UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Insects with Forensic and Agri-food Implications: *Fannia Pusio* (Diptera: Fanniidae) and *Tenebrio Molitor* (Coleoptera: Tenebrionidae)

Insectos con Implicaciones Forenses y Agroalimentarias: *Fannia Pusio* (Diptera: Fanniidae) y *Tenebrio Molitor* (Coleoptera: Tenebrionidae)

Dª Yolanda Bravo Pena

2021

Insects with forensic and agri-food implications: *Fannia pusio* (Diptera: Fanniidae) and *Tenebrio molitor* (Coleoptera: Tenebrionidae).

Insectos con implicaciones forenses y agroalimentarias: *Fannia pusio* (Diptera: Fanniidae) y *Tenebrio molitor* (Coleoptera: Tenebrionidae).

Yolanda Bravo Pena

2021

Escuela Internacional de Doctorado

Universidad de Murcia

Directores:

Dr. José Galián Albaladejo

Dr. Elena Romera Lozano

El trabajo titulado “Contribution to the development of applied entomology: forensic and agri-food use” realizado por Yolanda Bravo Pena en el Área de Biología Animal del Departamento de Zoología y Antropología Física de la Universidad de Murcia bajo la dirección de los Doctores José Galián Albaladejo y Elena Romera Lozano, como parte de los proyectos “Animal Phylogeny and Evolution” (19908-GERM-15) financiado por la Fundación Séneca en la convocatoria de Grupos de Excelencia Regional.

El trabajo titulado “Contribution to the development of applied entomology: forensic and agri-food use” realizado por Yolanda Bravo Pena en el Área de Biología Animal del Departamento de Zoología y Antropología Física de la Universidad de Murcia bajo la dirección de los Doctores José Galián Albaladejo y Elena Romera Lozano, como parte del Proyecto CGL2011-25298 de la Dirección General de Investigación Científica y Técnica, Ministerio de Economía y Competitividad.

El trabajo titulado “Contribution to the development of applied entomology: forensic and agri-food use” realizado por Yolanda Bravo Pena en el Área de Biología Animal del Departamento de Zoología y Antropología Física de la Universidad de Murcia bajo la dirección de los Doctores José Galián Albaladejo y Elena Romera Lozano, como parte del Proyecto Retos-Colaboración CDTI “Insectflour” (RTC-2017-5964-2) del Plan Estatal de Investigación Científica y Técnica y de Innovación del cual la autora ha disfrutado de un contrato laboral.

“La inteligencia sin ambición
es un pájaro sin alas”.
Salvador Dalí

Agradecimientos

He temido y deseado el momento de escribir estas palabras desde que comencé el doctorado. Cuando empiezas a redactar el apartado de los agradecimientos, es cuando recuerdas todo lo vivido durante esta fase, una etapa muy dura pero gratificante al mismo tiempo. Pertenecer al mundo científico hace sentir orgullo, un país sin investigación ni desarrollo lo empobrece, no hay más que verlo. Por esto mismo, el poco reconocimiento que recibe esta profesión en muchas ocasiones hace sentir frustración e impotencia. Los sentimientos encontrados durante estos años son contradictorios, pero el final de una tesis doctoral hace predominar la satisfacción personal. Sin embargo, su elaboración no habría sido posible sin la colaboración, apoyo, ayuda, pero, sobre todo, paciencia de muchas personas.

Primero agradecer a mi directora Elena Romera por despertarme la curiosidad en las moscas (siempre tan infravaloradas) y que ha dado pie a continuar en el mundo de los insectos. Agradecer a mi director José Galián por encauzar esta tesis que casi se queda en el olvido, por toda la paciencia en mi negatividad y, sobre todo, por aguantar mis TOCs. Gracias a ti, esta tesis se ha realizado.

Todo el mundo ha tenido la inspiración de un profesor cuya materia le ha empujado a elegir "esa carrera profesional". Yo quiero agradecerle a Marieta que haya conseguido que una asignatura me fascine, aunque la suspendiese una y otra vez, que me haya enseñado que lo importante es la curiosidad y la constancia. Gracias a esas virtudes, he conseguido llegar donde estoy. Aprovecho estas líneas para decirte que lo que más leo son obras de filósofos y, sintiéndolo mucho, Kant no es mi favorito.

A mis biólogos Majo, Rous y Pertico. Gracias a vosotros esos años de carrera los recuerdo con morriña y, a día de hoy, nos hacen reír cada vez que nos juntamos. Ese consejo de sabios que más que sabidurías compartíamos estupideces, la ficha de fisiología vegetal, la tensión de los problemas de genética y la emoción de no salir elegido, la crema de orujo. Eso es lo más enraizado en mi memoria que tengo, es así y me gusta.

Karol, mi forense zaragozana, fuiste la única del máster que entendiste mi afición por los bichos y si no fue así, por lo menos lo apoyaste. Sigo teniendo la tarántula y es de lo más apreciado que tengo. Gracias por estar ahí.

A mis compañeros de laboratorio de Dípteros Necrófagos Miriam, Mario, Beatriz y Alberto, gracias a vosotros el mundo “mosquil” fue muy entretenido. A mis compañeros del día a día, mi segunda familia, el departamento de Biología Animal: Mica (gracias por existir), Javi y Andrés (mis consejeros diarios), los apidólogos Nuria, Álvaro, Daniel, Vicente y Diego, Alejandro y Alberto (que hemos compartido orígenes mosquiles), gracias a todos vosotros por esas risas en los congresos, los pocos italianos a los que he podido ir, los viernes sin falta en el aperitivo y los juegos reunidos ocasionales. Sin todos vosotros, esta tesis hubiese sido mucho más pesada y mucho menos divertida. A Pepe Serrano, Pilar de la Rúa e Irene Muñoz por sus grandes consejos. A nuestra gran técnico de laboratorio Ana (mejor persona) por toda la ayuda y paciencia durante todos estos años, no cabe la menor duda que sin ti las tesis no saldrían adelante.

Pero no todos son los compañeros, a mis amigos Amado, Nati, Arantxa, Eri, Bea, Lu, Cristel, Evelyn … aunque no lo creáis sois parte de esto, por aguantar las comeduras de cabeza sobre mi futuro, por hablaros de bichos y mostrar interés o, simplemente, por estar ahí después de tantos años. Sois los mejores.

A mi familia de sangre y adoptiva, muchas gracias por todo el apoyo emocional que me ha dado la seguridad para seguir adelante. Papa, Franchu, gracias por todo. Gràcies per totes les paelles (i les cervesetes!) els caps de setmana que em carregaven les piles per a començar una nova.

Y por último, el que debería ser coautor de esta tesis, mi compañero de vida. Vi, has estado desde el comienzo de todo esto. Para ti no tengo palabras suficientes de agradecimiento. El apoyo, la fuerza moral, la confianza que has demostrado en mí, el aguante de la comedura de cabeza más tonta y sobre todo, hacerme reír en compañía de nuestra Suna cuando más lo necesitaba. Sois el pilar de este trabajo, incluso has participado en uno de los capítulos explicándome tu mundo de números. Os quiero.

Contents

Resumen (Spanish summary) ……... 1

Introduction ……..... 25

General introduction ……...……...…... 27

Forensic Entomology ……..……... 29

Fannia pusio (Diptera: Fanniidae) ……..…… 31

Identification methods ……..…….. 32

Molecular technique ……..... 32

Morphometric technique ……..…….…….. 34

Post-mortem interval estimation (PMI) ……..…….…….. 35

Isomegalen and isomorphen diagrams ……..……...……..…….. 36

Insects for Food and Feed ……...…... 38

Farming *Tenebrio molitor* to low-scale ……..……. 40

Anomalies found in insect rearing ……...……..…..… 43

Objectives ……..... 45

PART I

Chapter I. Barcoding identification and phylogeographic analysis of *Fannia pusio* (Diptera: Fanniidae) in the Iberian Peninsula ……...……..... 51

Abstract ……..... 53

Introduction ……..……. 55

Material and methods ……..……... 56

Results ……..……. 59

Discussion ……..…… 63

Chapter II. The head of *Fannia pusio* (Diptera: Fanniidae) as a novel source of morphometric data for the assessment of variation along geographic and biological lines ……..…….. 67

Abstract ……..…… 69

Introduction ……..…. 71

Material and methods ……..…... 72

Results ……..……. 75

Discussion ……..… 83

Chapter III. Development of *Fannia pusio* (Diptera: Fanniidae) under controlled temperature conditions and its enforcement in the estimate of the post-mortem interval (PMI) ……..…. 87

Abstract ……..… 89

Introduction ……..…. 91

Material and methods ……..……... 92

Results ……..……. 93

Discussion ……...……..… 98

PART II

Chapter IV. Production and economic feasibility studies in the elaboration of a project for farming the yellow mealworm, *Tenebrio molitor* ……...……...……. 103

Abstract ……...… 105

Introduction …… 107

Tenebrio molitor …...……... 108

Breeding of *T. molitor* ……... 109

Production of *T. molitor* ……...……. 111

Economic Feasibility ..…… 114

Investment …… 114

Finance ……..... 115

Operating Revenue ……... 116

Cost Analysis …… 116

Operating cash flow ……..… 118

Project profitability ……... 118

Chapter V. Expression analysis of *Lac2*, *TH* and *AANAT1* genes related to exoskeleton malformations in reared *Tenebrio molitor* (Coleoptera: Tenebrionidae) ……..…… 119

Abstract ……...…… 121

Introduction ……. 123

Material and methods ……... 124

Results ……. 127

Discussion ……...…… 128

Conclusions ……...…….. 133

References ……...…… 137

Appendix ……. 169

Table index

Table 1. Details of sampling zones of the Iberian Peninsula where the genus *Fannia* was found (see Figure 14) ……. 58

Table 2. Intraspecific and interspecific Kimura two-parameters distances of *Fannia* species collected in the sampling ……..…. 60

Table 3. Information on the geographic data of the sampling of each species and the number (N) of individuals collected ……... 73

Table 4. Description of the landmarks used in the analysis of *Fannia* head (see also Figure 18) ……..…. 75

Table 5. Results of Mahalanobis distances (*p*-value) and Cross-validation (%) between different *Fannia* species ……...…….. 79

Table 6. Results of the Mahalanobis distances (*p*-value) and Cross-validation (%) among *F. pusio* populations in terms of geographical distribution ……. 81

Table 7. Descriptive statistics on the length of the different stages of *F. pusio* at each constant temperature studied ……..…. 94

Table 8. Quadratic model regression, variance absorbed (R^2aj), coefficients of regression (F-value) and coefficients significance (*p*-value) of the relationship between the body length of *F. pusio* larvae (L) (mm) and the time (T) (h) at five constant temperatures (see Figure 30). * Indicates coefficients in the model are statistically significant ……. 96

Table 9. Investment and amortisation of the different start-up facilities. The amortisation is the amount for each year of the project's life, which is estimated to be 5 years …..... 115

Table 10. Quantities, price and format of *T. molitor* production during the 5-year life of the project. Production in different formats (live, dried and meal) expressed in kilograms (kg) and euros (€) ……..… 116

Table 11. Direct costs during the 5-year life of the project. The raw material for each type of insect for sale (live, dried and meal) is taken into account. Both larvae and adults are offered a diet of 90 % wheat bran and 10 % vegetable by-products …….. 117

Table 12. Indirect costs broken down by project year and total expressed in euros (€) .. ……..…….. 117

Table 13. Cash flow of the project expressed in euros (€) per year, showing the investment made, and the liquidity at the end of the project. Negative figures are indicated in red ……. 118

Table 14. Primers used for qRT-PCR experiments for *T. molitor*. E = Amplification efficience ……...……...… 126

Table 15. Stability values obtained from geNorm and NormFinder algorithms for all genes tested ……... 131

Figure index

Figure 1. Phylogenetic tree of insect diversity relationships. The timeline at the bottom of the tree relates the geological origin of the insect clades to the main geological and biological events (Misof *et al.*, 2014) ……..… 28

Figure 2. Observation of decomposition of human corpses: "Dance of the Death" (Stammler, 1460) (left); grave of Robert Touse who "expects the resurrection of the dead" (exact time of making unknow) (right) (Benecke, 2001) ……...…. 29

Figure 3. *Fannia pusio* adult (Quinn, 2018. College Station, Brazos County, Texas, USA) ……. 31

Figure 4. Ventral view of *Sarcopromusca pruna* (Diptera) as a vector of *D. hominis* eggs on the abdomen (Azevedo *et al*., 2007) ……... 32

Figure 5. Figure 5. Diagram of the steps to follow for the molecular DNA Barcoding technique (Sinsoma, https://sinsoma.com/en/technology/dna-barcoding/) ……. 33

Figure 6. Distribution of the landmark used to analyse the head of Chagasi Series species (Diptera: Psychodidae: Phlebotominae) (Godoy *et al*., 2018) ……..….. 34

Figure 7. Isomegalen diagram of *Lucilia sericata* (Diptera: Calliphoridae). The X-axis is time and is measured from egg hatching, represented in hours (at the top) and days (at the bottom). The Y-axis represents temperature which is measured in degrees Celsius (on the left) and in degrees Fahrenheit (on the right). Each line of the graph shows larval lengths (in millimetres) (Grassberger & Reiter, 2001) ……... 36

Figure 8. Isomorphen-diagram of *Lucilia sericata* (Diptera: Calliphoridae). Time is expressed in days on the X-axis and temperature expressed in degrees Celsius on the Y-axis. The space between each line represents each stage of the life cycle of the species: a = egg, b = first larval stage, c = second larval stage, d = third larval stage, e = prepupa, f = pupa, g = imago (Grassberger & Reiter, 2001) …… 37

Figure 9. Comparison of resources and environmental impact of insect farm and conventional farm. (A) Digestible biomass percentage, (B) Feed conversion ratio, (C) Greenhouse gas equivalent production per kilogram of body weight gain, (D) Ammonia pollution production per kilogram of body mass, (E) Global warming potential, (F) Energy use, (G) Land use, (H) Water use (Gahukar, 2016) ……..... 39

Figure 10. Larval stage of *T. molitor*, commonly called yellow mealworm ……..… 40

Figure 11. Phases of the holometabolous life cycle of *T. molitor* (Singh, 1975) ……..… 41

Figure 12. *T. molitor* farming systems. Breeding system: the adults are in tray B which has a porous surface that lets the newly hatched larvae fall from the eggs to tray C where they are collected. Rearing system: stacked trays with pores of different diameters to separate the larvae by size, the last tray is where the waste is collected (Morales-Ramos *et al.*, 2012) ……..… 42

Figure 13. *Callipogon relictus* pupaes with different deformations of teguments and limbs (Yi *et al*., 2017) ……. 43

Figure 14. Sampling points of the Iberian Peninsula where the genus *Fannia* was found. 1 (pink point) = Villanueva de Cameros (La Rioja), 2 (blue point) = Villar del Arzobispo (Valencian Community), 3 (blue point) = Catarroja (Valencian Community), 4 (orange point) = Campus of Espinardo (Region of Murcia), 5 (orange point) = Aguilas (Region of Murcia), 6 (green point) = Estremadura (Lisbon) (for more details see Table 1) ……... 57

Figure 15. NJ tree of mini–barcode of cox1 gene of *Fannia* species. Red = *F. pusio*, Blue = *F. leucosticta*, Green = *F. lepida*, Yellow = *F. aequilineata*, Purple = *F. canicularis*. Sequences with an asterisk are those obtained from the NCBI database for comparison ……..… 61

Figure 16. Haplotype network of mini–barcode of *Fannia* species collected in the Iberian Peninsula and sequences obtained from the NCBI database for comparison ……. 62

Figure 17. Haplotype network of mini–barcode of *cox1* of *Fannia pusio* individuals collected in the Iberian Peninsula and sequences obtained from the NCBI database for comparison. In addition, the individuals from the colony established in the laboratory (collected in Murcia) are separated from the individuals collected from the Region of Murcia ……...……. 63

Figure 18. Head of *Fannia pusio* female. The numbered points indicate the location of the 18 landmarks used for head measurements ……...… 74

Figure 19. Transformation Grid (left) and Wireframe (right) representation of shape variations between *Fannia* species based on Principal Component Analysis. *In the wireframe the turquoise outline characterizes the position of consensus landmarks, while the blue outline represents landmarks configurations ……..… 76

Figure 20. Discrimination of *Fannia* species with a *Hydrotaea floccosa* as outgroup based on Principal Component Analysis ……. 77

Figure 21. Transformation Grid (left) and Wireframe (right) representation of shape variations between *Fannia* species based on Canonical Variate Analysis. *In the wireframe the turquoise outline characterizes the position of consensus landmarks, while the blue outline represents landmarks configurations ……...…….. 77

Figure 22. Discrimination of *Fannia* species with *a Hydrotaea floccosa* as outgroup based on Canonical Variate Analysis …… 78

Figure 23. Geographical differences among *Fannia pusio* populations based on Principal Component Analysis …….. 80

Figure 24. Transformation Grid (left) and Wireframe (right) representation of shape variations among *Fannia pusio* populations in terms of geographical distribution based on Canonical Variate Analysis. *In the wireframe the turquoise outline characterizes the position of consensus landmarks, while the blue outline represents landmarks configurations ……. 80

Figure 25. Geographical differences between the populations of *Fannia pusio* from the four localities of the Iberian Peninsula where they were found (La Rioja, Lisbon, Region of Murcia and Valencian Community) based on the Canonical Analysis of Variables ……..… 81

Figure 26. Variation between a laboratory bred colony *versus* wild sample of *Fannia pusio* based on Principal Component Analysis …….. 82

Figure 27. Transformation Grid (left) and Wireframe (right) representation of shape variations between a laboratory bred colony versus wild samples of *Fannia pusio* based on Canonical Variate Analysis. *In the wireframe the turquoise outline characterizes the position of consensus landmarks, while the blue outline represents landmarks configurations ……. 82

Figure 28. Geometric morphometrics variation of *Fannia pusio* head landmarks based on Canonical Variable Analysis. Individuals from the colony reared under laboratory conditions are compared against wild-caught individuals …….. 83

Figure 29. Comparative results for the length (mm) on the y-axis of a) L1, b) L2, c) L3, d) Prepupae and e) Pupae at different temperatures (º C) on the x-axis. The legend indicates the minimum and maximum length values for each stage ……..… 95

Figure 30. Quadratic model regression curves between length (mm) on the y-axis and age samples (h) on the x-axis at a) 15 ºC, b) 20 ºC, c) 25 ºC, d) 30 ºC and e) 35 ºC (for more details see Tabe 8) ……..… 96

Figure 31. Time development variations in each instar stage of *F. pusio* reared at different temperatures within the maximum and minimum threshold ……... 97

Figure 32. a) Isomorphen-diagram of *F. pusio*: representation of the time expressed in hours (x-axis) required for the development of each stage of the biological cycle within each test temperature range (y-axis); b) Isomegalen-diagram of *F. pusio*: representation of the time expressed in hours (x-axis) needed to reach a certain length expressed in millimetres within each range of temperatures studied (y-axis) ……..... 98

Figure 33. Breeding system of *T. molitor*. A: Reproduction procedure, adults are deposited on top while first instar larvae newly hatched from eggs fall to the bottom through the screened bottom. B: Adults with food and water source in the upper box with screened bottom. C: Lower box where larvae are collected with food scraps ……. 110

Figure 34. A: Storage of first instar larvae on solid bottom to reach minimum size for screening, approximately 30 days. B: Oats are added to the tray so that the newly emerged adults have food and do not eat the pupae (approximately 10 days); and soil is added to maintain adequate moisture to prevent the pupae from drying out ……..... 111

Figure 35. Developmental system of *T. molitor* larvae. A: Larval development procedure, the larger larvae stay at the top while the smaller larvae fall through the screened bottom. In the last box with a solid bottom, unwanted droppings and food debris are collected, thus maintaining a hygienic environment. B: Larger larvae on top with a 0.85 mm sieve to allow smaller larvae to pass through. These larvae are collected in other trays with a 0.5 mm sieve through which unwanted droppings and food debris will pass. Finally, the last tray will collect the waste, which will be removed weekly ……..… 112

Figure 36. Calculation of the annual production schedule and life cycle of *T. molitor*. It is estimated that approximately 3 tonnes are produced in one year. *Lv: last larval stage; Pup: pupae; AD: adults ……...….. 113

Figure 37. a) Adult of *T. molitor* with deformed elytra and abdomen. b) Healthy adult of *T. molitor* without physical deficiencies ……...…… 125

Figure 38. Relative expression of *TH, Lac2* and *AANAT1* genes (control and anomalous samples) using the reference gene *RpS3*. Error bars represent the standard error for the normalised relative amount. Asterisks represent statistical significance (** = $p < 0.01$, *** = $p < 0.001$) ……..... 127

Figure 39. Metabolic pathway of *T. molitor* exoskeleton pigmentation adapted and modified from Mun *et al.* (2019). The enzymes in red are those that have been analysed in this work together with the expression graph differentiating between healthy and anomalous individuals …….. 129


species
Fannia
forensic
pusio
Diptera
genus
molitor
Iberian
identification
morphological
development
complete
Peninsula
molecular
individuals
Fanniidae
economic
fragment
analysed
many
insect
involved
study
analyses
results
work
feed
food
due
legal
behaviour
previously
specimens
insects
found
interest
family
conditions
addition
advanced
alternative
correct
feasibility
DNA
entomofauna
first
gene
bp
consumption
head
morphometric
variation
knowledge
abnormally
sclerotization
Tenebrio
belonging
sanitary
may
also
protein
human
broaden
However
information
melanisation
decomposition
structure
developing
clear
using
often
help
different
research
developed
collected
abnormal
provide
assessed
become
observed
common
great
abundant
production
showed
becoming

Resumen

Introducción general

Los artrópodos son el grupo más numeroso y diverso del reino animal con una riqueza de 5 a 10 millones de especies (Ødegaard, 2000) que se extiende por todo el planeta. Insecta es la clase más abundante y está compuesta por más de 5,5 millones de especies según estimaciones recientes (García-Robledo *et al.,* 2020). Aunque algunas tienen efectos nocivos (White, 1976), muchas otras son beneficiosas para el ser humano, los animales domésticos y los cultivos.

El análisis de estos efectos, tanto los perjudiciales como los beneficiosos, son objeto de estudio de la entomología aplicada. Dicha disciplina engloba el estudio de insectos y otros artrópodos de interés para el ser humano, ya sea por los productos que proporcionan o por el impacto negativo que causan en bienes importantes. Existen siete grandes ramas en las que los insectos nos benefician (Clark, 2009): i) la polinización en el ámbito ambiental (Gabriel & Tscharntke, 2007; Sawe *et al.*, 2020); ii) en la agricultura se utilizan para el control biológico, es decir, organismos entomófagos (parásitos, parasitoides, depredadores, patógenos o competidores) son empleados para la supresión de especies plaga (Chatterjee *et al*., 2009; Sethuraman *et al.,* 2020), y para la producción de fertilizantes (Arango-Gutiérrez & Agudelo-Bentacur, 2004; Piñero *et al*., 2018); iii) en la silvicultura también se emplean como control biológico (Sethuraman *et al.*, 2020); iv) en el ámbito sanitario tanto en animales como en humanos, el tratamiento larvario se está convirtiendo en una terapia emergente para la eliminación de tejidos necróticos (Forero-Becerra, 2011; Reyes-Parrado *et al*., 2020), aunque también se incluyen en este campo aspectos como las enfermedades causadas de forma pasiva y directa (reacciones alérgicas por picadura, contacto o inhalación, y el papel vectorial de las enfermedades); v) en el ámbito legal se pueden explotar como datadores forenses (Smith, 1986) y otras aplicaciones (Baz *et al,* 2015; Eon, 2019); vi) en el ámbito alimentario los insectos son cada vez más valiosos debido a su alto contenido en proteínas, así como en actividades tan antiguas como la astacicultura, la apicultura y la sericultura (Lizhang *et al*., 2008; Van Huis *et al.*, 2020); y vii) en la producción de piensos los insectos se están incorporando, vivos o como proteínas hidrolizadas en animales de compañía, acuicultura, aves de corral y cerdos (Van Huis *et al.,* 2020).

En este trabajo nos centramos en dos de las aplicaciones mencionadas de los insectos: 1) el uso legal o forense y 2) la producción de alimentos y piensos.

Entomología Forense

La entomología forense es una rama de las ciencias forenses que extrae conclusiones de la información proporcionada por los artrópodos. Se trata de una disciplina que se basa en el estudio de la interacción de una gran diversidad de diferentes especies de insectos y otros artrópodos con su entorno. Además, esta fauna manifiesta disparidades morfológicas, lo que resulta difícil de manejar para un no experto en entomología (Lutz *et al.*, 2021).

Son muchas las especies que intervienen en las investigaciones, pero las pertenecientes al orden Diptera son las más comunes, ya que son las primeras en colonizar el cuerpo. Las hembras adultas son atraídas por el cadáver para depositar sus huevos, ya que una vez que las larvas eclosionan se van alimentando de él (Smith, 1986). Sin embargo, se debe tener en cuenta que hay especies que acuden al cuerpo únicamente porque depredan insectos necrófagos (Gennard, 2007). Como ya se ha mencionado, una de las principales fuentes de invasión de tejidos se produce a través del orden Diptera que puede causar efectos tan perjudiciales como la miasis, parasitando tanto animales como humanos (Smith, 1986). Asimismo, al parasitar animales domésticos pueden llegar a propagar todo tipo de zoonosis (Grzywacz *et al.*, 2017b).

El primer caso documentado de entomología forense aparece en un manual jurídico chino del siglo XIII escrito por Sung Tz'u que explica el asesinato de un campesino. Médicos y juristas comenzaron a emplear esta ciencia como técnica complementaria en las investigaciones criminales. Los procesos de descomposición del cadáver (físicos y químicos) y todos los fenómenos que los acompañan, también llamaron la atención en el mundo del arte (Benecke, 2001). Sin embargo, no fue hasta 1894 cuando Megnin observó la atracción selectiva y ordenada de artrópodos hacia el cadáver ayudando a indicar el tiempo transcurrido desde la muerte, y los definió como "Escuadrones de la Muerte". Este descubrimiento dio lugar a la publicación de "La Faune des Cadavres: Application de l'Entomologie à la Médicine Légale", donde se describen las diferentes etapas de descomposición de un cadáver. En la actualidad existen diferentes escalas de descomposición, los patólogos forenses emplean una escala compuesta por cuatro estadios establecidos según el estado del cadáver (fresco, enfisematoso, putrefacción y esquelético); mientras que los entomólogos forenses describen cinco fases donde se

valora el estado de descomposición y los grupos de artrópodos asociados (fresco, enfisematoso, putrefacción temprana, putrefacción tardía y esquelético) (Gennard, 2007).

El establecimiento de la entomología forense como instrumento reconocido para la investigación de las escenas del crimen no se produjo hasta 1850. El cuerpo momificado de un recién nacido fue encontrado dentro de una chimenea detrás de la repisa de una casa. El Dr. Marcel Bergeret realizó la autopsia y descubrió larvas de mosca (*Sarcophaga carnaria* L) y varias especies de polillas. Llegó a la conclusión de que el cuerpo del bebé había sido sellado en 1848 y que los insectos accedieron a él en 1849. Como resultado, los habitantes anteriores a 1848 fueron acusados mientras que los ocupantes actuales fueron exonerados (Bergeret, 1855).

Esta disciplina se pudo perfeccionar y desarrollar como estudio científico a finales del siglo XX y principios del XXI. Analiza todos los aspectos relacionados con los insectos que afectan o implican aspectos legales, pero su ámbito de actuación va más allá de la mera identificación de la fauna entomológica propia de los cadáveres (entomología médico-legal) (Hall, 1990), que debe realizarse de forma correcta e inequívoca. La colonización de los cadáveres por parte de los insectos sigue un patrón característico que está modulado por las condiciones ambientales y la ubicación (enterrado, al aire libre o sumergido en el agua). También puede utilizarse para datar las especies protegidas, determinar cuándo se produjo la muerte e identificar la causa de la misma (Smith, 1986). Además de su utilidad para estimar aspectos del intervalo post-mortem (PMI), la determinación de la especie en relación con la distribución geográfica de esa especie puede ser útil para determinar la localización (Byrd & Sutton, 2019).

El orden Diptera es el primero en ser atraído por el cadáver, siendo las familias Calliphoridae, Sarcophagidae y Muscidae las más comunes (Smith, 1986; Gennard, 2007; Amendt *et al.*, 2009). Sin embargo, la familia Fanniidae también ocupa un lugar destacado en la medicina forense, pero menos conocida (Szpila, 2019).

Fannia pusio (Diptera: Fanniidae)

Fanniidae (Diptera) es una familia Calyptratae con aproximadamente 330 especies distribuidas en cinco géneros: *Fannia* Robineau-Desvoidy, 1830, *Piezura* Rondani, 1866, *Euryomma* Stein, 1899, *Australofannia* Pont, 1977 y *Zealandofannia* Domínguez & Pont,

2014 (Couri & Sousa, 2019). El género *Fannia* es el más amplio, abarcando hasta 60 especies (De Carvalho & Mello-Patiu, 2008) con las siguientes características (Al Gazi *et al*., 2004): i) tamaño pequeño; ii) tegumento oscuro; iii) abdomen predominantemente amarillo (Rozkosný *et al*., 1997); iv) seta dorsal submediana en la tibia posterior; v) vena corta CuA+1A como prolongación de la vena 2A del margen del ala (Pont, 1977).

Fannia pusio (Wiedemann, 1830) es una de las especies dentro del género *Fannia* y se conoce comúnmente como "mosca del estiércol de las gallinas" por su aparición habitual en las granjas de gallinas ponedoras (Al Gazi *et al*., 2004). Los caracteres taxonómicos más relevantes de *F. pusio* son la tibia trasera con largas setas en la superficie ventral en los machos, y la pilosidad de los parafrontales destacando alrededor del margen del ojo en las hembras (De Carvalho & Mello-Patiu, 2008). Aunque la especie tiene un origen neártico (Couri & Sousa, 2019) actualmente se encuentra en todo el mundo debido al transporte de ganado (Grzywacz & Prado e Castro, 2012). Tiene preferencia por las zonas cálidas (Smith, 1986; Bélo *et al.*, 1998) y fue en Brasil, distribución nativa de la especie (Marchiori *et al*., 2000), donde se empezó a considerar su importancia en medicina y veterinaria. Sin embargo, varios estudios la han encontrado durante todas las estaciones del año (Leandro & D'Almeida, 2005; Omar *et al*., 2008; Barbosa *et al*., 2009; Krüger *et al.*, 2010; Aballay *et al.*, 2012).

Se trata de una especie bien estudiada (véase De Carvalho *et al.*, 2002, 2003 y sus referencias) debido a su importancia económica y a sus hábitos sinantrópicos (Linhares, 1981; Leandro & D'Almeida, 2005). Sin embargo, son necesarios más estudios para mejorar el conocimiento de su biología, ecología y comportamiento (Szpila *et al*., 2019). El interés sanitario y legal alberga por su presencia en cadáveres animales y humanos (Mendes & Linhares, 2002). Además, parte de la importancia económica y sanitaria de la especie proviene del papel común de la hembra como huésped forético de huevos de *Dermatobia hominis* (la mosca de la botánica humana) que causa miasis en humanos y en animales domésticos (Gomes *et al*., 2002).

Para que las especies sean útiles desde el punto de vista forense es crucial su correcta identificación. En particular, las especies del género *Fannia* son muy similares entre sí a nivel morfológico (Couri & Sousa, 2019) y las claves taxonómicas basadas en la morfología suelen ser insuficientes (Grzywacz *et al.*, 2017b). Asimismo, en las claves de identificación de Muscidae y Fanniidae de Europa, las especies *F. canicularis* y *F. scalaris* son las más citadas (Pont, 2015; Grzywacz & Prado e Castro, 2012) mientras que

F. pusio no fue incluida pese a ser común en el sureste de la Península Ibérica. Por ello, en los estudios legales se utilizan técnicas más especializadas para la correcta identificación (Ekrem *et al.*, 2007; Dujardín, 2008; Kosmann, 2009; Alves-Rolo, 2010; Arnaldos *et al*., 2015; Yussef-Vanegas & Agnarsson, 2017; Onder *et al.*, 2019; Fuentes-López *et al*., 2020).

Métodos de identificación

La aplicación de los insectos en las ciencias forenses requiere de una identificación precisa, a pesar de que las pruebas entomológicas halladas en las investigaciones suelen estar dañadas, contaminadas y fase del ciclo biológico en las que las claves dicotómicas son limitadas (Zehner *et al.*, 2004). Por ello, las técnicas más potentes que se han desarrollado en las últimas décadas, tienen un mayor rango de fiabilidad y eficacia, y permiten identificaciones previas que tienen una aplicación importante en casos reales de investigaciones forenses.

Técnicas moleculares

Las técnicas moleculares ofrecen ventajas allí donde otros métodos son insuficientes. Uno de los mayores problemas son las muestras dañadas o mal conservadas que hacen inviable el reconocimiento de los fenotipos característicos de cada especie (Hoyos-López *et al.*, 2012; Li *et al*., 2015; Shi *et al*., 2017). El ADN está presente no sólo en huevos, larvas y adultos, sino también en las pupas vacías (cuando el adulto ha emergido) (Mazzanti *et al.*, 2010). Esto representa una enorme ventaja para utilizar este último estadio para la identificación de especies mediante técnicas moleculares, y poder emplearlo, así, con fines forenses.

La técnica más utilizada es el "barcoding" que utiliza un pequeño fragmento llamado "barcode" del gen de la subunidad uno de la citocromo oxidasa (*cox1*) con un tamaño de 658 pb (Hebert *et al*., 2003). Sin embargo, fragmentos más pequeños (240 pb) llamados “mini-barcode” están comenzando a emplearse por la dificultad de su amplificación en ciertas ocasiones (Meusnier *et al*., 2008, Lee *et al*., 2015; Grzywacz *et al*., 2017b; Yeo *et al.*, 2020). El barcode es la subunidad catalítica de la enzima que predomina en la mitocondria (Kerr *et al*., 2009; Alves-Rolo, 2010; Pérez & Henao, 2013) siendo de naturaleza haploide con herencia materna sin recombinación (Kosmann, 2009). Esto genera marcadores específicos de especie lo que proporciona la eficacia de esta

metodología (Harvey *et al.,* 2003). Es por ello que este gen se ha utilizado en el estudio de los orígenes y evolución de las poblaciones de insectos mediante el análisis de haplotipos (López-López *et al*., 2016; Hurtado-Burillo *et al.,* 2017; Silva *et al.*, 2020).

Las secuencias obtenidas en los trabajos científicos para todos los organismos se depositan en la base de datos del NCBI (National Centre for Biotechnology Information) como requisito previo a su publicación. Esas secuencias están disponibles para todos los científicos y pueden ser descargadas del NBCI usando la herramienta BLAST permitiendo ser incluidas en análisis posteriores con nuevos enfoques (Altschul *et al.*, 1997). De esta manera podemos comparar nuestras secuencias generadas con otras ya registradas procedentes del mismo taxón y de otros relacionados. Para ello se debe tener en cuenta el menor valor E y el mayor Score en la herramienta BLAST, es decir, maximizar las similitudes entre secuencias para construir filogramas de unión de vecinos que permitan la identificación de especies (Hebert *et al*., 2003).

El ADN es una de las herramientas de mayor crecimiento en las ciencias forenses que ha permitido identificar numerosas especies del orden Diptera aumentando la fiabilidad de los informes y juicios forenses (Coyle *et al*., 2005; Kosmann, 2009; Alves-Rolo, 2010; Boehme *et al*., 2012; Arnaldos *et al*., 2015; Grzywacz *et al*., 2017b; Fuentes-López *et al*., 2020, 2021). Sin embargo, otros campos relacionados con los insectos también han recurrido a técnicas moleculares como la genómica ambiental (Valentini *et al.*, 2009; Kang *et al.*, 2017), así como otros aspectos de importancia médica y veterinaria (Hoyos-López *et al*., 2012; Rodrigues *et al*., 2018; Onder *et al*., 2019).

La información genética obtenida también puede utilizarse para estudiar las relaciones filogeográficas entre diferentes especies y dentro de especies estrechamente relacionadas (Lambertini *et al*., 2006; Fuentes-López *et al*., 2021). Es un campo de estudio que aborda 1) la identificación de los patrones de distribución geográfica de los linajes, mediante la construcción de las genealogías de las poblaciones y los genes, y 2) la inferencia de los procesos que han originado esos patrones (Avise, 2000).

Técnica morfométrica

La morfometría geométrica (MG) es una metodología que junto con el análisis molecular da resultados muy efectivos de identificaciones a nivel de especie (Hurtado-Burillo, 2015). La MG estudia la morfología utilizando coordenadas cartesianas de puntos de

referencia que son capaces de recoger distintas variables de forma y se pueden analizar mediante diversas técnicas estadísticas (Bookstein, 1982).

Por tanto, es un potente enfoque para cuantificar variables como la forma biológica, la variación y la covariación de la forma (Zelditch *et al*., 2004) con otras variables o factores bióticos o abióticos (Webster & Sheets, 2017). Estos puntos de coordenadas corresponden a particularidades presentes en todos los individuos que siguen la misma disposición (Zelditch *et al*., 2004). La técnica solo analiza la forma ignorando el tamaño, la posición y la orientación del ejemplar y, por tanto, ayuda a entender muchas cuestiones no resueltas sobre la evolución y el desarrollo del organismo (Klingenberg, 2013).

La gran ventaja del método es que los datos pueden ser analizados de forma rutinaria y directa con programas estadísticos (Klingenberg, 2013). Dichos análisis son, el Análisis de Componentes Principales (PCA) para reducir la dimensionalidad de los datos y determinar las variables elegidas para el estudio (Dujardín *et al*., 2014); el Análisis de Variación Canónica (CVA) para explorar las diferencias entre grupos (Zelditch *et al.*, 2004); y "Transformation Grid" y "Wireframe" para representar los cambios de forma de PCA y CVA, mostrando las variaciones para la ubicación relativa de cada punto de referencia. Además, una reclasificación con validación cruzada (Refaeilzadeh *et al*., 2009) y distancia de Mahalanobis (McLachlan, 1999) con permutación por pares, también son necesarias para verificar los resultados (Wink-da-Silva *et al*., 2018).

Los insectos son uno de los grupos que más atención han recibido en la aplicación de la MG (Calle *et al*., 2008; Bolufe-Torres *et al*., 2014; Dujardín *et al.*, 2014; Gerard *et al.*, 2015) con especial énfasis en los dípteros (Loh *et al*., 2008; Hall *et al.*, 2014; Espra *et al.*, 2015; Grzywacz *et al*., 2017a; Macedo, 2017; Godoy *et al*., 20018; Mikery *et al*., 2019; Szpila *et al*., 2019). Además de ser una herramienta para diferenciar poblaciones, subespecies o incluso especies muy emparentadas, la morfometría, en cuanto a forma y dimensiones del exoesqueleto, también nos da información sobre el estilo de vida del animal (Menes-Hernández, 2004). Esta herramienta, junto con el análisis molecular, son metodologías complementarias para obtener una buena y adecuada identificación a nivel de especie (Grzywacz *et al.*, 2017a).

Estimación del intervalo post-mortem (PMI)

La forma en que los insectos parasitan y colonizan los cadáveres se debe a un reloj endógeno de origen genético que marca la pauta de sus ritmos circadianos (Smith, 1986). Este oscilador interno afecta a su comportamiento y etología, que depende de la temperatura, la humedad, la intensidad de la luz y la duración relativa entre el número de horas de luz y oscuridad entre otros factores. El ritmo circadiano permite estimar el tiempo transcurrido desde la muerte o, lo que es lo mismo, el PMI junto con la identificación del posible traslado del cuerpo y, en su caso, las características de las zonas de origen (Amendt *et al.*, 2009). El inicio del PMI coincide con el momento en el que ocurre la primera oviposición por parte del díptero, mientras que el final es el descubrimiento del cadáver y el reconocimiento del estadio vital de la especie colonizadora más antigua que lo infesta (Gennard, 2007).

Las mediciones longitudinales de las larvas a diferentes temperaturas son las que se utilizan para calcular el tiempo mínimo transcurrido desde la muerte (Cavallari *et al.*, 2015). Los estudios de casos forenses en la literatura han utilizado esas metodologías de cálculo del PMI para resolver investigaciones criminales (Dutra-Kirst, 2006; Barros *et al.*, 2008; Krüger *et al.*, 2010; Grzywacz & Prado e Castro, 2012; Faria *et al.*, 2013; Cavallari *et al.*, 2015; Defilippo *et al.*, 2019). Además, hay bibliografía existente donde se recogen curvas de crecimiento a distintas temperaturas de diferentes especies de dípteros que pueden emplearse como base científica (Reiter, 1984; Marchiori & Do Prado, 1996, 1999; Grassberger & Reiter, 2001; Grassberger *et al.*, 2003; Monteiro & Do Prado, 2006).

Existen varios métodos para calcular este intervalo, sin embargo, los diagramas de isomegalen e isomorfen son las herramientas más utilizadas ya que proporcionan información gráfica de forma sencilla y muy intuitiva.

Diagramas isomegalen e isomorfen

Por un lado, el isomegalen-diagrama mide el tiempo transcurrido desde la eclosión de los huevos del insecto hasta el estado de desarrollo que alcanza en el cadáver. Dicha medida se realiza mediante la longitud de las larvas, expresada en milímetros, y se representa frente a la temperatura. Además, el diagrama también muestra el tiempo que tardan los huevos en eclosionar en función de la temperatura (Gennard, 2007).

Por otro lado, el isomorfen-diagrama representa las fases del insecto desde la oviposición hasta la emergencia del imago. Las zonas entre las líneas del diagrama simbolizan un cambio en el ciclo vital que da lugar al siguiente estadio (Gennard, 2007). Esta herramienta es útil cuando se encuentran larvas o pupas migratorias en el cadáver, ya que la longitud no es un criterio significativo para estimar la edad (Grassberger & Reiter, 2001).

La correcta identificación de las especies de insectos es un punto crítico y decisivo para su adecuada aplicación. Sin embargo, es igualmente importante enriquecer los conocimientos sobre morfología, taxonomía, evolución, biogeografía, ecología e historia de vida. Por ello, esta primera parte de la tesis se centra en la combinación de diferentes métodos de identificación, tanto morfométricos como moleculares, de las especies del género *Fannia* recogidas en un muestreo por toda la Península Ibérica. Dados los resultados, nos centramos en la especie *Fannia pusio*, de la que se estableció una colonia en condiciones de laboratorio y se calcularon las curvas de crecimiento de su ciclo biológico a diferentes temperaturas. Además, esta especie se ha introducido en España, por lo que su interés y la necesidad de aumentar nuestro conocimiento sobre ella es un punto relevante. Finalmente, los datos recogidos en esta parte se aplicaron por primera vez en la elaboración de diferentes métodos para el cálculo del PMI.

