# Unveiling the Ecosystem Services of Insectivorous Bats through a Comprehensive Approach: Trophic Ecology, Methodology and Study Design

# *Saguzar Intsektiboroen Zerbitzu Ekosistemikoak argitara ekartzeko Hurbilketa Integrala: Ekologia Trofikoa, Metodologia eta Ikerketa Diseinua*

Unai Baroja Ibáñez de Maeztu

PhD thesis

Leioa, 2021

eman ta zabal zazu

Universidad del País Vasco

Euskal Herriko Unibertsitatea

*Cover photo made by Adrià López-Baucells and Oriol Massana.*

*Back cover photo by Jose Mena*

Maiteri

*“Gerturatu ahala konturatzen gara zeinen urrun gauden errealitatetik”*

# Unveiling the Ecosystem Services of Insectivorous Bats through a comprehensive approach:
## Trophic Ecology, Methodology and Study Design

# Saguzar Intsektiboroen Zerbitzu Ekosistemikoak argitara ekartzeko hurbilketa integrala:
## Ekologia Trofikoa, Metodologia eta Ikerketa Diseinua

A thesis submitted by Unai Baroja Ibáñez de Maeztu to the University of the Basque Country for the degree of Doctor of Philosophy, under the supervision of Dr. Inazio Garin Atorrasagasti.

Leioa, Basque Country, 2021

eman ta zabal zazu

Universidad del País Vasco
Euskal Herriko Unibertsitatea

# Edukien taula-Table of contents

## Argitalpenak - Publications

Article 1.

Baroja, U., Garin, I., Aihartza, J., Arrizabalaga-Escudero, A., Vallejo, N., Aldasoro, M., & Goiti, U. (2019). Pest consumption in a vineyard system by the lesser horseshoe bat (*Rhinolophus hipposideros*). *PLoS One*, 14(7), e0219265.

Article 2.

Baroja, U., Garin, I., Vallejo, N., Aihartza, J., Rebelo, H., & Goiti, U. (2021). Bats actively track and prey on grape pest populations. *Ecological Indicators*, 126, 107718.

Article 3.

Baroja, U., Garin, I., Vallejo, N., Caro, A., Ibáñez, C., Basso, A., Goiti, U. Molecular assays to reliably detect and quantify predation on a forest pest in bats faeces. *Scientific Reports. Under review.*

## Kolaborazioak – Collaborations

Aldasoro, M., Garin, I., Vallejo, N., Baroja, U., Arrizabalaga-Escudero, A., Goiti, U., & Aihartza, J. (2019). Gaining ecological insight on dietary allocation among horseshoe bats through molecular primer combination. *PLoS One*, 14(7), e0220081.

Vallejo, N., Aihartza, J., Goiti, U., Arrizabalaga-Escudero, A., Flaquer, C., Puig, X., ... & Garin, I. (2019). The diet of the notch-eared bat (*Myotis emarginatus*) across the Iberian Peninsula analysed by amplicon metabarcoding. *Hystrix, the Italian Journal of Mammalogy*, 30(1), 59-64.

## Kongresuak – Conferences

Baroja, U., Garin I., Goiti, U. & Aihartza J. (2016). *Respuesta de la actividad de los quirópteros a los cambios de la polilla de la vid (lobesia botrana) en dos viñedos del norte de la península ibérica: estudio preliminar.* SECEMU IV, Vairão [poster].

Baroja, U., Garin I., Goiti, U. & Aihartza J. (2017). *Response of bat activity to changes in a vineyard moth pest*. 14th EBRS, Donostia [poster].

Baroja, U., Garin I., Goiti, U. & Aihartza J. (2018). *Bats take advantage of numerous pest arthropods in vineyard systems: a metabarcoding approach*. IBAGRECO I, Évora. [oral communication].

Baroja, U., Garin I., Goiti, U. & Aihartza J. (2018). *Bakar bat askoren ehizan*. Natur Zientzien III. Topaketa, Gasteiz (awarded for the best poster) [poster].

Baroja, U., Garin I., Vallejo N., Aldasoro M., Goiti, U. & Aihartza J. (2019). *Saguzarren izurriekiko zaletasuna mahasti sistema batean: kontrol biologikorako oinarrizko hastapenak*. IkerGazte Ikertzaile Euskaldunen Kongresua, Baiona [oral communication].

Baroja, U., Garin I., Vallejo N., Aldasoro M., Goiti, U. & Aihartza J. (2019). *Pest Consumption in a Vineyard System by the lesser horseshoe bat*. 18th IBRC, Phuket [oral communication].

Baroja, U., Garin I., Vallejo N., Aldasoro M., Goiti, U. & Aihartza J. (2019). *Let's play tag! Bats actively track and prey on grape pest populations*. 15th EBRS, Turku [oral communication].

Baroja, U., Garin I., Vallejo N., Aldasoro M., Goiti, U. & Aihartza J. (2019). *Rapid, robust and reliable: a molecular assay for the quantification of the pine processionary moth in bat faeces*. 15th EBRS, Turku [poster].

# Dibulgazioa-Scientific dissemination

## Artikuluak – Articles

Baroja, U. (2019). "Zer dago afaltzeko?" Izurriak saguzarren menuan. *Elhuyar Zientzia eta Teknologia*, 335, 64-68.

## Irratia – Radio

Hiri Gorrian (Euskadi Irratia) – 2018/10/31

Mezularia (Euskadi Irratia) – 2019/10/25

Lau Haizetara (Bizkaia Irratia) – 2019/10/30

Egunon Bizkaia Magazine (Radio Popular) - 2019/10/30

La Casa de la Palabra (Radio Euskadi) - 2019/11/08

# Esker onak – Acknowledgments

Tesia bizitzako sentipen gazi-gozoz eta gorabeherez jositako garaia izan da, erabat. Hala ere, asko izan dira bidean lagundu didatenak, animatu, lagundu, sinistu eta baita lana konstruktiboki kritikatu dutenak ere, zuei, denei, eskerrik asko.

Hasteko, eskerrak eman nahiko nizkioke nire zuzendari izan den Inaziori, urte hauetan zehar irakatsitakoagatik eta ezin ahaztu estatistika eta modelizazioan erakutsitako grina, never give up!. Noski, Joxerra eta Urtziri landa lanean erakutsitakoagatik eta eskatu izan dudanean eskeinitako laguntzagatik. Ezin ahaztu lan taldeko *saguzarrologo* guztiak, gaur egun jarraitzen dutenak eta jada ez daudenak, eskerrik asko Lide, Aitor, Ane, Nerea eta Mireni, baita zuri ere Amaiur, atsedenaldiak eta bazkalorduak atseginagoak egiteagatik eta momentu latz horietan lasaitzeagatik. Eskerrik asko laginketetan etorritako guztiei, Ibarra, Itxaso, Egoitz (batez ere 7 orduz gidatzen egon eta ez lokartzeagatik) eta arrebari ere. Ana Diez Navajas esker mila izurrien bilketan egindako lanagatik. Gracias Elena por transmitirme toda tu pasión hacia el estudio de los quirópteros y por todos aquellos muestreos y “Bat Nights”.

Ezin ahaztu saguzarren gorotzak biltzea posible egin duten guztiak, eskerrik asko zuei ere, saguzar koloniak zeuden lekuetara sartzen uzteagatik, zuen parte-hartzea eta aldartea garrantzitsua izan da prozesu honetan. Gracias Carlos por enviarme tantas muestras, y a ti Juanele, por aquellas conversaciones sobre metabarcoding por e-mail, todavía no hemos tenido ocasión de hablar en condiciones…

Genetikan adituei, Irati eta Irantzuri, eskerrak eman nahiko nizkizueke zuen ezagutza transmititzeagatik, gomendioengatik, inbertitu duzuen denboragatik eta egindako lan guztiagatik. Amaia milesker egindako lan finagatik eta laginketetan pasa ditugun une horiengatik. Thank you to Andrea Basso for your advice on primer designing and your

collaboration. Dar gracias a Gonzalo por su colaboración y sus clases magistrales de estadística.

Gostaria de agradecer a Hugo e Vanessa por tudo o que pude aprender convosco durante a minha estadia no Porto e, claro, por me terem convidado para os vossos jantares, muito obrigado! Muchas gracias a Adrià Lopez-Baucells, Oriol Massana y Jose Mena por dejarme utilizar vuestras fotos en la tesis.

Eskerrik asko lagunei, lana, lana dela gogoratzeagatik eta eskainitako uneengatik, indarrak emateagatik eta trago batzuekin gauzak beste ikuspuntu bat dutela gogoratzeagatik. Zuek barik zail samarra izango litzateke.

Nola ez, familiari, eskainitako laguntzagatik eta sinisten laguntzeagatik, benetan eskerrik asko.

Azkenik, Maiteri. Urte hauetan guztietan zehar eman didazunagatik, egunerokotasunean bidea errazteagatik, bizi izan dugunagatik eta bizitzeke geratzen zaigunagatik.

*I am grateful to the following organizations which have funded my thesis: the Spanish Ministry of Economy and Competitiveness and the European Regional Development Fund (CGL-2015-69069P), as well as the Government of the Basque Country (IT754-13 and IT1163-19). This thesis was supported by a predoctoral grant of the Basque Government UB (PRE_2016_2_0186)*

## Laburpena

Artropodo fitofagoek nekazal ekoizpenean kalte oso larriak eragiten dituzte urtero eta izurrite hauen kontra modu jasangarrian aurre egiteko bideen artean eragile biologikoak proposatu izan dira. Saguzar intsektiboroak, haien dieta ohiturak direla eta, artropodoen harrapakari amorratuak dira, gau bakar batean beraien gorputz masa bezainbeste intsektu jan ditzaketelarik. Oraintsu arte ordea, ezinezkoa izan zaigu saguzarren dieta osatzen duten espezieen zerrenda ezagutzea, baina, azken hamarkadan teknika molekularren aurrerapenei esker, saguzarrek izurriteen dinamikan eduki dezaketen garrantzia agerian geratu da. Hala ere, badira oraindik ere bete beharreko hutsune ugari.

Tesi honetan saguzar eta izurrien arteko elkarrekintzak aztertu dira, horretarako hurbilketa integral bat burutu delarik: saguzarren ekologia trofikoa, izurrien sakabanaketa-ereduak, modelizazio espazio-tenporala eta izurrien detekzio eta kuantifikaziorako metodologia molekular ezberdinen konparaketa burutu da. Testuinguru honetan, tesiaren helburu orokorra izan da saguzar-intsektiboroek mahastietan eskaintzen dituzten zerbitzu-ekosistemikoak hobeto ulertzea eta teknika molekular ezberdinek saguzarren gorotzetan izurrien jarraipenerako duten eginkizuna aztertzea. Horretarako, hurrengo galderei erantzuten saiatu gara: batetik, zein den saguzarrek izurrien erregulatzaile gisa duten ahalmena eta zein izurri-espezie kontsumitzen dituzten; ea saguzarrek mahastietako izurrien populazioak jarraitu eta ustiatzen dituzten; saguzarrek denboran zehar ematen diren izurrien gorabeherekiko erantzuten duten; zein metodo molekular den egokiagoa helburu diren izurrien detekziorako, metabarcoding edo PCR metodoak eta azkenik, qPCR-a metodo aski sendoa eta fidagarria ote den pinu-beldarra *Thaumetopoea pityocampa* saguzarren gorotzetan kuantifikatzeko. Oro har, emaitzek aditzera ematen dute saguzarrek kultibo oso ezberdinetako izurriak kontsumitzeko ahalmena

dutela. Horrez gain, izurrien garai oparoenetan saguzarren aktibitatea altuena izateaz gain, izurrien agerpen maiztasunak saguzarren dietan ere garaienak izan ziren, nahiz eta espezie guztiek ez zuten maila berean jokatu. Gainera, bazka-gremio ezberdinetako saguzarrek positiboki erantzun zuten izurrien gorabeherekiko, saguzar-espezie ezberdinek, sinergikoki jokatuz, izurriak aire-espazioko maila ezberdinetan ustiatzeko ahalmena dutela jakinaraziz. Azkenik, hurbilketa molekularraren hautaketa (metabarcoding, cPCR edo qPCR) ikerketa-galderaren eta metodo bakoitzak dituen abantaila eta desabantailen arabera erabaki behar dela ondoriozta dezakegu.

## Summary

Herbivorous arthropods cause immense damage in crop production annually, and the use of biological agents has been lately proposed to counteract their adverse effects in a sustainable way. Owing to their dietary habits, insectivorous bats are considered among the most voracious suppressors of arthropod pests, in fact daily consumption of arthropods can reach values around 100% of the bat body mass amounting to thousands of insects. The advent of innovative molecular techniques in the last decade has revealed the importance of bats in pest dynamics. Yet, there are still a number of knowledge gaps.

In this thesis I studied the interaction between bats and pests, for which a multidisciplinary approach was carried out, including trophic ecology of bats, distribution patterns of pests, spatio-temporal modeling and the development and comparison of different molecular assays for pest detection and quantification purposes. In this context, the overall aim of the thesis was to better understand the ecosystem services of bats as pest suppressors and to empirically evaluate the performance of different molecular techniques (metabarcoding, cPCR and qPCR) for pest monitoring in bats' faeces. We therefore tried to answer the following research questions: which is the potential of bats as pest regulators and what pest species they consume? do bats track and exploit pest populations? do bats respond to pest abundances across the season? which molecular method is more suitable for target pest detection? Metabarcoding or PCR methods, and finally, is qPCR a robust and reliable method to quantify the pine processionary moth *Thaumetopoea pityocampa* in bats faeces?

Overall, the results imply that bats act as a suppressor of a wide array of agricultural pests. Further, during pest major irruptions, we found the highest bat activity levels and frequencies of grape pests in the diet of bats,

although not all bat species contributed equally to pest suppression. Bats of different foraging guilds positively responded to pest abundances, indicating distinct bat species may synergistically play a role at suppressing agricultural pests at broad scales of the aerospace.

Last but not least, deciding on which molecular approach to use (metabarcoding, cPCR or qPCR) depends on the research question and the advantages and disadvantages associated with each assay.

# Sarrera orokorra

## Izurriak eszenatoki Antropogenikoan: Mundu mailako kezka

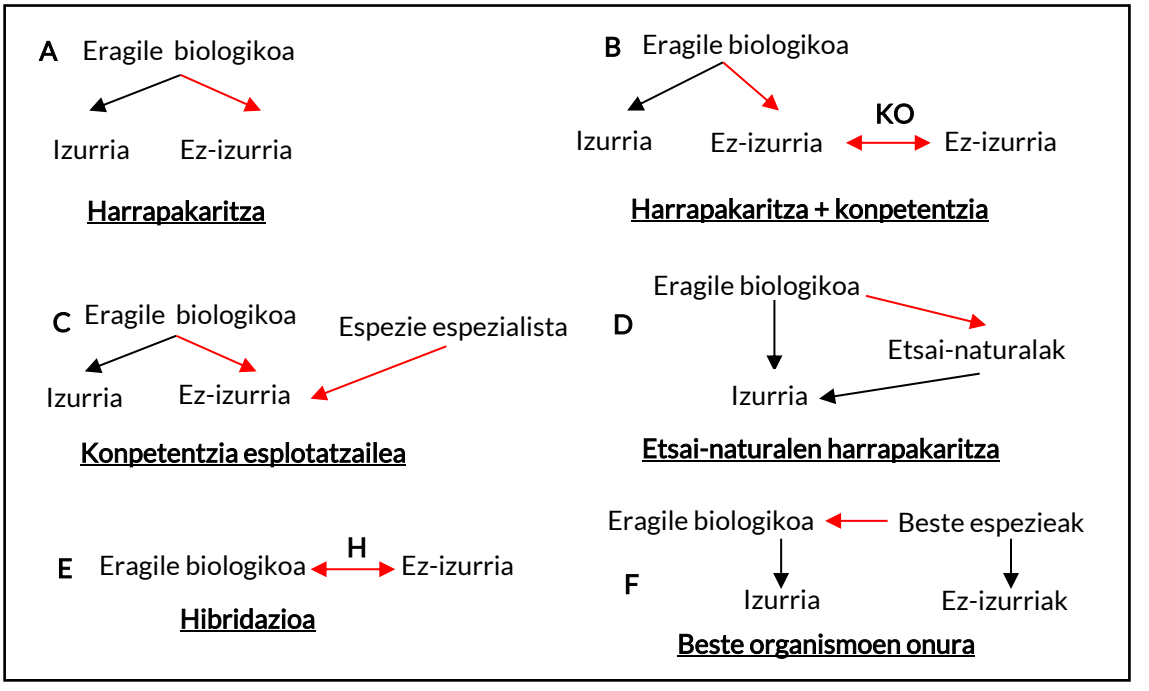
Gain-populazioak, elikagaien segurtasun ezak eta nekazaritza intentsiborako joerak bortizki eragiten dute mundu-mailako iraunkortasunaren lorpenean. Azken urteetako biztanleria-hazkundearen eta baliabideen gehiegizko kontsumoaren ondorioz, elikaduren produkzio-eskariei aurre egitea erronka handia bihurtu da. 2050 urterako, mundu-mailako giza biztanleria 10 mila milioi pertsonara iritsiko dela estimatzen da (UNDESA, 2017), eta horrek erronka gehigarriak ekarriko ditu: besteen artean, uzta emendatzeko zailtasunak (%60-110ko hazkuntza 2050erako, Pardey ***et al.***, 2014), era berean epe luzerako jasangarritasuna eta elikadura-segurtasuna bermatuz. Gaur egungo elikaduraren eskari handiak bat egin behar du ingurumen-inpaktuko politikekin; izan ere, oraindik nekazaritza intentsiboari eutsiz, gero eta habitat natural gehiago suntsitzen ari dira nekazal eremuak esanguratsuki emendatuz, horrek dakartzan paisaiaren sinplifikazioari erreparatu barik. Halaber, nekazaritzako izurrite kontsideratzen diren intsektu askok gogor astintzen dituzte munduko toki ezberdinetako laborantzak, jasotako uzta gutxituz, produkzio-kostuak emendatuz eta elikaduraren segurtasuna kolokan jarriz (Paini eta kideak, 2016; Savary eta kideak, 2019). Artropodoek bakarrik, urtero 550 bilioi dolar inguruko galera eragiten dute nekazaritzan (Paini eta kideak, 2016), baina badirudi, klimaren-aldaketaren ondorioz, kalteak are gehiago okertzeko joera izango dutela (Deutsch eta kideak, 2018). Oro har, tenperaturen igoeren ondorioz, handitu egiten da izurrite diren artropodoen tasa metabolikoa (hau da, elikatze tasaren emendioa), eta baita populazio-hazkundea ere. Hori horrela, luzatu ohi da izurrien bizi zikloaren fase aktiboa, urteko belaunaldi kopurua emendatuz eta nekazaritza-kalteak areagotuz (Dillon eta kideak, 2010; Forrest, 2016). Hori gutxi balitz, globalizazioak biziagotu egiten du espezie inbaditzaileen mundu-mailako hedapena eta nekazal-gune berrien kolonizazioa (Perrings eta kideak, 2005). Ikusita datozen urteotan izurrien presioak eta kostu ekonomikoek gora egingo dutela, baita, historikoki erabilitako ohiko kontrol-metodoek ingurugiroan duten eragina kontuan hartuta (neurri fisiko, kimiko eta biologikoen bidez, Thacker, 2002), nekazariek konponbide iraunkor eta eraginkorragoak bilatzera ekin diote; adibidez, eragile biologikoek izurrien ustiatzaile gisa eskaintzen dituzten zerbitzu ekosistemikoez baliatuz.

## Kontrol biologikoa: Epe luzerako estrategia jasangarrien bila

*"Organismo bizidunen erabilera izurri zehatzen populazio dentsitatean eragin edo suntsitzeko, haren kalteak kultiboetan murrizteko"* (Eilenberg eta kideak, 2001) definizioz ezaguna, kontrol biologikoa izurrien kontrako borrokan balio handiko alternatiba eraginkortzat jotzen da, kontuan izanik datozen urteotan "nekazaritza jasangarriari" bidea zabaldu behar diogula. Metodo biologikoak, gero eta indar handiagoa ari dira hartzen, hurrengo arrazoiak direla medio: 1) organismo inbaditzaileak hiltzeko duten ahalmena, 2) ingurumenarekin arduratsua den kontrol sistema bat da, 3) ezarpen kostuak ez dira altuak izaten, eta 4) kontsumitzaile askoren jarrera aldatzen ari da, pestizidarik erabiltzen ez duen nekazal-sistema baten alde apustu eginez (van Lenteren eta kideak, 2018). Kontrol biologikoarekin lotutako hainbat metodo ezberdin erabili izan dira; hauen artean, etsai-natural ez natiboen sarrera (**ENEN**, kontrol biologiko klasikoa), etsai-natural natiboen (**ENN**) populazioen kontserbazioa beraien bizi baldintzak hobetuz (kontserbazio bidezko kontrol biologikoa, **KBK**), biopestiziden ekoizpena (hau da, izurrien kontra borrokatzeko patogenoz osatutako produktu ez-kimikoak), edota intsektuekiko iraunkorrak diren laboreen landaketa transgenikoak aurki ditzakegu (Van Driesche eta Hoddle, 2009). Kontrol biologikorako bide horiek, oraindik gutxi erabiliak diren arren, berebiziko garrantzia izan dute historian zehar, baina, gaur egun guztiz beharrezkoak bilakatu dira ingurumenarekin bat egiten dutelako.

Tradizionalki, kontrol biologiko klasikoan oinarritutako metodoak sarri erabiltzen ziren izurriteekin akabatu edo hauek dentsitate maila baxuetan mantentzeko. **ENEN**-en sarrera masiboek ordea, hondamendi ekologiko larriak sor ditzake (begiratu berrikuspena, Hajek eta kideak, 2016). Zentzu honetan, eragile biologiko asko izurrien kontra oso eraginkorrak badira ere, dituzten albo-ondorioak kontuan hartu behar dira nahitaez. Adibidez, harrapakin anitzez baliatzen diren **ENEN**-ak helburu diren espezieez gain (izurrite diren intsektuak adibidez), ekologikoki garrantzia duten beste organismo ugari ustiatu ditzakete, eta ondorioz, espezie inbaditzaile bihurtu. Horrekin lotuta, Holt eta Hochberg (2001) eta Van Driesche eta Hoddle (2016) ezagutarazi zituzten izurrien kontrolerako erabiltzen diren eragile biologiko ezberdinek eduki ditzaketen efektu ekologiko larriak: besteak beste, izurrite ez diren beste organismoengan eragin zuzena izan ditzakete, hauek ehizatu

edota baliabide ezberdinekiko konpetentzia sortuz, konpetentzia esplotatzailea sortuz, beste etsai-natural potentzialak ehizatuz, edota espezie natiboekin hibridatuz (1. irudia).




**1. irudia.** Kontrol biologiko klasikoaren albo efektu posibleak izurriak ez diren gainerako organismoengan. Geziek harrapakaritza adierazten dute eta alde-biko geziek ordea, konpetentzia (KO) edo hibridazioa (H). Gezi gorriek izurriak ez diren gainerako organismoetan eragindako zeharkako efektuak adierazten dituzte.

Hori dela eta, teknika hauek ez dira gomendagarriak eta gutxitan erabili ohi dira gaur egun (baina begiratu, adibidez, Ferracini eta kideak, 2019). Bestalde, mikroorganismoen bidezko pestiziden erabileran oinarritutako biokontrolerako estrategiak arras eraginkorrak, izurri zehatzekiko espezifikoagoak eta zeharo merkeagoak dira intsektizida kimikoen aurrean (Van Driesche eta Hoddle, 2009). Azken hamarkadetan, *Bacillus thuringiensis* (**Bt**) bakteriotik, hainbat birusetatik eta onddoetatik eratorritako toxinen erabilera (biopestizidak) sustatu egin da alternatiba jasangarri gisa, izurrien populazioak hil edo kontrolatzeko duten ahalmena dela eta (1. taula). Hala ere, biopestiziden erabilera ez da %5era iristen izurrien kontra saldutako produktuen artean (Olson, 2015). Gainera, teknologia

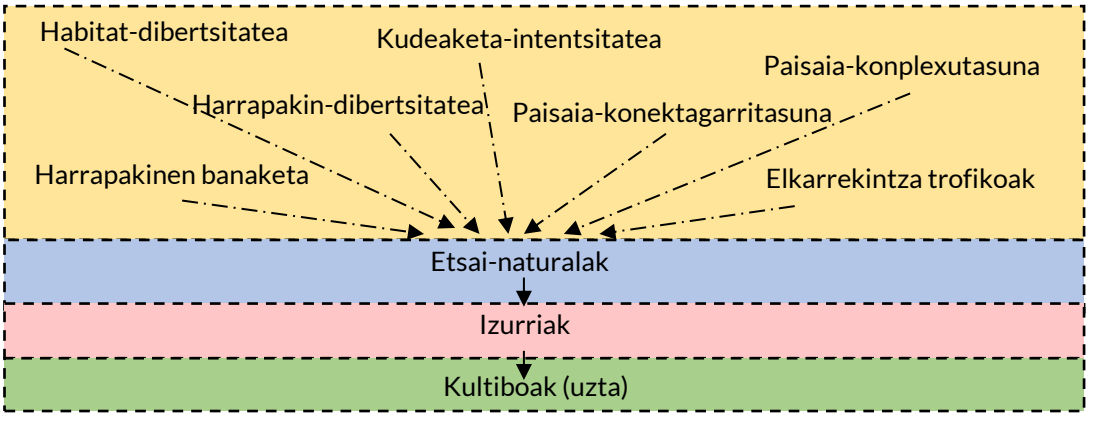
molekularrak, gene zehatzen adierazpena aldatuz, baimentzen du Bt-dun laboreak ekoiztea, eta ondorioz, izurriekiko babesa emendatzen da (adibidez, Lu eta kideak, 2012). Dena den, azken hamarkadan zenbait izurritek transgeniko hauekiko erresistentzia garatu dute (Tabashnik eta kideak, 2013) eta beraz, haien bideragarritasuna bertan behera geratu da.

**1. taula.** Biopestizida gisa erabilitako mikroorganismo ohikoenetarikoak *Kabaluk eta kideak (2010)*-en arabera.

| Biopestizidarako espeziea | Kaltetutako izurriak |
|---|---|
| **Bakterioak** | |
| *Bacillus thuringiensis* subsp. *kurstaki* | Lepidopteroen larba |
| *B. thuringiensis* subsp. *aizawai* | Lepidopteroen larba |
| *B. thuringiensis* subsp. *galleriae* | Lepidopteroen larba |
| *B. thuringiensis* subsp. *tenebrionis* | Koleopteroen larba |
| *B. thuringiensis* subsp. *israelensis* | Eltxo eta simulidoak |
| *B. sphaericus* | Eltxoen larbak |
| *B. moritai* | Dipteroak |
| **Birusak** | |
| Granuloviruses | Lepidopteroen larba |
| Nucleopolyhedroviruses | Lepidopteroen larba |
| **Onddoak** | |
| *Beauveria bassiana* | Euli zuriak, afidoak, trips-ak |
| *Lecanicillium muscarium* | Euli zuriak, afidoak, trips-ak |
| *Paecilomyces fumosoroseus* | Euli zuriak |

Azkenik, KBK epe luzerako hurbilketa integrala da. Neurri ezberdinen bidez nekazal sistemak berregituratu egiten dira, izurrien kontrolerako onuragarriak izan daitezkeen bertako harrapakari, parasitoide edota organismo patogenoen habitaten egoera esanguratsuki hobetuz (Van Driesche eta Hoddle, 2009). Oro har, nekazal sistemetako izurrien etsai-naturalen kopurua eta dibertsitatea emendatu egiten da inguruko habitat heterogeneotasuna emendatuz, nekazaritzako

kudeaketa intentsiboa murriztuz, edo paisaia-egitura konplexuetan (Begg eta kideak, 2017). Hala ere, badira KBK-rekin harreman estua duten beste hainbat faktore; besteak beste, ENN eta harrapakinen banaketa, gordeleku potentzialen eskuragarritasuna, habitat ezberdinen konektagarritasuna, elkarrekintza trofikoak, edo paisaiaren eragina. Guztiak dira bere horretan eskala anitzeko hurbilketa honen parte garrantzitsu (Veres eta kideak, 2013; Chailleux eta kideak, 2014; Schellhorn eta kideak, 2014) (2. irudia).




**2. irudia.** Kontserbazio bidezko kontrol biologikorako (KBK) kontuan hartu beharreko hainbat eragile ekologiko.

Aurretik aipatutako biokontrolerako estrategiek, modu isolatuan erabilita, ez dute nahikoa presio ezartzen izurrien populazioak maila jasangarrietan mantentzeko. Hori horrela, gero eta gehiago dira estrategia ezberdinen konbinazioez baliatzen direnak izurrien kalteak baretzeko. Adibide gisa, etsai-naturalez baliatuz, izurriak ustiatzen dituzten harrapakari natibo anitzen ekintza sinergikoa (adibidez, KBK sustatuz), eta noizbehinkako biopestiziden erabilera proposatu izan da (Torres eta Bueno, 2018) (3. irudia). Harrapakin-dentsitatearen menpeko harrapakariak oso preziatuak dira nekazaritzan, izan ere, harrapakin-ugaritasunaren arabera erantzuten dute (adibidez, izurrien eztanda masiboak), kasu batzuetan izurrien populazioak maila jasangarrietan mantentzeko gai direlarik. Hauen artean, harrapakari jeneralistak aurki daitezke.

Urte osoan zehar nekazal sistemetan modu iraunkorrean bizi diren harrapakari jeneralistak biokontrolatzaile oso eraginkorrak izan daitezke, besteak beste, beraien

izaera trofiko oportunistak beste harrapakin mota ugari ustiatzea baimentzen baitie izurrien eskasia garaietan (Ehler, 1998; Symondson eta kideak, 2002). Bestalde, izurrien estai-naturalek eraginkorrak izateko zenbait ezaugarri ezinbestekoak dira: 1) dispertsio gaitasun eta eremu berrien kolonizaziorako abilezia izurrien inbasioei aurre egiteko (Ehler eta Miller, 1978); 2) bazka-jokabide oportunista izurrien bat-bateko dentsitate-gorabeherei erantzuteko, eta 3) izurri-eskuragarritasunaren araberako erantzun positiboa (Symondson eta kideak, 2002). Hori horrela, zenbait saguzar intsektiboroen ezaugarri ekologikoek bat egiten dute goian aipatutakoekin eta, ondorioz, azken urteetan ikertzaile askok arreta ugaztun hegalari hauengan jarri dute.


**3. irudia.** Nekazal-sistema jasangarriaren eredua, KBK estrategian eta biopestizida bidezko ihinztapen selektiboan oinarrituz.

## Saguzar-Intsektiboroen Bazka-Ekologia eta Balio Ekonomikoa

Saguzar intsektiboroek (ezagutzen diren espezieen % 70 artropodoez elikatzen da, 980 espezie inguru, Simmons eta Cirranello, 2020) ekokokapena eta hegan modu aktiboan egiteko gaitasuna garatu dituzte eboluzioan zehar. Ezaugarri hauek ezinbestekoak izan dira gaueko bizimodura moldatu eta bertako intsektu talde ugariz baliatzeko (Jones eta Rydell, 2003). Are gehiago, saguzar intsektiboroen dibertsitate ekologiko altua zeharo lotuta dago saguzarren ekokokapen eta hegakerarekin erlazionatutako jokamolde, ezaugarri morfologiko eta sentsorialekin: hegakera-azkarreko saguzarretatik, hego estu eta luzedunak, frekuentzia-baxuko ekokokapen deiak igortzen dituztenak, eremu irekietan hegaz egiten duten artropodoez ehizatzera ondo moldatutakoak (adibidez, *Tadarida brasiliensis*, Lee eta McCracken, 2002), saguzar motel, baina distantzia laburretan oso abilak direnetara, hego-labur eta zabalekoak, ingurune itxietan harrapakin iheskorrak harrapatzeko gaituak (adibidez, *Rhinolophus hipposideros*, Abbott eta kideak, 2012). Hortaz, esan dezakegu saguzar-espezie bakoitzaren ezaugarriek hauek erabilitako nitxo-trofikoa baldintzatzen dutela, hots, saguzar-espezie bakoitza detektatzeko, ehizatzeko eta kontsumitzeko gai den harrapakin mota (Jones eta Rydell, 2003; Swartz eta kideak, 2003). Saguzar intsektiboro batzuk dietaren ikuspuntutik oso jeneralistak eta oportunistak diren bitartean (adibidez, Vesterinen eta kideak, 2013; Aldasoro eta kideak, 2019), beste hainbatek lehentasuna dute taxon espezifikoekiko, adibidez sits, kakalardo edo armiarmekiko (adibidez, *Plecotus macrobullaris*; Alberdi eta kideak, 2012, *Myotis myotis*; Pereira eta kideak, 2002, *Myotis emarginatus*; Goiti eta kideak, 2011). Bereziki, sitsak dira saguzarren ikuspuntutik talde esanguratsuenetariko bat (Jones eta Rydell, 2003). Saguzarren kontsumo tasa eta bazka ohiturak kontuan hartuta (egunean beraien gorputz masaren % 70 beste intsektu jan ditzakete, Kunz eta kideak, 1995), eta sitsak nekazal sistema askotako izurri nagusiak direla jakinda (Alford, 1999), saguzarrak nekazaritzako izurrien oinarrizko ustiatzailetzat jotzen dira (Russo eta kideak, 2018). Antza, nekazal-industriak 22,9 bilioi dolar aurrezten ditu urtean saguzarren harrapakaritzari esker (Boyles eta kideak, 2011). Dena den, saguzarren balio ekonomikoa zehazteko zenbait prozedura ekologiko kontuan hartu behar dira, adibidez, saguzarrek kontsumitzen dituzten izurri espezieen zerrenda, saguzar-

espezie bakoitzak jaten dituen izurri kopurua, eta saguzarren harrapakaritzak izurrien populazioetan duen eragina (Boyles eta kideak, 2013), besteak beste. Hurbilketa hauek ordea, zailtasun logistiko eta metodologikoz josita daude, eta nolabait, galarazi egiten digute saguzarrek duten balioa zehaztasunez neurtzea. Horren adibide, duela urte gutxi batzuk arte, saguzarren dieta lanak gorotzetan agertzen ziren digeritu gabeko harrapakinen arrastoen identifikazioan oinarritzen ziren (adibidez, Pereira eta kideak, 2002; Goiti eta kideak, 2003; Bontadina eta kideak, 2008). Dena den, oro har, sitsen eta beste hainbat intsektuen atal samurrak digestioan zehar zeharo deskonposatzen dira, eta horrek, eragotzi egiten du bisuzko espezieen identifikazioa. Hori dela eta, gorotzetan agertzen ziren harrapakinen arrastoen identifikazioa ordena edo familia mailan baino ez zen egiten (Whitaker eta kideak, 2009). Esan dezakegu beraz, ohiko metodoek ez zutela zehaztasun taxonomiko nahikorik adierazten saguzarrek izurrien ustiatzaile gisa eskaintzen dizkiguten zerbitzu ekosistemikoez jabetzeko (Boyles eta kideak, 2013).

## DNA metabarcoding-en etorrera: Aukerak eta Erronkak

DNA **metabarcoding**-en etorrerak aurrerapauso izugarria suposatu du bazka-ekologiaren esparruan, batez ere harrapakari eta harrapakinen arteko elkarrekintza trofikoak modu zehatzago batean ezagutzea baimendu duelako (Taberlet eta kideak, 2012). Testuinguru horretan, C-zitokromo oxidasa I (COI) hasleen garapenari esker, zeina espezieen artean nahikoa aldakorra den, baina, era berean, espezie beraren baitan aski kontserbakorra dena, eta "***High Throughput Sequencing***" sekuentziazio masiboari esker, kapaz gara ehundaka ingurumen-DNA (***eDNA***) lagin prozesatu (adibidez, gorotzak) eta saguzar zein beraien harrapakinak espezie mailan identifikatzeko (adibidez, Tournayre eta kideak, 2020). Hori horrela, **metabarcoding**-ek saguzarren dieta orokorra modu zehatzean erakusten du, izurriak diren harrapakinak eta izurriak ez direnak, eta hortaz, oso metodo baliagarria bilakatu da elkarrekintza-trofiko eta bazka-sareen inguruko ezagutza hobetzen duelako (Casey eta kideak, 2019). Halaber, azken urteotako sekuentziazio tekniken prezioen murrizketek, eta berriki publikatutako dieta-analisien protokoloen hobekuntzek (Alberdi eta kideak, 2018), **metabarcoding**-aren erabilera

ekologia-lanetan erabat emendatzea eragin dute. Horren erakusle dira azken hamarkadan gaiarekin lotuta publikatu diren 155-etik gorako ikerketa-lanak (Ando eta kideak, 2020). Ahalmen handia izan arren, oraindik ere badira kontuan hartu beharreko zenbait erronka tekniko eta metodologiko ugari:

a) Gorotz laginetan agertzen diren harrapakin sekuentzien presentzia/ausentzia datuen interpretazioan oinarritzen denez, dietaren ebaluazio kualitatiboa egitea baino ez du baimentzen.

b) Jomuga diren espezieak erreferentziazko datu-baseetan pobreki karakterizatuak egotekotan litekeena da sekuentzia horiek espezie mailan esleitu ezin izatea (Alberdi eta kideak, 2018).

c) Hasle egokien aukeraketa erabaki eztabaidagarria izan daiteke. Zenbait markatzailek talde taxonomiko oso ezberdinak anplifikatzeko gai dira, baina kontrara, hasle mota hauekin baxuagoa izaten da espezie-mailako ebazpena (Epp eta kideak, 2012). Gainera, hasle bakoitzak taxon espezifikoekiko alborapena erakuts dezake, sekuentzia horien presentzia balioetsiz eta beste batzuena gutxietsiz.

## Izurrien detekziorako estrategia alternatiboak: espezieekiko espezifikoak diren PCR metodoak.

Metodo zuzenduak, adibidez PCR konbentzionala (cPCR) eta PCR kuantitatiboa (qPCR), hasle espezifikoekin batera, maiz erabiltzen dira lagin mota ezberdinetan espezieen detekziorako (adibidez, Jarman eta kideak, 2004; Martin eta Rygiewicz, 2005; Czernik eta kideak, 2013; Nowakowska eta kideak, 2017; Knudsen eta kideak, 2019). **Metabarcoding** ez bezala, hurbilketa zuzendu hauek helburu diren espezieen detekzio zehatza eta arina ahalbidetzen dute eta gainera, hasleen alborapena ekiditen da. Hori horrela, espezie baten edo gutxi batzuen detekzioa xede den kasuetan metodo zuzenduak hobesten dira (Wood eta kideak, 2019). Detekzioaz gain, qPCR teknikak gorotzetako DNA-ren kuantifikazio edo semikuantifikaziorako ahalmena duela argudiatu izan da (Deagle, 2005, 2007; Gosh eta kideak, 2018), baina, gaur egun arte, saguzarren gorotzekin egin diren saiakera apurrek kale egin dute (McCracken eta kideak, 2005, 2012). Tesi honen garapena

beraz, batik bat lau faktore nagusik eragin dute: saguzar eta izurrien artean dagoen elkarrekintzaren ezagutza gabeziak, DNA metabarcoding-en dieta analisirako gaitasunak, saguzarrek izurrien ustiatzaile gisa eduki ditzaketen ahalmenak eta baita, izurrien monitorizaziorako metodo azkar, efiziente eta kuantitatiboak aurkitzeko beharrak ere.

## Eredutzat erabilitako laboreak eta espezieak

Tesia hiru hurbilketa ezberdinetan oinarritu da: 1) saguzar eta izurrien arteko elkarrekintzak batetik (**hurbilketa ekologikoa**, 1. eta 2. kapituluak), Iberiar Penintsulako mahastien sistema bat ardatz izanik; 2) izurrien detekziorako eta kuantifikaziorako teknika molekularren efizientzia bestetik (**hurbilketa metodologikoa**, 3. kapitulua), horretarako eredu-espeziea pinuetako prozesionaria (***Thaumetopoea pityocampa***) izanik, eta 3) saguzar-izurrien ikerketen diseinurako jarraibide eta muga metodologikoen berrikuspena (**diseinuari lotutako hurbilketa**, 4. kapitulua).

Eredutzat erabilitako laborea **mahastia** izan zen eta *Rhinolophus hipposideros* eta *T. pityocampa* tesiaren eredu-espezieak izan dira hurrengo arrazoiengatik:

1. Hurbilketa ekologikoa: mahastiak

**a) Hedadura eta garrantzia sozio-ekonomikoa:** munduko nekazal-lurren % 15 mahastiez estalita dago, guztira 7,4 milioi hektarea osatzen dituztelarik. Bereziki, Iberiar Penintsula da mundu-mailan mahastiez estalitako eremurik hedatuena (munduko mahasti-azaleraren % 16-a) eta beraz, ardo-ekoizle nabarienetarikoa ere (OIV, 2019). Horren ondorioz, garrantzia sozio-ekonomiko handia du gaur egun (Vivier eta Pretorius, 2002).

**b) Mahastietako izurriekin arazoak:** gorago aipatu bezala, paisaia-mailan aldaketa handiak eman dira, batez ere nekazaritzarako erabiltzen diren lurren kudeaketa intentsiboa areagotu egin baita. Horrek lur-gainazal eta landaredian aldaketak sortu ditu, artropodoen populazioetan eraginez eta ondorioz, izurrien berpizteak emendatuz. Zehazki, eskualde Palearktikoan badira galera ekonomiko garrantzitsuak eragiten dituzten zenbait sits espezie, besteak beste, *Lobesia*

*botrana, Sparganothis pilleriana, Eupoecilia ambiguella,* edo *Argyrotaenia ljungiana* (Ioriatti eta kideak, 2012; Thiéry eta kideak, 2018).

**c) Ezagutza mugatua mahastietako saguzar eta izurrien arteko elkarrekintzaren inguruan:** aurretik, saguzarrek mahastiak bazka-eremu gisa erabiltzen dituztela jakin arren (Stahlschmidt eta kideak, 2012; Kelly eta kideak, 2016; Froidevaux eta kideak, 2017), tesi honi hasiera eman genionean ez zegoen inolako ezagutzarik saguzarren eta haien harrapakin potentzialak diren mahastietako izurrien artean.

Gainera, lehenengo kapitulurako, ferra-saguzar txikia *R. hipposideros* aukeratu genuen eredu-espezie bezala. Badira hainbat faktore espeziea aproposa egiten dutenak tesiko galderei erantzuteko:

1.1 *R. hipposideros:*

a) Europan hedatuta dagoen espeziea da eta ikerketa-eremuan historikoki maiz agertu den saguzarra (Agirre-Mendi, 2003; Migens, 2007).

b) Lurretik gertu ehizatu ohi da, zuhaixken artetik (adibidez, mahats-mordoak) eta ehiza-estrategia ezberdinak erabil ditzakete habitataren arabera, harrapakin-mota anitzez baliatzea ahalbidetzen dielarik. Adibidez, airean harrapakinak ehizatu eta landaredi edo lur gainean mugimenduan dauden artropodoei eraso egin diezaiekete (Jones eta Rayner, 1989; Siemers eta Ivanova, 2004).

c) Beraien berezko ekokokapen-sistemak tamaina-txikiko sitsak (adibidez, xede diren izurriak) detektatu, espazioan kokatu eta sailkatzea ahalbidetzen die.

d) Aurretik egindako ikerketek adierazten dute espezie honen dieta nagusiki lepidopteroek (sitsak) eta dipteroek osatzen dutela (Lino eta kideak, 2014; Mitschunas eta Wagner, 2015; Galan eta kideak, 2018), eta ondorioz, saguzar-espeziea taxon hauen izurrien harrapakari potentziala kontsidera daiteke.

e) Udaberri-udan leialtasun handia erakutsi ohi du erabiltzen dituen gordelekuekiko eta horrek erraztu egiten du gorotzen bilketa eta dieta azterketak garai hauetan denboran zehar burutzea.

f) Kolonietan multzokatzeko joera dutenez gero, hainbat indibiduoren gorotz-lagin tamaina esanguratsuak lor daitezke.

2. Hurbilketa metodologikoa: *T. pityocampa.*

i. **Inpaktu sozio-ekonomikoa:** Mediterraneoko arroan zehar eta Ekialde Hurbilean sozio-ekonomikoki garrantzitsua den basoko izurria da (Kerdelhué eta kideak, 2009).

ii. **Hasleen diseinua eta balioztatze prozesuak:** erreferentziazko sekuentzien datu-baseetan (adibidez, BOLD eta NCBI) informazio franko dago espezie kongenerikoen (*Thaumetopoea* spp.) gainean. Horrek errazten ditu hasleen diseinua eta balioztatze prozesuak *in silico*.

iii. **Gainjartze geografikoa:** izurriaren eta ezagunak diren saguzar-kolonia askoren banaketa-eremuek bat egiten dute. Saguzarren hainbat gordeleku sitsaz izurriztatutako pinadietatik oso gertu aurkitzen ziren. Horrek emendatu egiten du *T. pityocampa*-ren DNA-dun gorotzen bilketa.

## COVID-19 eta saguzarrak jazartzeko arriskua

2019ko amaieran Txinan agertu zenetik, **COVID-19** mundu osoan zehar barreiatu da, eta 2020-ko hasieran Munduko Osasun Erakundeak (**MOE**) pandemia izendatu zuen. Saguzarrak bidegabeki jarraituak izan dira (ikusi MacFarlane eta Rocha, 2020) **SARS-CoV-2, COVID-19**-aren eragilea, eta aurretik Txinako ferra-saguzar espezie batean (*Rhinolophus affinis,* Zhou eta kideak, 2020) aurkitu izandako **Bat CoV RATG13** koronabirusaren arteko antzekotasun-maila altuagatik. Bi birusak estuki erlazionatuta egon arren (Zhou eta kideak, 2020), azterketa filogenetikoek birusak orain dela 40-70 urte elkarrengandik banandu zirela aitortzen dute (Boni ***et al.***, 2020), eta beraz, oraindik ere ez dakigu beste animalia talderik tartean egon daitekeen. Saguzarrak askotan gaixotasunen iturritzat jotzen dira, beharbada aurretik eman diren hainbat zoonosiekin izandako loturagatik, hala nola, **SARS**, **Ebola** edota **Nipah henipabirusa** (Brook eta Dobson, 2015). Hala eta guztiz ere, Mollentze eta Streicker (2020) frogatu zuten saguzarretan aurki daitezkeen birusen proportzioa ez

dela ohiz kanpokoa beste ugaztun taldeekin konparatuz gero, hots, saguzarretan aurki daitezkeen birus kopuruek bat egiten dute talde honetako espezieen dibertsitate altuarekin. Testuinguru honetan, hainbat zientzialari (adibidez, Fenton eta kideak, 2020; MacFarlane eta Rocha, 2020; Rocha eta kideak, 2020) eta gobernuz kanpoko erakunde (adibidez, Bat Conservation Trust, 2020; Bat Conservation International, 2020) lanean ari dira saguzarren kontserbazioan eta ekologikoki zein ekonomikoki eskaintzen dituzten onura eta zerbitzu ekosistemikoen komunikazioan, hauen kontra sor daitezkeen erantzun oldarkorrak ekiditeko. Zentzu honetan, tesi honek saguzarrek nekazaritzan duten eginkizuna argitu dezake, eta espero dut sortutako estereotipo negatiboei aurre egiteko ere lagungarri izatea.

## Tesiaren helburuak

Hitz gutxitan, tesiaren helburu orokorra da saguzar-intsektiboroek mahastietan eskaintzen dituzten zerbitzu-ekosistemikoak hobeto ulertzea eta PCR teknika ezberdinek saguzarren gorotzetan izurrien jarraipenerako duten eginkizuna aztertzea. Tesiko galdera nagusiak hurrengoak dira: a) zein da saguzarrek izurrien erregulatzaile gisa duten ahalmena eta zein izurri-espezie kontsumitzen dituzte?, b) saguzarrek mahastietako izurrien populazioak jarraitu eta ustiatzen dituzte?, c) saguzarrek denboran zehar ematen diren izurrien gorabeherekiko erantzuten dute?, d) zein metodo molekular da egokiagoa helburu diren izurrien detekziorako, metabarcoding edo PCR metodoak?, e) qPCR metodo aski sendoa eta fidagarria ote da *T. pityocampa* saguzarren gorotzetan kuantifikatzeko?

