht-May et al. 2012).

Beste intsektu espezie batzuetan, dietak esperma-produkzioan eragin positiboa erakusten du (Gage eta Cook 1994; Fedina eta Lewis 2006; Perez-Staples et al. 2008; McGraw et al. 2008; Bunning et al. 2015). Gure emaitzek aldiz, kontrakoa erakusten dute; hau da, *V. velutina*-n dietak oso eragin txikia duela espermatozoide -ekoizpenean. Hau, langileek larba fasean zehar emandako bazka, esperma-produkzioan oso garrantzitsua delako izan daiteke (Pech-May et al. 2012), agian emergitu ostean kontsumitutakoa baino garrantzi handiagokoa. Modu berean, baieztapen honek koloniaren efektua azaldu dezake, nahiz eta txikia izan, zeren eta larbak hazteko habiaren inguruan dauden baliabideak desberdinak izan baitaitezke. Nolanahi ere, adina eta dietaren artean elkarrekintza bat dago, bi dieten arteko espermatozoide-kopuru desberdintasuna handiagoa delako liztortzar berantiarretan gazteagoetan baino.

Berriro ere, gineen gantzetan gertatzen den moduan, adin zehatz batean, azukre ugariko bazkarekin elikatutako arrek espermatozoide kopuru altuagoak aurkezten dituzte. Hala ere, aldakortasun handiagoa ikus daiteke datuetan bazka pobrearekin

elikatutako arrekin alderatuz. Esan beharra dago, behatzailearen bidez ezin izan zela intsektuek hartutako bazka kantitatea kontrolatu, nahiz eta talde guztien indibiduoek bazka *ad libitum* eduki; beraz, talde bereko indibiduoen artean hartutako elikagai kantitatea desberdina izatea gerta liteke. Honek azal dezake taldeen barruan esperma-produkzioan ikus daitezkeen desberdintasunak.

Gure emaitzak kontuan hartuz, ezin dugu espermatozoide-kantitateak kutikularen profilean zuzenean eragina edukiko duenik ziurtatu, biak adinarekiko oso menpekoak direlako. Dena den, hipotetizatu dezakegu langileek profil kimikoa erabil dezaketela arren adina, eta beraz sexualki helduak diren ala ez, jakiteko. Modu honetan habiatik noiz bota behar dituzten (Monceau et al. 2013) jakin ahalko dute. Honela, habia bereko kideen arteko estalketa saihestuko da eta beraz, endogamia (Tabadkani et al. 2012). Esperimentu gehiago burutu behar dira espermatozoide-kantitateak arren kutikularen profil kimikoa kontrolatzen duen jakiteko. Horretarako, espermatogenesia guztiz inhibituta (hormona edo tenperatura altuen bidez adibidez) eta ez-inhibituta duten arren CHCak alderatzea eta ea honako ar hauek langileengan jarrera erasokorra eragiten duten ala ez *in vivo* probatzea beharrezkoa izango litzateke. Galdera irekita mantentzen da.

Ondorioak:

Bistakoa da arren kasuan liztortzarren adinaren araberako profil kutikularraren distribuzio argia dagoela. Alabaina, eskainitako dietak eta koloniak profil honetan eragin txikiagoa dute, koloniak dietak baino influentzia gehiago izanik. Esperma-

ekoizpenean, adina da berriz ere eraginkorrena, dietak eta koloniak jarraitzen diotelarik. Adina eta dietaren arteko elkarrekintza dago esperma-produkzioan, ar zaharrenak eta ondo elikatuak espermatozoide gehiago ekoizten dituztelarik. Gure emaitzen arabera, hipotetizatu dezakegu langileek arren profil kutikularra erabil dezaketela azken hauek sexualki helduak noiz diren jakiteko. Dena den, bai esperma-ekoizpena, bai profil kutikularra adinarekiko menpekoak izanda, ezin gara ziur egon lehenak bigarrena zuzenean aldatu dezakeen ala ez.

Chapter 5: Concluding remarks

The following conclusions can be drawn from the studies performed in this PhD thesis:

Chapter 2:

1. In order to better understand some biological aspects of the yellow-legged hornet *Vespa velutina*, it is necessary to discriminate between female castes, workers and gynes.

2. It is possible to distinguish morphologically *V. velutina* two females castes depending on their mesoscutum width (MW). Hornets with a MW of 4.5 mm or more are considered to be gynes, while those with a MW less than 4.5 mm are considered to be workers. This conclusion is confirmed by the CHCs profiles.

3. Dry weight (DW) parameter worked better than wet weight (WW) although neither of them is as accurate as MW, at least with young or not well fed gynes.

Chapter 3:

1. The chemical profiles of gynes of *V. velutina* present a clear distribution according to the age of the individuals. 0 and 9 days gynes groups present the most different cuticular profiles, while 3 and 6 days ones have intermediate profiles without a clear characterization.

2. Young gynes present lower molecular weight hydrocarbons in the cuticle than the oldest ones, making the first ones' cuticles more permeable for adsorbing external compounds. Oldest ones present the definitive cuticular profile.

3. The effect of the diet and the colony in the cuticular profile of gynes is less important than the age. Between the diet and the colony is the second one which has bigger influence, due to the unique chemical profile of each nest. There are not important CHCs related to rich and poor food.

4. There is no relation between the size and the fats contents of our samples. All the observed fat content differences are due to the independent variables. Among them, the diet is the most important. Those hornets fed with the rich food present higher fat contents.