Insectos con fines alimenticios

Los insectos han sido utilizados como alimento desde los inicios de la humanidad, pero el coste de su recolección era mayor que la energía que proporcionaban. Por ello, la entomofagia se estableció como un hábito basado en la oportunidad (Cortes-Ortiz *et al.*, 2016). Los países de las regiones tropicales y subtropicales son los que mantienen la entomofagia como costumbre ya que en estas zonas los insectos se reproducen y crecen rápidamente debido a las altas temperaturas y humedades durante todo el año. Pese a carecer de estas condiciones, la alta tasa reproductiva y el corto ciclo biológico convierten a los insectos en alternativa a la ganadería convencional reduciendo así, la huella ecológica (Pérez-Altamirano, 2019). Además, al tratarse de animales de sangre fría, no tienen que gastar energía para regular su temperatura (Cortes-Ortiz *et al*., 2016) por lo que la transformación de residuos biológicos la realizan de manera muy eficiente (Lainez, 2017).

El consumo de insectos como fuente no convencional o adicional tiene muchas ventajas (Guiné *et al*., 2021): i) un gran número de propiedades nutricionales (FAO, 2010a; Alves *et al.*, 2019) que pueden ser moduladas a través de la dieta (De Marco *et al*., 2015), ii) tienen un bajo impacto ambiental (Veldkamp *et al*., 2012; Biasato *et al*., 2016) convirtiéndolos en un ingrediente sostenible, y iii) pueden ser producidos en espacios reducidos (Gahukar, 2020). Todas estas características supondrían una gran oportunidad para sostener a los países pobres (Rumpold & Schlüter, 2013; Guiné *et al*., 2021).

La demanda de alimentos, especialmente de proteína animal, está creciendo rápidamente debido al desarrollo de la población (FAO, 2010a; Alves *et al*., 2019). Este hecho impulsa la investigación de nuevas alternativas de insumos proteicos (Cortes-Ortiz *et al.,* 2016). La Organización Mundial de la Salud (OMS) y la Organización de las Naciones Unidas para la Alimentación y la Agricultura (ONUAA) han revelado que el consumo de insectos podría ser una forma viable de abordar las deficiencias nutricionales. Es por ello que, desde enero de 2018, los insectos están clasificados como "nuevo alimento" por la Agencia Europea de Seguridad Alimentaria (AESA) donde la especie *Tenebrio molitor* Linnaeus, 1758 (Coleoptera: Tenebrionidae), comúnmente llamada "gusano amarillo de la harina", ha sido recientemente autorizada (EFSA *et al.,* 2021)

Hay otras especies de insectos según la AESA (2015) con potencial para ser consumidas en la UE: *Musca domestica* Linnaeus, 1758 (Diptera: Muscidae), *Hermetia illucens* Linnaeus, 1758 (Diptera: Stratiomyidae), *Tenebrio molitor* Linnaeus, 1758 (Coleoptera: Tenebrionidae), *Zophobas atratus* Fabricius, 1775 (Coleoptera: Tenebrionidae), *Alphitobius diaperinus* Panzer, 1797 (Coleoptera: Tenebrionidae), *Galleria mellonella* Linnaeus, 1756 (Lepidoptera: Pyralidae), *Achroia grisella* Fabricius, 1754 (Lepidoptera: Pyralidae), *Bombyx mori* Linnaeus, 1758 (Lepidoptera: Bombycidae), *Acheta domesticus* Linnaeus, 1758 (Orthoptera: Gryllidae), *Gryllodes sigillatus* Walker, 1869 (Orthoptera: Gryllidae), *Locusta migratora migratorioides* Linnaeus, 1758 (Orthoptera: Acrididae), *Schistocerca americana* Drury, 1770 (Orthoptera: Acrididae).

Granja de *Tenebrio molitor* a pequeña escala

El escarabajo amarillo de la harina, *Tenebrio molitor*, es una de las especies permitidas para el consumo humano y animal según la normativa antes mencionada (EFSA *et al*., 2021). Su perfil nutricional es valioso con una alta cantidad de proteína bruta (20 ─ 71

%), aminoácidos esenciales (46,3 %) y ácidos grasos (22 ─ 55 %) (Gasco *et al.*, 2019). Además, Bosch *et al.* (2014) establecen una digestibilidad de la materia orgánica del 90,85 % y de la proteína del 91,60 %; y la cantidad de quitina es un valor añadido por su actividad prebiótica sobre la microbiota intestinal (Bovera *et al*., 2015). Sin embargo, el uso de insectos en los piensos tiene pequeñas desventajas, ya que pueden provocar reacciones alérgicas (Bessa *et al*., 2020) y contener sustancias tóxicas. Van Broekhoven *et al*. (2017) y Niermans *et al*. (2019) vieron en sus estudios cómo las larvas de *T. molitor* degradan y excretan micotoxinas y metales pesados, por lo que este último problema podría resolverse.

El uso de subproductos industriales, agrícolas y ganaderos para alimentar insectos hace que la competitividad de los recursos con los humanos sea prácticamente nula (Viñeta-Valdelvira, 2017), por lo que la cría de *T. molitor* sigue sumando puntos como alternativa alimentaria. Esta práctica fomenta la economía circular, la sostenibilidad, reduce el impacto ambiental y la huella ecológica debido a las bajas emisiones de gases de efecto invernadero (Biasato *et al*., 2016). La alta eficiencia de conversión alimenticia es también una característica a valorar en su eficiencia productiva frente a la ganadería convencional (Garnett *et al.*, 2015; Cortes-Ortiz *et al*., 2016), así como su rápida reproducción y corto ciclo de vida (Pérez-Altamirano, 2019). Además, tienen un menor riesgo de zoonosis (Ramón-Vázquez, 2014), e incluso se han utilizado en la terapia contra el cáncer (Suo *et al*., 2016; Wu *et al.*, 2020).

T. molitor es un insecto holometábolo (Cruz-Lozano, 2005), que tiene una metamorfosis completa siguiendo un ciclo vital de cuatro etapas: huevo, larva, pupa e imago. Las larvas del primer instar eclosionan aproximadamente a los 10 días de ser ovipositados los huevos. Éstas se alimentan y completan las mudas necesarias hasta alcanzar un tamaño adecuado para llevar a cabo la pupación. El tiempo transcurrido desde la eclosión de la larva más pequeña hasta la pupa suele ser de unos tres meses. Pasados 10 días desde que se forman las pupas, emergen los adultos que alcanzan la madurez sexual a los 12 días aproximadamente. Una vez copulan, necesitan ocho días para ovipositar. De esta forma, el ciclo completo de la especie puede durar hasta seis meses, ya que un adulto puede vivir hasta tres meses en condiciones óptimas: 25 ─ 28 ºC de temperatura ambiente, 50 ─ 55 % de humedad relativa, ciclo de luz 12:12, agua y dieta *ad libitum* a base de cereales y restos vegetales (Bernad-González, 2019).

La información sobre la automatización y optimización de la cría de *T. molitor* ayuda a separar cada fase del ciclo biológico de manera mecánica (Morales-Ramos *et al*., 2012; Cortes-Ortiz *et al.*, 2016; Gahukar, 2016; Bernad-González, 2019; Cadinu *et al.*, 2020). En la oviposición se necesita el mayor número posible de huevos con el mayor porcentaje de viabilidad; en la fase larvaria se requiere el mayor tamaño posible con un tiempo rápido; y en la fase adulta se busca la mayor fertilidad posible de los individuos para asegurar el éxito de la reproducción (Morales-Ramos *et al*., 2012).

Por tanto, la cría de insectos requiere de baja tecnología y capital donde el espacio necesario es asequible incluso para pequeños agricultores que empleen módulos a pequeña escala.

Anomalías encontradas en la cría de insectos

La producción de cualquier ser vivo tanto a pequeña como a gran escala debe estar controlado y monitorizado. La higiene, la alimentación, el control de los distintos procesos biológicos y la manipulación en cría son los factores más importantes en la producción de insectos con fines alimenticios. Si estas condiciones se ignoran pueden dar lugar a malformaciones desconocidas o, incluso, la muerte del individuo. Las teratologías en artrópodos son bastante comunes (Socha & Sehnal, 1972) pero pueden estar ocasionadas por factores muy diferentes: cambios físicos resultantes de alteraciones hormonales (Bong *et al.*, 2018), productos químicos, alteraciones genéticas (Mun *et al.*, 2019) o enfermedades derivadas de infecciones bacterianas (Zeikus & Steinhaus, 1968). En *T. molitor* se han observado diferentes anomalías que, principalmente, afectan a la pupa y a la emergencia del adulto dando lugar a un individuo con el abdomen y los élitros incompletos (Zeikus & Steinhaus, 1968; Socha & Sehnal, 1972).

La esclerotización y melanización del exoesqueleto de los insectos es la primera barrera de defensa frente a patógenos invasores y factores de estrés ambiental (Hayakawa *et al*., 2018). Además, la pigmentación también está implicada en la comunicación, comportamiento de apareamiento, cripsis, mimetismo e interacciones entre depredador y presa (Fukatsu & Futahaski, 2016; Mun *et al*., 2019). Por tanto, el exoesqueleto y su correcto desarrollo es esencial para la respuesta inmune del insecto, así como para la resistencia a parásitos; una síntesis incorrecta de las proteínas implicadas en la formación de la cutícula puede provocar la muerte del individuo (Parkinson *et al.*, 2003; Hattori *et*

al., 2005; Hoegger *et al.*, 2006; Bettedi *et al.*, 2011; Strong & Claus, 2011; Balabanidou *et al*., 2018; Wang *et al.*, 2018). Es por ello, que el silenciamiento génico mediante RNA de interferencia de algunos de estos genes se ha propuesto para controlar plagas (Du *et al*., 2017; Matsumoto & Hattori, 2019; Liu *et al.*, 2020).

Múltiples genes intervienen en la ruta metabólica del endurecimiento y pigmentación del exoesqueleto (Mun *et al.*, 2019), y el correcto desarrollo del individuo depende del nivel de expresión. La *Laccase2* (*Lac2*) es una de las proteínas más importantes en dicha ruta metabólica ya que es la responsable del oscurecimiento y endurecimiento de la cutícula (Simon *et al.*, 2009); mientras que en la coloración intervienen *Arilalquilamina N-acetiltransferasa* (*AANAT1*) y *Tirosina Hidroxilasa* (*TH*) (Mun *et al.*, 2019). Para examinar los niveles de expresión génica se emplea la reacción en cadena de la polimerasa en tiempo real (RT-qPCR) como metodología principal. Existen trabajos donde se han visto alteraciones de dichas expresiones en diferentes condiciones (Lord *et al.*, 2010; Toutges *et al.*, 2010; Liu *et al.*, 2015; Sang *et al.*, 2015; García-Reina *et al.*, 2018; Mun *et al.*, 2019).

Debido a la escasez de proteína de origen animal se están estudiando sustitutos cuya producción sea sostenible y que, además, potencien la economía circular. Según la AESA los insectos son los principales candidatos con este fin y es, por esta razón, que las granjas de insectos están en auge, no sólo en diversos países de Europa, sino también en España. La puesta en marcha de instalaciones de este tipo está sacando a la luz a problemas de cría antes desconocidos. Es por ello, que es necesario aumentar el conocimiento de la biología y etología de las especies de interés alimentario, para permitir una producción eficiente y con la misma seguridad desde el punto de vista sanitario.

Objetivos

Los dos objetivos principales de esta tesis son la correcta identificación de especies de dípteros necrófagos mediante metodologías moleculares y morfométricas, y el correcto manejo y mantenimiento de especies de insectos comestibles en condiciones controladas de cría a baja escala. Esta información permitirá ampliar el conocimiento de la biología, ecología y etología de los insectos para su aplicación en dos ramas principales de la entomología aplicada: la entomología forense y los insectos con fines alimentarios.

Los resultados obtenidos se dividen en cinco capítulos organizados en dos partes. La primera parte se compone de los capítulos I, II y III, donde se identifican muestras de *Fannia pusio* recogidas en la Península Ibérica, y se analizan mediante técnicas moleculares y morfométricas para generar conocimiento que permita su posterior uso en el cálculo del intervalo post-mortem (PMI). La segunda parte se compone de los capítulos IV y V donde se optimiza la cría de *Tenebrio molitor* a pequeña escala realizando estudios técnicos y económicos; en este proceso, la visualización de malformaciones en la transformación de pupa a imago, nos lleva a analizar las diferencias de expresión génica entre individuos sanos y anómalos.

Capítulo I. Identificación por barcoding y análisis filogeográfico de *Fannia pusio* (Diptera: Faniidae) en la Península Ibérica.

El objetivo general de este capítulo es la identificación a nivel de especie mediante análisis molecular de individuos del género *Fannia* recogidos en un muestreo realizado en toda la Península Ibérica. Este capítulo se centra en *Fannia pusio* (Wiedemann, 1830) como la especie de Fanniidae más abundante en el muestreo.

Los objetivos específicos son:

- Aumentar el número de secuencias del género en la base de datos del GenBank.
- Comprobar la capacidad de la región mini-barcode *cox1* en la correcta identificación de *F. pusio* de la Península Ibérica.
- Identificar a *F. pusio* reconociendo sus peculiaridades genéticas, su patrón de distribución filogeográfica e inferir los procesos implicados.

Capítulo II. La cabeza de *Fannia pusio* (Diptera: Fanniidae) como nueva fuente de datos morfométricos para la evaluación de la variación a lo largo de líneas geográficas y biológicas.

El objetivo general de este estudio es complementar los resultados de los datos moleculares recogidos en el capítulo I con un análisis morfométrico, donde se evaluará la variación geográfica y biológica de la especie.

Los objetivos específicos son:

- Estudiar la cabeza de *F. pusio* como estructura taxonómica válida.
- Validar los puntos de referencia de la cabeza en el contexto de las diferencias geográficas y biológicas de *F. pusio.*

Capítulo III. Desarrollo de *Fannia pusio* (Diptera: Fanniidae) en condiciones de temperatura controlada y su aplicación en la estimación del intervalo post-mortem (PMI).

El objetivo general de este capítulo es evaluar, por primera vez, el efecto de la temperatura en el desarrollo de *F. pusio* proporcionando datos para que los especialistas forenses puedan determinar su idoneidad para una estimación más precisa del PMI.

Los objetivos específicos son:

- Obtener las longitudes de todos los estadios larvarios a diferentes temperaturas, desde la eclosión hasta la emergencia de los imagos.
- Calcular los diagramas de isomegalen e isomorfen, mediante la curva de crecimiento obtenida de la matriz de datos.

Capítulo IV. Estudios de producción y viabilidad económica en la elaboración de un proyecto de cría del gusano de la harina*, Tenebrio molitor.*

El objetivo general de este estudio es analizar las condiciones óptimas para la cría y el manejo de *T. molitor* en una instalación de producción a pequeña escala que permita a los emprendedores con algún espacio disponible a convertirse en pequeños ganaderos de insectos.

Los objetivos específicos son:

- Calcular la producción anual de una pequeña instalación de *T. molitor* en condiciones óptimas en las dimensiones disponibles, según las etapas del ciclo vital de la especie.
- Calcular la viabilidad económica de la instalación para establecer su rentabilidad.

Capítulo V. Análisis de la expresión de los genes *Lac2, TH* y *AANAT1* relacionados con las malformaciones del exoesqueleto en *Tenebrio molitor* (Coleoptera: Tenebrionidae) criado.

El objetivo general del estudio es analizar la expresión génica de tres genes relacionados con la formación de la cutícula de *T. molitor* en individuos con un desarrollo de la cutícula normal frente a individuos con desarrollo anómalo. La expresión alterada de estos genes, que codifican proteínas esenciales para la vida adulta, puede conducir a un desarrollo anómalo de la cutícula con las correspondientes limitaciones para la supervivencia y la reproducción. Una mejor comprensión de los mecanismos genéticos implicados ayudará a mejorar los sistemas de producción minimizando los costes.

Los objetivos específicos de este trabajo son:

- Verificar los niveles de expresión de las proteínas *Lac2, AANAT1* y *TH* en adultos normales y malformados mediante RT-qPCR.
- Discutir la relación de estas malformaciones con el manejo de la producción y el estrés de la colonia.

Resúmenes de capítulos

La información reunida en esta tesis aumenta el conocimiento de dos especies con objetivos diferentes en entomología aplicada. Los resultados obtenidos en la Parte I aportan información que pone de manifiesto la utilidad potencial de *Fannia pusio* en entomología forense, mientras que los resultados de la Parte II abarcan información sobre *Tenebrio molitor*, una especie recientemente aprobada para fines alimentarios.

Capítulo I. Identificación por barcoding y análisis filogeográfico de *Fannia pusio* (Diptera: Fanniidae) en la Península Ibérica.

Fannia es un género de dípteros perteneciente a la familia Fanniidae con aproximadamente 300 especies; son atraídos por la materia orgánica en descomposición y presentan hábitos necrófagos, lo que arroja luz sobre cuestiones criminológicas. Además de la importancia jurídica, tienen interés médico y económico, ya que son capaces de causar miasis en animales domésticos y en el hombre. Por estas razones, su identificación a nivel de especie basada en caracteres morfológicos en ocasiones es difícil debido a la mala conservación de las muestras en los cadáveres en descomposición. Una alternativa para superar esta situación es el código de barras de ADN mediante el análisis molecular de un fragmento de 658 pb del genoma mitocondrial (*cox1*). Sin embargo, las secuencias de este fragmento pueden estar contaminadas con restos de ADN externos, por diferentes motivos. Una alternativa es el uso de regiones cortas e internas (mini-barcode); en este trabajo mostramos cómo un fragmento más pequeño de 240 pb es capaz de identificar correctamente las especies del género *Fannia* que se recogieron en un estudio en toda la Península Ibérica.

La identificación molecular mostró que *Fannia pusio* (Wiedemann, 1830) fue la especie más abundante recogida. Además, se han identificado mutaciones particulares que permiten diferenciar los haplotipos de los individuos del noreste y del sureste. Las secuencias disponibles en las bases de datos analizadas junto con los resultados de este trabajo sugieren que la especie es originaria de América y que posteriormente se introdujo en la Península Ibérica a través de Portugal, sufriendo una continua expansión de su área de distribución.

Capítulo II. La cabeza de *Fannia pusio* (Diptera: Fanniidae) como nueva fuente de datos morfométricos para la evaluación de la variación a lo largo de líneas geográficas y biológicas.

Fannia Robineau-Desvoidy, 1830 es el género más diverso de la familia Fanniidae (Diptera) con 288 especies que incluyen muchas de interés sanitario, económico y legal. La homogeneidad morfológica dentro del género dificulta a menudo la determinación de las especies. La mejor opción para una correcta identificación es la combinación del análisis molecular con los estudios morfometría. La variación en la forma de una selección de caracteres corporales es evaluada por la Morfometría Geométrica empleando la cabeza como estructura innovadora. El sexo debe tenerse en cuenta como una covariable clave en este tipo de estudios, ya que *Fannia*, como muchos otros dípteros, tiene una estructura de cabeza sexualmente dimórfica, con machos holópticos y hembras dicópticas. En primer lugar, analizamos un conjunto de ejemplares de *Fannia* sp. muestreados en la Península Ibérica (2012 – 2015), de los cuales *F. pusio* (Wiedemann, 1830) se estableció como la especie más abundante. Nuestros análisis proporcionaron información morfológica significativa donde *F. pusio* muestra una clara variación morfométrica intraespecífica a lo largo de un eje este-oeste en toda la Península Ibérica. Un patrón similar se obtuvo de una colonia criada en laboratorio frente a muestras silvestres.

Capítulo III. Desarrollo de *Fannia pusio* (Diptera: Fanniidae) en condiciones de temperatura controlada y su aplicación en la estimación del intervalo post-mortem (PMI).

Fannia pusio (Wiedemann, 1830) es una especie perteneciente a la familia Fanniidae de gran interés forense, sanitario y veterinario. Las peculiaridades de comportamiento que presenta esta especie pueden aportar información adicional para futuras investigaciones. La aplicación de la entomología en el ámbito forense se ha centrado especialmente en los primeros taxones colonizadores de cadáveres en la fase inicial de descomposición. Sin embargo, las especies que aparecen en fases más avanzadas pueden contribuir a ampliar los conocimientos, como es el caso de *F. pusio*. Además, tiene la capacidad de colonizar

cadáveres enterrados, condición en la que los dípteros de mayor tamaño son incapaces de hacerlo.

En este trabajo se estudia el comportamiento de *F. pusio* en un rango de temperaturas que va desde los 5 ºC hasta los 40 ºC. Se han realizado diferentes análisis estadísticos para estudiar su comportamiento y los datos recogidos se han aplicado para calcular el intervalo post-mortem (PMI) mediante los diagramas de isomorfen e isomegalen. Por lo tanto, los resultados mostrados en este manuscrito ayudan, junto con la bibliografía existente de otras especies, a ampliar el conocimiento de la entomofauna que se da en las investigaciones forenses.

Capítulo IV. Estudios de producción y viabilidad económica en la elaboración de un proyecto de cría del gusano de la harina, *Tenebrio molitor.*

La demanda de alimentos aumenta considerablemente debido al incremento de la población. Además, dicha necesidad también nace de la competencia generada por el uso de proteína animal en piensos. Para paliar la escasez, los insectos se han convertido en una alternativa prometedora en vista de su alto contenido en proteínas, grasas y fibras. A su vez, es una fuente de alimento sostenible y de bajas emisiones de carbono, dando lugar a una reducción de la huella ecológica que suele estar tan castigada por el ganado convencional. Recientemente, La Agencia Europea de Seguridad Alimentaria ha aceptado al gusano amarillo de la harina (*Tenebrio molitor*) como especie segura para alimentación humana. Esto conlleva a que se convierta en el principal candidato para la elaboración del estudio de viabilidad de una granja de insectos. En este trabajo se analizan las condiciones óptimas de la cría de *T. molitor*, su manejo en dichas condiciones, el cálculo de la producción anual en las instalaciones y su viabilidad económica para conocer la rentabilidad del proyecto.

Capítulo V. Análisis de la expresión de los genes *Lac2, TH* y *AANAT1* relacionados con las malformaciones del exoesqueleto en *Tenebrio molitor* (Coleoptera: Tenebrionidae) criado.

En el correcto desarrollo de los insectos influye la esclerotización y melanización del exoesqueleto, que interviene en la función inmunitaria del individuo. Con el auge de las

granjas de insectos, las anomalías que antes se desconocían son ahora más frecuentes. Nuestra granja de *Tenebrio molitor* (Coleoptera: Tenebrionidae) es una instalación a pequeña escala, donde la producción de esta especie es rutinaria. Se ha observado en muchas ocasiones que el proceso de metamorfosis no se completa, dando lugar a individuos anómalos con diferentes teratologías. Los más comunes son los adultos con un desarrollo anómalo que muestran el abdomen y los élitros incompletos.

Tres de los genes implicados en la vía metabólica de la esclerotización y melanización (*TH*, *AANAT1* y *Lac2*) se analizan mediante RT-qPCR, utilizando un gen de referencia previamente validado (*Rps3*), para determinar el nivel de expresión de estos genes. Se eligieron individuos anómalos con la misma malformación e individuos normales y se utilizaron como controles.

Conclusiones

Capítulo I. Identificación por barcoding y análisis filogeográfico de *Fannia pusio* (Diptera: Fanniidae) en la Península Ibérica.

Un muestreo exhaustivo en la Península Ibérica muestra la presencia del género *Fannia* del cual, *F. pusio* es la especie predominante.

- Un mini-barcode de 240 pb ha demostrado ser útil para la identificación de las especies de *Fannia*, contribuyendo a la investigación forense y médica.
- Las mutaciones particulares diferencian los haplotipos del noreste y del sureste de la Península Ibérica.
- *F. pusio* se originó en América y se introdujo en la Península Ibérica a través de Portugal, sufriendo posteriormente una continua expansión del área de distribución.

Capítulo II. La cabeza de *Fannia pusio* (Diptera: Fanniidae) como nueva fuente de datos morfométricos para la evaluación de la variación a lo largo de líneas geográficas y biológicas.

- Los puntos de referencia de la cabeza de *F. pusio* proporcionan información morfométrica válida para los estudios de identificación de la especie.
- Las áreas parafacial y fronto-orbital de *F. pusio* son las estructuras de la cabeza más variables.
- La variación de las condiciones ambientales en la Península Ibérica parece haber originado en *F. pusio* la diferenciación intraespecífica entre las poblaciones del este y del oeste.
- Los individuos de *F. pusio* criados en el laboratorio presentan particularidades morfométricas que los diferencian de los recolectados en la naturaleza, probablemente debido a las condiciones ambientales estables y a la elevada endogamia en la colonia de laboratorio.

Capítulo III. Desarrollo de *Fannia pusio* (Diptera: Fanniidae) en condiciones de temperatura controlada y su aplicación en la estimación del intervalo post-mortem (PMI).

- Los resultados obtenidos indican que el rango de viabilidad para el desarrollo en *F. pusio* oscila entre 15 ºC y 35 ºC, siendo 25 ºC la temperatura en la que el estrés de los individuos parece ser menor.
- Los diagramas de isomegalen e isomorfen dan resultados coherentes, mostrando un crecimiento más acelerado con tallas más pequeñas a temperaturas más altas.
- *F. pusio* parece ser un buen indicador estacional que proporciona información útil en las investigaciones forenses.

Capítulo IV. Estudios de producción y viabilidad económica en la elaboración de un proyecto de cría del gusano de la harina, *Tenebrio molitor*.

- La información recopilada y la proyección realizada proporcionan datos optimizados para poner en marcha una granja de *T. molitor* a pequeña escala, basándose en el ciclo biológico de la especie y en el espacio disponible de la instalación.
- Se obtiene por primera vez una estimación de la producción anual de *T. molitor* y su viabilidad económica, proporcionando resultados rentables para su desarrollo.
- Los cálculos muestran un VAN de 82.468,87 € y una TIR del 27,50 %, proporcionando datos para una vida útil del proyecto de cinco años.

Capítulo V. Análisis de la expresión de los genes *Lac2, TH* y *AANAT1* relacionados con las malformaciones del exoesqueleto en *Tenebrio molitor* (Coleoptera: Tenebrionidae) criado.

Los individuos anómalos muestran una sobreexpresión del gen *TH* y una subexpresión de los genes *AANAT1* y *Lac2*.

- El patrón de expresión obtenido podría alterar la vía metabólica de melanización y esclerotización de los adultos, aumentando los niveles de dopamina en la hemolinfa y provocando una alteración del desarrollo normal.

- El patrón de expresión encontrado en los individuos anómalos podría estar correlacionado con el estrés generado por la manipulación diaria, provocando un aumento de la dopamina en la hemolinfa.

Introduction

General introduction

Arthropods are the most numerous and diverse group in the animal kingdom with a species richness of 5 to 10 million species (Ødegaard, 2000) that extends over the entire planet. Insecta is the most abundant class and consists of more than 5.5 million species according to recent estimates (García-Robledo *et al.*, 2020). Although some species have harmful effects (White, 1976), many others are beneficial to humans, domestic animals and crops.

The analysis of these effects, both harmful and beneficial, is the subject of study of applied entomology. This discipline encompasses the study of insects and other arthropods of interest to humans, either for the products they provide or for the negative impact they cause on important goods. There are seven major branches where insects benefit us (Clark, 2009): i) In the ecological field it is worth mentioning services of pollination (Gabriel & Tscharntke, 2007; Sawe *et al.*, 2020); ii) in agriculture they are used for biological control, i.e. entomophagous organisms (parasites, parasitoids, predators, pathogens or competitors) are used for the suppression of insect pest species (Chatterjee *et al.*, 2009; Sethuraman *et al.*, 2020) and for fertilizer production (Arango-Gutiérrez & Agudelo-Bentacur, 2004; Piñero *et al.*, 2018); iii) in forestry they are also used as biological control (Sethuraman *et al.*, 2020); iv) in both animal and human health, larval treatment is becoming an emerging therapy for the removal of necrotic tissues (Forero-Becerra, 2011; Reyes-Parrado *et al.*, 2020), although aspects such as passively and directly caused diseases (allergic reactions caused by stinging, contact or inhalation, and the vectorial role of diseases); v) in the legal field they can be exploited as forensic daters (Smith, 1986) and other applications (Baz *et al.*, 2015; Eon, 2019); vi) in the food field, insects are increasingly valuable due to their high protein content, as well as in such ancient activities as astaciculture, beekeeping, and sericulture (Lizhang *et al.*, 2008; Van Huis *et al.*, 2020); and vii) in the feed production insects are being incorporated, either live or as hydrolysed proteins in pets, aquaculture, poultry and pigs (Van Huis *et al.*, 2020).

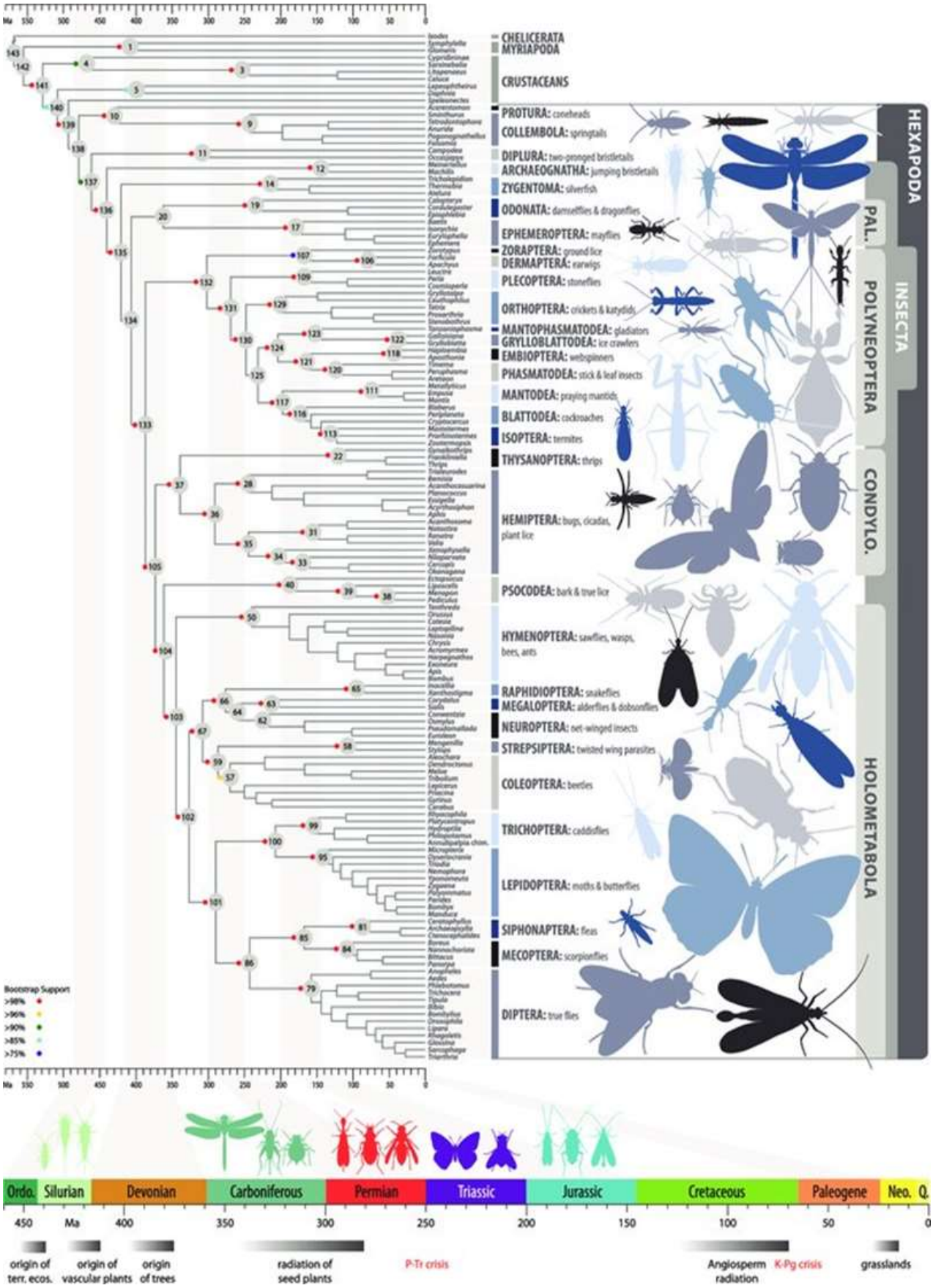


Figure 1. Phylogenetic tree of insect diversity relationships. The timeline at the bottom of the tree relates the geological origin of the insect clades to the main geological and biological events (Misof *et al.*, 2014).

In this work we focus on two of the above-mentioned applications of insects: 1) legal or forensic use and 2) food and feed production.

Forensic Entomology

Forensic entomology is a branch of forensic science that draws conclusions from information provided by insects. It is a discipline based on the study of the interactions of a great diversity of different species with their environment. Furthermore, this fauna manifests morphological disparities, which is difficult to handle for a non-expert in entomology (Lutz *et al.*, 2021).

Many species are involved in the investigations, but those belonging to the order Diptera are the most common, as they are the first to colonise the corpse. Adult females are attracted to the carcass to lay their eggs and, once the larvae hatch, feed on the carcass (Smith, 1986). However, it should be noted that other species only come to the body because they prey on necrophagous insects (Gennard, 2007). As mentioned, one of the main sources of tissue invasion is through the order Diptera which can cause such detrimental effects as myiasis, parasitising both animals and humans (Smith, 1986). Also, by parasitising domestic animals they can spread all kinds of zoonoses (Grzywacz *et al.*, 2017b).

The first documented case of forensic entomology appears in a 13th century Chinese legal manual written by Sung Tz'u explaining the murder of a farmer. Doctors and jurists began to use this science as a complementary technique in criminal investigations. The processes of corpse decomposition (physical and chemical) and all the accompanying phenomena also attracted attention in the art world (Fig. 2) (Benecke, 2001). However, it was not until 1894 that Megnin observed the selective and orderly attraction of arthropods to the corpse helping to indicate the time elapsed since death, and defined them as "Death Squads". This discovery led to the

Figure 2. Observation of decomposition of human corpses: "Dance of the Death" (Stammler, 1460) (left); grave of Robert Touse who "expects the resurrection of the dead" (exact time of making unknow) (right) (Benecke, 2001).

publication of "La Faune des Cadavres: Application de l'Entomologie à la Médicine Légale", which describes the different stages of decomposition of a corpse. Forensic pathologists use a scale composed of four stages established according to the state of the corpse (fresh, emphysematous, putrefactive and skeletal); while forensic entomologists describe five stages where the state of decomposition and the associated arthropod groups are assessed (fresh, emphysematous, early putrefaction, late putrefaction and skeletal) (Gennard, 2007).

The establishment of forensic entomology as a recognised tool for crime scene investigation did not occur until 1850. The mummified body of a new born was found inside a chimney behind the mantelpiece of a house. Dr. Marcel Bergeret performed the autopsy and discovered fly larvae (*Sarcophaga carnaria* L) and several species of moths. He concluded that the baby's body had been sealed in 1848 and that the insects gained access to it in 1849. As a result, the previous to 1848 inhabitants were indicted while the current occupants were exonerated (Bergeret, 1855).

This discipline could be perfected and developed as a scientific study at the end of the 20th century and the beginning of the 21st century. It analyses all aspects related to insects that affect or involve legal aspects, but its scope goes beyond the mere identification of the entomological fauna of corpses (Medico-Legal Entomology) (Hall, 1990), which must be carried out correctly and unequivocally. The colonisation of corpses by insects follows a characteristic pattern that is modulated by environmental conditions and location (buried, outdoors or submerged in water). It can also be used to date of protected species, determine when death occurred and identify the cause of death (Smith, 1986). In addition to its usefulness in estimating aspects of the post-mortem interval (PMI), determining the species in relation to the geographic distribution of that species can be useful in determining location (Byrd & Sutton, 2019).

The order Diptera is the first to be attracted to the corpse, with the families Calliphoridae, Sarcophagidae and Muscidae being the most common (Smith, 1986; Gennard, 2007; Amendt *et al.*, 2009). However, the family Fanniidae also figures prominently in forensic medicine, but is less well known (Szpila, 2019).

Fannia pusio (Diptera: Fanniidae)

Fanniidae (Diptera) is a Calyptrate family with approximately 330 species distributed in five genera: *Fannia* Robineau-Desvoidy, 1830, *Piezura* Rondani, 1866, *Euryomma* Stein, 1899, *Australofannia* Pont, 1977 and *Zealandofannia* Domínguez & Pont, 2014 (Couri & Sousa, 2019). The genus *Fannia* is the largest, comprising up to 60 species (De Carvalho & Mello-Patiu, 2008) with the following (Al Gazi *et al.,* 2004): i) small size; ii) dark integument; iii) predominantly yellow abdomen (Rozkosný *et al*., 1997); iv) submedian dorsal seta on the posterior tibia; v) short vein CuA+1A as a prolongation of vein 2A of the wing margin (Pont, 1977).

Fannia pusio (Wiedemann, 1830) is one of the species within the genus *Fannia* and is commonly known as the "chicken dung fly" because of its common occurrence on laying hen farms (Al Gazi *et al.,* 2004). The most relevant taxonomic characters of *F. pusio* (Fig. 3) are hind tibia with long setae on ventral surface in males, and pilosity of the parafrontal hairs protruding around the eye margin in females (De Carvalho & Mello-Patiu, 2008). Although the species has a Nearctic origin (Couri & Sousa, 2019) it is currently found worldwide due to livestock transport (Grzywacz & Prado e Castro, 2012). It has a preference for warm areas (Smith, 1986; Bélo *et al.,* 1998) and it was in Brazil, the native distribution of the species (Marchiori *et al*., 2000), that its importance in medicine and veterinary medicine began to be considered. However, several studies have found it during all seasons of the year (Leandro & D'Almeida, 2005; Omar *et al.,* 2008; Barbosa *et al*., 2009; Krüger *et al*., 2010; Aballay *et al*., 2012).

Figure 3. *Fannia pusio* adult (Quinn, 2018. College Station, Brazos County, Texas, USA).

It is a well-studied species (see De Carvalho *et al.,* 2002, 2003 and references therein) due to its economic importance and synanthropic habits (Linhares, 1981; Leandro & D'Almeida, 2005). However, further studies are needed to improve knowledge of their biology, ecology and behaviour (Szpila *et al.,* 2019). The sanitary and legal interest harbours for its presence in animal and human carcasses (Mendes & Linhares, 2002). Part of the economic and sanitary importance of the species stems from the common role of the female as a phoretic host for eggs of *Dermatobia hominis* (the human botfly) (Fig. 4) that causes myiasis in humans and some domestic animal species (Gomes *et al.*, 2002).

For species to be forensically useful, correct identification is crucial. In particular, species of the genus *Fannia* are very similar to each other morphologically (Couri & Sousa, 2019) and taxonomic keys based on morphology are often insufficient (Grzywacz *et al.*, 2017b). Likewise, in the identification keys of Muscidae and Fanniidae from Europe, the species *F. canicularis* and *F. scalaris* are the most cited (Pont, 2015; Grzywacz & Prado e Castro, 2012), while *F. pusio* was not included despite being common in the southeast of the Iberian Peninsula. Therefore, more specialised techniques are used in legal studies for correct identification (Ekrem *et al.*, 2007; Dujardín, 2008; Kosmann, 2009; Alves-Rolo, 2010; Arnaldos *et al.*, 2015; Yussef-Vanegas & Agnarsson, 2017; Onder *et al.*, 2019; Fuentes-López *et al.*, 2020).

Figure 4. Ventral view of *Sarcopromusca pruna* (Diptera) as a vector of *D. hominis* eggs on the abdomen (Azevedo et al., 2007).

Identification methods

The application of insects in forensic science requires correct identification, as mentioned above. In addition, entomological evidence found in investigations is often damaged, contaminated and in a pre-imaginal state, where dichotomous keys are limited (Zehner *et al.*, 2004). Therefore, the more powerful techniques that have been developed in recent decades have a greater range of reliability and efficacy.

Molecular techniques

Molecular techniques offer advantages where other methods are insufficient. One of the biggest problems is damaged or poorly preserved samples that make it unfeasible to recognize the characteristic phenotypes of each species (Hoyos-López *et al*., 2012; Li *et al*., 2015; Shi *et al*., 2017). DNA is present not only in eggs, in each larval stage and in adults, but also in empty pupae (when the adult has emerged) (Mazzanti *et al*., 2010). This represents an enormous advantage for using the latter stage for species identification by molecular techniques, and thus for forensic purposes.

The most commonly used technique is "barcoding" which uses a small fragment called “barcode” of the cytochrome oxidase subunit one gene (*cox1*) with a size of 658 bp

(Hebert *et al*., 2003). However, smaller fragments (240 bp) called "mini-barcode" are starting to be used because of the difficulty of extraction in certain occasions (Meusnier *et al*., 2008, Lee *et al*., 2015; Grzywacz *et al*., 2017b; Yeo *et al*., 2020). Barcode is the catalytic subunit of the enzyme that predominates in mitochondrial (Kerr *et al.*, 2009; Alves-Rolo, 2010; Pérez & Henao, 2013) and is haploid in nature with maternal inheritance without recombination (Kosmann, 2009). This generates species-specific markers which provides the efficacy of this methodology (Harvey *et al.*, 2003). This is why this gene has been used in the study of the origins and evolution of insect populations through haplotype analysis (López-López *et al*., 2016; Hurtado-Burillo *et al.*, 2017; Silva *et al.*, 2020).

Sequences obtained in scientific works for all organisms are deposited in the NCBI (National Centre for Biotechnology Information) database (Fig. 5) as a prerequisite for publication. These sequences are available to all scientist and can be downloaded from NBCI using the BLAST tool (Altschul *et al*., 1997) allowing them to be included in subsequent analyses with new approaches producing new data for publications. In this way we can compare with new approaches. In this way we can compare our generated sequences with other already recorded sequences from the same and related taxa. To do so, the lowest E-value and the highest Score in the BLAST tool must be taken into account, i.e., maximising the similarities between sequences (Hebert *et al*., 2003) to construct neighbour-joining phylograms for species identification.

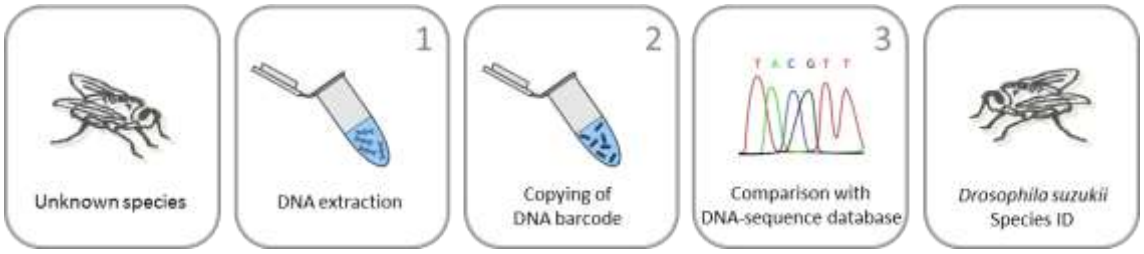


Figure 5. Diagram of the steps to follow for the molecular DNA Barcoding technique (Sinsoma, https://sinsoma.com/en/technology/dna-barcoding/).