Tesia lau kapitulu nagusitan egituratzen da hurrengo helburuak zehaztu direlarik:

– **1. kapitulua.**

Ikerketa ugarik, artoa, kotoia, arroza edota makadamia intxaurren nekazal eremuetan kalteak sortzen dituzten izurriak aurkitu dituzte saguzarren dietan. Hala ere, era honetako ikerketak ez dira burutu mahastietan. Gainera, lan mota hauek ez dira denboran zehar burutu, momentu konkretu batean baizik. Saguzarrek momentuko harrapakinen eskuragarritasunaren arabera dietaren

konposizioa drastikoki alda dezaketela kontuan izanik, baliteke izurriak ehizatzeko duten ahalmena gutxietsi izana. Kapitulu honetan *R. hipposideros*-en dieta aztertu genuen mahatsaren hazkuntza-denboraldi osoan zehar, espezie honek mahastietako eta beste hainbat nekazal guneetako izurrien kontra bete dezakeen funtzioa zehazteko.

– **2. kapitulua.**

Saguzarrak mugikortasun handiko animaliak izanik, gauero hainbat kilometroko desplazamenduak egin ditzakete harrapakinetan ugariak diren bazka-toki egokien bila. Gaur egun, nekazal gune askotan labore bakarra lantzen dute, horrek daraman paisaiaren sinplifikazioari erreparatu barik. Sinplifikazio horrek labore horien izurri diren populazioak ezartzea eta sakabanatzea baimentzen die. Horren ondorioz, nekazaritza-eremuetan bazkatzen diren saguzar-espezie askoren harrapakin-iturri nagusia intsektu-izurriak izan ohi dira. Hori horrela, saguzar-espezie ezberdinek modu oportunista batean jokatzen duten eta positiboki izurrien dentsitateekiko erantzuten duten aztertu genuen bi modutan: batetik, saguzarrak izurriak ugari diren sailetan elkartuz (agregazio-erantzuna) eta izurrien kontsumoa beraien ugaritasun balioetara egokituz (erantzun funtzionala).

– **3. kapitulua.**

Oro har, harrapakarien dieta lanetan gorotzetako DNA erauzi eta metabarcoding teknika aplikatzen da dietan agertzen diren izurriak identifikatzeko. Azken urteotan, teknika honi lotutako kostuak murriztu badira ere, lagin tamaina handiekin edo erreplikekin lan egitean, kostuak zeharo igotzen dira. Bestalde, PCR metodoak (adibidez, cPCR eta qPCR) hasle espezifikoekin batera teknika azkar, efiziente eta errentagarriak dira saguzarren dietan helburu diren espezie konkretuen detekziorako (adibidez, *T. pityocampa*). Horrez gain, qPCR teknika saguzarren dietaren parte diren harrapakinak kuantifikatzeko erabil daitekeela susmatzen da. Hortaz, qPCR-ak kuantifikaziorako duen ahalmena eta balioa ebaluatu ziren. Azkenik, PCR metodo ezberdinen detekzio-efizientziak konparatu

eta bata edo bestea erabiltzearen abantailak eta desabantailen inguruan eztabaidatu genuen.

– **4. kapitulua.**

Saguzarrek nekazaritzako produkzioan eta izurrien kontrako kudeaketan laguntzen dutela argi geratu da azken urteotan. Urtero, gero eta gehiago dira saguzar eta izurrien arteko elkarrekintzak aztertzen dituzten lanak, nekazaritza-mota ezberdinetara eta munduko txoko ezberdinetara zabalduz. Hala ere, hurbilketa hauetan guztietan ikerlariek aurre egin behar diete muga metodologiko eta erronka ugariri. Horrela izanik, kapitulu honetan ikerketa prozesuan aurki daitezkeen muga eta zailtasun garrantzitsuenak identifikatu, ebaluatu eta berrikusi genituen etorkizunean egin daitezkeen antzeko ikerlanen diseinua hobetu eta ikerlariak bideratzeko.

# Chapter 1

# Bats as potential control agents of pests in vineyards: *Rhinolophus hipposideros* as a case study

*Photo by Oriol Massana and Adrià López-Baucells*

An article on this Chapter was published in *PLoS One*:
Baroja, U., Garin, I., Aihartza, J., Arrizabalaga-Escudero, A., Vallejo, N., Aldasoro, M., & Goiti, U. (2019). Pest consumption in a vineyard system by the lesser horseshoe bat (*Rhinolophus hipposideros*). *PLoS One , 14*(7), e0219265

# Bats as potential control agents of pests in vineyards: *Rhinolophus hipposideros* as a case study

## Abstract

Herbivorous arthropods cause immense damage in crop production annually. Consumption of these pests by insectivorous animals is of significant importance to counteract their adverse effects. Insectivorous bats are considered amongst the most voracious predators of arthropods, some of which are known crop pests. In vineyard-dominated Mediterranean agroecosystems, several crops are damaged by the attack of insect pests. This chapter aimed 1) to explore the diet and pest consumption of the lesser horseshoe bat *Rhinolophus hipposideros* and 2) analyse whether the composition of pest species in its diet changes throughout the season. We employed a dual-primer DNA metabarcoding analysis of DNA extracted from faeces collected in three bat colonies of a wine region in Southwestern Europe during the whole active period of most pest species. In all, 395 arthropod prey species belonging to 11 orders were detected; lepidopterans and dipterans were the most diverse orders in terms of species. Altogether, 55 pest species were identified, 25 of which are known to cause significant agricultural damage and eight are regarded as pests affecting grapevines. The composition of pest species in faeces changed significantly with the season, thus suggesting several periods should be sampled to assess the pest consumption by bats. Overall, the results imply that *R. hipposideros* acts as a suppressor of a wide array of agricultural pests in Mediterranean agroecosystems. Therefore, management measures favouring the growth of *R. hipposideros* populations should be considered.

**Keywords**: Crop Pests, Insectivorous Bats, *Rhinolophus hipposideros*, Pest Consumption

## Laburpena

Artropodo herbiboroek urtean kalte esanguratsuak eragiten dituzte nekazaritzako produkzioan. Izurri hauek kontsumitzen dituzten animalia intsektiboroek duten eginkizuna beraz, funtsezkoa izan daiteke nekazaritzan sortzen dituzten galerei aurre egiteko. Saguzar intsektiboroak artropodoen jatun handienen artean sailka daitezke, hauetako asko izurri ezagunak direlarik. Mahastiez gailendutako nekazal-sistema mediterraneotan intsektuek kalteak eragiten dituzte zenbait laboreetan. Kapitulu honetan hurrengo helburuak zehaztu dira: 1) ferra-saguzar txikiaren *Rhinolophus hipposideros* dieta eta izurriak kontsumitzeko ahalmena aztertu eta 2) izurri espezieen konposizioa dietan sasoian zehar aldatzen den analizatu. Horretarako, Europa Hego-mendebaldeko mahastien sistema batean, hiru kolonietatik saguzarren gorotzak bildu genituen izurri gehienak aktibo dauden denboraldi osoa zehar, ondoren DNA erauzi eta metabarcoding analisia burutu genuen bi hasle molekular erabiliz. Guztira, 11 artropodo ordena ezberdinetako 395 harrapakin espezie detektatu ziren; lepidopteroak eta dipteroak izan ziren talderik anitzenak espezie kopuruei erreparatuz. Denetara, 55 izurri-espezie identifikatu ziren, horietatik 25 nekazaritzan kalte esanguratsuak sortzeko gaitasuna dutenak eta 8 dira mahastiei eragiten dietenak. Izurri espezieen konposizioa saguzarren gorotzetan esanguratsuki aldatu zen sasoi ezberdinetan, nolabait aditzera emanez hainbat denboraldi lagintzea beharrezkoa dela saguzarren izurriak kontsumitzeko ahalmena xedatzeko. Orokorrean, emaitzek iradokitzen dute *R. hipposideros* nekazal sistema mediterraneoetan izurri-mota anitzen ustiatzailetzat jarduten dela. Ondorioz, *R. hipposideros*-en populazioen hazkundea faboratuko duten neurriak gogoan hartu beharko litzateke.

**Hitz gakoak**: Izurriak, Saguzar Intsektiboroak, *Rhinolophus hipposideros*, Izurrien Kontsumoa

## Introduction

The increasingly tight regulation of chemical pesticide use in agriculture, the rapidly developed resistance by pests, and the rising consumer awareness for sustainably produced crops (Jensen ***et al.***, 2016) have stimulated growing attention on the importance of biological suppression as a pest management tool (Zehnder ***et al.***, 2007; Naranjo ***et al.***, 2015). The annual crop damaged by herbivorous arthropods (mainly lepidopteran larvae) is estimated between 10-26% globally (Oerke, 2006; Culliney, 2014). Further, rising temperatures due to climate change may benefit insect pests, resulting in higher yield losses (Bale ***et al.***, 2002; Deutsch ***et al.***, 2018).

Consumption of arthropod pests by insectivorous animals is of major importance (Kunz ***et al.***, 2011; Wenny ***et al.***, 2011). Owing to their dietary habits, insectivorous bats are considered among the most voracious suppressors of arthropod pests (Russo ***et al.***, 2018); in fact, daily consumption of arthropods can reach values of over 70% of the bat body mass (Kunz ***et al.***, 1995) amounting to thousands of insects (Fenton, 1992). Both bats' energy demand and arthropod abundance increase during warm months in temperate regions (Kurta ***et al.***, 1989). Further, bats can respond to a wide diversity of arthropod pests: flying or non-flying, diurnal or nocturnal, and prey of various sizes. The recently developed molecular techniques like DNA metabarcoding (Arrizabalaga-Escudero ***et al.***, 2015) have extended our ability to detect particular insect species in the diet of bats and several studies have reported the presence of certain pests detrimental to corn, pecan orchards, macadamia orchards, cotton and rice (Cleveland ***et al.***, 2006; Brown ***et al.***, 2015; Maine and Boyles, 2015; Puig-Montserrat ***et al.***, 2015; Aizpurua ***et al.***, 2018; Weier ***et al.***, 2019). However, these studies only provided a snapshot of the pest consumption at a given point in time because they did not cover extensive periods and samples were not taken regularly. Importantly, bats can drastically modify their diet composition in response to changes in prey availability (McCracken ***et al.***, 2012; Almenar ***et al.***, 2013) due to pests' cyclic fluctuations, which entail sudden variations in pest numbers over time (Welch and Hardwood, 2014). Despite this knowledge, studies showing the pest consumption of bats within intensive agroecosystems over time are limited (but see McCracken ***et al.***, 2012).

Among fruit crops, grapes have the largest cultivated area and the highest global revenue (Vivier, 2002). The crop is attacked during spring and summer by several pests and pathogens. In Europe, for instance, four pest species can severely damage vine grapes (Voigt, 1972; Bărbuceanu and Andriescu, 2009; Ioratti ***et al.***, 2015; Rusch ***et al.***, 2015; Thiéry ***et al.***, 2016): the European grapevine moth (*Lobesia botrana*), the grape berry moth (*Eupoecilia ambiguella*), the leaf rolling tortrix (*Sparganothis pilleriana*) and the spotted wing drosophila (*Drosophila suzukii*). Altogether, the four can cause significant yield losses (Ioratti ***et al.***, 2012; Ioratti ***et al.***, 2015; Moschos, 2016).

Bats use vineyards for commuting and foraging (Stahlschmidt ***et al.***, 2012; Kelly ***et al.***, 2016; Froidevaux ***et al.***, 2017). The lesser horseshoe bat (*Rhinolophus hipposideros*), commonly reported in vineyard systems (Agirre-Mendi, 2003; Barros, 2014; Froidevaux ***et al.***, 2017), shows a remarkably adaptable foraging behaviour. Hunting close to vegetation, it is able to catch prey by aerial hawking, gleaning fluttering prey from vegetation or even pouncing at prey on the ground (Jones and Rayner, 1989; Siemers and Ivanova, 2004). Its echolocation system consists of broadband and constant frequency components in combination, allowing horseshoe bats excellent detection, localization and classification of prey (von der Emde and Schnitzler, 1990; Tian and Schnitzler, 1997; Schnitzler ***et al.***, 2003). Previous studies revealed that *R. hipposideros*' diet mainly comprises Diptera and Lepidoptera (Bontadina ***et al.***, 2008; Lino ***et al.***, 2014; Mitschunas and Wagner, 2015; Galan ***et al.***, 2018), including species regarded as pests (Galan ***et al.***, 2018). Given that moths comprise major agricultural pests damaging crops worldwide (Alford, 1999), it is essential to decipher the feeding habits of insectivorous bats within intensive agroecosystems to better understand the ecosystem services provided by these insectivores so that sustainable and more responsible agroecosystem management policies will be implemented. Unfortunately, studies showing bat-pest trophic interactions are still lacking in human-modified vineyard landscapes.

Consequently, we aimed to study the diet and pest consumption of *R. hipposideros* dwelling within a vineyard-dominated Mediterranean agroecosystem during the active period of most pest species through metabarcoding of DNA extracted from the faeces of three bat colonies.

# Material and methods

## Study area

From late May to late September 2017, we collected faeces in three maternity colonies of *R. hipposideros* from Rioja wine region (Southwestern Europe). Two of the colonies, Rivas (42˚36' N 2˚45' W) and Leza (42˚33' N 2˚38' W), roosted in human-made buildings and consisted of 80 and 13 individuals on average respectively through the sampling season. The third roost, occupied by 16 bats, is a winery's cellar located in Haro (42˚35' N 2˚49' W). The region is characterized by a continental Mediterranean climate with hot, dry summers and cold winters with annual mean rainfall around 500 mm. The landscape is dominated by grapevine with more than 13.000 cultivated hectares (52% of the area) (D.O.C Rioja, 2019), interspersed with other minor cultivations (e.g., olive groves, almond trees, cereal fields, and vegetable gardens or fruit orchards). Additionally, patches of riparian forests of *Populus nigra, P. alba*, *Alnus glutinosa*, *Fraxinus angustifolia* and *Sambucus* sp.; Mediterranean trees and shrubs like *Quercus ilex*, *Q. faginea* or *Q. coccifera*; a few stands of pine plantations of *Pinus nigra*, *P. pinaster*, *P. halepensis* and *P. sylvestris*; and rivers, lakes and urban settlements complete the landscape.

## Faecal samples collection

We placed stool-collecting nets under each colony two weeks before starting dropping sampling. We collected bat faeces every two weeks from late May to late September to cover the adult stage of most pest species present in the study region (Alford, 2016). The Leza roost was only occupied for a certain period, allowing faecal collection only from July to mid-August. *R. hipposideros* exclusively inhabited each roost and the collecting nets were cleaned after every sampling. Pellets were dried at 40ºC and then stored at -80ºC until processed. The number of analysed samples varied with colony size, but a minimum of 20 pellets and an average of 25 pellets were pooled per sample for each colony and two-week period. Nonetheless, if the colony-size was large, two additional samples were gathered to completely characterize the diet of a bat colony at a given period (Mata ***et al.***, 2018). We

homogenised each faecal sample in a buffer solution before DNA extraction. The study was carried out on private lands, and we obtained permission from owners to conduct our field sampling. No animal ethics clearance was required for this study because samples were passively collected and did not involve manipulating endangered or protected species.

## DNA extraction, library preparation and sequencing

DNA was extracted using the DNeasy PowerSoil Kit (Qiagen) following the manufacturer's instructions with modifications suggested by (Zeale ***et al.***, 2011). Extraction blanks were included in each extraction round. Two cytochrome oxidase I gene (COI) primer sets were used for each faecal sample to maximise the diversity due to the primer-specific taxonomic bias. We used the 157 bp primer set "**Zeale**" ZBJ-ArtR2c and ZBJ-ArtF1c (Zeale ***et al.***, 2011) and the 133 bp "**Gillet**" modified forward primer LepF1 (Hebert ***et al.***, 2004) and modified reverse primer EPT-long-univR (Hajibabaei ***et al.***, 2011) described in (Gillet ***et al.***, 2015). Combining these two primer sets is the most cost-effective means of characterizing diets that may include a high diversity of prey taxa (Esnaola ***et al.***, 2018). We followed the 16S Metagenomic Sequencing Library Preparation protocol by Illumina® (Illumina, 2013) with slight modifications. For the first amplification process, we followed the Qiagen 2X kit protocol, using 12.5 µL Qiagen Multiplex PCR kit 2x, 1.25 µL forward primer (10 µM), 1.25 µL reverse primer (10 µM), 8 µL $H_2O$ and 2 µL DNA for a total volume of 25 µL for each sample and primer set. Each primer set was subjected to different PCR cycling conditions (Table S1.1.). PCR negative controls were used. Then, PCR products were migrated in agarose gel electrophoresis to test the efficiency and homogeneity of amplification. Amplicons were bead-purified with CleanPCR kit (CleanNA, PH Waddinxveen, The Netherlands), and a second PCR reaction was performed to attach dual indices and Illumina sequencing adapters using the Nextera XT Index Kit. Once indexed and adapters attached, samples were bead-purified, fluorometrically quantified, and pooled at equal molarities to sequence in an Illumina MiSeq with 5%. DNA library construction and sequencing

processes were done at Genomics and Proteomics General Service (SGIker) of the University of the Basque Country.

## Bioinformatics processing

Paired-end reads were merged and quality-filtered using USEARCH v.10 (Edgar, 2010), considering only sequences with a minimum 50bp overlap and discarding sequences with quality values inferior to Q30. We demultiplexed reads according to primers and trimmed adapter and primer sequences using **Cutadapt** (Martin, 2011). Sequences in samples identical to those in the corresponding extraction blanks were removed and the remaining sequences clustered into haplotypes using **USEARCH's** *-fastx_uniques* command. Singletons and chimeras were discarded. The remaining haplotypes were quality-filtered and collapsed into zero-radius operational taxonomic units (ZOTUs); an amplicon sequencing error-correction method used to infer accurate biological template sequences (Edgar, 2016). We manually compared ZOTUs from the overall samples against reference sequences within the BOLD systems database (**www.boldsystems.org**). The species-level assignment was conceded when query sequences matched the reference sequences above 98.5% similarity value (Razgour ***et al.***, 2011). When the haplotype coincided with more than one species belonging to the same genera, we made a genus-level assignment; if the haplotype coincided with species belonging to different genera, we only included species present in the Iberian Peninsula.

## Determining the pest category

We categorised pests found in bat diets based on crop diet, prevalence areas (within or outside the Iberian Peninsula), and according to the potential damage and economic impact they cause (Alford, 1999, 2012, 2016, Hill, 2008; Lakatos ***et al.***, 2014; Ministerio de Agricultura, 2014). As a result, pests were classified as follows: a) minor grapevine pests: species affecting vineyard production but not causing economically severe losses or yield reduction; b) major grapevine pests: species that

may critically affect vineyard production with a potentially high economic impact, and c) minor or major pests of other crops.

### Data analysis

Since samples come from different locations and periods, we tested for space-time interaction as well as spatial (colonies) and temporal (two-week periods) effects on the pest species composition in the bats' diet. We first Hellinger-transformed (Legendre and Gallagher, 2001) presence/absence data of pests and then a two-way ANOVA without replication was performed (Legendre ***et al.***, 2010) using *STImodels* function with 9999 random permutations in STI 3.1.1 package (Legendre ***et al.***, 2012) for R (R Core Team, 2018).

## Results

We generated 2053 ZOTUs from libraries built with Zeale and Gillet primers, of which 761 (37%) were identified at the species level and assigned to 401 taxa (Table S1.2.). Altogether, DNA sequences retrieved with both primer sets from the faeces of *R. hipposideros* were assigned to 393 arthropod species: among them, 25 are considered major pests and 29 minor pests (Table 1). One pest, *Philaenus spumarius,* is a vector of the plant pathogen *Xylella fastidiosa*, but it remains unclear whether it is a major or minor pest. Most of the 55 pest species were lepidopterans (n=47), followed by four dipterans, three hemipterans, and one coleopteran. Among all the pest species detected in bats' diet, six major pests and two minor pests were potentially harmful to grapevines. The remaining insects are regarded as pests of other crop types.

The Rivas colony accounted for 51 out of the 55 pest species, while Leza and Haro accounted for 21 and 16 pest species, respectively. The sum of pest species across colonies did not add up to 55 because some pests were detected at more than one site. The list of identified pest species was different with each primer set (Table 1),

and thus, the number of detected pest species increased combining the output of the two primer sets (Figure S1.1.).

**Table 1.** List of pest species identified in faeces of *R. hipposideros*, affected hostplants, their corresponding pest category and the primer set with which they were detected. Species affecting grapevine are highlighted in bold. Asterisks refer to species considered as pest locally in the Iberian Peninsula. Primer set(s): (G) Gillet, (Z) Zeale, (GZ) both.

| Order | Species | Family | Damaged plants | Pest category[a] | Primer |
|---|---|---|---|---|---|
| Lepidoptera | *Acleris schalleriana* | Tortricidae | Guelder-rose, ornamentals | Minor | GZ |
| | *Acleris variegana* | Tortricidae | Rosaceous | Major | GZ |
| | *Aleimma loeflingiana* | Tortricidae | Oak, hornbeam, maple | Minor* | GZ |
| | *Anacampsis populella* | Gelechiidae | Poplar, willow | Minor* | GZ |
| | *Ancylis achatana* | Tortricidae | Fruit trees (apple, plum...) | Minor | GZ |
| | *Archips podana* | Tortricidae | Polyphagous (trees, shrubs, rosaceous) | Major* | G |
| | *Archips rosana* | Tortricidae | Hazelnut, rosaceous | Major* | GZ |
| | *Argyresthia abdominalis* | Argyresthiidae | Oak | Minor | G |
| | *Argyresthia sorbiella* | Argyresthiidae | Rowan (*Sorbus aucuparia*) | Minor | G |
| | *Argyresthia spinosella* | Argyresthiidae | Damson and plum | Minor | GZ |
| | *Bedellia somnulentella* | Bedellidae | Convulvalaceous crops | Minor | GZ |
| | *Calliteara pudibunda* | Erebidae | Beech, hop, fruit trees | Minor* | Z |
| | *Clepsis consimilana* | Tortricidae | Many trees and shrubs (Rosaceae) | Minor | G |
| | ***Cnephasia incertana*** | Tortricidae | Polyphagous (**grapevine**) | Minor | GZ |
| | *Cydia fagiglandana* | Tortricidae | Beech, oak, chestnut | Minor* | GZ |
| | *Cydia pomonella* | Tortricidae | Apple, pear, quince… | Major* | GZ |
| | *Cydia splendana* | Tortricidae | Chestnut, walnut | Minor* | GZ |
| | ***Ditula angustiorana*** | Tortricidae | Polyphagous, fruit crops (**grapevine**) | Minor | Z |
| | ***Ephestia parasitella*** | Pyralidae | Polyphagous (**grapevine**) | Major* | GZ |
| | *Exoteleia dodecella* | Gelechiidae | Pine trees | Major* | GZ |
| | *Gypsonoma aceriana* | Tortricidae | Poplar | Major* | GZ |
| | *Hedya nubiferana* | Tortricidae | Apple, pear, almond… | Minor* | GZ |
| | *Hedya ochroleucana* | Tortricidae | Rosaceous (apple...) | Minor | Z |
| | *Hedya pruniana* | Tortricidae | Rosaceous (plum, cherry) | Minor | G |
| | ***Lobesia botrana*** | Tortricidae | **Grapevine** | Major* | GZ |

**Table 1.** (continuation).

| Order | Species | Family | Damaged plants | Pest category | Primer |
|---|---|---|---|---|---|
| Lepidoptera | *Mythimna unipuncta* | Noctuidae | Cereals | Major* | Z |
| | *Neosphaleroptera nubilana* | Tortricidae | Plum, apple and apricot | Minor | Z |
| | *Notocelia uddmanniana* | Tortricidae | Blackberry, boysenberry… | Major | GZ |
| | *Oecophora bractella* | Oecophoridae | Currant, mulberry tree | Minor | G |
| | *Orthotaenia undulana* | Tortricidae | Trees and shrubs | Minor | GZ |
| | *Parornix devoniella* | Gracillariidae | Hazelnut | Minor | G |
| | ***Peridroma saucia*** | Noctuidae | Polyphagous (**grapevine**) | Major* | GZ |
| | *Phtorimaea operculella* | Gelechiidae | Solanaceae family (potato) | Major* | Z |
| | *Phyllonorycter messaniella* | Gracillariidae | Trees, fruit trees | Minor | G |
| | *Plutella xylostella* | Plutellidae | Brassicaceous crops | Major* | GZ |
| | *Prays oleae* | Praydidae | Olive | Major* | GZ |
| | *Recurvaria leucatella* | Gelechiidae | Apple, pear | Minor | GZ |
| | *Recurvaria nanella* | Gelechiidae | Fruit trees (apple, pear…) | Major | G |
| | *Rhyacionia buoliana* | Tortricidae | Pine trees | Major* | GZ |
| | *Rhyacionia pinicolana* | Tortricidae | Pine trees | Minor* | G |
| | ***Sparganothis pilleriana*** | Tortricidae | **Grapevine** | Major* | GZ |
| | *Spilonota ocellana* | Tortricidae | Apple, pear, quince | Minor* | Z |
| | ***Spodoptera exigua*** | Noctuidae | Polyphagous (**grapevine**) | Major* | G |
| | *Thaumetopoea pityocampa* | Notodontidae | Pine trees | Major* | GZ |
| | *Tischeria ekebladella* | Tischeriidae | Chestnut | Minor | GZ |
| | *Udea ferrugalis* | Pyralidae | Plum, gooseberry… | Minor | GZ |
| | *Ypsolopha scabrella* | Ypsolophidae | Apple, pear, cherry, plum | Minor | GZ |
| Diptera | *Delia platura* | Anthomyiidae | Polyphagous (cereals) | Major* | GZ |
| | ***Drosophila suzukii*** | Drosophilidae | Polyphagous, fruit crops (**grapevine**) | Major* | GZ |
| | *Tipula oleracea* | Tipulidae | Horticultural crops | Minor* | Z |
| | *Tipula paludosa* | Tipulidae | Horticultural crops, cereals | Major* | G |
| Hemiptera | *Adelphocoris lineolatus* | Miridae | Polyphagous | Major | G |
| | *Fieberiella florii* | Cicadellidae | Vector of phytoplasmic diseases | Major | G |
| | *Philaenus spumarius* | Aphrophoridae | Vector of *Xylella fastidiosa* | Unknown | G |
| Coleoptera | *Curculio glandium* | Curculionidae | Oak | Major* | Z |

Time had a statistically significant effect on the pest composition observed in the bats' diet ($F$ = 1.839; $R^2$= 0.458; $p$= <0.001). Some grapevine pest species such as *Lobesia botrana*, *Sparganothis pilleriana*, *Peridroma saucia* and *Drosophila suzukii* were regularly consumed throughout the sampling period. In contrast, others occurred in the bats' diet only occasionally (Figure 1).

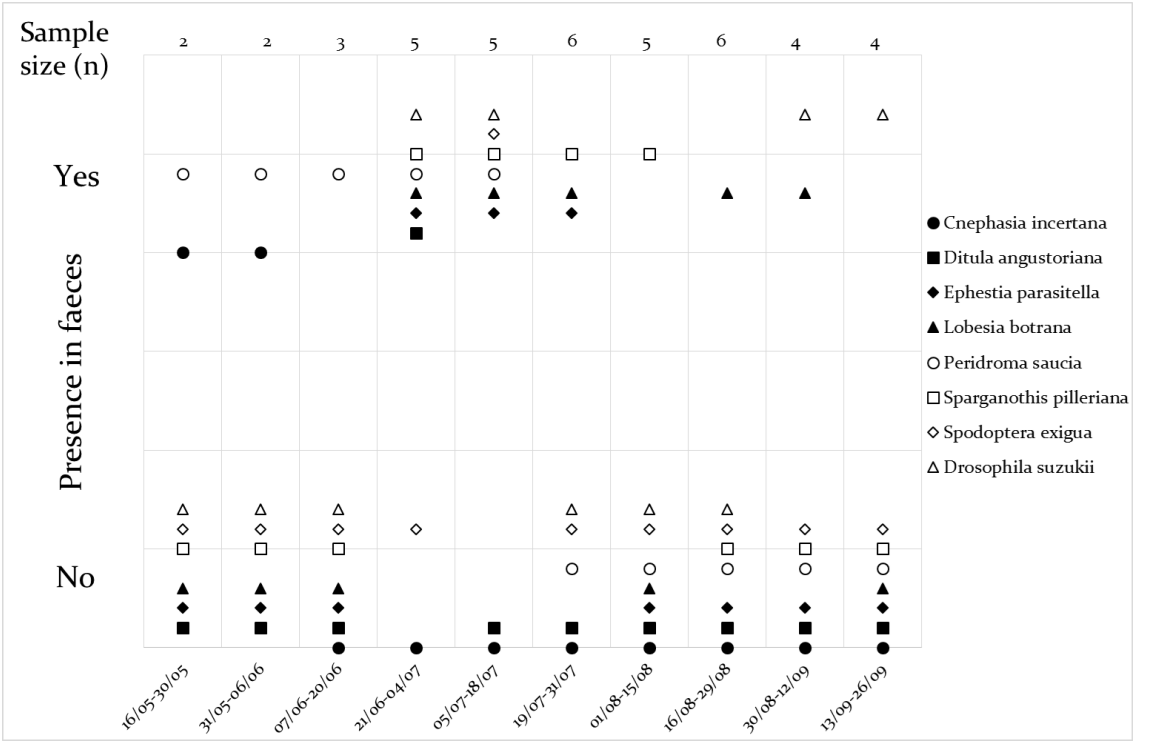



**Figure 1.** Presence of grapevine pests in faeces of the lesser horseshoe bat *R. hipposideros*.

There was no space-time interaction though ($F$= 0.994; $R^2$= 0.137; $p$= 0.49) and pest composition in diet did not significantly differ among colonies ($F$= 0,798; $R^2$= 0.044; $p$= 0.83). Most prey consisted of members of the Lepidoptera family (66% of identified species), some of which have auditory defensive mechanisms against bats (Figure S1.2.), followed by Diptera (20%). The remaining prey species belonged to the orders Ephemeroptera, Neuroptera, Trichoptera, Hemiptera, Hymenoptera, Coleoptera, Araneae, Plecoptera and Blattodea, but their species richness was low (<2%). Within Lepidoptera, 85% of species were micromoths, belonging primarily to the families Tortricidae, Gelechiidae, Coleophoridae and Pyralidae (50.6% of

identified Lepidoptera altogether, Figure S1.2.). Finally, 15 ZOTUs were assigned to taxa considered non-prey species: namely, Chiroptera (*R. hipposideros*), Rodentia, fungus (Mucorales, Eurotiales and Rickettsiales), mite (Trombidiformes, Mesostigmata and Sarcoptiformes), tick (Ixodida) and moss (Orthotrichales).

## Discussion

The 55 pest species consumed by *R. hipposideros* included insects affecting diverse types of crops including grapevines (e.g., *L. botrana, S. pilleriana, D. suzukii*), fruit trees (e.g., *Acleris variegana, Cydia pomonella*), olive groves (e.g., *Prays oleae*), cereals (e.g., *Tipula paludosa, Mythimna unipuncta*), vegetables (e.g., *Spodoptera exigua*) or forest plantations (e.g., *Thaumetopoea pityocampa, Rhyacionia buoliana*).

We also found diurnal pest species —for instance, *Delia platura*— in the bats' faeces; this can be explained by either the ability of rhinolophids to detect fluttering insects resting on the vegetation during the night (Bell and Fenton, 1984; Siemers and Ivanova, 2004) or because diurnal prey are regularly still active at dusk, co-occurring with the emergence of *R. hipposideros* (Bontadina ***et al.***, 2002; Zahn ***et al.***, 2008). Further, the pest species consumed by bats changed with the season. For instance, while *Cnephasia incertana* was consumed during May and June, *D. suzukii* was consumed during July and September. These patterns can be attributed to the phenology of each insect species. Insect adaptations to environmental changes (e.g., the change in weather patterns with season) will determine the number of insect generations per year. Thus, the season in which the adult stages of insects appear (Tauber and Tauber, 1981). In vineyards, for instance, *L. botrana* completes between three and four generations during the flying season from April to September, whereas *S. pilleriana* has just one generation from June to August (Bărbuceanu and Andriescu, 2009; Rusch ***et al.***, 2015).

Several studies on bat diets have detected DNA of pest arthropods, ranging with some finding only a few species (Galan ***et al.***, 2018; Kemp ***et al.***, 2019) and others finding many (44 in *Miniopterus schreibersii* and *Tadarida basiliensis* (Aizpurua ***et al.***, 2018; Krauel ***et al.***, 2018)). The sampling period in each of these studies did not

cover more extended periods than our study, and the sampling date was randomly chosen. Our results revealed that the composition of pest species in bat diets varied with season. Consequently, assessing pest consumption by bats in the area demands sampling bat diets over several seasons. We covered almost the whole vegetative period of grapevine in this geographic area and the flying phases of several pest species of grapevines in temperate regions, providing representative data on the bat-pest interaction in vineyards. Although our research was focused on a vineyard-dominated agroecosystem, we found insect pests associated with other crops— this may be linked with the different habitat requirements of prey through different life stages (Arrizabalaga-Escudero ***et al.***, 2015). Whereas the larval host plant of a given prey species may be associated with forest trees or shrubs, adults can occur in diverse habitats like pastures or crops due to their dispersal abilities and varying trophic needs or phenology (Murakami ***et al.***, 2008; Betzholtz and Ftanzén, 2011; Slade ***et al.***, 2013).

This study reaffirms the value of metabarcoding diet analyses for unveiling the interactions of bats with agroforestry pests. Moreover, such studies are valuable tools for the timely detection of insect pests and potentially harmful arthropod species, which is fundamental to avoid irreparable damage to the crops (Maslo ***et al.***, 2017). In this context, our results suggest that the detectability of potential pests is, to some extent, primer-dependant. Whereas some pests were detected just with the Gillet primers, others were only detected with Zeale's (Table 1). Therefore, combining complementary primer sets is critical to determine the full or the most comprehensive taxonomic range of prey consumed by predators (Elbrecht and Leese, 2017; Esnaola ***et al.***, 2018). Finally, Lepidoptera and Diptera were the most diverse taxa of *R. hipposideros* diet, as found in previous studies based on morphological identification of prey remains (Arlettaz ***et al.***, 2000; Bontadina ***et al.***, 2008; Lino ***et al.***, 2014). However, in contrast with prior research, we observed high diversity of moth species. Among the 269 lepidopteran species detected, we mainly found species belonging to the so-called group of micromoths or small-size moths. However, we also detected moths with very different traits, such as size, flight patterns, and evasive/defensive strategies, including those with the capacity to hear bat echolocation calls (Figure S1.2.). This finding confirms that *R. hipposideros* is

well adapted to detect and prey on small size lepidopterans in accordance to its high-frequency calls (Jones, 1995, 1999; Fenton, 1999), and it can overcome the defensive mechanisms of moths (Jones, 1992), which comprise the major agricultural pests that damage crops globally (Alford, 1999).

## Conclusions

This chapter reveals the pest consumption of *Rhinolophus hipposideros* within vineyard agroecosystems, and consequently, points at the potential ecosystem service provided by the species in a modified agricultural landscape. Secondly, due to its putative contribution to crop production, this bat should be integrated into pest management practices, for example, promoting the establishment of new populations. Looking forward, the application of organic farming practices (Wickramasinghe ***et al.***, 2003), bat roosts protection initiatives and the construction of artificial roosts (Alcalde ***et al.***, 2017) will be essential steps to strengthen these bat populations. Further, to gain insight into the interaction of bats and pests, the variation of pest consumption should be investigated across the bat community and along the life cycle of pests sharing the agroecosystem. Deciphering how bats respond to changes in pest communities is of particular importance not only to characterise the foraging behaviour of bats against pests, but also to manage the negative impacts of pests through consumption by insectivorous bats

# Chapter 2

# Unveiling the foraging responses of bats to grape pests

An article on this Chapter was published in *Ecological Indicators*: Baroja, U., Garin, I., Vallejo, N., Aihartza, J., Rebelo, H., & Goiti, U. (2021). Bats actively track and prey on grape pest populations. *Ecological Indicators*, 126, 107718.

# Unveiling the foraging responses of bats to grape pests

## Abstract

There is growing evidence about the role of insectivorous bats against agricultural pests in various crops. Nevertheless, little research addressed bat assemblages' aggregational and functional responses to changes in pest availability across a spatio-temporal scale. Therefore, we examined the activity and diet habits of different bat species using DNA metabarcoding by simultaneously monitoring the relative abundance of two major pests (the European grapevine moth, *Lobesia botrana*, and the leaf rolling tortrix, *Sparganothis pilleriana*) through the grape growing season, in a vineyard region of the Iberian Peninsula. During pest major irruptions, we found the highest bat activity levels and frequencies of grape pests in the diet of bats, although not all bat species contributed equally to pest suppression. Bats of different foraging guilds positively responded to pest abundances, indicating distinct bat species may synergistically play a role at suppressing agricultural pests at broad scales of the aerospace. For instance, narrow space foragers exploiting major irruptions in the grape interior, edge space foragers hampering pest dispersion at a local scale, and open space foragers preventing infestations of new grapevine patches at broader scales. Yet, our study exposed the current methodological constraints regarding pest dispersion dynamics, acoustic monitoring of bats' foraging activity, or the unfeasibility of metabarcoding to reliably quantify prey abundance in bats diet, and thus further improvement in these issues is required in order to gain insight on the agroecological interactions between bats and pests.

**Keywords**: Grape pests, Bats, Aggregational response, Functional response, Ecosystem services, Foraging guilds

## Laburpena

Gero eta nabarmenagoa da saguzar intsektiboroek labore anitzetan betetzen duten funtsezko egitekoa. Halarik ere, oso ikerketa gutxik heldu dio saguzarrek izurrien eskuragarritasunarekiko erakusten duten erantzuna aztertzeari. Bada, Iberiar Penintsulako mahastien sistema batean, saguzar-espezie ezberdinen aktibitatea eta dieta ohiturak arakatu genituen DNA metabarcoding bidez, garai berean bi izurri garrantzitsuren ugaritasun balio erlatiboen (mahats mordoaren sitsa, *Lobesia botrana,* eta mahastietako pirala, *Sparganothis pilleriana*) jarraipena egin zelarik. Izurrien garai oparoenetan saguzarren aktibitatea altuena izateaz gain, izurrien agerpen maiztasunak saguzarren dietan ere garaienak izan ziren, nahiz eta espezie guztiek ez zuten maila berean jokatu. Bazka-gremio ezberdinetako saguzarrek positiboki erantzun zuten izurrien gorabeherekiko, aditzera emanez saguzar-espezie ezberdinek, sinergikoki jokatuz, izurriak aire-espazioko maila ezberdinetan ustiatzeko ahalmena dutela. Adibidez, espazio itxietako bazka-biltzaileak mahastien barrualdean izurriak ehizatuz, ertzetako bazka biltzaileak izurrien dispertsioa maila lokalean eragotziz, eta espazio irekietako bazka biltzaileak mahasti berrien inbasioa eskala zabalagoetan saihestuz. Nolanahi ere, ikerketa honek agerian uzten ditu mota honetako ikerlanek dituzten zailtasun metodologikoak, izurrien dispertsio-dinamikak, saguzarren aktibitatearen monitorizazio akustikoarekin lotutakoak edota metabarcoding bidez, saguzarren dietan agertzen diren harrapakinen ugaritasuna modu fidagarrian neurtzeko bideraezintasuna. Hori horrela, muga hauen inguruko hobekuntzak beharrezkotzat jotzen dira saguzar eta izurrien arteko elkarrekintzak hobeto uler daitezen.

**Hitz gakoak:** Mahats-izurriak, Saguzarrak, Agregazio erantzuna, Erantzun funtzionala, Zerbitzu ekosistemikoak, Bazka-gremio

## Introduction

Insect pests are responsible for substantial annual losses in crop production (16-26%; Oerke, 2006; Culliney, 2014). More sustainable pest suppression solutions to conventional chemical pesticide use are in demand by consumers and as a measure to tackle the biodiversity crisis caused by intensive farming. Those alternatives stand primarily on the spraying of the *Bacillus thuringiensis* toxin (Ifoulis and Savopoulou-Soultani, 2004), pheromone-based mating disruption techniques (Louis and Schirra, 2001; Louis ***et al.***, 2002), the introduction of egg parasitoids (Moreau ***et al.***, 2009) and on identifying, preserving and promoting the contribution of local natural enemies (Pickett and Bugg, 1998, Begg ***et al.***, 2017). Unlike specialist predators, generalists that show temporal persistence can be efficient biocontrol agents in these agroecosystems since, as opportunistic feeders, they may shift between different pest outbreaks feeding on alternative prey while main pest species are scarce or rare (Ehler, 1998; Symondson ***et al.***, 2002). Besides, natural enemies of pests to be effective in agroecosystems should have, 1) a rapid colonizing and dispersal ability to track sudden spatio-temporal pest invasions (Ehler and Miller, 1978); 2) opportunistic feeding behaviour that elicits the exploitation of highly changeable pest outbreaks, and 3) an aggregative response to pest availability (Symondson ***et al.***, 2002). The ecological features of some insectivorous bats not only comply with these requirements, but bats have regularly been documented to display an aggregative and functional response to pest bursts (McCracken ***et al.***, 2012; Charbonnier ***et al.***, 2014; Puig-Montserrat ***et al.***, 2015; Korine ***et al.***, 2020). Note that monoculture crops surrounded by a simplified landscape cover the vast majority of current agricultural fields. Thereby, a substantial part of the prey source of bats in these crop systems often comes from insect pests (Symondson ***et al.***, 2002; Segoli and Rosenheim, 2012). Assuming that predators' distribution, abundance, and activity depend on the spatial dispersion, patchiness, and variation of food accessibility (Resource Dispersion Hypothesis; Carr and MacDonald, 1986), we should expect predator-prey interactions to be directly influenced by the aggregational and functional responses of predators to prey density (Abrams and Ginzburg, 2000; Bayliss and Choquenot, 2002). The aggregational response implies that predators' density and activity patterns are

coupled with prey numbers in patches with high food supply (Hassel and May, 1974), whereas the functional response asserts that predators adjust their intake rate to prey abundance (Goss-Custard ***et al.***, 2006). Large populations of prey are spatio-temporally structured, meaning that successful predators should be able to reach bountiful feeding patches. Bats can forage over many kilometres in a single night (Müller ***et al.***, 2012), but they also eavesdrop on successful foragers, resulting in an increasing number of bats that feed rapidly on newly available resources (Gillam, 2007; Cvikel ***et al.***, 2015). However, because flying is energetically expensive, some bats may have a limited commuting range, limiting their choice of foraging areas. For instance, while bats such as *Tadarida brasiliensis* or *Miniopterus schreibersii* can commute nightly to foraging sites located at up to tens of kilometres away from the day roost (Best and Geluso, 2003; Vincent ***et al.***, 2010), others (e.g., *Pipistrellus pipistrellus* or *Myotis lucifugus*) show limited nightly mobility (Henry ***et al.***, 2002; Davidson-Watss and Jones, 2006) and consequently, these species may not encounter potentially profitable but distant foraging sites.

The advent of DNA metabarcoding has extended our ability to detect pest species among bats' prey, revealing that bats frequently consume pest arthropods (Aizpurua ***et al.***, 2018; Krauel ***et al.***, 2018; Baroja ***et al.***, 2019a). In most cases, bats attack adult aerial arthropods, preventing them from laying eggs and preventing subsequent growth of larvae, which usually are responsible for crop damage (Russo ***et al.***, 2018). Pest exploitation by bats, though, does not necessarily imply bats performing a pest regulation function. To guarantee control over insect populations, bats should track pest abundance by increasing their activity and intake rate due to sudden pest irruptions (Russo ***et al.***, 2018). So far, few works have addressed these issues, though, and some revealed that bats positively respond to pest abundances (McCracken ***et al.***, 2012; Charbonnier ***et al.***, 2014; Puig-Montserrat ***et al.***, 2015; Korine ***et al.*** 2020). Yet, some critical issues have been overlooked. For example, previous research tended to focus on the bat's diet or on activity levels to assess bat-pest interactions, but both features are reasonably important. Despite this, very few studies have simultaneously studied bats' diet, activity patterns, and pest dynamics throughout the whole active period of any insect pest (but see Charbonnier ***et al.***, 2021).

Grapevine crops, covering 7.4 million hectares, account for 15% of agricultural lands worldwide (OIV, 2019) and are thus considered one of the fruit crops with the highest economic importance (Vivier and Pretorious, 2002). They are regularly attacked by arthropod pests, resulting in huge yield reduction and consequent economic losses (Ioriatti ***et al.***, 2012; Thiéry ***et al.***, 2014, 2018; Delbac and Thiéry, 2016). The European grapevine moth (*Lobesia botrana*: Lepidoptera, Tortricidae; hereafter "LB") and the leaf rolling tortrix (*Sparganothis pilleriana*: Lepidoptera, Tortricidae; hereafter "SP") are among the most destructive insects of grapevine in the Palearctic. Production losses are caused by direct damage to the plant and the subsequent infection by fungi, such as *Botrytis cinerea* and *Aspergillus* sp., which cause grape cluster rotting (Moschos, 2005; Moschos, 2006; Ioratti ***et al.***, 2012). Besides, the species are rapidly responding to climate change by prolonging their active periods and spreading northward (Martín-Vertedor ***et al.***, 2010; Gutierrez ***et al.***, 2018). With this in mind, and in light of the potential pest regulatory function of insectivorous bats, the present paper aims to determine the bat-pest agroecological interactions in a vineyard system by analysing the aggregative and functional responses of bats to changes in pests' populations. To test this, we studied the spatio-temporal activity and diet habits of different bat species by simultaneously monitoring the relative abundance of the pests within a vineyard region in the northern Iberian Peninsula (Southwestern Europe). We hypothesized that bat activity and pest consumption patterns are associated with seasonal changes in LB and SP imagoes' activity.

## Material and methods

### Study region

The study was conducted in the Rioja wine region (42˚ 32' N, 2˚ 34' W), one of the most important wine-growing areas in Southwestern Europe (Figure 1).

The region has a continental Mediterranean climate with average annual temperatures around 13˚C and low mean annual rainfall (500 mm/year). The land use is mainly devoted to agriculture, where vineyards predominate (52% of the total

area). In 2017, during the grape-growing season from late April to the end of September, we simultaneously surveyed bats' activity, diet, and the flight dynamics of LB and SP within the region.