5. The age is the variable which had bigger influence on the fats chemical characterization. It would be very interesting to identify them, to try to understand the biological importance of the variation in fats proportion on *Vespa velutina.*

6. Even if some fats and CHCs present a slightly relation, we cannot conclude that one can modify the other because both of them are very age dependent.

Chapter 4:

1. The cuticle chemical profiles of males of *V. velutina* present a clear distribution according to the age of the individuals. 0 and 12 days males groups present the most different cuticular profile, while 3 and 6 days ones have intermediate profiles without a clear characterization.

2. Young males present lower molecular weight hydrocarbons in the cuticle than the oldest ones, making the first ones' cuticles more permeable for adsorbing

external compounds. Oldest ones present the definitive cuticular profile.

3. The effect of the diet and the colony in the cuticular profile of males is less important than the age. Between the diet and the colony is the second one which has bigger influence, due to the unique chemical profile of each nest. There are not important CHCs related to rich and poor food.

4. The age is the variable with bigger influence in the sperm production, followed by the diet and colony. An interaction between the age and the diet on the sperm production exist, the oldest males fed with rich food producing more sperm.

5. We cannot conclude that sperm production can modify the CHCs profile because both of them are very age dependent.

5. Kapitulua: Ondorioak

Hauek dira PhD tesi honetatik ateratako ondorioak:

2. Kapitulua:

1. *Vespa velutina* liztortzar asiarraren hainbat aspektu biologiko ezagutzeko, eme kasten (langile eta gine) arteko desberdintzapena egitea beharrezkoa da.

2. *V. velutina*-ren bi eme kastak morfologikoki desberdindu daitezke mesoeskuteloaren zabaleran (MW) oinarrituz. 4.5 mm-ko edo gehiagoko MWa duten indibiduoak gineak kontsideratuko dira eta 4.5 mm baino gutxiagokoek aldiz, langileak. Ondorio hau CHCen emaitzez dago baieztatua.

3. Pisu lehorrak (DW) pisu hezeak (WW) baino hobeto funtzionatzen du nahiz eta ez bata, ez bestea MW bezain ona ez izan, behintzat gine gazteetan edo gaizki elikatutakoetan.

3. Kapitulua:

1. *V. velutina* gineen profil kimikoek indibiduoen adinaren araberako distribuzio argi bat aurkezten dute. 0 eta 9 eguneko gineen taldeek kutikula ezberdinenak aurkezten dituzte eta 3 eta 6 egunekoek aldiz, tarteko profilak, karakterizazio argi gabekoak.

2. Gine gazteek helduek baino pisu molekular txikiagoko hidrokarburoak aurkezten dituzte kutikulan. Honek, lehenengoen kutikula iragazkorragoa izatea eragiten du, modu honetan kanpoko konposatuak xurgatzea errazagoa delarik. Liztortzar helduek behin-betiko profila aurkezten dute.

3. Gineen profil kutikularrean, dieta eta koloniaren eragina adinarena baino garrantzi gutxiagokoa da. Dieta eta koloniaren artean bigarrena da influentzia gehiago duena, habia bakoitzak berezko usaina duelako. Ez dago bazka aberatsa eta pobrearekiko espezifikoak diren CHC garrantzitsurik.

4. Gure laginetan ez dago gineen tamaina eta gantz kantitatearen arteko erlaziorik. Behatutako gantz kopuruaren desberdintasunak aldagai independenteei dagokie. Azukrean aberatsa den bazkarekin elikatutako gineetan behatzen da gantz kopuru handiena.

5. Adina da gantzen karakterizazio kimikoan gehien eragiten duen aldagaia. Oso interesgarria suertatuko litzateke gantz hauek identifikatzea haien proportzio-aldaketen garrantzi biologikoa ulertzeko.

6. Nahiz eta zenbait gantz eta CHC batzuen artean erlazio sotil bat egon, ezin dugu ondorioztatu batak bestea aldatu dezakeenik, biak adinarekiko oso menpekoak direlako.

4. Kapitulua:

1. *V. velutina* arren profil kimikoek indibiduoen adinaren araberako distribuzio argi bat aurkezten dute. 0 eta 9 eguneko arren taldeek kutikula ezberdinenak aurkezten dituzte eta 3 eta 6 egunekoek aldiz, tarteko profilak, karakterizazio argi gabekoak.

2. Ar gazteek helduek baino pisu molekular txikiagoko hidrokarburoak aurkez-

ten dituzte kutikulan. Honek, lehenengoen kutikula iragazkorragoa izatea eragiten du, modu honetan kanpoko konposatuak xurgatzea errazagoa delarik. Liztortzar helduek behin-betiko profila aurkezten dute.

3. Arren profil kutikularrean, dieta eta koloniaren eragina adinarena baino garrantzi gutxiagokoa da. Dieta eta koloniaren artean bigarrena da influentzia gehiago duena, habia bakoitzak berezko usaina duelako. Ez dago bazka aberatsa eta pobrearekiko espezifikoak diren CHC garrantzitsurik.

4. Adina da esperma-produkzioan gehien eragiten duen aldagaia, bazka eta kolonia atzetik daudelarik. Adina eta dietaren arteko interakzioa beha daiteke esperma-produkzioan, ar helduagoek eta hobeto elikatutakoek espermatozoide gehiago ekoiztuz.

5. Ezin dugu ondorioztatu esperma-produkzioak CHCen profila alda dezakeenik, biak adinarekiko oso menpekoak baitira.