This technique has allowed the identification of numerous species of the order Diptera in forensic cases (Coyle *et al.*, 2005; Kosmann, 2009; Alves-Rolo, 2010; Boehme *et al.*, 2012; Arnaldos *et al.*, 2015; Grzywacz *et al.*, 2017b; Fuentes-López *et al*., 2020, 2021). However, other insect-related fields have also turned to molecular techniques such as

environmental genomics (Valentini *et al*., 2009; Kang *et al*., 2017), as well as other aspects of medical and veterinary importance (Hoyos-López *et al*., 2012; Rodrigues *et al.*, 2018; Onder *et al*., 2019).

The genetic information obtained can also be used to study phylogeographic relationships between different species and within closely related species (Lambertini *et al*., 2006; Fuentes-López *et al.,* 2021). It is a field of study that addresses 1) the identification of geographic distributions patterns of genealogical lineages, by constructing genealogies of populations and genes, and 2) the inference of the processes that have originated those patterns (Avise, 2000).

Morphometric technique

Geometric morphometrics (GM) is a methodology that together with molecular analysis gives very effective results for species-level identifications (Hurtado-Burillo, 2015). GM studies morphometric using Cartesian coordinates (landmarks) of reference points that are capable of collecting different shape variables and can be analysed using various statistical techniques (Bookstein, 1982). It is therefore a powerful approach to quantify variables such as biological shape, variation and covariation shape (Zelditch *et al*., 2004) with other biotic or abiotic variables or factors (Webster & Sheets, 2017). These coordinate points correspond to particularities present in all individuals that follow the same arrangement (Zelditch *et al*., 2004). The technique only analyses shape while ignoring the size, position and orientation of the specimen and therefore helps to understand many unresolved questions about the evolution and development of the organism (Klingenberg, 2013).

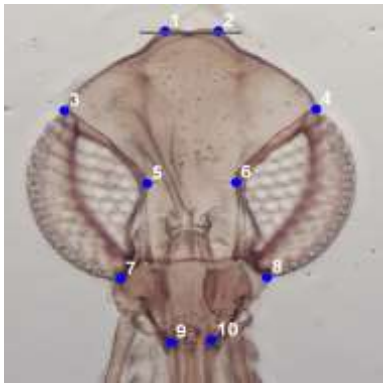


Figure 6. Distribution of the landmark used to analyse the head of Chagasi Series species (Diptera: Psychodidae: Phlebotominae) (Godoy *et al*., 2018).

The great advantage of the method is that the data can be analysed routinely in a directly manner with statistical software (Klingenberg, 2013). Such analyses are Principal Component Analysis (PCA) to reduce the dimensionality of the data and determines the variables chosen for the study (Dujardín *et al.*, 2014); Canonical Variation Analysis (CVA) to explore differences between groups (Zelditch *et al*., 2004); and Transformation Grids and Wireframe to represent PCA and CVA shape changes, showing the variations

for the relative location of each reference point. In addition, a cross-validated reclassification (Refaeilzadeh *et al*., 2009) and Mahalanobis distance (McLachlan, 1999) with pairwise permutation are also necessary to verify the results (Wink-da-Silva *et al*., 2018).

Insects are one of the groups that have received most attention in the application of GM (Calle *et al.,* 2008; Bolufe-Torres *et al.,* 2014; Dujardín *et al*., 2014; Gerard *et al.*, 2015) with special emphasis on Diptera (Loh *et al.,* 2008; Hall *et al.,* 2014; Espra *et al.*, 2015; Macedo, 2017; Grzywacz *et al.*, 2017a; Godoy *et al.*, 2018; Mikery *et al*., 2019; Szpila *et al*., 2019). In addition, to being a tool for differentiate populations, subspecies or even closely related species, morphometric, in terms of shape and dimensions of the exoskeleton, also gives us information about the animal's lifestyle (Menes-Hernández, 2004). This tool, together with molecular analysis, are complementary methodologies to obtain a good and adequate identification at species level (Grzywacz *et al.,* 2017a).

Post-mortem interval estimation (PMI)

The way insects parasitise and colonise corpses is due to an endogenous clock of genetic origin that they all possess and that sets the pattern of their circadian rhythms. This internal oscillator affects their behaviour and ethology, which depends on temperature, humidity, light intensity and the relative duration between the number of hours of light and darkness, among other factors. The circadian rhythm allows estimation of the time since death or, in other words, the PMI together with the identification of the possible movement of the body and, if applicable, the characteristics of the areas of origin (Amendt *et al*., 2009). The beginning of the PMI coincides with the time when the first oviposition by the dipteran occurs, while the end is the discovery of the corpses and the recognition of the life stage of the oldest infesting colonising species (Gennard, 2007).

Longitudinal measurements of larvae at different temperatures are used to calculate the minimum time since death (Cavallari *et al.,* 2015). Forensic case studies in the literature have used such PMI calculation methodologies to solve criminal investigations (Dutra-Kirst, 2006; Barros *et al.,* 2008; Krüger *et al*., 2010; Grzywacz & Castro, 2012; Faria *et al*., 2013; Cavallari *et al*., 2015; Defilippo *et al.,* 2019). In addition, there is existing literature where growth curves at different temperatures of different dipteran species are

collected and can be used as a scientific basis (Reiter, 1984; Marchiori & Do Prado, 1996, 1999; Grassberger & Reiter, 2001; Grassberger *et al.*, 2003; Monteiro & Do Prado, 2006).

There are several methods for calculate this interval, the isomegalen and isomorphen diagrams are the most commonly used tools as they provide graphical information in a simple and very intuitive way.

Isomegalen and isomorphen diagrams

On the one hand, the isomegalen-diagram measures time elapsed from the hatching of the insect eggs to the stage of development reached in the corpse. This is measured by the length of the larvae, expressed in millimetres, and plotted against temperature. In addition, the diagram also shows the time it takes for the eggs to hatch as a function of temperature (Gennard, 2007).

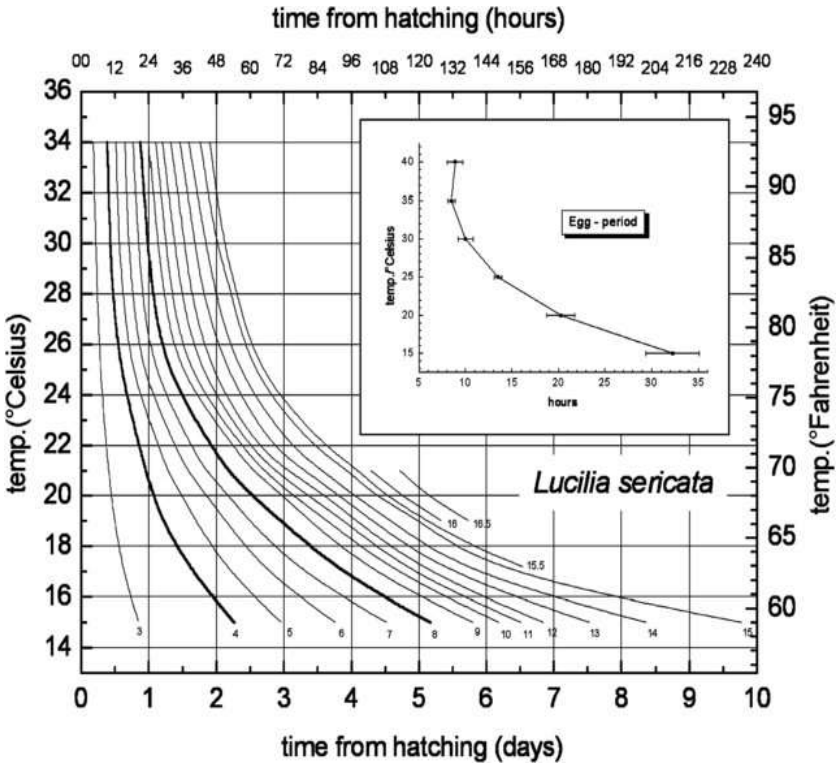


Figure 7. Isomegalen-diagram of *Lucilia sericata* (Diptera: Calliphoridae). The X-axis is time and is measured from egg hatching, represented in hours (at the top) and days (at the bottom). The Y-axis represents temperature which is measured in degrees Celsius (on the left) and in degrees Fahrenheit (on the right). Each line of the graph shows larval lengths (in millimetres) (Grassberger & Reiter, 2001).

On the other hand, the isomorphen-diagram represents the stages of the insect from oviposition to the emergence of the imago. The areas between the lines of the diagram

symbolise a change in the life cycle leading to the next stage (Gennard, 2007). This tool is useful when larvae or migrating pupae are found in the carcass, as length is not a significant criterion for age estimation (Grassberger & Reiter, 2001).

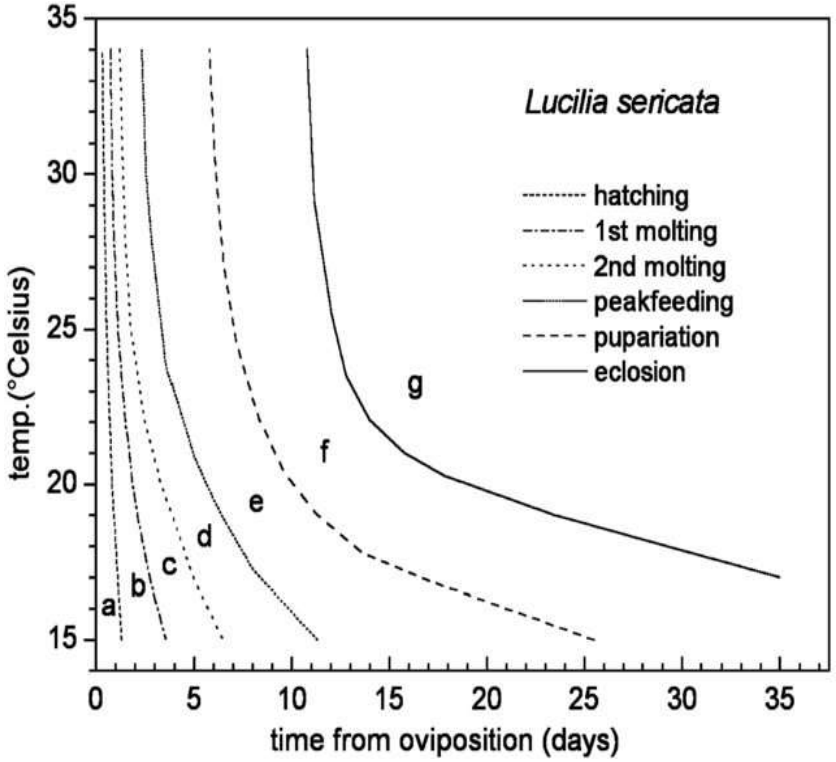


Figure 8. Isomorphen-diagram of *Lucilia sericata* (Diptera: Calliphoridae). Time is expressed in days on the X-axis and temperature expressed in degrees Celsius on the Y-axis. The space between each line represents each stage of the life cycle of the species: a = egg, b = first larval stage, c = second larval stage, d = third larval stage, e = prepupa, f = pupa, g = imago (Grassberger & Reiter, 2001).

The correct identification of insect species is a critical and decisive point for their proper application. However, enriching knowledge on morphology, taxonomy, evolution, biogeography, ecology and life history are equally important. Therefore, this first part of the thesis focuses on the combination of different methods of identification, both morphometric and molecular, of species of the genus *Fannia* collected in a survey throughout the Iberian Peninsula. Given the results, we focus on the species *Fannia pusio*, of which a colony was established under laboratory conditions and the growth curves of its biological cycle at different temperatures were calculated. Moreover, this species has been recently introduced to Spain, so its interest and the need to increase our knowledge of it is a relevant point. Finally, the data collected in this part were applied for the first time in the elaboration of different methods for the calculation of the PMI.

Insects for food purposes

Insects have been used as food since the beginning of humanity, but the cost of collecting them was greater than the energy they provided. Therefore, entomophagy was established as a habit based on opportunity (Cortes-Ortiz *et al*., 2016). The countries that still maintain entomophagy as a habit are those located in tropical and subtropical regions. In these areas, insects reproduce rapidly and tend to grow more due to the combination of high temperatures and humidity throughout the year. But generally, insect breeding is fast due to its high reproductive rate and short biological cycle compared to mammals, reducing the ecological footprint (Pérez-Altamirano, 2019). In addition, they have high food conversion efficiency by transforming biological waste (Lainez, 2017) because they are cold-blooded and therefore do not have to expend energy to regulate their temperature (Cortes-Ortiz *et al.*, 2016).

The consumption of insects as a non-conventional or additional source has many advantage (Guiné *et al.*, 2021): i) a large number of nutritional properties (FAO, 2010a; Alves *et al*., 2019) which can be modulated through the diet (De Marco *et al*., 2015), ii) insect production has a low environmental impact (Veldkamp *et al.*, 2012; Biasato *et al*., 2016) makes them a sustainable ingredient, and iii) insects can be produced in small spaces (Gahukar, 2020). Figure 9 shows a graphical comparison of all resources and environmental impact between an insect farm and conventional farms, with insects being the most efficient. All these characteristics would be a great opportunity to sustain poor countries (Rumpold & Schlüter, 2013; Guiné *et al.*, 2021).

Demand for food, especially animal protein, is growing rapidly due to the population development (FAO, 2010a; Alves *et al.*, 2019). This fact encourages research into new alternatives for protein inputs (Cortes-Ortiz *et al.*, 2016). The World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) have revealed that insect consumption could be a viable way to address nutritional deficiencies by being used as human food and livestock feed. Since January 2018 insects are classified as a "novel food" by European legislation. However, the European Food Safety Agency (EFSA) has recently concluded that the specie *Tenebrio molitor* Linnaeus, 1758 (Coleoptera: Tenebrionidae), commonly called "yellow mealworm", is safe as food and feed (EFSA *et al*., 2021).

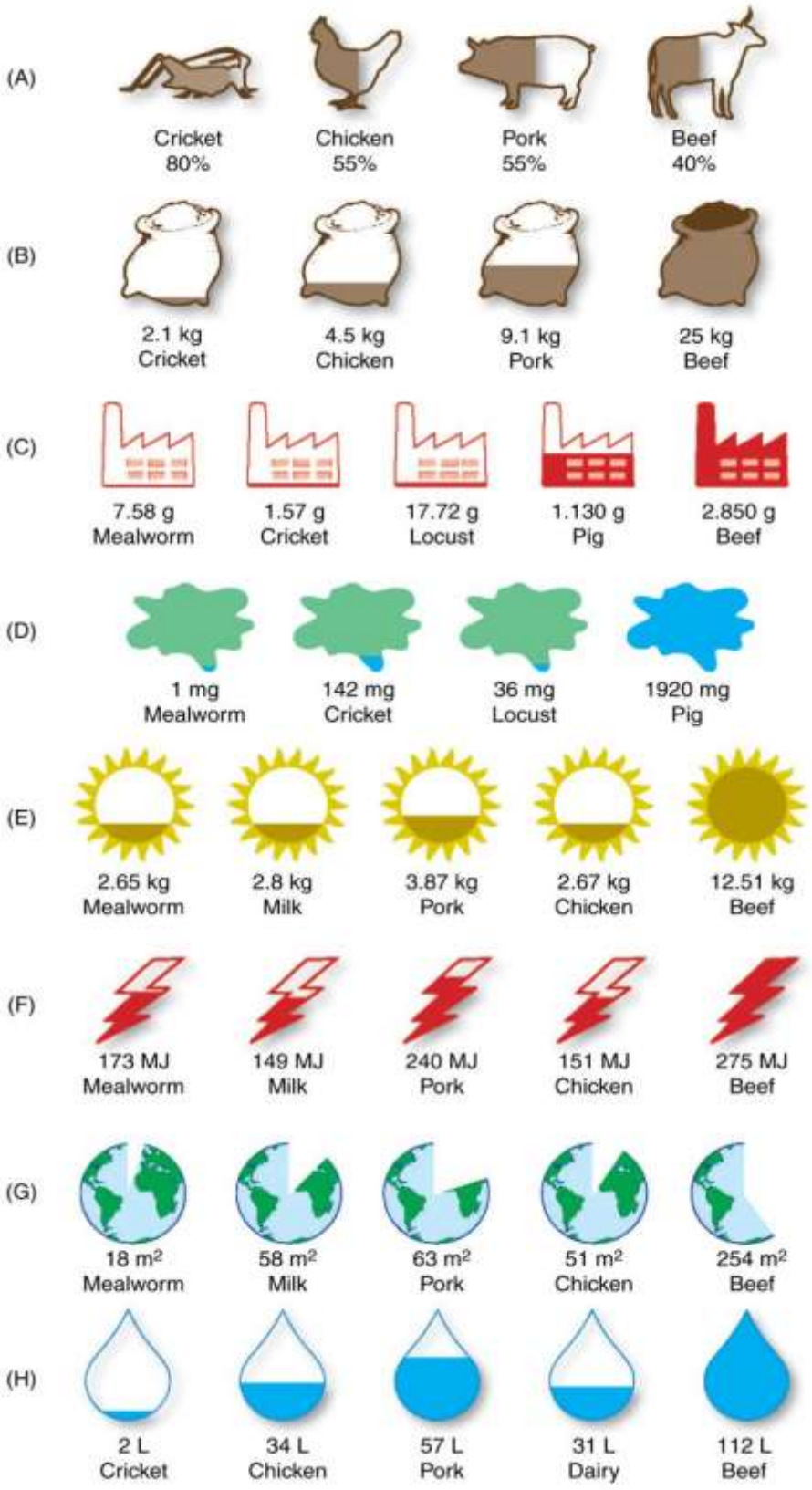


Figure 9. Comparison of resources and environmental impact of insect farm and conventional farm. (A) Digestible biomass percentage, (B) Feed conversion ratio, (C) Greenhouse gas equivalent production per kilogram of body weight gain, (D) Ammonia pollution production per kilogram of body mass, (E) Global warming potential, (F) Energy use, (G) Land use, (H) Water use (Gahukar, 2016).

There are other insect species according to EFSA (2015) with potential to be consumed in the EU, but are not authorised for the time being. These are *Musca domestica* Linnaeus, 1758 (Diptera: Muscidae), *Hermetia illucens* Linnaeus, 1758 (Diptera: Stratiomyidae), *Tenebrio molitor* Linnaeus, 1758 (Coleoptera: Tenebrionidae), *Zophobas atratus* Fabricius, 1775 (Coleoptera: Tenebrionidae), *Alphitobius diaperinus* Panzer, 1797 (Coleoptera: Tenebrionidae), *Galleria mellonella* Linnaeus, 1756 (Lepidoptera: Pyralidae), *Achroia grisella* Fabricius, 1754 (Lepidoptera: Pyralidae), *Bombyx mori* Linnaeus, 1758 (Lepidoptera: Bombycidae), *Acheta domesticus* Linnaeus, 1758 (Orthoptera: Gryllidae), *Gryllodes sigillatus* Walker, 1869 (Orthoptera: Gryllidae), *Locusta migratora migratorioides* Linnaeus, 1758 (Orthoptera: Acrididae), *Schistocerca americana* Drury, 1770 (Orthoptera: Acrididae).

Farming *Tenebrio molitor* to low-scale

The yellow mealworm beetle, *Tenebrio molitor* (Fig. 10), is one of the species permitted for human and animal consumption according to the aforementioned regulations (EFSA *et al.*, 2021). Its nutritional profile is valuable with a high amount of crude protein (20 – 71 %), essential amino acids (46.3 %), and fatty acids (22 – 55 %) (Gasco *et al*., 2019). Furthermore, Bosch *et al.* (2014) set a digestibility of organic matter of 90.85 % and protein of 91.60 %; and the amount of chitin is an added value due to its prebiotic activity on gut microbiota (Bovera *et al*., 2015). However, the use of insects in feed has minor disadvantages as they can cause allergic reactions (Bessa *et al*., 2020) and contain toxic substances. Van Broekhoven *et al.* (2017) and Niermans *et al.* (2019) saw in their studies how *T. molitor* larvae degrade and excrete mycotoxins and heavy metals so the latter problem could be solved.

Figure 10. Larval stage of *T. molitor*, commonly called yellow mealworm.

Breeding of *T. molitor* has numerous other advantages as a food alternative. The use of industrial, agricultural and livestock by-products to feed insect's income that the competitiveness of resources with humans is practically nil (Viñeta-Valdelvira, 2017). This inspires circular economy, encourages sustainability, reduced environmental impact,

and decrease the ecological footprint due to the low greenhouse gas emissions (Biasato *et al.*, 2016). The high feed conversion efficiency of insects is also a feature to be valued in their production efficiency compared to conventional livestock (Garnett *et al.*, 2015; Cortes-Ortiz *et al*., 2016) as well as their rapid reproduction and short life cycle (Pérez-Altamirano, 2019). Additionally, they have a lower risk of zoonosis (Ramón-Vázquez, 2014), and even been used in cancer therapy (Petit *et al*., 2005; Suo *et al*., 2016; Wu *et al*., 2020).

This species is a holometabolous insect (Cruz-Lozano, 2005), which has a complete metamorphosis following a four-stage life cycle: egg, larva, pupa and imago (Fig. 11). Once the larva stops feeding and has completed all its moults, it moves on to the pupal stage. The process from egg hatching and larval growth to pupation can take up to four months. After about 10 days, the adult emerges from the pupa and reaches sexual maturity after 12 days. After copulation, eggs are laid within eight days. The full cycle of this species can be up to six months as an adult can live up to three months under optimal conditions. Those conditions are 25 – 28 ºC ambient temperature, 50 – 55 % relative humidity, 12:12 light cycle, water and diet *ad libitum* based on cereals and plant remains (Bernad-González, 2019).

Figure 11. Phases of the holometabolous life cycle of *T. molitor* (Singh, 1975).

Information on the automation of small-scale breeding of this species is already collected in several papers (Morales-Ramos *et al*., 2012; Cortes-Ortiz *et al*., 2016; Gahukar, 2016; Bernad-González, 2019; Cadinu *et al*., 2020). There is a mechanism that facilitates the management of each phase of the biological cycle for better collection of eggs oviposited by adults, subsequent hatching of larvae and growth. Figure 12 shows the two management systems, the reproduction system and the rearing system. In this way, the separation of phases to optimise each phase of the life cycle is more mechanical and can handle quantities where one manpower is insufficient. In oviposition, the largest possible number of eggs with the highest percentage of viability is needed; in the larval phase, the largest possible size is required with a fast time; and in the adult phase, the greatest possible fertility of the individuals is sought in order to ensure successful reproduction (Morales-Ramos *et al.*, 2012).

Therefore, insect farming requires low-tech and low-capital activities. The space required for insect production is affordable even for small farmers creating small-scale modules.

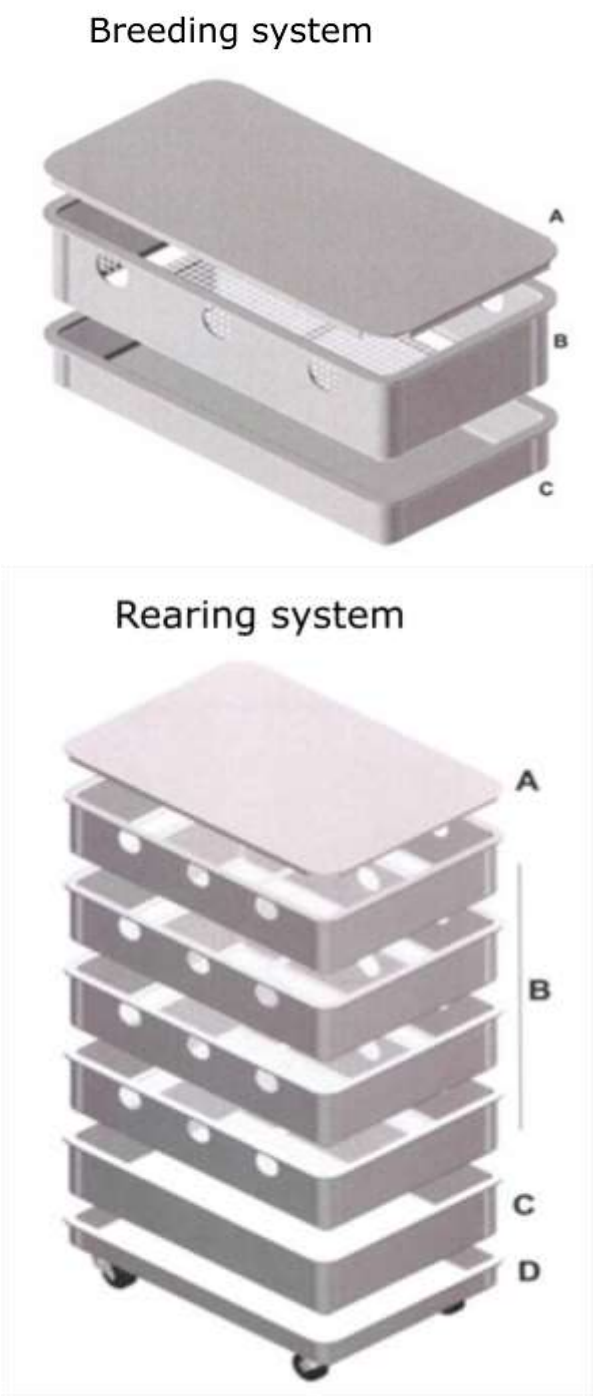


Figure 12. *T. molitor* farming systems. Breeding system: the adults are in tray B which has a porous surface that lets the newly hatched larvae fall from the eggs to tray C where they are collected. Rearing system: stacked trays with pores of different diameters to separate the larvae by size, the last tray is where the waste is collected (Morales-Ramos *et al.*, 2012).

Anomalies found in insect rearing

The production of any living creature, whether on a small or large scale, must be perfectly controlled. Hygiene, feeding, control of the different biological processes and rearing manipulation are the most important factors in the production of insects for food. If these conditions are ignored, they can lead to unknown malformations or even death of the insect. Teratologies in arthropods are quite common (Socha & Sehnal, 1972) (Fig. 13) but can be caused by many different factors: physical alterations resulting from hormonal disturbances (Bong *et al.*, 2018), spontaneity, chemicals, genetic alterations (Mun *et al*., 2019) or diseases resulting from bacterial infections (Zeikus & Steinhaus, 1968). Different anomalies have been observed in *T. molitor*, mainly affecting the pupa and adult emergence, resulting in an individual with incomplete abdomen and elytra (Zeikus & Steinhaus, 1968; Socha & Sehnal, 1972).

Figure 13. *Callipogon relictus* pupaes with different deformations of teguments and limbs (Yi *et al*., 2017).

Sclerotization and melanisation of the insect exoskeleton is the first defence barrier against invading pathogens and environmental stressors (Hayakawa *et al.*, 2018). In addition, pigmentation is also involved in communication, mating behaviour, crypsis, mimicry and predator-prey interactions (Fukatsu & Futahaski, 2016; Mun *et al.*, 2019). Therefore, the exoskeleton and its correct development is essential for the insect's immune response, as well as for resistance to parasites; poor synthesis of proteins involved in cuticle formation can lead to the death of the individual (Parkinson *et al.*, 2003; Hattori *et al*., 2005; Hoegger *et al.*, 2006; Bettedi *et al.*, 2011; Strong & Claus, 2011; Balabanidou *et al*., 2018; Wang *et al*., 2018). Therefore, RNA interference gene silencing of some of these genes has been proposed to control pests (Du *et al*., 2017; Matsumoto & Hattori, 2019; Liu *et al*., 2020).

Multiple genes are involved in the metabolic pathway of hardening and pigmentation of the exoskeleton (Mun *et al*., 2019), and the correct development of the individual depends on the level of expression. *Laccase2* (*Lac2*) is one of the most important proteins in this metabolic pathway as it is responsible for cuticle darkening and hardening (Simon *et al*.,

2009); while *Arylalkylamine N-acetyltransferase* (*AANAT1*) and *Tyrosine Hydroxylase* (*TH*) are involved in colouring (Mun *et al*., 2019). To examine gene expression levels, real-time chain reaction (RT-qPCR) is used as the main methodology. Some studies have shown alterations in these expressions depending on the conditions (Lord *et al*., 2010; Toutges *et al*., 2010; Liu *et al*., 2015; Sang *et al*., 2015; García-Reina *et al*., 2018; Mun *et al*., 2019).

Due to the shortage of animal protein, substitutes are being studied whose production is sustainable and, in addition, enhances the circular economy. According to the European Food Safety Authority (EFSA), insects are the main candidates for this purpose, which is why insect farms are booming. The implementation of such facilities is giving rise to previously unknown rearing problems. Therefore, increasing knowledge of the biology and ethology of the species is necessary.

Objectives

The two main objectives of this thesis are the correct identification of necrophagous dipteran species by means of molecular and morphometric methodologies, and the correct management and maintenance of edible insect species in controlled low-scale rearing conditions. This information will allow us to broaden the knowledge of the biology, ecology and ethology of insects for its application in two main branches of applied entomology: forensic entomology and insects for food and feed purposes.

The results obtained are divided into five chapters organised in two parts. The first part is composed of chapters I, II and III, where samples of *Fannia pusio* collected in the Iberian Peninsula, are identified and analysed by means of molecular and morphometrics techniques for generating knowledge that allows its subsequent use in the calculation of the post-mortem interval (PMI). The second part is made up of chapters IV and V where the breeding of *Tenebrio molitor* on a small scale is optimised accomplishing technical and economic studies; in this process, the visualisation of malformations in the transformation from pupae to imago, lead us to analyse gene expression differences between healthy and anomalous individuals.

Chapter I. Barcoding identification and phylogeographic analysis of *Fannia pusio* (Diptera: Fanniidae) in the Iberian Peninsula

The general aim of this chapter is the identification to species level by molecular analysis of individuals of the genus *Fannia* collected in a survey carried out throughout the Iberian Peninsula. We will focus on *Fannia pusio* (Wiedemann, 1830) as the most abundant species in the sampling.

The specific objectives are:

- To increase the number of sequences of the genus in the GenBank database.
- To test the capacity of the mini-barcode *cox1* region in the correct identification of *F. pusio* from the Iberian Peninsula.
- To identify *F. pusio* by recognising its genetic peculiarities, its phylogeographic pattern distribution and the inference of the process involved.

Chapter II. The head of *Fannia pusio* (Diptera: Fanniidae) as a new source of morphometric data for the evaluation of variation along geographical and biological lines.

The general objective of this study is to complement the results of the molecular data collected in chapter I with a morphometric analysis, where the geographical and biological variation of the species will be assessed.

The specific objectives are:

- To study the head of *F. pusio* as a valid taxonomic structure.
- To validate the head landmarks in the context of the geographical and biological differences of *F. pusio*.

Chapter III. Development of *Fannia pusio* (Diptera: Fanniidae) under temperature-controlled conditions and its application in post-mortem interval (PMI) estimation.

The general objective of this chapter is to assess for the first time, the effect of temperature on the development of *F. pusio* providing data for forensic specialists to determine its suitability for a more accurate PMI estimation.

The specific objectives are:

- To obtain the lengths of all larval stages at different temperatures, from hatching to imagoes emergence.
- To calculate isomegalen and isomorphen diagrams, by means of the growth curve obtained from the data matrix.

Chapter IV. Production and economic feasibility studies in the development of a rearing project for the yellow mealworm, *Tenebrio molitor*.

The overall objective of this study is to analyse the optimal conditions for the rearing and management of *T. molitor* in a small-scale production facility to encourage entrepreneurs with limited space available to become small-scale farmers.

The specific objectives are:

- To calculate the annual production of a *T. molitor* small facility under optimal conditions in the available dimensions, according to life cycle stages of the species.
- To calculate the economic feasibility of the facility in order to establish its profitability.

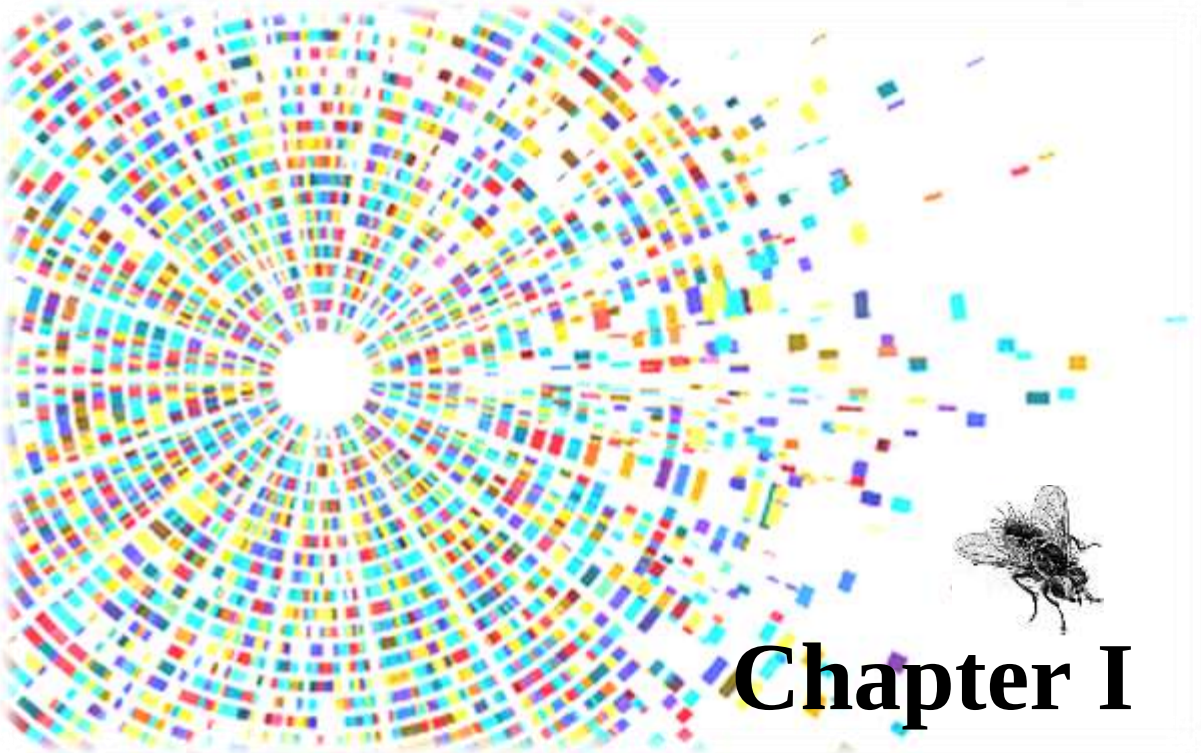
Chapter V. Expression analysis of *Lac2, TH* and *AANAT1* genes related to exoskeleton malformations in reared *Tenebrio molitor* (Coleoptera: Tenebrionidae).

The general objective of the study is to analyse the gene expression of three genes related to the formation of the cuticle of *T. molitor* in individuals with normal *versus* anomalous cuticle development. The altered expression of these genes, which encode essential proteins for adult life, can lead to an anomalous development of cuticle with the corresponding limitations for survival and reproduction. A better understanding of the genetic mechanisms involved will help to improve production systems while minimising costs.

The specific objectives of this work are:

- To verify the expression levels of the proteins *Lac2, AANAT1*, and *TH* in normal and malformed adults using RT-qPCR.
- To discuss the relationship of these malformations to the production handling and the stress of the colony.

Part I



Chapter I

Barcoding identification and phylogeographic analysis of *Fannia pusio* (Diptera: Fanniidae) in the Iberian Peninsula

Chapter I

Barcoding identification and phylogeographic analysis of *Fannia pusio* (Diptera: Fanniidae) in the Iberian Peninsula

Abstract

Fannia is a genus of diptera belonging to the family Fanniidae with approximately 300 species; they are attracted to decaying organic matter and exhibit necrophagous habits, thus shedding light on criminological issues. In addition to legal importance, they have medical and economic concern, as they are capable of causing myiasis in domestic animals and humans. For these reasons, their identification to species level based on morphological characters is sometimes difficult due to poor preservation of samples in decomposing corpses. An alternative to overcome this situation is DNA barcoding by molecular analysis of a 658 bp fragment of the mitochondrial genome (*cox1*). However, the sequences of this fragment may be contaminated with external DNA debris, due to different reasons. An alternative is the use of short, internal regions (mini-barcodes); in this work we show how a smaller fragment of 240 bp is able to correctly identify the species of the genus *Fannia* that were collected in a survey throughout the Iberian Peninsula.

Molecular identification showed that *Fannia pusio* (Wiedemann, 1830) was the most abundant species collected. In addition, particular mutations have been identified, that allows to differentiate haplotypes of individuals from northeast and southeast. The available sequences in the databases analysed together with the results of this work suggest that the species originates from America and it was subsequently introduced into the Iberian Peninsula via Portugal, undergoing a continuous expansion of its distribution area.

Keywords

Forensic Entomology, *cox1*, Haplotype Network, Mini–Barcode, Molecular Analysis, NJ tree.

Introduction

Fannia Robineau-Desvoidy, 1830 is a genus of diptera belonging to the family Fanniidae with importance in the legal, medical, and economic fields. They are attracted to decaying organic matter (Grzywacz *et al.*, 2017b) exhibiting necrophagous habits that decompose carrion and corpses (Pérez & Henao, 2013). The feeding habits and biology together with the decomposition of carrion shed light on criminological issues (Amendt *et al.*, 2011). Although there are no cases in Spain, it is worth noting the sanitary and economic interest of this genus in Latin America due to its distinctiveness of being an egg carrier for the human botfly, *Dermatobia hominis* (Linnaeus, 1781) which cause myiasis in both humans and livestock (Bélo *et al.*, 1998; Al Gazi *et al.*, 2004). It is the most diverse genus in the family Fanniidae (Diptera), with 288 species (Couri & Sousa, 2019); it is native to America but currently distributed in many parts of the world due to the transportation of livestock (Al Gazi *et al.*, 2004; Grzywacz & Prado e Castro, 2012). The high biodiversity of this genus makes the correct species identification much needed (Grzywacz *et al.*, 2017b).

DNA barcoding is a molecular instrument that is assisting in the taxonomic classification of organisms, particularly in difficult groups, that has attracted much attention from insect taxonomists to achieve correct identification (Pérez & Henao, 2013; Li *et al.*, 2015; Shi *et al.*, 2017). This method uses a small fragment called "DNA barcode" which consists of the Cytochrome c Oxidase subunit I gene (*cox1*); it is the catalytic subunit of the predominant enzyme in the mitochondrial genome, and easy to amplify and sequence (Kerr *et al.*, 2009; Alves-Rolo, 2010). A wide variety of molecular markers have been used in insects with forensic (Coyle *et al.*, 2005; Kosmann, 2009; Alves-Rolo, 2010; Arnaldos *et al.*, 2012; Boehme *et al.*, 2012; Grzywacz *et al.*, 2017b; Fuentes-Lopez *et al.*, 2020), medical and veterinary importance (Hoyos-López *et al.*, 2012; Rodrigues *et al.*, 2018; Onder *et al.*, 2019) as well as from the point of view of environmental genomics (Valentini *et al.*, 2009; Kang *et al.*, 2017). In turn, many dipteran families were analyzed using this same methodology, such as the Calliphoridae (Yusseff-Vanegas & Agnarsson, 2017), Culicidae (Guo *et al.*, 2018; Osório *et al.*, 2018), Drosophilidae (Liu *et al.*, 2017; Liu & Chen, 2018), Muscidae (Renaud *et al.*, 2012), Sarcophagidae (Arnaldos *et al.*, 2015; Buenaventura *et al.*, 2018), Tephritidae (Li *et al.*, 2018) and Fanniidae which is the subject of this study (Grzywacz *et al.*, 2017b).

This tool typically uses 658 base pairs, which is the so-called "barcode"; however, these sequences can be contaminated with external DNA remnants due to poor preservation in decomposing remains (Couri & Sousa, 2019). For this reason, there are studies where smaller fragments called "mini-barcodes" are being used as an alternative (Meusnier *et al*., 2008; Grzywacz *et al.*, 2017b). In this study, a 240 bp fragment (Lee *et al*., 2015; Yeo *et al*., 2020) was used as a discriminant molecular marker showing *Fannia pusio* (Wiedemann, 1830) as the most abundant species in the sample. In addition, this gene has been used in the study of the origins and evolution of insect populations through haplotype analysis (López-López *et al*., 2016; Hurtado-Burillo *et al*., 2017; Silva *et al*., 2020), specifically in the order Diptera (Pfeiler *et al*., 2013; Izumitani *et al.*, 2016; Rampasso *et al*., 2017; Iglesias *et al*., 2018; Fuentes-López *et al*., 2020).

The aim of this work is the identification to species level by molecular analysis of individuals of the genus *Fannia* collected in a survey carried out throughout the Iberian Peninsula, focusing on *Fannia pusio* as the most abundant species in the survey. The specific objectives are: 1) to increase the number of sequences of the genus in the GenBank database, 2) to test the capacity of the mini-barcode *cox1* region in the correct identification of *F. pusio* from the Iberian Peninsula, 3) to identify *F. pusio* by recognizing its genetic peculiarities, its phylogeographic distribution pattern and to infer the processes involved. Therefore, this work will show the advantages and shortcomings of the mini-barcode method for the identification of fanniids species of forensic interest.

Materials and methods

Sampling

Samples for this study were collected using bottle traps baited with pig liver and blood (Couri & Sousa, 2019), by members of the Department of the Zoology and Physical Anthropology research group of the University of Murcia. The areas sampled in Spain were covered in summer and autumn 2012, while the area of Lisbon was sampled in autumn 2015. Although intensive sampling was carried out throughout the Iberian Peninsula, only the points marked on the map represent localities where specimens of the genus *Fannia* were found (Fig. 14).

Figure 14. Sampling points of the Iberian Peninsula where the genus *Fannia* was found. 1 (pink point) = Villanueva de Cameros (La Rioja), 2 (blue point) = Villar del Arzobispo (Valencian Community), 3 (blue point) = Catarroja (Valencian Community), 4 (orange point) = Campus of Espinardo (Region of Murcia), 5 (orange point) = Aguilas (Region of Murcia), 6 (green point) = Estremadura (Lisbon) (for more details see Table 1).

The traps were placed in peri-urban areas of each locality (Table 1) for 3–6 days. All individuals collected in the decaying liver and blood were washed in 70 % ethanol and subsequently preserved in absolute ethanol until analysis. Furthermore, a laboratory *Fannia pusio* colony, maintained in controlled laboratory conditions (25 ºC, 60 % relative humidity, 12:12 light cycle). Sugar and water *ad libitum* was also provided to the colony. The collection was obtained from larvae recovered from a cat carcass in advanced stages of decomposition that was found near an urban area 11 km from Murcia (Region of Murcia, Spain) in September. The identification of specimens was corroborated by Dr. Andrej Grzywacz, an expert in the family Fanniidae and Muscidae, and subsequently at a molecular level by barcoding analysis. The samples considered here were from generation 165, which was obtained after approximately four years of laboratory breeding of the same lineage.