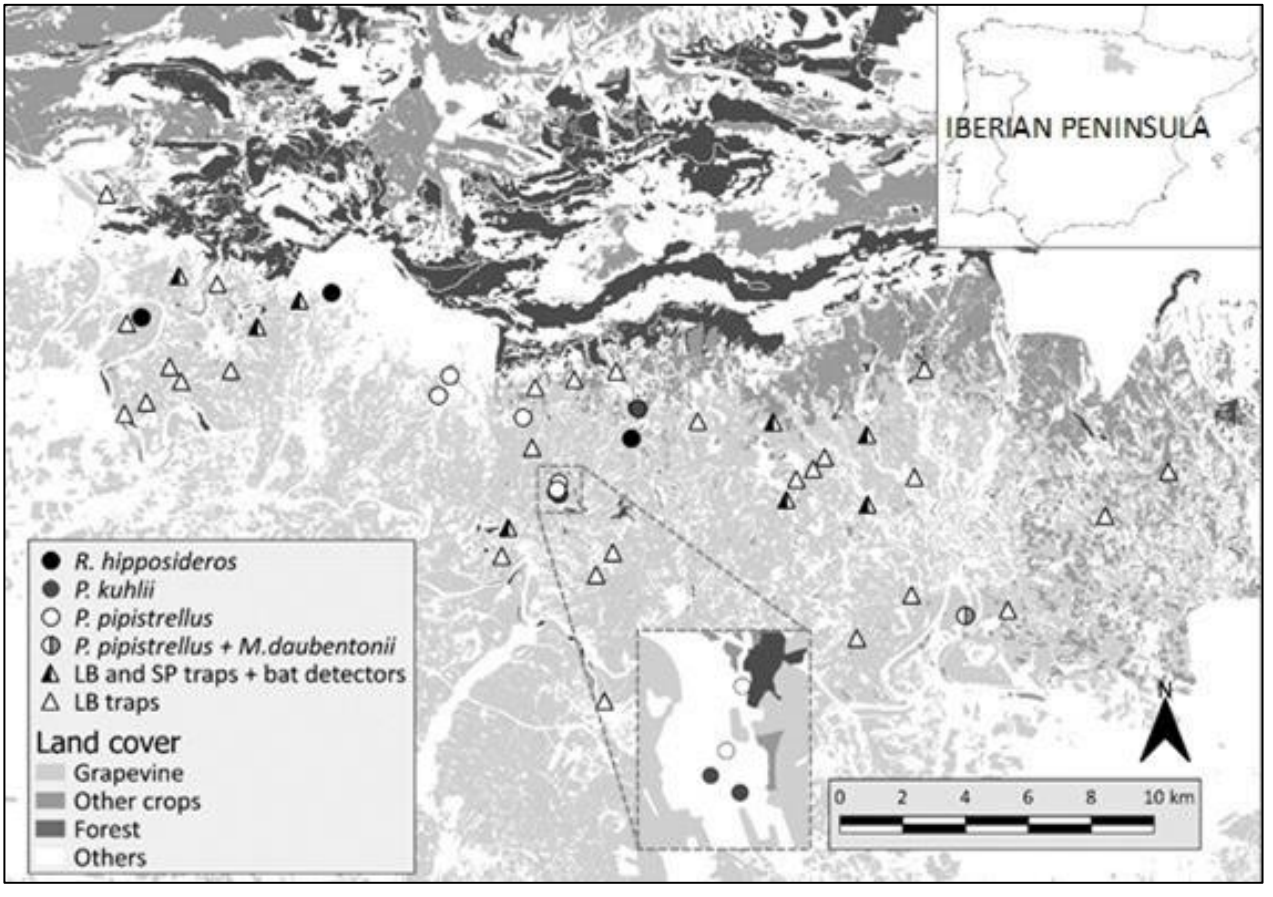



**Figure 1**. Map of the study area showing land cover and locations of bats roosts, detectors and pheromone traps within the Rioja wine region (Southwestern Europe). LB= *Lobesia botrana*; SP= *Sparganothis pilleriana*.

## Insect pest surveys

We deployed delta traps baited with synthetic sex pheromone (ECONEX) in the interior of eight randomly selected grapevine patches to lure adult male LB and SP moths. Traps were placed one meter above the ground, and we checked them and replaced sticky inserts once every two weeks. We monthly substituted pheromone lures following the manufacturer's instructions. In our analysis, we also analysed

data from additional LB traps deployed and surveyed at a regional scale by a public agency. Overall, we obtained 35 and eight data points for LB and SP, respectively, in each sampling time (Figure 1).

## Bat surveys

We placed eight automatic ***D500X*** ultrasound detectors (Pettersson Elektronik AB) alongside the moth traps (Figure 1) to passively record the first four hours of bat activity after sunset (4896 recording hours in total). We set the detectors to record 3-second-long sequences at 300 kHz sampling rate and very high sensitivity. The location of detectors was stationary throughout the study period. We measured the number of bats passes for every two weeks and site (bat activity) to indicate relative bat activity (Walsh ***et al.***, 2004). We scanned recorded files with filtering software (***Kaleidoscope*** v. 4.5.4, Wildlife Acoustics), discarding files with ≤2 bat calls, insect noise, and non-biological sounds such as rain (Rydell ***et al.***, 2017). Then, we visually identified bat calls to the species level using ***BatSound*** v. 4.0.3. (Pettersson Electronic, AB) based on identification criteria by Barataud, (2015), Russ (2012), and Russo and Jones (2002), and grouped species according to foraging guilds following Denzinger and Schnitzler (2013): open space foragers (bats that exploit airborne insects flying far from the background, e.g., *Tadarida teniotis*, *Nyctalus* spp., *Eptesicus serotinus*), edge space foragers (bats that exploit the airborne prey near the edges of vegetation, above the ground and water surfaces, e.g., *Barbastella barbastellus*, *Miniopterus schreibersii, Hypsugo savii* and *Pipistrellus* spp.) and narrow space foragers (bats that prey upon insects positioned on or close to vegetation or the ground, e.g., *Rhinolophus* spp., *Plecotus* spp. and *Myotis* spp.). Several bats, such as some *Myotis* spp. and *Plecotus* spp., could not be reliably identified to species, and thus, they were classified at the genus level. Likewise, *Pipistrellus kuhlii* and *P. nathusii* exhibit similar call structures and overlap in spectrogram shape and sound frequency. Nevertheless, the former is relatively common and abundant, whereas the latter has only been recorded on very few occasions in the region (Aihartza and Garin, 2002). Therefore, these calls were assigned to *P. kuhlii*. Similarly, identifying calls of *Nyctalus leisleri* and *N. noctula*

was not always feasible; thereby, some bat passes were identified as *Nyctalus* spp. Finally, given the overlap in call structure and frequency of maximum energy between *Pipistrellus pygmaeus* and *M. schreibersii* as well as between *N. leisleri* and *E. serotinus* (Papadatou ***et al.***, 2008; Russo and Papadatou, 2014), several passes were classified as sonotypes *P. pygmaeus-M. schreibersii* and *N. leisleri-E. serotinus*. We also counted the "feeding buzzes" (calls emitted by bats before tackling the prey) as a measure of bat foraging activity (Gillam, 2007).

## Faecal sample collection

We identified 13 maternity roost sites exclusively inhabited by one of the following bat species: *Rhinolophus hipposideros* (three roosts), *P. kuhlii* (three), *P. pipistrellus* (six) and *Myotis daubentonii* (one). We placed collecting sheets beneath bat colonies and gathered faecal droppings every two weeks during the roost occupancy period through the grape growing season (Table S2.1.). Collecting sheets were replaced after every two weeks. Bat droppings were kept in 15 ml tubes, dried at 40˚C, and then stored frozen at -80˚C until processed. For analysis, an average of 24.2 (±5) pellets was pooled per colony and two-week period in each sample. Nevertheless, we included more samples in those periods where pest outbreaks occurred, gathering 439 samples in total (Table S2.1.). Finally, we homogenised each sample in a buffer solution before DNA extraction. No animal ethics clearance was required for this study because samples were passively and non-invasively collected, not involving manipulation of endangered or legally protected species.

## DNA extraction, library preparation and sequencing

DNA was extracted from samples using the ***Dneasy PowerSoil Kit*** (Qiagen) following the manufacturer's protocol with a few modifications (see Baroja ***et al.*** 2019a, b). We included extraction blanks in every extraction round. We used a combination of two cytochrome oxidase I gene (COI) primer sets to PCR amplify DNA from each sample to reduce primer-specific taxonomic bias (Elbrecht and Leese, 2015, Aldasoro ***et al.***, 2019). The first primer set, hereafter called "Zeale", targeted arthropod prey DNA (Zeale ***et al.***, 2011). The second, henceforth called

"Gillet", amplified both bat and prey DNA sequences (Gillet ***et al.***, 2015). For the amplification process, we followed the Qiagen 2X kit protocol using 12.5 µL ***Qiagen Multiplex PCR kit*** 2X, 1.25 µL forward primer (10 µM), 1.25 µL reverse primer (10 µM), 8 µL H2O and 2 µL DNA for a total volume of 25 µL for each sample and primer set. Each primer set was subjected to different PCR cycling conditions (Table S2.2). Then, PCR products were migrated in agarose gel electrophoresis to test the efficiency and homogeneity of amplification. PCR negative controls were included in every amplification round, and all blank extractions were checked for contamination. Amplicons were bead-purified with ***CleanPCR*** kit (CleanNA, PH Waddinxveen, The Netherlands). A second PCR reaction was performed to assign a unique dual combination of tags and Illumina sequencing adapters to the amplicons, using the ***Nextera XT Index Kit***, following the guide for metagenomics sequencing library by Illumina (Illumina, 2013) with some modifications. Once indexed and adapters attached, samples were bead-purified, fluorometrically quantified, and pooled at equal molarities to finally sequence in an Illumina ***MiSeq*** with 5%.

DNA library construction and sequencing processes were done at the Genomics and Proteomics General Service (SGIker) of the University of the Basque Country.

## Bioinformatic procedures

After sequencing, paired-end reads were merged and quality-filtered and primers removed using ***Usearch*** v.10 (Edgar, 2010) and ***Cutadapt*** (Martin, 2011). Sequences in samples identical to those in the corresponding extraction blanks were removed and the remaining sequences clustered into haplotypes using ***USEARCH's*** - *fastx_uniques* command. Singletons and chimaeras were discarded, and the remaining haplotypes collapsed into zero-radius operational taxonomic units (ZOTUs), an amplicon sequencing error-correction method used to infer accurate biological templates sequences (Edgar, 2016). We manually assigned taxon to ZOTUs by comparing them against the reference Barcode of Life Database, BOLD (Ratnasingham and Hebert, 2007). We used the taxonomic assignment criteria of Razgour ***et al.*** (2011) with slight modifications as follows: a) when query sequences matched to a single reference species above 98.5% similarity value, we assigned it to

such species; b) when the haplotype coincided with more than one species (> 98.5%) belonging to the same genus, we ascribed it to the genus-level, and c) when it matched to several species of different genera (>98.5%), we only included those present in the Iberian Peninsula. Only arthropod DNA sequences were considered as potential prey items. Afterwards, we calculated the % frequency of occurrences (%FOO) and percentage of occurrences (POO) of diet content for each sampling period and bat species. The former refers to the number of samples that contain a given food item divided by the total number of samples multiplied by 100. The latter is %FOO, rescaled so that the sum across all food items is 100% (Deagle ***et al.***, 2019).

## Data analysis

### Spatio-temporal dispersion patterns of pests

On the one hand, we evaluated the distribution patterns of pests via the Morisita`s index and the Standardized Morisita index proposed by Morisita (1959) and Smith-Gill (1975), which estimates the degree of spatial dispersion and intraspecific aggregation level of populations. Hence, we studied clumpiness of SP and LB populations, for which we used *dispindmorisita ()* function of the *vegan* package in R (Oksanen ***et al.***, 2007). The index values ranged from -1 to 1 (< -0.5= uniform; -0.5≤ ≤0.5= random; > 0.5= aggregated). On the other hand, given the spatio-temporal variation of insect populations (Hassel ***et al.***, 1991), we estimated the recommended sample size (number of insect traps; from Krebs 1999, equation 7.17) in every two weeks under different levels of precision (defined as the closeness of repeated measurements to the same item, Krebs 1999) for the densities of LB in the study area. We did not perform the sample size estimation analysis for SP due to the low number of traps.

### Distance buffers

We created distance buffers around each recording station (0 km – "local"–, 2 km – "short"–, 5 km – "medium"–, 10 km – "long" – and whole study area – "regional"–) and

bat roost (5 km – "medium"–, 10 km – "long"– and whole study area – "regional"–) using QGIS version 3.0.3 (QGIS Development Team, 2018). These buffers were used to estimate the relative mean abundance of LB and SP within the area surrounding each monitoring location, to decipher the bat response to grape pest abundances at different spatial scales. The scales analysed were limited by the number of traps within each buffer.

## Statistical analysis

All the statistical analysis were carried out in R Studio v.1.2.5042 and R v.3.6.1. (R Core Team, 2019). Modelling the responses of predators to prey density fluctuations over spatio-temporal scales entails methodological issues, such as spatial and/or temporal dependency structures that must rigorously be regarded (Zuur ***et al.***, 2017). Not accounting for spatio-temporal dependencies results in biased parameter estimates and p-values (Zuur ***et al.***, 2017), which may lead to erroneous conclusions (Carroll and Pearson, 2000). Current statistical tools to deal with spatial and temporal correlation structures of binary or count data are rather limited in a frequentist setting (but see g*lmmPQL* and *GLMM* functions of *MASS* [Ripley ***et al.***, 2013] and *lme4* [Bates ***et al.***, 2018] packages, respectively). To overcome these issues, we modelled the spatio-temporal dependency of our data through a Bayesian approach based on the Integrated Nested Laplace Approximation (INLA, Rue ***et al.***, 2009). We used *R-INLA* package in R (Bakka ***et al.***, 2018), which has proven very useful as multiple tools capable of handling with dependencies are available for spatio-temporal models.

We carried out the analyses on the four species that constitute the bulk of the bat activity in the study area (i.e., *P. kuhlii*, *P. pipistrellus*, *Hypsugo savii* and *T. teniotis*, totalling 89% of bat calls). We tested for the associations between the measured bat species' activity and relative mean abundance of pests (i.e., LB and SP) at the different distance ranges (0 km, 2 km, 5 km, 10 km and overall region). We checked different plausible distribution models for count data (Poisson and negative binomial) and selected them through DIC and WAIC values (Zuur ***et al.***, 2017), overdispersion check and model residuals' graphical plots for visual comparison of

distributions. We included "site" (vineyard plot) as random effect and temporal correlation was modelled as a function of sampling date, adopting a random walk term of the first order, to account for spatial and temporal dependencies, and modelled the bat activity pattern with a negative binomial distribution, a log link and diffuse or uninformative priors. We further tested for spatial and temporal autocorrelation in the residuals of the selected models using ***variogram ()*** and ***acf()*** functions of the respective ***gstat*** and ***stats*** R packages (Pebesma and Heuvelink, 2016; R Core Team, 2019). Concerning the relationship between the abundance of pests at each distance category (5 km, 10 km and overall region) and their consumption frequency by bats, we included "site" (bat roost) as a random effect and modelled the relationship with a binomial distribution, a logit link and diffuse priors. Nonetheless, some ordinary binomial models had an excess number of zeros (e.g., presence of LB in the diet of *R. hipposideros*) and in such cases, zero-altered (or hurdle) binomial (ZAB) models were implemented (Yee, 2015). Hurdle models are based on the assumption that zero counts are generated from a different process (binary) than are positive counts (Hilbe, 2011). The binary component is generally estimated using a Bernoulli distribution model on the presence and absence data whereas the positive count component is estimated using a zero-truncated binomial distribution model on the non-zero data (Hilbe, 2011, Zuur ***et al.***, 2017). For the rest, we checked for spatio-temporal dependencies as described above. In line with the **Bayesian framework**, we assessed the support for each parameter in the models by examining the 95% credible intervals. Precisely, we considered as important or significant the covariates whose coefficient density distributions did not contain zero between quantiles 0.025 and 0.975 in their intervals.

# Results

## Bat activity

We recorded 9440 passes of at least 17 species (Table 1) and 400 feeding buzzes. Most of the recordings belonged to open and edge space foragers such as *P. kuhlii* (42%), *P. pipistrellus* (32%), *H. savii* (11%) and *T. teniotis* (4%), which showed the highest

foraging activity levels (Table 1). To a lesser extent, we also recorded *Nyctalus lasiopterus*, *N. leisleri*, *N. noctula*, *E. serotinus*, *M. schreibersii*, *P. pygmaeus*, *B. barbastellus*, *R. ferrumequinum*, *R. euryale* and *R. hipposideros*, as well as some bats from *Myotis* and *Plecotus* genera. The highest bat activity levels occurred during June and July, along with the pests' major outbreaks (Figure 2). For the rest of months, bat activity remained at lower levels, except for *P. pipistrellus*, with an activity peak during LB third generation in August.

**Table 1.** Overall bat activity (number of bat calls) and number of feeding buzzes in the study area. Numbers in brackets correspond to the percentage of the total bat activity.

| Bat taxa | Bat calls (%) | Feeding buzzes |
|---|---|---|
| **Open space foragers:** | | |
| *Nyctalus* spp.* | 198 (2) | 1 |
| *T. teniotis* | 342 (4) | 6 |
| **Edge space foragers:** | | |
| *P. kuhlii* | 3941 (42) | 179 |
| *P. pipistrellus* | 3047 (32) | 163 |
| *P. pygmaeus* | 59 (<1) | 2 |
| *H. savii* | 1021 (11) | 30 |
| Others | 55 (<1) | 1 |
| **Narrow space foragers:** | | |
| *Myotis* spp. | 99 (<1) | 0 |
| *Plecotus* spp. | 178 (2) | 0 |
| *Rhinolophus hipposideros* | 20 (<1) | 0 |
| Other *Rhinolophus* | 4 (<1) | 0 |
| **Sonotypes:** | | |
| *P. pygmaeus-M. schreibersii* | 479 (5) | 18 |
| **Total bat activity** | **9440** | **400** |

*We included the sonotype *N. leisleri-E. serotinus*.

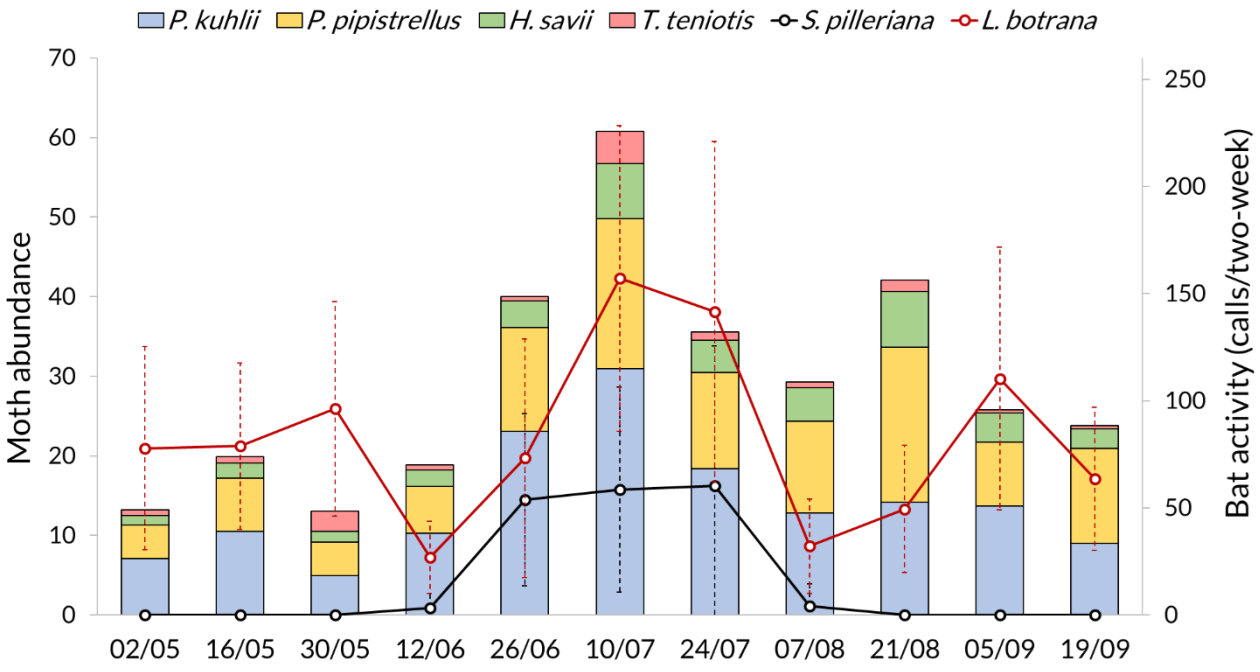



**Figure 2.** Two-week mean abundance values of *L. botrana* (three generations) and *S. pilleriana* (single generation, July) (left Y-axis) and mean of the bat activity (right Y-axis) from May to September 2017.

## Diets of bats

Overall, 5550 ZOTUs were generated from DNA extracted and successfully amplified belonging to 436 faecal samples. Out of them, 2724 corresponded to 969 arthropod species from 14 orders (Table S1.1.). The dietary spectrum of the four bat species was dominated by Lepidoptera and Diptera, which accounted for most of the identified ZOTUs and their occurrences (Figure 3). Among the most regularly consumed species *Emmelina monodactyla*, *Aproaerema anthyllidela*, *S. pilleriana*, *Metzneria hilarella*, *Cydia fagiglandana* or *Agrotix trux* prevailed within Lepidoptera whereas *Culex pipiens* and *Psychoda albipennis* predominated amongst Diptera (Figures S2.1., S2.2., S2.3. and S2.4.). Ephemeropteran species diversity was poor but a few species, such as *Choroterpes picteti*, *Ephoron virgo*, *Caenis luctuosa or C. pusilla* were rather frequent (Figures S2.1., S2.2., S2.3. and S2.4.),

primarily in the diet of *M. daubentonii*, whose second most consumed prey were ephemeropterans (Figure 3).

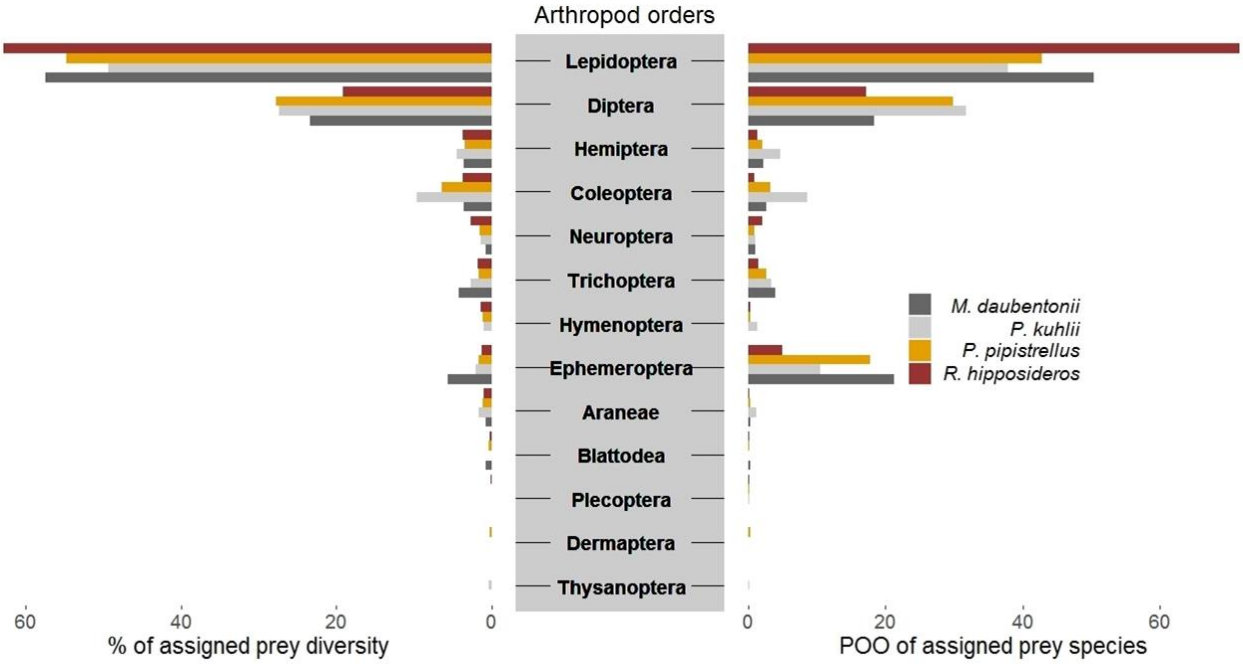



**Figure 3.** Percentage of assigned prey diversity and percentage of occurrence (POO) of prey orders in the diet of each bat species.

The rest of orders were recorded at a much lower frequency (<10%) but yet, again, some species were frequently preyed upon by some of the bat species — *Hydropsyche exocellata*, *Lepidostoma hirtum* (Trichoptera), *Ophonus ardosiacus* (Coleoptera) or *Psammotettix confinis* (Hemiptera) (Figures S2.1., S2.2., S2.3. and S2.4.). We did not find hymenopterans in *M. daubentonii* nor blattodeans in *P. kuhlii* and some other taxa were only detected in a single bat species' diet: for instance, Thysanoptera was only consumed by *P. kuhlii;* and Dermaptera solely by *P. pipistrellus*. In addition, during LB and SP main outbreak period (June-July) some non-pest arthropod species appeared frequently in the diet of bats (Figures S2.5., S2.6., S2.7. and S2.8.). A few samples contained non-prey DNA traces —including human (*Homo sapiens*), mouse (*Apodemus sp.*), rat (*Rattus sp.*), fungi (Eurotiales, Mucorales, Pythiales), bacteria (Rickettsiales, Xanthomonadales) and mite (Mesostigmata, Sarcoptiformes, Trombidiformes) —, which were considered

environmental pollution. Sequences in blank samples corresponded mainly to potential prey taxa (Table S2.1).

### Spatio-temporal distribution of pests and sample size estimation

The spatial distribution of LB and SP showed some degree of aggregation (Morisita index > 1 with $p < 0.0001$ and Standardized Morisita index > 0.5; Table S2.3.) with the south-eastern corner of the study region as the main spot of pest abundance. Nevertheless, the locations of moth hotspots partially changed over time (Figures S2.9., S2.10., S2.11., S2.12. and S2.13.). The sample size estimation analysis revealed that the number of traps required for estimating the population density of LB varied with time and, as expected, increased abruptly at greater precision levels (Figure S2.14.). The power analysis suggested that the 35 traps used across all study sites encompassed pest densities with precision values between 30% and 50%, respectively. About 92 to 282 traps would be needed for a precision of 20% depending on mean and variance data for every two weeks (Figure S2.14.).

## The response of bats to grapevine pests

### Aggregational response

The aggregational response of the bats to LB and SP abundance measured over the various distance buffers from detectors was generally weak (Table S2.4.). Greater SP densities triggered a significant increase in the activity of *P. kuhlii* (95% CI: 0.003, 0.045) and *H. savii* (95% CI: 0.004, 0047) at the local scale. For the rest of the bats, we did not find any effect within any distance range (Table S2.4.). Besides, *P. kuhlii* activity increased when LB abundances raised over two, five and ten kilometres (95% CI: 0.004, 0.027; 95% CI: 0.005, 0.031; and 95% CI: 0.003, 0.029, respectively) but not at the local (95% CI: -0.003, 0.022) and regional scales (95% CI -0.008, 0.068).

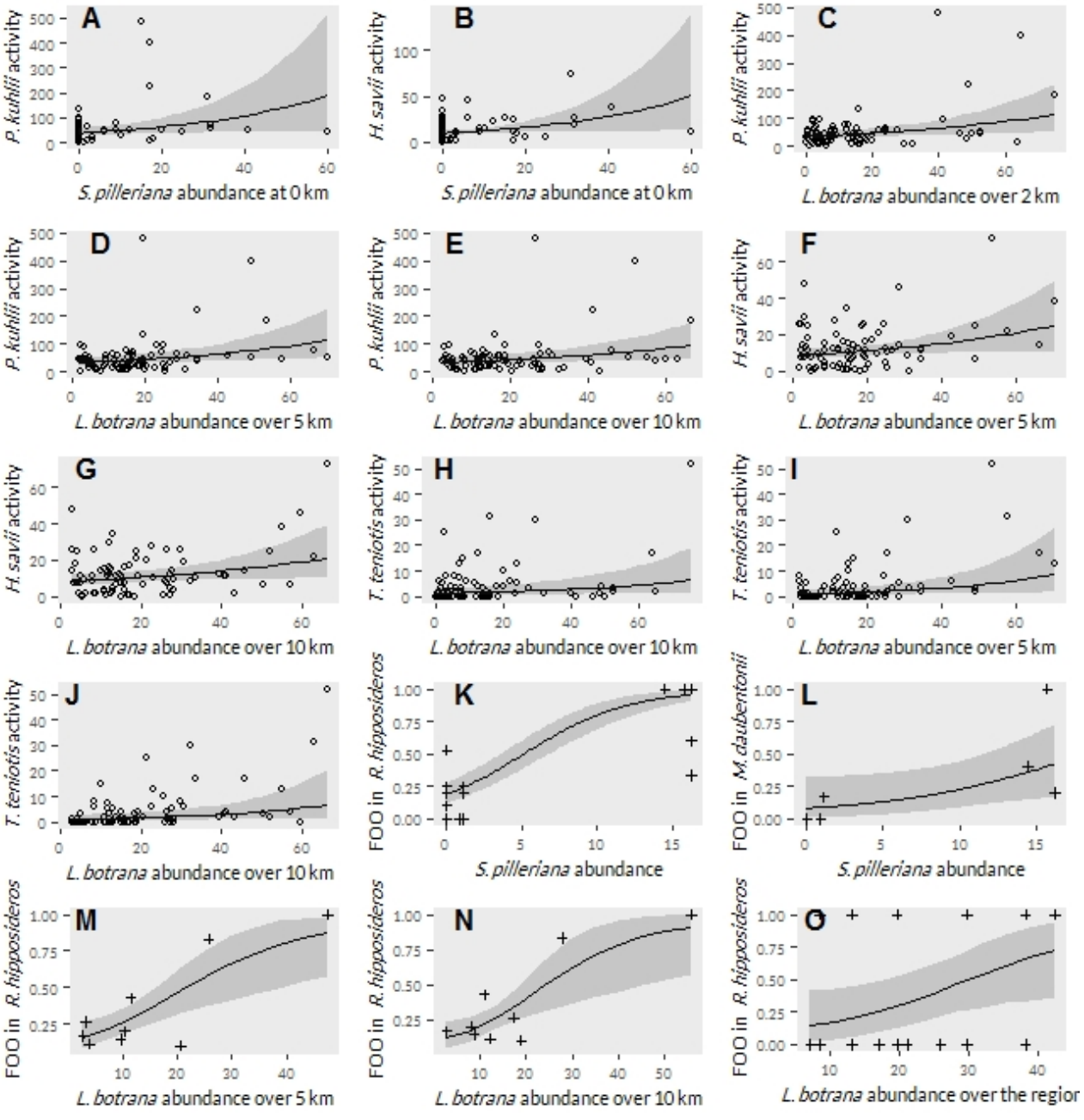



**Figure 4.** Relationships between the density of *L. botrana* and *S. pilleriana* over different distance buffers and the activity of bats (number of calls/two-week, A-J) as well as pest abundances against their frequency of occurrence (FOO) in the diet of *R. hipposideros* and *M. daubentonii* (K-O).

Similarly, *H. savii* and *T. teniotis* exhibited positive activity responses at low, medium and long scales (*H. savii* at 5 km, 95% CI: 0.002, 0.030; 10 km, 95% CI: 0.004, 0.029; and *T. teniotis* at 2 km, 95% CI: 0.004, 0.036; 5 km, 95% CI: 0.008, 0.05; 10 km, 95% CI: 0.002, 0.049) but not at the local (*H. savii*, 95% CI: -0.002, 0.029; *T. teniotis*, 95% CI: -0.011, 0.035), low (only *H. savii,* 95% CI: -0.001, 0.021) and regional scales (*H. savii,* 95% CI: -0.017, 0.031; *T. teniotis*, 95% CI: -0.093, 0113). By contrast, we could not find such effect within any distance range in the activity of *P. pipistrellus*. Plots of models with significant effects of pest densities are shown in Figure 4.

## Functional response

DNA from SP was found in all surveyed bat species and it was detected in 33% of all bat species samples: in 10% of *P. kuhlii*, in 18% of *P. pipistrellus* and *M. daubentonii,* and even in 56% of *R. hipposideros*, where SP reached one of the highest frequency occurrences of all prey taxa in its diet (Figure S2.1.). Further, during the SP outbreak (July 2017) we found the highest frequency occurrences of SP in the bats' diet: for instance, 95% of R. *hipposideros* faecal samples contained the pest, 36% of *M. daubentonii*, 22% of *P. pipistrellus* and 20% of *P. kuhlii*. In some instances, bats also consumed SP when low or null abundances of it were recorded in traps (Figure 5). All but *M. daubentonii* consumed LB, but only 10% of overall samples contained it: FOO ranged from 3.7% (*P. pipistrellus)* to 13% (*P. kuhlii*) and 18% (*R. hipposideros*). Amid the first generation of LB, we did not detect it in bat faeces, but during the second generation (Figure 5) FOO of LB raised to 27% in *R. hipposideros*, 17% in *P. kuhlii* and 5% in *P. pipistrellus*. Lastly, during the third-generation consumption rates dropped to 13% in *R. hipposideros*, 6% in *P. pipistrellus* and zero in *P. kuhlii*. We observed a strong association between the abundance of SP and its FOO in the diets of *R. hipposideros* (95% CI: 0.22, 0.37) and *M. daubentonii* (95% CI: 0.01, 0.33), but not in *P. kuhlii* (95% CI: -0.10, 0.66) and *P. pipistrellus* (95% CI: -0.01, 0.10). Further, the zero-truncated binomial distribution model showed an important association between the occurrence of LB in the *R. hipposideros* diet and its abundance at the medium (95% CI: 0.04, 0.15) and long scales (95% CI: 0.04, 0.17) but not, though, at the regional scale (95% CI: -0.02, 0.07). Nevertheless, the probability of presence of LB in

the diet increased when greater numbers of LB were measured at regional scale (95% CI: 0.02, 0.17). On the contrary, we found no important effect of LB availability in *P. pipistrellus* diet within any distance scale (medium, 95% CI: -0.03, 0.07; long, 95% CI: -0.04, 0.07; regional, 95% CI: -0.05, 0.07). Finally, the model on *P. kuhlii* did not fit the data, probably due to limited sample size. Plots of models with significant effects of pest abundances are shown in Figure 4.

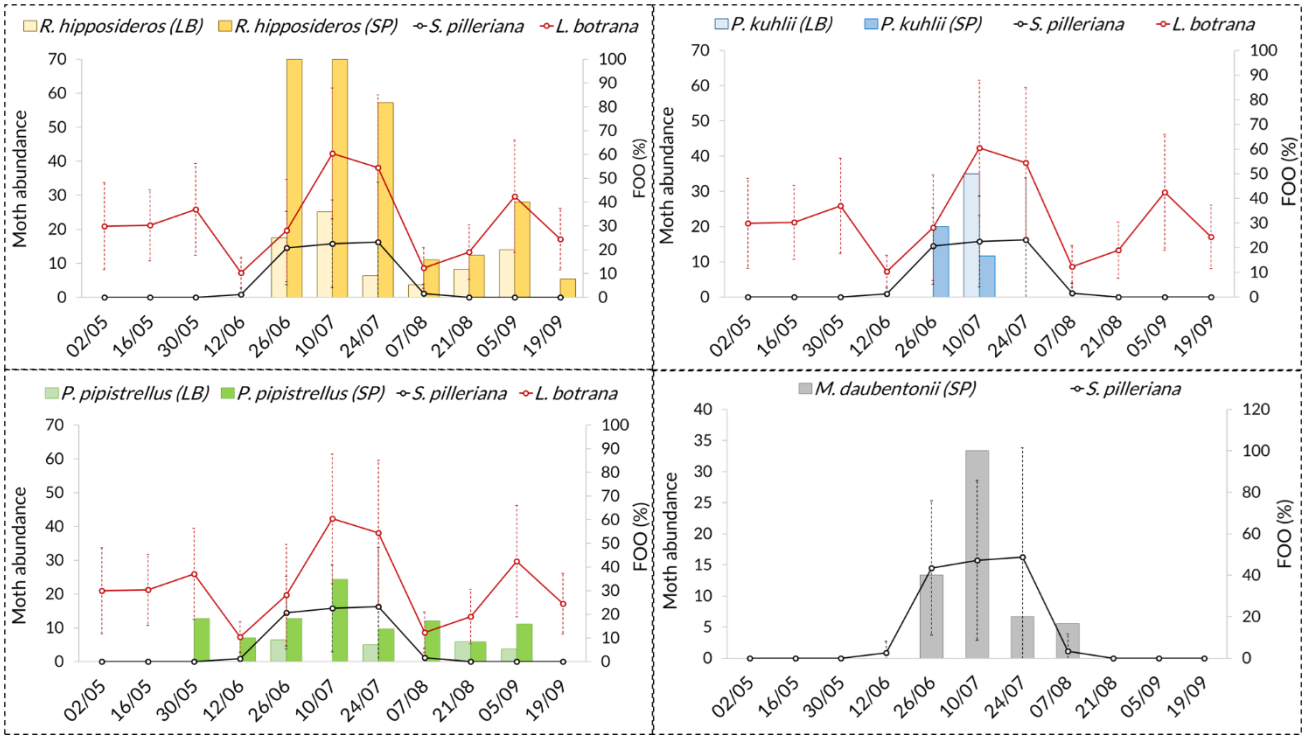



**Figure 5.** Two-week mean and standard deviation (SD) of abundance values of *L. botrana* and *S. pilleriana* (left Y-axis) and their % frequency of occurrence in the bats' diet (right Y-axis) from May to September 2017. Consumed pest is shown in brackets.

## Discussion

We provide proof of different predation rates and responses by a bat ensemble on two economically important pests of grape through their seasonal fluctuations. The observed pattern suggests that not all bat species contribute equally to that response. Based on our findings, *R. hipposideros* were the primary bats that prey

upon of SP and LB moths. Their diet showed a much greater frequency of pest moths than any other diet of the bat species inspected. Moreover, the consumption rates of both grape pests were positively associated with their abundances in vineyards, indicating that *R. hipposideros* tracked SP and LB densities. Besides, other insectivorous bats such as *P. kuhlii, H. savii, T. teniotis* and *M. daubentonii* were also responsive to at least one of the grape pests, and they are likely to exert predation pressure on the adult stages of moths.

## Spatio-temporal insect dynamics

Our results indicated that population dynamics of LB and SP within the study area showed an aggregated distribution like previously pointed by Sciarretta ***et al.***, (2008) and Peláez ***et al.***, (2006), with the south-eastern corner of the study region showing the highest density hotspots. These spatially structured patterns may likely answer to habitat suitability, due to slight local variations in temperature, humidity and wind strength or direction, which have a direct influence on the larval development and adult emergence (Weiss ***et al.***, 1993; Rank ***et al.***, 2020). In fact, the clumped pattern of pest populations leads to an exponential decrease in the provided level of precision and a concomitant increase in the required sample size (Subramanyam and Harei, 1990). The number of traps used in our study did not enable precise population density estimates. As many as 282 traps would be needed for accurate population calculations at the regional scale. The relatively low detection ranges of pheromone traps, together with the spatial heterogeneity of LB populations, highlight the need to augment the monitoring efforts by wine producers and public agencies. Uncertain spatiotemporal estimates of pest populations may lead to the application of misleading pest control treatments, resulting in the loss of human and economic resources. For our research purposes, though, such high degree of certainty was not critically necessary, especially considering that the number of traps used appropriately matched the number of expected generations of pests and their corresponding peak and slack periods with previous observations in the region (Ortega-Lopez ***et al.***, 2014).

## Bat-pest interactions

The highest overall bat activity was found during mid-summer (July), co-occurring with the peaks of SP and LB (second generation) moth emergence. This increase in the bat activity, along with the positive aggregational response of some species (e.g., *P. kuhlii*, *H. savii*, and *T. teniotis*) to pest abundances, suggests bat species of different ecological and morphological features plausibly track abundances of SP and LB moths. Nevertheless, we did not observe strong species-specific responses and therefore, we claim that distinct bat species may synergistically play a role at suppressing grape pests, rather than the action of specific-species alone. The diet confirmed the consumption of both grape pests by at least some of the bat species, which primarily consumed them during pest irruptions, and thereby strengthened the role of bats as pest suppressors. Within the bat assemblage that we investigated, our results confirm *R. hipposideros* as the major candidate pest suppressor (Baroja ***et al.***, 2019a), primarily because pest consumption frequencies were greater than for the other bat species, and there was a strong association between the abundances of SP and/or LB and their frequency of occurrences in its diet. *R. hipposideros*, a narrow space hunter, typically flies close to the vegetation in cluttered environments, which suggests that SP and LB encounters may preferentially occur on the crop canopy, or between grapevine rows at or close to ground level. Conversely, despite the increased activity levels of *P. kuhlii* during grape pest irruptions, it showed low consumption frequencies of grapevine pests. Previous work defined *P. kuhlii* as an opportunistic predator, feeding upon a vast array of prey taxa (Goiti ***et al.***, 2003; Cohen ***et al.***, 2020). As a consequence, prey and pests other than SP and LB that are also plentiful in agroecosystems (e.g., *Culex* spp. mosquitoes, Puig-Montserrat ***et al.***, 2020) may contribute significantly to this bat´s diet.

*Pipistrellus pipistrellus* was the second most active bat in vineyards overall, but we neither observed an elevated activity during pest irruptions nor a responsive behaviour to their abundance. Accordingly, the species did not functionally respond to grape pests. This may relate to various factors: firstly, *P. pipistrellus* is a synanthropic bat that forages in a wide variety of habitat types (Russ and Montgomery, 2002), including urban areas, treelines and forest and park edges, some

of which were rather abundant just around the roosts; secondly, the species forages over relatively short distance ranges (< 3 km) from roosts (Davidson-Watts and Jones, 2006). Further, we found aggregated dispersion of grape moths and, thus, *P. pipistrellus* populations might not reach pest-abundant but distant areas. Besides, our findings on its diet composition revealed some ephemeropterans (e.g., *Choroterpes picteti, Ephoron virgo, Caenis luctuosa and Caenis pusilla*) and dipterans (e.g., *Psychoda albipennis* and *Paramormia ustulata*) being frequent prey, especially during the LB and SP outbreak period from July. Finally, we found DNA traces of SP in *P. pipistrellus* faeces 2-4 weeks earlier and after the peak of the pest recorded by our traps, which suggests that the number of traps used to monitor SP did not entirely capture neither the temporal nor the spatial dynamics of the regional population.

*Myotis daubentonii* predominantly forages in riparian habitats or in the nearby (Swift and Racey, 1983), generally not farther than a few kilometres away from the roost (Nardone ***et al.***, 2015). Previous research found mostly Diptera, but also Trichoptera and Lepidoptera as their prevailing food items (Vesterinen **et al.,** 2013; 2016). Thus, given the fondness for riparian environments and the feeding habits of *M. daubentonii*, we would expect low or null encounter rates with detrimental insects flying over grape clusters. However, the species exhibited a functional response to SP densities and consumed the pest during the moth outbreak (mid-summer). Given there were grapevines nearby the roost surrounded by the Ebro River, *M. daubentonii* would likely prey upon SP over water or in the vineyards adjacent to water bodies during its burst. *Tadarida teniotis* also exhibited pest density-responsive behaviour. Although we could not survey its diet, *T. teniotis* is a typical open space hunter that forages in open areas. While females primarily consumed large migratory moths that fly at high altitudes, males fed on smaller but more abundant sedentary moths flying closer to the ground than females (Mata ***et al.***, 2016) where encounters with swarms of LB are more likely to occur. Even if the intense narrowband and low-frequency echolocation calls of *T. teniotis* appear to be a specialization for long-range detection of relatively large insects (Rydell and Arlettaz, 1994), they can also detect swarms of small-size insects (e.g., *L. botrana*) from long distances (Boonman ***et al.***, 2019).

Lastly, *H. savii,* a typical representative bat of the Mediterranean, has expanded northwards its distributional range, presumably due to temperature increase and the species' ability to settle in synanthropic environments (e.g., vineyards) (Uhrin ***et al.***, 2016; Ancillotto ***et al.***, 2018). Its ability to fly long distances in search of food, the capability to exploit locally abundant aerial prey sources and its generalist and opportunistic foraging requirements (Beck, 1995; Kipson ***et al.***, 2018), enable *H. savii* to take advantage of high concentrations of swarming insects, such as sudden pest irruptions. Accordingly, we should not disregard the potential contribution of *H. savii* as pest suppressor in vineyards. Unfortunately, we could not monitor its diet. The characteristic roosting behaviour of the species to commonly hide in rock crevices underline the intrinsic difficulties to monitor the diet of *H. savii*.

## Guilds

Bats of every foraging strategy, open (*T. teniotis*), edge (*H. savii, P. kuhlii* and *M. daubentonii*) and narrow (R. *hipposideros*) space foragers showed a response, at least in a certain degree, to pest abundances. Although we cannot confirm the consumption of grape pests by open space foragers, these bats are known to prey upon other pests that are usually observed close to vegetation (Garin ***et al.***, 2019). The predation by different bat guilds would synergistically suppress these pests' populations, narrow space bats preying upon the moths on the canopy and at ground level in the crop interior, and edge and open space foragers hunting in the crop edges or above the grapevine canopy, likely hampering pests from spreading and interrupting the infestation of new grape patches. Further research in the vertical aerial space use by pests and the temporal diet analysis of bats from as many members of foraging guilds as possible would offer a more precise picture of the interaction between bats and pests in crops.

## Distance buffers

Some bats are expected to respond to changes in prey communities at a more localised scale, while others are more likely to forage over larger hunting grounds

and respond to regional-scale changes. As such, *R. hipposideros*, a highly manoeuvrable bat, positively responded to densities of LB measured up to ten kilometres from the roost. Previous research though, showed that it generally covers shorter foraging distances from the roost. However, given that their congeneric species (*R. euryale* and *R. mehelyi*; Goiti ***et al.***, 2006; Salsamendi ***et al.***, 2012) are capable of travelling long foraging distances, it is likely that *R. hipposideros* can travel longer distances in search of suitable foraging areas than has hitherto been recorded. On the other hand, *T. teniotis, H. savii* and *P. kuhlii* exhibited responsive behaviours to SP and/or LB from local to large distance ranges, highlighting the flexible foraging behaviour of these highly mobile species to travel broad range distances in search of profitable areas (Marques ***et al.***, 2004, Uhrin ***et al.***, 2016; Ancillotto ***et al.***, 2018), potentially tracking sudden swarms of grape moths. Generally, the ability of a bat of any guild to suppress a given pest seemed to change with its spatial and foraging behaviour, particularly the distance to outbreaks and the availability of other profitable prey. For highly mobile animals like bats, foraging implies trade-off decision making between the energy gains foraging in prey-abundant areas, against the energy loss while commuting to such locations. In general terms, to promote ecosystem services provided by bats agri-environmental schemes should be oriented to multi-scale management planning (Kalda ***et al.***, 2015).

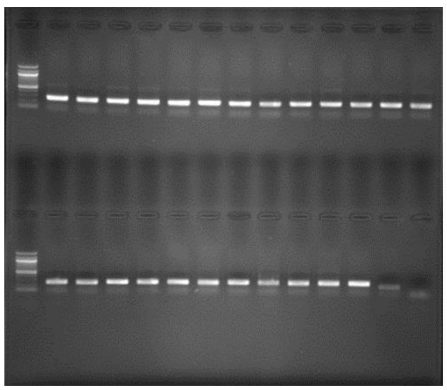
## Implications and future directions

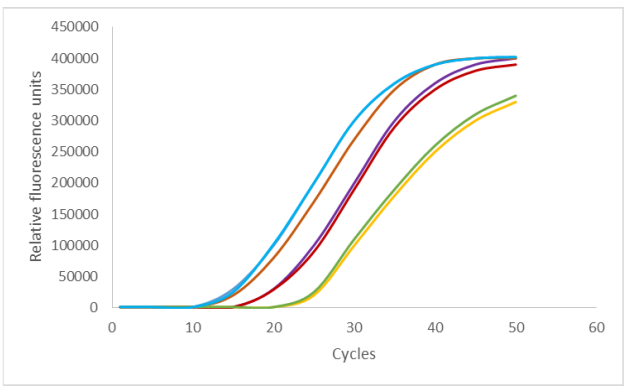
Generally, the ability of bats to aggregate and exploit grape pest irruptions but at the same time to persist and rely on diversified and alternative arthropods when pest numbers are low, confirms their opportunistic nature and highlights the pest suppression value of bats as generalist predators (Symondson ***et al.***, 2002; Snyder and Ives, 2003). Particularly, major damage levels are reached during the carpophagous phase (second and third generations of larvae) in which yield losses are especially critical (Moschos, 2006). Interestingly, bats might exert predation pressures mainly on the second generation of adult moths and therefore, they provide direct as well as indirect benefits to grapes. Estimating the economic importance of bats in vineyards is key to make policy-makers and the general public

understand about the ecological, economic and social benefits of insectivorous bats. As such, Rodríguez-San Pedro ***et al.***, (2020) by exclusion field experiments, reported direct evidence of a reduction in grapevine pest infections by bats and their benefits to wine production in vineyards located in central Chile. However, given that Europe represents almost 50% of the world's vine-covered area (Eurostat, 2017), further efforts to economically quantify the contribution of bats as pest suppressors in this crop must be also addressed in this continent. Conservation measures and management strategies to promote bat communities and their ecosystem services in vineyards must be thoroughly deliberated. This may be done, for instance, conserving native forest remnants and shrubs, strengthening habitat heterogeneity, promoting artificial wetlands and increasing artificial roosting opportunities in vineyards (Stahlschmidt ***et al.***, 2012; Kelly ***et al.***, 2016; Rodríguez-San Pedro, 2019). Further, more intensive agricultural activities entail lower pest predation pressure by bats (Aizpurua and Alberdi, 2020) and therefore, management strategies that favour environmental sustainability may magnify the ecosystem services of bats in farmland.