DNA extraction and PCR amplification

Due to the small size of the individuals, we had to use the complete body for DNA extraction. Only the head was removed to prevent eye pigments from interfering with the DNA amplification process. The CCDB Glass Fiber Plate DNA extraction protocol was

used (Ivanova *et al.*, 2006). Briefly, 100 µl of insect lysis buffer and 10 µl of proteinase K were added to the plate, and maintained in an incubator at 56 °C overnight. Subsequently, 100 µl of binding mix was added and all this was transferred to a PALL2 plate. Two washes were performed with 200 µl of protein wash buffer and 600 µl of wash buffer, and left in the incubator at 56 °C for 30 minutes. Additionally, 100 µl of water previously warmed up to 56 °C was added. After extraction, the DNA concentration was verified by Nanodrop (NanoDrop ™ Thermo Scientific ™ spectrophotometers).

Map point	Locality	Exact area	Coordinates	Elevation (m)
1	La Rioja	Villanueva de Cameros	42°10′04″N; 2°39′00″W	1265
2	Valencian Community	Villar del Arzobispo	39°44'7"N; 0°49'40"W	520
3	Valencian Community	Catarroja	39°24'0"N; 0°24'0"W	8
4	Region of Murcia	Campus of Espinardo	38°01′06″N; 1°10′12″W	150
5	Region of Murcia	Águilas	37º24'22"N; 1°34'58"W	326
6	Estremadura	Campus of Lisbon	38º75'26"N; -9º15'82"W	84

Table 1. Details of sampling zones of the Iberian Peninsula where the genus *Fannia* was found (see Figure 14).

Amplification of the *cox1* barcode region was performed in a 2720 thermocycler (Applied Biosystems, Foster City, USA) using a KAPA BIOSYSTEMS PCR kit (Wilmington, USA). The PCR cocktail contains buffer, 0.2 µM of each primer, LCO1490 and HCO2198 as described by Folmer *et al*. (1994) and 0.3 U / tube of Taq polymerase. Once the cocktail is prepared, 10.5 µl of the reaction mix and 2 µl of DNA were added to the PCR plate. The PCR program consists of an initial activation of 95 °C for 3 min, followed 30 cycles of 30 sec at 94 °C, 30 sec at 55 °C, and 1 min at 72 °C, with a final extension of 5 min at 72 °C. The PCR product was then electrophoretically checked in a 2 % agarose stained with RED SAFE (iNtRON Biotechnology, Seongnam, South Korea).

Sequencing of cox1 and molecular analyses

The samples were sequenced at Macrogen (Amsterdam, The Netherlands) and the *cox1* gene fragment was obtained. However, the sequences contained gaps and were contaminated, so manual editing using GENEIOUS 7.1.3. (Kearse *et al.,* 2012), clean sequences of 240 bp (mini-barcode) were gained. In addition, sequences included in the NCBI (National Centre for Biotechnology Information) database were incorporated using the BLAST tool (Altschul *et al.,* 1997). The alignment was made using MUSCLE (Edgar, 2004) and it is free-gaps translating to amino acid sequences correctly. The sequences were uploaded to GenBank databases where a label was assigned (Supplementary Table 1 of Appendix).

A Neighbour Joining (NJ) tree with a 1000 bootstrap and the statistical analysis were calculated with MEGA–X 10.0.5 software (Tamura *et al.,* 2007). The Kimura two-parameter (K2P) model (Kimura, 1980), the most widely used in phylogenetic studies (Ekrem *et al.,* 2007; Kosmann, 2009; Arnaldos *et al.,* 2015; Borges *et al.,* 2016; Grzywacz *et al.,* 2017b; Emmam *et al.,* 2020; Fuentes-López *et al.,* 2020), was used as nucleotide substitution model for estimating genetic differences and phylogenetic relationships. The mitochondrial lineage of the samples was decisive for analysing whether there was separation in the different groups by means of the K2P pairwise distance analysis. The haplotype networks were obtained with the PopArt software (Leigh & Bryant, 2015) using the median binding algorithm (Bendelt *et al.,* 1999).

Results

Genomic DNA was isolated from 78 samples belonging to the genus *Fannia* (Diptera: Fanniidae). The present work provides a good species-level identification of *Fannia aequilineata* Ringdahl, 1945 (N = 2), *Fannia canicularis* Linnaeus, 1761 (N = 5), *Fannia lepida* Wiedemann, 1817 (N = 2), *Fannia leucosticta* Meigen, 1838 (N = 4) and *F. pusio* (N = 65), using only an internal and shorter 240 bp fragment of the *cox1* gene (mini–barcode). All sequences were uploaded into the GenBank systems with the numerical references MT527094 – MT527174. In addition, for correct identification and subsequent comparative study, sequences from the NCBI database of the different species studied were incorporated (Supplementary Table 1 in the Appendix).

In the NJ tree of *cox1* a clear separation of the studied species was observed with a support value of 100 %. Within the genus *Fannia*, each species studied in this work presents a monophyletic relationship (Fig. 15). The distance analysis shows an intraspecific distance of 0 – 4.3 % and an interspecific distance of 7.9 – 21.6 % (Table 2). The distances showed a clear gap of 3.6 %, thus indicating that the species were correctly separated. The greatest intraspecific distances appeared for *F. canicularis*, followed by *F. leucosticta* and *F. pusio* and with 4.3 %, 3.4 %, and 3 % respectively (Table 2). On the other hand, the greatest interspecific distances were for *F. leucosticta* with F. *aequilineata* (19.4 %), *F. leucosticta* with *F. lepida* (19.1 %), and *F. leucosticta* with *F. canicularis* (18.3 %) (Table 2) which were the most distant in the NJ tree (Fig. 15). Furthermore, the shortest interspecific distance occurred between *F. aequilineata* and *F. canicularis* with 7.9 % (Table 2) indicating a close relationship in the NJ tree.

Maximum distances (intraspecific)	**Minimum distances (interspecific)**				
	F. aequilineata	*F. canicularis*	*F. lepida*	*F. leucosticta*	*F. pusio*
0	*F. aequilineata*	0.079	0.140	0.194	0.155
0.043	*F. canicularis*		0.142	0.183	0.162
0.008	*F. lepida*			0.191	0.157
0.034	*F. leucosticta*				0.157
0.030	*F. pusio*				

Table 2. Intraspecific and interspecific Kimura two-parameters distances of *Fannia* species collected in the sampling.

The haplotypic network (Fig. 16) shows a clear distribution of haplotypes of the analysed species of the genus *Fannia*. Focusing on *F. pusio*, the wide distribution of a common haplotype (1) was observed, which includes all the regions studied in the Iberian Peninsula, with the exception of the Valencian Community. It can be seen how the latter locality showed a haplotype that was connected to another (2) that has not been detected in the USA or in La Rioja (Spain).

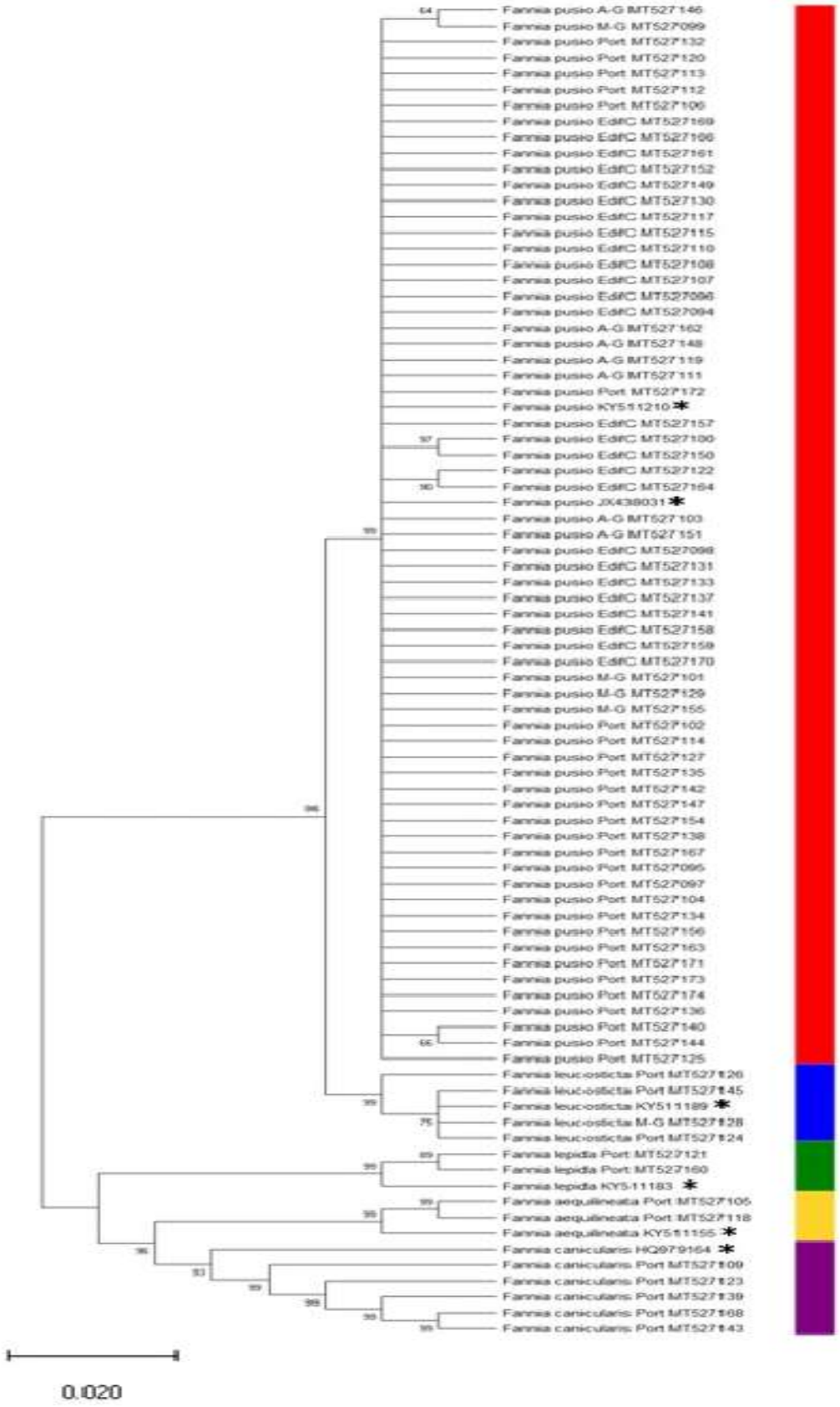


Figure 15. NJ tree of mini–barcode of *cox1* gene of *Fannia* species. Red = *F. pusio,* Blue = *F. leucosticta*, Green = *F. lepida*, Yellow = *F. aequilineata*, Purple = *F. canicularis*. Sequences with an asterisk are those obtained from the NCBI database for comparison.

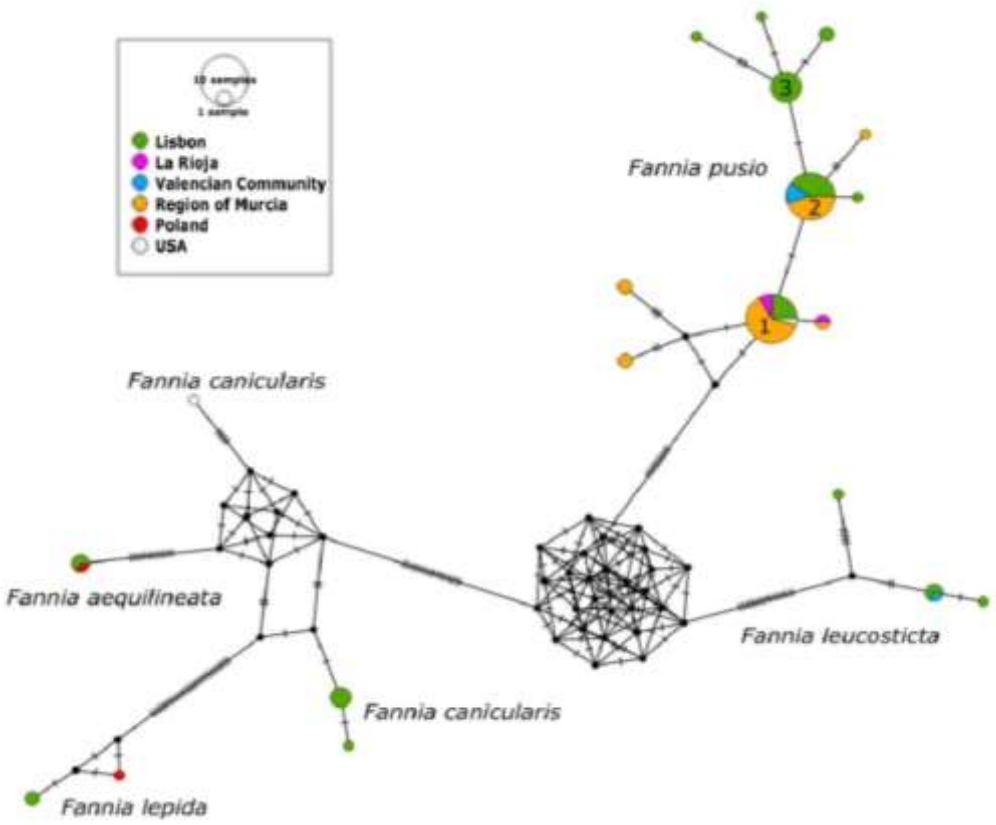


Figure 16. Haplotype network of mini–barcode of *Fannia* species collected in the Iberian Peninsula and sequences obtained from the NCBI database for comparison.

The localities that showed the highest number of haplotypes were Lisbon with seven and Region of Murcia with six, sharing both haplotype 1 and haplotype 2. On the one hand, a new haplotype (3) occurred in Lisbon, which connected with haplotype 2 and gave rise to three other new haplotypes. It should be noted that in the Region of Murcia, the individuals established and maintained in laboratory conditions were also included, as this is where they were collected (see Materials and methods). However, in Figure 17 the same analysis was performed but classifying the sequences belonging to the individuals bred in the laboratory and the samples collected in the wild during the sampling. Both groups of samples also shared haplotypes 1 and 2 as in the previous case (Fig. 16). Five haplotypes of the laboratory specimens and three of the wild type were observed. The main difference is that the wild type specimens collected in the Region of Murcia show haplotypes arising from 1, while the individuals from the laboratory arise from both 1 and 2 (Fig. 17).

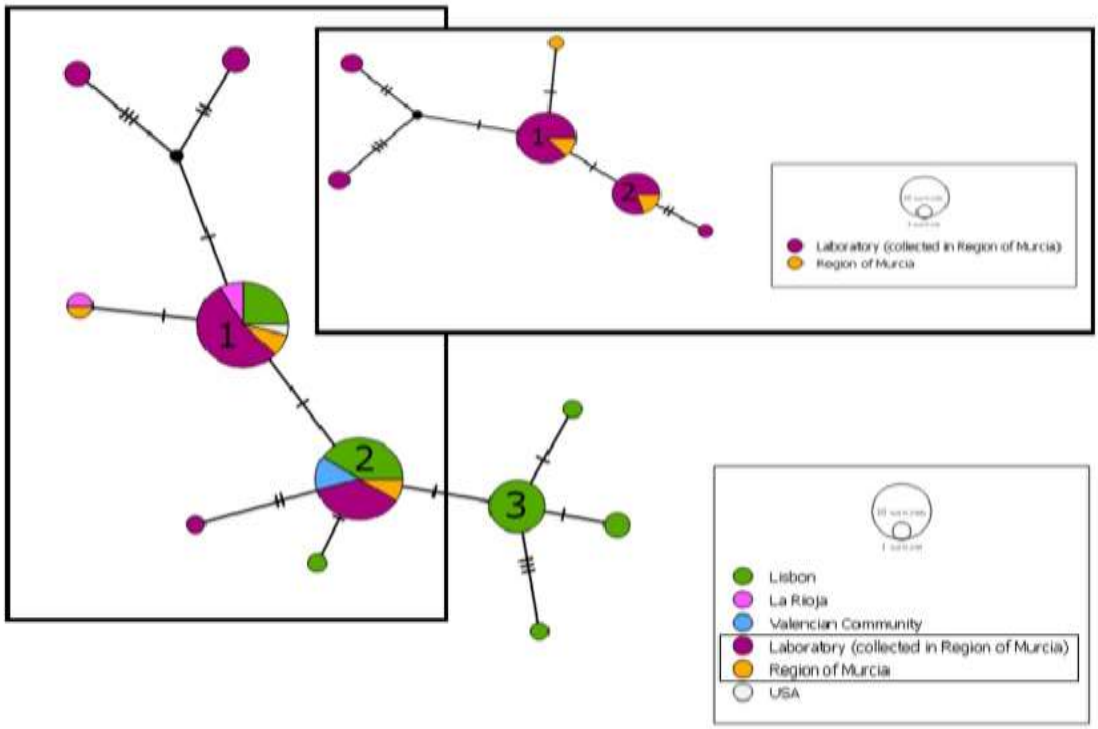


Figure 17. Haplotype network of mini–barcode of *cox1* of *Fannia pusio* individuals collected in the Iberian Peninsula and sequences obtained from the NCBI database for comparison. In addition, the individuals from the colony established in the laboratory (collected in Murcia) are separated from the individuals collected from the Region of Murcia.

Discussion

To our knowledge, there are studies based on morphology of fanniids species (Domínguez & Roig-Juñent, 2008; Ochoa, 2014), however, there are few published molecular analyses. Only Grzywacz et al. (2017b) have provided find *cox1* sequences available in GenBank databases. As it is well known, the attention in fanniids is concentrated not only in the work undertaken in the forensic field (Grzywacz et al. 2017b), but also in the sanitary, medical, and economic fields (Barták et al. 2016) due to their association with animals and humans (Rozkosny et al. 1997). For these reasons, their molecular identification constitutes substantial improvement in practical knowledge, which can be used to carry out good management and prevention policies (Hoyos-López et al. 2012).

The individuals collected in the sampling had some taxonomic structures damaged, making it impossible to identify the species using keys based on morphology (Rozkosny et al. 1997). The use of a barcoding analysis offers a quick identification method to overcome this difficulty (Arnaldos et al. 2015; Fuentes-López et al. 2020) even with

smaller fragments (mini–barcode) of the *cox1* gene (Meusnier et al. 2008; Reibe et al. 2009; Guo et al. 2012; Lee et al. 2015; Grzywacz et al. 2017b; Yeo et al. 2020). In our study we used a mini–barcode of 240 bp which was adequate for a correct identification of the species of the genus *Fannia*, showing a clear gap in the distance analysis of 3.6% (Table 2) (Meier et al. 2006). Only 85 species of fanniids are known in Europe (Barták et al. 2016), so that, the scarcity of data on this group (Grzywacz et al. 2017b) makes studies such as this one necessary. *Fannia* is a genus of Diptera of recent diversification (Pont, 1977) that has been subjected to a rapid radiation (Wiegmann et al. 2001), thus resulting in less genetic variability among species, many of which are closely related (Fig. 2). The results obtained by Grzywacz et al. (2017b) corroborate these hypotheses.

The sampling presented in this manuscript was carried out at various locations in the Iberian Peninsula (Fig. 14), where *F. pusio* was the predominant species. This information is relevant since in previous studies this species was almost absent (Grzywacz & Prado e Castro, 2012). The presence of this species in the Iberian Peninsula has been explained as a consequence of entries through Portugal via livestock transportation (Baz *et al.*, 2015). These statements are corroborated by the results obtained, as shown in Figure 16 where the existence of a common haplotype for all the regions studied can be observed. Furthermore, sequences from the NCBI database were included in the analysis (only available from USA samples) and it is clear that they share the haplotype 1 with the exception of the Valencian Community (Fig. 16).

It is known that rapidly diverging species, as in this case (Wiegmann et al. 2001), can form hybrid species through genetic introgression (Genner & Turner, 2011). After the entry of *F. pusio* in the Iberian Peninsula, pioneers may have undergone mutations that generated new haplotypes such as that carried by individuals from Lisbon, the Valencian Community and the Region of Murcia. However, the specimens from La Rioja harbor only haplotype 1 and another haplotype separated by one mutation. It could be said that there is a clear difference between individuals of *F. pusio* from the northeast (La Rioja) and the southeast (Valencian Community) of Spain. Unfortunately, the presence of *F. pusio* in Spain was not detected by previous studies (Velásquez et al. 2010; Grzywacz & Prado e Castro, 2012), which makes it difficult to propose more sound hypotheses.

Another issue that could be controversial is the recommendation to avoid the use of individuals from an established colony under laboratory conditions for DNA barcoding studies (Paz *et al.*, 2011), particularly in long-term established colonies which would have

a homogeneous diversity of COI haplotypes due to inbreeding. However, in our short-term colony (165 generations) the results obtained show the opposite (Fig. 17) where five haplotypes are present, two of which are shared with the specimens collected in the Region of Murcia in the wild. In this particular case, inbreeding should not be a limitation in the study of the haplotypic diversity of *F. pusio*, as much more time should be required for haplotype homogenization in captive colonies.

Chapter II

The head of *Fannia pusio* (Diptera: Fanniidae) as a new source of morphometric data for the evaluation of variation along geographical and biological lines.

Chapter II

The head of *Fannia pusio* (Diptera: Fanniidae) as a novel source of morphometric data for assessing of variation along geographic and biological lines

Abstract

Fannia Robineau-Desvoidy, 1830 is the most diverse genus in the family Fanniidae (Diptera), with 288 species, many of which are include many of sanitary, economic and legal interest. The morphological homogeneity within the genus often makes species determination difficult. The best option for correct identification is to combine molecular and morphometric analyses. The variation in the shape of a selection of body characters can be assessed by Geometric Morphometrics using the head as an innovative structure. Sex must be accounted for as a key covariate in this kind of study, since *Fannia*, as many other Diptera, has a sexually dimorphic head structure, with holoptic males and dicoptic females. Firstly, we analysed a set of *Fannia* sp. specimens sampled across the Iberian Peninsula (2012–2015), of which *Fannia pusio* (Wiedemann, 1830) was found to be the most abundant species. This species Our analyses provide significant morphological information. *Fannia pusio* exhibits clear intraspecific morphometric variation along an Iberian-wide East-West axis. A similar pattern emerged when comparing a laboratory-bred colony and wild samples.

Keywords: Biological Variability, Geographical Variability, Geometric Morphometrics, Head landmarks, Iberian Peninsula.

Introduction

Insects are one of the groups that have received most attention in the application of Geometric Morphometrics (GM) (Dujardín *et al*., 2014; Gerard *et al*., 2015), particularly the Diptera (Espra *et al*., 2015; Grzywacz *et al*., 2017a; Macedo, 2017; Mikery *et al*., 2019; Szpila *et al.*, 2019). Morphology, in terms of shape and dimensions of the exoskeleton, gives us information about an animal's lifestyle (Menes-Hernández, 2004).

Wings are an ideal biological structure for this type of analysis due to the taxonomic information they provide (Grzywacz *et al.*, 2017a; Sontingun *et al*., 2017), but other structures such as the head have also received attention (Baylac *et al*., 2003; Khamis *et al*., 2012; De Souza *et al*., 2015; Godoy *et al*., 2018). These types of studies allow us to establish the degree of diversity at both inter (Fuentes-López, 2018) and intra-populational levels (Menes-Hernández, 2004), and to determine the history of the species establishing the processes that explain the evolutionary patterns observed in organisms (Bustamante *et al.*, 2004).

Fannia Robineau-Desvoidy, 1830 is the most diverse genus in the family Fanniidae (Diptera), with 288 species. The morphological similarities among its species become a significant hurdle for reliable identification. *Fannia* spp. share the following characteristics (Al Gazi *et al*., 2004): i) small size; ii) dark integument; iii) predominantly yellow abdomen (Rozkosný *et al*., 1997); iv) dorsal submedian seta on the hind tibia; v) short vein CuA+1A as an extension of vein 2A of the margin of the wing (Pont, 1977).

Fannia pusio (Wiedemann, 1830), commonly known as the "chicken dung fly" because of its common appearance in laying hen farms, is a species of great sanitary, economic and legal interest. The originally Nearctic species (Couri & Sousa, 2019) is currently found worldwide thanks to the transportation of livestock. Part of the species' economic and sanitary interest stems from the female's common role as the phoretic host of the eggs of *Dermatobia hominis*, the human botfly, which causes myasis in humans and other animals (Gomes *et al*., 2002). Females can be easily identified due to the sexuality dimorphic eye arrangement in *F. pusio*: they are dichoptic with the eyes well separated by the frons (Domínguez & Pont, 2014). On the other hand, the legal interest refers to the forensic field, as this species exploits decaying organic matter, both animal and human (De Souza *et al.*, 2008; Grzywacz & Prado e Castro, 2012; Vasconcelos & Araujo, 2012).

For all these reasons, a correct identification is essential. The best option is to use molecular analysis with morphological tools like Geometric Morphometrics, which use the shape variability of body characters (Bookstein, 1982). Landmark-based morphological analysis has been successful in examining the morphological variations in different animal groups, including the cranial morphology of rodents (Vallejo *et al.*, 2017) and carapaces of zooplankton (Wong *et al.,* 2018; Hethke & Weeks, 2020). This tool has not yet been used as an identification methodology in the genus *Fannia*, so the present study is the first in its field. However, Grzywacz *et al*. (2017) used GM as an alternative to the classical morphology in Muscidae due to the fact that the identification of adults is considered difficult. In that paper, the wings were chosen as the study structure, and it was concluded that this method facilitates identification compared to more difficult and time-consuming approaches, with a very high success rate in terms of results.

From the data obtained, we formulated two main questions: i) is the head of *F. pusio* a structure of enough taxonomic resolving power? and furthermore, ii) does the documented distribution of head landmarks match the geographical and biological differences in *F. pusio*?

Materials and methods

Sampling

The individuals analysed in this work were collected over the course of several collection trips made throughout the Iberian Peninsula from 2012 to 2015 (sampling design detailed in Fuentes-López, 2018). Eighty-one specimens were keyed to the family level using the keys provided by Szpila (2012) and identified as Fanniidae. The geographical information on the samples analysed in this study is presented in table 3. We collected the following samples: *F. aequilineata* (N = 2), *F. canicularis* (N = 5), *F. lepida* (N = 2), *F. leucosticta* (N = 4), *F. monilis* (N = 1), *F. pusio* (N = 65) and *Hydrotaea floccosa* (N = 2); *F. pusio* was by far the most common species recorded. In addition to the field sampling, a colony of *F. pusio* was stabilized under controlled laboratory conditions in the Laboratory of Necrophagous Diptera at the University of Murcia (Spain): 25°C, 65 % relative humidity and a 12:12 light cycle. Adults were given water and sugar ad libitum, supplemented with canned cat food to induce oviposition (Couri, 1991). The choice of this product for obtaining eggs was a consequence of the poor success of other substrates—e.g., dog and human faeces and chicken and pig liver (D'Almeida, 1994). The samples considered here

were from the 165th generation, which was obtained after about four years of laboratory breeding of the same lineage. Initially, 30 individuals were sampled for a study on the morphogeometric differences between lab-raised individuals (domestic) versus wild-captured specimens (wild type). These samples were also preserved, although four of the lab-raised specimens were removed from the study due to excessive damage. Finally, 26 domestic samples were used together with 39 wild type samples collected in the sampling.

Species	N	Collection date	Country	Region	Latitude	Longitude	Elevation
F. aequilineata	2	28-09-2015	Portugal	Lisbon	38.75818	-9.15804	79 m.
F. canicularis	5	28-09-2015	Portugal	Lisbon	38.75818	-9.15804	79 m.
F. lepida	2	28-09-2015	Portugal	Lisbon	38.75818	-9.15804	79 m.
F. leucosticta	3	28-09-2015	Portugal	Lisbon	38.75818	-9.15804	79 m.
	1	22-07-2012	Spain	Valencian Community	38.45880	-0.77851	403 m.
F. monilis	1	28-09-2015	Portugal	Lisbon	38.75818	-9.15804	79 m.
F. pusio	28	4-09-2012	Spain	Region of Murcia	38.02773	-1.17556	150 m.
	30	28-09-2015	Portugal	Lisbon	38.75818	-9.15804	79 m.
	3	23-06-2012	Spain	La Rioja	42.09369	-2.56187	1265 m.
	4	19-07-2014	Spain	Valencian Community	38.45880	-0.77851	403 m.

Table 3. Information on the geographic data of the sampling of each species and the number (N) of individuals collected.

Molecular analysis

To verify the identification at the species level, we performed a molecular analysis of the *cox1* gene of the mitochondrial genome, which was described elsewhere. Briefly, DNA extraction was performed using the CCDB Glass Fiber Plate DNA extraction protocol (Ivanova *et al*., 2006). Amplification of the *cox1* barcode region was performed on a 2720 Thermal Cycler (Applied Biosystems, Foster City, USA) using a PCR kit from KAPA BIOSYSTEMS (Wilmington, USA) (Folmer *et al*., 1994). Finally, the samples were sequenced at Macrogen (Amsterdam, Netherlands). The software GENEIOUS 7.1.3 was used to manually edit the sequences (Kearse *et al*., 2012) and the alignment was

performed using MUSCLE (Edgar, 2004). The sequences were uploaded to GenBank under reference codes MT527094–MT527174.

Data analysis

As stated in the introduction, wings are generally the best insect structure for this type of study. In our particular case, *Fannia* has a small body size, which compounded the poor state of conservation of some samples, making the wings impossible to use. We therefore decided to use the better conserved heads of the 81 samples (Fig. 18) to search for useful landmarks (described in Table 4). The strongly dimorphic sexual character of *Fannia* made it a straightforward choice to focus only on one sex for a meaningful analysis. We focused our analysis on the more frequently caught females, which have a stronger tendency to enter the traps in their search for moist and nutritious substrate to deposit their eggs on (Domínguez & Pont, 2014). To examine the variation in head shapes in the samples studied, data files were generated with a STEMI-200-C stereoscopic (Fisher Scientific, Madrid, Spain) calibrated with the SPOT 4.6 AdvancedTM program. TpsDig2 v.2.31 and tpsUtil32 v.1.73 were used to digitize the landmarks (Fig. 18).

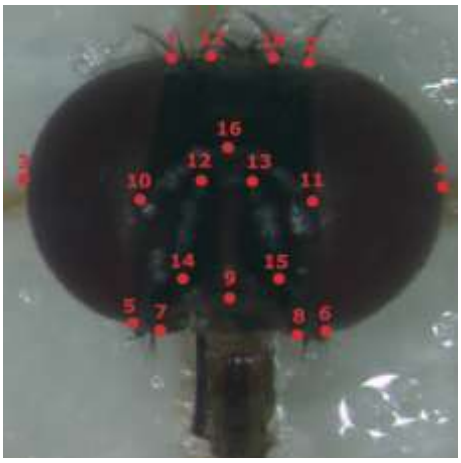


Figure 18. Head of *Fannia pusio* female. The numbered points indicate the location of the 18 landmarks used for head measurements.

The resulting numerical data were analysed with MorphoJ statistical software (Klingenberg, 2013). Based on the results of Fuentes-López (2018) on three species of Lucilia, we selected 18 landmarks. It should be noted that landmarks 12–15 (Table 4) are antennal and thus mobile relative to the head capsule, so these landmarks were analysed separately to not introduce any artifacts. The plot of the two first relative warps shows the scores of each specimen in that shape space, as well as the shape changes explained by each axis. Principal Component Analysis (PCA) was performed to reduce the dimensionality of the data and to determine the variables chosen for this study (Dujardín *et al*., 2014). We followed up with a Canonical Variate Analysis (CVA) to explore the differences among groups (Zelditch *et al.*, 2004). Transformation Grids and Wireframes representing the PCA and CVA shape changes, showing variations for the relative location of each landmark, were presented. Finally, a reclassification with Cross-

validation (Refaeilzadeh *et al.*, 2009) and Mahalanobis distance (McLachlan, 1999) with permutation for pairwise was made to verify our results (Wink-da-Silva *et al.*, 2018).

Landmarks	Description of the landmarks
1	Right eye upper margin
2	Left eye upper margin
3	Right eye lateral margin
4	Left eye lateral margin
5	Right eye lower margin
6	Left eye lower margin
7	Lower right margin of the mouth
8	Lower left margin of the mouth
9	Lower margin of the clypeus
10	Interior angle of right eye
11	Interior angle of left eye
12	Flagellum base of the right antennae
13	Flagellum base of the left antennae
14	Flagellum apex of the right antennae
15	Flagellum apex of the left antennae
16	Upper margin of the frontal suture
17	Upper final of right orbital bristles line
18	Upper final of left orbital bristles line

Table 4. Description of the landmarks used in the analysis of *Fannia* head (see also Figure 18).

Results

Three sets analyses were performed. The first concerned the species of the genus *Fannia* present in the initial sample (N = 81) and was intended to test if the head is a suitable structure for this type of analysis and if the chosen landmarks are adequate. *Hydrotaea floccosa* (Muscidae) was used as an outgroup, allowing the effectiveness of the analysis to be tested. Identification at the species level was achieved through molecular analysis of DNA sequences (*cox1*), which are now available on GenBank. The other two analyses were performed only on a sub-sampling of *F. pusio* (N = 65). One was geography based, to interpret the morpho-geometric differences according to the sampling locations (Table 3). The other was based on the biological of the species to test for differences between our domestic lineage and the samples collected in the wild.

Search for suitable head landmarks in the genus Fannia

As previously mentioned, for the comparative study of the *Fannia* species present in the sampling, we recovered 81 individuals (Table 3). First, the Principal Component Analysis (PCA) showed great differences in the landmarks located in the parafacial zone. This area

is where the antennae and interocular space are located. Specifically, the landmarks that show this difference ordered by degree of variation are: 10, 11, 13, 12, 14 and 15. These differences can be observed in the Transformation Grid and the Wireframe (Fig. 19). The projection of the geometric configuration of the landmarks in the tangent space is shown in figure 20. On the other hand, according to the Canonical Variate Analysis (CVA), the landmarks that show most differences between species were: 5, 6 and 16. These landmarks cover the lower margins of the eyes and the ptilinal suture respectively (Fig. 21). As portrayed in figure 22, these results allow us to differentiate most of the species. However, the samples of *F. lepida* overlap with *F. leucosticta.* As observed in table 5, the statistics contradict the graphic results obtained. Statistically significant differences of *p*-value < 0.05 were observed between *F. pusio* and all other species, except for *F. canicularis* and *F. leucosticta*. However, the cross validation obtained was higher than 75% in all comparisons between *F. pusio* and the other species.

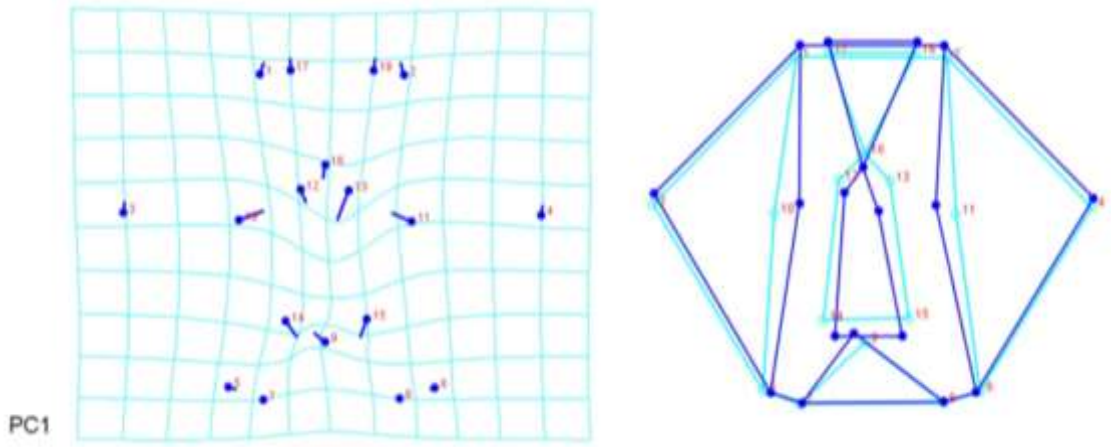


Figure 19. Transformation Grid (left) and Wireframe (right) representation of shape variations between *Fannia* species based on Principal Component Analysis. *In the wireframe the turquoise outline characterizes the position of consensus landmarks, while the blue outline represents landmarks configurations.

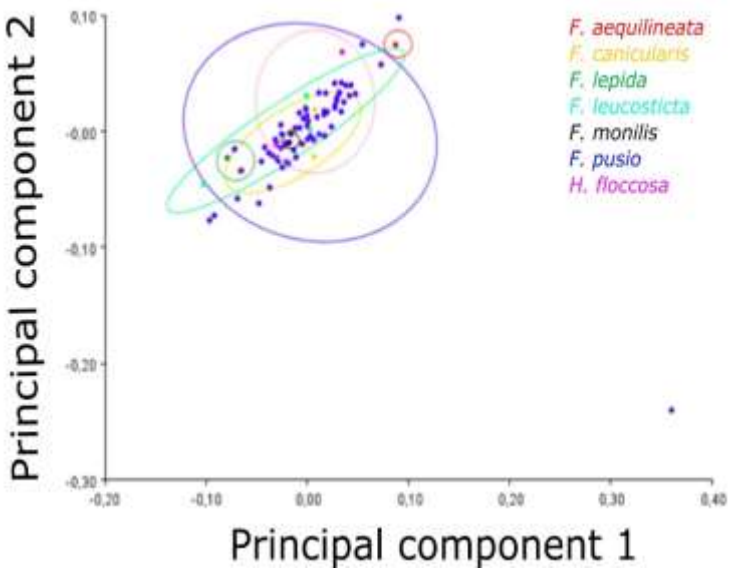


Figure 20. Discrimination of *Fannia* species with a *Hydrotaea floccosa* as outgroup based on Principal Component Analysis.

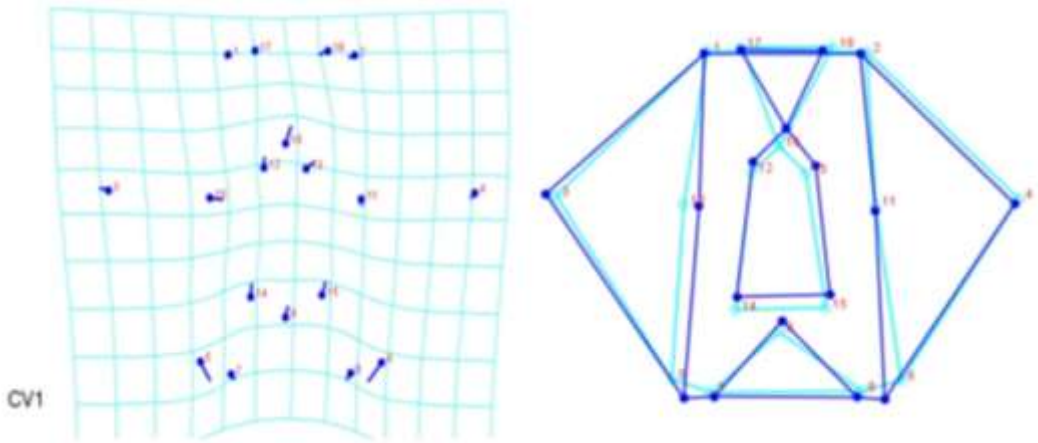


Figure 21. Transformation Grid (left) and Wireframe (right) representation of shape variations between *Fannia* species based on Canonical Variate Analysis. *In the wireframe the turquoise outline characterizes the position of consensus landmarks, while the blue outline represents landmarks configurations.

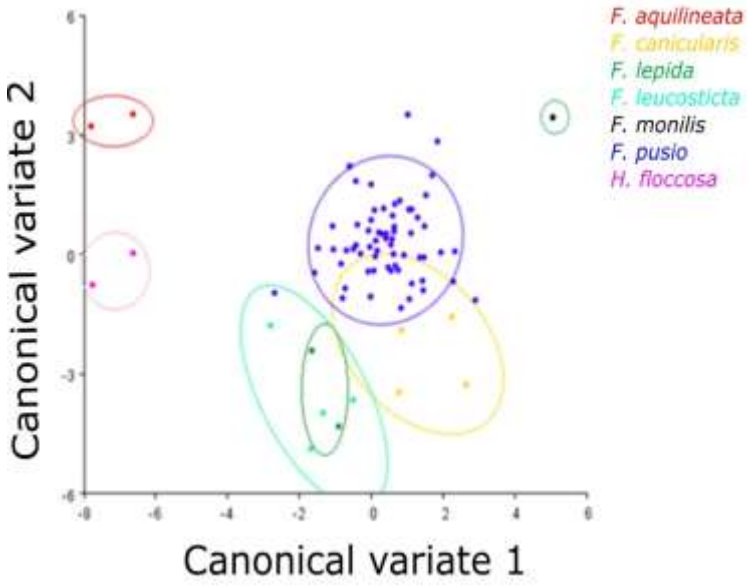


Figure 22. Discrimination of Fannia species with *a Hydrotaea floccosa* as outgroup based on Canonical Variate Analysis.

Regarding the differences among other species comparisons, no statistical significance was obtained with a $p > 0.05$. However, the pairs *F. aequilineata – F. leucosticta* and *F. leucosticta – F. monilis* showed a percentage higher than 75 % in the cross validation (Table 5). Most of our samples belonged to *F. pusio* and it is in this species where statistically significant differences are actually observed. This led us to carry out two further analyses in this species, the first according to the locations where *F. pusio* was collected (Table 4) to evaluate whether geography has an explanatory role in the morpho-geometric differences found in the species. In the second analysis, we evaluated whether environmental fluctuations affected the GM parameters of the species. To this end, we compared the previously considered individuals of *F. pusio* with a set of lab-reared flies originating from a colony kept under constant laboratory conditions for several years.

Species	Mahalanobis distance	Cross-validation
F. aequilineata – F. canicularis	*p – value* > 0.05	71.43 %
F. aequilineata – H. floccose	*p – value* > 0.05	25 %
F. aequilineata – F. lepida	*p – value* > 0.05	50 %
F. aequilineata – F. leucosticta	*p – value* > 0.05	83.33 %
F. aequilineata – F. monilis	*p – value* > 0.05	33.33 %
F. aequilineata – F. pusio	*p – value* < 0.01**	97.01 %
F. canicularis – H. floccosa	*p – value* > 0.05	57.14 %
F. canicularis – F. lepida	*p – value* > 0.05	57.14 %
F. canicularis – F. leucosticta	*p – value* > 0.05	44.44 %
F. canicularis – F. monilis	*p – value* > 0.05	50 %
F. canicularis – F. pusio	*p – value* > 0.05	75.71 %
H. floccosa – F. lepida	*p – value* > 0.05	50 %
H. floccosa – F. leucosticta	*p – value* > 0.05	16.67 %
H. floccosa – F. monilis	*p – value* > 0.05	33.33 %
H. floccosa – F. pusio	*p – value* < 0.01**	94.03 %
F. lepida – F. leucosticta	*p – value* > 0.05	16.67 %
F. lepida – F. monilis	*p – value* > 0.05	33.33 %
F. lepida – F. pusio	*p – value* < 0.05*	97.01 %
F. leucosticta – F. monilis	*p – value* > 0.05	80 %
F. leucosticta – F. pusio	*p – value* > 0.05	84.06 %
F. monilis – F. pusio	*p – value* > 0.05	98.48 %

Table 5. Results of Mahalanobis distances (p-value) and Cross-validation (%) between different *Fannia* species.