# Chapter 3

# Species-specific PCR methods as reliable pest monitoring tools: prey Detection and Quantification






An article on this Chapter was submitted for publication in *Scientific Reports*: Baroja, U., Garin, I., Vallejo, N., Caro, A., Ibáñez, C., Basso, A., Goiti, U. Molecular assays to reliably detect and quantify predation on a forest pest in bats faeces. *Scientific Reports.*

# Species-specific PCR methods as reliable tools for prey monitoring: pest detection and quantification


## Abstract

Targeted molecular methods such as conventional PCR (cPCR) and quantitative PCR (qPCR), combined with species-specific primers and probes, are widely applied for pest species detection. Besides, the potential of qPCR to quantify DNA in samples makes it an invaluable molecular tool to infer the predation levels on specific prey by analysing predators' stools. Nevertheless, studies on the diet of bats failed to find any empirical relationship, and it remains to be evaluated. Thus, we developed and evaluated two species-specific PCR assays to detect and quantify DNA of a major forest pest, the pine processionary, ***Thaumetopoea pityocampa,*** in bats' faeces. Further, we empirically compared a range of different known DNA concentrations (***input***) of the target species mixed with mocks and bat faecal samples against DNA abundances yielded by **qPCR** (***output***) for a quantitative assessment. Overall, **cPCR** showed a lower detection rate than qPCR, but augmenting the replicate effort from one to three replicates led to a greater increase in the detection rate of the cPCR (from 57% to 80%) than the qPCR (from 90% to 99%). The quantitative experiment results showed a highly significant correlation between the input and output DNA concentrations ($t=10.84$, $p<0.001$) with a mean slope value of 1.05, indicating the accuracy of our qPCR assay to estimate DNA abundance of *T. pityocampa* in bat faeces. The framework of this study can be taken as a model to design similar assays applicable to other species of interest, such as agricultural pests or insects of public health concern.


**Keywords**: *Thaumetopoea pityocampa*, Molecular assays, PCR, Bats, Pest Monitoring

## Laburpena

Metabarcoding eta PCR metodo espezifikoak, adibidez PCR konbentzionala (cPCR) eta PCR kuantitatiboa (qPCR), zunda eta hasle espezifikoekin batera, maiz erabiliak dira izurrien detekziorako. Horrez gain, qPCR-ak DNA kuantifikatzeko ahalmena du, eta horrek balio handia izan dezake, izan ere, harrapakarien gorotz-laginak analizatuz harrapakin espezifikoekiko duten harrapakaritza-maila kalkulatzeko erabil baitaiteke. Dena den, orain arte saguzarren dietan oinarrituta egindako ikerketa esperimentalek ez dute inolako harremanik aurkitu, eta beraz ebaluatzeke dago. Hori dela eta, lan honetan bi PCR entsegu espezifiko garatu eta ebaluatu genituen basoetako izurria den pinu-beldarraren, ***Thaumetopoea pityocampa,*** DNA saguzarren gorotzetan detektatu eta kuantifikatzeko. Gainera, ebaluazio kuantitatiborako, izurriaren DNA kontzentrazio jakinak ("***input***") intsektuen "***mock***" laginetan eta saguzarren gorotzetan nahastu genituen lehenengo eta ondoren, qPCR bidez lortutako DNA kontzentrazioekiko ("***output***") konparatu genituen. Orokorrean, cPCR-ak qPCR-ak baino detekzio-tasa baxuagoa erakutsi zuen, baina erabilitako erreplika kopurua batetik hirura emendatzean detekzio-tasa gehiago igo zen cPCR-an (% 57-tik % 80-ra) qPCR-an baino (% 90-etik % 99-ra). Saiakuntza kuantitatiboaren emaitzek adierazi zuten "input" eta "output" DNA kontzentrazioen artean korrelazio positibo esanguratsua dagoela ($t=10.84$, $p<0.001$) bi aldagaien erlazioaren maldaren balioa 1,05 izanik, aditzera emanez diseinatutako qPCR metodoaren zehaztasuna *T. pityocampa*-ren DNA saguzarren gorotzetan kuantifikatzeko. Hurbilketa molekularraren hautaketa (**metabarcoding, cPCR edo qPCR**) ikerketa-galderaren eta metodo bakoitzak dituen abantaila eta desabantailen arabera erabaki behar da. Ikerketa honetan garatutako modeloa eredutzat erabil daiteke interesekoak izan daitezkeen beste espezieen detekziorako, besteak beste nekazaritzako izurrien edota osasun kezka bilakatzen diren hainbat intsektuen jarraipenerako.

**Hitz gakoak**: *Thaumetopoea pityocampa*, Entsegu molekularrak, PCR, Saguzarrak, Izurrien monitorizazioa

## Introduction

The pine processionary moth, *Thaumetopoea pityocampa* (Figure 1), is a significant forest pest of coniferous trees in the Western Palearctic. The larvae not only defoliate trees causing significant growth reduction and leading to severe economic losses (Buxton, 1983; Gatto ***et al.***, 2009), but its urticating setae are also considered a risk to human and animal health (Battisti ***et al.***, 2017; Moneo ***et al.***, 2015). The species is rapidly responding to climate change prolonging its active period and spreading northward (Battisti ***et al.***, 2005; Kerdelhué ***et al.*** 2009, Robinet ***et al.***, 2013) Among its natural predators, insectivorous bats are common predators of adult moths (Auger-Rozenberg ***et al.***, 2015) with a vast array of bat species preying on *T. pityocampa* (Garin ***et al.***, 2019). These bat species increase their hunting activity at pine stand edges where *T. pityocampa* swarms (Charbonnier ***et al.***, 2014). Some of these bat species have a broad foraging range (Goiti ***et al.***, 2006; Russo ***et al.***, 2005; Vincent ***et al.***, 2010) being able to reach prey-abundant distant areas. During the breeding period, female bats increase their food intake imposed by pregnancy´s high energetic demands (Rydell, 1989), which coincides with the high resource availability period (e.g., outbreak of *T. pityocampa*). In summary, these flying mammals may precisely track the abundance of *T. pityocampa* and other species of interest.

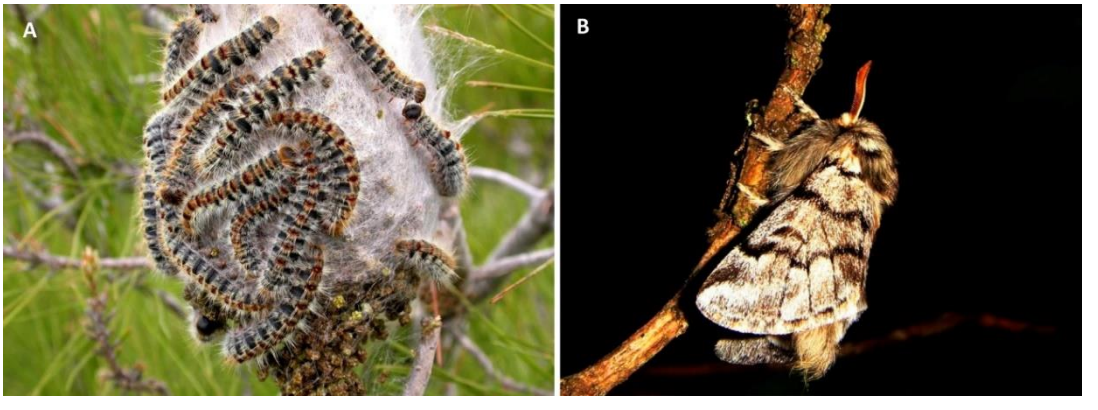



**Figure 1.** Larval (A) and adult stages (B) of the pine processionary moth, *Thaumetopoea pityocampa*. Photos by João Coelho and John H. Gent.

Current molecular techniques for the identification of bat prey in diet studies are primarily based on the metabarcoding of prey DNA in faeces (e.g., Aldasoro ***et al.***, 2019; Baroja ***et al.***, 2019; Vallejo ***et al.***, 2019). The high throughput sequencing of thousands of PCR amplicons in parallel renders this technique a powerful tool to study animals' diet with a high diversity of consumed taxa (Bohmann ***et al.***, 2011; Pompanon ***et al.***, 2012). Nonetheless, specialised laboratory equipment and personnel is required and the budget needed to process the data makes it impractical in many research settings. Moreover, metabarcoding brings an imbalanced amplification of the prey's DNA attributed to the differential affinity of the primer sets across taxa, which can potentially lead to false negative results of some prey (Elbrecht & Leese, 2017). Likewise, as most insectivorous bats have diverse diets, the large number of DNA sequences from multiple taxa binding to universal primers can potentially disguise the presence of other prey (Pompanon ***et al.***, 2012; Evans ***et al.***, 2016; Harper ***et al.***, 2018). In that sense, although it is tempting to assume that prey DNA sequence proportions or read counts recovered from faecal samples are representative of predator's diet proportions, digestion and amplification biases impair quantitative interpretations of the data, especially in samples containing many taxa (Deagle ***et al.***, 2019; Piñol ***et al.***, 2015). Consequently, faithful results of metabarcoding restricts to the qualitative assessment of the species list in the faecal samples. It is therefore desirable that cheaper, targeted approaches are developed for studies which aim to detect a single species. Whereby a single-species detection is aimed, DNA metabarcoding may fail to reliably capture the species of interest leading biased results. Alternatively, the combination of target species-specific primers with PCR-based methods provides greater detection than metabarcoding. While the species-specific primers only target the species of interest, metabarcoding uses universal primers that bind to multiple species' DNA, where more common templates are more likely to be amplified, potentially preventing the detection of rare species (Evans ***et al.***, 2016; Harper et a., 2018). Among targeted approaches, conventional PCR (cPCR) and real-time or quantitative PCR (qPCR) methods allow the amplification of minute amounts of template DNA even when the target is mixed with large amounts of non-target DNA, such as in animal scat [265]. (Jarman ***et al.***, 2004).

Work from several fields have succeeded in the direct detection of target species using targeted PCR methods from environmental samples, for instance, water (Piggot, 2016; Knudsen ***et al.***, 2019), stomach content (Jarman ***et al.***, 2004), predator identification from its scats (Kaňuch ***et al.***, 2007, Czernik ***et al.***, 2013), plant tissue (Martin and Rygiewicz, 2005) or soil samples (Nowakowska ***et al.***, 2017). When working with environmental DNA (eDNA) several factors directly influence target species' detection, primarily the low abundance genetic material and quality of samples. Hence, the detectability of a single species in environmental samples by PCR assays can sometimes lead to false-negative results (Schultz & Lance, 2015). In addition, choosing the proper PCR strategy may sometimes result in a hard striking decision since both assays have their benefits and drawbacks (Bott ***et al.***, 2010). Although cPCR is one of the most widespread and affordable molecular technique, it allows the amplification of a target sequence only at a qualitative level. In contrast, qPCR monitors the amplification process in real-time and hence it provides a qualitative and quantitative assessment of the aimed sequence, but at a much higher cost. Another determining factor is the number of replicates used. Increasing PCR replicates significantly improves the detection probability by reducing the risk of false negatives and yielding more reliable results (Schmidt ***et al.***, 2013). Making all these decisions (choosing PCR strategy or the number of replicates included) will involve a trade-off between the financial costs, logistical feasibility, and the risk of inaccurate results.

Despite the potential of qPCR to quantify DNA in samples, results obtained from these analyses have not yet shown correlations with the biomass of prey consumed by bats (McCracken ***et al.***, 2005; 2012). Indeed, several factors, such as degradation of dietary DNA during digestion or primer binding biases attributed to highly diverse prey diets, might hinder the potential of qPCR as a quantitative measure of predation.

In light of the above, the main goals of this chapter were to 1) develop rapid, easy and cost-effective PCR-based methods for detecting *T. pityocampa* in bat stools, 2) to compare the detection rates yielded by both cPCR and qPCR strategies along with different replicates and 3) to assess the accuracy of qPCR as a tool for estimating the abundance of *T. pityocampa* consumed by insectivorous bats.

# Material and methods

## Primer design

For cPCR, we designed specific primer sets to *T. pityocampa* from the 3' end of TRNK to the 5' of ATP8 regions of the mitochondrial DNA using PerlPrimer software (Marshall, 2004, see Table 1).

**Table 1.** Primer pairs and probes used in the study, the coding region, the primer sequence, the melting temperature, and the amplicon size.

| Primer name | Assay | Primers | Region | Sequence 5'-3' | Tm (°C) | Product (bp) |
|---|---|---|---|---|---|---|
| Tpit_cF | cPCR | $_c$F | TRNK-ATP8 | TCTAATGAAACTATTAACAC | 48 | 131 |
| Tpit_cR | | $_c$R | | ATAATAATCAATTAATGGGC | 48 | |
| Tpit_qF | qPCR | $_q$F | ATP6 | ATTATTCGACCCGGTACTTTGG | 62 | 89 |
| Tpit_qR | | $_q$R | | ATAACTCTCTTAAGAAGAACAGGACC | 62 | |
| Tpit_qP | | $_q$P | | ACGATTAACAGCAAACATAATTGCCGGAC | 68 | |

For that, we first downloaded all the available sequences from their congeneric species (*Thaumetopoea* spp., see Simonato ***et al.***, 2013) on ***GenBank*** (www.ncbi.nlm.nih.gov/genbank/) and BOLD (www.boldsystems.org). Then we aligned them using MAFFT (Katoh and Standley, 2013), and we identified binding sites. Finally, we verified primers' specificity *in silico* against available sequence data in the ***GenBank*** nucleotide database (www.ncbi.nlm.nih.gov/genbank/). For qPCR, we designed *T. pityocampa* specific primers and Taq-man probes optimised for qPCR reactions using the ***PrimerQuest*** Design Tool (Integrated DNA Technologies, www.eu.idtdna.com/) for the amplification of mitochondrial gene ***ATP6*** (Table 1) with the following custom parameters:

- Primers: melting temperature (Tm) of 62˚C, GC content of 50%, and 22 bp length.

- Probe: Tm of 68˚C, GC content of 50%, and 24 bp length.

The specificity of the assemblage of primers and probes suggested by the software was checked and verified *in silico* against available sequence data (**www.ncbi.nlm.nih.gov/genbank/**). We selected the primers, probe and amplicon sequences specific to *T. pityocampa* (100% similarity value, Table 1).

## DNA sampling

Primers were empirically tested in both fresh *T. pityocampa* samples and bat faecal samples to test their efficiency by both PCR techniques.

Fresh *T. pityocampa* male moths were collected from the Basque Country (Southwestern Europe) using pheromone-baited G-traps (Econex, Murcia, Spain). Traps were suspended from trees at the height of ~ 4 m. Additionally, we placed a couple of light traps during a single night to capture moths other than *T. pityocampa*. Insects were then collected and stored at -80 ˚C until processed. A couple of hind legs from individuals of *T. pityocampa* and other moth species were used for DNA extraction (Table S3.1.). The whole extraction process was carried out according to the protocol described by Aljanabi and Martinez (1997, Supplementary Information 1) with slight modifications. In fact, after washing the DNA pellets with 70% ethanol, we centrifuged them for 10 min, 4 ˚C at 13000 rpm, dried them at 60 ˚C for 30 min and resuspended them in 30 µL dd$H_20$ overnight. Likewise, we collected bat droppings in 2014, 2016, 2017 and 2018 underneath bat colony clusters of different species (*Myotis crypticus, M. daubentonii*, *M. emarginatus*, *Miniopterus schreibersii*, *Pipistrellus kuhlii*, *P. pipistrellus*, *Rhinolophus euryale*, *R. hipposideros*, *R. ferrumequinum-M. emarginatus* and *Tadarida teniotis*) throughout different Iberian Peninsula regions (Basque Country and Andalusia, Figure S3.1.). Stools were then dried at 40 ˚C and stored at -80 ˚C until processed. DNA from these faecal samples was extracted as explained in Baroja ***et al.*** (2019), and extraction products were stored at -20ºC. An aliquot from these DNA extracts was further analysed through metabarcoding according to the protocol described in Chapter 2. The aforementioned samples were classified as follows (for further information, see Table S3.1.):

1. *C+*, DNA samples from fresh *T. pityocampa* (positive controls),
2. *C-*, DNA samples from fresh moths other than *T. pityocampa* (negative controls),
3. *Th+*, DNA samples from bat faeces containing traces of *T. pityocampa* according to metabarcoding (sequences matching 98.5% similarity value (Razgour ***et al.***, 2011) with *T. pityocampa* were considered),
4. *Th-*, DNA samples from bat faeces in which traces of the pest were not found.

   Similarly, we aimed to evaluate whether the developed PCR assays are more sensitive than metabarcoding at detecting *T. pityocampa.* Thus, we divided *Th-* samples into two subgroups:

4.1 $Th_{out}$-, samples collected in non-forest habitats and out of the *T. pityocampa* flight period

4.2. $Th_{in}$-, samples collected within forest habitats throughout the *T. pityocampa* flight period.

## cPCR and qPCR

In the qPCR framework, A260/280 ratio is routinely used for purity assessment of DNA, which is an indication of its presence and quality (Page and Gomez-Curet, 2011). Impure samples have ratios outside an acceptable range and they are therefore discarded from the analysis. Thus, in in all, we selected 184 samples for analysis (*C+*, n=6; *C-*, n= 18; *Th+*= 96; *Th-*= 63), but only the samples meeting some purity criteria were retained (A260/A280 ratio 1.77±0.24; n= 169). We only kept quantification data from qPCR runs with $R^2$ values of ≥ 0.98 and slopes between 3.19 and 3.71, which indicated 86-105% efficiency of qPCR reactions.

## Qualitative assessment

To validate the detection efficiency of both PCR techniques, the size of each sample group was as follows (Table S3.1.):

- *C+*: 5 samples
- *C-*: 7 samples
- *Th+*: 83 samples
- $Th_{out}$-: 22 samples
- $Th_{in}$-: 21 samples

We assayed each sample in triplicate, and we included a negative PCR with each round. For cPCR, we compared several PCR protocols and master mixes in an initial pilot study, and finally, we selected the KAPA HiFi HotStart ReadyMix PCR Kit, which performed best. The amplifications were conducted in 25 µL containing 12.5 µL KAPA HiFi ReadyMix, 1.25 µL of each primer (10 µM), 9 µL Milli-Q $H_2O$ and 1 µL of template DNA per sample. The reaction conditions included: 95 ˚C for 3 min, followed by 10 cycles of 95˚C for 30 s, 50˚C (-0.5˚C/ cycle ramp) for 30 s and 72˚C for 30 s, 25 cycles of 95˚C for 30 s, 48˚C for 30 s, 72˚C for 30 s and ended by 72˚C for 10 min. PCR products were migrated with a DNA 5K Reagent kit in a LabChip GX Touch 24 Nucleic Acid Analyzer. For qPCR, before amplification processing, we quantified DNA from each sample by a NanoDrop 8000 and based on this quantification, we adjusted the DNA concentration to 10 ng/µL. Afterward, qPCR reactions were performed in a CFX96 Touch Real-Time PCR system (Bio-Rad) using 5 µL NZY qPCR Probe Master Mix (2x) mixture, 0.4 µL of each primer (10 mM), 0.1 µL of probe (10 mM), 3.1 µL nuclease-free $H_2O$ and 1 µL template DNA for a total volume of 10 µL. The amplification conditions were: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 s and 62˚C for 45 s. In order to test primers' specificity, some PCR products (cPCR, n= 5; qPCR, n=7) were purified and sequenced in forward and reverse directions using either an ABI3730XL or an ABI3700 Genetic Analyser (Applied Biosystems). The resulting forward and reverse sequences were assembled using BioEdit 7.1 (Hall, 2011) or Geneious 8.0.2 (Kearse ***et al.***, 2012) and checked for errors and ambiguities.

## Quantitative assessment

We assessed qPCR's quantification accuracy in two types of media: moths mocks and bat faecal samples (Table S3.2.). We prepared mock mixtures composed of DNA

from combinations of seven common prey species of bats (*Lobesia botrana* and *Cydia funebrana* [Tortricidae], *Synthymia fixa*, *Catocala nymphagoga*, *Lymantria dispar*, *Noctua pronuba*, *Eremobia ochroleuca* [Noctuidae]) with equal amounts of DNA. We also incorporated faecal samples collected in non-forest habitats in which DNA traces of *T. pityocampa* were not found by metabarcoding ($Th_{out}$-) belonging to different bat species (i.e., *Rhinolophus hipposideros*, *Pipistrellus pipistrellus*, *P. kuhlii*, *Myotis daubentonii*, Figure S3.1), to include DNA of a broader range of prey associated with the particular feeding habits of each bat species. DNA of samples was quantified by a NanoDrop 8000, and subsequently, it was diluted to adjust DNA concentrations to specific gradients (assays with 1, 0.2, 0.04, 0.008, 0.00016 and 0.000032 ng/µL DNA, Figure 2). Finally, we empirically compared known concentrations of target DNA (hereafter called "input DNA") and estimated concentrations by qPCR (hereafter called "output DNA").

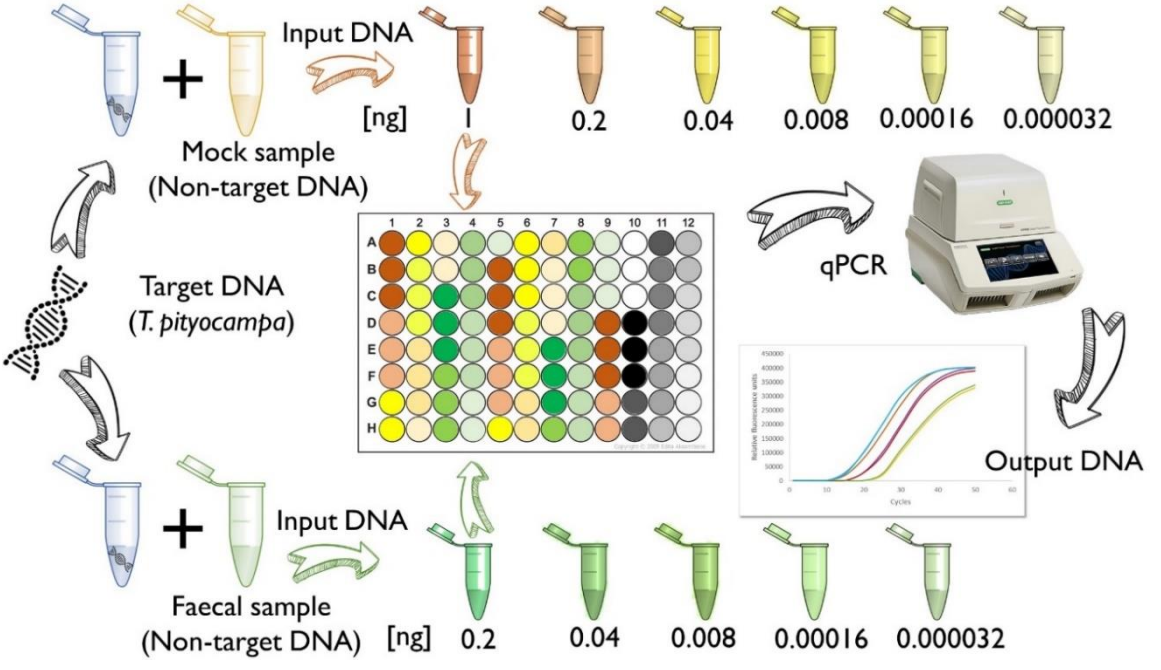



**Figure 2.** Overview of the experimental workflow for the quantitative assessment of qPCR.

For the experiment, we tested 1) 29 mock sample mixtures, i.e., fresh DNA from *T. pityocampa* (*C+*) mixed with DNA of other moths (*C-*) and 2) 35 faecal sample

mixtures, mixed DNA from fresh *T. pityocampa* (*C+*) and bat faeces without *T. pityocampa* (*Th$_{out}$-*) (Figure 2). In faecal mixtures, the *T. pityocampa* DNA concentration of 1 ng/µL was not used due to problems in maintaining molarity values. The amplification process was carried out following the same qPCR protocol described above. Besides, we also constructed standard curves in triplicate using a five-fold dilution series of targeted templates, from 3.3 x $10^6$ to 5.2 x $10^{10}$ copies per µL, to predict the abundance of target DNA present in tested samples.

## Data analysis

All the statistical analyses were carried out in R Studio v. 1.4.1103 and R v.4.0.3. (R Core Team, 2020). We conducted further analysis for each of the following questions:

- **The effect of replication effort on detection probabilities.** As described by Piggot (2016), an increase in the PCR replication effort leads to an increased likelihood of detecting a target present in the sample. Accordingly, we aimed to evaluate the detection probabilities over the three replicates, for which we used the following equation for each of the replicates:

$$D/(D+ND),$$

where D is the number of detections and ND is the number of non-detections.

- **The relationship between DNA quantity and detection probability by cPCR and qPCR.** We should expect a higher detection rate in those samples with a higher target DNA concentration. Hence, we examined if there is a relationship between the average DNA abundance estimated from triplicates of *Th+* samples by qPCR and the detection rates by each PCR assay over the three replicates, for which we fitted logistic regression models (quasi-binomial family) using *glm* function of the *stats* package in R (R Core Team, 2020).

- **The relationship between input and output DNA concentrations.** We tested whether the input DNA concentrations correlated with the average concentrations estimated by triplicated samples in both mock and faecal samples and determined

our qPCR technique's accuracy to predict target DNA concentrations. For that, we compared abundances yielded by qPCR against the expected DNA concentrations. Ideally, we should expect a positive correlation with a slope near 1. Hence, we initially fitted a linear regression model, but the residual spread increased along with the explanatory variable, which violated the homogeneity of variance assumption of our linear regressions. Therefore, we fitted a generalised least squares (GLS) model with a combined variance structure using the *varComb* function of the *nlme* package (Pinheiro ***et al.***, 2021), allowing an increase in the residual spread for larger **input** DNA concentrations as well as a different spread per sample type (mock or faecal). Afterwards, we carried out a likelihood ratio test for the fixed component selection, comparing nested models with three possible explanatory variables: run, sample type and input DNA. The test indicated that neither run nor sample type were important variables affecting the yielded DNA by qPCR. Therefore, the final model only included input DNA as an explanatory variable. We plotted all the models using the *ggplot2* package (Wickham, 2016).

## Results

Sequence analysis of the qPCR products revealed that all the *T. pityocampa* amplicons were 100% identical to the expected 89 bp fragment (Figure S3.2.). cPCR products also exclusively matched to *T. pityocampa,* but they did not show the same consistency as qPCR. Indeed, only two reverse sequences from cPCR sequencing analysis were 99% and 95% similar to the 131 bp fragment sequence, respectively. The rest of the sequences were not long enough to assess the adscription of the fragment. Overall, none of the negative controls showed signs of amplification and all the positive controls and their corresponding replicates exhibited amplification signals in both PCR assays (Figure S3.3., Table S3.1.).

cPCR showed a lower detection rate than qPCR for *Th+* samples (Figure 3), besides the increasing number of test replicates had a bigger impact on the detectability rate of the cPCR than the qPCR. Both assays tested negative for $Th_{out}$- samples.

Further, some samples tested with cPCR showed some nonspecific bands of different DNA fragment lengths together with the targeted band (Table S3.1.).

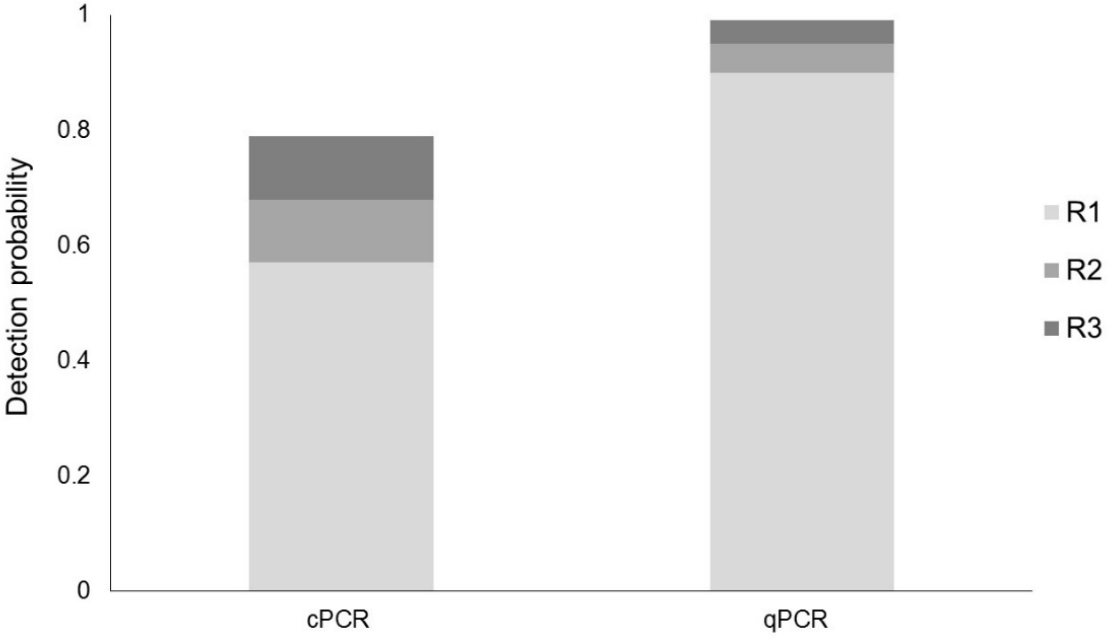



**Figure 3.** Detection probability of *T. pityocampa* DNA traces in faecal samples for each PCR assay as we increased the replicate effort (R).

Higher DNA concentrations of *T. pityocampa* in *Th+* samples resulted in an increased detection probability by the qPCR ($t=2.21$, $p< 0.05$), whereas that correlation did not hold for the cPCR assay ($t=1.16$, $p=0.25$) (Figure 4).

The results from the quantitative assessment experiment showed there is a highly significant correlation between the input and output DNA concentrations ($t=10.84$, $p<0.001$) with a mean slope value of 1.05 (95% CI, 0.85 to 1.23) (Figure 5). This output indicated that the average DNA concentration estimated from qPCR triplicates represented the DNA concentration present in the sample within a range of 85% and 123%.

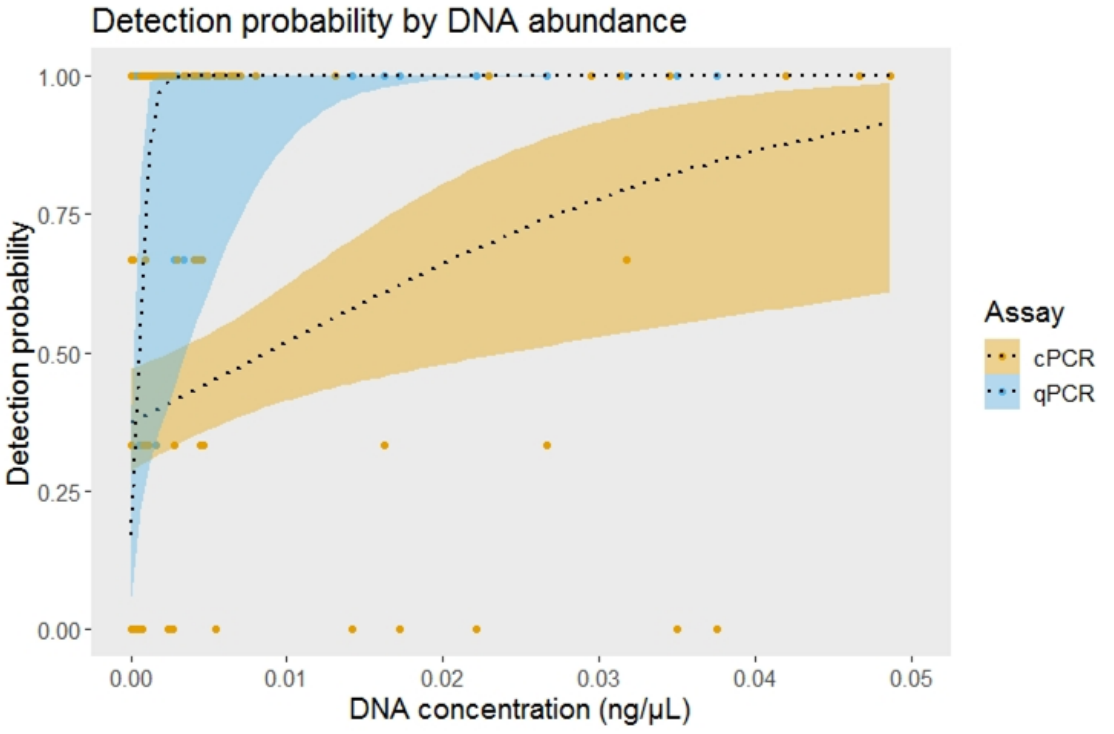



**Figure 4.** The relationship between the detection probability and DNA concentration for cPCR and qPCR. The dotted lines indicate the fitted regression model and the coloured bands the 95% confidence interval.

## Discussion

We observed that the performance of two primer sets and PCR methods differed, affecting their efficiency to detect DNA traces of *T. pityocampa* in bat faeces. Studies on predator-prey trophic interactions are currently among the main highlights of ecological research (e.g., Arrizabalaga-Escudero ***et al.***, 2019; Curstdotter ***et al.***, 2019; Michalko ***et al.***, 2019; Baroja ***et al.***, 2021). In this context, finding robust molecular methods to reliably identify and quantify DNA of prey remains in predators' faeces is a fundamental issue, for instance, to gain insight into the ecosystem services provided by predators as pest suppressors.

Both PCR assays exclusively identified *T. pityocampa,* as revealed by the sequencing analysis, indicating they are highly specific to our target. In some samples, though, cPCR produced a predominant target band of 131 bp with multiple

nonspecific bands of different DNA fragment length, which suggests a reduction in the efficiency of the amplification, likely due to non-optimal amplification conditions, such as sub-optimal annealing temperatures (Sun ***et al.***, 2020), MgCl2 concentration, primer concentration or PCR cycling conditions (Roux, 2009).

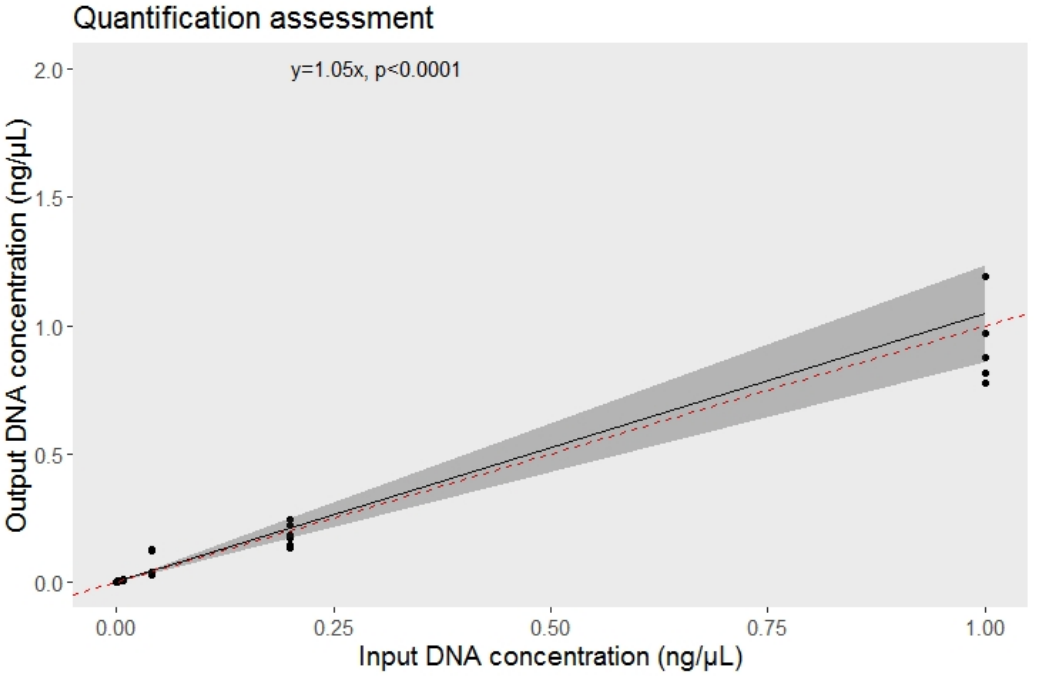



**Figure 5.** The relationship between the input and output DNA concentration. The grey band indicates the 95% confidence interval and the dotted red line represents a slope of 1.

## Comparison of cPCR and qPCR

In line with previous findings of Piggot (2016), Xia ***et al.*** (2018) and Yang ***et al.*** (2020) qPCR had a higher sensitivity than cPCR. It proved to be a more reliable and robust assay to detect *T. pityocampa* in bat faecal samples for two main reasons:

1. **Replication effort**. Increasing the number of replicates leads to an improvement in the DNA detection probability. Nonetheless, while cPCR requires up to three replicates to detect *T. pityocampa* in the 80% of *Th+* samples, qPCR detected the target in 90% of the samples with a single replicate and 99% of them with three replicates. The only sample not tested positive by any of the PCR assays

was processed in 2014, and only 8 copies of the target sequence were found that time by metabarcoding. Hence, we cannot discard that the DNA degradation in such an old sample might have hampered the detection of *T. pityocampa*.

2. **Detectability and DNA concentration**. Ideally, a robust PCR method for species monitoring should reliably detect the target species, increasing the detection probability as the target DNA concentration in the sample rises (Mauvisseau ***et al.***, 2019). In line with this, the qPCR assay results showed a positive relationship between DNA concentration and the detection probability, which suggests that a higher incidence of *T. pityocampa* in bats' diet entails a greater likelihood of detection. In contrast, the lack of such correlation in cPCR denotes that the detectability of *T. pityocampa* might be influenced by other disturbing factors, such as the presence of PCR inhibitors in some samples. In fact, they affect more severely to cPCR than qPCR owing to its less sensitive detection mechanisms (Smith and Osborn, 2009; Xia ***et al.***, 2018), which in turn, prompt false-negative results (King ***et al.***, 2008), and a higher unpredictability.

## Diet quantification by qPCR

Our study also demonstrates the potential of qPCR assays to quantify DNA in bat faecal samples and, as a result, infer the predation levels on specific prey. We undertook extensive validation steps using mocks and faecal samples from a vast array of bat species, suggesting that the developed qPCR assay is robust for quantifying *T. pityocampa* in the bat faeces irrespective of the sample source. Further methodological studies should focus on developing or improving current molecular tools for the simultaneous identification and quantification of highly diverse polyphagous predators' diets, where dozens of species occur at a time (e.g., Baroja ***et al.***, 2019). The framework of this study can be taken as a model to design similar assays applicable to other species of interest, such as agricultural pests or insects of public health concern. Further, the incorporation of the multiplex qPCR, including Taq-man probes with various dyes, will enable the simultaneous amplification of multiple prey targets in a single reaction (Burbank and Ortega, 2018; e.g., up to five species with the CFX96 Real-Time PCR Detection System used in the

study). Because of the affinity differences between primers and their target sequences (Knudsen ***et al.***, 2019), quantitative interpretations from dietary data must focus on the intraspecific variations between samples rather than on interspecific differences.

# 4. Kapitulua

## Eztabaida orokorra

# Eztabaida orokorra

Tesi honetan zehar mahastietako saguzarren bazka-ekologiaz eta nekazaritzan eskaintzen dituzten zerbitzu ekosistemikoez aritu izan naiz batik bat. Azken hamarkadan, ikerketa gutxi batzuk saguzarrek nekazaritzan dituzten onurak agerian utzi dituzte eta ondorioz, asko dira mundu mailan antzeko ikerketak nekazal-sistema ezberdinetan burutzen ari direnak. Halaber, saguzar intsektiboroen eta izurrien arteko elkarrekintzak aztertzeko, hurrengo erreminta metodologikoak maiz erabiliak dira: izurrien ugaritasun balioak feromona tranpen bidez; saguzarren aktibitatea ultrasoinuen detektore bidez; eta izurrien kontsumoa saguzarren gorotzen DNA metabarcoding bidez. Hala eta guztiz ere, hurbilketa hauek ezartzen dituzten ikerlanek hainbat muga metodologikoei aurre egin behar izaten diete.

## Muga metodologikoak eta saguzar-izurri ikerlanen diseinurako ondorioak

Maila espazio-tenporal batean egiten diren izurri eta saguzarren ikerlanetan ikerlariek erronka ugari aurki ditzakete eta ezinbestean, hainbat erabaki kritiko hartu behar izaten dituzte (1. irudia). Hauek behar bezala ezagutzea eta identifikatzea guztiz beharrezkoa da ikerketen metodologiak eta diseinua optimizatze aldera.

### Intsektuen monitorizazioa

Izurri diren intsektuen sakabanaketa eta gorabeheren dinamikak ezagutzea interes handia duen gaia da, ez bakarrik intsektuen jokamolde eta ekologiaz antzemateko, baita harrapakinak ugari diren eskualdeak eta garaiak identifikatzeko ere, adibidez izurrien eta harrapakari potentzialen artean egon daitezkeen harremanak analizatzeko. Oro har, Intsektu-izurrien populazioen jarraipena feromona-tranpa edo argi ultramoredun tranpen bitartez egiten da (adibidez, de Torrez eta kideak, 2019). Lehenak, nahiz eta emeak bakarrik harrapatzen dituen, merkeak dira, espeziekiko espezifikoak, eta ia ez dute esku-hartzerik, ez mantenu-esfortzurik

eskatzen eta beraz, epe-luzeko intsektuen monitorizazioa pasiboki egitea baimentzen dute; bigarrenek ordea, interesekoak izan ez daitezkeen beste hainbat espezie erakartzen dituzte, etengabe energiaz hornitzea eskatzen dute eta intsektuez betetzeko joera dute tranpak noizean behin berrikusiz gero (Hegazi eta Khafagi, 2005; Neethirajan eta kideak, 2007). Gure ikerketan, mahats-izurrien zenbaketak korrelazionatuta egon ziren saguzarren aktibitate eta espezie batzuen izurrien dietan zuten agerpen maiztasunarekin, nolabait iradokiz erabilitako tranpa kopuruek *S. pilleriana* eta *L. botrana*-ren eskuragarritasun balioak modu fidagarrian islatzen dituztela. Hala ere, beste sits-espezieekin lan egin zuten beste ikerketa batzuetan feromona-tranpen erabilera ez zen nahikoa izan intsektuen populazioak modu zehatzean estimatzeko (Krauel eta kideak, 2018; de Torrez eta kideak, 2019). Erabilitako monitorizazio-teknika edozein dela ere, xede den espeziearen gaineko sakabanaketa espazialaren informazioa ezagutzea ezinbestekoa da ikerketaren diseinurako (1. irudia). Hiru sakabanaketa-patroi definitu daitezke (Krebs, 1999): zorizko-sakabanaketa, populazioa osatzen duten indibiduoek espazioko edozein lekutan egoteko probabilitate berdina dutenean; sakabanaketa uniformea, populazioaren barneko konpetentzia bortitzak bultzatuta indibiduoak modu uniformean barreiatzen direnean eta multzokatze-sakabanaketa, populazioaren indibiduoek leku edo eremu konkretuetan elkartzeko joera dutenean. Azken hau, intsektu-izurrien artean ohikoa izaten da (Taylor eta kideak, 1978; Liebhold eta kideak, 2013; Sciarreta eta Trematerra, 2014), izan ere, hauek joera izaten dute baldintza biotiko eta abiotikoak egokiagoak diren txokoetan biltzeko. Alabaina, multzokatzeko joera izaten duten populazioetan aldakortasun-espazial handiagoa egoten da, eta populazioen tamainaren estimek zehaztasun maila baxuagoa izaten dute. Ondorioz, laginketa-esfortzu handiagoa (adibidez, tranpa gehiago erabiltzea) behar izaten da zehaztun maila jakin batean populazioaren tamaina kalkulatzeko. Gure kasuan, ez genuen izurri-populazioen gaineko sakabanaketaren inolako aurretiko informaziorik eta beraz, izurri-populazioen estimazioak ez ziren uste bezain zehatzak izan. Ondorioz, zailtasunak izan genituen saguzar eta izurri-populazioen arteko elkarrekintza trofikoen inguruko modelo sendoak eraikitzeko. Hori horrela, ikergai diren izurri-populazioen dinamiken gaineko ikerketa pilotuak egitea ezinbestekoa da, laginketa esfortzua modu egokian doitu eta aldakortasun-espaziala gutxitzeko.

# Saguzarren monitorizazioa

## 1. Monitorizazio akustikoa

Saguzar aktibitatearen detekzio akustikoa eta monitorizazioa alborapenaz eta erronkaz beteriko gaia da (ikusi Walters eta kideak, 2013), besteak beste, lan-kostuekin edota espezieen detekzioarekin eta identifikazioarekin lotuak.

### 1.1. Kostuak

Gurea bezalako espazialki egituratutako epe luzeko monitorizazio programek detektore automatiko ugari aldi berean eta eremu ezberdinetan erabiltzea eskatzen dute, baina historikoki, monitorizazio programa hauen kostu altuak muga garrantzitsua bilakatu izan dira. Hainbat detektore erosteko, kudeatzeko, jarraipena egin eta grabazioen datu sortak prozesatzeko beharrezkoa den aurrekontua handiegia izaten da ikerketa askorentzako. Bereziki, aipatutako muga hauek gure ikerketaren diseinua kilometro gutxi batzuetako eskualde batean burutzera murriztu zuten eta beraz, harrapakari-harrapakinen arteko elkarrekintzen ikerketak eskala espazio-tenporal zabalagoetan gauzatzea zail samarra izaten da. Berriki merkaturatutako kostu-baxuko detektoreek saguzarren monitorizazioa eskala handiagotan burutzea ahalbidetzen dute, baina laginketa esfortzua emendatzeak, mantenu eta prozesatze kostuen (adibidez, datuen analisia) gorakada esanguratsua ere suposatzen du (Hill eta kideak, 2018).