Geographical differences among F. pusio populations

The PCA shows that the landmarks with the greatest difference in order of variation are: 13, 10, 11, 9, 14, 15 and 12. All of them are points arranged along the parafacial area, where the antennae and interocular space are located. A further visualization of these differences is offered in figure 19. The projection of the geometric landmark configuration onto the tangent space is shown in figure 23. According to the CVA, the landmarks that bear the most differences between species are: 9, 14, 12 and 15. These points also reflect the parafacial area (Fig. 24). As can be gleaned from figure 25, these results are sufficient to differentiate between the sites where *F. pusio* was found. In the case of the individuals from the Region of Murcia and the Valencian Community, there is extensive overlap. Further statistical analysis was applied for full validation (Table 6). They reflect statistical significance in shape difference between the Lisbon – Region of

Murcia ($p < 0.01$), while cross-validation shows us a considerable percentage in all of them (> 60 %), except between the Region of Murcia – Valencian Community.

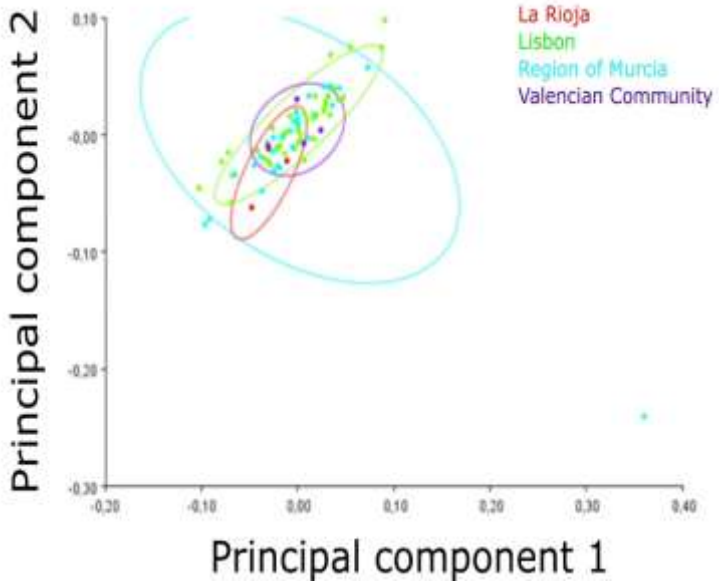


Figure 23. Geographical differences among *Fannia pusio* populations based on Principal Component Analysis.

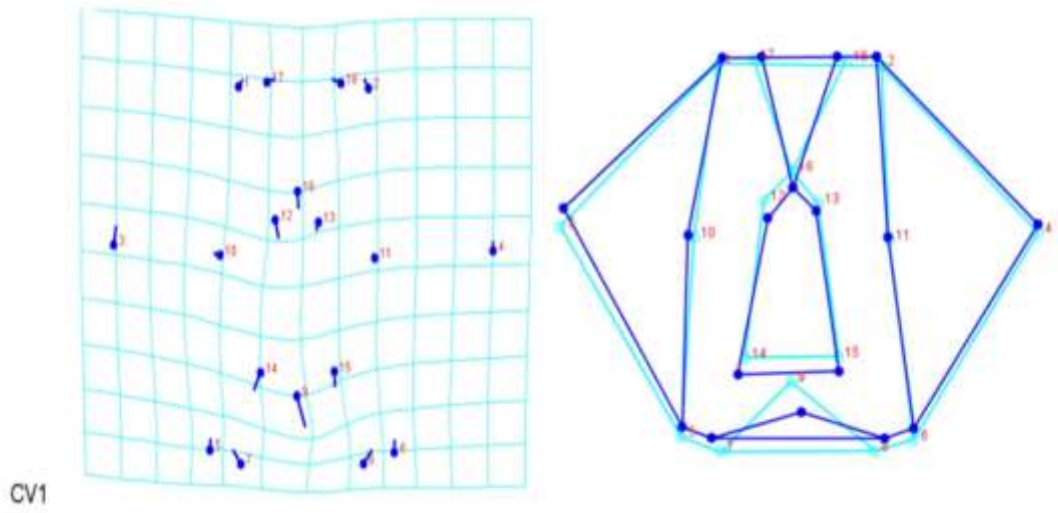


Figure 24. Transformation Grid (left) and Wireframe (right) representation of shape variations among *Fannia pusio* populations in terms of geographical distribution based on Canonical Variate Analysis. *In the wireframe the turquoise outline characterizes the position of consensus landmarks, while the blue outline represents landmarks configurations.

	Lisbon La Rioja	Lisbon R. Murcia	Lisbon Valencian C.	La Rioja R. Murcia	La Rioja Valencian C.	R. Murcia Valencian C.
Mahalanobis	$p > 0.05$	$p < 0.01$**	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
Cross-validation	71.86 %	69.49 %	62.5 %	78.79 %	66.67 %	39.39 %

Table 6. Results of the Mahalanobis distances (*p*-value) and Cross-validation (%) among *F. pusio* populations in terms of geographical distribution.

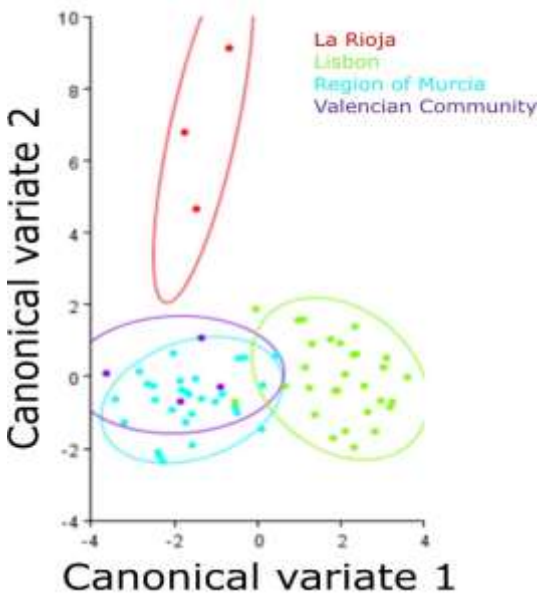


Figure 25. Geographical differences between the populations of *Fannia pusio* from the four localities of the Iberian Peninsula where they were found (La Rioja, Lisbon, Region of Murcia and Valencian Community) based on the Canonical Analysis of Variables.

Variations between a laboratory bred colony versus wild samples of F. pusio

Regarding the ecology of the species, some differences were observed; The PCA shows variation in the same landmarks as in the geographical comparison: 13, 10, 11, 9, 14, 15 and 12 (points of the parafacial area) that can be observed in the Transformation Grid and the wireframe (Fig. 19). However, the projection on the scatter diagram varies (Fig. 26). On the other hand, in the CVA the landmarks present another order of variation: 9, 10, 15 and 13, also from the parafacial area, as shown in figure 27. Figure 28 shows the alterations that exist between the subspace projections of individual landmark configurations according to environmental and life-history differences. Furthermore, in the same manner as with the previous analyses, statistical testing was performed.

Mahalanobis distances returned a very high statistical significance ($p < 0.01$) and cross-validation with a considerably high percentage (72.3 %).

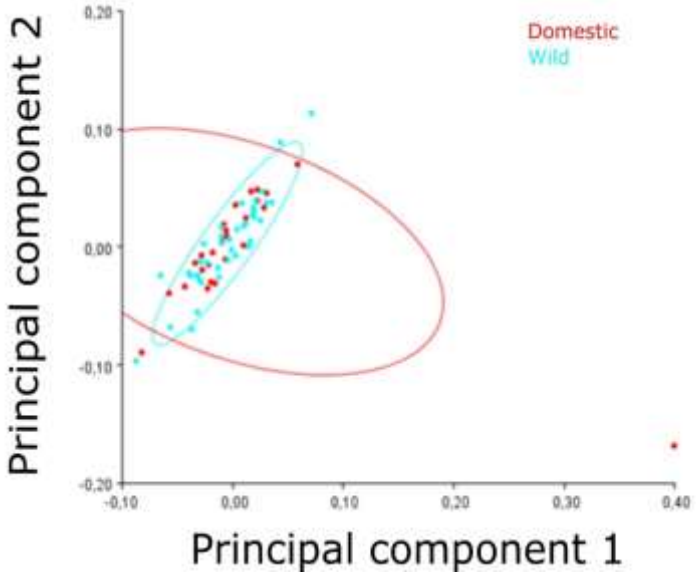


Figure 26. Variation between a laboratory bred colony *versus* wild sample of *Fannia pusio* based on Principal Component Analysis.

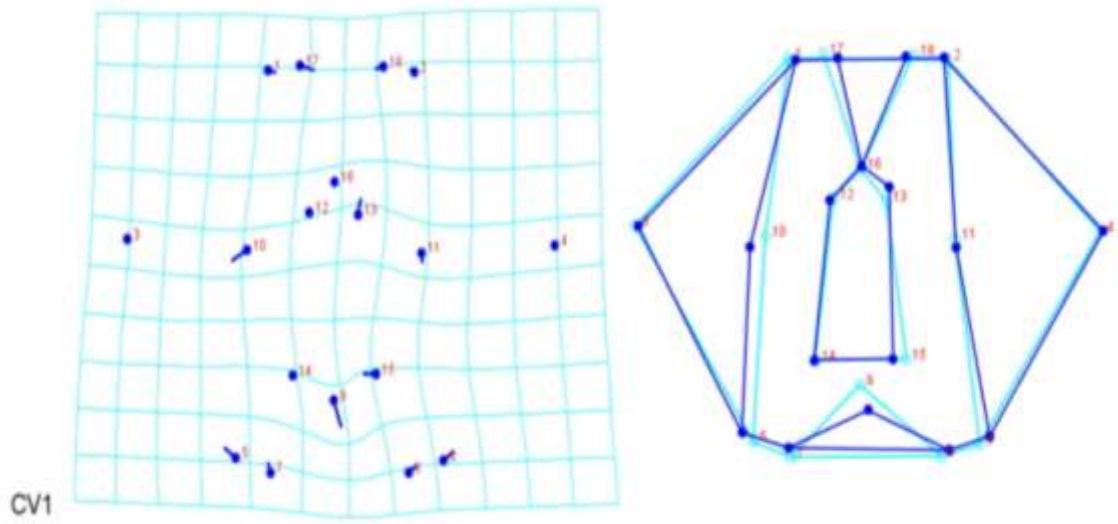


Figure 27. Transformation Grid (left) and Wireframe (right) representation of shape variations between a laboratory bred colony versus wild samples of *Fannia pusio* based on Canonical Variate Analysis. *In the wireframe the turquoise outline characterizes the position of consensus landmarks, while the blue outline represents landmarks configurations.

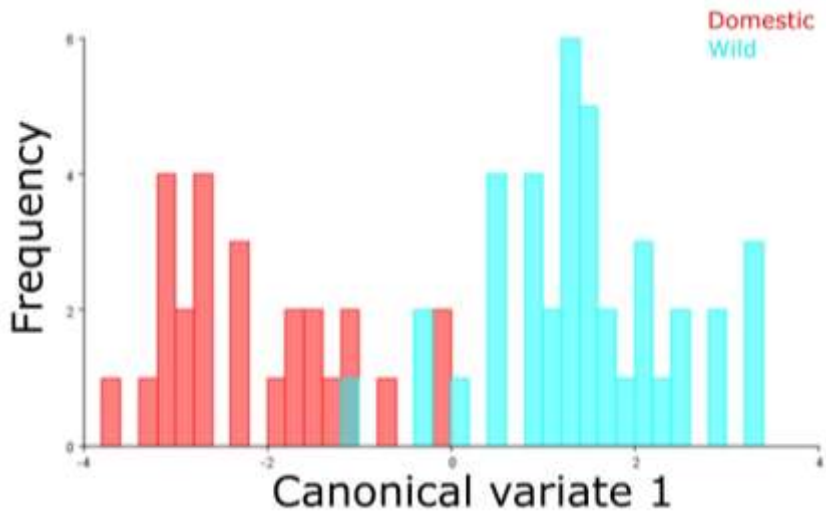


Figure 28. Geometric morphometrics variation of *Fannia pusio* head landmarks based on Canonical Variable Analysis. Individuals from the colony reared under laboratory conditions are compared against wild-caught individuals.

Discussion

The present work provides a case study of the application of GM on the species *F. pusio*, and alleviates the scarcity of information in the scientific literature on this important Dipteran family (Szpila *et al.*, 2019). The samples used were collected all throughout the Iberian Peninsula. Despite the fact that the wings are the Diptera structure from which the most taxonomic information regarding morphometric applications has been obtained (Grzywacz *et al.*, 2017a), other structures may also hold promise, and in this work, we assess the usefulness of the head (De Souza *et al.*, 2015; Fuentes-López, 2018).

We reported for the first time the application of GM to aid in the differentiation of *Fannia* species morphology and assess the intraspecific variability of *F. pusio* from head landmarks, focusing on the parafacial area. As mentioned above, most *Fannia* are morphologically very similar and GM may deliver an important tool for defining some ambiguously or hither to unidentifiable specimens (Dobigny *et al.*, 2002). The possibility to identify them with GM suggests that shape is more relevant than size (Sumruayphol & Chaiphongpachara, 2019).

Results of our data analysis showed a clear differentiation among species, except for the pairs *F. pusio – F. canicularis* and *F. pusio – F. leucosticta* ($p > 0.05$). In the latter case, we know that the lack of differences between the species may also be associated with close phylogenetic relationship—within the same subgroup (pusio-group)—which is

classified in higher canicularis-group described by Chilcott (Wang *et al*., 2016). However, members of the pair *F. pusio* – *F. canicularis* are phylogenetically separate taxa and yet offer no clearly differentiated results. It is possible that, in this case, the inability of the analysis to reflect differences between them could result from the low number of specimens of (N = 5) available in our sampling. However, comparison between species belonging to different families, such as *H. floccosa* (Muscidae) – *F. pusio* (Fanniidae), even though we found a low number of the former (N = 2), allowed us to demonstrate clear differentiation with a $p < 0.01$ and a reclassification with cross validation of 94.03%.

We view the following alternative conclusions as the most likely: i) the structures and landmarks chosen are efficient for differentiating at the family level (De Souza *et al.*, 2015), but not as good at the genus level. Alternatively, ii) it could be inferred that the species that show this overlap ($p > 0.05$) have a close phylogenetic relationship (Dos Santos *et al*., 2003) and therefore present smaller measurable morphometric differences.

The *Fannia* species from which the greatest number of samples could be analysed in this work was *F. pusio*. The sampling was carried out from June to September (Table 3), with the bulk of specimens collected in September. This was been observed in the samplings carried out in other studies (De Carvalho *et al.*, 2003; Carles-Tolrá, 2006; Grzywacz & Prado e Castro, 2012; Monteiro *et al.*, 2014), confirming that this *Fannia* species predominates in autumn (Smith, 1986; Bélo *et al.*, 1998). Based on the samples available, we focused on intraspecific variations according to geographic location and differences in lifestyle, comparing wild caught samples to a laboratory population. These two approaches analysed through GM will help better understand the variability that this species carries (Webster & Sheets, 2017). This goal has medical, economic and forensic importance. First, *F. pusio* is a vector of myasis in humans and cattle; furthermore, it is a useful indicator species in forensic entomology, since it is known to be present in animal remains and human corpses (Grzywacz *et al*., 2017a; Szpila *et al*., 2019). Furthermore, Nuñez-Rodriguez & Liria (2017) already observed that GM provides the means to differentiate ecological conditions and geographic range in forensic entomology, thus helping in criminal investigations.

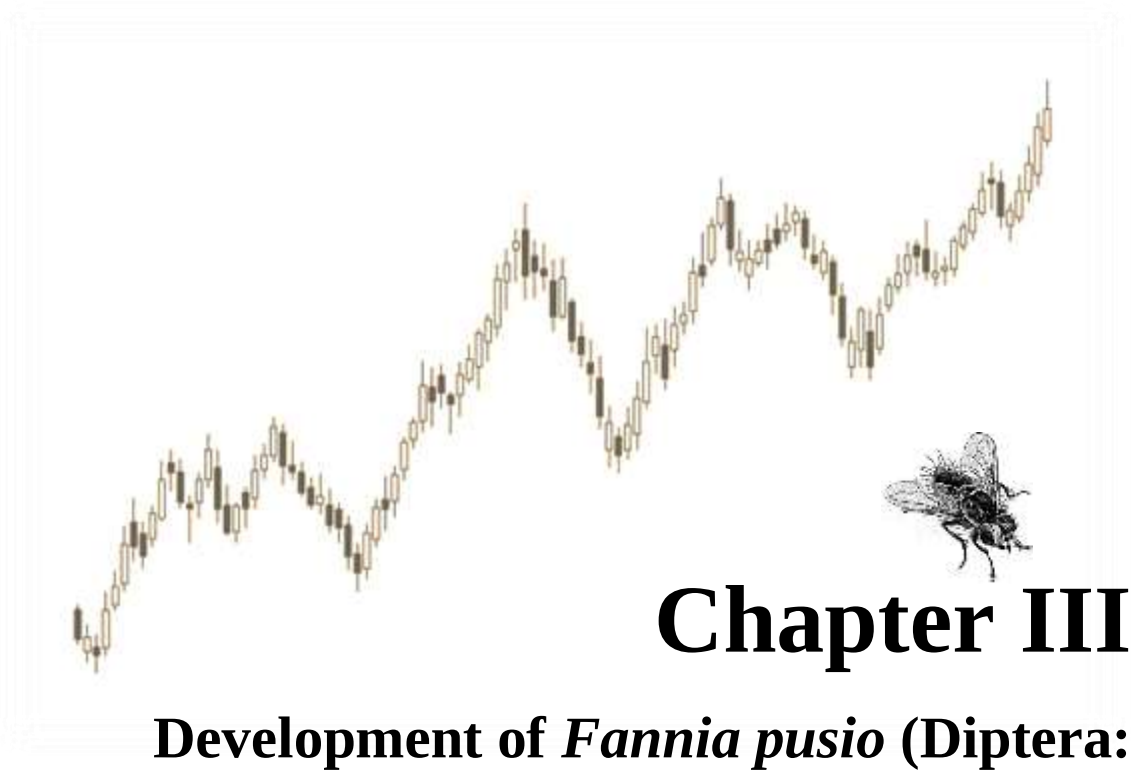
Regarding the geographic range of *F. pusio*, different phenomena are observed. First, the specimens collected in the Valencian Community and the Region of Murcia do not show GM differentiation ($p > 0.05$; cross-validation of 39.39 %) apart from indicating overlap

(Fig. 25). The explanation could be the small sample size obtained in the Valencian Community; however, a low number of samples was also obtained in La Rioja and the analysis does in this case show a great difference to the rest of the locations. Therefore, we understand that there is a close relationship between the samples of the Valencian Community and the Region of Murcia, which is plausible since they are geographically closely located in the East of the Iberian Peninsula where environmental conditions are very similar.

Consequently, the morpho-geometric differentiation along the observed Iberian locations ranges among the Northeast (La Rioja), Southeast (Valencian Community + Region of Murcia) and Southwest (Lisbon), giving a cross validation > 60 % among all (Table 5). Nevertheless, at statistically significant difference is found between Lisbon - Murcia Region with a *p*-value in the Mahalanobis distance < 0.01 (Table 6). Intraspecific differentiation is a clear example of how abiotic factors affect individual development (Pacheco *et al*., 2017; Sumruayphol & Chaiphongpachara, 2019); and GM represents the best option to analyse population segregation (Mikery *et al.*, 2019). From these data it can be inferred that the environmental conditions shaped by the Atlantic Ocean in Lisbon and the Mediterranean Sea in the Region of Murcia may result in morpho-geometric changes to the species. The areas bordering the Atlantic are colder and less humid, while the Mediterranean areas are much warmer and wetter. These climatic variations provide morphometric alterations among populations of the same species (Hajd *et al*., 2014; Espra *et al*., 2015; Fuentes-López, 2018).

Regarding the comparison between samples of lab-raised *F. pusio* under constant conditions and those collected in the wild, we see that there is no overlap between the domestic and the wild samples (Fig. 28). This is a fairly noticeable component within the same species, with statistically significant differences among individuals' flies ($p < 0.01$; cross validation > 70 %) (Table 6), indicating that environmental fluctuations also affect the morphology of the species. Although all samples belong to the same species, the variation in shape could be affected by environmental conditions instead of genetic drift and evolutionary divergences (Arias *et al*., 2017). Another aspect to take into account is the high inbreeding of the colony since it is a 165th generation. Alternatively, the fact that domestic individuals are raised ad libitum and with the absence of predators might provide advantageous conditions for their development (Riaño *et al.*, 2008).

In the different analyses carried out in this paper, all the results show the variation in the parafacial and fronto-orbital zones. As previously mentioned, the landmarks fixed at the apices and base of the antennas study the differences between them and not with the rest of the structure of the head. This should be clear since they are two mobile organs and can change their position. These results differ from those obtained by Godoy *et al.* (2018) where it shows differences in landmarks fixed in the contour of the head instead of in internal areas. It should be noted that this work is innovative, with a relatively scarce bibliographical background to draw form, especially regarding the Fanniidae. As mentioned by Szpila *et al.* (2019), more studies should be performed on the Fanniidae using GM to improve our knowledge of the family. This study has provided some proof that previously unused structures, such as the head, are useful to discriminate between species and even to find differences within the same species, along geographic and ecological axes of variation (Garzón & Schweigmann, 2018).



Chapter III

Development of *Fannia pusio* (Diptera: Fanniidae) under temperature-controlled conditions and its application in post-mortem interval (PMI) estimation.

Chapter III

Development of *Fannia pusio* (Diptera: Fanniidae) under temperature-controlled conditions and its application in post-mortem interval (PMI) estimation

Abstract

Fannia pusio (Wiedemann, 1830) is a species belonging to the family Fanniidae of great forensic, sanitary and veterinary interest. The behavioural peculiarities exhibited by this species may provide additional information for further research. The application of entomology in the forensic field has focused especially on the first colonising taxa of corpses in the early stage of decomposition. However, species that appear at more advanced stages can help to broaden knowledge, as is the case with *F. pusio*. In addition, it has the ability to colonise buried bodies, a condition where larger dipterans are unable to do so.

In this work, the behaviour of *F. pusio* is studied in a temperature range from 5 ºC to 40 ºC. Different statistical analyses have been carried out to study its behaviour and the data collected have been applied to calculate the post-mortem interval (PMI) using the isomorphen and isomegalen diagrams. Therefore, the results shown in this manuscript help, together with the existing bibliography of other species, to broaden the knowledge of entomofauna occurring in forensic investigations.

Keywords: Forensic importance, Growth rate, Isomegalen-diagram, Isomorphen-diagram, Sanitary importance, Veterinary importance.

Introduction

Forensic entomology studies the association of arthropods in evidence with judicial examinations (Grzywacz *et al.*, 2017b). These arthropods colonise the corpse in a specific order according to the stage of decomposition (faunal succession) and climatic conditions (Barrios & Wolff, 2011). The data obtained following this behaviour are essential for the determination of the post-mortem interval (PMI) (Amendt *et al.*, 2011; Charabidze, 2012; Cavallari *et al*., 2015; Faris et al., 2020; Wang *et al.*, 2020).

There are various methodologies for the estimation of PMI, however, the isomorphen and isomegalen diagrams are the simplest and most intuitive techniques. Diptera are the order of insects with the greatest application in forensics as they are the first colonisers (Amendt *et al.*, 2011). That is why there are many studies carried out on different families: Calliphoridae (Benecke & Lessig, 2001; Oliveira-Costa & Mello-Patiu, 2004; Michaud & Moreau, 2009), Muscidae (Benecke & Lessig, 2001; García-Rojo *et al.*, 2009), Sarcophagidae (Nassu *et al*., 2014) and Fanniidae (Barros *et al*., 2008; Amat, 2010; Matuszewski *et al*., 2010; Quiroga & Domínguez, 2010; Aballay *et al*., 2012; Akbarzadeh *et al.*, 2012; Grzywacz & Prado e Castro, 2012; Baz *et al*., 2015; Cavallari *et al.*, 2015). The most commonly used species for PMI estimation are those belonging to the first colonising families (Benecke & Lessig, 2001; García-Rojo *et al.*, 2009; Nassu *et al*., 2014; Defilippo *et al*., 2019). However, there is also a need for research on insect species that occur in the most advanced stages of decomposition due to their increasingly frequent occurrence (Dias-Vasconcelos *et al*., 2017). Fanniidae is one of the examples that highlight this need, since despite preferring very advanced stages of decomposition, there is evidence of their appearance in the early stages (Amat *et al.*, 2013).

In this work we will focus on *Fannia pusio* (Wiedemann, 1830), a species belonging to the family Fanniidae. This species was established under controlled environmental conditions in the laboratory after being found in a cat carcass in an advanced state of decomposition in an urban area of the Region of Murcia (Spain). It is also known as the "chicken dung fly" due to its presence on laying hen farms. They are native to the Nearctic and Neotropical regions (Smith, 1986; Bélo *et al.*, 1998; Couri & Sousa, 2019), but are widespread in tropical and warm temperate areas of the Old World. However, they have adapted to survive low temperatures and wet periods (Linhares, 1978; Leandro & D'Almeida, 2005). Therefore, *F. pusio* is considered a synanthropic species (Monteiro &

Do Prado, 2006) and is found in all seasons (De Souza & Linhares, 1997; Barbosa *et al*., 2009; Krüger *et al.*, 2010; Aballay *et al*., 2012; Faria *et al*., 2013).

The forensic interest of this species is its ability to colonise buried bodies that have been partially protected from colonisation by larger diptera, which provides a notable added point of forensic interest (Introna *et al.*, 2011; Grzywacz *et al*., 2017b). The sanitary and economic interest of this species is also relevant as it acts as an egg carrier of Dermatobia hominis, which causes myiasis in both domestic animals and humans (Gomes *et al*., 2002; De Carvalho *et al*., 2003; Espindola & Couri, 2004; Amat, 2010; Quiroga & Domínguez, 2010; Amat *et al.*, 2013; Velásquez *et al.*, 2013). Unfortunately, there is still no studies on the effect of temperature on the development of *F. pusio* (Faris *et al.*, 2020). Therefore, the aim of this work is to evaluate the effect of temperature on its development, for the first time, providing data to specialists for determining its suitability for a more accurate PMI determination. The importance of this information for medical, veterinary and forensic purposes (Marchiori, 2014) is also discussed.

Materials and methods

A colony of *F. pusio* was established under controlled environmental conditions (25 ºC of temperature, 60 % relative humidity and a 12:12 light cycle) in Laboratory of Necrophagous Diptera at the University of Murcia (Spain). Water and sugar *ad libitum*, supplemented with caned cat food, were provided to adults (Couri, 1991) for three hours to induce oviposition and obtain eggs (Marchiori & Do Prado, 1996). Canned cat food was chosen after unsuccessfully testing various substrates such as dog and human faeces and chicken and pork liver (D'Almeida, 1994). Its composition is based on meat and meat by-products, fish and fish by-products and minerals, similar to that used by Marchiori & Do Prado (1996).

Once the eggs were obtained, they were moved to cages (25 cm x 25 cm x 10 cm) with this substrate and sand to allow larvae growth and pupariation, after which the pupae were returned to the adult cage (40 cm x 30 cm x 30 cm). The process was repeated as necessary. The eggs cages were kept at constant temperatures (eight different temperatures were tested: 5 ºC, 10 ºC, 15 ºC, 20 ºC, 25 ºC, 30 ºC, 35 ºC and 40 ºC) and 60 % of relative humidity, until hatching and pupae were transferred thereafter to plastic jars to rear to adults. Three replicates were carried out for each experiment and twenty

individuals were sampled twice a day, until more than 50 % of larvae had reached the pupae stage. Immediately upon collection, samples (larvae, prepupae, pupae) were immersed in boiling water for a few seconds, and preserved in 70 % ethanol. Each individual was measured according to the protocol described by Byrd & Allen (2001) and Charabidze *et al*. (2008). Data analysis was performed using SPSS v20 statistical software (IBM, Armonk, New York, USA) where analysis of variance (ANOVA) was carried out to evaluate the effect on response variability and statistical significance.

Longitudinal measurements of the specimens were used to calculate PMI using the isomorphen and isomegalen diagrams. These methodologies are graphical representations that cover information from oviposition until the pre-imaginal stages are reached (Richards *et al.,* 2008; Bambaradeniya *et al.,* 2018). The isomorphen represents the duration of development of the various stages at different temperatures where each line shows the transition time of each stage according to temperature; the spaces between these lines are the periods corresponding to each stage. Whereas, the isomegalen represents the development time needed to reach certain lengths versus temperature. Each line groups larvae of the same size within each temperature range studied (Reiter, 1984; Gennard, 2007).

Results

This study is based on the longitudinal data of 8908 individuals (including eggs, larvae, prepupae and pupae) of *F. pusio* established under laboratory conditions at different temperatures. Two types of analysis were carried out, a statistical analysis comparing the length and time of each stage of the life cycle versus temperature (see Table 7), and their application to the calculation of the PMI by isomegalen and isomorphen diagrams.

Statistical comparisons

The results are summarized in Table 7 with the measurements of each stage and the time each one has taken depending on the temperature studied. However, at 5 ºC, 10 ºC and 40 ºC it has been observed that adult *F. pusio* can survive but oviposition does not occur. Thus, the viable temperature range for the species is from 1 5ºC to 35 ºC and this is where further analysis will be focused.

Temperature (°C)	Mean length ± SD (mm)	95 % Confidence interval (mm)	Minimum-maximal length (mm)	Total sample (N)	Time since hatching (h./ days)
Eggs					
25	0.74 ± 0.01	0.72–0.75	0.59–0.91	57	–
L1					
15	1.07 ± 0.03	1.01–1.12	0.44–1.91	163	9–165 h/0.4–6.9 d
20	1.07 ± 0.28	1.01–1.12	0.44–1.91	163	9–69 h/0.4–2.9 d
25	1.25 ± 0.04	1.18–1.33	0.58–2.39	124	9–68 h/0.5–2.8 d
30	0.82 ± 0.03	0.75–0.89	0.36–1.63	81	9–33 h/0.4–1.4 d
35	0.83 ± 0.03	0.78–0.89	0.47–1.64	75	9–33 h/0.4–1.4 d
L2					
15	1.94 ± 0.04	1.87–2.01	1.23–3.18	156	9–237 h/0.4–9.9 d
20	1.94 ± 0.04	1.87–2.01	1.24–3.18	156	9–69 h/0.4–2.9 d
25	2.75 ± 0.05	2.65–2.85	1.52–3.67	90	9–84 h/1.5–3.5 d
30	1.68 ± 0.09	1.50–1.86	0.74–3.67	70	12–81 h/0.9–3.4 d
35	1.31 ± 0.02	1.26–1.35	0.68–1.95	118	9–45 h/0.4–1.9 d
L3					
15	5.40 ± 0.04	5.34–5.48	2.14–7.13	816	81–549 h/3.4–22.9 d
20	5.39 ± 0.04	5.32–5.46	2.14–7.13	825	33–285 h/1.4–11.9 d
25	5.11 ± 0.06	4.99–5.23	2.15–7.15	369	36–189 h/1.5–7.9 d
30	5.01 ± 0.07	4.95–5.22	1.13–7.00	371	21–165 h/1.9–6.9 d
35	4.73 ± 0.06	4.60–4.86	1.00–7.24	535	9–537 h/0.4–22.4 d
Prepupae					
15	4.87 ± 0.02	4.82–4.92	3.24–6.22	690	137–549 h/5.7–22.9 d
20	4.87 ± 0.02	4.82–4.91	3.24–6.22	690	105–285 h/4.4–11.9 d
25	5.39 ± 0.04	5.31–5.46	3.53–6.95	346	96–224 h/4–9.3 d
30	4.77 ± 0.05	4.68–4.87	3.15–6.89	214	57–165 h/2.4–6.9 d
35	4.96 ± 0.03	4.90–5.03	2.52–6.30	371	57–537 h/2.4–22.4 d
Pupae					
15	4.07 ± 0.04	3.99–4.15	2.27–5.10	167	321–537 h/13.4–22.4d
20	4.08 ± 0.04	4.00–4.20	2.27–5.40	199	129–285 h/5.4–11.9 d
25	4.57 ± 0.03	4.50–4.63	3.05–6.05	139	92–248 h/3.8–10.3 d
30	4.07 ± 0.41	3.92–4.22	2.53–5.54	94	81–165 h/3.4–6.9 d
35	4.25 ± 0.05	4.15–4.36	2.13–5.94	219	81–720 h/3.4–30 d

Table 7. Descriptive statistics on the length of the different stages of *Fannia pusio* at each constant temperature studied.

Comparative analyses are carried out of the stages (expressed in length, mm) in relation to the mentioned temperatures. The length of the first instar larvae (L1) varies for all the temperatures tested (ANOVA $F = 28.189$, $p < 0.01$) with the lengths at 15 ºC and 20 ºC being very similar. The largest L1 was obtained at 25 ºC and the smaller larvae cannot be determined at what temperature they are established since at 30 ºC and 35 ºC rather similar data are observed (Fig. 29, Table 7). The same applies to the second instar larvae (L2) (ANOVA $F = 94.464$, $p < 0.01$); at 25 ºC the largest larvae are also obtained and at 35 ºC, clearly, the smallest (Fig. 29, Table 7).

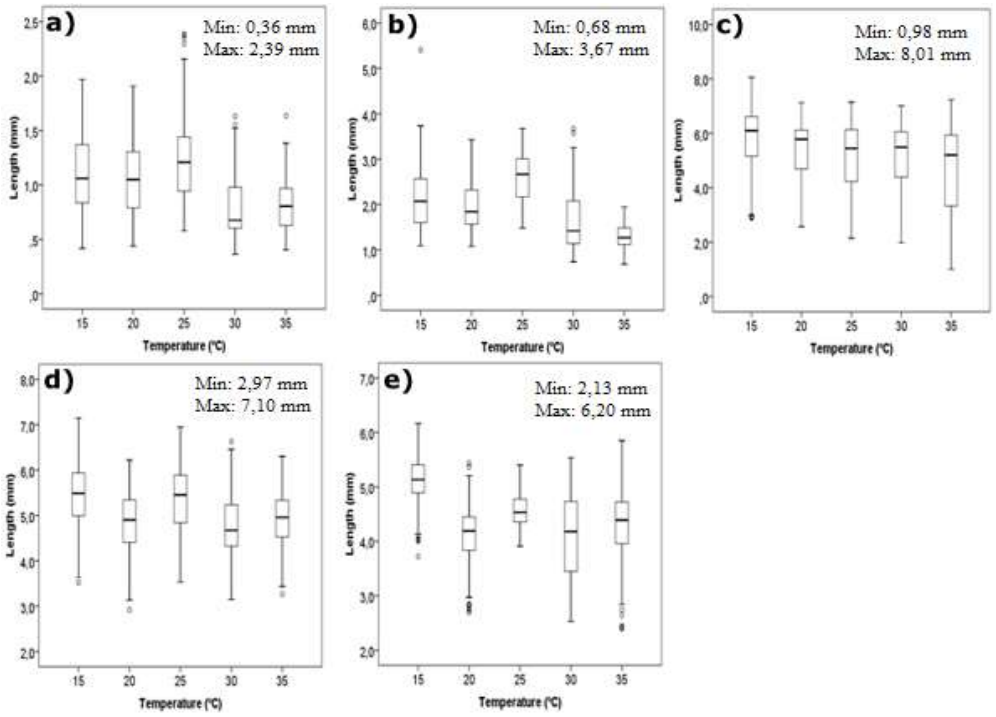


Figure 29. Comparative results for the length (mm) on the y-axis of a) L1, b) L2, c) L3, d) Prepupae and e) Pupae at different temperatures (º C) on the x-axis. The legend indicates the minimum and maximum length values for each stage.

Third instar larvae (L3) represent a very extended period and, in addition, there is no homogeneity of variances at all temperatures (ANOVA F = 89.053, $p < 0.01$). Figure 1 shows an average more or less similar to all temperatures, with the largest larvae at 15 ºC and the smallest at 35 ºC (Table 7). These results are in line with those obtained in the prepupae since these two states (L3 and prepupae) (ANOVA F = 105.841, $p < 0.01$) are developed in a synchronized way (Fig. 29, Table 7).

Finally, significant differences were observed in the length of pupae at all the temperatures tested (ANOVA F = 142.512, $p < 0.01$). In this state the largest larvae are also observed at 15 ºC, while the smallest are between 30 ºC and 35 ºC (Fig. 29). When measuring pupae, the curvature that modifies the length of individuals must be taken into account. The highest number of curved pupae (70.2 %) was obtained at 30 ºC; while the lowest number (6.9 %) was obtained at 25 °C.

The relationship between the body length of the *F. pusio* larvae and the time from egg hatching to pupation (age of specimens) varies with different temperatures, being at 30 ºC and 35 ºC similar. An inverse relationship between the viable temperature range and

development time (Fig. 30). At higher temperatures the development time to complete it is much shorter than at lower temperatures. The inverse relationship shown by these two variables can be observed in the regression analyses with high-quality models of explained variance of more than 95 % and $p < 0.01$ (Table 8).

Temperature	Quadratic model regression	R^2aj.	F-value	*p*-value
15 ºC	$L = 0.028*T - 3.228E-005*T^2$	0,968	F = 5889.85	$p < 0.01$*
20 ºC	$L = 0.051*T - 0.001*T^2$	0,969	F = 2695.14	$p < 0.01$*
25 ºC	$L = 0.056*T - 0.0001*T^2$	0,967	F = 2013.09	$p < 0.01$*
30 ºC	$L = 0.078*T - 0.00001*T^2$	0,951	F = 934012	$p < 0.01$*
35 ºC	$L = 0.083*T - 0.0001*T^2$	0,961	F = 1820.34	$p < 0.01$*

Table 8. Quadratic model regression, variance absorbed (R^2aj), coefficients of regression (F-value) and coefficients significance (*p*-value) of the relationship between the body length of *F. pusio* larvae (L) (mm) and the time (T) (h) at five constant temperatures (see Figure 30). * Indicates coefficients in the model are statistically significant.

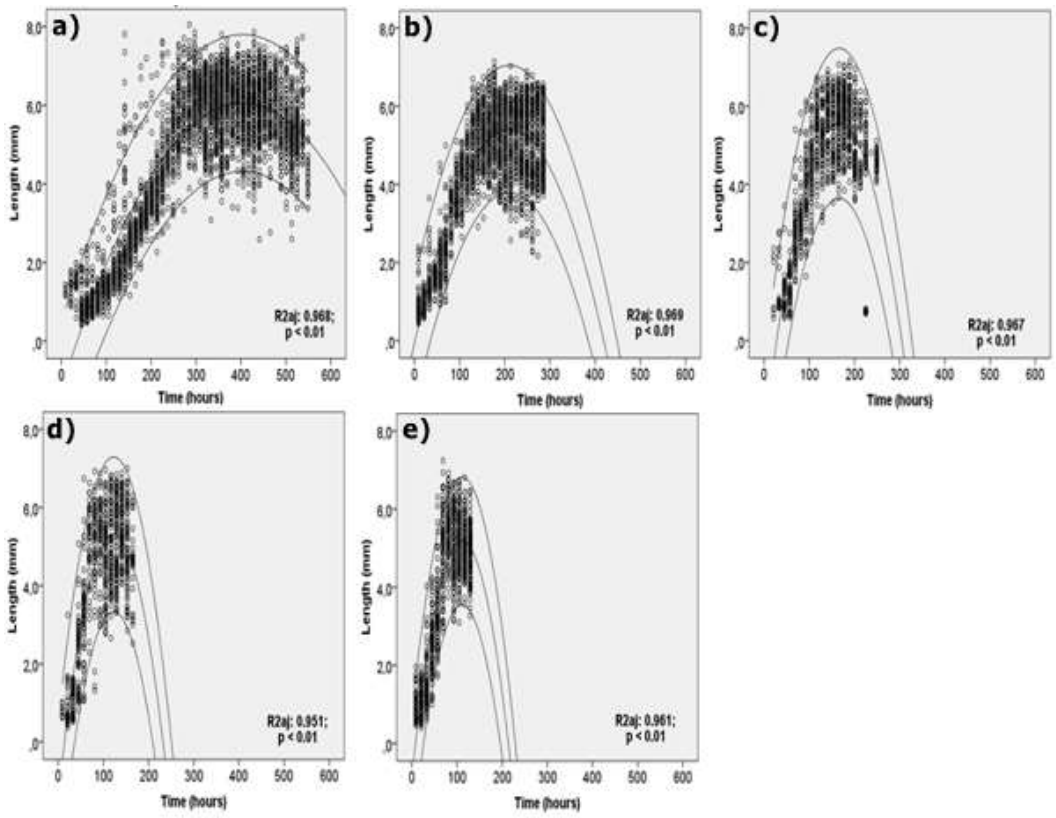


Figure 30. Quadratic model regression curves between length (mm) on the y-axis and age samples (h) on the x-axis at a) 15 ºC, b) 20 ºC, c) 25 ºC, d) 30 ºC and e) 35 ºC (for more details see Tabe 8).

Application in the calculation of the PMI

First, figure 31 displays the time required for each stage of *F. pusio* to complete its cycle at each temperature within its viable range. It can be seen how at high temperatures this time is less, while at low temperatures the time is greater. However, at 35 ºC a slowing down of the pupal stage can be seen, prolonging the complete time of development more than at 30 ºC.

The inverse variation between temperature and time can also be seen in the isomorphen (Fig. 32a) and the isomegalen (Fig. 32b) diagrams. That is, at a lower temperature, the development of *F. pusio* takes longer, while at higher temperatures, it takes less time. The eggs development is not altered by the temperatures analysed; however, we have documented that 10 ºC and 40 ºC are the limits in which clutch was obtained and larvae were able to hatch and develop (Fig. 32).