### 1.2. Detekzio-probabilitatea

Ultrasoinuen detektore bidezko saguzar-espezie ezberdinen detekzio-probabilitatea espezie bakoitzak duen ekokokapen-deien ezaugarrien araberakoa da (Jones eta Teeling, 2006; Meyer eta kideak, 2011). Saguzarrek igortzen dituzten deien frekuentziak, intentsitateak eta norabideak eragin zuzena dute detekzio-probabilitatean. Horrela, energia handiko, transmisio zabaleko edo tonu baxuko deiak detektatzea samurragoa den bitartean, frekuentzia altuko, intentsitate baxuko edo norabidezkoak diren deiak oharkabean joateko arrisku handiagoa dute

(Schnitzler eta Kalko 2001; Walters eta kideak, 2013). Zentzu honetan, espazio irekietan ehizatzen diren saguzarrek edo landarediaren gainetik habitaten ertzetan ehizatzen direnek frekuentzia baxuko edo ertaineko, transmisio zabaleko eta intentsitate altuko deiak igortzen dituzte eta beraz, aski ondo detektatu eta monitorizatu daitezke. Aitzitik, espazio itxietan ehizatzen diren saguzarrak detektatzea erronka handiagoa izan ohi da norabide altuko, intentsitate baxuko edo frekuentzia altuko deiak igortzen dituztelako. Gure Ikerketan, akustikoki grabatutako 17 saguzar espezietatik (2. kapitulua), lauk baino ez zituzten nahiko grabazio eduki analisi estatistikoetan barneratzeko. Hauetako batzuk, besteak beste, *B. barbastellus*, *Myotis* eta *Plecotus* generoko espezieak edo rhinolofidoak (*Rhinolophus* spp.) detektorez detektatzeko zailak dira duten ekokokapen sistemagatik, eta ondorioz, ohikoa izaten da beraien aktibitate-tasa gutxiestea. Adibide gisa, gure ikerketa-eremuan *R. hipposideros* maiz ehizan ibiltzen den espezie arrunta da, bertako gordelekuen okupatze datuek eta dieta-analisiek azaldu bezala; detektoreen bidez ordea, oso gutxitan detektatu genuen. Bereizketa argi honek agerian uzten du gaur egungo monitorizazio sistemek dituzten alborapen eta muga akustikoak aitortzearen garrantzia eta beharra. Horrez gain, errazen detektatu genituen saguzarrak kezka-txikiko kontserbazio-egoeran aurkitzen dira eta beraz, ezinezkoa zitzaigun ikertutako mahasti-sistema saguzarren kontserbazioaren ikuspuntutik baloratzea. Tamalez, espezie iheskor horiek ebaluatzeko egin beharreko laginketa-esfortzua kasik ezinezkoa litzateke. Hau kontuan izanik, mota hauetako ikerketek funtsean muga handiak dituztela onartu beharra daukagu, eta litekeena dela saguzar-espezie orokorrenekin eta akustikoki detektagarritasun handiagoa dutenekin bakarrik lan egin ahal izatea.

## 1.3. Identifikazioa

Saguzarren identifikazioa erronka handia izan daiteke igortzen dituzten deien aldakortasun intraespezifiko eta antzekotasun interespezifiko handiak direla eta. Europan adibidez, saguzar deien gida oso garatuak egonda ere, eta espezieen dibertsitatea oso altua ez den arren (Russo eta Jones, 2002; Russ, 2012; Barataud, 2015), zailtasun handiak daude saguzar-espezie batzuk identifikatzerako orduan.

Espezie ezberdinek deien estruktura, itxura eta antzeko frekuentzia tarteak partekatzen dituzte eta horrek zaildu egiten du saguzar askoren arteko bereizketa (Preatoni eta kideak, 2005). Horren eraginez, ikerketan zehar grabatutako deien portzentaje esanguratsu bat ezin izan zen espezie-mailan identifikatu, genero edo sonotipo mailan baizik, eta horrek ere, espezie ezberdinen detektagarritasun mugak bezala, areagotu zituen lagin tamainarekin sortutako kezkak.

Azken urteotan ordea, saguzarren jarraipena hobetzeko aurrerapausoak eman dira. Besteak beste, saguzar deien identifikazio automatikoan oinarritutako hainbat ***software*** hobetu dituzte, aurretik kontuan hartu izan ez ziren aldagai anitz barneratuz eta saguzar-espezie gehiagoren deien lista emendatuz (Russo eta Voigt, 2016). **Identifikatzaile automatiko** hauek duten abantaila nagusia da identifikazio estandarizatuak eta objektiboak eskaintzen dituztela (Rydell eta kideak, 2017) eta, eskuzko identifikazioarekin konparatuz, askoz denbora gutxiago inbertitzea eskatzen dute, batez ere datuak epe-luzean zehar bildu badira. Halarik ere, hurbilketa hauek oraindik ez dira gai zalantza gabeko espezieen sailkapena egiteko dei ezberdinen ezaugarriak gainjartzen direlako, eta hortaz, eztabaida sortu da ***software*** hauen erabilera hutsak ekar ditzakeen identifikazio-akatsen arriskuen inguruan. Honek ondorio larriak ekar ditzake harrapakari-harrapakinen arteko elkarrekintzak saguzar komunitatearen baitan zehazterako orduan, ikerketa ekologiko asko identifikatzeko errazak diren saguzarrekiko guztiz alboratuak daudelarik. Egoera honen aurrean, zuhurtasunez jokatzea eta identifikazioak norberak egitea edota modu automatikoan eginez gero, deiak behar bezala berrikustea gomendatzen dugu. Azkenik, aipatu beharra dago espezieen aberastasuna altuagoa den heinean hauek bereizteko zailtasunak ere emendatuz doazela, dei ezberdinen ezaugarriak haien artean antzekoagoak baitira. Hala ere, badira identifikatzeko errazak eta ugariak diren zenbait taxon, adibidez *Pipistrellus* generoko espezieak modu fidagarrian sailkatu daitezke (Rydell eta kideak, 2017). Horrek identifikazio-denbora asko aurreztea suposa dezake, bereziki detekzio-esfortzu handia eskatzen duten ikerketetan.

## 2. Dietaren monitorizazioa

**Metabarcoding**-ean oinarritutako ikerketan jakintza-alor eta trebetasun anitz praktikan jarri ohi dira, besteak beste, landa eta ikerketa diseinuarekin lotutako ezagutza, biologia molekularra, bioinformatika eta estatistikarekin zerikusia dutenak (Zinger eta kideak, 2019). Laginen bilketatik, datuen analisira (1. irudia), prozesuan zehar hartu beharreko erabaki bakoitzean alborapena sor daiteke, eta hein handi batean ondorio ekologiko ez fidagarrietara eraman. Horregatik, erabaki bakoitza zuhurtziaz eta ikerketa prozesuan zehar egon daitezkeen distortsio-faktoreak minimizatuz hartzea ezinbestekoa da.

### 2.1. Gorotzen bilketa

Saguzar-espezie ezberdinek askotariko ohiturak dituzte babeslekuen edota ehiza-lekuen erabilerari dagokionez, eta beraz, kasu askotan erronka handia izan daiteke saguzar-talde zabal bateko gorotz-laginak eskuratzea. Saguzar-espezie batzuk eskuragarri dauden koba, mina, elizetako bobeda edo saguzar-kutxak bezalako babeslekuetan elkartzen diren bitartean, beste espezie asko iristeko zailak edo eskuraezinak diren lekuetan ezkutatzen dira, adibidez, zuhaitzetako zulo garaiak edota kobazulo sakonak eta estuak (ikusi Kunz eta kideak, 2003). Hala ere, ikerketa-eremuko saguzar-espezieen lista ezagutzea behar beharrezko baldintza da, lagin-bilketaren bideragarritasuna zehazten duelako eta baita lortu daitezkeen ondorioen osotasun-maila. Bada, saguzarren gorotz-bilketarako bi hurbilketa nagusi erabiltzen dira: saguzar kolonien azpian biltzaileak jarriz gorotz-laginen bilketa pasiboa eta ez inbaditzailea (adibidez, Bohmann eta kideak, 2011) edo sare bidezko harrapaketa aktiboa (adibidez, Garin eta kideak, 2019). Gorotzen bilketa pasiboa aukerarik egokiena da eskuragarri dauden babeslekuetako saguzarren laginak eskuratzeko. Kasu hauetan, lagin-multzo handi bat lortzea erraza izaten da eta animalien manipulazioa eta asaldura ekiditen dira, era berean, laginak epeluzera bildu ahal izateko probabilitatea emendatzen delarik (adibidez, eragindako asaldurengatik babeslekutik ihes egiteko arriskuak gutxitu). Dena den, ikerketa eremuan zehar dauden babeslekuen bilaketa trinkoa behar izaten da trukean. Espezie-anitzeko sistemetan, laginak biltzeko estrategia muga logistikoen (adibidez, babeslekuen eskuragarritasuna) eta espezieak izkutatzen diren babeslekuen

kokapenaren gaineko ezagutzaren araberakoa izango da. Babeslekuetatik jasotako laginen kopurua nahikoa ez den kasuetan, laginketa harrapaketen bidez osatu daiteke, nahiz eta estrategia honen bidez saguzarrak epe-luzean harrapatzea gero eta zailagoa izaten den, denboraren poderioz saguzarrek sareak ekiditen baitituzte. Gure ikerketan, babesleku eskuragarrietan soilik zauden saguzar-espezieen jarraipena egin ahal izan genuen, besteen artean, *R. hipposideros* gizakiek eratutako eraikinetan, *Pipistrellus* spp. saguzar-kutxa eta begi-bistako arrakaletan eta *M. daubentonii* arroketako zuloetan, baina aitortu beharra dago mahastietan aktibo izan ziren *T. teniotis* edo *H. savii* bezalako saguzarren ohitura trofikoen gaineko informazio oso baliagarria galdu genuela. Hau kontuan izanda, babesleku oso ezberdinetan izkutatzen diren saguzar-espezie anitzen gorotzekin diharduten ikerketek, adibidez bazka-gremio ezberdinetako saguzarrek izurri zehatz batekiko duten elkarrekintza trofikoa konparatzeko, edo saguzar espezie bakarrak denboran zehar dituen elikadura-ohiturak neurtzeko, hainbat mugei aurre egin behar izaten diete. Gainera, kasu askotan bildutako datuak desorekatuak egotea edo laginketa-esfortzua esanguratsuki emendatzea suposa dezakeelarik.

## 2.2. Laginen tratamendua: banatzea edo taldekatzea?

Laginak bildu ostean, hurrengo pausua laborategian hartu beharreko laginen tratamenduaren gaineko erabakiak finkatzea da: laginak **indibidualki** prozesatu, **gutxi batzuk bildu** edo laginak **multzo handitan** pilatu. Erabaki hauek saguzarren dietan detekta daitezkeen harrapakinen dibertsitatean eragina badute ere (Mata eta kideak, 2019; Andriollo eta kideak, 2019), ikerketa-eredu bakoitzak erabiltzen duen **lagin-unitatearen** araberakoa izan behar da ezinbestean: saguzar **indibiduoa** edo saguzar **kolonia**. Hori horrela, laginak indibiduoetatik batzen direnean, gorotzak indibidualki edo gutxi batzuk bilatuz prozesa daitezke, baina laginak kolonien babeslekuetatik biltzen direnean, indibiduoak bereiztea ezinezkoa da eta gorotzen multzoak dira prozesatu beharrekoak (adibidez, Andriollo eta kideak, 2019). Tesian zehar adibidez, saguzar-kolonia ugari lagindu genituen maila espazio-tenporalean zehar (guztira 439 lagin-multzoetatik eratorritako 10607 gorotz), eta beraz, kolonia osatzen zuten saguzarren aldakortasuna eta elikadura-ohiturak

osotasunean adierazteko beharrezkoa izan zen lagineko gorotz-kopuru esanguratsu bat erabiltzea. Horren ordez gorotzak bakanki analizatu izan bagenitu, indibiduo solteen dietaren gaineko ezagutza emendatuko litzateke, baina ordean, ez genuke saguzar koloniaren dieta behar bezala adieraziko.

## 2.3. Erreplikak

Metabarcoding bidez egindako dieta lanetan, erreplikek, biologiko zein teknikoek, eragina handia izan dezakete dietan aurkitzen diren harrapakinen zerrendan (Mata eta kideak, 2019). Aurreko atalarekin lotuta, erreplikazio biologikoez ari garenean, lagin unitateko erabiltzen ditugun biologikoki ezberdinak diren azpi-laginei egiten diegu erreferentzia (adibidez, saguzar-kolonia baten dieta irudikatzeko erabiltzen diren gorotz-sorta unitateak), lagin unitate bakoitzak izan dezakeen heterogeneotasuna (adibidez, espaziala eta tenporala) kontuan hartzeko helburuz (Alberdi eta kideak, 2018). Bestalde, erreplika teknikoak lagin berdinaren neurketa errepikatuak dira (adibidez, lagin batentzako erabiltzen diren erauzketa edo PCR kopurua, Blainey eta kideak, 2014) eta prozesu hauetan zehar sortzen den estokastizitateari aurre egiteko erabili ohi dira (Alberdi eta kideak, 2018). Dena den, kontuan izanik lagin-unitate ezberdinen artean dagoen dietaren aldakortasuna lagin-unitatearen baitan egon daitekeena baino handiagoa dela (Mata eta kideak, 2019), erreplika biologikoek eragin handiagoa izaten dute erreplika teknikoen aldean. Horregatik, eta errepliken erabilerak suposatzen dituen lan-karga eta kostuen emendioa kontuan hartuta, erreplika teknikoei baino, lehentasuna eman beharko litzaioke erreplika biologikoen erabilerari.

## 2.4. Hasleen aukeraketa eta estrategia molekularra

Ingurumen-laginetatik eratorritako izurri-espezie ezberdinen DNA metodo espezifikoen bidez edo komunitate-mailako metodoen bidez detekta daiteke (adibidez, Varadínová eta kideak, 2015; Piggott, 2016; Harper eta kideak, 2018). Lehena, **hasle espezifikoen** eta PCR konbentzional (**cPCR**) edo PCR kuantitatiboaren (**qPCR**) erabileran oinarritzen da, jomuga diren espezieak **kualitatiboki** edo

**kuantitatiboki** ebaluatzeko (Deagle, 2005, 2007; Gosh eta kideak, 2018). Azkenak, hasle unibertsalak erabiltzen ditu komunitate osoak barneratuz, honen adibide da DNA metabarcoding. Bata edo bestea aukeratzea ikerketa galdera eta baliabide ekonomikoen araberakoa da. Adibidez, izurri espezie gutxi batzuk helburu diren kasuetan hurbilketa espezifikoen erabilera zentzukoagoa dirudi, baina horren ordez, esfortzu handiagoa egin behar da hasleen diseinuan eta balidazioan, eta baita anplifikazio protokoloen optimizazioan; bestalde, ikerketaren helburu nagusia baldin bada ekosistema, sare trofiko eta komunitatearen funtzionamenduaz jakitea, metabarcoding bezalako teknikak eskaintzen dituen emaitzek osotasun handiagoz erantzungo dio planteatzen diren galderei. Teknika bakoitzak dituen abantailak, mugak eta erabilerak eztabaidatzen dira jarraian 3. kapituluan lortutako emaitzetan oinarrituz (1.taula).

## - Metabarcoding

Metabarcoding-ek entsegu bakoitzean aldi berean ehundaka laginen prozesamendu eta sekuentziazioa ahalbidetzen du (Alberdi eta kideak, 2018). Hori dela eta, azken hamarkadan DNA metabarcoding-en erabilerak egundoko bultzada izan du harrapakari **polifagoen** dieta aztertzeko. Besteen artean, aparteko aukerak eskaintzen ditu **bazka-sareetan** ematen diren elkarrekintza trofikoen inguruan sakontzeko (Sow eta kideak, 2020). Halarik ere, sare trofikoen gainean informazio oso baliagarria eskaintzen duen arren, eztabaida honen testuinguruan esan beharra dago gabeziak ere badituela. Adibidez, harrapakin espezie konkretuak detektatu nahi direnean, metodologia honen zehaztasuna eta detekziorako sentikortasuna baxuak izan daitezke (Wood eta kideak, 2019). Gure ikerketaren emaitzek esaterako, aditzera eman zuten cPCR edo qPCR tekniken bidez positibo adierazitako laginen % 10 baino gehiago ez zirela metabarcoding bidez detektatu (Table S3.1). Hortaz, esan dezakegu espezie dibertsitatearen edo saguzar eta harrapakinen arteko elkarrekintzen inguruko ekologia hobeto ulertu nahi badugu edo baita izurrien kontsumoan eragiten duten faktoreak (adibidez, beste harrapakin potentzialekiko zaletasuna) ezagutu nahi baditugu, metabarcoding da teknika egokia.

Metabarcoding-en testuinguru honetan, **hasleen hautaketak** eragin esangarria du bukaeran lortzen den espezieen zerrendan eta beraz, hainbat faktore arretaz kontuan hartu behar dira hasleekin lotutako alborapenak gutxitu eta kasu bakoitzean hasle egokienak aukeratzeko:

- **Eskualde markatzailea:** lehen pausua helburu diren taxonak barneratzen dituen eskualde markatzailea erabakitzea izan behar da. Oro har, c zitokromo oxidasa I azpi-unitatea (**COI**) maiz erabiltzen den gene eskualdea da, izan ere DNA sekuentzien online datu-baseetan (adibidez, BOLD systems eta Genbank) artropodoen liburutegi zabala dago eskuragarri. Beste gene batzuk ere (adibidez, 16S edo 18S) erabili ohi dira, baina hauen kasuan, datu-baseak ez dira horren hedatsuak eta sekuentzia asko ezin izaten dira espezie mailaraino identifikatu (Alberdi eta kideak, 2017; Giebner eta kideak, 2020).

- **Anplikoi luzera:** gorotzetan DNA zatiak oso degradatuak egoten dira eta luzera handiko anplikoiak (>200 bp) detektatzea arraroa izaten da. Baina alderantziz, anplikoi oso laburrek (<100 bp) espezifikotasun arazoak sortzen dituzte (Meusnier eta kideak, 2008) eta ondorioz, espezie mailako harrapakinen identifikazioa zaila izaten da. Hori dela eta, gure gomendioa da **100-200 bp-ko** luzerako anplikoia anplifikatzen dituen hasleak erabiltzea, ikerketa gehienetan egin ohi den bezala.

- **Dibertsitate taxonomikoa:** ikerketaren helburuen arabera, taxonomikoki **tarte zabaleko** edo **tarte estukoak** diren hasleak interesa dakizkiguke. Lehenak taxon ugari anplifikatzeko gai dira eta beraz, aproposak izaten dira harrapakin dibertsitate handia detektatzea xede duten dieta lanetan. Gainera, laginak saguzarren babeslekuetatik biltzen diren kasuetan, bereziki garrantzitsua izaten da harrapakin-espeziea identifikatzea, izan ere intereseko laginak beste saguzar espezieen gorotzekin kutsatuak egon daitezke (Forin-Wiart eta kideak, 2018). Taxonomikoki tarte estukoak diren hasleek ordea, talde taxonomiko jakinekiko afinitate altuagoa izaten dute (adibidez, Lepidoptera eta Diptera, Zeale eta kideak, 2011). Markatzaile molekular hauek bereziki mesedegarriak dira dietan taxon espezifikoak detektatu nahi badira, esaterako saguzarrek kontsumitutako izurrite diren lepidopteroak edo eltxo espezieak. Modu honetan ere, interesekoak ez diren eta dietan ohikoak izan daitezkeen DNA sekuentziak hasleekin lotzeko

konpetentzia ekiditen dugu. Ondorioz, interesekoak diren espezieen detektagarritasuna emendatzen da (Harper eta kideak, 2018).

- **Hasle kopurua:** hasle ezberdinen konbinazioari esker interesekoa den zatia anplifikatzeko aukerak emendatzeaz gain (Zhang eta kideak, 2018), harrapakin dibertsitate altuko harrapakarien dietak zehatzago irudikatu daitezke (Esnaola eta kideak, 2018). Hala ere, hasle bat baino gehiago erabiltzeak suposatzen duen kostuen igoera dela eta, etorkizuneko ikerketek hasle bakar efizienteen erabilera kontuan hartuko beharko lukete, hau da harrapakinak, zein harrapakarien sekuentziak anplifikatzeko gai direnak hain zuzen (Tournayre eta kideak, 2020).

Testuinguruz aldatuz, metodo espezifikoen alorrean, hasle eta zunda espezifikoen eta PCR metodoen (cPCR or qPCR) konbinazioa, saguzarren gorotzetan interesekoak diren espezieen jarraipena egiteko alternatiba fidagarri, arina eta merkea da, 3. kapituluan adierazi bezala.

- cPCR

cPCR-ko emaitzek (3. kapitulua) erakutsi zuten metodoak % 80-ko eraginkortasuna duela, beti ere lagineko hiru erreplika erabiltzen badira. Modu berean, erreplika-esfortzuaren emendioaren ondorioz, *T. pityocampa*-ren detektagarritasuna ere emendatzen da, 3. irudian (3. kapitulua) adierazi bezala. Teknika hau onuragarria izan daiteke ikerketa pilotuetan erabiltzeko, besteak beste, haragoko analisietara jo aurreko lehen pausu bezala. Horrez gain, kudeaketaren ikuspuntutik, izurri-espezieak saguzar gorotzen bitartez garaiz detektatzeko ere baliagarria izan daiteke (Maslo eta kideak, 2017), izurrien populazioak baso eta nekazal landaketetan finkatu eta zabaldu baino lehenago beharrezko neurriak hartu eta kalteak murrizteko. Azkenik, metodo honen abantaila nagusietako bat bere prezio baxuan datza (0,17€/erreplika, Purcell eta kideak, 2016), batez ere qPCR (1,50€/erreplika, Purcell eta kideak., 2016) eta metabarcoding (5,30-8,60€/lagina, Behrens-Chapui eta kideak, 2021) teknikekin konparatuz gero, eta hortaz, ia edozein ikerketak ordain dezakeena. Dena dela, metodoaren sentikortasuna dela eta, ez dugu gomendatzen izurrien jarraipen zehatza behar den ikerketetan erabiltzea.

- qPCR

3. kapituluan *T. pityocampa* izurriarekin erakutsi bezala, qPCR-ak saguzarren dietan agertzen diren intereseko harrapakin espezieak maila kualitatiboan eta kuantitatiboan aztertzeko ahalmena du. Esan dezakegu beraz, metodo honek erabilera bikoitza duela; batetik, dietan harrapakinen presentzia-ausentziak egiaztatzeko erabil daiteke, baina baita saguzarrek kontsumitutako intereseko espezieen biomasa kalkulatzeko hurbilketa orokor gisa. Horrez gain, metodo honek % 90eko detekzio ratioa erakutsi zuen erreplika bakarra erabilita eta beraz, kostuak gutxitzeko aukera ere ematen du, emaitzen fidagarritasuna maila onargarri batean mantenduz. Hala eta guztiz ere, kuantifikazioari begira, lagineko hiru erreplika erabiltzea beharrezkoa da errepliken arteko aldakortasunak duen eragina gutxitu, eta emaitzen sinesgarritasuna bermatzeko.

**2. taula.** Ikerketan erabilitako teknika molekularren abantailak, mugak eta erabilerak.

| Teknika | Abantailak | Mugak | Erabilera |
|---|---|---|---|
| Metabarcoding | -Dietako harrapakinen ikuspegi zabala.<br>-Dietaren inguruko aurretiko ezagutza ez da behar.<br>-Entseguko hainbat lagin analizatzeko aukera (≈384).<br>-Jada diseinatuta egoten diren hasle unibertsalen erabilera. | -qPCR baino sentikortasun maila baxuagoa espezie bakarraren detekziorako.<br>-Organismoen sekuentziak erreferentziazko datu-baseetan egon behar dira.<br>-Prozedura garestia (5,30-8,60€/lagina).<br>-Lan-zama handia eskatzen du (liburutegiaren prestaketa, sekuentziazioa, bioinformatika) | -Dibertsitate altuko dieta lanak<br>-Sare-trofiko konplexuak |

**2. taula.** (jarraipena)

| Teknika | Abantailak | Mugak | Erabilera |
|---|---|---|---|
| cPCR | -Espezifikoa.<br>-Prozedura merkea (0,17€/erreplika). | -PCR osteko prozedurak (denbora eskatzen du, kutsadura gertatzeko aukera).<br>-Sentikortasun baxua.<br>-Hiru erreplika, gutxienez.<br>-Kualitatiboa (presentzia-ausentzia)<br>-Intereseko izurriaren hasle espezifikoen diseinua ez da beti posible izaten. | -Ikerketa pilotuak:<br>analisi sakonetara jo aurreko lehen pausu bezala.<br>-Dietan intereseko espeziearen ikuspegi orokorra prezio baxuan. |
| qPCR | -Espezifikotasun eta sentikortasun altuak.<br>-Analisi azkarra (PCR osteko prozedurarik ez).<br>-Kualitatiboa eta kuantitatiboa<br>-Hainbat lagin analizatzeko aukera (≈3 erreplikadun 120 lagin, 384ko PCR plakan ). | -Prozedura erlatiboki garestia (1,50€/erreplika)<br>-Intereseko izurriaren hasle eta zunda espezifikoen diseinua ez da beti posible izaten. | -Harrapakin zehatzekiko harrapakaritza-maila erlatiboa. |

## 2.5 Erabaki bioinformatikoak - Akatsak ezabatzea eta identifikaziorako irizpide taxonomikoak

DNA-ren sekuentziazioan oinarritutako dieta analisietan prozedura ugari egin behar izaten dira. Prozesuan zehar, ehundaka lagin paraleloan analizatzen dira, laginketetatik hasita, laborategiko lana (DNA erauzketa, anplifikazioa, liburutegiaren prestaketa eta sekuentziazioa) eta pausu bioinformatikoak burutzera, eta horrek, akatsak eta kutsadura sortzeko aukerak emendatzen ditu. Hori horrela, erauzketa, anplifikazio eta liburutegiaren prestaketari dagozkien protokoloetako bakoitzean kontrol negatiboak gehitzea, eta ondoren gainerako laginekin batera sekuentziatzea guztiz gomendagarria da. Kontrol negatiboek

kutsadura egon den aztertzea baimentzen dute eta ondorioz, sekuentzia horiek analisitik baztertzeko aukera ematen digute positibo faltsuak ekiditeko (Aizpurua eta kideak, 2018; Alberdi eta kideak, 2018). Sekuentziazioaren ostean beraz, jasotako datuekin zelan jokatu erabaki behar dugu: kontrol negatiboetan agertzen diren sekuentziak gainerako laginetatik ezabatu (negatibo faltsuak emendatu) edo sekuentzia horiek mantendu ustezko informazio baliagarri hori ez galtzeko (positibo faltsuak emendatu). Orokorrean, negatibo faltsuak gutxitzea baino, lehentasuna eman ohi zaio positibo faltsuak murrizteari, hain zuzen ere taxon baten ausentziak (negatibo faltsua) ez duelako ziurtatzen espeziea hor ez dagoenik, baina akatsez taxon bat dagoela esatea (positibo faltsua) askoz larriagoa izan daiteke. Kasu gehienetan, kontrol negatiboek erraztasun handiagoarekin kutsatzen dira ugariak eta arruntak diren taxonen DNA-rekin, batik bat laginetan dagoen DNA totalaren proportzio handi bat betetzen dutelako (Jensen eta kideak, 2015), besteak beste, harrapakariaren edo saguzarrek gehien kontsumitutako harrapakinen DNA. Sekuentzia horiek kendu edo mantentzearen erabakia ikerketaren helburuen edo interesen araberakoa da, baina saguzar-izurrien testuinguruan bi hurbilketa proposatuko nituzke: batetik, edozein erabaki hartu baino lehen kontroletan agertzen diren taxonen identifikazioa burutu eta bestetik, gorago aipatu bezala, estrategia kontserbakorrago bat jarraitu eta kontroletan agertzen diren sekuentziak gainerako laginetatik ezabatu. Sentsu berean, ZOTU/OTU-en identifikazio prozesuan erabilitako irizpideek dietan agertzen diren espezieen zerrenda baldintzatzen dute (ikusi Alberdi eta kideak., 2017). Egun arte ordea, oraindik ere ez dago adostasunik espezieen identifikaziorako erabili beharreko hurbilketa eta sekuentzien antzekotasun-atarien inguruan, kasu askotan modu arbitrarioan ezartzen direlarik (Ando eta kideak, 2020): antzekotasun-atari ahuletatik (Kolkert eta kideak, 2020, espezie maila % 97-tik gorako antzekotasuna eta e-balioa $<1^{e-20}$), zorrotzetara (Wray eta kideak, 2018, espezie maila % 99-tik gorako antzekotasuna). Antzekotasun-atari ahulen erabilerak, lortutako sekuentziak modu ezegokian identifikatzeko aukerak emendatzen dituen bitartean, antzekotasun-atari oso hertsietan oinarritzeak zailtasunak ekar ditzake ZOTU/OTU-en espezie-mailako identifikazioa burutzerako orduan. Kontuan izanik positibo faltsuak ekiditeari lehentasuna eman behar diogula eta **saguzar-izurri elkarrekintzen** ikerketen fidagarritasuna eta

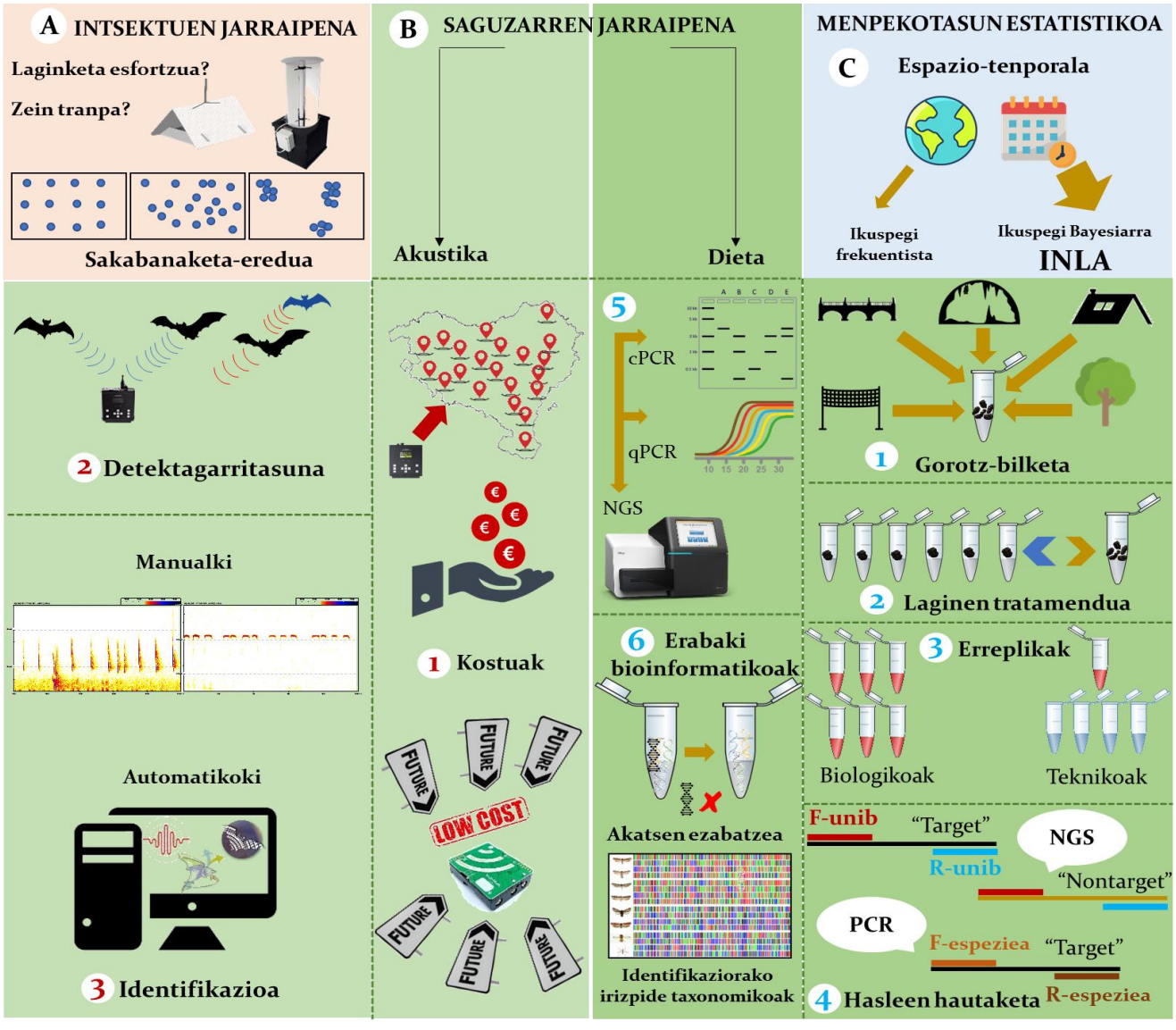
erreplikagarritasuna bermatze aldera, beharrezkoa da izurrien identifikaziorako sistemak estandarizatzea antzekotasun-atari hertsiak erabiliz.

## Menpekotasun estatistikoa

Saguzarrek izurri populazioetan harrapakaritza-presioa ezartzen dutela argi dago, baina presio hori aldakorra izan daiteke momentu eta espazioaren arabera (Krauel eta kideak, 2018; Baroja eta kideak, 2019). Mota honetako saguzar-izurrien arteko elkarrekintzak maila espazio-tenporal batean modelizatzeak zailtasun-metodologikoak eduki ditzake, adibidez denbora eta/edo espaziorekiko menpekotasuna duten datuei aurre egin behar izatea (Zuur eta kideak, 2017). Gaur egun **R programak** sakabanaketa normaleko datuen **korrelazio tenporal eta espazialari** aurre egiteko erreminta ugari eskaintzen dituen arren, ikerketa ekologikoetan sakabanaketa normala ez duten datuen (adibidez, binomialak edo zenbaketak) nagusitasuna erabatekoa da. Sakabanaketa ez-normalaren testuinguruan, korrelazio horiei aurre egiteko eskuragarri dauden pakete eta erreminta konputazionalak nahiko mugatuak dira [baina ikusi *MASS* (Ripley eta kideak, 2013) eta *lme4* (Bates eta kideak, 2018) paketeek eskainitako *glmmPQL* eta *GLMM* funtzioak].

Tesian zehar, modelo mistoentzako era askotako baliabide, sakabanaketa, modeloen egitura eta tresna konputazionalak erabili genituen R-n, baina hauetako inork lortu zuen 2. kapituluko datuen **menpekotasun espazio-tenporalari** jarkitzea. Egoera honen aurrean, harrapakari eta harrapakinen elkarrekintzen gaineko ikerketa askok aldagai espazio-tenporalak **ausazko efektu** gisa barneratzen dituzte korrelazioei aurre egiteko. Hauen erabilera ordea, **ez da batere gomendagarria**, lortutako modeloen parametroek eta p-balioek alborapena erakusten baitute (Zuur eta kideak, 2017), eta horrek ondorio ekologiko desegokietara eraman gaitzake (Carroll eta Pearson, 2000). Hori dela eta, praktika hau baztertu genuen. Azkenean, **hurbilketa Bayesiar** bat erabiltzea erabaki genuen (Rue eta kideak, 2009). Ikuspegi "**frekuentistarekin**" alderatuta, ikuspegi Bayesiarrean ez dago parametroen balio finkorik, eta horren ordez, modeloen parametroen probabilitateak kalkulatzen dira gure datuetan oinarrituz (Box eta Tiao, 2011), edo beste modu batera esanda, proposatutako hipotesiarekiko

probabilitatea kalkulatzen da. Ikuspuntu teoriko batetik, emaitzen interpretazioa zertxobait ezberdina da, baina ikuspuntu praktiko batetik, hurbilketa honek eskaintzen dituen abantailak anitzak dira. Besteen artean, azken urteotako aurrerapen konputazionalak, adibidez *R-INLA software*-aren garapena (Bakka eta kideak, 2018), oso eraginkorrak bilakatu dira modu erraz batean korrelazio tenporalari eta espazialari aurre egiteko eskaintzen dituzten tresna konputazional ugariei esker.




**1. Irudia.** Saguzar eta izurrien arteko elkarrekintzak maila espazio-tenporalean aztertzeko diseinatutako ikerketetan aurki daitezkeen zailtasun eta erabakien eskema.

## Aukerak eta etorkizuneko erronkak

Tesian zehar konturatu naiz zer nolako erronka handia izan daitekeen maila espazio-tenporal ezberdinetan saguzar eta intsektuekin erlazionatutako ikerketa ekologikoek dakartzaten konplexutasunei aurre egitea. Hau idaztearen helburua ordea, ez da gainerako ikerlariak saguzar eta izurrien arteko elkarrekintzak aztertzearen zailtasunak aipatuz gogogabetzea, horren ordez, helburua da hasiberritan ari diren ikerlariak bultzatzea eta komunitate zientifikoa nolabait bideratzea prozesuan zehar erabaki egokiak har daitezen. Azken urteotako aurrerapen analitiko eta molekularrek erakusten dute  badagoela oraindik ere muga hauei aurre egiteko hurbilketa eta metodologia berrientzako tartea. Ordura arte ostera, ziurtatu beharko dugu muga metodologiko hauek kontuan hartzen ditugula ondorio ezegokiak ekiditeko.

# 5. Kapitulua

# Ondorioak

## Ondorioak

Tesi honek saguzar intsektiboroek izurrien kontsumitzailetzat eskaintzen dituzten zerbitzu ekosistemikoak hobeto ulertzeko ezagutza handitu du. Honako hauek dira ondorio nagusiak:

1. *R. hipposideros* garrantzia handiko izurri-espezie ugariz baliatzen da, adibidez mahatsaren hazkuntza-garaian *Lobesia botrana*, *Sparganothis pilleriana*, *Ephestia parasitella, Peridroma saucia* edo *Spodoptera exigua* izurriak kontsumitzen ditu, saguzar espezie honek potentzialki eskaintzen dituen zerbitzu ekosistemikoak agerian geratzen direlarik. Ondorioz, espezieak kultiboen produkzioan eduki ditzakeen onurak direla eta, saguzar-espeziea izurrien kudeaketarako plangintzen barruan kontuan hartu beharko litzateke.

2. Metabarcoding testuinguruan, izurri potentzialen detektagarritasuna hasleen araberakoa da, hein handi batean. Zenbait **izurri** "***Gillet***" hasleekin detektatu ziren soilik, eta beste hainbat ordea, "***Zeale***" hasleekin. Hasle osagarriak konbinatzea beraz, funtsezkoa da harrapakarien dieta osotasunean deskribatzeko.

3. *R. hipposideros*-en dieta-ohiturak denboran zehar aldatzen dira. Hau kontuan izanik, saguzarren izurriak kontsumitzeko ahalmena aztertzeko laginketak hainbat aldietan zehar burutzea gomendatzen dugu.

4. *L. botrana*-k eta *S. pilleriana*-k multzokatzeko joera dute. Ikerketa eremuan batez ere hego-ekialdean aurkitu ziren izurrien ugaritasun balio altuenak. Feromona tranpen detektagarritasun-tarte baxuak eta izurri populazioen heterogeneotasun espazialak agerian uzten du monitorizazio-esfortzua emendatzeko beharra.

5. Saguzarrek izurrien gorakadak ematen diren partzeletan elkartzeko eta hauek ustiatzeko ahalmena dute, baina baita izurrien populazioak urriak diren garaietan harrapakin alternatiboez baliatzeko ere. Modu horretan, ikertutako saguzarrek duten izaera oportunista gailendu daiteke eta harrapakari jeneralistek izurrien ustiatzaile gisa betetzen duten balioa agerian geratzen da.

6. Gremio ezberdinetako saguzar-espezieek positiboki erantzuten dute *L. botrana* eta *S. pilleriana* gorabeherekiko; batetik, izurriak ugariak diren partzeletan pilatuz eta bestetik, izurrien kontsumoa beraien aberastasun balioetara egokituz. Argi geratzen da beraz, saguzar-espezie ezberdinek, sinergikoki jokatuz, izurriak aire-espazioko maila ezberdinetan ustiatzeko ahalmena dutela.

7. Saguzar-espezie ezberdinen izurriekiko erantzuna maila oso lokaletik, distantzia luzeagoko eskualdetara eman zen, beraz, saguzarrek bazkaleku egokiak bilatzeko distantzia tarte zabalak egin ditzaketela agerian geratzen da.

8. Laginketan zehar saguzarren babeslekuetako gorotzen bilketa pasiboaz gain, sare bidezko harrapaketa saioak egitea ere gomendagarria da iheskorrak diren saguzar espezieen laginak ere kontuan hartzeko.

9. qPCR-a cPCR-a baino teknika sentikorragoa da. *T. pityocampa*-ren detekziorako metodo fidagarriagoa dela ikusi genuen bi arrazoiengatik:

**a) Erreplikazio-esfortzua:** cPCR-ak hiru erreplika behar izan zituen *T. pityocampa* Th+ laginen % 80-an baino ez detektatzeko; qPCR-ak ostera, erreplika bakarrarekin kasuen % 90-ean detektatzeko gai izan zen eta % 99-an hiru erreplikekin.

**b) Detektagarritasuna eta DNA kontzentrazioa:** qPCR-ak DNA kontzentrazio eta detekzio-probabilitatearen arteko erlazio positiboa erakutsi zuen, baina cPCR-an ez zen erlazio esangarririk aurkitu.

10. qPCR metodoak *T. pityocampa*-ren DNA saguzarren gorotzetan kuantifikatzeko ahalmena du, eta ondorioz, harrapakin espezifikoetan ezartzen duten harrapakaritza maila ezagutzeko.

11. Metodo espezifiko (cPCR edo qPCR) zein metabarcoding analisiek hainbat abantaila eta desabantaila dituzte. Haien erabilera ikerketa-galderen eta baliabide ekonomikoen araberakoa da.

12. Eskala espazio-tenporal batean burututako saguzar-izurrien gaineko ikerketak erronka handia izan daitezke eta prozesuan zehar erabaki kritikoak hartzea eskatze dute. Hori dela eta, ikerketen diseinuari begira, ikerlariek aurki ditzaketen muga metodologikoak ezagutzea beharrezkoa da, besteak beste:

- Intsektuen sakabanaketa espazialaren ereduak.
- Saguzarren monitorizaziorako ereduak: akustika eta dietaren bidezko jarraipena.
- Menpekotasun estatistikoa.

# Erreferentziak

# Erreferentziak

Abbott, I. M., Harrison, S., & Butler, F. (2012). Clutter-adaptation of bat species predicts their use of under-motorway passageways of contrasting sizes–a natural experiment. *Journal of Zoology*, 287(2), 124-132.

Abrams, P. A., & Ginzburg, L. R. (2000). The nature of predation: prey dependent, ratio dependent or neither?. *Trends in Ecology & Evolution*, *15*(8), 337-341.

Agirre-Mendi, P. T. (2003). Protección de refugios de quirópteros (Mammalia: Chiroptera) en la Comunidad Autónoma de La Rioja: Resultados de las campañas de 1998, 1999, 2000 y 2001. *Zubia*, (21), 63-70.

Aihartza, J. R., & Garin, I. (2002). Distribución de los murciélagos de los géneros Pipistrellus, Hypsugo y Eptesicus (Mammalia, Chiroptera) en el País Vasco Occidental. *Munibe*, 53, 229-244.

Aizpurua, O., Budinski, I., Georgiakakis, P., Gopalakrishnan, S., Ibañez, C., Mata, V., ... & Zrncic, V. (2018). Agriculture shapes the trophic niche of a bat preying on multiple pest arthropods across Europe: Evidence from DNA metabarcoding. *Molecular Ecology*, *27*(3), 815-825.

Alberdi, A., Garin, I., Aizpurua, O., & Aihartza, J. (2012). The foraging ecology of the mountain long-eared bat *Plecotus macrobullaris* revealed with DNA mini-barcodes. *PLoS One*, 7(4), e35692.

Alberdi, A., Aizpurua, O., Gilbert, M. T. P., & Bohmann, K. (2017). Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution,* 9(1), 134-147.

Alberdi, A., Aizpurua, O., Gilbert, M. T. P., & Bohmann, K. (2018). Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution*, 9(1), 134-147.

Aldasoro, M., Garin, I., Vallejo, N., Baroja, U., Arrizabalaga-Escudero, A., Goiti, U., & Aihartza, J. (2019). Gaining ecological insight on dietary allocation among horseshoe bats through molecular primer combination. *PloS One*, 14(7), e0220081.

Alford, D. V. (1999). *A textbook of Agricultural Entomology*. Blackwell Science Ltd.Ancillotto, L., Budinski, I., Nardone, V., Di Salvo, I., Della Corte, M., Bosso, L., ... & Russo, D. (2018). What is driving range expansion in a common bat? Hints from thermoregulation and habitat selection. *Behavioural processes*, 157, 540-546.

Aljanabi, S. M., & Martinez, I. (1997). Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Research*, 25(22), 4692-4693.

Ando, H., Mukai, H., Komura, T., Dewi, T., Ando, M., & Isagi, Y. (2020). Methodological trends and perspectives of animal dietary studies by noninvasive fecal DNA metabarcoding. *Environmental DNA*.

Andriollo, T., Gillet, F., Michaux, J. R., & Ruedi, M. (2019). The menu varies with metabarcoding practices: A case study with the bat *Plecotus auritus*. *PloS One*, 14(7), e0219135.

Arrizabalaga-Escudero, A., Merckx, T., García-Baquero, G., Wahlberg, N., Aizpurua, O., Garin, I., ... & Aihartza, J. (2019). Trait-based functional dietary analysis provides a better insight into the foraging ecology of bats. *Journal of Animal Ecology*, 88(10), 1587-1600.

Auger-Rozenberg, M. A., Barbaro, L., Battisti, A., Blache, S., Charbonnier, Y., Denux, O., ... & Roques, A. (2015). Ecological responses of parasitoids, predators and associated insect communities to the climate-driven expansion of the pine processionary moth. In *Processionary moths and climate change: an update* (pp. 311-357). Springer, Dordrecht.

Bakka, H., Rue, H., Fuglstad, G. A., Riebler, A., Bolin, D., Illian, J., ... & Lindgren, F. (2018). Spatial modeling with R-INLA: A review. Wiley *Interdisciplinary Reviews: Computational Statistics*, 10(6), e1443.

Barataud, M. (2015). *Acoustic Ecology of European Bats. Species identification, habitat studies and hunting behaviour*. Biotope Editions, Mèze and National Museum of Natural History, Paris.

Baroja, U., Garin, I., Aihartza, J., Arrizabalaga-Escudero, A., Vallejo, N., Aldasoro, M., & Goiti, U. (2019a). Pest consumption in a vineyard system by the lesser horseshoe bat (*Rhinolophus hipposideros*). *PloS One*, 14(7), e0219265.

Baroja, U., Garin, I., Aihartza, J., Arrizabalaga-Escudero, A., Vallejo, N., Aldasoro, M., & Goiti, U. (2019b). DNA extraction protocol. protocols.io. dx.doi.org/10.17504/protocols.io.2ptgdnn

Baroja, U., Garin, I., Vallejo, N., Aihartza, J., Rebelo, H., & Goiti, U. (2021). Bats actively track and prey on grape pest populations. *Ecological Indicators*, 126, 107718.

Bat Conservation International. (2020). Bats and COVID-19 Overview. Available from: https://www.batcon.org/wp-content/uploads/2020/08/Bats-and COVID19 Overview.pdf

Bat Conservation Trust. (2020). COVID-19 and Bats. Available from: https://www.bats.org.uk/about-bats/bats-and-disease/covid-19-and-bats, accessed December, 7, 2020.

Bates, D., Maechler, M., Bolker, B., Walker, S., Christensen, R. H. B., Singmann, H., ... & Green, P. (2018). Package 'lme4'. Version, 1, 17.

Battisti, A., Stastny, M., Netherer, S., Robinet, C., Schopf, A., Roques, A., & Larsson, S. (2005). Expansion of geographic range in the pine processionary moth caused by increased winter temperatures. *Ecological applications*, *15*(6), 2084-2096.

Battisti, A., Larsson, S., & Roques, A. (2017). Processionary moths and associated urtication risk: Global change–driven effects. *Annual review of entomology*, *62*, 323-342.

Bayliss, P., & Choquenot, D. (2002). The numerical response: rate of increase and food limitation in herbivores and predators. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *357*(1425), 1233-1248.

Beck, A. (1995). Fecal analyses of European bat species. *Myotis*, 32(33), 109-119.

Begg, G. S., Cook, S. M., Dye, R., Ferrante, M., Franck, P., Lavigne, C., ... & Quesada, N. (2017). A functional overview of conservation biological control. *Crop Protection*, 97, 145-158.

Behrens-Chapuis, S., Herder, F., & Geiger, M. F. (2021). Adding DNA barcoding to stream monitoring protocols–What's the additional value and congruence between morphological and molecular identification approaches?. *PLoS One*, 16(1), e0244598.

Best, T. L., & Geluso, K. N. (2003). Summer foraging range of Mexican free-tailed bats (*Tadarida brasiliensis mexicana*) from Carlsbad Cavern, New Mexico. *The Southwestern Naturalist*, 48(4), 590-596.

Blainey, P., Krzywinski, M., & Altman, N. (2014). Points of significance: replication. *Nature Methods*, 11(9), 879.

Bohmann, K., Monadjem, A., Noer, C. L., Rasmussen, M., Zeale, M. R., Clare, E., ... & Gilbert, M. T. P. (2011). Molecular diet analysis of two African free-tailed bats (Molossidae) using high throughput sequencing. *PloS one*, *6*(6), e21441.

Bott, N. J., Ophel-Keller, K. M., Sierp, M. T., Rowling, K. P., McKay, A. C., Loo, M. G., ... & Deveney, M. R. (2010). Toward routine, DNA-based detection methods for marine pests. *Biotechnology advances*, 28(6), 706-714.

Boni, M. F., Lemey, P., Jiang, X., Lam, T. T. Y., Perry, B. W., Castoe, T. A., ... & Robertson, D. L. (2020). Evolutionary origins of the SARS-CoV-2 sarbecovirus lineage responsible for the COVID-19 pandemic. *Nature Microbiology*, 5(11), 1408-1417.

Bontadina, F., Schmied, S. F., Beck, A., & Arlettaz, R. (2008). Changes in prey abundance unlikely to explain the demography of a critically endangered Central European bat. *Journal of Applied Ecology*, 45(2), 641-648.

Boonman, A., Fenton, B., & Yovel, Y. (2019). The benefits of insect-swarm hunting to echolocating bats, and its influence on the evolution of bat echolocation signals. *PLoS Computational Biology*, 15(12), e1006873.

Boyles, J. G., Cryan, P. M., McCracken, G. F., & Kunz, T. H. (2011). Economic importance of bats in agriculture. *Science*, 332(6025), 41-42.

Boyles, J. G., Sole, C. L., Cryan, P. M., & McCracken, G. F. (2013). On estimating the economic value of insectivorous bats: prospects and priorities for biologists. In *Bat evolution, ecology, and conservation* (pp. 501-515). Springer, New York, NY.

Brook, C. E., & Dobson, A. P. (2015). Bats as 'special'reservoirs for emerging zoonotic pathogens. *Trends in microbiology*, 23(3), 172-180.

Burbank, L. P., & Ortega, B. C. (2018). Novel amplification targets for rapid detection and differentiation of *Xylella fastidiosa* subspecies fastidiosa and multiplex in plant and insect tissues. *Journal of microbiological methods*, 155, 8-18.

Buxton, R. D. (1983). Forest management and the pine processionary moth. *Outlook on Agriculture*, *12*(1), 34-39.

Carr, G. M., & Macdonald, D. W. (1986). The sociality of solitary foragers: a model based on resource dispersion. *Animal Behaviour*, *34*(5), 1540-1549.

Carroll, S. S., & Pearson, D. L. (2000). Detecting and modeling spatial and temporal dependence in conservation biology. *Conservation Biology*, 14(6), 1893-1897.

Casey, J. M., Meyer, C. P., Morat, F., Brandl, S. J., Planes, S., & Parravicini, V. (2019). Reconstructing hyperdiverse food webs: Gut content metabarcoding as a tool to disentangle trophic interactions on coral reefs. *Methods in Ecology and Evolution*, 10(8), 1157-1170.

Chailleux, A., Mohl, E. K., Teixeira Alves, M., Messelink, G. J., & Desneux, N. (2014). Natural enemy-mediated indirect interactions among prey species: potential for enhancing biocontrol services in agroecosystems. *Pest Management Science*, 70(12), 1769-1779.

Charbonnier, Y., Barbaro, L., Theillout, A., & Jactel, H. (2014). Numerical and functional responses of forest bats to a major insect pest in pine plantations. *PloS One*, *9*(10), e109488.

Charbonnier, Y., Papura, D., Touzot, O., Rhouy, N., Sentenac, G., & Rusch, A (2021). Pest control services provided by bats in vineyard landscapes. *Agriculture, Ecosystems & Environment*, 306, 107207.

Clarke, L. J., Soubrier, J., Weyrich, L. S., & Cooper, A. (2014). Environmental metabarcodes for insects: in silico PCR reveals potential for taxonomic bias. *Molecular Ecology Resources*, *14*(6), 1160-1170.Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460-2461.

Cohen, Y., Bar-David, S., Nielsen, M., Bohmann, K., & Korine, C. (2020). An appetite for pests: Synanthropic insectivorous bats exploit cotton pest irruptions and consume various deleterious arthropods. *Molecular Ecology*, 29(6), 1185-1198.

Culliney, T. W. (2014). Crop losses to arthropods. In *Integrated Pest Management* (pp. 201-225). Springer, Dordrecht.

Curtsdotter, A., Banks, H. T., Banks, J. E., Jonsson, M., Jonsson, T., Laubmeier, A. N., ... & Bommarco, R. (2019). Ecosystem function in predator–prey food webs—confronting dynamic models with empirical data. *Journal of Animal Ecology*, 88(2), 196-210.

Cvikel, N., Berg, K. E., Levin, E., Hurme, E., Borissov, I., Boonman, A., ... & Yovel, Y. (2015). Bats aggregate to improve prey search but might be impaired when their density becomes too high. *Current Biology*, 25(2), 206-211.

Czernik, M., Taberlet, P., Świsłocka, M., Czajkowska, M., Duda, N., & Ratkiewicz, M. (2013). Fast and efficient DNA-based method for winter diet analysis from stools of three cervids: moose, red deer, and roe deer. *Acta Theriologica*, 58(4), 379-386.

Davidson-Watts, I., & Jones, G. (2006). Differences in foraging behaviour between *Pipistrellus pipistrellus* (Schreber, 1774) and *Pipistrellus pygmaeus* (Leach, 1825*). Journal of Zoology*, 268(1), 55-62.

de Torrez, E. C. B., Brown, V. A., McCracken, G. F., & Kunz, T. H. (2019). Sympatric Bat Species Prey Opportunistically on a Major Moth Pest of Pecans. *Sustainability*, 11(22), 6365.

Deagle, B. E., Tollit, D. J., Jarman, S. N., Hindell, M. A., Trites, A. W., & Gales, N. J. (2005). Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions. *Molecular Ecology*, 14(6), 1831-1842.

Deagle, B. E., Gales, N. J., Evans, K., Jarman, S. N., Robinson, S., Trebilco, R., & Hindell, M. A. (2007). Studying seabird diet through genetic analysis of faeces: a case study on macaroni penguins (*Eudyptes chrysolophus*). *PLoS One*, 2(9), e831.

Deagle, B. E., Chiaradia, A., McInnes, J., & Jarman, S. N. (2010). Pyrosequencing faecal DNA to determine diet of little penguins: is what goes in what comes out? *Conservation Genetics*, 11(5), 2039-2048.

Deagle, B. E., Thomas, A. C., McInnes, J. C., Clarke, L. J., Vesterinen, E. J., Clare, E. L., ... & Eveson, J. P. (2019). Counting with DNA in metabarcoding studies: How should we convert sequence reads to dietary data? *Molecular Ecology*, 28(2), 391-406.

Delbac, L., & Thiéry, D. (2016). Damage to grape flowers and berries by *Lobesia botrana* larvae (Denis & Schiffernüller) (Lepidoptera: Tortricidae), and relation to larval age. *Australian Journal of Grape and Wine Research*, *22*(2), 256-261.

Denzinger, A., & Schnitzler, H. U. (2013). Bat guilds, a concept to classify the highly diverse foraging and echolocation behaviors of microchiropteran bats. *Frontiers in physiology*, 4, 164.

Deutsch, C. A., Tewksbury, J. J., Tigchelaar, M., Battisti, D. S., Merrill, S. C., Huey, R. B., & Naylor, R. L. (2018). Increase in crop losses to insect pests in a warming climate. *Science*, 361(6405), 916-919.

Dillon, M. E., Wang, G., & Huey, R. B. (2010). Global metabolic impacts of recent climate warming. *Nature*, 467(7316), 704-706.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460-2461.

Edgar, R. C. (2016). UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. *BioRxiv*, 081257.

Ehler, L. E., & Miller, J. C. (1978). Biological control in temporary agroecosystems. *Entomophaga*, 23(3), 207-212.

Ehler, L. E. (1998). Invasion Biology and Biological Control. *Biological control* 13(2): 127-133.

Eilenberg, J., Hajek, A., & Lomer, C. (2001). Suggestions for unifying the terminology in biological control. *BioControl*, 46(4), 387-400.

Elbrecht, V., & Leese, F. (2015). Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass—sequence relationships with an innovative metabarcoding protocol. *PloS One*, 10(7).

Elbrecht, V., & Leese, F. (2017). Validation and development of COI metabarcoding primers for freshwater macroinvertebrate bioassessment. *Frontiers in Environmental Science*, *5*, 11.

Epp, L. S., Boessenkool, S., Bellemain, E. P., Haile, J., Esposito, A., Riaz, T., ... & Stenøien, H. K. (2012). New environmental metabarcodes for analysing soil DNA: potential for studying past and present ecosystems. *Molecular Ecology*, 21(8), 1821-1833.

Esnaola, A., Arrizabalaga-Escudero, A., González-Esteban, J., Elosegi, A., & Aihartza, J. (2018). Determining diet from faeces: Selection of metabarcoding primers for the insectivore Pyrenean desman (*Galemys pyrenaicus*). *PloS one*, 13(12), e0208986.

Eurostat. (2017). Vineyards in the EU – statistics. https://ec.europa.eu/eurostat/statisticsexplained/index.php/Vineyards_in_the_EU_-_statistics. (Accessed 14 September 2020).

Evans, N. T., Olds, B. P., Renshaw, M. A., Turner, C. R., Li, Y., Jerde, C. L., ... & Lodge, D. M. (2016). Quantification of mesocosm fish and amphibian species diversity via environmental DNA metabarcoding. *Molecular Ecology Resources*, 16(1), 29-41.

Fenton, M. B., Mubareka, S., Tsang, S. M., Simmons, N. B., & Becker, D. J. (2020). COVID-19 and threats to bats. *FACETS*, 5: 349–352.

Ferracini, C., Ferrari, E., Pontini, M., Saladini, M. A., & Alma, A. (2019). Effectiveness of *Torymus sinensis*: a successful long-term control of the Asian chestnut gall wasp in Italy. *Journal of Pest Science*, 92(1), 353-359.

Ficetola, G. F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., ... & Rayé, G. (2015). Replication levels, false presences and the estimation of the presence/absence from DNA metabarcoding data. *Molecular Ecology Resources*, *15*(3), 543-556.

Forin-Wiart, M. A., Poulle, M. L., Piry, S., Cosson, J. F., Larose, C., & Galan, M. (2018). Evaluating metabarcoding to analyse diet composition of species foraging in anthropogenic landscapes using Ion Torrent and Illumina sequencing. *Scientific Reports*, 8(1), 1-12.

Forrest, J. R. (2016). Complex responses of insect phenology to climate change. *Current Opinion in Insect Science*, 17, 49-54.

Froidevaux, J. S., Louboutin, B., & Jones, G. (2017). Does organic farming enhance biodiversity in Mediterranean vineyards? A case study with bats and arachnids. *Agriculture, Ecosystems & Environment*, 249, 112-122.

Galan, M., Pons, J. B., Tournayre, O., Pierre, E., Leuchtmann, M., Pontier, D., & Charbonnel, N. (2018). Metabarcoding for the parallel identification of several hundred predators and their prey: Application to bat species diet analysis. *Molecular Ecology Resources*, 18(3), 474-489.

Garin, I., Aihartza, J., Goiti, U., Arrizabalaga-Escudero, A., Nogueras, J., & Ibáñez, C. (2019). Bats from different foraging guilds prey upon the pine processionary moth. *PeerJ*, 7, e7169.

Gatto, P., Zocca, A., Battisti, A., Barrento, M. J., Branco, M., & Paiva, M. R. (2009). Economic assessment of managing processionary moth in pine forests: A case-study in Portugal. *Journal of Environmental Management*, *90*(2), 683-691.

Ghosh, B., Crosbie, P. B., Nowak, B. F., & Bridle, A. R. (2018). A highly sensitive, non-invasive qPCR-based strategy for direct quantification of Yersinia ruckeri in fish faeces. *Journal of Fish Diseases*, 41(9), 1421-1428.

Giebner, H., Langen, K., Bourlat, S. J., Kukowka, S., Mayer, C., Astrin, J. J., ... & Fonseca, V. G. (2020). Comparing diversity levels in environmental samples: DNA sequence capture and metabarcoding approaches using 18S and COI genes. *Molecular Ecology Resources*, 20(5), 1333-1345.

Gillam, E. H. (2007). Eavesdropping by bats on the feeding buzzes of conspecifics. *Canadian Journal of Zoology*, 85(7), 795-801.

Gillet, F., Tiouchichine, M. L., Galan, M., Blan, F., Némoz, M., Aulagnier, S., & Michaux, J. R. (2015). A new method to identify the endangered Pyrenean desman (Galemys pyrenaicus) and to study its diet, using next generation sequencing from faeces. *Mammalian Biology*, 80(6), 505-509.

Goiti, U., Vecin, P., Garin, I., Saloña, M., & Aihartza, J. R. (2003). Diet and prey selection in Kuhl's pipistrelle *Pipistrellus kuhlii* (Chiroptera: Vespertilionidae) in south-western Europe. *Acta Theriologica*, 48(4), 457-468.

Goiti, U., Aihartza, J. R., Almenar, D., Salsamendi, E., & Garin, I. (2006). Seasonal foraging by *Rhinolophus euryale* (Rhinolophidae) in an Atlantic rural landscape in northern Iberian Peninsula. *Acta Chiropterologica*, 8(1), 141-155.

Goiti, U., Aihartza, J., Guiu, M., Salsamendi, E., Almenar, D., Napal, M., & Garin, I. (2011). Geoffroy's bat, *Myotis emarginatus*, preys preferentially on spiders in multistratified dense habitats: a study of foraging bats in the Mediterranean. *Journal of Vertebrate Biology*, 60(1), 17-24.

Ghosh, B., Crosbie, P. B., Nowak, B. F., & Bridle, A. R. (2018). A highly sensitive, non-invasive qPCR-based strategy for direct quantification of *Yersinia ruckeri* in fish faeces. *Journal of Fish Diseases*, 41(9), 1421-1428.

Goss-Custard, J. D., West, A. D., Yates, M. G., Caldow, R. W., Stillman, R. A., Bardsley, L., ... & Eichhorn, G. (2006). Intake rates and the functional response in shorebirds (Charadriiformes) eating macro-invertebrates. *Biological Reviews*, *81*(4), 501-529.

Gutierrez, A. P., Ponti, L., Gilioli, G., & Baumgärtner, J. (2018). Climate warming effects on grape and grapevine moth (*Lobesia botrana*) in the Palearctic region. *Agricultural and Forest Entomology*, *20*(2), 255-271.

Hajek, A. E., Hurley, B. P., Kenis, M., Garnas, J. R., Bush, S. J., Wingfield, M. J., ... & Cock, M. J. (2016). Exotic biological control agents: A solution or contribution to arthropod invasions?. *Biological Invasions*, 18(4), 953-969.

Hall, T., Biosciences, I., & Carlsbad, C. (2011). BioEdit: an important software for molecular biology. GERF Bull Biosci, 2(1), 60-61.

Harper, L. R., Lawson Handley, L., Hahn, C., Boonham, N., Rees, H. C., Gough, K. C., ... & Hänfling, B. (2018). Needle in a haystack? A comparison of DNA metabarcoding and targeted qPCR for detection of the great crested newt (*Triturus cristatus*). *Ecology and Evolution*, 8(12), 6330-6341.

Harvey, J. B., Hoy, M. S., & Rodriguez, R. J. (2009). Molecular detection of native and invasive marine invertebrate larvae present in ballast and open water environmental samples collected in Puget Sound. *Journal of Experimental Marine Biology and Ecology*, *369*(2), 93-99.

Hassell, M. P., & May, R. M. (1974). Aggregation of predators and insect parasites and its effect on stability. *The Journal of Animal Ecology*, 567-594.

Hassell, M. P., Comins, H. N., & Mayt, R. M. (1991). Spatial structure and chaos in insect population dynamics. *Nature*, 353(6341), 255-258.

Hegazi, E. M., & Khafagi, W. E. (2005). Unexpected mass collection of the olive moth, *Prays oleae* Bern. by non-traditional black light traps. *IOBC wprs Bulletin*, 28(9), 109.

Henry, M., Thomas, D. W., Vaudry, R., & Carrier, M. (2002). Foraging distances and home range of pregnant and lactating little brown bats (*Myotis lucifugus*). *Journal of Mammalogy*, 83(3), 767-774.

Hilbe, J. M. (2011). *Negative binomial regression*. Cambridge University Press.

Hill, R., Allen, L., & Bucklin, A. (2001). Multiplexed species-specific PCR protocol to discriminate four N. Atlantic Calanus species, with an mtCOI gene tree for ten Calanus species. *Marine Biology*, *139*(2), 279-287.

Hill, A. P., Prince, P., Piña Covarrubias, E., Doncaster, C. P., Snaddon, J. L., & Rogers, A. (2018). AudioMoth: Evaluation of a smart open acoustic device for monitoring biodiversity and the environment. *Methods in Ecology and Evolution*, 9(5), 1199-1211.

Holt, R. D., & Hochberg, M. E. (2001). Indirect interactions, community modules and biological control: a theoretical perspective. *Evaluating indirect ecological effects of biological control*, 125, 13-37.

Ifoulis, A. A., & Savopoulou-Soultani, M. (2004). Biological control of *Lobesia botrana* (Lepidoptera: Tortricidae) larvae by using different formulations of *Bacillus thuringiensis* in 11 vine cultivars under field conditions. *Journal of Economic Entomology*, *97*(2), 340-343.

Illumina, I. (2013). 16S Metagenomic sequencing library preparation. Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System, 1-28. Available: https://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html

Ioriatti, C., Lucchi, A., & Varela, L. G. (2012). Grape berry moths in Western European vineyards and their recent movement into the New World. In *Arthropod Management in Vineyards:* (pp. 339-359). Springer, Dordrecht.

Illumina, I. (2013). 16S Metagenomic sequencing library preparation. Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System, 1-28. Available: https://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html

Jarman, S. N., Deagle, B. E., & Gales, N. J. (2004). Group-specific polymerase chain reaction for DNA-based analysis of species diversity and identity in dietary samples. *Molecular Ecology*, 13(5), 1313-1322.

Jensen, R. H., Mollerup, S., Mourier, T., Hansen, T. A., Fridholm, H., Nielsen, L. P., ... & Vinner, L. (2015). Target-dependent enrichment of virions determines the reduction of high-throughput sequencing in virus discovery. *PloS One*, 10(4), e0122636.

Jones, G., & Rayner, J. M. (1989). Foraging behavior and echolocation of wild horseshoe bats *Rhinolophus ferrumequinum* and *R. hipposideros* (Chiroptera, Rhinolophidae). *Behavioral Ecology and Sociobiology*, 25(3), 183-191.

Jones, G., & Rydell, J. (2003). *Attack and defense: interactions between echolocating bats and their insect prey*. Pp 301-345 In: Kunz T. H and Fenton M. B. (Eds), Bat Ecology. The University Chicago Press, Ltd., London.

Jones, G., & Teeling, E. C. (2006). The evolution of echolocation in bats. *Trends in Ecology & Evolution*, 21(3), 149-156.

Kabaluk, J. T., Svircev, A. M., Goettel, M. S., & Woo, S. G. (Eds.). (2010). *The use and regulation of microbial pesticides in representative jurisdictions worldwide* (p. 99). International Organization for Biological Control of Noxious Animals and Plants (IOBC).

Kalda, O., Kalda, R., & Liira, J. (2015). Multi-scale ecology of insectivorous bats in agricultural landscapes. *Agriculture, Ecosystems & Environment*, 199, 105-113.

Kaňuch, P., Hájková, P., Řehák, Z., & Bryja, J. (2007). A rapid PCR-based test for species identification of two cryptic bats *Pipistrellus pipistrellus* and *P. pygmaeus* and its application on museum and dropping samples. *Acta Chiropterologica*, *9*(1), 277-282.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., ... & Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organisation and analysis of sequence data. *Bioinformatics*, 28(12), 1647-1649.

Kelly, R. M., Kitzes, J., Wilson, H., & Merenlender, A. (2016). Habitat diversity promotes bat activity in a vineyard landscape. *Agriculture, Ecosystems & Environment*, 223, 175-181.

Kerdelhué, C., Zane, L., Simonato, M., Salvato, P., Rousselet, J., Roques, A., & Battisti, A. (2009). Quaternary history and contemporary patterns in a currently expanding species. *BMC Evolutionary Biology*, 9(1), 220.

King, R. A., Read, D. S., Traugott, M., & Symondson, W. O. C. (2008). Molecular analysis of predation: a review of best practice for DNA-based approaches. *Molecular Ecology*, 17(4), 947-963.

Kipson, M., Šálek, M., Lučan, R., Uhrin, M., Maxinová, E., Bartonika, T., ... & Naďo, L. (2018). Foraging habitat, home-range size and diet of a Mediterranean bat species, Savi's pipistrelle. *Acta Chiropterologica*, 20(2), 351-360.

Knudsen, S. W., Ebert, R. B., Hesselsøe, M., Kuntke, F., Hassingboe, J., Mortensen, P. B., ... & Møller, P. R. (2019). Species-specific detection and quantification of

environmental DNA from marine fishes in the Baltic Sea. *Journal of Experimental Marine Biology and Ecology*, 510, 31-45.

Kolkert, H., Andrew, R., Smith, R., Rader, R., & Reid, N. (2020). Insectivorous bats selectively source moths and eat mostly pest insects on dryland and irrigated cotton farms. *Ecology and Evolution*, 10(1), 371-388.

Korine, C., Niv, A., Axelrod, M., & Dahan, T. (2020). Species richness and activity of insectivorous bats in cotton fields in semi-arid and mesic Mediterranean agroecosystems. *Mammalian Biology*, 100(1), 73-80.

Krauel, J. J., Brown, V. A., Westbrook, J. K., & McCracken, G. F. (2018). Predator–prey interaction reveals local effects of high-altitude insect migration. *Oecologia*, 186(1), 49-58.

Krebs, C. J. (1999). *Ecological methodology*. New York. 2nd edition. Benjamin Cummings.

Kunz, T. H., Whitaker, J. O., & Wadanoli, M. D. (1995). Dietary energetics of the insectivorous Mexican free-tailed bat (*Tadarida brasiliensis*) during pregnancy and lactation. *Oecologia*, 101(4), 407-415.

Kunz, T. H., Lumsden, L. F., & Fenton, M. B. (2003). Ecology of cavity and foliage roosting bats. *Bat ecology*, 1, 3-89.

Lee, Y. F., & McCracken, G. F. (2002). Foraging activity and food resource use of Brazilian free-tailed bats, *Tadarida brasiliensis* (Molossidae). *Ecoscience*, 9(3), 306-313.

Liebhold, A. M., McCullough, D. G., Blackburn, L. M., Frankel, S. J., Von Holle, B., & Aukema, J. E. (2013). A highly aggregated geographical distribution of forest pest invasions in the USA. *Diversity and Distributions*, 19(9), 1208-1216.

Lino, A., Fonseca, C., Goiti, U., & Pereira, M. J. R. (2014). Prey selection by *Rhinolophus hipposideros* (Chiroptera, Rhinolophidae) in a modified forest in Southwest Europe. *Acta Chiropterologica*, 16(1), 75-83.

Louis, F., & Schirra, K. J. (2001). Mating disruption of *Lobesia botrana* (Lepidoptera: Tortricidae) in vineyards with very high population densities. *IOBC Wprs Bulletin*, *24*(2), 75-80.

Louis, F., Schmidt-Tiedemann, A., & Schirra, K. J. (2002). Control of *Sparganothis pilleriana* Schiff. and *Lobesia botrana* Den. & Schiff. in German vineyards using sex pheromone-mediated mating disruption. *IOBC/WPRS Bulletin*, *25*, 1-9.

Lu, Y., Wu, K., Jiang, Y., Guo, Y., & Desneux, N. (2012). Widespread adoption of Bt cotton and insecticide decrease promotes biocontrol services. *Nature*, 487(7407), 362-365.

MacFarlane, D., & Rocha, R. (2020). Guidelines for communicating about bats to prevent persecution in the time of COVID-19. *Biological Conservation*, 108650.

Marshall, O. J. (2004). PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. *Bioinformatics*, 20(15), 2471-2472.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. Journal*, *17*(1), 10-12.

Martin, K. J., & Rygiewicz, P. T. (2005). Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC Microbiology*, 5(1), 28.

Martín-Vertedor, D., Ferrero-García, J. J., & Torres-Vila, L. M. (2010). Global warming affects phenology and voltinism of *Lobesia botrana* in Spain. *Agricultural and Forest Entomology*, *12*(2), 169-176.

Marques, J. T., Rainho, A., Carapuço, M., Oliveira, P., & Palmeirim, J. M. (2004). Foraging behaviour and habitat use by the European free-tailed bat *Tadarida teniotis*. *Acta Chiropterologica*, 6(1), 99-110.

Maslo, B., Valentin, R., Leu, K., Kerwin, K., Hamilton, G. C., Bevan, A., ... & Fonseca, D. M. (2017). Chirosurveillance: The use of native bats to detect invasive agricultural pests. *PloS One*, 12(3), e0173321.

Mata, V. A., Rebelo, H., Amorim, F., McCracken, G. F., Jarman, S., & Beja, P. (2019). How much is enough? Effects of technical and biological replication on metabarcoding dietary analysis. *Molecular Ecology*, 28(2), 165-175.

Mauvisseau, Q., Burian, A., Gibson, C., Brys, R., Ramsey, A., & Sweet, M. (2019). Influence of accuracy, repeatability and detection probability in the reliability of species-specific eDNA based approaches. *Scientific reports*, 9(1), 1-10

McCracken, G. F., Brown, V. A., Eldridge, M., & Westbrook, J. K. (2005). The use of fecal DNA to verify and quantify the consumption of agricultural pests. *Bat Research News*, 46, 195-196.

McCracken, G. F., Westbrook, J. K., Brown, V. A., Eldridge, M., Federico, P., & Kunz, T. H. (2012). Bats track and exploit changes in insect pest populations. *PLoS One*.

Meusnier, I., Singer, G. A., Landry, J. F., Hickey, D. A., Hebert, P. D., & Hajibabaei, M. (2008). A universal DNA mini-barcode for biodiversity analysis. *BMC genomics*, 9(1), 1-4.

Michalko, R., Pekár, S., & Entling, M. H. (2019). An updated perspective on spiders as generalist predators in biological control. *Oecologia*, 189(1), 21-36.

Migens, E. (2007). *Rhinolophus hipposideros* (Bechstein, 1800). *Atlas y Libro Rojo de los Mamíferos Terrestres de España. Ministerio de Medio Ambiente. Dirección General para la Biodiversidad-SECEM-SECEMU*. Madrid, 139-143. Gisbert, J. (Eds). Ministerio de Medio Ambiente.

Mitschunas, N., & Wagner, M. (2015). Diet of the lesser horseshoe bat (*Rhinolophus hipposideros*) in Central Germany and its seasonal and site-specific variation. *Acta Chiropterologica*, 17(2), 379-392.

Meyer, C. F., Aguiar, L. M., Aguirre, L. F., Baumgarten, J., Clarke, F. M., Cosson, J. F., ... & Kalko, E. K. (2011). Accounting for detectability improves estimates of species richness in tropical bat surveys. *Journal of Applied Ecology*, 48(3), 777-787.

Mollentze, N., & Streicker, D. G. (2020). Viral zoonotic risk is homogenous among taxonomic orders of mammalian and avian reservoir hosts. *Proceedings of the National Academy of Sciences*, 117(17), 9423-9430.

Moneo, I., Battisti, A., Dufour, B., García-Ortiz, J. C., González-Muñoz, M., Moutou, F., ... & Roques, A. (2015). Medical and veterinary impact of the urticating processionary larvae. In *Processionary moths and climate change: an update* (pp. 359-410). Springer, Dordrecht.

Moreau, J., Richard, A., Benrey, B., & Thiéry, D. (2009). Host plant cultivar of the grapevine moth *Lobesia botrana* affects the life history traits of an egg parasitoid. *Biological Control*, *50*(2), 117-122.

Morisita, M. (1959). Measuring of the dispersion of individuals and analysis of the distributional patterns. *Mem. Fac. Sci. Kyushu Univ. Ser. E*, 2(21), 5-235.

Moschos, T. (2005). Yield loss quantification and assessment of economic injury level for the anthophagous generation of the European grapevine moth *Lobesia botrana* Den. et Schiff.(Lepidoptera: Tortricidae). *International Journal of Pest Management*, *51*(2), 81-89.

Moschos, T. (2006). Yield loss quantification and economic injury level estimation for the carpophagous generations of the European grapevine moth *Lobesia botrana* Den. et Schiff.(Lepidoptera: Tortricidae). *International Journal of Pest Management*, *52*(02), 141-147.

Müller, J., Mehr, M., Bässler, C., Fenton, M. B., Hothorn, T., Pretzsch, H., ... & Brandl, R. (2012). Aggregative response in bats: prey abundance versus habitat. *Oecologia*, *169*(3), 673-684.

Nardone, V., Cistrone, L., Di Salvo, I., Ariano, A., Migliozzi, A., Allegrini, C., ... & Russo, D. (2015). How to be a male at different elevations: Ecology of intra-sexual segregation in the trawling bat *Myotis daubentonii*. *PLoS One*, 10(7), e0134573.

Neethirajan, S., Karunakaran, C., Jayas, D. S., & White, N. D. G. (2007). Detection techniques for stored-product insects in grain. *Food Control*, 18(2), 157-162.

Nowakowska, J. A., Malewski, T., Tereba, A., & Oszako, T. (2017). Rapid diagnosis of pathogenic Phytophthora species in soil by real-time PCR. *Forest Pathology*, 47(2), e12303.

Oerke, E. C. (2006). Crop losses to pests. *The Journal of Agricultural Science*, *144*(1), 31-43.

OIV. (2019). 2019 Statistical Report on World Vitiviniculture. In Balance 2019 de la OIV sobre la situación vitivinícola mundial. http://www.oiv.int/es/actualidad-de-la-oiv/balance-2019-de-la-oiv-sobre-la-situacion-vitivinicola-mundial (Accessed July 3, 2020).

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M. H. H., Oksanen, M. J., & Suggests, M. A. S. S. (2007). The vegan package. *Community Ecology Package*, 10, 631-637.

Olson, S. (2015). An analysis of the biopesticide market now and where it is going. *Outlooks on pest management*, 26(5), 203-206.

Ortega-Lopez, V., Amo-Salas, M., Ortiz-Barredo, A., & Diez-Navajas, A. M. (2014). Male flight phenology of the European grapevine moth *Lobesia botrana* (Lepidoptera: Tortricidae) in different wine-growing regions in Spain. *Bull Entomol Res*, 104, 566-575.

Page, A., & Gomez-Curet, I. (2016). Assuring Reliability of qPCR & RT-PCR Results: Use of Spectrophotometry on Nucleic Acid Samples Before Experiment Improves Outcome. *Genetic Engineering & Biotechnology News*, 31(16), 26-26.

Paini, D. R., Sheppard, A. W., Cook, D. C., De Barro, P. J., Worner, S. P., & Thomas, M. B. (2016). Global threat to agriculture from invasive species. *Proceedings of the National Academy of Sciences*, 113(27), 7575-7579.

Papadatou, E., Butlin, R. K., & Altringham, J. D. (2008). Identification of bat species in Greece from their echolocation calls. *Acta Chiropterologica*, 10(1), 127-143.

Pardey, P. G., Beddow, J. M., Hurley, T. M., Beatty, T. K., & Eidman, V. R. (2014). A bounds analysis of world food futures: global agriculture through to 2050. *Australian Journal of Agricultural and Resource Economics*, 58(4), 571-589.

Pebesma, E., & Heuvelink, G. (2016). Spatio-temporal interpolation using gstat. *RFID Journal*, 8(1), 204-218.

Peláez, H., Maraña, R., Vasquez de Prada, P., Puras, A., & Santiago, Y. (2006). Local population behaviour of *Lobesia botrana* Denis & Schiffermüller (Lepidoptera: Tortricidae). *Bol. San. Veg., Plagas*, 32, 189-197.

Pereira, M. J. R., Rebelo, H., Rainho, A., & Palmeirim, J. M. (2002). Prey selection by *Myotis myotis* (Vespertilionidae) in a Mediterranean region. *Acta Chiropterologica*, 4(2), 183-193.

Perrings, C., Dehnen-Schmutz, K., Touza, J., & Williamson, M. (2005). How to manage biological invasions under globalization. *Trends in Ecology & Evolution*, 20(5), 212-215.

Pickett, C. H., & Bugg, R. L. (Eds.). (1998). *Enhancing biological control: habitat management to promote natural enemies of agricultural pests*. Univ of California Press.

Piggott, M. P. (2016). Evaluating the effects of laboratory protocols on eDNA detection probability for an endangered freshwater fish. *Ecology and evolution*, *6*(9), 2739-2750.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., & Team, R. C. (2021). *nlme: Linear and Nonlinear Mixed Effects Models*. R package version, 3(57), 1-89.

Piñol, J., Mir, G., Gomez-Polo, P., & Agustí, N. (2015). Universal and blocking primer mismatches limit the use of high-throughput DNA sequencing for the quantitative metabarcoding of arthropods. *Molecular Ecology Resources*, 15(4), 819-830.

Pompanon, F., Deagle, B. E., Symondson, W. O., Brown, D. S., Jarman, S. N., & Taberlet, P. (2012). Who is eating what: diet assessment using next generation sequencing? *Molecular ecology*, *21*(8), 1931-1950.

Preatoni, D. G., Nodari, M., Chirichella, R., Tosi, G., Wauters, L. A., & Martinoli, A. (2005). Identifying bats from time-expanded recordings of search calls: Comparing classification methods. *The Journal of wildlife management*, 69(4), 1601-1614.

Puig-Montserrat, X., Torre, I., López-Baucells, A., Guerrieri, E., Monti, M. M., Ràfols-García, R., ... & Flaquer, C. (2015). Pest control service provided by bats in

Mediterranean rice paddies: linking agroecosystems structure to ecological functions. *Mammalian Biology*, *80*(3), 237-245

Puig-Montserrat, X., Flaquer, C., Gómez-Aguilera, N., Burgas, A., Mas, M., Tuneu, C., ... & López-Baucells, A. (2020). Bats actively prey upon mosquitoes and other deleterious insects in rice paddies: Potential impact on human health and agriculture. *Pest Management Science*.

Purcell, R. V., Pearson, J., Frizelle, F. A., & Keenan, J. I. (2016). Comparison of standard, quantitative and digital PCR in the detection of enterotoxigenic Bacteroides fragilis. *Scientific reports*, 6(1), 1-8.

QGIS Development Team (2018). QGIS Geographic Information System v3.0.3 Girona. Open Source Geospatial Foundation. URL http://qgis.org

Rank, A., Ramos, R. S., da Silva, R. S., Soares, J. R. S., Picanço, M. C., & Fidelis, E. G. (2020). Risk of the introduction of *Lobesia botrana* in suitable areas for *Vitis vinifera*. *Journal of Pest Science*, 1-13.

Ratnasingham, S., & Hebert, P. D. N. (2007). Barcoding. BOLD: The barcode of life data system (www. barcodinglife. org). *Molecular Ecology Notes*, 7(3), 355-364.

Razgour, O., Clare, E. L., Zeale, M. R., Hanmer, J., Schnell, I. B., Rasmussen, M., ... & Jones, G. (2011). High-throughput sequencing offers insight into mechanisms of resource partitioning in cryptic bat species. *Ecology and Evolution*, 1(4), 556-570.

R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Ripley, B., Venables, B., Bates, D. M., Hornik, K., Gebhardt, A., Firth, D., & Ripley, M. B. (2013). Package 'mass'. Cran R, 538.

Robinet, C., Rousselet, J., Pineau, P., Miard, F., & Roques, A. (2013). Are heat waves susceptible to mitigate the expansion of a species progressing with global warming? *Ecology and evolution*, *3*(9), 2947-2957.

Rocha, R., Aziz, S. A., Brook, C. E., Carvalho, W. D., & Cooper-Bohannon, R. (2020). Bat conservation and zoonotic disease risk: a research agenda to prevent misguided persecution in the aftermath of COVID-19. *Animal Conservation*.

Rodríguez-San Pedro, A., Rodríguez-Herbach, C., Allendes, J. L., Chaperon, P. N., Beltrán, C. A., & Grez, A. A. (2019). Responses of aerial insectivorous bats to landscape composition and heterogeneity in organic vineyards. *Agriculture, Ecosystems & Environment*, 277, 74-82.

Rodríguez-San Pedro, A., Allendes, J. L., Beltrán, C. A., Chaperon, P. N., Saldarriaga-Córdoba, M. M., Silva, A. X., & Grez, A. A. (2020). Quantifying ecological and economic value of pest control services provided by bats in a vineyard landscape of central Chile. *Agriculture, Ecosystems & Environment*, 302, 107063.

Roux, K. H. (2009). Optimisation and troubleshooting in PCR. *Cold Spring Harbor Protocols*, 2009(4).

Rue, H., Martino, S., & Chopin, N. (2009). Approximate Bayesian inference for latent Gaussian models by using integrated nested Laplace approximations. *Journal of the Royal Statistical Society: Series b* (statistical methodology), 71(2), 319-392.

Rue, H., Riebler, A., Sørbye, S. H., Illian, J. B., Simpson, D. P., & Lindgren, F. K. (2017). Bayesian computing with INLA: a review. *Annual Review of Statistics and Its Application*, 4, 395-421.

Russ, J. (2012). *British bat calls: a guide to species identification*. Pelagic publishing.

Russ, J. M., & Montgomery, W. I. (2002). Habitat associations of bats in Northern Ireland: implications for conservation. *Biological Conservation*, 108(1), 49-58.

Russo, D., & Jones, G. (2002). Identification of twenty-two bat species (Mammalia: Chiroptera) from Italy by analysis of time-expanded recordings of echolocation calls. *Journal of Zoology, 258*(1), 91-103.

Russo, D., Almenar, D., Aihartza, J., Goiti, U., Salsamendi, E., & Garin, I. (2005). Habitat selection in sympatric *Rhinolophus mehelyi* and *R. euryale* (Mammalia: Chiroptera). *Journal of Zoology, 266*(3), 327-332.

Russo, D., & Papadatou, E. (2014). Acoustic identification of free-flying Schreiber's bat *Miniopterus schreibersii* by social calls. *Hystrix, the Italian Journal of Mammalogy*, 25(2), 119-120.

Russo, D., & Voigt, C. C. (2016). The use of automated identification of bat echolocation calls in acoustic monitoring: A cautionary note for a sound analysis. *Ecological Indicators*, 66, 598-602.

Russo, D., Bosso, L., & Ancillotto, L. (2018). Novel perspectives on bat insectivory highlight the value of this ecosystem service in farmland: research frontiers and management implications. *Agriculture, Ecosystems & Environment*, 266, 31-38.

Rydell, J. (1989). Site fidelity in the northern bat (*Eptesicus nilssoni*) during pregnancy and lactation. *Journal of mammalogy*, *70*(3), 614-617.

Rydell, J., & Arlettaz, R. (1994). Low-frequency echolocation enables the bat *Tadarida teniotis* to feed on tympanate insects. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 257(1349), 175-178.

Rydell, J., Nyman, S., Eklöf, J., Jones, G., & Russo, D. (2017). Testing the performances of automated identification of bat echolocation calls: A request for prudence. *Ecological Indicators*, *78*, 416-420.

Salsamendi, E., Arostegui, I., Aihartza, J., Almenar, D., Goiti, U., & Garin, I. (2012). Foraging ecology in Mehely's horseshoe bats: influence of habitat structure and water availability. *Acta Chiropterologica*, 14(1), 121-132.

Savary, S., Willocquet, L., Pethybridge, S. J., Esker, P., McRoberts, N., & Nelson, A. (2019). The global burden of pathogens and pests on major food crops. *Nature Ecology & Evolution,* 3(3), 430-439.

Schellhorn, N. A., Bianchi, F. J. J. A., & Hsu, C. L. (2014). Movement of entomophagous arthropods in agricultural landscapes: links to pest suppression. *Annual Review of Entomology*, 59, 559-581.

Schlatter, R. P., Matte, U., Polanczyk, C. A., Koehler-Santos, P., & Ashton-Prolla, P. (2015). Costs of genetic testing: Supporting Brazilian public policies for the

incorporating of molecular diagnostic technologies. *Genetics and molecular biology*, 38(3), 332-337.

Schmidt, B. R., Kery, M., Ursenbacher, S., Hyman, O. J., & Collins, J. P. (2013). Site occupancy models in the analysis of environmental DNA presence/absence surveys: a case study of an emerging amphibian pathogen.*Methods in Ecology and Evolution*, *4*(7), 646-653.

Schnitzler, H. U., & Kalko, E. K. (2001). Echolocation by insect-eating bats: we define four distinct functional groups of bats and find differences in signal structure that correlate with the typical echolocation tasks faced by each group. *Bioscience*, 51(7), 557-569.

Schultz, M. T., & Lance, R. F. (2015). Modeling the sensitivity of field surveys for detection of environmental DNA (eDNA).*PloS One*, *10*(10), e0141503.

Sciarretta, A., Zinni, A., Mazzocchetti, A., & Trematerra, P. (2008). Spatial analysis of *Lobesia botrana* (Lepidoptera: Tortricidae) male population in a Mediterranean agricultural landscape in central Italy. *Environmental Entomology*, 37(2), 382-390.

Sciarretta, A., & Trematerra, P. (2014). Geostatistical tools for the study of insect spatial distribution: practical implications in the integrated management of orchard and vineyard pests. *Plant protection science*, 50(2), 97-110.

Segoli, M., & Rosenheim, J. A. (2012). Should increasing the field size of monocultural crops be expected to exacerbate pest damage? *Agriculture, Ecosystems & Environment*, *150*, 38-44.

Shiel, C. B., Shiel, R. E., & Fairley, J. S. (1999). Seasonal changes in the foraging behaviour of Leisler's bats (*Nyctalus leisleri*) in Ireland as revealed by radio-telemetry. *Journal of Zoology*, 249(3), 347-358.

Siemers, B. M., & Ivanova, T. (2004). Ground gleaning in horseshoe bats: comparative evidence from *Rhinolophus blasii*, *R. euryale* and *R. mehelyi*. *Behavioral Ecology and Sociobiology*, 56(5), 464-471.

Simmons, N. B., & Cirranello, A. L. (2020). Bat Species of the World: A taxonomic and geographic database. https://batnames.org), accessed November, 13, 2020.

Simonato, M., Battisti, A., Kerdelhué, C., Burban, C., Lopez-Vaamonde, C., Pivotto, I., ... & Negrisolo, E. (2013). Host and phenology shifts in the evolution of the social moth genus *Thaumetopoea*. *PLoS One*, 8(2), e57192.

Smith, C. J., & Osborn, A. M. (2009). Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiology Ecology*, 67(1), 6-20.

Smith-Gill, S. J. (1975). Cytophysiological basis of disruptive pigmentary patterns in the leopard frog *Rana pipiens*. II. Wild type and mutant cell-specific patterns. *Journal of Morphology*, 146(1), 35-54.

Snyder, W. E., & Ives, A. R. (2003). Interactions between specialist and generalist natural enemies: parasitoids, predators, and pea aphid biocontrol. *Ecology*, 84(1), 91-107.

Sonawane, G. G., & Tripathi, B. N. (2013). Comparison of a quantitative real-time polymerase chain reaction (qPCR) with conventional PCR, bacterial culture and ELISA for detection of Mycobacterium avium subsp. paratuberculosis infection in sheep showing pathology of Johne's disease. *SpringerPlus*, *2*(1), 45.

Sow, A., Haran, J., Benoit, L., Galan, M., & Brévault, T. (2020). DNA Metabarcoding as a Tool for Disentangling Food Webs in Agroecosystems. *Insects*, 11(5), 294.

Stahlschmidt, P., Pätzold, A., Ressl, L., Schulz, R., & Brühl, C. A. (2012). Constructed wetlands support bats in agricultural landscapes. *Basic and Applied Ecology*, 13(2), 196-203.

Subramanyam, B., & Harein, P. K. (1990). Accuracies and sample sizes associated with estimating densities of adult beetles (Coleoptera) caught in probe traps in stored barley. *Journal of Economic Entomology*, 83(3), 1102-1109.

Sun, C., Cheng, Y., Pan, Y., Yang, J., Wang, X., & Xia, F. (2020). Efficient polymerase chain reaction assisted by metal–organic frameworks. *Chemical Science*, 11(3), 797-802.

Swift, S. M., & Racey, P. A. (1983). Resource partitioning in two species of vespertilionid bats (Chiroptera) occupying the same roost. *Journal of Zoology*, 200(2), 249-259.

Symondson, W. O. C., Sunderland, K. D., & Greenstone, M. H. (2002). Can generalist predators be effective biocontrol agents?. *Annual Review of Entomology*, 47(1), 561-594.

Swartz, S. M., Freeman, P. W., & Stockwell, E. F. (2003). *Ecomorphology of bats: comparative and experimental approaches relating structural design to ecology*. Pp. 257-300. In: Kunz, T. H. and Fenton, M. B. (Eds), Bat ecology. The University Chicago Press, Ltd., London.

Tabashnik, B. E., Brévault, T., & Carrière, Y. (2013). Insect resistance to Bt crops: lessons from the first billion acres. *Nature biotechnology*, 31(6), 510-521.

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., & Willerslev, E. (2012). Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology*, 21(8), 2045-2050.

Taylor, L. R., Woiwod, I. P., & Perry, J. N. (1978). The density-dependence of spatial behaviour and the rarity of randomness. *The Journal of Animal Ecology*, 383-406.

Thacker, J. R. (2002). *An introduction to arthropod pest control*. Cambridge University Press.

Thiéry, D., Monceau, K., & Moreau, J. (2014). Different emergence phenology of European grapevine moth (*Lobesia botrana*, Lepidoptera: Tortricidae) on six varieties of grapes. *Bulletin of Entomological Research*, *104*(3), 277-287.

Thiéry, D., Louâpre, P., Muneret, L., Rusch, A., Sentenac, G., Vogelweith, F., ... & Moreau, J. (2018). Biological protection against grape berry moths. A review. *Agronomy for Sustainable Development*, 38(2), 15.

Torres, J. B., & Bueno, A. D. F. (2018). Conservation biological control using selective insecticides–a valuable tool for IPM. *Biological Control*, 126, 53-64.

Tournayre, O., Leuchtmann, M., Filippi-Codaccioni, O., Trillat, M., Piry, S., Pontier, D., ... & Galan, M. (2020). In silico and empirical evaluation of twelve metabarcoding primer sets for insectivorous diet analyses. *Ecology and Evolution*.

Turner, C. R., Miller, D. J., Coyne, K. J., & Corush, J. (2014). Improved methods for capture, extraction, and quantitative assay of environmental DNA from Asian bigheaded carp (*Hypophthalmichthys* spp.). *PloS one*, *9*(12), e114329.

Uhrin, M., Hüttmeir, U., Kipson, M., Estók, P., Sachanowicz, K., Bücs, S., ... & Maxinová, E. (2016). Status of Savi's pipistrelle *Hypsugo savii* (Chiroptera) and range expansion in Central and south-eastern Europe: a review. *Mammal Review*, 46(1), 1-16.