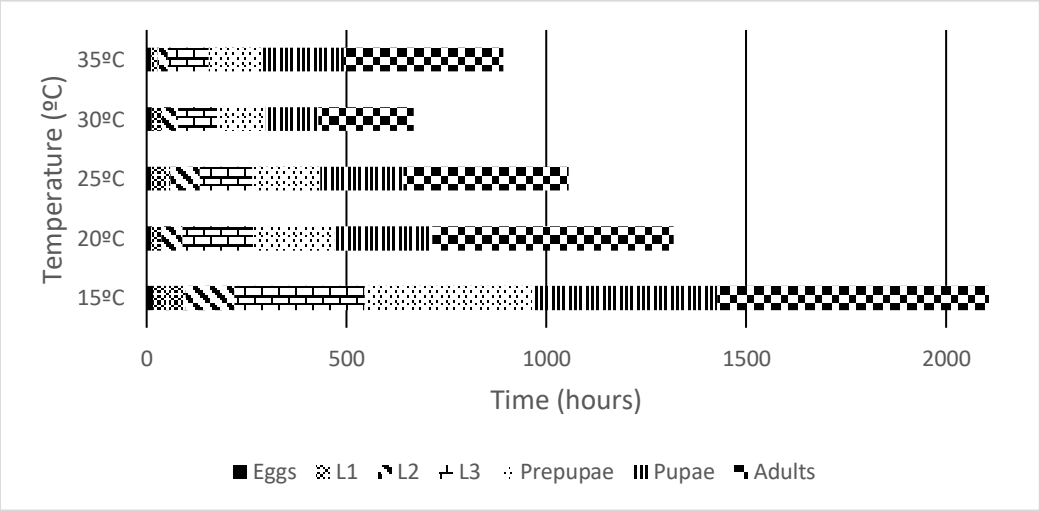


Figure 31. Time development variations in each instar stage of *F. pusio* reared at different temperatures within the maximum and minimum threshold.

The isomorphen-diagram shows a similar behaviour in L1 and L2, undergoing the same drop at 20 ºC. L3 and prepupae also show the same behaviour pattern. However, the results of the pupal stage do show differences, with a decrease in the development time at 25 ºC to rise again at 30 ºC. This means that the adult's emergence is also prolonged in time (Fig. 32a). In addition, isomegalen has other peculiarities; at 20 ºC a very noticeable

acceleration of development is observed for the smallest lengths (1 ─ 4 mm.), but for individuals of 5 mm a deceleration was registered (Fig. 32b).

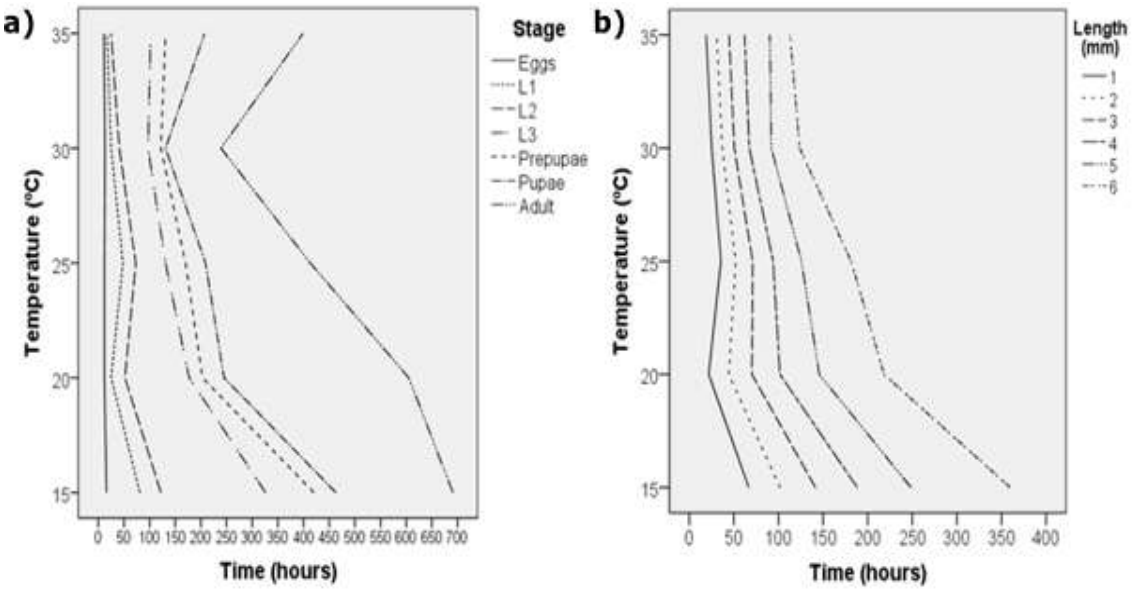


Figure 32. a) Isomorphen-diagram of *F. pusio*: representation of the time expressed in hours (x-axis) required for the development of each stage of the biological cycle within each test temperature range (y-axis); b) Isomegalen-diagram of *F. pusio*: representation of the time expressed in hours (x-axis) needed to reach a certain length expressed in millimetres within each range of temperatures studied (y-axis).

Discussion

There are few studies on the oogenesis, oviposition and hatching of *F. pusio* eggs (Linhares, 1978; D'Almeida, 1994; Marchiori & Do Prado, 1996, 1999) and on their use to calculate the post-mortem interval (Aballay *et al.*, 2012). In this work, the behaviour of *F. pusio* at different temperatures was studied for its subsequent application in the calculation of PMI. It has been observed that the viable temperature range of the species is between 15 ºC and 35 ºC, below and above these thresholds adults can barely survive but oviposition does not occur. It has also been found that the minimum time of embryonic development is 19 hours and eggs begin to hatch between 9 ─ 12 hours, results which differ from those presented by Marchiori & Do Prado (1996). These differences could be due to the sample size of the analyses since Marchiori & Do Prado (1996) used only 10 individuals, whereas we have used 20 individuals per cage. Regarding the immature stages, unexpected results were also obtained. The non-existent differences

between eggs and L1 larvae suggest that the youngest larvae seek a good place to feed, in which they remain until reaching the minimum size required to undergo the moult to L2 (Fig. 29, Table 7). Furthermore, the largest individuals were L3 and the time that this species spends to enter the pupal stage is generally longer than in other dipteran families such as Calliphoridae, Sarcophagidae or Muscidae, regardless of temperature (Reiter, 1984; Grassberger & Reiter, 2001; Grassberger *et al*., 2003; Defilippo *et al*., 2019).

In this work, remarkable data previously unknown have been obtained. In the pupal stage, a natural dehydration occurs, which causes tissue contraction. The higher the temperature, the lower the relative humidity, the more pronounced this curvature is, affecting pupal viability. In addition, the larval masses of larger larvae increase the temperature of their microenvironment due to their own metabolism (Highley & Haskell, 2001; Charabidze *et al*., 2011; Díaz-Martín *et al*., 2014). This is why, at 30 ºC and 35 ºC, the highest percentage of curvature and non-viability was obtained. These results improve at 25 ºC where larger and more viable larvae are observed (Table 7, Fig. 29), coinciding with the predominant temperature in their areas of origin (Pont, 1977; De Carvalho *et al*., 2003).

Insects are ectothermic animals and their metabolic activity depends on environmental conditions (Grassberger & Reiter, 2001; Nassu *et al.,* 2014). Therefore, the forensic activity of the different dipteran species depends on these conditions. Often, they must save energy to keep their metabolic processes active in stressful situations such as low temperatures, which leads to a slowdown in development (Fig. 31). However, at extremely high temperatures it behaves in the same way (Velásquez *et al*., 2013; Díaz-Martín *et al*., 2013) as can be observed in *F. pusio* at 35 °C. These same behaviours can be observed in the PMI calculation diagrams (Fig. 32) which coincides with the behaviour of other species (Reiter, 1984; Grassberger & Reiter, 2001; Grassberger *et al*., 2003; Díaz-Martín *et al.*, 2014; Yanmanee *et al*., 2016; Wang *et al.*, 2017; Yang *et al*., 2017; Defilippo *et al.*, 2019; Zhang *et al.,* 2019). However, *F. pusio* presents certain peculiarities that unfortunately cannot be contrasted due to the lack of studies in this species.

Both the isomorphen (Fig. 32a) and isomegalen diagrams (Fig. 32b) show the same patterns. The only variation shown in the egg phase by temperature is the hatching time, as the morphology does not vary. The results obtained for L1 and L2 show a comparable pattern of behaviour with an increase in developmental speed and length at 20 ºC. L3 and prepupae also grow in the same way with a decreasing rate as temperature increases. In

addition, pupae and adults accelerate their development at 30 °C, which is not the case for the other temperatures tested. All these behaviours are consistent with other species of forensic interest (Bambaradeniya *et al.*, 2018). In other words, the two methodologies used to calculate the PMI are complementary to each other and show the same patterns of behaviour.

In summary, this work expands the knowledge of the behaviour of *F. pusio* at different temperatures, offering data relevant to its forensic application that have been little studied so far. The peculiarities shown by this species may offer additional information in the resolution of legal cases.

Part II

Chapter IV

Production and economic feasibility studies in the development of a rearing project for the yellow mealworm, *Tenebrio molitor*.

Chapter IV

Production and economic feasibility studies in the development of a rearing project for the yellow mealworm, *Tenebrio molitor*.

Abstract

The demand for food is increasing considerably due to the increase in population. Furthermore, this need also arises from the competition generated by the use of animal protein in feed. To overcome the shortage, insects have become a promising alternative in view of their high protein, fat and fibre content. At the same time, it is a sustainable, low-carbon source of feed, resulting in a reduction of the ecological footprint that is often so severely punished by conventional livestock.

The European Food Safety Agency has recently accepted the mealworm (*Tenebrio molitor*) as a safe species for human consumption. This leads to it becoming the leading candidate for the development of an insect farm feasibility study. In this work, the optimal conditions for the breeding of *T. molitor* are analysed, as well as its management under those conditions, the calculation of the annual production in the facilities and its economic feasibility in order to know the profitability of the project.

Keywords: Breeding, Edible insects, Management, Maintenance, Rearing, Scaled-up production.

Introduction

Agricultural intensification, population pressures, poverty, urbanisation and lifestyle changes have reshaped our food production and consumption to a limit that is beginning to be detrimental to human and environmental health (Dalton & Al-Zubiedi, 2019), increasing the demand for food considerably (FAO, 2010a; Alves *et al*., 2019). The World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) have revealed that insect consumption could be a viable way to fill nutritional gaps by using insects as food and feed. Insects thus become a promising alternative to fishmeal and soybean meal offered to conventional livestock and aquaculture, respectively (Grau *et al.*, 2017; Van Broekhoven *et al*., 2017; Ao *et al*., 2019; Pérez-Altamirano, 2019; Shafique *et al.*, 2021). This substitution curbs competition as it increases protein availability (Rumpold & Schlüter, 2013). Aligned with this trend, insect farms are starting to be set up for large-scale rearing (Morales-Ramos *et al*., 2012; Ramón-Vázquez, 2014; Cortes-Ortiz *et al*., 2016; Viñeta-Valdelvira, 2017; Ribeiro *et al*., 2018; Alves *et al*., 2019; Bernad-González, 2019; Koutsos *et al*., 2019; Cadinu *et al*., 2020).

Insects have many advantages when considering their production as a food alternative: high quantity and quality of protein, essential amino acids and minerals (Ravzanaadii *et al*., 2012; Grau *et al.*, 2017), low competitiveness in terms of food as insects are fed on biological waste, reduced need for water (Viñeta-Valdelvira, 2017), lower environmental impact for the reduced emission of greenhouse gases (Biasato *et al.*, 2016), high efficiency in feed conversion (Garnett *et al*., 2015; Cortes-Ortiz *et al*., 2016), a reduced risk of zoonoses (Ramón-Vázquez, 2014), and even a potential use in cancer therapy (Petit *et al.*, 2005; Suo *et al.*, 2016; Wu *et al.*, 2020). Likewise, insect breeding is fast due to its high reproductive rate and short biological cycle compared to mammals (Pérez-Altamirano, 2019).

However, the use of insects in food has small disadvantages as they can cause allergic reactions (Bessa *et al*., 2020) and contain toxic substances, but Van Broekhoven *et al*. (2017) and Niermans *et al.* (2019) saw in their studies how the larvae of *Tenebrio molitor* (Coleoptera: Tenebrionidae), commonly called yellow mealworm, degrade and excrete mycotoxins and heavy metals.

The legal framework in charge of regulating the food and feed chains has evolved favourably towards the progressive incorporation of insects into food and feed. Since January 2018 there has been a new Regulation (EU) 2015/2283 on "novel food". This regulation amends regulation (EU) 1169/2011 and repeals regulation (EC) 258/97 and regulation (EC) 852/2001. However, the European Food Safety Agency has recently accepted *T. molitor* as a safe species for human consumption. They are intended to make it easier for businesses to market novel foods in the European Union while maintaining a high level of food safety.

Novel food is defined as "foods or novel food ingredients not consumed in the European Union in significant quantities before 15 May 1997 (date of entry into force of the first regulation on novel foods)" (Bernhardt, 2018). However, in some European countries there still exist a legal void regarding entomophagy in humans. With regard to animal feed, Regulations (EU) 142/2011 and (EC) 999/2001 on by-products of animal origin not authorized for human consumption have been amended.

The aim of this work is to analyse the optimal conditions for the rearing and management of *T. molitor* in a small-scale production facility with information obtained from existing literature, and its economic viability, in order to encourage entrepreneurs with little available space to become small-scale farmers. In addition, we calculated the annual production to establish the profitability of the project.

Tenebrio molitor

The mealworm beetle, whose common name comes from their unwanted appearance in grain stores, were already used as pet food in their larval stage. It is known as a model organism in immunity studies and its complete sequence of the mitochondrial genome has been published (Liu & Wang, 2014). The availability of information on T. molitor with respect to other edible insects, allows the improvement of the development of mass breeding (Grau *et al*., 2017). It is a good candidate for the development of an insect farm project to obtain high quality protein in a sustainable way (Finke, 2002; Bovera *et al.*, 2015; Grau *et al*., 2017; Hussain *et al.*, 2017; Iaconisi *et al.*, 2017; Khan *et al*., 2017; Gasco *et al*., 2019).

Designing diets from plant by-products allows us to create a circular economy while shaping the nutritional profile of the insect (De Marco *et al.*, 2015). This nutritional

profile contains raw protein (20 – 71 %), total essential amino acids (46.3 %) and fatty acids (22 – 55 %), as the most remarkable nutrients (Gasco *et al*., 2019), in addition to a high digestibility of organic matter (90.85 %) and protein (91.60 %) (Bosch *et al.*, 2014). It is considered highly nutritious and more reliable than other orders of edible insects as substitutive of meat and fish meal and soya derivatives (Viñeta-Valdelvira, 2017). Moreover, the amount of chitin in the larval stage, which corresponds to the stage that they are consumed, represents an added value due to its prebiotic activity on the intestinal microbiota (Bovera *et al*., 2015).

T. molitor is a holometabolous insect (Cruz-Lozano, 2005), i.e., it has a complete metamorphosis following a cycle of four phases during its life: egg, larva which can vary from seven to twenty stages depending on environmental conditions pupa and imago (Park *et al.,* 2014). Once it has stopped feeding and has completed all its moulting, it goes into the pupal stage. After approximately 10 days, the adult emerges from the pupa and reaches sexual maturity after 12 days approximately. Later copulation, the eggs are laid over a period of eight days. The complete cycle of the *T. molitor* can reach 6 months under steady state conditions, since an adult can live up to three months in optimal conditions (Ludwig & Fiore, 1960; Urs & Hopkins, 1973; Ribeiro *et al*., 2018).

Breeding of T. molitor

The optimal conditions of *T. molitor* for its adequate development cycle are 26 – 28 ºC ambient temperature, 50 – 55 % relative humidity, and a diet for both adults and larvae based on flour bran ad libitum and vegetables remains as a source of water. In addition, it prefers dark areas to light ones, so they should be located in areas located in semi-darkness with a 12:12 hour light cycle (Ribeiro *et al*., 2018; Bernad-González, 2019).

T. molitor colony was started from a stock purchased at La Grillería (Valencia, Spain) in 2017. The proposed rearing system is based on the descriptions of Morales-Ramos *et al.* (2012) and Cortés-Ortiz *et al.* (2016) where 800 adult individuals are placed in a 60 x 40 x 12 cm box with a sieved bottom of 0.85 mm diameter. A 1 kg of wheat bran was added to serve as food and oviposition substrate. In addition, 100 grams of vegetable and fruit scraps together with 25 ml of water were provided as a water source, which was changed three times a week to avoid fungal proliferation, as well as the removal of dead individuals (Eilenberg *et al*., 2015). Adults oviposited on the sides of the box and on the bran flakes.

Once the first larval stages hatched, they fell through the mesh into the lower tray with the same dimensions (60 x 40 x 12 cm) but with an unmodified bottom (Fig. 33). The wheat bran and vegetable scraps that fell to the bottom also served as food for the newly hatched larvae.

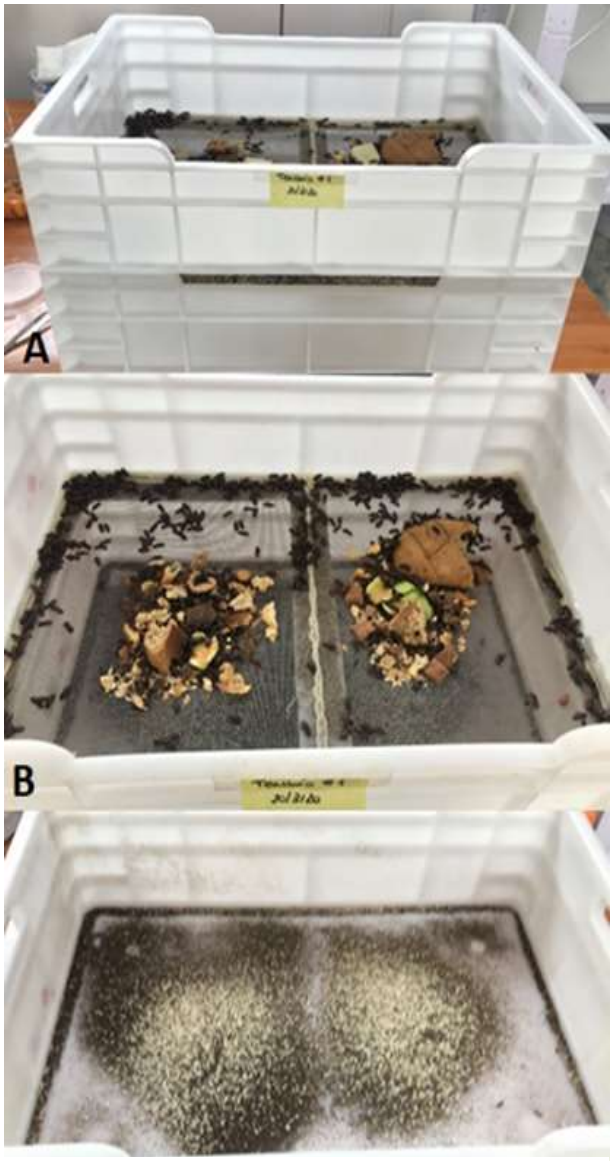


Figure 33. Breeding system of *T. molitor*. A: Reproduction procedure, adults are deposited on top while first instar larvae newly hatched from eggs fall to the bottom through the screened bottom. B: Adults with food and water source in the upper box with screened bottom. C: Lower box where larvae are collected with food scraps.

First instars were collected weekly from the lower tray and placed in plastic boxes (42 x 9 x 9 x 10 cm) where they remained for a period of 30 days (Fig. 34A) until they reached a size larger than the diameter of the mesh. After this period, the three-week-old larvae were transferred to another box with the same design as the adults (60 x 40 x 12 cm dimension + 0.85 mm diameter sieved bottom) (Fig. 35) to separate the larger larvae from the smaller ones. One kg of wheat bran and 100 grams of non-perishable vegetables (e.g., potato peelings, carrot peelings, bean pods) are added and replenished as needed. At the bottom is another tray of the same dimensions (60 x 40 x 12 cm) but with a 0.5 mm diameter sieved bottom that retains the smallest larvae and allows unwanted excrement particles and food scraps to pass through. In this way, a hygienic environment is maintained in which droppings and food debris fall through three stacked boxes to an unmodified bottom tray that is cleaned weekly.

As mentioned above, *T. molitor* has a great plasticity, so the development time varies and different larval stages coexist in each box. The larvae of the last stage must be separated from the rest to avoid cannibalism (Weaver & McFarlane, 1990; Morales-Ramos *et al.,* 2012). For this reason, once a month the larvae are subjected to a screening of different pores to separate them by size and thus speed up the distinction of the different stages. Larger larvae were transferred to a box with a solid bottom and oats to allow pupation. The remaining larvae were returned to the system created for rearing to continue their development. Pupae were collected daily using a 3.5 mm diameter sieve and separated from the larvae into a separate box (42 x 9 x 10 cm) to complete metamorphosis undisturbed (Fig. 34B). Oats are added to the tray so that the newly emerged adults have food and do not eat the pupae; and soil is added to maintain adequate moisture to prevent the pupae from drying out. After the emergence of the adults (approximately 10 days), they were separated daily in order to have them by age and thus synchronise sexual maturation.

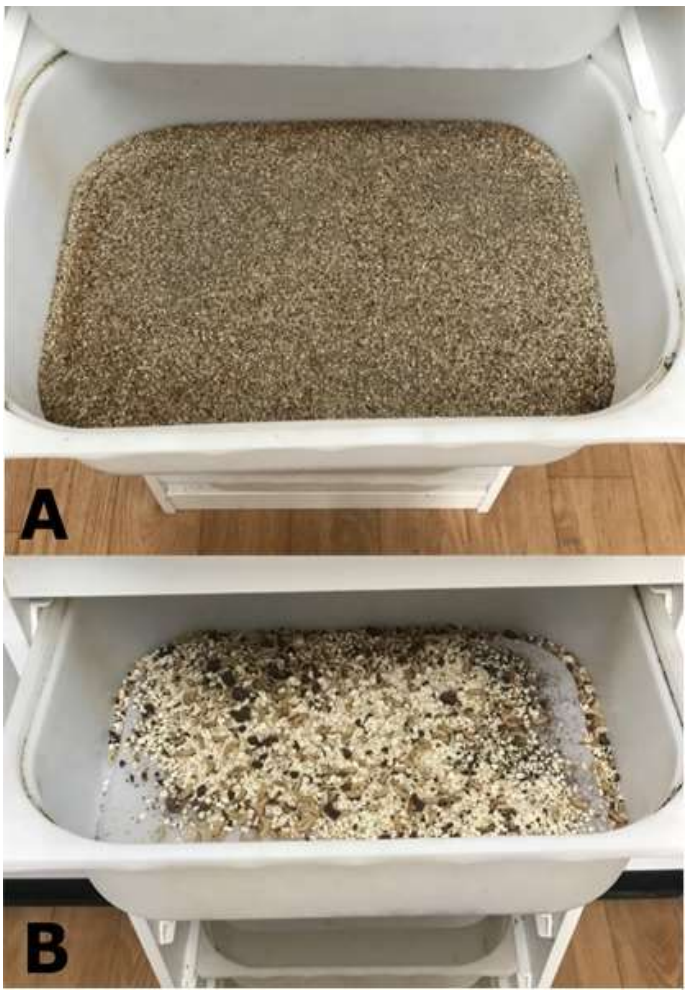


Figure 34. A: Storage of first instar larvae on solid bottom to reach minimum size for screening, approximately 30 days. B: Oats are added to the tray so that the newly emerged adults have food and do not eat the pupae (approximately 10 days); and soil is added to maintain adequate moisture to prevent the pupae from drying out.

Production of T. molitor

Three worksite modules of 36 m^3 (6 m long, 2.4 m wide and 2.5 m high) are needed to complete a production facility for *T. molitor*. This design has been projected on the basis

of our own experience in maintaining this species in the facilities of the Veterinary Farm at the University of Murcia.

Figure 35. Developmental system of *T. molitor* larvae. A: Larval development procedure, the larger larvae stay at the top while the smaller larvae fall through the screened bottom. In the last box with a solid bottom, unwanted droppings and food debris are collected, thus maintaining a hygienic environment. B: Larger larvae on top with a 0.85 mm sieve to allow smaller larvae to pass through. These larvae are collected in other trays with a 0.5 mm sieve through which unwanted droppings and food debris will pass. Finally, the last tray will collect the waste, which will be removed weekly.

One of them will be used for the production of the insect and will house 280 boxes inside. Half of these boxes will be used for breeding stock where the eggs will be collected, and the other half for the growth and development of the larvae that have hatched from the eggs. In the second module there will be a freezer cabinet for the storage of perishable food, a refrigerator for the storage of the weekly food, a storage room for the material and a sink for the cleaning of the material. Finally, the third module will contain the machinery necessary for processing the larvae: drying machine, crushing machine and sieving machine.

To start the colony, a number of 800 individuals in each adult box with approximately 400 females is proposed. The product of 84 boxes with 800 adults will be 67,200 individuals, approximately 33,660 females. This would be equivalent to the number used in the "Pre-Status" (Fig. 36) with a duration of about 3 months. If each can lay about 150 eggs, we will start with 5,049,000.

From here, the annual production of *T. molitor* amounts to 3.61 tons (Fig. 36). The biological cycle is completed after approximately six months, under the conditions already mentioned. In this way, two production batches are obtained each year, 510.3 kg and 3,100.7 kg adding up to the above-mentioned total. In these quantities, a 10 % decrease in each stage of the insect has already been taken into account, since there are always casualties, and another 10 % of individuals destined to maintain the farm, where the exploitation is progressively increased.

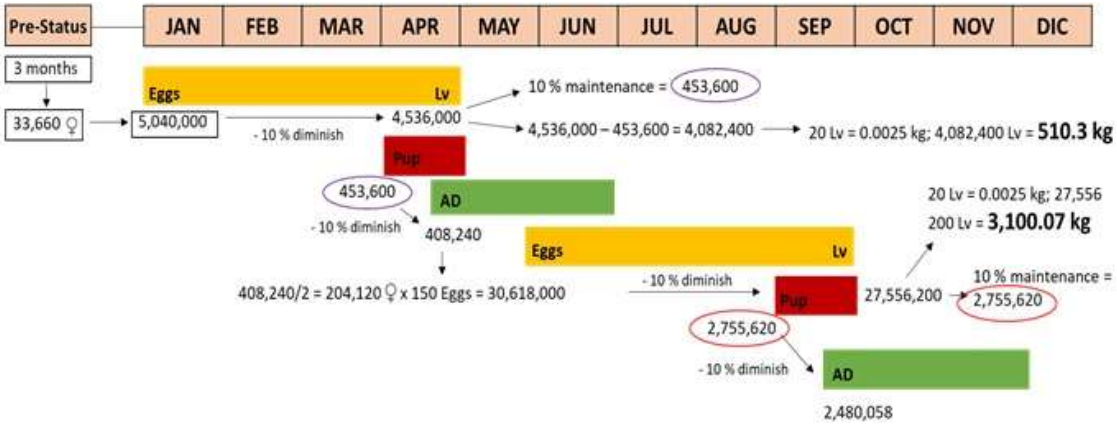


Figure 36. Calculation of the annual production schedule and life cycle of *T. molitor*. It is estimated that approximately 3 tonnes are produced in one year. *Lv: last larval stage; Pup: pupae; AD: adults.

The product to be sold is the live and dried insect as pet food, and insect meal as a supplement in animal feeds. Before slaughter, the insects are left without food for 24 hours to remove any internal residue naturally. This process must be carried out at low temperatures as the lethargy prevents cannibalism due to lack of food (Viñeta-Valdelvira, 2017). Slaughter shall be carried out by freezing between 5 and - 20 ºC for processing of the product other than the live insect. This procedure is supported by Regulation (EC) 1069/2009 and its implementation (EU) 142/2011 classifying insect meal in category three as feed (Bernad-González, 2019).

The three forms for selling *T. molitor* occur in the last larval stage, when the insect reaches its largest size. To process the dry insect and insect meal, they must undergo a bulking process for 5 minutes and a subsequent one at 55 ºC for 24 hours (Klunder *et al.*, 2012). However, the insect meal needs to be milled, which can be done by various methods. According to Bernad-González (2019) the most commonly used method for grinding is

three consecutive steps: 100 ºC for 95 minutes, 110 ºC for 55 minutes and 120 ºC for 13 minutes.

As we know, insects are rich in nutrients and have an intestinal microflora that provides a medium for the growth of unwanted microorganisms. That is why the processing and storage of the dry insect in flour format must be adequate. Many microorganisms are inactivated by heat, but many others are resistant. After processing at high temperatures, it would be most appropriate to store it at - 20 ºC (Klunder *et al.*, 2012).

Economic Feasibility

The study of the economic feasibility of a project is a necessary starting point. This analysis will use different economic indicators that will show us if the project is profitable or not. To do this, you must first calculate the ordinary cash flows which are the net outflows and inflows of money that the project has in a given period.

Therefore, the indicators to be calculated to carry out this section are:

1) Annual net asset value (NPV): this procedure makes it possible to calculate the present value of future cash flows arising from an investment. The methodology consists of discounting all the cash flows at the present time.
2) Internal rate of return (IRR): the geometric average of the expected future returns on such an investment and implies the assumption of an opportunity to reinvest.
3) Payback: a static investment valuation criterion that allows a given project to be selected on the basis of how long it will take to recover the initial investment through cash flows.

The study of the economic feasibility extracted from a simulated Profit and Loss statement. The development of the project through his parts:

Investment

The initial investment amounts to a total of thirty-six thousand five hundred and fifty euros (36,550 €). Table 1 details the necessary installations with their price per unit and the number of pieces of equipment. Here we can see the non-current assets that will remain in the project for more than one year, so that the annual depreciation amounts to 3,615 €. The estimated useful life of the project is 5 years.

When the project comes to an end, we value the non-current asset at a balance amount of 18,475 € recoverable, i.e., the residual value (Table 9). This value can be transferred to a new project or even recovered by selling the assets.

Structural investment	€	Nº	Investment	Depreciation (x 5 years)	Residual Value
Modules	5,000	3	15,000	750	11,250
PVC Curtains	150	3	450	45	225
Air-conditioning unit	400	3	1,200	240	0
Air extractor	300	3	900	180	0
Fridge	400	1	400	80	0
Chest freezer	1,000	1	1,000	100	500
Boxes	9	280	2,520	504	0
Drying machine	5,000	1	5,000	500	2,500
Installation	1,500	1	1,500	300	0
Grinding machine	8,000	1	8,000	800	4,000
Air extractor installation	80	1	80	16	0
Drying machine installation	200	1	200	40	0
Grinding machine installation	300	1	300	60	0
TOTAL	**22,339**		**36,550**	**3,615**	**18,475**

Table 9. Investment and amortisation of the different start-up facilities. The amortisation is the amount for each year of the project's life, which is estimated to be 5 years.

Finance

The funding for this project comes from different ways. We have set an initial contribution of the 40 % of the Working Equity (36,550 €) which the associates will disburse.

A further 40 % comes from a grant of "Centro para el Desarrollo Tecnológico Industrial" (CDTI). The strategic R&D projects within this type of funding are large applied research and business development projects to create or significantly improve the production process, product, or service. They must also prove a differential technological aspect over existing technologies on the market. These projects must be strategic nature for the company. They must involve R&D activities with an incentive effect that stimulate the development and incorporation of new technological knowledge into products, processes or services.

The balance 20 % comes from the Animal Biology Research Group of the Faculty of Veterinary, as it is a spin-off from the University of Murcia, the get integrated into the funding for further development and employment of the university's researchers.

Operating Revenue

The source of income is the sale of live and processed *T. molitor* (dried insect and meal) for animal feed. The price can vary significantly, from 1.50 €/50 g, 19 €/kg to 17 € for 5 kg (La Grilleria, live pet food). We have set the price at 10 €/kg live, 20 €/kg dry for pet food and 30 €/kg meal for animal and human food.

In the section "Production of *T. molitor*" we have calculated the yield of a colony of *T. molitor* kept under controlled conditions (Fig. 36). Table 10 shows a summary of production and revenue for the different sales formats (live, dried and meal). It should be noted that 60 % of the total production has been allocated to the live format, 20 % to the dried format and the remaining 20 % to meal. As we can see, *T. molitor* increases geometrically until the third year, when we can stabilise production and focus on improving quality, increasing income.

YEAR	1	2	3	4	5	Total
kg (live)	2,166	3,720	3,900	4,080	4,170	18,036
€ (live)	21,660	37,944	66,300	70,747.20	73,753.96	27,0405.16
kg (dry)	274.36	471.20	494	516.80	528.20	2,284.56
€ (dry)	5,487.20	9,612.48	9,880	10,542.72	10,990.79	46,513.19
kg (flour)	216.60	372	390	408	417	1,803.60
€ (flour)	6,498	11,383.20	11,700	12,484.80	13,015.40	55,081.40

Table 10. Quantities, price and format of *T. molitor* production during the 5-year life of the project. Production in different formats (live, dried and meal) expressed in kilograms (kg) and euros (€).

Cost Analysis

Through the cost analysis, we divided the structural, direct and indirect costs, consisting of the difference if the cost is involved in the production or a structural cost that the company has, not directed related to the kg produced.

The Direct Costs are detailed in table 11. Here we include the kilograms of raw material consumed for each insect format which amounts to a total of 1,679.26 €. The cost of

transporting the raw material is also specified with a total of 3,399.01 € for the estimated production. Labour is the biggest expense at 214,316 €, as only one person is employed in the first year, but from the second year onwards another person will be needed due to the increase in production.

Direct costs	Year 1	Year 2	Year 3	Year 4	Year 5	TOTAL
Live insect	105.19	184.26	197.05	210.26	219.20	915.96
Dry insect	35.06	61.42	65.68	70.88	73.07	305.32
Insect in flour	35.06	61.42	65.68	70.88	73.07	305.32
Diminish	17.53	30.71	32.84	35.04	36.53	152.66
Raw material consumption	192.84	337.82	361.25	385.48	401.86	1,679.26
Manpower	22,780	46,471	47,400	48,349	49,316	214,316
Transport	400	663	728.28	795.91	811.82	3,399.01

Table 11. Direct costs during the 5-year life of the project. The raw material for each type of insect for sale (live, dried and meal) is taken into account. Both larvae and adults are offered a diet of 90 % wheat bran and 10 % vegetable by-products.

The rest of the costs involved in production are Indirect Costs and are shown in table 12. These are the structure costs where mainly supplies are detailed with a total of 19,243.29 €. This section also takes into account the depreciation detailed in table 9 where 3,615 € per year of the project was available but, as already mentioned, these are paid at the beginning of the project and hence the need for financing.

YEAR	1	2	3	4	5	TOTAL
Air conditioning	1,800	1,836	1,872.72	1,910.17	1,948.38	9,367.27
Air extractor	500	510	520.20	530.60	541.22	2,602.02
Lighting	450	459	468.18	477.54	487.09	2,341.82
Consumption of the drying machine	207.36	356.12	373.36	390.59	399.20	1,726.63
Contracted power	335.54	342.25	349.09	356.07	363.20	1,746.15
Grinding machine	33.49	57.51	60.29	63.08	64.47	278.37
Water	360	367.20	374.54	382.03	389.68	1,181.04
TOTAL	3,686.39	3,560.88	4,018.38	4,110.08	3,867.56	19,243.29

Table 12. Indirect costs broken down by project year and total expressed in euros (€).

Operating cash flow

We need an investment of 36,550 € to start the project. Table 13 shows how this investment is recovered every year. In the last year of the project (5th) of the 51,657 € recovered, 33,182 € is from the production and sale of insects and the remaining 18,475 € are from return on the initial investment obtained from the property and machinery of the operation.

	Funding	1	2	3	4	5	**Settlement**
Operating cash flow	-36,550	5,843	6,558	27,432	31,005	51,657	
						33,182	18,475
Liquidity	-36,550	-30,707	-24,148	3,284	34,289	78,104	

Table 13. Cash flow of the project expressed in euros (€) per year, showing the investment made, and the liquidity at the end of the project. Negative figures are indicated in red.

The project is generating profit every year, and it is recovering the amount invested. We need almost three years to recover the funding amount, but one of our project's exciting points is that it is profitable since it gets started. When the project is over, we generate 33.182 € from the operation, and we recover the 18.475 € from the liquidation of the assets not depreciated.

Project profitability

After the economic feasibility study, two data are obtained (NPV and IRR) that show the viability and profitability of the project. In our case we have obtained an NPV of 82,468.87 € and an IRR of 27.50 %.

Chapter V

Expression analysis of *Lac2*, *TH* and *AANAT1* genes related to exoskeleton malformations in reared *Tenebrio molitor* (Coleoptera: Tenebrionidae)

Chapter V

Expression analysis of *Lac2, TH* and *AANAT1* genes related to exoskeleton malformations in reared *Tenebrio molitor* (Coleoptera: Tenebrionidae).

Abstract

The proper development of insects is strictly dependent on the sclerotization and melanisation of the exoskeleton, which is involved in the immune function of the individual. With the increased presence of insect farms due to their recent approval for animal and human consumption, previously unknown abnormalities are becoming more and more frequent. Abnormal individuals with different teratologies have been observed as a result of a partially complete metamorphosis process of *Tenebrio molitor* (Coleoptera: Tenebrionidae) in a small-scale rearing facility. The absence of a complete developed abdomen and elytra is showed among the most common abnormally developed adults.

Three of the genes involved in the metabolic pathway of sclerotization and melanisation (*TH, AANAT1,* and *Lac2*) were analysed by RT-qPCR, using a previously validated reference gene (*Rps3*), to determine gene differences between abnormally developing and normally developing control specimens. The results showed clear distinctions between the two groups and the possibility that a hormonal mismatch or an epigenetic change is responsible for these malformations is assessed. This work provides a first insight into the genetic basis of *T. molitor* abnormal development, and highlights the necessity of further research to elucidate all the molecular mechanisms involved to achieve a more efficient production of this insect species for food and feed.

Keywords

Dopamine, Incomplete development, Insect farm, Pupation development, RT-qPCR.

Introduction

The envelope and melanisation of the insects exoskeleton is the first defence barrier against invading pathogens and environmental stressors such as droughts or predators (Hayakawa *et al.*, 2018). The four arthropod sub-phyla (Chelicerata, Myriapoda, Crustacea and Insecta) present structural similarities in their cuticle (Asano *et al.*, 2019). that has allowed them to be one of the first terrestrial colonizers (Engel & Grimaldi, 2004; Kenrick *et al*., 2012). In addition, body colour is involved in communication, mating behaviour, crypsis, mimicry and predator-prey interactions (Fukatsu & Futahaski, 2016; Mun *et al.*, 2019). Different kind of chemical pigments are responsible for the darkening and hardening of the cuticles of many insects (Arakane *et al*., 2009; Mun *et al*., 2019).

One of the most important proteins related to the insect cuticle formation process (darkening and hardening) is the *Laccase2* (*Lac2* gene) (Simon *et al.*, 2009), although it also appears in the formation of eggs and pupae (Arakane *et al.*, 2005; Asano *et al*., 2019). Other putative functions are the involvement in cellulose digestion in some termite species (Coy *et al*., 2010; Geng *et al.*, 2016), immune response, iron metabolism or resistance against parasites and insecticides (Parkinson *et al*., 2003; Hattori *et al.*, 2005; Hoegger *et al.*, 2006; Bettedi *et al.*, 2011; Strong & Claus, 2011; Balabanidou *et al*., 2018; Wang *et al*., 2018). All arthropods studied so far have this protein and its reduced synthesis results in the death of specimens. That is why it has been proposed for gene silencing for pest control (Du *et al.*, 2017; Matsumoto & Hattori, 2019).

The *Lac2* is also involved in the pigmentation process of the cuticle. Mun *et al*. (2019) observed that the expression of this gene in *Tenebrio molitor* (Coleoptera: Tenebrionidae) is activated on the fourth-sixth day of pupation but remained at lower levels during the adult stage. Other enzymes such as arylalkylamine N-acetyltransferase (*AANAT1*), and tyrosine hydroxylase (*TH*) also participate in this process, in particular in the tyrosine-mediate cuticle tanning pathway (Noh *et al*., 2016b). *AANAT1* is consistently expressed in the pupal and adult stages, while *TH* is expressed in all moulting periods of larvae and pupae showing the highest levels just after adult emergence (Mun *et al.*, 2019).

Although *T. molitor* is one of the best studied species both histochemical and genetically (Zeikus & Steinhaus, 1968; Socha & Sehnal, 1972; Liu & Wang, 2014; Bong *et al*., 2018; Mun *et al.*, 2019), and its whole genome is now published (Liu & Wang, 2014), information on specific references genes for RT-qPCR studies on this species is still

needed. The anomalies undergone by this species are increasingly documented as small- and large-scale farming is becoming standardised due to recent approval for food and feed purposes (EFSA *et al.,* 2021).

The aim of the study is to analyse the gene expression of three genes related to the formation of the cuticle of *T. molitor* in individuals with normal versus anomalous cuticle development. The altered expression of these genes, which encode essential proteins for adult life, can lead to an anomalous development of cuticle with the corresponding limitations for survival and reproduction. A better understanding of the genetic mechanisms involved will help to improve production systems while minimising costs. To test this hypothesis, the objective of this work is to analyse the expression levels of proteins *Lac2, AANAT1*, and *TH* in normal and anomalous adults using RT-qPCR

Material and methods

Specimens

Tenebrio molitor were collected from a breeding facility of the University of Murcia and reared under controlled conditions: 24 – 28 °C temperature, 47 – 50 % relative humidity and a photoperiod L:D 12 h: 12 h. The specimens are established in differentiated plastic boxes for larvae and adults with 60 x 40 x 12, L x W x H cm dimensions each; approximately 1500 individuals live in the larvae boxes while approximately 700 individuals live in the adult box. They have an ad libitum food supply (oat flakes) and the water source is ingested from the fresh fruit offered to them every two days. For the analysis we collected 10 adult individuals without physical deficiencies (control) and another 10 adults with deformed elytra and abdomen (anomalous) (Fig. 37), all of them at the same age, i.e., newly emerged from the pupa. Each individual was given small perforations in the abdomen with an entomological needle and was immersed in 2 µl of RNA later (Qiagen, Crawley, UK). Finally, they are kept at 5 ºC until the RNA extraction process.

RNA extraction

A total of 20 samples were homogenised in 350 µl Buffer RLT with TissueRuptor II (Qiagen) and RNA extraction was performed on ice using the RNeasy Mini Kit (Qiagen) following manufacturer protocols. 50 µl of were used to the final elution of total RNA.

Before storing it at – 80 ºC, the RNA concentration was quantified by NanoDrop 1000 from Thermo Fisher Scientific® (Waltham, MA, USA).

Figure 37. a) Adult of *T. molitor* with deformed elytra and abdomen. b) Healthy adult of T. molitor without physical deficiencies.

cDNA synthesis

Equal concentrations of RNA were used for all samples to perform the cDNA synthesis. This standardisation was carried out by diluting each sample to a concentration of 100 µg/µl with RNase free water. 2 µl of total RNA were used to perform the cDNA synthesis, using the Reverse Transcription Kit of Quantitect (Qiagen) according to the manufacturer protocol. Samples were then stored at – 20ºC until following procedures.