UNDESA. (2017). World population projected to reach 9.8 billion in 2050, and 11.2 billion in 2100. United Nations Department of Economic and Social Affairs. Retrieved from https://www.un.org/development/desa/en/news/population/world-population prospects-2017.html

Van Driesche, R., & Hoddle, M. (2009). *Control of pests and weeds by natural enemies: an introduction to biological control*. John Wiley & Sons.

Van Driesche, R., & Hoddle, M. S. (2016). Non-target effects of insect biocontrol agents and trends in host specificity since 1985. *CAB Reviews*, 11(44), 1-66.

Van Lenteren, J. C., Bolckmans, K., Köhl, J., Ravensberg, W. J., & Urbaneja, A. (2018). Biological control using invertebrates and microorganisms: plenty of new opportunities. *BioControl*, 63(1), 39-59.

Varadínová, Z., Wang, Y. J., Kučerová, Z., Stejskal, V., Opit, G., Cao, Y., ... & Li, Z. H. (2015). COI barcode based species-specific primers for identification of five species of stored-product pests from genus *Cryptolestes* (Coleoptera: Laemophloeidae). *Bulletin of entomological research*, *105*(2), 202-209.

Veres, A., Petit, S., Conord, C., & Lavigne, C. (2013). Does landscape composition affect pest abundance and their control by natural enemies? A review. *Agriculture, Ecosystems & Environment*, 166, 110-117.

Vesterinen, E. J., Lilley, T., Laine, V. N., & Wahlberg, N. (2013). Next generation sequencing of fecal DNA reveals the dietary diversity of the widespread

insectivorous predator Daubenton's bat (*Myotis daubentonii*) in Southwestern Finland. *PloS One*, 8(11), e82168.

Vesterinen, E. J., Ruokolainen, L., Wahlberg, N., Peña, C., Roslin, T., Laine, V. N., ... & Lilley, T. M. (2016). What you need is what you eat? Prey selection by the bat *Myotis daubentonii*. *Molecular Ecology*, 25(7), 1581-1594.

Vincent, S., Nemoz, M., & Aulagnier, S. (2010). Activity and foraging habitats of *Miniopterus schreibersii* (Chiroptera: Miniopteridae) in southern France: implications for its conservation. *Hystrix, the Italian Journal of Mammalogy*, 22(1).

Vivier, M. A., Pretorius I. S. (2002). Genetically tailored grapevines for the wine industry. *TRENDS in Biotechnology*, 20(11): 472-478.

Walsh, A. L., Barclay, R. M., & McCracken, G. F. (2004). Designing bat activity surveys for inventory and monitoring studies at local and regional scales. *Bat echolocation research: tools, techniques and analysis. Bat Conservation International, Austin,* 157-165.

Walters, C. L., Collen, A., Lucas, T., Mroz, K., Sayer, C. A., & Jones, K. E. (2013). Challenges of using bioacoustics to globally monitor bats. In *Bat evolution, ecology, and conservation* (pp. 479-499). Springer, New York, NY.

Weiss, S. B., Murphy, D. D., Ehrlich, P. R., & Metzler, C. F. (1993). Adult emergence phenology in checkerspot butterflies: the effects of macroclimate, topoclimate, and population history. *Oecologia*, 96(2), 261-270.

Whitaker, J.O., McCracken, G.F., Siemers B.M., 2009. *Food habits analysis of insectivorous bats*. In: Kunz, T., H., Parsons, S. (Eds.), Ecological and Behavioural Methods for the Study of Bats, Second ed. The Johns Hopkins University Press, Maryland.

Wickham, H. (2016). *Ggplot2: Elegrant graphics for data analysis*. Springer.

Wood, S. A., Pochon, X., Laroche, O., von Ammon, U., Adamson, J., & Zaiko, A. (2019). A comparison of droplet digital polymerase chain reaction (PCR), quantitative PCR and metabarcoding for species-specific detection in environmental DNA. *Molecular Ecology Resources*, 19(6), 1407-1419.

Wray, A. K., Jusino, M. A., Banik, M. T., Palmer, J. M., Kaarakka, H., White, J. P., ... & Peery, M. Z. (2018). Incidence and taxonomic richness of mosquitoes in the diets of little brown and big brown bats. *Journal of Mammalogy*, 99(3), 668-674.

Xia, Z., Johansson, M. L., Gao, Y., Zhang, L., Haffner, G. D., MacIsaac, H. J., & Zhan, A. (2018). Conventional versus real-time quantitative PCR for rare species detection. *Ecology and Evolution*, 8(23), 11799-11807.

Yang, T. B., Liu, J., & Chen, J. (2020). Compared with conventional PCR assay, qPCR assay greatly improves the detection efficiency of predation. *Ecology and Evolution*, 10(14), 7713-7722.

Yee, T. W. (2015). Zero-Inflated, Zero-Altered and Positive Discrete Distributions. In *Vector Generalized Linear and Additive Models* (pp. 469-497). Springer, New York, NY.

Zeale, M. R., Butlin, R. K., Barker, G. L., Lees, D. C., & Jones, G. (2011). Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. *Molecular Ecology Resources*, 11(2), 236-244.

Zhang, G. K., Chain, F. J., Abbott, C. L., & Cristescu, M. E. (2018). Metabarcoding using multiplexed markers increases species detection in complex zooplankton communities. *Evolutionary Applications*, 11(10), 1901-1914.

Zhou, P., Yang, X. L., Wang, X. G., Hu, B., Zhang, L., Zhang, W., ... & Chen, H. D. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*, 579(7798), 270-273.

Zinger, L., Bonin, A., Alsos, I. G., Bálint, M., Bik, H., Boyer, F., ... & De Barba, M. (2019). DNA metabarcoding—Need for robust experimental designs to draw sound ecological conclusions. *Molecular Ecology*, 28(8), 1857-1862.

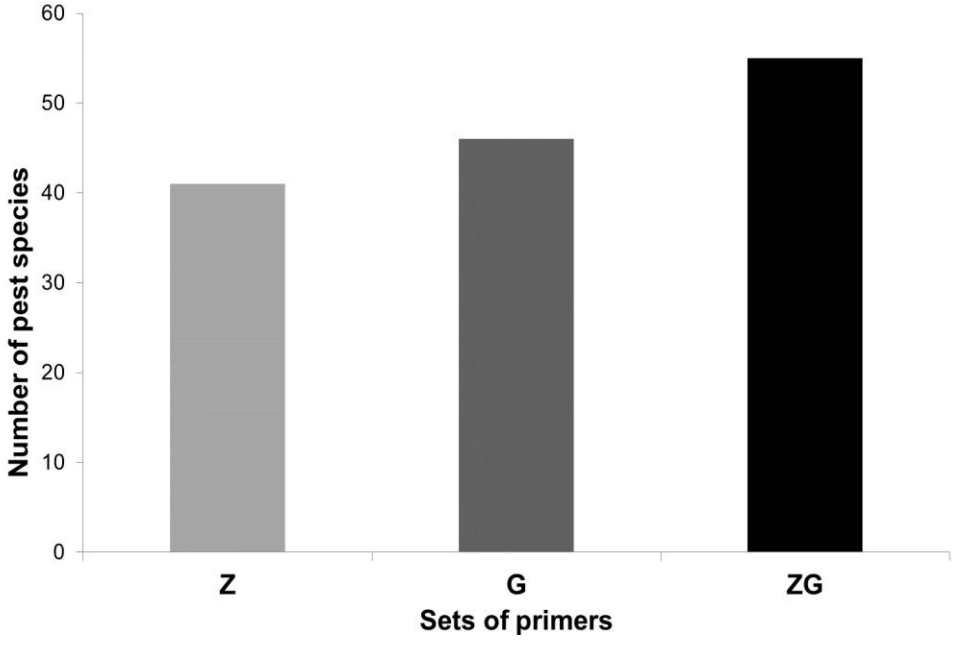
Zuur, A. F., Ieno, E. N., & Saveliev, A. A. (2017). *Spatial, Temporal and Spatial-Temporal Ecological Data Analysis with R-INLA*. Highland Statistics Ltd, 1.

# Eranskina
# Informazio gehigarria

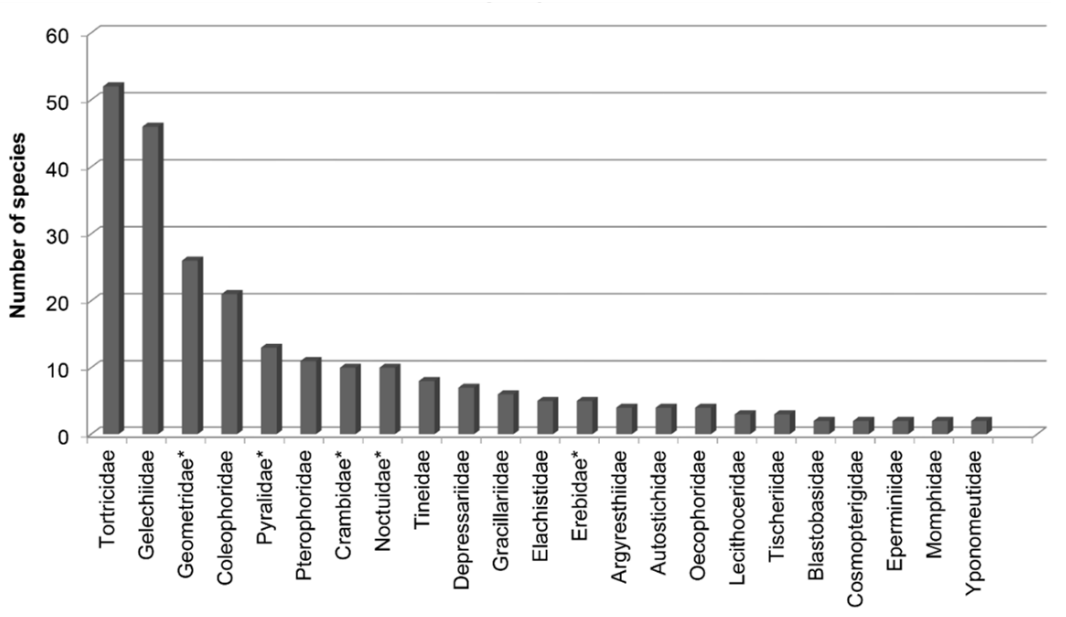
# E.1. 1 kapitulua

**Atal honetan 1. kapituluari dagozkion hurrengo edukiak landu dira:**

- **S1.1. Irudia.** Hasle bakoitzarekin (Z = ZBJ-ArtR2c eta ZBJ-123 ArtF1c; G = LepF1 eta EPT-long-univR) eta hain konbinazioz detektatutako izurri-espezieak.
- **S1.2. Irudia.** *R. hipposideros*-en dietan aurkitutako lepidopteroen familiak eta talde taxonomiko bakoitzari esleitutako espezieen kopuruak.
- **S1.1. Taula.** Bi hasleen PCR baldintzen xehetasunak.
- **S1.2. Taula.** *R. hipposideros*-en gorotzetan hasle bakoitzarekin aurkitutako espezieen zerrenda.




**S1.1. Irudia.** Hasle bakoitzarekin (Z = ZBJ-ArtR2c eta ZBJ-123 ArtF1c; G = LepF1 eta EPT-long-univR) eta hain konbinazioz detektatutako izurri-espezieak.

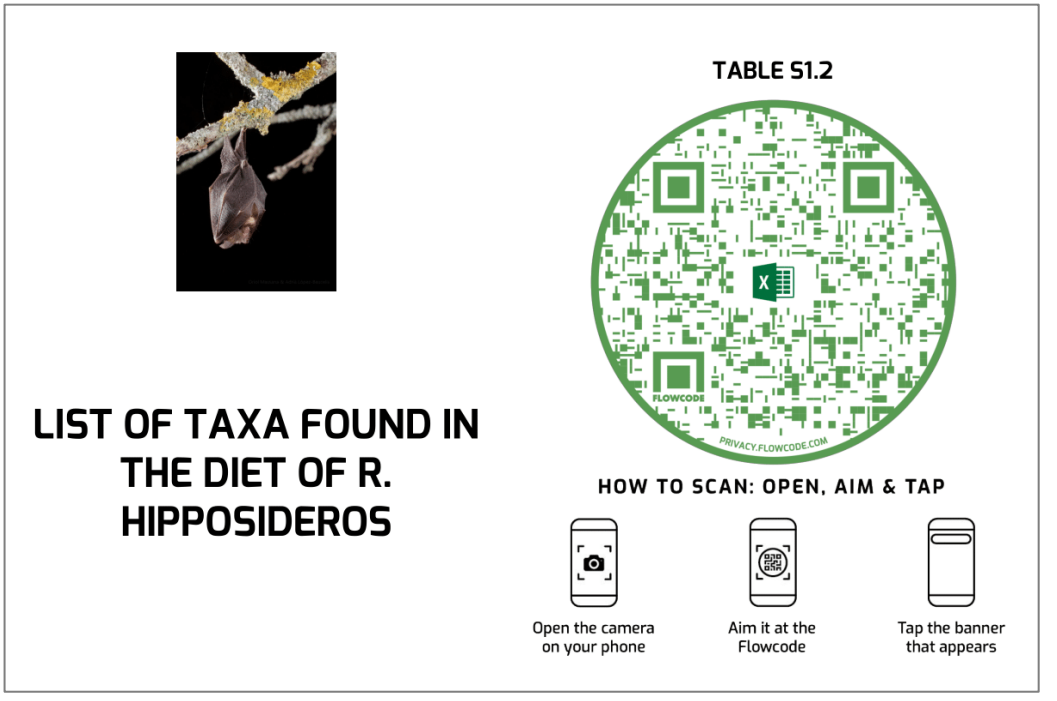



**S1.2. Irudia.** *R. hipposideros*-en dietan aurkitutako lepidopteroen familiak eta talde taxonomiko bakoitzari esleitutako espezieen kopuruak. Espezie bakarreko familiak ez dira adierazi, hauen artean Adelidae, Alucitidae, Batrachedridae, Bedelliidae, Glyphipterigidae, Hepialidae, Lasiocampidae, Limnephilidae, Lypusidae, Nepticulidae, Notodontidae, Nymphalidae, Plutellidae, Praydidae, Psychidae eta Ypsolophidae. Entzumenezko defentsa mekanismoak dituzten familiak izartxo batez (*) adierazi dira.

**S1.1. Taula.** Bi hasleen PCR baldintzen xehetasunak.

| PCR faseak | Zeale | Gillet |
|---|---|---|
| **Hasiera** | 95°C—10min | 95°C—15 min |
| **Desnaturalizazioa** | 16 ziklo:<br>95°C—30seg<br>61°C—30seg | 40 ziklo:<br>94°C—30seg<br>45°C—45seg |
| | 72°C—30seg | 72°C—30seg |
| **Suberapena** | 24 ziklo:<br>95°C—30seg<br>53°C—30seg | |
| | 72°C—30seg | |
| **Hedapena** | 72°C—10min | 72°C—10min |

**S1.2. Taula.** *R. hipposideros*-en gorotzetan hasle bakoitzarekin aurkitutako espezieen zerrenda. https://doi.org/10.1371/journal.pone.0219265.s004



# E.2. 2. kapitulua

- **Atal honetan 2. kapituluari dagozkion hurrengo edukiak landu dira:**
- **S2.1. Irudia.** *R. hipposideros*-en dietan maizen agertutako harrapakin-espezieen agerpen maiztasunak (FOO).

  **S2.2. Irudia**. *P. kuhlii*-ren dietan sarrien agertutako harrapakin-espezieen agerpen maiztasunak (FOO).

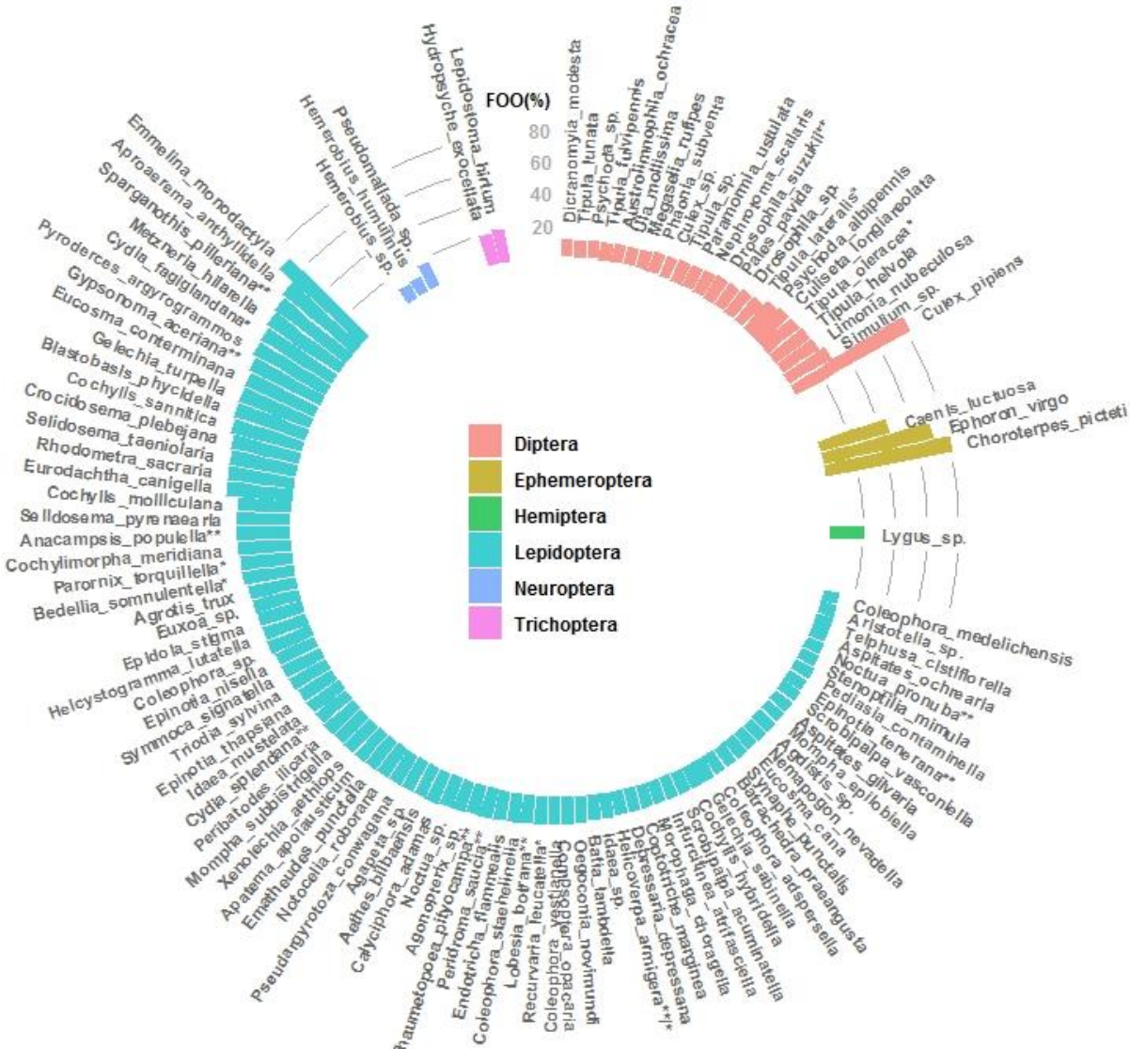
  **S2.3. Irudia.** *P. pipistrellus*-en dietan sarrien agertutako harrapakin-espezieen agerpen maiztasunak (FOO).

  **S2.4. Irudia**. *M. daubentonii*-ren dietan sarrien agertutako harrapakin-espezieen agerpen maiztasunak (FOO).
- **S2.5. Irudia.** *R. hipposideros*-en dietan izurrien garai oparoenean (Ekaina-Uztaila) maizen agertutako taxonak.
- **S2.6. Irudia.** *P. kuhlii*-ren dietan izurrien garai oparoenean (Ekaina-Uztaila) maizen agertutako taxonak.
- **S2.7. Irudia.** *P. pipistrellus*-en dietan izurrien garai oparoenean (Ekaina-Uztaila) maizen agertutako taxonak.
- **S2.8. Irudia.** *M. daubentonii*-ren dietan izurrien garai oparoenean (Ekaina-Uztaila) maizen agertutako taxonak.
- **S2.9. Irudia.** *L. botrana*-ren sakabanaketa espaziala ikerketa eremuan (1-4 asteak).
- **S2.10. Irudia.** *L. botrana*-ren sakabanaketa espaziala ikerketa eremuan (5-8 asteak).
- **S2.11. Irudia.** *L. botrana*-ren sakabanaketa espaziala ikerketa eremuan (9-11 asteak).
- **S2.12. Irudia.** *S. pilleriana*-ren sakabanaketa espaziala ikerketa eremuan (4-6 asteak).
- **S2.13. Irudia.** *S. pilleriana*-ren sakabanaketa espaziala ikerketa eremuan (7-8 asteak).
- **S2.1. Taula.** Gorotzen laginketaren gaineko informazioa eta xehetasunak.
- **S2.2. Taula.** Bi hasleen PCR baldintzen xehetasunak.

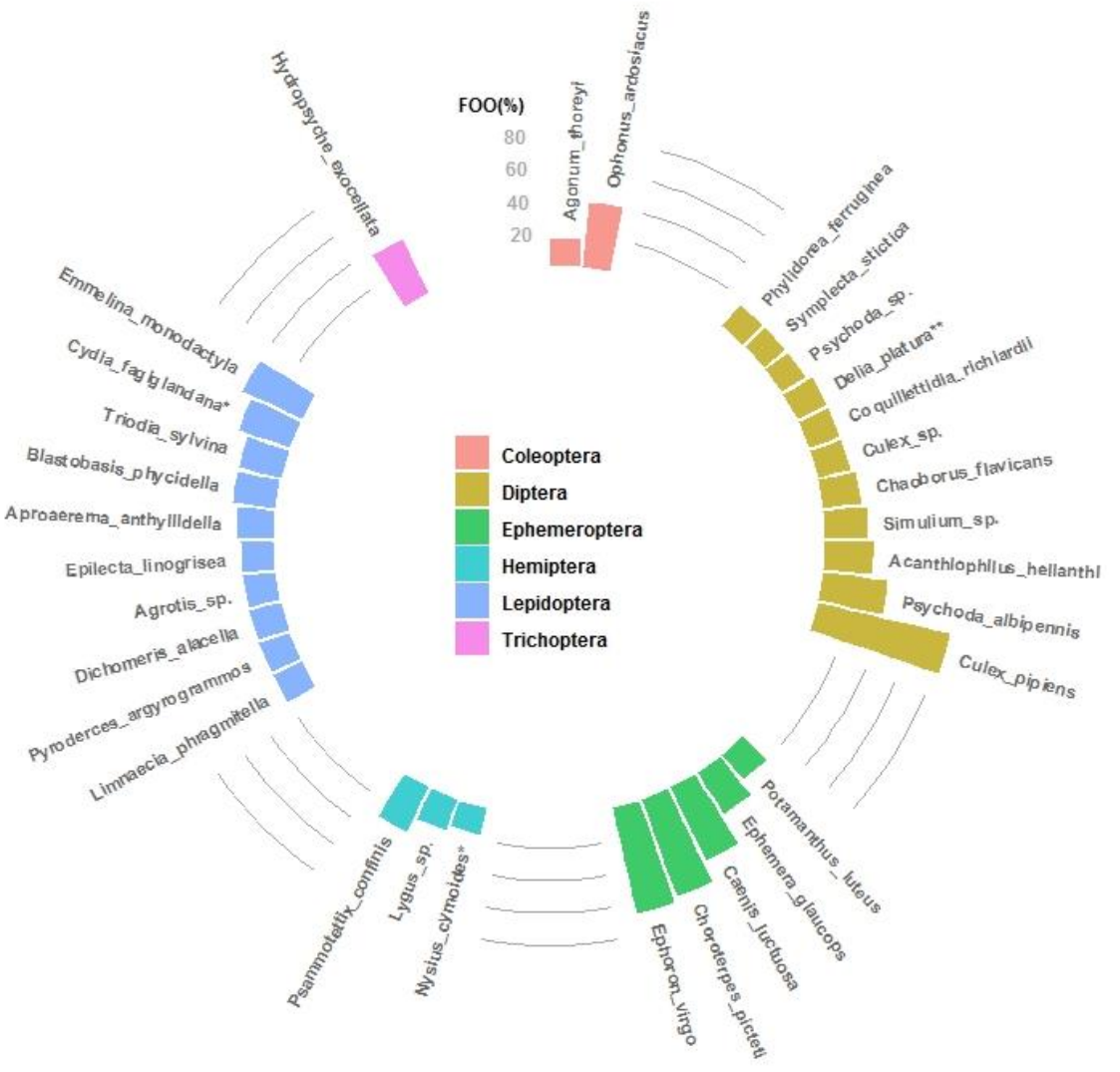
- S.2.3. Taula. *L. botrana* eta *S. pilleriana*-ren Morisita eta Morisita estandarizatuaren sakabanaketa-indizeak.

S2.4. Taula. Ikertutako saguzarren gorotzetan detektatutako harrapakari eta harrapakin espezieak eta agerpen-maiztasunak (FOO).

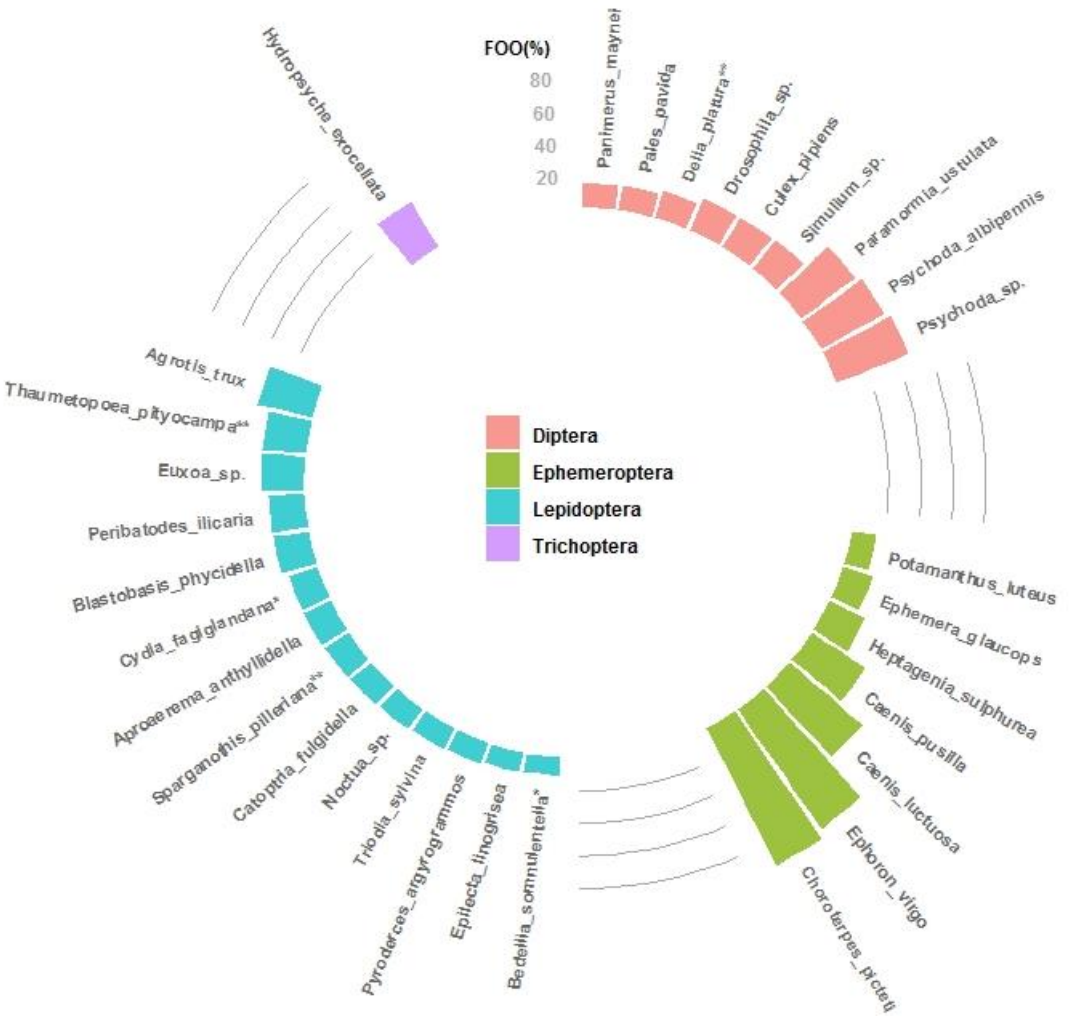
Informazio gehigarria. Eraikitako modelo estatistikoen R-kodeak.



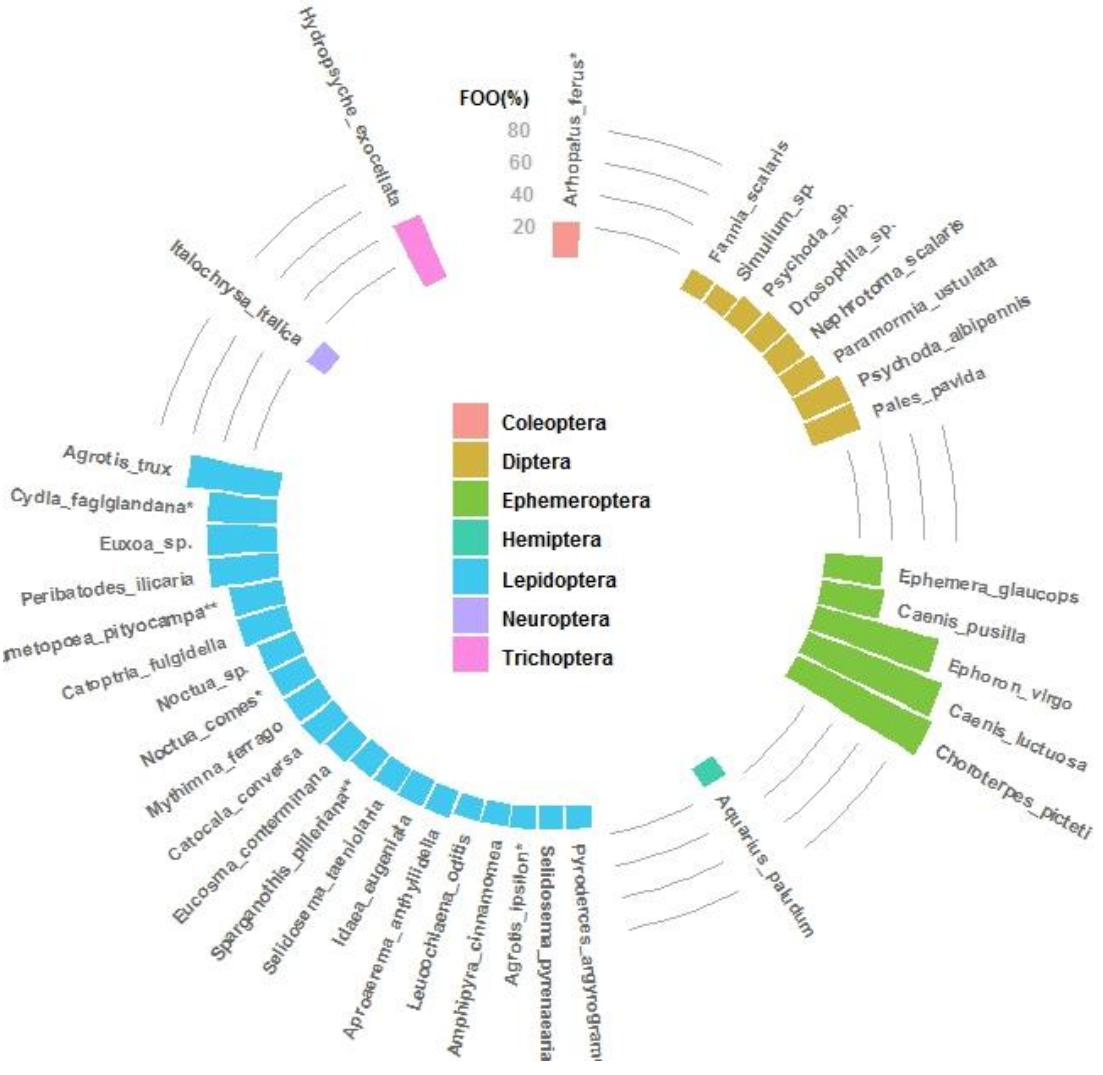

S2.1. Irudia. *R. hipposideros*-en dietan sarrien agertutako harrapakin-espezieen agerpen maiztasunak (FOO).



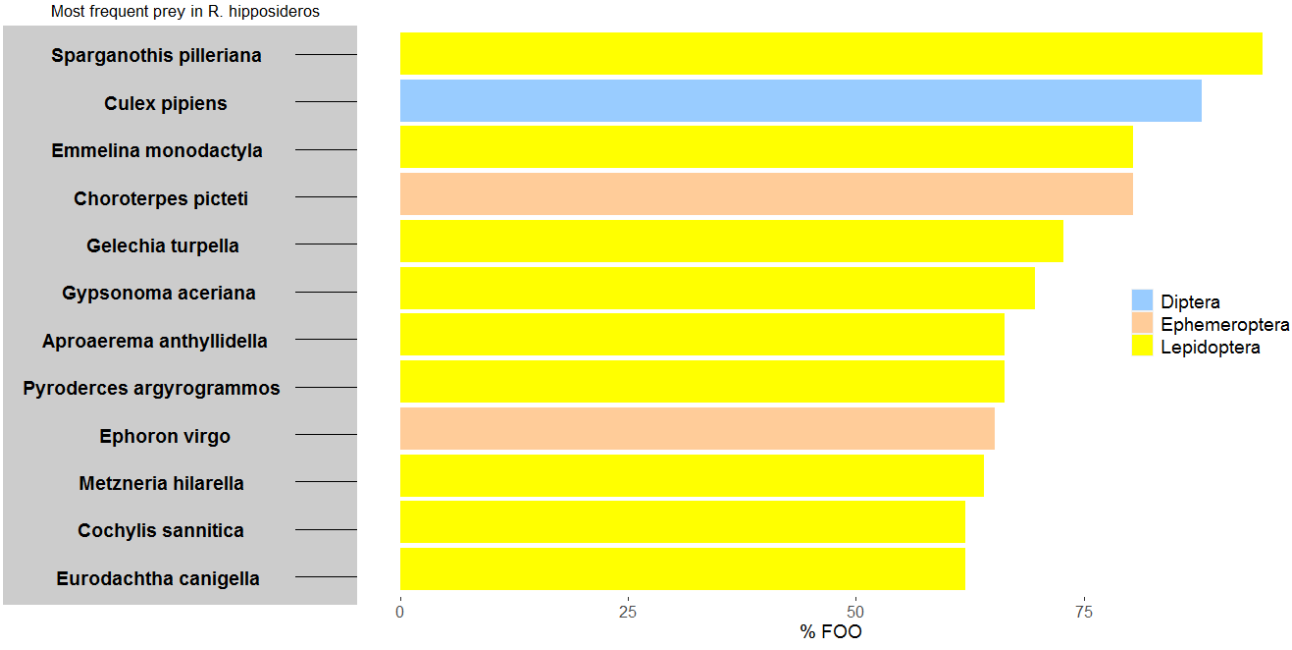

**S2.2. Irudia.** *P. kuhlii*-ren dietan sarrien agertutako harrapakin-espezieen agerpen maiztasunak (FOO).



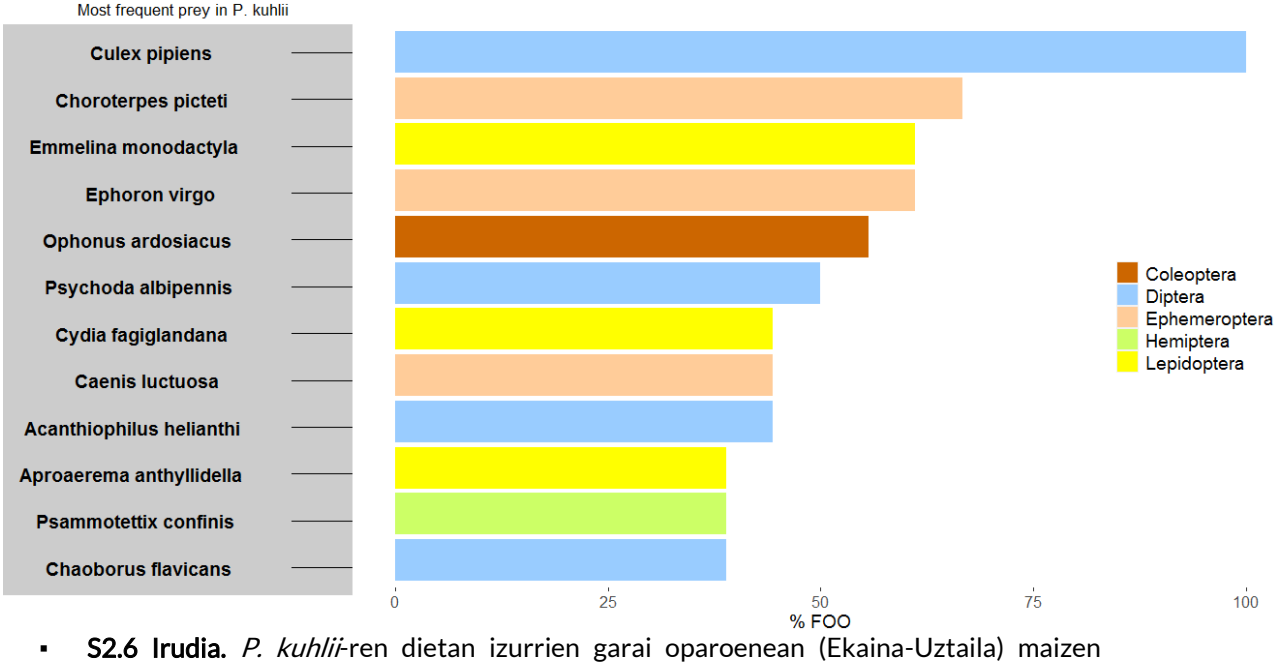

**S2.3. Irudia.** *P. pipistrellus*-en dietan sarrien agertutako harrapakin-espezieen agerpen maiztasunak (FOO).



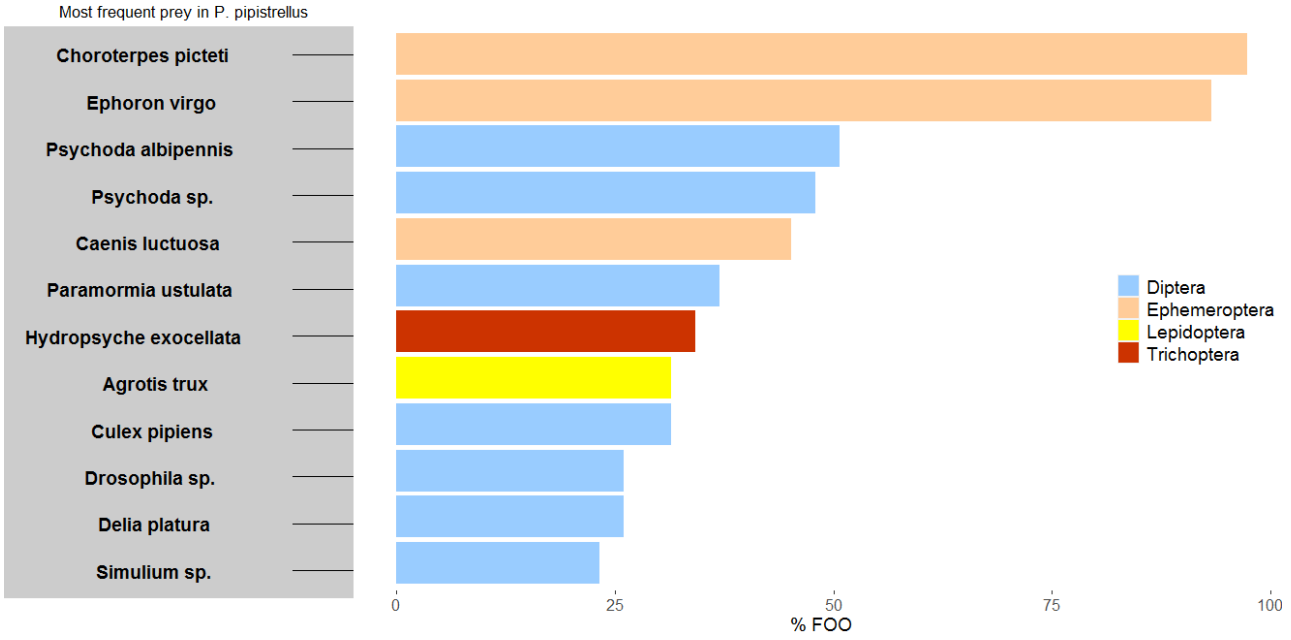

**S2.4. Irudia.** *M. daubentonii*-ren dietan sarrien agertutako harrapakin-espezieen agerpen maiztasunak (FOO).




- **S2.5 Irudia.** *R. hipposideros*-en dietan izurrien garai oparoenean (Ekaina-Uztaila) maizen agertutako taxonak.

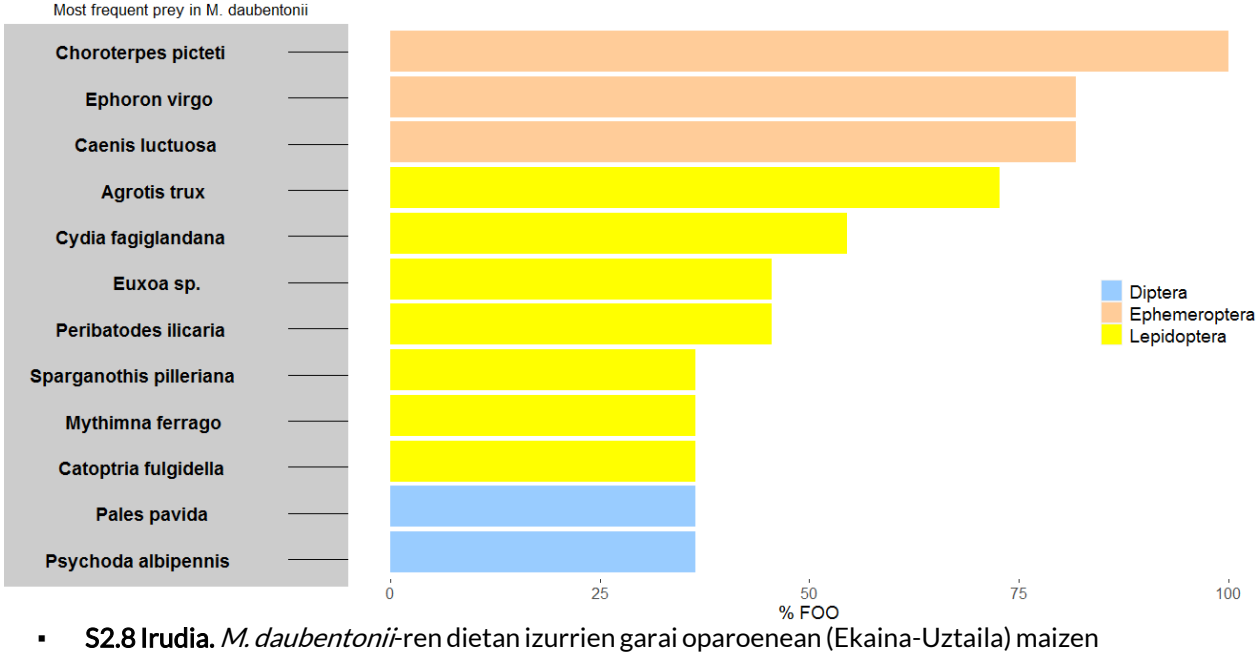



- **S2.6 Irudia.** *P. kuhlii*-ren dietan izurrien garai oparoenean (Ekaina-Uztaila) maizen agertutako taxonak.