RT-PCR

Prior to real-time PCR experiments, validation of each primer and reference gene must be performed to ensure a correct data interpretation and reliability of the results (Thellin *et al.*, 1999; Radonic *et al.*, 2004; García-Reina *et al.*, 2018). *T. molitor* target genes *TH, Lac2, AANAT1, DDC* (DOPA decarboxylase), *ADC* (aspartate 1-decarboxylase), *ebony* (N-β-alanyldopamine synthase) and, *Y-y* (yellow-y) were tested using primer from Mun *et al.* (2019), and three additional pair of primers were designed for *Lac2, ATPase* and *GADPH* genes from sequences obtained from the NCBI database using Primer Express 3 software (Applied Biosystems®). Five reference genes were tested: *18S RNA* (18S ribosomal RNA) and *L27a* (60S ribosomal protein L27a) from *T. molitor* (Mun *et al.*, 2019); *Actβ* (B-actin), *RPL13a* (60S ribosomal protein L13a) and *RpS3* (40S ribosomal protein S3) from *T. castaneum* (Lord *et al.*, 2010; Liu *et al.*, 2015). A serial diluted pool of cDNA was amplified in triplicate to evaluate the amplification efficiencies of all genes

by the standard curve method in a StepOnePlusTM instrument using SYBR® Green (Applied Biosystem®). Melt curves analyses and negative controls were carried out to check the specificity of the amplifications and genes with good efficiencies (Table 14) were used for RT-qPCR experiments in the same equipment with 1.5 µl of cDNA for each sample. The PCR conditions were: one cycle at 95 ºC for 2 min, and 40 cycles at 95 ºC for 15 s and 60 ºC for 30 s. For each condition tested, 10 individuals were considered as 10 biological replicates, and 10 technical replicates were used from each one of them.

Gene	Gene name	E (%)	R^2	Primers
TH	Tyrosine hydroxylase	79.6	0.994	F-5'-TCTTGGTCACATGCCCTTAC-3' R-3'-CTTTCACGACTCCGCTTTCT-5'
AANAT1	Arylalkylamine N-acetyltrasferase	84.5	0.985	F-5'-GAGCTGGGCTGTGGTATAATG-3' R-3'-CTTCTCCGTTGACCTTGTAGTC-5'
Lac2	Laccase 2	97.279	0.984	F-5'- CCCTCTGGACGCGAGATGTA-3' R-3'- CAACACGCCCTTGTCAATGT-5'
RpS3	Ribosomal protein S3	83.184	0.999	F-5'-GTGGTCGTTTCTGGCAAACT-3' R-3'- CAACACTCCTTGCCTCAACA-5'

Table 14. Primers used for qRT-PCR experiments for *T. molitor*. E = Amplification efficiency.

Data analysis

For all further analyses, the threshold cycle (C_T) raw values obtained with the software 7500 v.2.0.5 (Applied Biosystem®) were used, considering gene efficiencies corrections. As *RpS3* and *Actβ* were the only candidate reference genes available, their stability was evaluated before expression analyses using geNorm (Vandesompele *et al.*, 2002) and NormFinder (Andersen *et al.*, 2004) algorithms, adding the rest of the target genes data to the analysis (Table 15). Expression values were obtained as MeanΔC_T and statistical significance between healthy and anomalous individuals was assessed with a Student's t-test using GraphPad Prism version 5.00 software (GraphPad Software, San Diego California USA, www.graphpad.com).

	Stability values	
	geNorm	**NormFinder**
Laccase2	2,820	1,640
AANAT1	1,998	0,985
TH	2,195	0,971
Actβ	2,902	1,524
RpS3	1,779	0,617

Table 15. Stability values obtained from geNorm and NormFinder algorithms for all genes tested.

Results

Three target genes (*TH, AANAT1,* and *Lac2*) and two candidate reference genes (*RpS3 and Actβ*) showed proper amplification and efficiency values and thus were selected for further analyses. The other genes tested (*DDC, ADC, ebony, Y-y, ATPase, GADPH, 18S RNA, L27a* and *RPL13a*) did not provided an efficiency within the acceptable range to be considered viable for RT-PCR (Table 14), so they were discarded. *RpS3* was selected as reference gene for expression analysis according to the results obtained with geNorm and NormFinder tools (Table 15). This gene was the only that showed low stability values and therefore the most recommended for using as a reference gene. *Actβ* was discarded as it did not show proper stability values.

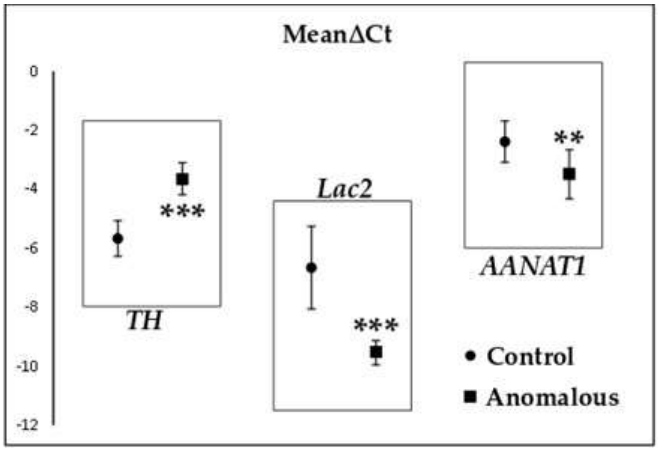


Figure 38. Relative expression of *TH, Lac2* and *AANAT1* genes (control and anomalous samples) using the reference gene *RpS3*. Error bars represent the standard error for the normalised relative amount. Asterisks represent statistical significance (** = $p < 0.01$, *** = $p < 0.001$).

Finally, *TH, Lac2,* and *AANAT1* genes were analysed for expression using *RpS3* as a reference gene (Fig. 38). Statistically significant changes in the expression of the three

genes were observed for the anomalous individuals. *Lac2* ($p < 0.001$) and *AANAT1* ($p < 0.01$) were downregulated, whereas *TH* ($p < 0.001$) was upregulated, in anomalous individuals in comparison to control individuals.

Discussion

Deformity in arthropods arises for a variety of reasons: bacterial infections (Zeikus & Steinhaus, 1968), physical disturbances (Bong *et al*., 2018) or stressful conditions (Rolff *et al*., 2019) resulting in hormonal, chemicals and genetic alterations (Mun *et al*., 2019) due to exposure to toxic compounds (Pierzynowska *et al*., 2017), or epigenetics modifications such as methylations, histone modification and non-coding RNAs (Burggren, 2017).

In colony maintenance, *T. molitor* individuals are continuously handled. Pupae are separated from larvae to optimise biological development, but this leads to a change in the original position adopted by the larva. Daily manipulation produces a stress condition in the pupa (Bong *et al*., 2018), which induces dopamine production, while juvenile hormone (JH) degradation decreases (Gruntenko & Rauschanbach, 2008). However, an increased frequency of moulting is also an unfavourable stress factor for the development of the individual (Bong *et al*., 2018).

This hormonal cascade is the biochemical basis of the metamorphosis process where JH acts simultaneously with ecdysone, responsible for insect growth and moulting (Connat *et al*., 1991; Rolff *et al*., 2019). In addition, behind the hormonal action there are molecular mechanisms that translate hormonal signals into cellular, tissue and morphological changes (Bellés, 2021). Grutenko *et al*. (2005) observed that an increase in dopamine concentration in the haemolymph of young *Drosophila virilis* females corresponded with an increase in JH and a decrease in ecdysone. This series of events, with augmented dopamine in the haemolymph (Noguchi *et al*., 1995; Verlinden, 2018), could be responsible for the physical alterations visible at the time of adult emergence (Fig. 37). Furthermore, in anomalous individuals, overexpression of *TH* and underexpression of *AANAT1* and *Lac2* are observed, favouring the increase in dopamine (Fig. 39). The correlation between malformations and *Lac2* deficiency has also been shown in Lepidoptera where wing deformation was observed (Pierzynowska *et al*., 2017).

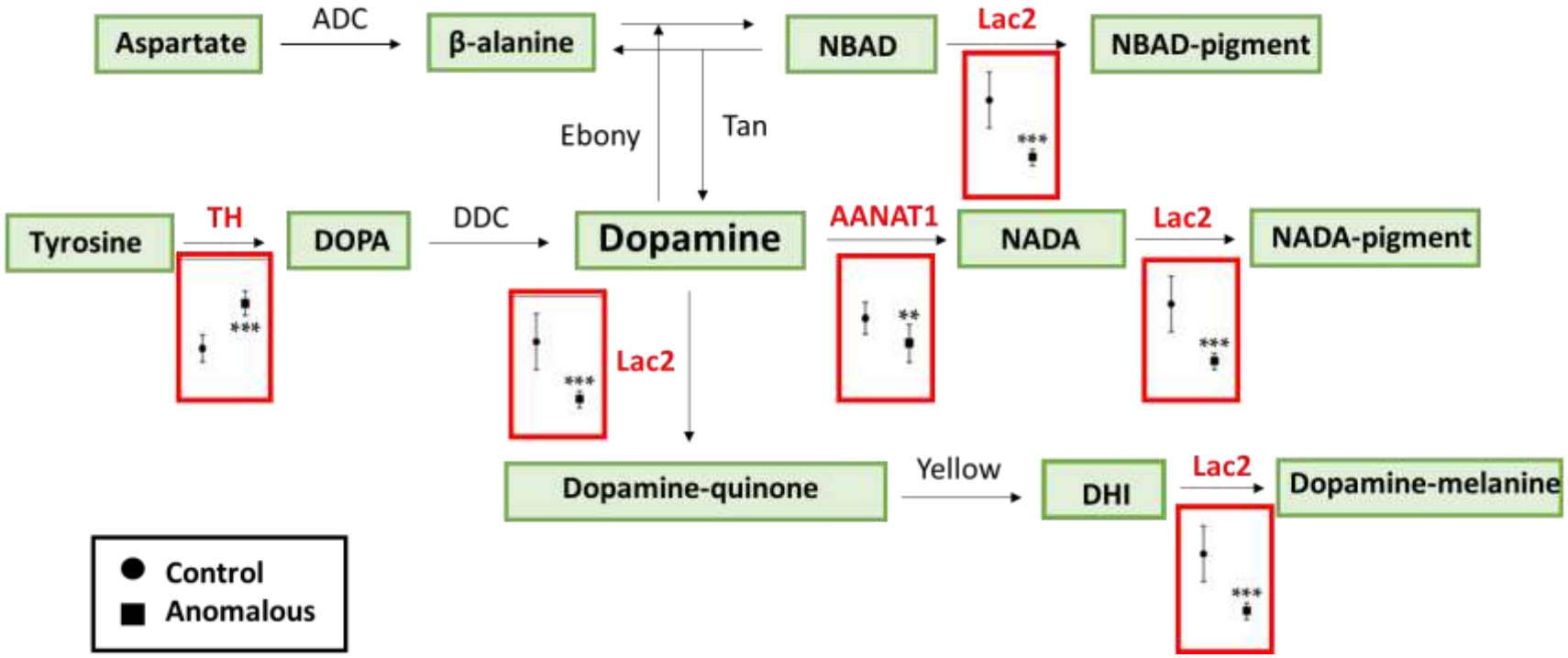


Figure 39. Metabolic pathway of *T. molitor* exoskeleton pigmentation adapted and modified from Mun *et al.* (2019). The enzymes in red are those that have been analysed in this work together with the expression graph differentiating between healthy and anomalous individuals.

The three genes whose expression was analysed in this paper (*TH, AANAT1*, and *Lac2*) are involved in the sclerotization and pigmentation of the exoskeleton of T. molitor (Mun *et al.,* 2019). Gene expression analysis showed that anomalous individuals had lower expression of *AANAT1* and *Lac2* than control individuals, as expected. However, the TH expression in the anomalous versus the control individuals is much higher (Fig. 38). This causes the metabolic pathway to be disrupted and accumulate overproduction of dopamine, as mentioned above. Additionally, information on specific reference genes for *T. molitor* is scarce so that *RpS3* was chosen as it is commonly used in the order Coleoptera (Lord *et al*., 2010; Toutges *et al.,* 2010; Liu *et al.,* 2015; Sang *et al*., 2015).

Other work has shown that decreased sclerotization occurs due to reduced *TH* function (Gorman & Arakane, 2010) and that overexpression of *TH* leads to melanism in many insects (Liu *et al.,* 2015). However, our results show that anomalous individuals with irregular sclerotization and pigmentation have increased *TH* expression. It is an essential survival gene in holometabolous insects that mediates in cuticle sclerotization and immunity (Verlinden, 2018) found in the secretory cells of the cuticle (Gorman & Arakane, 2010). This knowledge is being used in pest control by applying RNA interference silencing this gene (Liu *et al.,* 2020).

The malformations found in our individuals are similar to those described by Zeikus & Steinhaus (1968) where different teratologies of *T. molitor* are reported. Such physiological responses may be related to the environmental factors such as the relative humidity, the ambient temperature, the mechanical damage to larvae and pupae, the change in pupal microclimate (Yi *et al*., 2017), the photoperiod, nutrition or viral infection (Burggren, 2017). All of these factors may be producing epigenetic changes in the insects (Jaenisch & Bird, 2003). Although the concentration of toxic compounds presents in the food provided to *T. molitor* (agriculture by-product) have not been measured in this work, small amounts could be sufficient to cause methylations in their DNA, leading to the abnormalities found. In addition, the high energy generated in the metamorphosis process could lead to further methylation (Guo *et al*., 2019). Although methylation levels in insects are very low, they affect key genes, producing disturbance in individual phenotypes, even across multiple generations (Burggren, 2017). New experiments to test these hypotheses from an epigenetic point of view, could highlight the molecular basis of the metamorphosis process.

Unfortunately, there is little information on epigenetic changes in insects caused by physicochemical environmental factors (Maleszka, 2008; Maeno & Tanaka, 2011; Chen *et al*., 2015; Cridge *et al.*, 2015). This work contributes to add knowledge to the explanation of malformations found in the rearing of *T. molitor*, an increasingly standardised activity due to its recent authorisation for animal and human consumption.

Conclusions

Chapter I. Barcoding identification and phylogeographic analysis of *Fannia pusio* L. in the Iberian Peninsula

- Exhaustive sampling in the Iberian Peninsula shows the presence of the genus *Fannia* of which, *F. pusio* is the predominant species.
- A 240 bp mini-barcode has shown to be useful for the identification of *Fannia* species, contributing to forensic and medical research.
- Particular mutations differentiate the haplotypes from the north-east and south-east of the Iberian Peninsula.
- *F. pusio* originated in the Americas and it was introduced into the Iberian Peninsula via Portugal, subsequently undergoing a continuous range expansion.

Chapter II. The head of *Fannia pusio* (Diptera: Fanniidae) as a new source of morphometric data for the evaluation of variation along geographical and biological lines.

- Head landmarks of *F. pusio* provide valid morphometric information for species identification studies.
- The parafacial and fronto-orbital areas of *F. pusio* are the most variable head structures.
- Variation in environmental conditions in the Iberian Peninsula seem to have originated in *F. pusio* the intraspecific differentiation between the east and west populations.
- Individuals of *F. pusio* reared in the laboratory show morphometric particularities that differentiate them from those collected in the wild, probably due to the stable environmental conditions and the high inbreeding level in the laboratory colony.

Chapter III. Development of *Fannia pusio* (Diptera: Fanniidae) under temperature-controlled conditions and its application in post-mortem interval (PMI) estimation.

- The results obtained indicate that the viability range for development in *F. pusio* oscillates between 15 ºC and 35 ºC, being 25 ºC the temperature in which the stress of the individuals seems to be lower.

- The isomegalen and isomorphen diagrams give coherent results, showing more accelerated growth with smaller sizes at higher temperatures.
- *F. pusio* seem to be a good seasonal indicator providing useful information in forensic investigations.

Chapter IV. Production and economic feasibility studies in the development of a rearing project for the yellow mealworm, *Tenebrio molitor*.

- The information gathered and the projection made provide optimised data for starting a small-scale *T. molitor* farm based on the biological cycle of the species and the available space of the facility.
- An estimate of the annual production of *T. molitor* and its economic feasibility is obtained for the first time, providing cost-effective results for its development.
- Calculations show an NPV of 82,468.87 € and an IRR of 27.50 %, providing data for a five-year profitable project life.

Chapter V. Expression analysis of *Lac2*, *TH* and *AANAT1* genes related to exoskeleton malformations in reared *Tenebrio molitor* (Coleoptera: Tenebrionidae).

- Anomalous individuals show an overexpression of *TH* gene and an underexpression of *AANAT1* and *Lac2* genes.
- The expression pattern obtained could alter the metabolic pathway of melanisation and sclerotization of adults, increasing the levels of dopamine in the haemolymph and leading to a disruption of normal development.
- The expression pattern found in anomalous individuals could be correlated with the stress generated by the daily handling, causing an increase of dopamine in the haemolymph.

References

Aballay FH, Domínguez MC, & Campón FF. (2012). Adult Fanniidae associated to pig carcasses during the winter season in a semiarid environment: Initial examination of their potential as complementary PMI indicators. *Forensic Science International*, 219: 284.e1–284.e4.

Akbarzadeh K, Rafinejad J, Nozari J, Rassi Y, Sedaghat MM, & Hosseini M. (2012). A modified trap for adult sampling of medically important flies (Insecta: Diptera). *Journal of Arthropod-Borne Diseases*, 6: 119–128.

Al Gazi ADF, Bélo M, & Dos Santos JM. (2004). Microscopia eletrônica de varredura de duas espécies de *Fannia* Robineau-Desvoidy (Diptera, Fanniidae). *Revista Brasileira de Entomologia*, 48: 169–180.

Altschul SF, Madden TL, Schaffer AA, Zhang Z, Miller W, & Lipman DJ. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 25: 3389–3402.

Alves AV, De Lima FF, Da Silva TG, De Oliveira VS, Kassuya CAL, & Sanjinez-Argandoña EJ. (2019). Safety evaluation of the oils extracted from edible insects (*Tenebrio molitor* and *Pachymerus nucleorum*) as novel food for humans. *Regulatory Toxicology and Pharmacology*, 102: 90–94.

Alves-Rolo ES. (2010). *DNA barcoding and forensic entomology: a molecular approach for diptera species' identification*. Master's Thesis. Universidade de Lisboa, Faculdade de Ciências.

Ao Z, Yoo JS, Wu Z, & Kim IH. (2019). Can dried mealworm (*Tenebrio molitor*) larvae replace fish meal in weaned pigs? *Livestock Science*. doi: 10.1016/j.livsci.2020.104103

Amat E. (2010). Notes on necrophagous flies (Diptera: Calyptratae) associated to fish carrion in Colombian Amazon. *Acta Amazonica*, 40: 397–400.

Amat E, Ramírez-Mora MA, Buenaventura E, & Gómez-Piñerez LM. (2013). Temporal variations in abundance of carrion fly families (Diptera, Calyptratae) in an anthropized Andean valley of Colombia. *Acta Zoologica Mexicana*, 29: 463–472.

Amendt J, Campobasso CP, Goff ML, & Grassberger M. (2009). *Current concepts in forensic entomology*. Springer Dordrecht Heidelberg London New York. Library of Congress Control Number: 2009943054.

Andersen CL, Jensen JL, & Ørntoft TF. (2004). Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research*, 64: 5245–5250

Arakane Y, Muthukrishnan S, Beeman RW, Kanost MR, & Kramer KJ. (2005). *Laccase 2* is the phenoloxidase gene required for beetle cuticle tanning. *Proceedings of the National Academy of Sciences of the Unites States of America*, 102: 11337─11342.

Arakane Y, Lomakin J, Beeman R., Muthukrishnan S, Gehrke SH, Kanost MR, & Kramer KJ. (2009). Molecular and functional analyses of amino acid decarboxylases involved in cuticle tanning in *Tribolium castaneum*. *Journal of Biological Chemistry*, 284: 16584─16594.

Arango-Gutiérrez GP, & Agudelo-Bentacur L. (2004). Valor biológico de las cucarachas en el compost. *Revista Lasallista de Investigación*, 1: 96–97.

Arias AR, Fuente ALC, Gómez A, Cacere MC, Rolón M, Gómez MCV, & Villalba C. (2017). Morphometric wings similarity among sylvatic and domestic populations of *Triatoma infestans* (Hemiptera: Reduviidae) from the Gran Chaco Region of Paraguay. *American Journal of Tropical Medicine and Hygiene*, 97: 481–488.

Arnaldos MI, Ruiz C, Torres B, Begoña I, García MD, González-Mora D, & Serrano J. (2015). DNA barcoding of two forensically important fleshflies species (Diptera: Sarcophagidae) from Spain and notes on barcoding success within genus *Sarcophaga* Meigen, 1826. *Ciencia Forense*, 12: 73–90.

Asano T, Seto Y, Hashimoto K, & Kurushima H. (2019). Mini-review an insect-specific system for terrestrialization: Laccase-mediated cuticle formation. *Insect Biochemistry and Molecular Biology*, 108: 61─70.

Avise JC. (2000). Phylogeography: The History and Formation of Species. Harvard University Press, Cambridge, Massachusetts, ISBN 0-674-66638-0.

Azevedo RR, Duarte JLP, Ribeiro PB, & Krüger RF. (2007). Occurrence of *Sarcopromusca pruna* (Diptera) in Southern Brazil as a vector of *Dermatobia hominis* (Diptera) eggs. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, 59: 1348–1350

Balabanidou V, Grigoraki L, & Vontas J. (2018). Insect cuticle: a critical determinant of

insecticide resistance. *Current Opinion in Insect Science*, 27: 68─74.

Bambaradeniya YTB, Karunaratne WAI, Tomberlin JK, Goonerathne I, & Kotakadeniya RB. (2018). Temperature and tissue type impact development of *Lucilia cuprina* (Diptera: Calliphoridae) in Sri Lanka. *Journal of Medical Entomology*, 5: 285–291.

Barbosa RR, Mello-Patiu CA, Mello RP, & Queiroz MMC. (2009). New records of calyptrate dipterans (Fanniidae, Muscidae and Sarcophagidae) associated with the decomposition of domestic pigs in Brazil. *Memórias do Instituto Oswaldo Cruz*, 104: 923–926.

Barták M, Preisler K, Kubík S, Sulákova H, & Sloup V. (2016). Fanniidae (Diptera): new synonym, new records and an updated key to males of European species of *Fannia*. *ZooKeys*, 593: 91–115.

Barrios M, & Wolff M. (2011). Initial study of arthropods succession and pig carrion decomposition in two freshwater ecosystems in the Colombian Andes. *Forensic Science International*, 212: 164–172.

Barros AS, Dutra F, & Ferreira R. (2008). Insects of forensic importance from Rio Grande do Sul state in southern Brazil. *Revista Brasileira de Entomologia*, 52: 641–646.

Baylac M, Villemant C, & Simbolotti G. (2003). Combining geometric morphometrics with pattern recognition for the investigation of species complexes. *Biological Journal of the Linnean Society*, 80: 89–98.

Baz A, Botías C, Martín-Vega D, Cifrián B, & Díaz-Aranda LM. (2015). Preliminary data on carrion insects in urban (indoor and outdoor) and periurban environments in central Spain. *Forensic Science International*, 248: 41–47.

Bellés X. (2021). La evolución de la metamorfosis en los insectos. *Investigación y Ciencia*, 18–27.

Bélo M, Alves SM, & Pires DJ. (1998). Flutuações e diversidade de espécies de dípteros em granja de galinhas poedeiras. *Revista Boikos, PUC-Campinas*, 12: 34–44.

Benecke M. (2001). A brief history of forensic entomology. *Forensic Science International*, 120: 2–14.

Benecke M, & Lessig R. (2001). Child neglect and forensic entomology. *Forensic Science International*, 120: 155–159.

Bergeret M. (1855). Infanticide. Momification naturelle du cadavre. *Annuals of Hygiene and Legal Medicine* 4: 443–452.

Bernad-González V. (2019). *Proyecto de una granja de insectos en el T.M. de Villamayor de Gállego (Zaragoza).* Trabajo Fin de Máster. Universidad de Zaragoza, España.

Bernhardt C. 'Novel food': towards greater protection of food innovation (in Spanish). Retrieved from https://www.garrigues.com/es_ES/noticia/novel-foods-hacia-una-mayor-proteccion-de-la-innovacion-en-los-alimentos. (Consulted 2018, Jan 12).

Bessa LW, Pieterse E, Marais J, & Hoffman LC. (2020). Why for feed and not for human consumption? The black soldier fly larvae. *Comprehensive Review in Food Science and Food Safety*, 19: 2747–2763.

Bettedi L, Aslam MF, Szular J, Mandilaras K, & Missirlis F. (2011). Iron depletion in the intestines of Malvolio mutant flies does not occur in the absence of a multicopper oxidase. *Journal of Experimental Biology*, 214: 971─978.

Biasato I, De Marco M, Rotolo L, Renna M, Lussiana C, Dabbou S, Capucchio MT, Biasibetti E, Costa P, Gai F, Pozzo L, Dezzutto D, Bergagna S, Martínez S, Tarantola M, Gasco L, & Schiavone A. (2016). Effects of dietary *Tenebrio molitor* meal inclusion in free-range chickens. *Journal of Animal Physiology and Animal Nutrition*, 100: 1104─1112.

Boehme P, Amendt J, & Zehner R. (2012). The use of COI barcodes for molecular identification of forensically important fly species in Germany. *Parasitology Research*, 110: 2325–32.

Bolufe-Torres R, Benítez-Álvarez L, Rodríguez-Ochoa A, Morales-Martínez A, & Mederos-Ferrer EA. (2014). Fluctuating asymmetry in the venation wing pattern of *Apis mellifera* in two farm of queen bee of Cuba. *Apiciencia*, 16: 49–60.

Bong LJ, Neoh KB, & Yoshimura T. (2018). Developmental irregularity and abnormal elytra formation in the oriental wood borer induced by physical disturbance. *Journal of Insect Science*, 18: 1–6.

Bookstein FL. (1982). On the cephalometric of skeletal change. *American Journal of Orthodontics and Dentofacial Orthopedics*, 82: 177–98.

Borges LMS, Hollatz C, Lobo J, Cunha AM, Vilela AP, Calado G, Coelho R, Costa ACC, Ferreira MSG, Costa MHC, & Costa FC. (2016). With a little help from DNA barcoding: investigating the diversity of Gastropoda from the Portuguese coast. *Scientific Reports*, 6: 20226.

Bosch G, Zhang S, Oonincx DGAB, & Hendriks WH. (2014). Protein quality of insects as potential ingredients for dog and cat foods. *Journal of Nutritional Science,* 3: 1–4.

Bovera F, Piccolo F, Gasco L, Marono S, Loponte R, Vassalotti G, Mastellone V, Lombardi P, Attia YA, & Nizza A. (2015). Yellow mealworm larvae (*Tenebrio molitor*, L.) as possible alternative to soybean meal in broiler diets. *British Poultry Science*, 1–7.

Buenaventura E, Valverde-Castro C, Wolff M, Triana-Chavez O, & Gómez-Palacio A. (2018). DNA barcoding for identifying synanthropic flesh flies (Diptera, Sarcophagidae) of Colombia. *Acta Tropical,* 182: 291–297.

Burggren, WW. (2017). Epigenetics in insects: mechanisms, phenotypes and ecological and evolutionary implications. *Advances in Insect Physiology,* 53: 1─30.

Bustamante DM, Monroy C, Menes M, Rodas A, Schettino PMS, Rojas G, Pinto N, Guhl F, & Dujardín JP. (2004). Metric variation among geographic populations of the chagas vector *Triatoma dimidiata* (Hemiptera: Reduviidae: Triatominae) and related species. *Journal of Medical Entomology*, 41: 296–301.

Byrd JH, & Allen JC. (2001). The development of the black blow fly, *Phormia regina* (Meigen). *Forensic Science International*, 120: 79–88.

Byrd J, & Sutton L. (2019). Forensic entomology for the investigator. *WIREs Forensic Science*, 2: e1370.

Cadinu LA, Barra P, Torre F, Delogu F, & Madau FA. (2020). Insect rearing: potential, challenges, and circularity. *Sustainability,* 12: 4567.

Calle DA, Quiñones ML, Erazo HF, & Jaramillo N. (2008). Discriminación por morfometría geométrica de once especies de *Anopheles* (*Nyssorhynchus*) presentes en Colombia. *Biomédica*, 28: 371–385.

Carles-Tolrá M. (2006). Fanniidae: Familia nueva de dípteros para Andorra (Diptera). *Boletín de la Sociedad Entomológica Aragonesa*, 38: 161–162.

Cavallari ML, Baltazar FN, Nihei SS, Muñoz DR, & Tolezano JE. (2015). Dipterofauna associated with Sus scrofa Linné, 1758, carcasses in urban and coastal regions of São Paulo State, Brazil. Psyche. Article ID: 983980.

Charabidze D. (2012). La biologie des insectes nécrophages et leur utilisation pour dater le décès en entomologie médico-légale. *Annales de la Société Entomologique de France*, 48: 239–252.

Charabidze D, Bourel B, Hedouin V, & Morvan G. (2008). Forenseek, un programme de simulation dédié à l'entomologie médico-légale. *Journal de la Médecine Légale, Droit Médical*, 51 : 131–140

Charabidze D, Bourel B, & Gosset D. (2011). Larval-mass effect: Characterisation of heat emission by necrophagous blowflies (Diptera: Calliphoridae) larval aggregates. *Forensic Science International*, 211: 61–66.

Chatterjee S, Isaia M, & Ventuno E. (2009). Spiders as biological controllers in the agroecosystem. *Journal of Theoretical Biology*, 258: 352–362.

Chen B, Li S, Ren Q, Tong X, Zhang X, & Kang L. (2015). Paternal epigenetic effects of population density on locust phase-related characteristics associated with heat-shock protein expression. *Molecular Ecology*, 24: 851–862.

Clark FMJ. (2009). *Bugs and the Victorians*. Yale University Press. pp. 26–27.

Cortes-Ortiz JA, Ruiz AT, Morales-Ramos JA, Thomas M, Rojas MG, Tomberlin JK, Han R, Giroud L, & Jullien RL. (2016). Insects as sustainable food ingredients. *Insect Mass Production Technologies*, 153–201.

Connat JL, Delbecque JP, Glitho I, & Delachambre J. (1991). The onset of metamorphosis in *Tenebrio molitor* larvae (Insecta, Coleoptera) under grouped, isolated and starved conditions. *Journal of Insect Physiology*, 37: 653–662.

Couri MS. (1991). Influência da dieta no desenvolvimento oogênico de *F. pusio* (Wiedemann, 1830) (Dip. Fanniidae). *Revista Brasileira de Zoologia*, 7: 85–88.

Couri MS, & Sousa VR. (2019). First record of Fanniidae (Diptera) from New Caledonia. *Anais da Academia Brasileira de Ciências*, 91: e20181117.

Coy MR, Salem TZ, Denton JS, Kovaleva ES, Liu Z, Barber DS, Campbell JH, Davis DC, Buchman GW, Boucias DG, & Scharf ME. (2010). Phenol-oxidizing laccases from the termite gut. *Insect Biochemistry and Molecular Biology*, 40: 723─732.

Coyle HM, Lee C, Lin W, Lee HC, & Palmbach TM. (2005). Forensic botany: using plant evidence to aid in forensic death investigation. *Croatian Medical Journal*, 46: 606–612.

Cridge AG, Leask MP, Duncan EJ, & Dearden PK. (2015). What do studies of insect polyphenisms tell us about nutritionally-triggered epigenomic changes and their consequences? *Forum of Nutrition*, 7: 1787–1797.

Cruz-Lozano J. (2005). Entomología: Morfología y fisiología de los insectos. Universidad Nacional de Colombia. ISBN978-958-701-731-1

Dalton S, & Al-Zubiedi H. (2019). *The Mealworm Farm Project a low-cost household mealworm farm*. Doctoral thesis. McGill University, Canada.

D'Almeida JM. (1994). Ovipositional substrates used by Calyptrate Diptera in Tijuca Forest, Rio de Janeiro. *Memórias do Instituto Oswaldo Cruz*, 89: 261–264

De Carvalho CJB, Moura MO, & Ribeiro PB. (2002). Chave para adultos de dípteros (Muscidae, Fanniidae, Anthomyiidae) associados ao ambiente humano no Brasil. *Revista Brasileira de Entomologia*, 46: 107–114

De Carvalho CJB, Pont AC, Couri MS, & Pamplona D. (2003). A catalogue of the Fanniidae (Diptera) of the Neotropical Region. *Zootaxa*, 219: 1–32.

De Carvalho CJB, & Mello-Patiu CAM. (2008). Key to the adults of the most common forensic species of Diptera in South America. *Revista Brasileira de Entomologia*, 52: 309–406.

Defilippo F, Grisendi A, Savoldelli S, Torri D, Dottori M, & Bonilauri P. (2019). Effect of temperature and diet on *Plodia interpunctella* (Lepidoptera: Pyralidae) development with special reference to isomegalen diagram and accumulated degree days. *Journal of Entomological and Acarological Research*, 51: 7855.

De Marco M, Martínez S, Hernandez F, Madrid J, Gai F, Rotolo L, Belforti M, Bergero D, Katz H, Dabbou S, Kovitvadhi A, Zoccarato I, Gasco L, & Schiavone A. (2015). Nutritional value of two insect larval meals (*Tenebrio molitor* and *Hermetia illucens*) for broiler chickens: Apparent nutrition digestibility, apparent ileal amino acid digestibility and apparent metabolizable energy. *Animal feed Science and Technology*, 209: 211–218.

De Souza AM, & Linhares AX. (1997). Diptera and Coleoptera of potential forensic importance in South-eastern Brazil. Brazil. *Medical and Veterinary Entomology*, 11: 8–12.

De Souza ASB, Dutra-Kirst F, & Ferreira-Krüger R. (2008). Insects of forensic importance from Rio Grande do Sul state in Southern Brazil. *Revista Brasileira de Entomologia*, 52: 641–646.

De Souza DA, Wang Y, Kaftanoglu O, De Jong D, Amdam GV, Gonçalves LS, & Francoy TM. (2015). Morphometric identification of queens, workers and intermediates in vitro reared honey bees (*Apis mellifera*). *PLOS ONE*, 10: e0123663.

Dias-Vasconcelos S, Leonel-Costa D, & Leandro-Oliveira D. (2017). Entomological evidence in a case of a suicide victim by hanging: first collaboration between entomologists and forensic police in north-eastern Brazil. *Australian Journal of Forensic Science*, 1–9.

Díaz-Martín B, Rodríguez AL, & Bordas MIS. (2014). Primeros resultados sobre desarrollo de *Calliphora vicina* (Diptera: Calliphoridae) bajo condiciones controladas de temperatura. *Ciencia Forense*, 11: 241–260.

Dobigny G, Baylac M, & Denys C. (2002). Geometric morphometrics, neural networks and diagnosis of sibling *Taterillus* species (Rodentia, Gerbillinae). *Biological Journal of the Linnean Society*, 77: 319–327.

Domínguez MC, & Roig-Juñent SA. (2008). A phylogeny of the family Fanniidae Schnabl (Insecta: Diptera: Calyptratae) based on adult morphological characters, with special reference to the Austral species of the genus *Fannia*. *Invertebrate Systematics*, 22: 563–587.

Domínguez MC & Pont AC. (2014). Fanniidae (Insecta: Diptera). Fauna of New Zealand 71, 91 pp.

Dos Santos CM, Jurberg K, Galvao C, Silva-Rocha D & Rodriguez-Fernandez J. (2003). Estudo morfométrico do Gênero *Panstrongylis* Berg, 1879 (Hemiptera, Reduviidae, Triatominae). *Memórias do Instituto Oswaldo Cruz*, 98: 939–944.

Du MH, Yan ZW, Hao YJ, Yan ZT, Si FL, Chen B, & Qiao L. (2017). Suppression of *Laccase 2* severely impairs cuticle tanning and pathogen resistance during the pupal metamorphosis of *Anopheles sinensis* (Diptera: Culicidae). *Parasites and Vectors*, 10: 171.

Dujardín JP. (2008). Morphometrics applied to medical entomology. *Infection, Genetics and Evolution*, 8: 875–890.

Dujardín JP, Kaba D, Solano P, Dupraz M, McCoy KD, & Jaramillo N. (2014). Outline-based morphometrics, an overlooked method in arthropod studies? *Infection, Genetics and Evolution*, 28: 704–714.

Edgar RC. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 32: 1792–1797.

EFSA. (2015). European Food Safety Authority. Scientific Opinion on a risk profile related to production and consumption of insects as food and feed. *EFSA Journal*, 13: 4257

EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA), Turck D, Castenmiller J, De Henauw S, Hirsch-Ernst K, Kearney J, Maciuk A, Mangelsdorf I, McArdle HJ, Naska A, Pelaez C, Pentieva K, Siani A, Thies F, Tsabouri S, Vinceti M, Cubadda F, Frenzel T, Heinonen M, Marcheli R, Neuhäuser-Berthold M, Poulsen M, Maradona MP, Schlatter JR, Van Loveren H, Ververis E, & Knutsen K. (2021). Safety of dried yellow mealworm (*Tenebrio molitor* larva) as a novel food pursuant to Regulation (EU) 2015/2283. *EFSA Journal*. doi: 10.2903/j.efsa.2021.6343

Eilenberg J, Vlak JM, Nielsen-LeRoux C, Cappellozza S, & Jensen AB. (2015). Diseases in insects produced for food and feed. *Journal of Insects as Food and Feed*, 1: 87–102.

Ekrem T, Willassen E, & Stur E. (2007). A comprehensive DNA sequence library is essential for identification with DNA barcodes. *Molecular Phylogenetics and Evolution*, 43: 530–542.

Emmam AK, Ibrahim HE, Helmi A, & Sharaf A. (2020). Identification of some Egyptian leafhopper species (Hemiptera: Cicadellidae) using DNA barcoding. *Biologia*. doi: 10.2478/s11756-019-00384-y.

Espindola CB, & Couri MS. (2004). *Fannia flavicincta* Stein (Dip. Fanniidae): a new vector of *Dermatobia hominis* (Linnaeus Jr.) (Dip. Cuterebridae). *Revista Brasileira de Zoologia*, 21: 115–116.

Espra AS, Tabugo SRM, Torres MAJ, Gorospe JG, Manting MME, & Demayo CG. (2015). Describing dimorphism in wing shapes in the blowfly *Lucilia sericata* Meigen (Diptera: Calliphoridae) using geometric morphometrics. *Advances in Environmental Biology*, 9: 64–70.

Eon SS. (2019). Taxonomic and ecological study on necrophagous insect and its forensic application. Kangwon National University, South Korea.

FAO. (2010a). Forest insects as food: humans bite back. Retrieved from https://doi.org/ISBN978-92-5-106488-7 (Consulted 2020, Sept 20).

Faria LS, Paseto ML, Franco FT, Perdigao VC, Capel G, & Mendes J. (2013). Insects breeding in pig carrion in two environments of a rural area of the State of Minas Gerais, Brazil. *Neotropical Entomology*, 42: 216–222.

Faris AM, West WR, Tomberlin JK, & Tarone AM. (2020). Field validation of a development data set for *Cochliomya macellaria* (Diptera: Calliphoridae): estimating insect age based on development stage. *Journal of Medical Entomology*, 57: 39–49.

Finke MD. (2002). Complete nutrient composition of commercially raised invertebrates used as food for insectivores. *Zoo Biology*, 21: 269–285.

Folmer O, Blac, M, Hoeh W, Lutz R, & Vrijenhoek R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3: 294–299.

Forero-Becerra EG. (2011). Miasis en salud pública y salud pública veterinaria. *Revista Sapuvet de Salud Pública*, 2: 95–132.

Fuentes-López A. (2018). Molecular analysis of necrophagous Diptera of the Iberian Peninsula. Doctoral thesis. Universidad de Murcia, España.

Fuentes-López A, Ruiz C, Galián J, & Romera E. (2020). Molecular identification of forensically important fly species in Spain using COI barcodes. *Science & Justice*, 60: 293–302.

Fuentes-López A, Rebelo MT, Romera E, López-López A, & Galián J. (2021). Genetic diversity of *Calliphora vicina* (Diptera: Calliphoridae) in the Iberian Peninsula based on cox1, 16S and ITS2 sequences. *Biological Journal of the Linnean Society*. doi: 10.1093/biolinnean/blaa109

Fukatsu T, & Futahashi R. (2016). Editorial overview: Molecular physiology: molecular basis 38 of insect colours and patterns. *Current Opinion in Insect Science,* 17: 6─8.

Gabriel D, & Tscharntke T. (2007). Insect pollinated plants benefit from organic farming. *Agriculture, Ecosystems & Environment*, 118: 43–48.

García-Reina A, Rodríguez-García M, & Galián J. (2018). Validation of reference genes for quantitative real-time PCR in tiger beetles across sexes, body parts, sexual maturity and immune challenge. *Scientific Reports,* 8: 10743.

García-Robledo C, Kuprewicz EK, Baer CS, Clifton E, Hernández GG, & Wagner DL. (2020). The Erwin equation of biodiversity: From little steps to quantum leaps in the discovery of tropical insect diversity. *Biotropica*, 52: 590–597.

García-Rojo AM, Honotaro L, González M, & Téllez A. (2009). Determinación del intervalo post-mortem mediante el estudio de la sucesión de insectos en dos cadáveres hallados en el interior de una finca rústica de Madrid. *Cuadernos de Medicina Forense*, 15: 137–145.

Garnett T, Röös E, & Little D. (2015). Lean, green, mean, obscene...? What is efficiency? And is it sustainable? Animal production and consumption reconsidered. *Food Climate Research Network*, 1–48.

Garzón MJ, & Schweigmann N. (2018). Morphometrics variation of the *Aedes albifasciatus* (Diptera: Culicidae) wings in three populations from different ecoregions of Argentina. *Journal of Medical Entomology*. doi: 10.1093/jme/tjy096.

Gasco L, Biasato I, Dabbou S, Schiavone A, & Gai F. (2019). Animals fed insect-based diets: state-of-the-art on digestibility, performance and product quality. *Animals,* 9: 170.

Gahukar RT. (2016). Edible insects farming: efficiency and impact on family livelihood, food security, and environment compared with livestock and crops. *Insects as Sustainable Food Ingredients*, 85–111.

Gahukar RT. (2020). Edible insects collected from forests for family livelihood and wellness of rural communities: A review. *Global Food Security*, 25: 100348.

Geng A, Wu J, Xie RR, Li X, Chang FX, & Sun JZ. (2016). Characterization of a laccase from a wood-feeding termite, *Coptotermes fomosanus. Insect Science*, 25: 251–258.

Gennard DE. (2007*)*. Forensic Entomology: an introduction. Chichester, England; Hoboken, NJ: John Wiley & Sons.

Genner MJ, & Turner GF. (2011). Ancient Hybridization and Phenotypic Novelty within Lake Malawi's Cichlid Fish Radiation. *Molecular Biology and Evolution*, 29: 195–206.

Gerard M, Michez D, Fournier D, Maebe K, Smagghe G, Biesmeijer JC, & Meulemeester TD. (2015). Discrimination of haploid and diploid males of *Bombus terrestris* (Hymenoptera; Apidae) based on wing shape. *Apidologie*, 46: 644–653.

Godoy RE, Shimabukuro PHF, Dos Santos TV, Pessoa FAC, Da Cunha AEFL, Santos FKM, Vilela ML, Rangel EF, & Galati EAB. (2018). Geometric morphometry of the head in sand flies (Diptera: Psychodidae: Phlebotominae), an alternative approach to taxonomy studies. *Zootaxa*, 4504: 566–576.

Gomes PR, Koller WW, Gomes A, Carvalho CJB, & Zorzatto R. (2002). Dípteros fanídeos vetores de ovos de *Dermatobia hominis* em Campo Grande, Mato Grosso do Sul. *Pesquisa Veterinária Brasileira*, 22: 114–118.

Gorman MJ & Arakane Y. (2010). Tyrosine hydroxylase is required for cuticle sclerotization and pigmentation in *Tribolium castaneum*. *Insect Biochemistry and Molecular Biology*, 40: 267–273.

Grassberger M, & Reiter C. (2001). Effect of temperature on *Lucilia sericata* (Diptera: Calliphoridae) development with special reference to the isomegalen- and isomorphen-diagram. *Forensic Science International*, 120: 23–36.

Grassberger M, Friedrich E, & Reiter C. (2003). The blowfly *Chrysomya albiceps* (Wiedemann) (Diptera: Calliphoridae) as a new forensic indicator in Central Europe. *International Journal of Legal Medicine*, 117: 75–81.

Grau T, Vilcinkskas A, & Joop G. (2017). Sustainable farming of the mealworm *Tenebrio molitor* for the production of food and feed. *Zeitschrift für Naturforschung*, 72: 337–349.

Grisales-Ochoa DL. (2014). Evolução e biogeográfica de Fanniidae (Insecta, Diptera), com ênfase nas linhagens do componente noroeste neotropical. Trabajo Fin de Máster. Universidad de Curitiba, Paraná, Brasil.

Gruntenko NE, & Rauschenbach IY. (2008). Interplay of JH, 20E and biogenic amines under normal and stress conditions and its effect on reproduction. *Journal of Insect Physiology*, 54: 902–908.

Grzywacz A, & Prado e Castro C. (2012). New records of *Fannia* Robineau-Desvoidy (Diptera: Fanniidae) collected on pig carrion in Portugal with additional data on the distribution of *F. conspecta* Rudzinski, 2003. *Entomologica Fennica*, 23: 169–176.

Grzywacz A, Ogiela J, & Tofilski A. (2017). Identification of Muscidae (Diptera) of medico-legal importance by means of wing measurements. *Parasitology Research*, 116: 1495–1504.

Grzywacz A, Wyborska D, & Piwczynski M. (2017). DNA barcoding allows identification of European Fanniidae (Diptera) of forensic interest. *Forensic Science International*, 278: 106–114.

Guiné RPF, Correia P, Coelho C, & Costa CA. (2021). The role of edible insects to mitigate challenges for sustainability. *Open Agriculture,* 6: 24–36

Guo YD, Cai JF, Meng FM, Chang YF, Gu Y, Lan LM, Liang L, & Wen JF. (2012). Identification of forensically important flesh flies based on a shorter fragment of the cytochrome oxidase subunit I gene in China. *Medical and Veterinary Entomology*, 26: 307–313.

Guo YD, Song Z, Luo L, Wang Q, Zhou G, Yang D, Zhong D, & Zheng X. (2018). Molecular evidence for new sympatric cryptic species of *Aedes albopictus* (Diptera: Culicidae) in China: A new threat from Aedes albopictus subgroup? *Parasites and Vectors,* 11: 228.

Guo B, Yin J, Hao W, & Jiao M. (2019). Polyurethane foam induces epigenetic modification of mitochondrial DNA during different metamorphic stages of *Tenebrio molitor. Ecotoxicology and Environmental Safety,* 183: 109461.

Hajd LH, Sauvage F, Ninio C, Depaquit J, & Augot D. (2014). Wing geometry as a tool for discrimination of *Obsoletus* group (Diptera: Ceratopogonidae: Culicoides) in France. *Infection, Genetic and Evolution*, 21: 110–117.

Hall RD. (1990). *Medico-criminal entomology*, pp. 1-8. Catts EP, Haskell NH (eds.). Entomology and death: A procedural guide. Joyce's Print Shop, Clemson.

Hall MJR, MacLeod N, & Wardhana AH. (2014). Use of wing morphometrics to identify populations of the Old-World screwworm fly, *Chrysomya bezziana* (Diptera: Calliphoridae): A preliminary study of the utility of museum specimens. *Acta Tropica*, 1385: 549–555.

Harvey ML, Dadour IR, & Gaudieri S. (2003). Mitochondrial DNA cytochrome oxidase I gene: potential for distinction between immature stages of some forensically important fly species (Diptera) in western Australia. *Forensic Science International*, 131: 134–139

Hattori M, Konishi H, Tamura Y, Konno K, & Sogawa K. (2005). Laccase-type phenoloxidase in salivary glands and watery saliva of the green rice leafhopper, *Nephotettix cincticeps. Journal of Insect Physiology*, 51: 1359–1365.

Hayakawa Y, Sawada M, Seki M, Sirasoonthorn P, Shiga S, Kamiya K, Minakuchi C, & Miura K. (2018). Involvement of *laccase2* and *yellow-e* genes in antifugal host defense of the model beetle, *Tribolium casteneum. Journal of Invertebrate Pathology*, 151: 41–49.

Hebert PDN, Cywinska A, Ball SL, & de Waard JR. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B*, 270: 313–321.

Hethke M, & Weeks SC. (2020). Population density effects on carapace growth in clam shrimp: implications for palaeontological studies. *Zoological Studies*, 59: 33.

Hoegger PJ, Kilaru S, James TY, Thacker JR, & Kües GD. (2006). Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. *FEBS Journal*, 273: 2308–2326.

Hoyos-López R, Uribe SS, & Vélez I. (2012). Tipificación de especímenes colombianos de *Lutzomyia longipalpis* (Diptera: Psychodidae) mediante “Código de barras”. *Revista Colombiana de Entomología*, 38: 134–140.

Hurtado-Burillo M. (2015). Molecular and morphometric characterization of the genus *Scaptotrigona* (Apidae: Melliponini) in Mesoamerica. Doctoral thesis. Universidad de Murcia, España.

Hurtado-Burillo M, May-Itzá WdJ, Quezada-Euán JJG, De La Rua P, & Ruiz C. (2017). Multilocus species delimitation in Mesoamerican *Scaptotrigona* stingless bees (Apidae: Meliponini) supports the existence of cryptic species. *Systematic Entomology*, 42: 171–181

Hussain I, Khan S, Sultan A, Chand N, Khan R, Alam W, & Ahmad N. (2017). Meal worm (*Tenebrio molitor*) as potential alternative source of protein supplementation in broiler. *International Journal of Bioscience*, 10: 255–262.

Iaconisi V, Marono S, Parisi G, Gasco L, Genovese L, Maricchiolo G, Bovera F, & Piccolo G. (2017). Dietary inclusion of *Tenebrio molitor* larvae meal: Effects on growth performance and final quality treats of blackspot sea bream (*Pagellus bogaraveo*). *Aquaculture*, 476: 49–58.

Iglesias PP, Soto IM, Soto EM, Calderón L, Hurtado J, & Hasson E. (2018). Rapid divergence of courtship song in the face of neutral genetic homogeneity in the cactophilic fly *Drosophila buzzatii*. *Biological Journal of the Linnean Society*, 125: 321–332

Introna F, De Donno A, Santoro V, Corrado S, Romano V, Porcelli F, & Campobasso CP. (2011). The bodies of two missing children in an enclosed underground environment. *Forensic Science International*, 207: e40-7.

Ivanova NV, Dewaard JR, & Hebert PDN. (2006). An inexpensive automation-friendly protocol for recovering high-quality DNA. *Molecular Ecology Notes*, 6: 998–1002.

Izumitani HF, Kusaka Y, Koshikawa S, Toda MJ, & Katoh T. (2016). Phylogeography of the subgenus *Drosophila* (Diptera: Drosophilidae): evolutionary history of faunal divergence between the old and the new worlds. *PLOS One*, 11: e0160051

Jaenisch R, & Bird A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Genetics*, 33: 245–254.

Kang Y, Deng Z, Zang R, & Long W. (2017). DNA barcoding analysis and phylogenetic relationship of tree species in tropical cloud forests. *Scientific Reports*, 7: 12564.

Kearse M, Moir R, Wilson A, Stones-Havas S, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, & Drummond A. (2012). Geneious basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatic*, 28: 1647–1649.

Kenrick P, Wellman CH, Schneider H, & Edgecombe GD. (2012). A timeline for terrestrialization: Consequences for the carbon cycle in the Palaeozoic. *Philosophical Transaction of the Royal Society B: Biological Sciences*, 367: 519–536.

Kerr KCR, Birks SM, Kalyakin MV, Red'kin Y, Koblik EA, & Hebert PDN. (2009). Filling the gap – COI barcode resolution in eastern Palaearctic's birds. *Frontiers in Zoology*, 6: 29.

Khamis FM, Masiga DK, Mohamed SA, Salifu D, de Meyer M, & Ekesi S. (2012). Taxonomic Identity of the Invasive Fruit Fly Pest, *Bactrocera invadens*: Concordance in Morphometry and DNA Barcoding. *PLOS ONE*, 7: e44862.

Khan S, Khan RU, Alam W, & Sultan A. (2017). Evaluating the nutritive profile of three insect meals and their effects to replace soya bean in broiler diet. *Journal of Animal Physiology and Animal Nutrition*, 102: e662–e668.

Kimura M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16: 111–120.

Klingenberg CP. (2013). Visualizations in geometric morphometrics: How to read and how to make graphs showing shape changes. *Hystrix*, 24: 15–24.

Klunder HC, Wolkers-Rooijackers J, Korpela JM, & Nout MJR. (2012). Microbiological aspects of processing and storage of edible insects. *Food Control*, 26: 628–631

Kosmann C. (2009). *Código de barras (DNA Barcode) de dípteros de interesse forense*. Master's thesis. Universidade Federal do Panamá, Curitiba, Brasil.

Koutsos L, McComb A, & Finke M. (2019). Insect composition and uses in animal feeding applications: a brief review. *Annales Entomologicae Fennicae*. doi: 10.1093/aesa/saz033

Krüger RF, Kirst FD, & De Souza ASB. (2010). Rate of development of forensically-important Diptera in southern Brazil. *Revista Brasileira de Entomologia*, 54: 624–629.

Lainez EM. (2017). *Influencia de diferentes dietas en la composición nutricional del insecto comestible Tenebrio molitor y estudio de su pardeamiento*. Trabajo Fin de Grado. Universidad pública de Navarra, España.

Lambertini C, Gustafsson MHG, Frydenberg J, Lissner J, Speranza M, & Brix H. (2006). A phylogeographic study of the cosmopolitan genus *Phragmites* (Poaceae) based on AFLPs. *Plant Systematics and Evolution*, 258: 161–182.

Leandro MJ, & D'Almeida JM. (2005). Levantamento de Calliphoridae, Fanniidae, Muscidae e Sarcophagidae em um fragmento de mata na Ilha de Governador, Rio de Janeiro, Brasil. *Iheringia, Série Zoologia, Porto Alegre*, 95: 337–381.

Lee PS, Sing KW, & Wilson JJ. (2015). Reading mammal diversity from flies: The persistence period of amplifiable mammal mtDNA in blowfly guts (*Chrysomya megacephala*) and a new DNA mini–barcode target. *PLOS ONE*, 10: e0123871.

Leigh JW, & Bryant D (2015) PopART: Full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, 6: 1110–1116

Li X, Yang Y, Henry RJ, Rossetto M, Wang Y, & Chen S. (2015). Plant DNA Barcoding from gene to genome. *Biological Reviews*, 99: 157–166.

Li D, Waite DW, Gunawardana DN, McCarthy B, Anderson D, Flynn A, & George S. (2018). DNA barcoding and real-time PCR detection of *Bactrocera xanthodes* (Tephritidae: Diptera) complex. *Bulletin of Entomological Research*, 109: 1–9.

Linhares AX. (1978). Estudos sobre eclosão em ovos de *Fannia pusio* Wiedemann (Diptera; Muscidae) em laboratório. *Ciência e Cultura*, 30: 603.

Linhares AX. (1981). Synantropy of Muscidae, Fanniidae and Anthomyiidae (Diptera) in the city of Campinas, Sao Paulo, Brasil. *Revista Brasileira de Entomologia*, 25: 231–243.

Liu LN, & Wang CY. (2014). Complete mitochondrial genome of yellow mealworm (*Tenebrio molitor*). *Zoological Research*, 35: 537–545.

Liu YQ, & Chen HW. (2018). The genus *Scaptodrosophila* Duda part II: the *coracina* species group from East Asia, with morphological and molecular evidence (Diptera, Drosophilidae). *ZooKeys*, 736: 119–148.

Liu S, Rao XJ, Li MY, Feng MF, He MZ, & Li SG. (2015). Identification of candidate chemosensory genes in the antennal transcriptome of *Tenebrio molitor* (Coleoptera: Tenebrionidae). *Comparative Biochemistry and Physiology*, 13: 44–51.

Liu YQ, Gao QS, & Chen HW. (2017). The genus *Scaptodrosophila* Duda part I: the brunnea species group from the Oriental Region, with morphological and molecular evidence (Diptera, Drosophilidae). *ZooKeys*, 671: 87–118.

Liu SH, Yang BJ, Wang AY, Luo J, & Tang J. (2020). RNA interference of tyrosine hydroxylase caused rapid mortality by impairing cuticle formation in *Nilaparvata lugens* (Hemiptera: Delphacidae). *Pest Management Science*, 76: 2225–2232

Lizhang W, Viejo-Montesinos JL, & Dinghong Y. (2008). Los insectos como fuente de alimento: análisis del contenido en proteína y grasa de 100 especies. *Boletim do Museu Municipal do Funchal*, 14: 55–70.

Loh R, David JR, Debat V, & Bitner-Mathé BC. (2008). Adaptation to different climates results in divergent phenotypic plasticity of wing size and shape in an invasive drosophilid. *Journal of Genetics*, 83: 209–217.

López-López A, Hudson P, & Galián J. (2016). Islands in the desert: species delimitation and evolutionary history of *Pseudotetracha* tiger beetles (Coleoptera: Cicindelidae: Megacephalini) from Australian salt lakes. *Molecular Phylogenetics and Evolution*, 101: 279–285

Lord JC, Hartzer K, Toutges M, & Oppert B. (2010). Evaluation of quantitative PCR reference genes for gene expression studies in *Tribolium castaneum* after fungal challenge. *Journal of Microbiological Methods*, 80: 219–221.

Ludwig D, & Fiore C. (1960). Further studies on the relationship between parental age and the life cycle of the mealworm, *Tenebrio Molitor*. *Annals of the Entomological Society of America*, 53: 595–600.

Lutz L, Verhoff MA, & Amendt J. (2021). To be there or not to be there, that is the question—On the problem of delayed sampling of entomological evidence. *Insects*, 12: 48.

Macedo MP. (2017). Morfometria geométrica alar como ferramenta para a identificação de três espécies de califorídeos (Diptera: Calliphoridae) no Cerrado Brasileiro, *EntomoBrasilis*, 10: 9–13.

Maeno K, & Tanaka S. (2011). Phase-specific responses to different qualities of food in the desert locust, *Schistocerca gregaria*: developmental, morphological and reproductive characteristics. *Journal of Insect Physiology*, 57: 514–520.

Maleszka R. (2008). Epigenetic integration of environmental and genomic signals in honey bees: the critical interplay of nutritional, brain and reproductive networks. *Epigenetics* 3: 188–192.

Marchiori CH. (2014). Diptera of economic and sanitary importance collected on different substrates in southern Goiás, Brazil. *Qeios,* 1: 4–9.

Marchiori CH, & Do Prado AP. (1996). Efeito da temperatura no desenvolvimento dos estágios imaturos de *F. pusio* (Wiedemann, 1830) (Dip. Fanniidae), em laboratório. *Revista Brasileira de Biologia*, 56: 93–98.

Marchiori CH, & Do Prado AP. (1999). Tabelas de vida de *F. pusio* (Wiedemann) (Dip.: Fanniidae). *Anais da Sociedade Entomológica do Brasil*, 28: 557–563.

Marchiori CH, Silva CG, Caldas ER, Vieira CIS, Almeida KGS, Teixeira FF, & Linhares AX. (2000). Artrópodes associados com carcaça de suíno em Itumbiara, Sul de Goiás. *Arquivos do Instituto Biológico*, 67: 167–170.

Matsumoto Y, & Hattori M. (2019). Characterization of multicopper oxidase genes in the green rice leafhopper, *Nephotettix cincticeps* (Hemiptera: Cicadellidae), with focus on salivary gland-specific genes. *Archives of Insect Biochemistry and Physiology*, 102: 1–17.

Matuszewski S, Bajerlein D, Konwerski S, & Szpila K. (2010). Insect succession and carrion decomposition in selected forests of Central Europe. Part 2. Composition and residency patterns of carrion fauna. *Forensic Science International*, 195: 42–51

Mazzanti M, Alessandrini F, Tagliabracci A, Wells JD, & Campobasso CP. (2010). DNA degradation and genetic analysis of empty puparia: Genetic identification limits in forensic entomology. *Forensic Science International*, 195: 99–102.

McLachlan GF. (1999). Mahalanobis distance. *Resonance,* 4: 20–26.

Meier R, Shiyang K, Vaidya G, & NG PKL. (2006). DNA Barcoding and taxonomy in Diptera: a tale of hight intraspecific variability and low identification success. *Systematic Biology*, 55: 715–728.

Mendes J, & Linhares AX (2002). Cattle dung breeding Diptera in pastures in Southeaster Brazil: diversity, abundance and seasonality. *Memórias do Instituto Oswaldo Cruz*, 97: 37–41.

Menes-Hernández M. (2004). Diferencias métricas entre poblaciones de *Triatoma dimidiata Latreille* (Hemiptera: Reduviidae: Triatominae) de México, Centro América y Colombia: Efecto de la procedencia geográfica y el ecotopo. Trabajo Fin de Grado. Universidad de San Carlos de Guatemala.

Meusnier I, Singer GAC, Landry JF, Hickey DA, Hebert PDN, & Hajibabaei M. (2008). A universal DNA mini–barcode for biodiversity analysis. *BMC Genomics*, 9: 214.

Michaud JP, & Moreuau G. (2009). Predicting the visitation of carcasses by carrion-related insects under different rates of degree-day accumulation. *Forensic Science International*, 185: 78–83.

Mikery OF, Rebollar-Téllez EA, Cruz-López LC, Marina CF, & Castillo A. (2019). Traditional and geometric morphometry analyses of *Lutzomyia cruciata* (Diptera: Psychodidae: Phlebotominae) populations of Chiapas, Mexico. *Journal of Medical Entomology*. doi: 10.1093/jme/tjy227

Misof B, Liu S, Meusemann K, Peters RS, Donath A, & Mayer C. (2014). Phylogenomic resolves the timing and pattern of insect evolution. *Science*, 346: 763–767.

Monteiro MR, & Do Prado AP. (2006). Moscas sinantrópicas (Dip. Cyclorrhapha) e seus parasitoides microhimentópteros (Insec. Hymenoptera) num plantel avícola de Monte Mor, São Paulo, Brasil. *Revista Brasileira de Parasitologia Veterinária*, 15: 49–57.

Monteiro TT, Da Silva EM, & Bravo FR. (2014). Levantamento taxonômico e sazonalidade de Calliphoridae, Muscidae e Fanniidae (Insecta: Diptera) em Feira de Santana, Bahia, Brasil. *EntomoBrasilis*, 7: 171–177.

Morales-Ramos JA, Guadalupe-Rojas M, Kay S, Shapiro-Ilan DI, & Tedders WL. (2012). Impact of adult weight, density, and age on reproduction of *Tenebrio molitor* (Coleoptera: Tenebrionidae). *Journal of Entomological Science*, 47: 208─220.

Mun S, Noh MY, Kramer KJ, Muthukrishnan S, &Arakane Y. (2019). Gene functions in adult cuticle pigmentation of the yellow mealworm, *Tenebrio molitor*. *Insect Biochemistry and Molecular Biology,* 117: 103291.

Nassu MP, Thyssen PJ, & Linhares AX. (2014). Development rate of immatures of two fly species of forensic importance: *Sarcophaga* (Liopygia) *ruficornis* and *Microcerella halli* (Diptera: Sarcophagidae). *Parasitology Research*, 113: 217–222.

Niermans K, Woyzichovski J, Kröncke N, Benning R, & Maul R. (2019). Feeding study for the mycotoxin zearalenone in yellow mealworm (*Tenebrio molitor*) larvae—investigation of biological impact and metabolic conversion. *Mycotoxin Research*, 35: 231─242

Noguchi H, Hayakawa Y, & Downer RG. (1995). Elevation of dopamine levels in parasitized insect larvae. *Insect Biochemistry and Molecular Biology*, 25: 197–201.

Noh MY, Muthukrishnan S, Kramer JK, & Arakane Y. (2016b). Cuticle formation and pigmentation in beetles. *Current Opinion in Insect Science,* 17: 1–9.

Nuñez-Rodriguez JA, & Liria J. (2017). Geometric Morphometrics sexual dimorphism in three forensically-important species of Blow Fly (Diptera: Calliphoridae). *Life*, 4: 272–284.

Ødegaard F. (2000). How many species of arthropods? Erwin's estimate revised. *Biological Journal of the Linnean Society,* 71: 583–597.

Oliveira-Costa J, & Mello-Patiu CA. (2004). Application of Forensic Entomology to estimate of the post-mortem interval (PMI) in homicide investigations by the Rio de Janeiro Police Department in Brazil. *Journal of Forensic Medicine and Toxicology*, 5: 40–44.

Omar B, Kurahashi H, Jeffery J, & Yasohdha N. (2008). A new record of *Fannia pusio* (Wiedemann) (Diptera: Fanniidae) from Malaysia. *Tropical Biomedicine,* 24: 99–100

Onder Z, Yildirim A, Duzlu O, Arslan MO, Sari B, Tasci GT, Ciloglu A, Aydin NP, Inci A, & Adler PH. (2019). Molecular characterization of black flies (Diptera: Simuliidae) in areas with pest outbreaks and simuliotoxicosis in Northeast Anatolia Region, Turkey. *Acta Tropica,* 199: 105149.

Osório HC, Zé-Zé L, Nieto M, Silva S, Marques F, Silva AS, & João-Alves M. (2018). Detection of the Invasive Mosquito Species *Aedes* (Stegomyia) *albopictus* (Diptera: Culicidae) in Portugal. *International Journal of Environmental Research and Public Health*, 15: 820.

Pacheco MA, González R, & Brochero HL. (2017). *Anopheles darlingi* Root 1926 (Diptera: Culicidae): variaciones morfométricas en alas y patas de poblaciones en Colombia. *Biomédica,* 37: 124–134.

Park JB, Ho CW, Hyun KS, Jung JH, Soo HY, Seok LY, & Jun KN. (2014). Developmental characteristics of *Tenebrio molitor* larvae (Coleoptera: Tenebrionidae) in different instars. *International Journal of Industrial Entomology*, 28: 5–9.

Parkinson NM, Conyers CM, Keen JN, MacNicoll AD, Smith I, & Weaver RJ. (2003). cDNAs encoding large venom proteins from the parasitoid was *Pimpla hypochondriaca* identified by random sequence analysis. *Comparative Biochemistry and Physiology – C Toxicology and Phamarcology,* 134: 513–520.

Paz A, Gonzalez MA, & Crawford AJ. (2011). DNA barcode of life: An introduction and perspective. *Acta Biológica Colombiana,* 16: 161–175.

Pérez-Altamirano A. (2019). Valoración nutricional de harinas de cucarachas de Madagascar (*Gromphadorhina portentosa*) y tenebrios (*Tenebrio molitor*), para su uso en acuicultura. Tesis Doctoral. Universidad Autónoma de Aguascalientes, México.

Pérez JP, & Henao DC. (2013). Código de barras genético para la identificación de dípteros de importancia forense. *Revista Facultad de Ciencias Forenses y de la Salud de Colombia*, 9: 51–66.

Pfeiler E, Richmond MP, Riesgo-Escovar JR, Téllez-García AA, Johnson S, & Markow TA. (2013). Genetic differentiation, speciation, and phylogeography of cactus flies (Diptera: Neriidae: Odontoloxozus) from Mexico and south-western USA. *Biological Journal of the Linnean Society,* 110: 245–256

Piñero JC, Shivers T, Byers PL, & Johnson H-Y. (2018). Insect-based compost and vermicompost production, quality and performance. *Renewable Agriculture and Food Systems,* 35: 102–108.

Pont AC. (1977). A revision of Australian Fanniidae (Diptera: Calyptrata). *Australian Journal of Zoology*, 51: 1–60.

Quiroga NI, & Dominguez NC. (2010). A new species of the genus *Fannia* Robineau-Desvoidy (Diptera: Fanniidae) belonging to the canicularis species group, collected on pig carrion in the Yungas of the province of Jujuy, Argentina. *Journal of Studies on Neotropical Fauna and Environment*, 45: 95–100.

Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, & Nitsche A. (2004). Guideline to reference gene selection for quantitative real-time PCR. *Biochemical and Biophysical Research Communications*, 313: 856–862.

Ramón-Vázquez JI. (2014). *Plan de negocio: Creación de una empresa de producción de harinas de insectos para consumo animal*. Trabajo Fin de Máster. Universidad Politécnica de Valencia, España.

Rampasso AS, Markow TANN, & Richmond MP. (2017). Genetic and phenotypic differentiation suggests incipient speciation within *Drosophila arizonae* (Diptera: Drosophilidae). *Biological Journal of the Linnean Society,* 122: 444–454

Ravzanaadii N, Kim SH, Choi WH, Hong SJ, & Kim NJ. (2012). Nutritional value of mealworm, *Tenebrio molitor* as food source. *International Journal of Industrial Entomology*, 25: 93–98.

Refaeilzadeh P, Tang L, & Liu H. (2009). Cross-Validation. In: Liu L. Özsu MT. (eds) *Encyclopaedia of Database Systems*. Springer, Boston, MA.

Reibe S, Schmitz J, & Madea B. (2009). Molecular identification of forensically important blowfly species (Diptera: Calliphoridae) from Germany. *Parasitology Research*, 106: 257–261.

Reiter C. (1984). On the growing behaviour of the maggots of blue blowfly *Calliphora vicina*. *Zeitschrift für Rechtsmedizin*, 91: 295–308.

Renaud AK, Savage J, & Adamowicz SJ. (2012). DNA barcoding of Northern Nearctic Muscidae (Diptera) reveals high correspondence between morphological and molecular species limits. *BMC Ecology*, 12: 24.

Reyes-Parrado A, Arrivillaga-Henríquez J, Oviedo M, Scorza-Dagert JV, & Ron-Garrido L. (2020). Larval therapy with *Musca domestica* in the treatment of leishmania ulcer in a murine model. *Acta Biológica Colombiana,* 25: 82–95.

Riaño HC, Jaramillo N, & Dujardín J-P. (2008). Growth changes in *Rhodnius pellescens* under simulated domestic and sylvatic conditions. *Infection, Genetic and Evolution*, 9: 162–168.

Ribeiro N, Abelho M, & Costa R. (2018). A review of the scientific literature for optimal conditions for mass rearing *Tenebrio molitor* (Coleoptera: Tenebrionidae). *Journal of Entomological Science*, 53: 434–454.

Richards CS, Paterson ID, & Villet MH. (2008). Estimating the age of immature *Chrysomya albiceps* (Diptera: Calliphoridae), correcting for temperature and geographical latitude. *International Journal of Legal Medicine,* 122: 271–279.

Rodrigues BL, Carvalho-Costa LF, Pinto IS, & Rebêlo JMM. (2018). DNA barcoding reveals hidden diversity of sand flies (Diptera: Psychodidae) at fine and broad spatial scales in Brazilian regions for Leishmaniasis. *Journal Medical Entomological*, 55: 893–901.

Rolff J, Johnston PR, & Reynolds S. (2019). Complete metamorphosis of insects. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 374: 20190063.

Rozkosný R, Gregor F, & Pont AC. (1997). *The European Fanniidae (Diptera).* Acta scientiarum naturalium Academiae Scientiarum Bohemicae, Brno 31: 80 pp.

Rumpold BA, & Schlüter O. (2013). Nutritional composition and safety aspects of edible insects. *Molecular Nutrition and Food Research,* 57: 802─823

Sang W, He L, Wang X-P, Zhu-Salzman K, & Lei C-L. (2015). Evaluation of reference genes for RT-qPCR in *Tribolium castaneum* (Coleoptera: Tenebrionidae) under UVB stress. *Molecular Ecology and Evolution,* 44: 418–425.

Sawe T, Nielse A, Totland Ø, Macrice S, & Eldegard K. (2020). Inadequate pollination services limit watermelon yields in northern Tanzania. *Basic and Applied Ecology,* 44: 35–45.

Sethuraman A, Janzen FJ, Weisrock DW, & Obrycki JJ. (2020). Insights from population genomics to enhance and sustain biological control of insect pests. *Insects,* 11: 482.

Shafique L, Abdel-Latif HMR, Hassan F, Alagawany M, Naiel MAE, Dawood MAO, Yilmaz S, & Liu Q. (2021). The feasibility of using yellow mealworms (*Tenebrio molitor*): Towards a sustainable aquafeed industry. *Animals*, 11: 811.

Shi ZY, Yang CQ, Hao MD, Wang XY, Ward RD, & Zhang AB. (2017). FuzzyID2: A software package for large dataset species identification via barcoding and metabarcoding using Hidden Markov Models and fuzzy set methods. *Molecular Ecology Resources*, 18: 666–675.

Silva SE, Seabra SG, Carvalheiro LG, Nunes VL, Marabuto E, Mendes R, Rodrigues ASB, Pina-Martins F, Yurtsever S, Laurentino TG, Figueiredo E, Rebelo MT, & Paulo OS. (2020). Population genomics of *Bombus terrestris* reveals high but unstructured genetic diversity in a potential glacial refugium. *Biological Journal of the Linnean Society*, 129: 259–272

Simon JD, Peles D, Wakamatsu K, & Ito S. (2009). Current challenges in understanding melanogenesis: bridging chemistry, biological control, morphology, and function. *Pigment Cell & Melanoma Research*, 22: 563–579.

Singh P. (1975). Yellow mealworm, *Tenebrio molitor* Linnaeus, life cycle. *New Zealand Department of Scientific and Industrial Research Information Series,* 105: 1–3.

Smith KGV. (1986). *A Manual of Forensic Entomology*. Trustees of the British Museum (Natural History). London. 205pp.

Socha R, & Sehnal F. (1972). Inhibition of adult development in *Tenebrio molitor* by insect hormones and antibiotics. *Journal of Insect Physiology,* 18: 317–337

Sontingun N, Sukontason KL, Zajac BK, Zehner R, Sukontason K, Wannasan A, & Amendt J. (2017). Wing morphometrics as a tool in species identification of forensically important blow flies of Thailand. *Parasites Vectors,* 10: 229.

Strong PJ, & Claus H. (2011). Laccase: A review of its past and its futuree in bioremediation. *Critical Reviews in Environmental Science and Technology,* 41: 373–434.

Sumruayphol S, & Chaiphongpachara T. (2019). Geometric morphometrics as a tool for three species identification fireflies (Coleoptera: Lampyridae) in Thailand. *Biodiversitas*, 20: 2388–2395.

Suo H, Sun P, Wang C, Peng D, & Zhao X. (2016). Apoptotic effects of insect tea in HepG2 human hepatoma cells. *CYTA – Journal of Food*, 14: 169–175.

Szpila K. (2012). Key for identification of European and Mediterranean blowflies (Diptera, Calliphoridae) of medical and veterinary importance – adult flies. In: Gennard D. (ed.) *Forensic entomology, an introduction*, II edition. Willey-Blackwell.

Szpila K, Zmuda A, Akbarzadeh K, & Tofilski A. (2019). Wing measurement can be used to identify European blow flies (Diptera: Calliphoridae) of forensic importance. *Forensic Science International*, 296: 1–8.

Tamura K, Dudley J, Nei M, & Kumar S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis. Version 4.0. *Molecular Biology and Evolution*, 24: 1596–1599.

Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, & Heinen E. (1999). Housekeeping genes as internal standards: use and limits. *Journal of Biotechnology*, 75: 291–295.

Toutges MJ, Hartzer K, Lord J, & Oppert B. (2010). Evaluation of reference genes for quantitative polymerase chain reaction across life cycle stages and tissue types of *Tribolium castaneum*. *Journal of Agricultural and Food Chemistry*, 58: 8948–8951.

Urs KCD, & Hopkins TL. (1973). Effect of moisture on growth rate and development of two strains of *Tenebrio molitor* L. (Coleoptera, Tenebrionidae). *Journal of Stored Products Research*, 8: 291–297.

Van Broekhoven S, Mota-Gutiérrez J, De Rijk TC, & De Nijs M. (2017). Degradation and excretion of the *Fusarium* toxin deoxynivalenol by an edible insect, the yellow mealworm (*Tenebrio molitor* L.). *World Mycotoxin Journal*, 10: 1–8.

Van Huis A, Oonincx DGAB, Rojo S, & Tomberlin J. (2020). Insects as feed: house fly or black soldier fly? *Journal of Insects as Food and Feed*, 6: 221─229.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe, & Speleman F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology, 8: 532─542.

Valentini A, Miquel C, Nawaz MA, Bellemain E, Coissac E, Pompanon F, Gielly L, Cruaud C, Nascetti G, Wincker P, Swenson JE, & Taberlet P. (2009). New perspectives

in diet analysis based on DNA barcoding and parallel pyrosequencing: the trnL approach. *Molecular Ecology Resources*, 9: 51─60.

Vallejo RM, Guerrero JA, & González-Cózatl FX. (2017). Patterns of differentiation and disparity in cranial morphology in rodent species of the genus *Megadontomys* (Rodentia: Cricetidae). *Zoological Studies,* 56: 14.

Vasconcelos SD, & Araujo MCS. (2012). Necrophagous species of Diptera and Coleoptera in northeaster Brazil: state of the art and challenges for the Forensic Entomologist. *Revista Brasileira de Entomologia*, 56: 7–14.

Velásquez Y, Magaña C, Martínez-Sánchez A, & Rojo S. (2010). Diptera of forensic importance in the Iberian Peninsula: larval identification key. *Medical and Veterinary Entomology,* 24: 293–308.

Veldkamp T, Van Duinkerken G, Van Huis A, Lakemond CMM, Ottevanger E, Bosch G, & Van Boekel MAJS. (2012). Insects as a sustainable feed ingredient in pig and poultry diets-a feasibility study. *Lelystad: Wageningen UR Livestock Research,* Report 638.

Verlinden H. (2018). Dopamine signalling in locusts and other insects. *Insect Biochemistry and Molecular Biology*, 97: 40–52

Viñeta-Valdelvira J. (2017). Diseño y acondicionamiento de una nave para la producción de insectos como piensos alimentarios. Trabajo Fin de Máster. Universitat Jaume I, España.

Wang Y, Li LL, Wang JF, Wang M, Yang LJ, Tao LY, Zhang YN, Hou YD, & Hou ZL. (2016). Development of the green bottle fly *Lucilia illustris* at constant temperatures. *Forensic Science International,* 267: 136–144.

Wang Y, Wang JF, Zhang YN, Tao LY, & Wang M. (2017). Forensically important *Boettcherisca peregrima* (Diptera: Sarcophagidae) in China: development pattern and significance for estimating post-mortem interval. *Journal of Medical Entomology*, 54: 1491–1497.

Wang Y, Zhang YN, Liu C, Hu GL, Wang M, Yang LJ, Chu J, & Wang F. (2018). Development of *Aldrichina grahami* (Diptera: Calliphoridae) at constant temperatures. *Journal of Medical Entomology*. doi: 10.1093/jme/tjy128

Wang M, Wang Y, Hu G, Wang Y, Xu W, Wu M, & Wang J. (2020). Development of *Lucilia sericata* (Diptera: Calliphoridae) under constant temperatures and its significance for the estimation of time of death. *Journal of Medical Entomology*. doi: 10.1093/jme/tjaa046

Weaver DK, & McFarlane JE. (1990). The effect of larval density on growth and development of *Tenebrio molitor*. *Journal of Insect Physiology*, 36: 531.

Webster MM, & Sheets HD. (2017). *Practical Introduction to Landmark-Based Geometric Morphometrics*. Cambridge University Press.

White TCR. (1976). Weather, food and plagues of locusts. *Oecologia*, 22: 119─134

Wiegmann BM, Trautwein MD, Winkler IS, Barr NB, Kim J-W, Lambkin C, Bertone MA, Cassel BK, Bayless KM, Heimberg AM, Wheeler BM, Peterson KJ, Pape T, Sinclair J, Skevington JH, Blagoderov V, Caravas J, Kutty SN, Shmidt-Ott U, Kampmeier GE, Thompson FC, Grimaldi DA, Beckenbach AT, Courtney GW, Friedrich M, Meier R, & Yeates D. (2001). Episodic radiations in the fly tree of life. *Proceedings of the National Academy of Sciences*, 108: 5690–5695

Wink-da-Silva R, Diniz MMCSL, Morrelli MT, & Wilke ABB. (2018). Wing morphometric variability in *Aedes aegypti* (Diptera: Culicidae) from different urban built environments. *Parasites and Vectors*, 11: 1–9.

Wong JY, Chan KYK, & Chan BKK. (2018). Phylogenetic, ecological and biomechanical constraints on larval form: A comparative morphological analysis of barnacle nauplii. *PLOS ONE*, 13: e0206973.

Wu RA, Ding Q, Lu H, Tan H, Sun N, Wang K, He R, Luo L, Ma H, & Li Z. (2020). Caspase 3-mediated cytotoxicity of mealworm larvae (*Tenebrio molitor*) oil extract against human hepatocellular carcinoma and colorectal adenocarcinoma. *Journal of Ethnopharmacology*, 250: 112438.

Yang L, Wang Y, Li L, Wang J, Wang M, Zhang Y, Chu J, Liu K, Hou Y, & Tao L. (2017). Temperature-depend development of *Parasarcophaga similis* (Meade, 1876) and its significance in estimating post-mortem interval. *Journal of Forensic Science*, 62: 1234–1243.

Yanmanee S, Husemann M, Benbow ME, & Suwannapong G. (2016). Larval development rates of *Chrysomya rufifaces* Macquart, 1842 (Diptera: Calliphoridae) within its native range in South-East Asia. *Forensic Science International*, 266: 63–67.

Yeo D, Srivathsan A, & Meier R. (2020). Longer is not always better: optimizing barcode length for large-scale species discovery and identification. *Systematic Biology*. doi: 10.1093/sysbio/syaa014

Yi DA, Kuprin AV, & Bae YJ. (2017). Morphological anomalies of endangered Korean relict long-horned beetle *Callipogon relictus* (Cerambycidae, Coleoptera) during ontogenesis and possible causes of their ocurrence. *ZooKeys*, 714: 53–60.

Yusseff-Vanegas SZ, & Agnarsson I. (2017). DNA-barcoding of forensically important blow flies (Diptera: Calliphoridae) in the Caribbean Region. *PeerJ*, 5: e3516.

Zhang Y, Wang Y, Sun J, Hu G, Wang M, Amendt J, & Wang J. (2019). Temperature-dependent development of the blow fly *Chrysomya pinguis* and its significance in estimating post-mortem interval. *The Royal Society*, 6: 190003.

Zehner R, Amendt J, Schütt S, Sauer J, Krettek R, & Povoln D. (2004). Genetic identification of forensically important flesh flies (Diptera: Sarcophagidae). *International Journal of Legal Medicine,* 118: 245─247.

Zeikus RD, & Steinhaus EA. (1968). Teratology of the beetle *Tenebrio molitor.* II. The development and gross description of the pupal-winged adult. *Journal of Invertebrate Pathology*, 11: 8─24

Zelditch ML, Swiderski DL, Sheets HD, & Kink WL. (2004). *Geometric Morphometrics for Biologists*. ISBN: 978-0-12-778460-1

Appendix

Supplementary Table 1: GenBank code, species, location of sampling and date of sampling. * The sequences at the end of the table that have an asterisk are those incorporated from the NCBI database.

GenBank Code	Species	Location of Sampling	Data of Sampling
MT527094	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527095	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527096	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527097	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527098	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527099	*F. pusio*	Elda (Valencian Community, Spain)	22-jul-12
MT527100	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527101	*F. pusio*	Catarroja (Valencian Community, Spain)	19-jul-14
MT527102	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527103	*F. pusio*	Aguilas (Region of Murcia, Spain)	08-sep-12
MT527104	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527105	*F. aequilineata*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527106	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527107	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527108	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527109	*F. canicularis*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527110	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527111	*F. pusio*	Villanueva de Cameros (La Rioja, Spain)	23-jun-12
MT527112	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527113	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527114	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527115	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527117	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12

MT527118	*F. aequilineata*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527119	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527120	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527121	*F. lepida*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527122	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527123	*F. canicularis*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527124	*F. leucosticta*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527125	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527126	*F. leucosticta*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527127	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527128	*F. leucosticta*	Elda (Valencian Community, Spain)	22-jul-12
MT527129	*F. pusio*	Catarroja (Valencian Community, Spain)	19-jul-14
MT527130	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527131	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527132	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527133	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527134	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527135	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527136	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527137	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527138	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527139	*F. canicularis*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527140	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527141	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527142	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527143	*F. canicularis*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527144	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527145	*F. leucosticta*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527146	*F. pusio*	Villanueva de Cameros (La Rioja, Spain)	23-jun-12

MT527147	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527148	*F. pusio*	Aguilas (Region of Murcia, Spain)	08-sep-12
MT527149	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527150	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527151	*F. pusio*	Aguilas (Region of Murcia, Spain)	08-sep-12
MT527152	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527154	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527155	*F. pusio*	Catarroja (Valencian Community, Spain)	19-jul-14
MT527156	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527157	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527158	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527159	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527160	*F. lepida*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527161	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527162	*F. pusio*	Villanueva de Cameros (La Rioja, Spain)	23-jun-12
MT527163	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527164	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527166	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527167	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527168	*F. canicularis*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527169	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527170	*F. pusio*	Campus of Espinardo (Region of Murcia, Spain)	04-sep-12
MT527171	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527172	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527173	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
MT527174	*F. pusio*	Estremadura (Lisbon, Portugal)	28-sep-15
KY511155*	*F. aequilineata*	Plock (Poland)	28-may-16
HQ979164*	*F. canicularis*	Massachusetts (USA)	30-sep-10
KY511183*	*F. lepida*	Las Piwnicki (Torun, Poland)	28-sep-14

KY511189*	*F. leucosticta*	Vairao (Porto, Portugal)	07-oct-15
JX438031*	*F. pusio*	Campo Grande (Lisbon, Portugal)	04-aug-11
KY511210*	*F. pusio*	Snook (Brazos Co., Texas, USA)	Jul-14