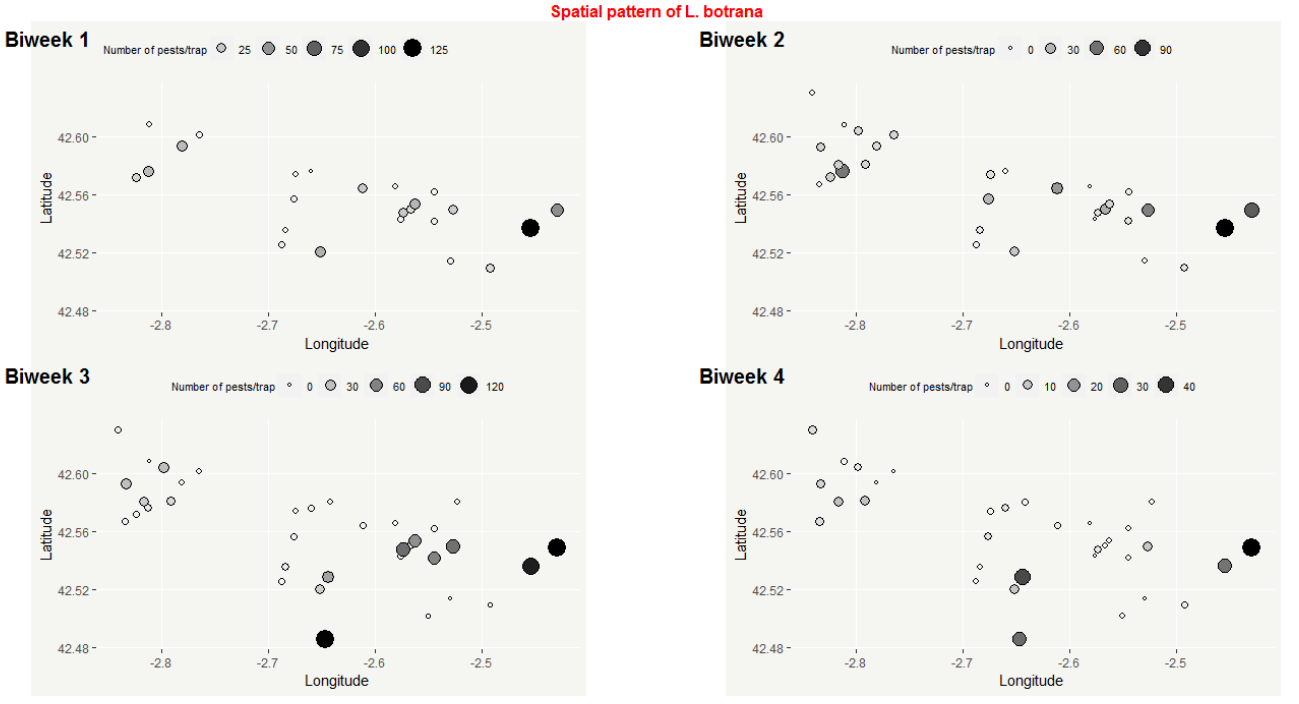



**S2.7 Irudia.** *P. pipistrellus*-en dietan izurrien garai oparoenean (Ekaina-Uztaila) maizen agertutako taxonak.

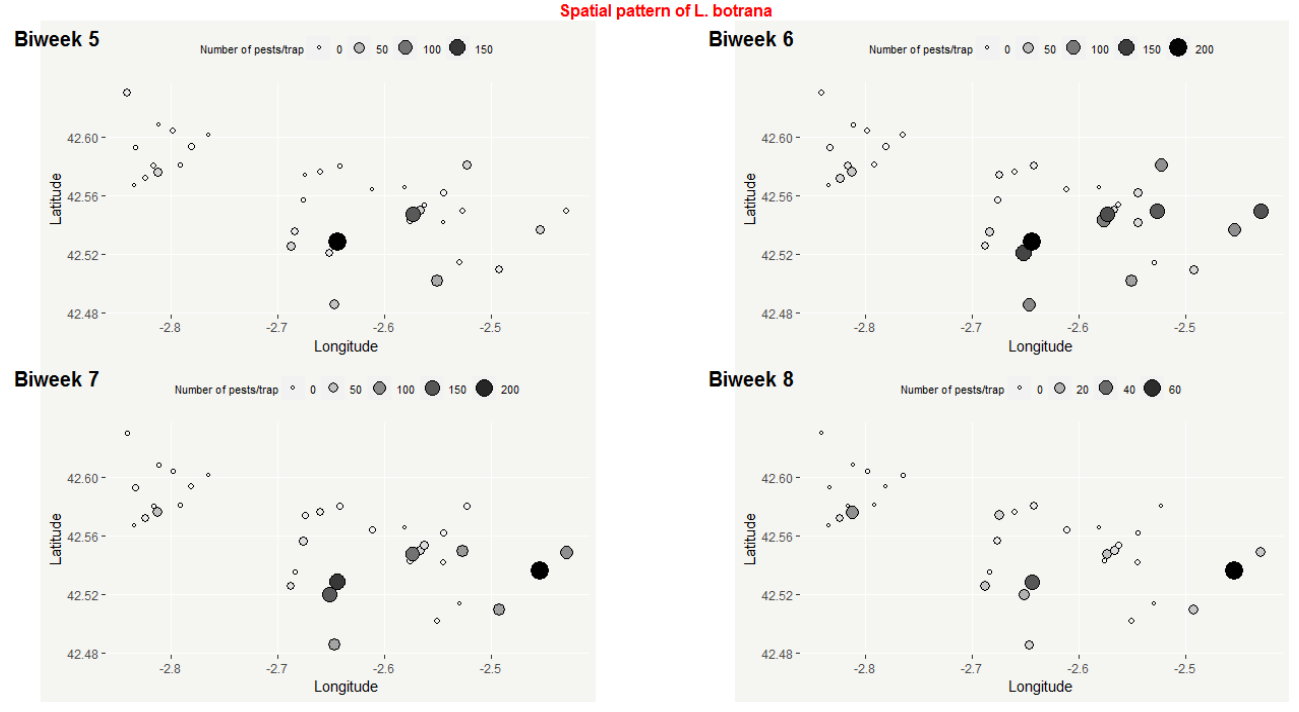
▪



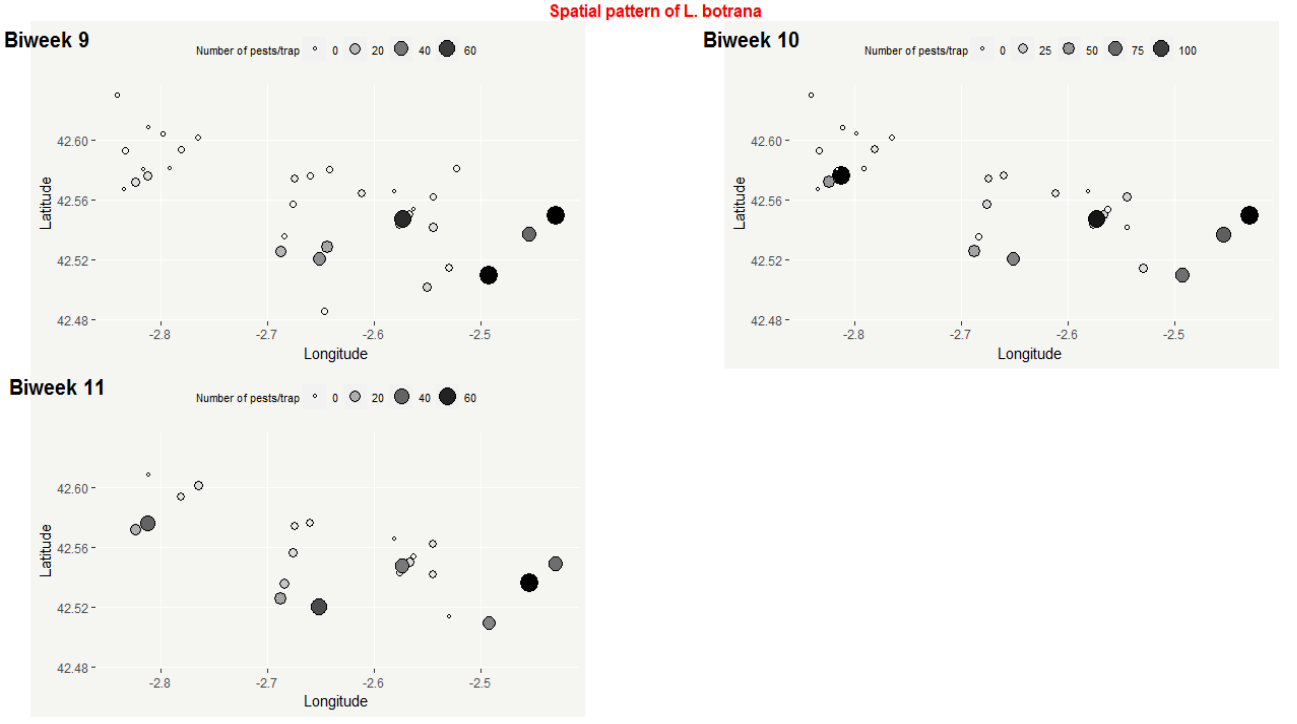

▪ **S2.8 Irudia.** *M. daubentonii*-ren dietan izurrien garai oparoenean (Ekaina-Uztaila) maizen agertutako taxonak.



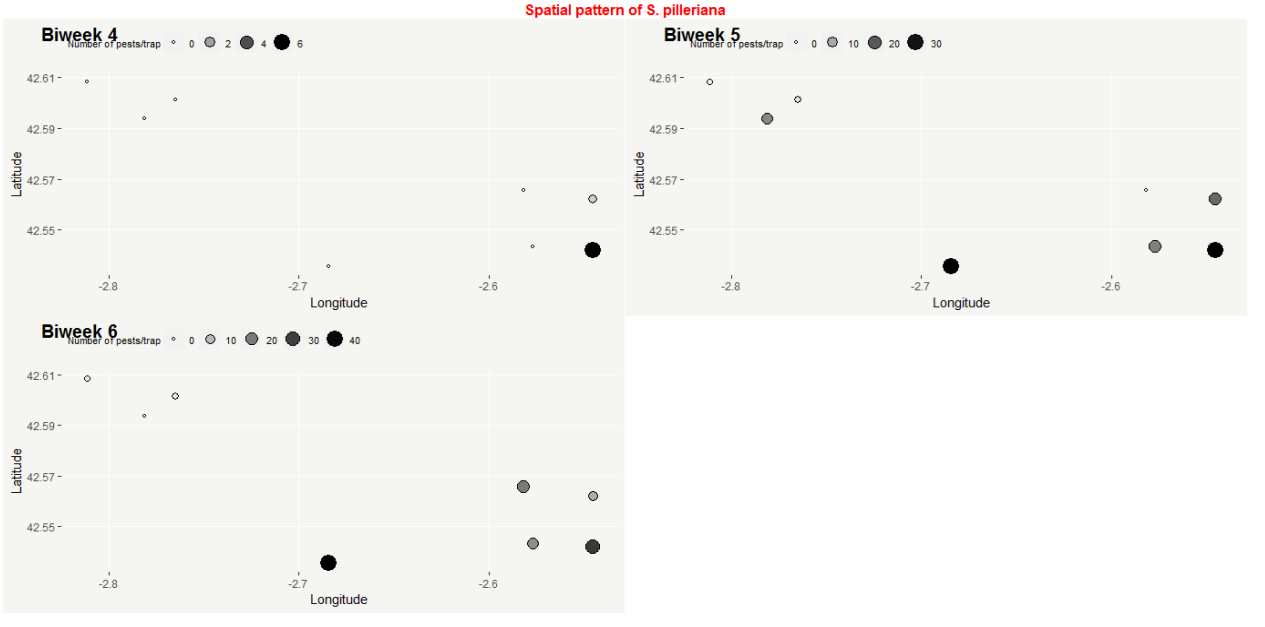

**S2.9 Irudia.** *L. botrana*-ren sakabanaketa espaziala ikerketa eremuan (1-4 asteak).



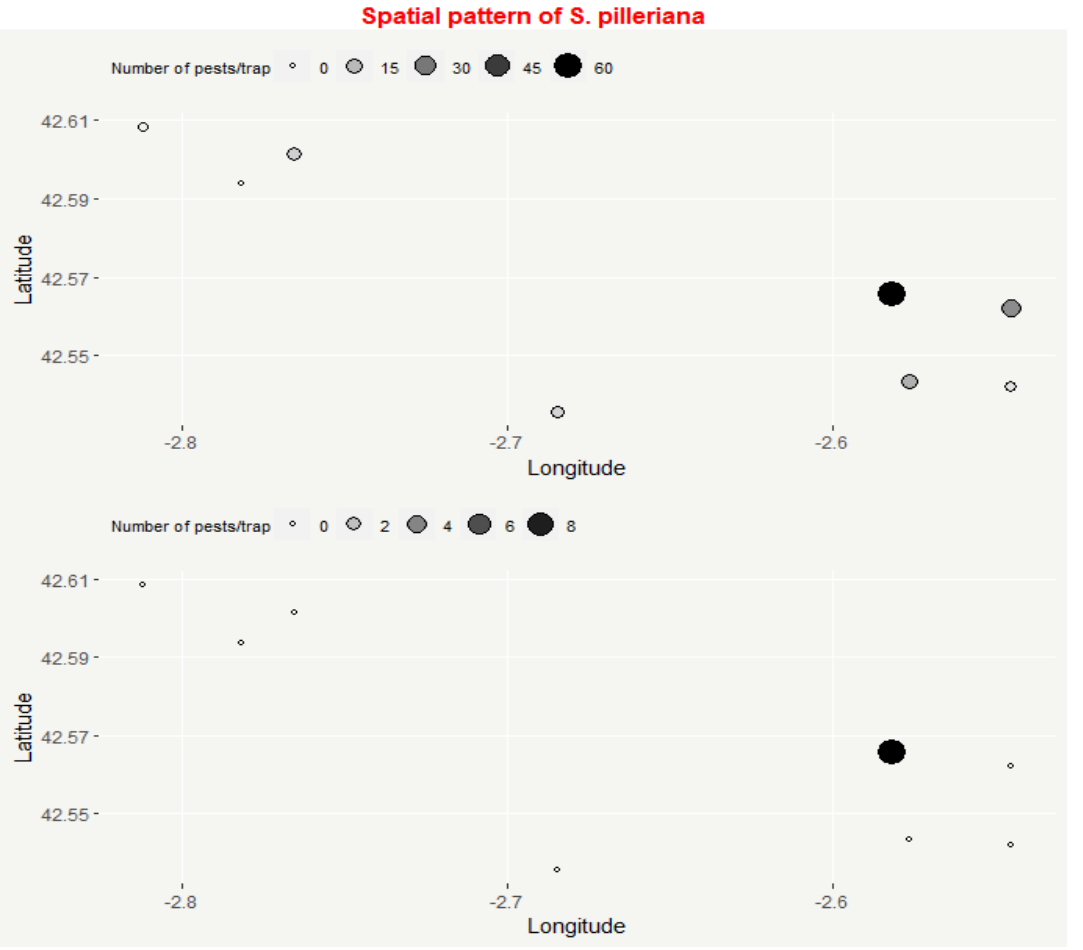

**S2.10 Irudia.** *L. botrana*-ren sakabanaketa espaziala ikerketa eremuan (5-8 asteak).



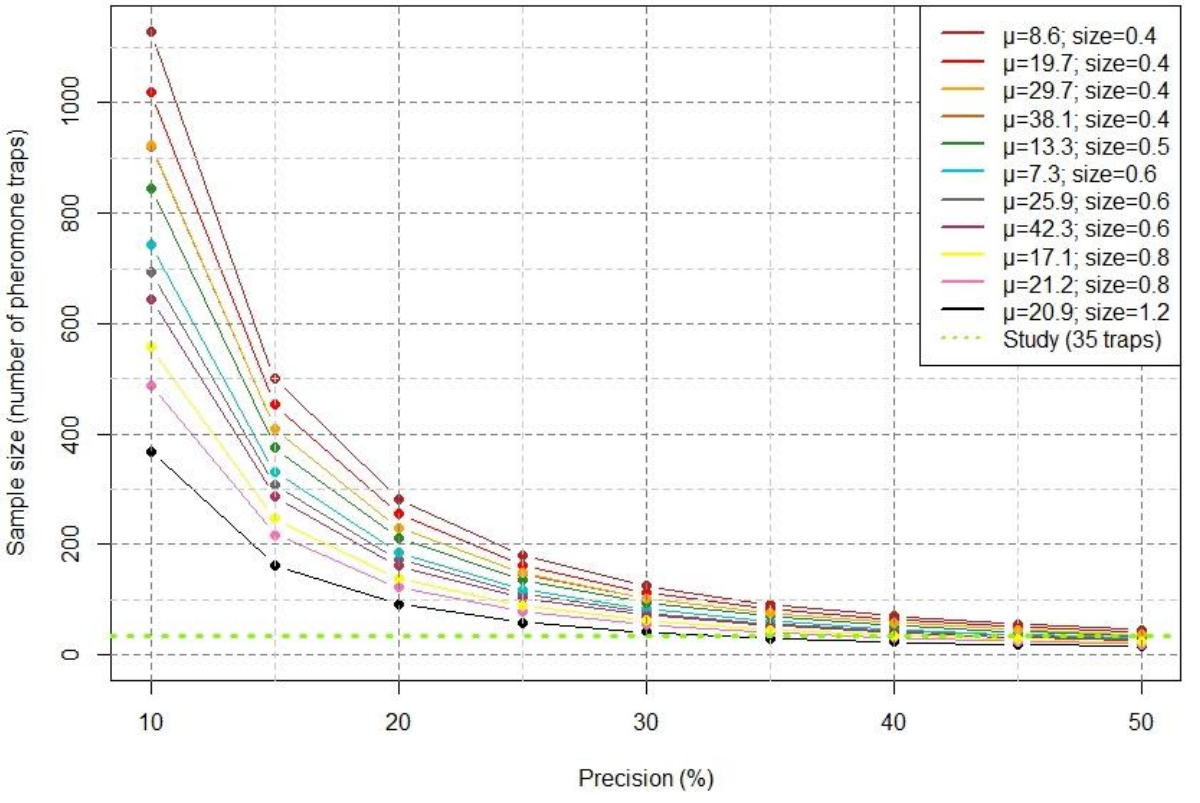

**S2.11 Irudia.** *L. botrana*-ren sakabanaketa espaziala ikerketa eremuan (9-11 asteak).




**S2.12 Irudia.** *S. pilleriana*-ren sakabanaketa espaziala ikerketa eremuan (4-6 asteak).




**S2.13 Irudia.** *S. pilleriana*-ren sakabanaketa espaziala ikerketa eremuan (7-8 asteak).




**S14 Irudia.** Estimatutako lagin tamainaren (intsektuen tranpa kopurua) eta zehaztasun-mailaren ("*precision*") arteko erlazioa. Lagin tamaina kalkulatzeko 7.17 ekuazioa erabili da (Krebs, 1999).

**S2.1. Taula**. Gorotzen laginketaren gaineko informazioa eta xehetasunak.

| Saguzar espeziea | Lagin guztiak | *Rhinolophus hipposideros* | | | *Pipistrellus pipistrellus* | | | | | | *P. kuhlii* | | | *Myotis daubentonii* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kolonia | - | RIV | HA | LEZ | SAM | AB | VILL | BERR | PU | MAN | VIROOF | MAR | LEBB | MAN |
| Koordenatuak | - | 42°36' N 2°45' W | 42°35' N 2°49' W | 42°33' N 2°38' W | 42° 34' N 2° 40' W | 42° 34' N 2° 42' W | 42° 32' N 2° 39' W | 42° 32' N 2° 39' W | 42° 34' N 2° 42' W | 42° 30' N 2° 30' W | 42° 32' N 2° 39' W | 42° 32' N 2° 39' W | 42° 33' N 2° 38' W | 42° 30' N 2° 30' W |
| Laginketa aldia | 10 Mai-27 Ira 2017 | 19 Mai-27 Ira | 15 Eka-27 Ira | 7 Eka-13 Ira | 28 Api- 27 Ira | 10 Mai-27 Ira | 10 Mai-27 Ira | 1 Abu-13 Ira | 8 Eka- 27 Ira | 31 Mai-12 Ira | 8 Eka-1 Abu | 8 Eka- 1 Abu | 5 Uzt- 27 Ira | 8 Eka- 30 Abu |
| Bildutako laginak | 439 | 60 | 106 | 25 | 45 | 38 | 29 | 14 | 32 | 33 | 7 | 10 | 12 | 28 |
| Gorotzak lagineko (±DE) | 24.2 (±5) | 29.5 (±2.2) | 28.6 (±3.4) | 23.6 (±3.1) | 24 (±1.8) | 22.7 (±2.7) | 21.5 (±5.2) | 22.7 (±3.9) | 23.1 (±2.2) | 20.4 (±4.2) | 23.5 (±2.7) | 20.7 (±4.8) | 20.1 (±4.6) | 19.2 (±3.5) |
| Batezbesteko lagin masa (mg) | 78.3 (±13.4) | 74.13 (±6.7) | 74.1 (±14.2) | 63.9 (±12) | 84.20 (±14.1) | 78.4 (±13.3) | 78.3 (±12.3) | 75.7 (±13.6) | 78.9 (±12.7) | 80.9 (±14.3) | 77.2 (±11.9) | 80.2 (±16.3) | 78.7 (±15.2) | 113.7 (±19.6) |
| **Gorotzak guztira** | 10607 | 1740 | 2915 | 593 | 1087 | 843 | 603 | 323 | 663 | 660 | 164 | 203 | 253 | 560 |

**S2.2. Taula.** Bi hasleen PCR baldintzen xehetasunak.

| PCR faseak | Zeale | Gillet |
|---|---|---|
| **Hasiera** | 95ºC—10min | 95ºC—15 min |
| **Desnaturalizazioa** | 16 ziklo:<br>95ºC—30seg<br>61ºC—30seg | 40 ziklo:<br>94ºC—30seg<br>45ºC—45seg |
| | 72ºC—30seg | 72ºC—30seg |
| **Suberapena** | 24 ziklo:<br>95ºC—30seg<br>53ºC—30seg | |
| | 72ºC—30seg | |
| **Hedapena** | 72ºC—10min | 72ºC—10min |

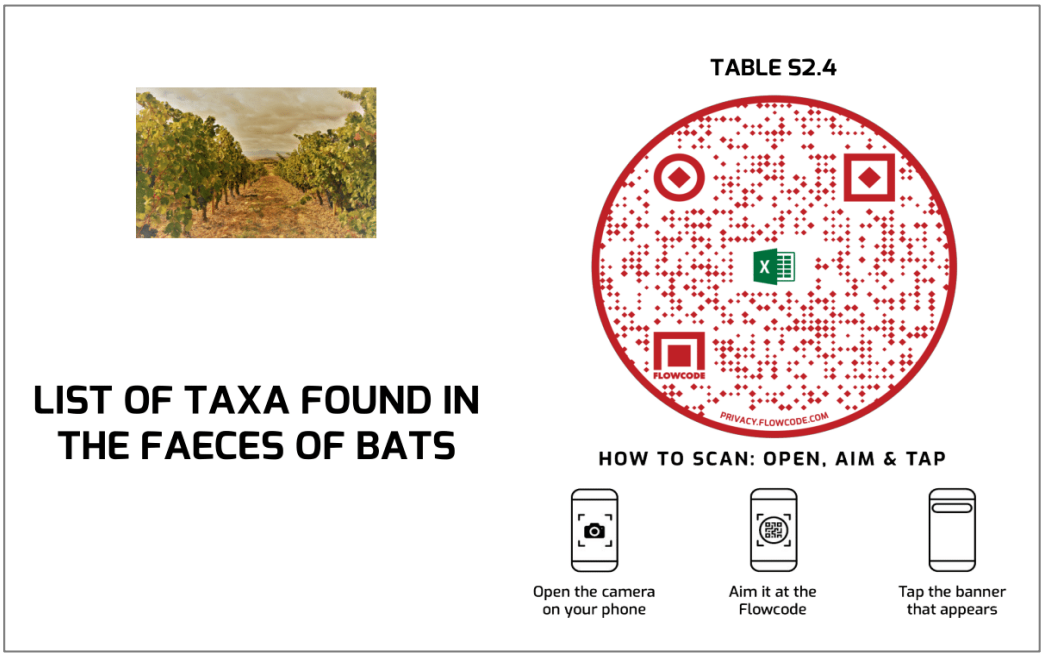
**S2.3. Taula**. *L. botrana* eta *S. pilleriana*-ren Morisita eta Morisita estandarizatuaren sakabanaketa-indizeak populazioen dentsitatearen arabera. NA: daturik ez.

| *L. botrana* | | | |
|---|---|---|---|
| Garaia (biastea) | Morisita indizea | Morisita estandarizatua | *p*-balioa |
| 1 | 2.40 | 0.529 | < 0.0001 |
| 2 | 2.26 | 0.521 | < 0.0001 |
| 3 | 2.95 | 0.528 | < 0.0001 |
| 4 | 3.30 | 0.535 | < 0.0001 |
| 5 | 4.58 | 0.552 | < 0.0001 |
| 6 | 2.44 | 0.521 | < 0.0001 |
| 7 | 3.15 | 0.531 | < 0.0001 |
| 8 | 3.85 | 0.542 | < 0.0001 |
| 9 | 3.48 | 0.537 | < 0.0001 |
| 10 | 2.82 | 0.532 | < 0.0001 |
| 11 | 2.06 | 0.524 | < 0.0001 |

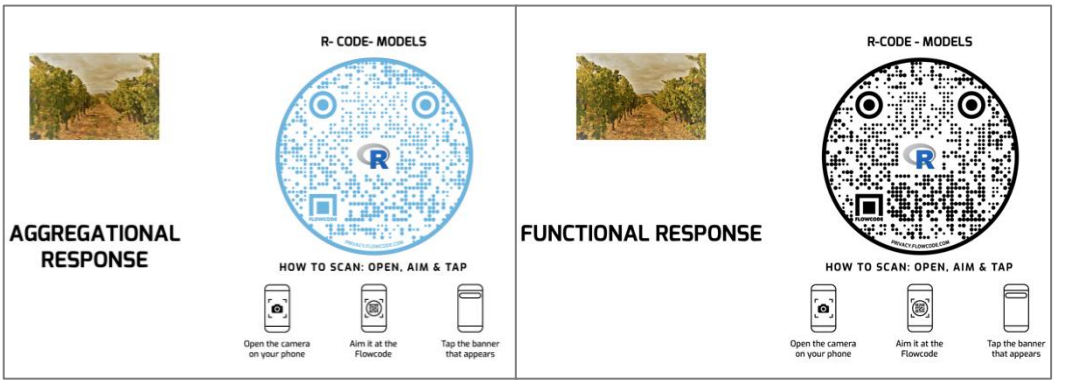
**S2.3. Taula.** (jarraipena).

| *S. pilleriana* | | | |
|---|---|---|---|
| Garaia (biastea) | Morisita indizea | Morisita estandarizatua | *p*-balioa |
| 1 | NA | NA | NA |
| 2 | NA | NA | NA |
| 3 | NA | NA | NA |
| 4 | 5.71 | 0.790 | < 0.0001 |
| 5 | 1.61 | 0.540 | < 0.0001 |
| 6 | 1.70 | 0.550 | < 0.0001 |
| 7 | 2.20 | 0.580 | < 0.0001 |
| 8 | 8.00 | 1.000 | < 0.0001 |
| 9 | NA | NA | NA |
| 10 | NA | NA | NA |
| 11 | NA | NA | NA |

**S2.4. Taula.** Ikertutako saguzarren gorotzetan detektatutako harrapakari eta harrapakin espezieak eta agerpen-maiztasunak (FOO).



- **Informazio gehigarria**. Eraikitako modelo estatistikoen R-kodeak.




## E.3. 3. kapitulua.

**Atal honetan 3. kapituluari dagozkion hurrengo edukiak landu dira:**

- **Informazio gehigarria 1.** Sitsen DNA erauzketarako protokoloa.
- **S3.1. Taula.** Ikerketan erabilitako laginak, taldea, espeziearen izena eta metabarcoding eta PCR metodoen erreplika ezberdinetan (R) lortutako emaitza [presentzia (+) edo ausentzia (-)].
- **S3.2. Taula.** Entsegu kuantitatiboan erabilitako laginak, lagin mota eta *T. pityocampa*-ren "input" eta "output" kontzentrazioak, erreplika ezberdinetan (R).
- **S3.1. Irudia.** Laginketa-eremuen kokapen geografikoa eta entsegu kualitatiborako leku bakoitzean erabilitako lagin kopurua.
- **S3.2. Irudia.** qPCR produktuen DNA sekuentziazioaren emaitzak: anplikoi guztietan *T. pityocampa* sekuentzia berdina aurkitu zen.
- **S3.3. Irudia.** cPCR produktuen anplifikazio-bandak.

- **Informazio gehigarria 1.** Sitsen DNA erauzketarako protokoloa.

  Hurrengo pausuak jarraitu genituen erauzte **jario laminarreko kanpaia** barruan:

  1.- Digestioa

  1.1. Jarri laginak (sitsaren 2 hanka/...) 2ml-ko tutu esteril batean.

  1.2. Gehitu **Digestio Bufferra-ren 400 µl** tutura.

  1.3. Gehitu silica bolatxoak.

  1.4. "Polytron Tissue Homogenizer" erabiliz 20 segunduz **homogeneizatu**, (prozesua bi aldiz errepika daiteke).

  1.5. Gehitu **20% SDS-ren 40 µl** (% 2 azkeneko kontzentrazioa).

  1.6. Gehitu **20 mg/ml proteasa** K-ren **8 µl** (400 µg/ml azkeneko kontzentrazioa). Ondo nahastu

  1.7. **Inkubatu 55-65º C-tan** gau osoan zehar (RPM=85).

  2.- DNA-ren erauzketa

  2.1. Gehitu **6 M NaCl-ren 300 µl** lagin bakoitzera.

  2.2 **30 segundoz** laginak nahastu (**vortex-a**) abiadura maximoan.

  2.3. Tutuak **30 minutuz zentrifugatu** 10000 G-ko potentzian= rcf (9703 rpm).

  2.4. **Goiko soberakina** 1.5 ml-ko tutu berrietara transferitu (interfaseko soberakina ekidin).

  3.- DNA-ren prezipitazioa

  3.1. Lagin bakoitzari **isopropanol** bolumen berdina gehitu. Ondo nahastu (eskuz).

  3.2. **Inkubatu: -20º C**-tan ordu batez.

  3.3. **Zentrifugatu** (20 minutuz, 4º C, 10000 G-potentzian = rcf (9703 rpm)).

3.4. Alkohol-soberakina pipeta batez kendu.

4.- DNA-ren garbiketa

4.1. Gehitu **70% Etanol 700 µl** tutura.

4.2. **Zentrifugatu** (10 minutuz, 13000 rpm, 4 ºC-tan).

4.3. Alkohol soberakina pipeta batez kendu.

4.4. Tutuak zabalik utzita gera daitekeen alkohol soberakina **lurrundu** (30 minutuz 60º C-tan).

4.5. Gehitu **dd$H_2O$ 30 µl** DNA erauzkinera.

4.6. Giro tenperatuan inkubatu 5 orduz gutxienez (hobe gau osoan zehar).

**S3.1. Taula.** Ikerketan erabilitako laginak, taldea, espeziearen izena eta metabarcoding eta PCR metodoen erreplika ezberdinetan (R) lortutako emaitza [presentzia (+) edo ausentzia (-)].

| | | | | cPCR | | | qPCR | | |
|---|---|---|---|---|---|---|---|---|---|
| **Lagina** | **Taldea** | **Espeziea** | **Metabarcoding** | **$R^1$** | **$R^2$** | **$R^3$** | **$R^1$** | **$R^2$** | **$R^3$** |
| Th1 | C+ | Tpi | NA | + | + | + | + | + | + |
| Th2 | C+ | Tpi | NA | + | + | + | + | + | + |
| Th3 | C+ | Tpi | NA | + | + | + | + | + | + |
| Th4 | C+ | Tpi | NA | + | + | + | + | + | + |
| Th5 | C+ | Tpi | NA | NA | NA | NA | + | + | + |
| Lep1 | C- | *Sfix* | NA | - | - | - | - | - | - |
| Lep2 | C- | *Cnym* | NA | - | - | - | - | - | - |
| Lep3 | C- | *Cnym* | NA | - | - | - | - | - | - |
| Lep4 | C- | *Ldisp* | NA | - | - | - | - | - | - |
| Lep5 | C- | *Ldisp* | NA | - | - | - | - | - | - |
| Lep6 | C- | *Npro* | NA | - | - | - | - | - | - |
| Lep7 | C- | *Eocr* | NA | - | - | - | - | - | - |
| 619va9 | $Th^+$ | Msc | + | - | - | - | + | + | + |
| 621msc10 | $Th^+$ | Msc | + | - | - | - | + | + | + |
| 621msc4 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 621msc5 | $Th^+$ | Msc | + | - | + | + | + | + | + |
| 621msc8 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 717va1 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 717va10 | $Th^+$ | Msc | + | - | - | - | + | + | + |
| 717va2 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 717va3 | $Th^+$ | Msc | + | - | - | - | + | + | + |
| 717va4 | $Th^+$ | Msc | + | - | - | - | + | + | + |
| 717va6 | $Th^+$ | Msc | + | - | - | - | + | + | + |
| 717va7 | $Th^+$ | Msc | + | - | - | - | + | + | + |
| 717va8 | $Th^+$ | Msc | + | - | - | - | + | + | + |

**S3.1 Taula.** (jarraipena)

| | | | | cPCR | | | qPCR | | |
|---|---|---|---|---|---|---|---|---|---|
| **Lagina** | **Taldea** | **Espeziea** | **Metabarcoding** | **$R^1$** | **$R^2$** | **$R^3$** | **$R^1$** | **$R^2$** | **$R^3$** |
| 717va9 | $Th^+$ | Msc | + | - | + | + | + | + | + |
| 720mor11 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 720mor2 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 720mor3 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 720mor4 | $Th^+$ | Msc | + | - | + | + | + | + | + |
| 720mor5 | $Th^+$ | Msc | + | - | + | + | + | + | + |
| 720mor7 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 720mor9 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 728mor1 | $Th^+$ | Msc | + | - | - | + | + | + | + |
| 728mor10 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 728mor3 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 728mor4 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 728mor5 | $Th^+$ | Msc | + | - | + | + | + | + | + |
| 728mor8 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 728mor9 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 730msc1 | $Th^+$ | Msc | + | - | + | + | + | + | + |
| 731va1 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 731va10 | $Th^+$ | Msc | + | - | - | + | - | + | + |
| 731va2 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 731va5 | $Th^+$ | Msc | + | - | - | + | + | + | + |
| 731va6 | $Th^+$ | Msc | + | - | - | + | + | + | + |
| 73mor10 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 73mor2 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 73mor3 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 73mor5 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 73mor7 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 73mor9 | $Th^+$ | Msc | + | + | + | + | + | + | + |

**S3.1 Taula.** (jarraipena)

| | | | | cPCR | | | qPCR | | |
|---|---|---|---|---|---|---|---|---|---|
| **Lagina** | **Taldea** | **Espeziea** | **Metabarcoding** | **$R^1$** | **$R^2$** | **$R^3$** | **$R^1$** | **$R^2$** | **$R^3$** |
| 76msc2 | $Th^+$ | Msc | + | - | - | + | + | + | + |
| 76msc6 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 816 mor9 | $Th^+$ | Msc | + | + | + | + | - | - | + |
| 816mor1 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 816mor2 | $Th^+$ | Msc | + | - | - | - | + | + | + |
| 816mor3 | $Th^+$ | Msc | + | - | + | + | + | + | + |
| 816mor4 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 816mor5 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 816mor6 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 816msc13 | $Th^+$ | Msc | + | + | + | NA | + | + | + |
| 817va10 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 817va5 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 817va6 | $Th^+$ | Msc | + | - | - | + | + | + | + |
| 829 mor1 | $Th^+$ | Msc | + | + | + | + | - | + | + |
| 829 mor3 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 829 mor5 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 829 mor6 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 829 mor7 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 829 mor8 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 829 mor9 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 911 mor2 | $Th^+$ | Msc | + | + | + | + | + | + | NA |
| 915 va9 | $Th^+$ | Msc | + | + | + | + | + | + | + |
| 721reu2 | $Th^+$ | Reu | + | - | - | - | - | - | - |
| 721reu3 | $Th^+$ | Reu | + | + | + | + | + | + | + |
| 721reu4 | $Th^+$ | Reu | + | NA | NA | NA | + | + | + |
| 721reu8 | $Th^+$ | Reu | + | + | + | + | + | + | + |
| 77reu2 | $Th^+$ | Reu | + | + | + | + | + | + | + |

**S3.1. Taula.** (jarraipena

| | | | | cPCR | | | qPCR | | |
|---|---|---|---|---|---|---|---|---|---|
| **Lagina** | **Taldea** | **Espeziea** | **Metabarcoding** | **R¹** | **R²** | **R³** | **R¹** | **R²** | **R³** |
| 77reu3 | $Th^{+}$ | Reu | + | + | + | + | + | + | + |
| 77reu5 | $Th^{+}$ | Reu | + | + | + | + | + | + | + |
| 77reu6 | $Th^{+}$ | Reu | + | + | + | + | + | + | + |
| 721ajd | $Th^{+}$ | Rfe&Mem | + | - | - | + | - | + | + |
| 817spa | $Th^{+}$ | Rfe&Mem | + | - | + | + | + | + | + |
| 824an | $Th^{+}$ | Rfe&Mem | + | - | - | - | + | + | + |
| 831bad | $Th^{+}$ | Rfe&Mem | + | - | - | - | + | + | + |
| 831spc | $Th^{+}$ | Rfe&Mem | + | - | - | - | + | + | + |
| 83baa | $Th^{+}$ | Rfe&Mem | + | - | - | - | + | + | + |
| 83bab | $Th^{+}$ | Rfe&Mem | + | - | - | - | + | + | + |
| 96an | $Th^{+}$ | Rfe&Mem | + | - | - | - | + | + | + |
| 717tte1 | $Th^{+}$ | Tte | + | - | + | + | + | + | + |
| 717tte2 | $Th^{+}$ | Tte | + | - | - | + | - | - | + |
| 717tte6 | $Th^{+}$ | Tte | + | + | + | + | + | + | + |
| 717tte8 | $Th^{+}$ | Tte | + | - | - | + | + | + | + |
| 717tte9 | $Th^{+}$ | Tte | + | - | - | - | + | + | + |
| 621mab | $Th^{-OUT}$ | Mda | - | - | - | - | - | - | - |
| 621mac | $Th^{-OUT}$ | Mda | - | - | - | - | - | - | - |
| 621mv | $Th^{-OUT}$ | Pku | - | - | - | - | - | - | - |
| 531ab | $Th^{-OUT}$ | Ppi | - | - | - | - | - | - | - |
| 621ab | $Th^{-OUT}$ | Ppi | - | - | - | - | - | - | - |
| 67ab | $Th^{-OUT}$ | Ppi | - | - | - | - | - | - | - |
| 510sa | $Th^{-OUT}$ | Ppi | - | - | - | - | - | - | - |
| 523sa | $Th^{-OUT}$ | Ppi | - | - | - | - | - | - | - |
| 621sa | $Th^{-OUT}$ | Ppi | - | - | - | - | - | - | - |
| 68sa | $Th^{-OUT}$ | Ppi | - | - | - | - | - | - | - |
| 510vel | $Th^{-OUT}$ | Ppi | - | - | - | - | - | - | - |

**S3.1. Taula.** (jarraipena).

| | | | | cPCR | | | qPCR | | |
|---|---|---|---|---|---|---|---|---|---|
| **Lagina** | **Taldea** | **Espeziea** | **Metabarcoding** | **$R^1$** | **$R^2$** | **$R^3$** | **$R^1$** | **$R^2$** | **$R^3$** |
| 531vel | $Th^{-OUT}$ | Ppi | - | - | - | - | - | - | - |
| 621vel | $Th^{-OUT}$ | Ppi | - | - | - | - | - | - | - |
| 68vel | $Th^{-OUT}$ | Ppi | - | - | - | - | - | - | - |
| 620haa | $Th^{-OUT}$ | Rhi | - | - | - | - | - | - | - |
| 621le | $Th^{-OUT}$ | Rhi | - | - | - | - | - | - | - |
| 531rva | $Th^{-OUT}$ | Rhi | - | - | - | - | - | - | - |
| 531rvb | $Th^{-OUT}$ | Rhi | - | - | - | - | - | - | - |
| 620rva | $Th^{-OUT}$ | Rhi | - | - | - | - | - | - | - |
| 620rvb | $Th^{-OUT}$ | Rhi | - | - | - | - | - | - | - |
| 67rva | $Th^{-OUT}$ | Rhi | - | - | - | - | - | - | - |
| 67rvb | $Th^{-OUT}$ | Rhi | - | - | - | - | - | - | - |
| 817fra | $Th^{-IN}$ | Mcry | - | - | - | - | - | - | + |
| 817lac | $Th^{-IN}$ | Mem | - | - | - | - | - | - | - |
| 522msc1 | $Th^{-IN}$ | Msc | - | - | - | - | - | - | - |
| 522msc2 | $Th^{-IN}$ | Msc | - | - | - | - | - | - | - |
| 522msc3 | $Th^{-IN}$ | Msc | - | - | - | - | - | - | - |
| 69va2 | $Th^{-IN}$ | Msc | - | - | - | - | - | - | - |
| 69va3 | $Th^{-IN}$ | Msc | - | - | - | - | - | - | - |
| 924mor2 | $Th^{-IN}$ | Msc | - | + | + | + | + | + | + |
| 924mor3 | $Th^{-IN}$ | Msc | - | - | + | + | - | - | - |
| 924mor4 | $Th^{-IN}$ | Msc | - | - | + | + | + | + | + |
| 924msc2 | $Th^{-IN}$ | Msc | - | - | - | - | - | - | - |
| 924msc3 | $Th^{-IN}$ | Msc | - | - | - | - | - | - | - |
| 713zea | $Th^{-IN}$ | Pku | - | - | - | - | - | - | - |
| 914ajc | $Th^{-IN}$ | Rfe&Mem | - | - | - | - | - | - | + |
| 615an | $Th^{-IN}$ | Rfe&Mem | - | NA | NA | NA | - | - | - |
| 622spa | $Th^{-IN}$ | Rfe&Mem | - | - | - | - | - | - | - |

**S3.1. Taula.** (jarraipena).

| | | | | cPCR | | | qPCR | | |
|---|---|---|---|---|---|---|---|---|---|
| **Lagina** | **Taldea** | **Espeziea** | **Metabarcoding** | **$R^1$** | **$R^2$** | **$R^3$** | **$R^1$** | **$R^2$** | **$R^3$** |
| 622spd | $Th^{-IN}$ | Rfe&Mem | - | - | - | - | - | - | - |
| 720le | $Th^{-IN}$ | Rfe&Reu | - | - | - | - | - | - | - |
| 817ern | $Th^{-IN}$ | Rhi | - | - | - | - | - | - | - |
| 629go | $Th^{-IN}$ | Rhi | - | - | - | - | - | - | - |
| 824go | $Th^{-IN}$ | Rhi | - | - | - | - | - | - | - |

**Taldea**: $C^+$: TP laginak; $C^-$: TP laginak ez direnak, $Th^+$: NGS bidez TP detektatutako gorotz-lagina; $Th^{-OFP}$: NGS bidez TP detektatu ez den gorotz-lagina (TP hegalditik kanpo); $Th^{-FP}$: NGS bidez TP detektatu ez den gorotz-lagina (TP-ren hegaldi-garaian). **Espezieak**: Tpi: *Thaumetopoea pityocampa*; Sfix: *Synthymia fixa*; Cnym: *Catocala nymphagoga;* Ldisp: *Lymantria dispar*; Npro: *Noctua pronuba*; Eocr: *Eremobia ochroleuca;* Mcry: *Myotis crypticus*; Mda: *M daubentonii*; Mem: *M. emarginatus*; Msc: *Miniopterus schreibersii*; Pku: *Pipistrellus kuhlii*; Ppi: *P. pipistrellus*; Reu: *Rhinolophus euryale*; Rfe: *R. ferrumequinum*; Rhi*: R. hipposideros*; Tte: *Tadarida teniotis.* NA: datuak ez daude eskuragarri.

**S3.2. Taula.** Entsegu kuantitatiboan erabilitako laginak, lagin mota, laginari dagokion espeziea eta *T. pityocampa*-ren "input" eta "output" kontzentrazioak, erreplika ezberdinetan (R). Espeziea, Tpi: *T. pityocampa*; Rhi: *R. hipposideros*; Pku: *P. kuhlii*; Ppi: *P. pipistrellus*; Hainbat: espezie ezberdinen DNA-ren nahasketa.

| | | | [DNA]"input"(ng/µL) | [DNA]qPCR(ng/µL) | | | |
|---|---|---|---|---|---|---|---|
| **Lagina** | **Mota** | **Espeziea** | **$R^{123}$** | **$R^1$** | **$R^2$** | **$R^3$** | **Mean** |
| Th6 | "Target" | Tpi | 5 | | | | |
| Th6 | "Target" | Tpi | 1 | | | | |
| Th6 | "Target" | Tpi | 0.2 | | | | |
| Th6 | "Target" | Tpi | 0.04 | | | | |
| Th6 | "Target" | Tpi | 0.008 | | | | |

**S3.2. Taula.** (jarraipena)

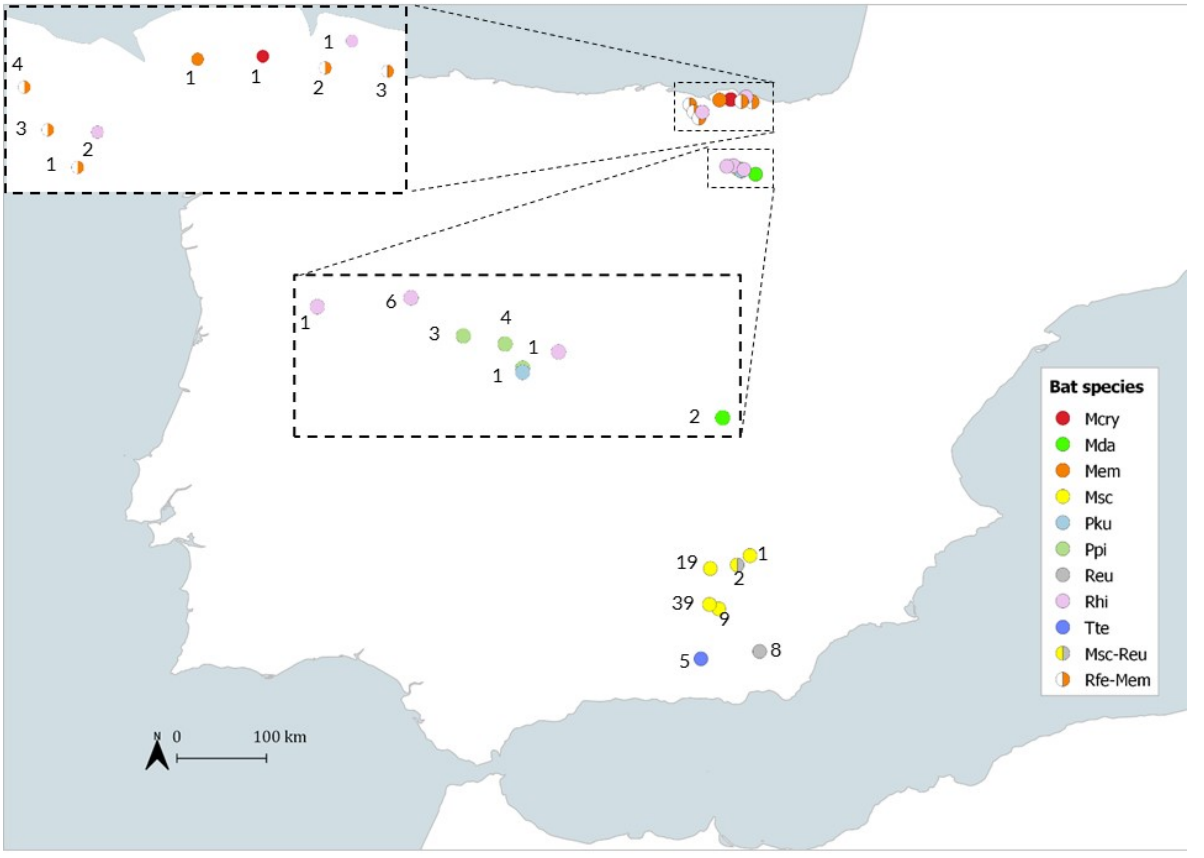
| | | | [DNA]"input"(ng/µL) | [DNA]qPCR(ng/µL) | | | |
|---|---|---|---|---|---|---|---|
| **Lagina** | **Mota** | **Espeziea** | **$R^{123}$** | **$R^1$** | **$R^2$** | **$R^3$** | **Mean** |
| Th6 | "Target" | Tpi | 0.00016 | | | | |
| Th6 | "Target" | Tpi | 0.000032 | | | | |
| M1A[1] | "Mock" | Hainbat | 1 | 0.61 | 0.63 | 1.67 | **0.97** |
| M1A[2] | "Mock" | Hainbat | 1 | 0.31 | 1.45 | 0.87 | **0.88** |
| M1A[3] | "Mock" | Hainbat | 1 | 0.92 | 0.22 | 1.30 | **0.82** |
| M1B[1] | "Mock" | Hainbat | 1 | 0.36 | 0.75 | 1.21 | **0.77** |
| M1B[2] | "Mock" | Hainbat | 1 | 1.38 | 0.18 | 2.01 | **1.19** |
| M2A[1] | "Mock" | Hainbat | 0.2 | 0.13 | 0.20 | 0.19 | **0.17** |
| M2A[2] | "Mock" | Hainbat | 0.2 | 0.17 | 0.04 | 0.34 | **0.18** |
| M2A[3] | "Mock" | Hainbat | 0.2 | 0.06 | 0.43 | 0.23 | **0.24** |
| M2B[1] | "Mock" | Hainbat | 0.2 | 0.03 | 0.10 | 0.28 | **0.13** |
| M2B[2] | "Mock" | Hainbat | 0.2 | 0.16 | 0.06 | 0.30 | **0.17** |
| M3A[1] | "Mock" | Hainbat | 0.04 | 0.018 | 0.036 | 0.068 | **0.040** |
| M3A[2] | "Mock" | Hainbat | 0.04 | 0.110 | 0.024 | 0.237 | **0.124** |
| M3A[3] | "Mock" | Hainbat | 0.04 | 0.029 | 0.223 | 0.130 | **0.127** |
| M3B[1] | "Mock" | Hainbat | 0.04 | 0.007 | 0.025 | 0.056 | **0.029** |
| M3B[2] | "Mock" | Hainbat | 0.04 | 0.023 | 0.006 | 0.069 | **0.033** |
| M4A[1] | "Mock" | Hainbat | 0.008 | 0.0040 | 0.0059 | 0.0135 | **0.008** |
| M4A[2] | "Mock" | Hainbat | 0.008 | 0.0056 | 0.0013 | 0.0156 | **0.007** |
| M4A[3] | "Mock" | Hainbat | 0.008 | 0.0024 | 0.0185 | 0.0072 | **0.009** |
| M4B[1] | "Mock" | Hainbat | 0.008 | 0.0018 | 0.0075 | 0.0088 | **0.006** |
| M4B[2] | "Mock" | Hainbat | 0.008 | 0.0106 | 0.0031 | 0.0194 | **0.011** |
| M5A[1] | "Mock" | Hainbat | 0.00016 | 0.00007 | 0.00007 | 0.00029 | **0.00014** |
| M5A[2] | "Mock" | Hainbat | 0.00016 | 0.00012 | 0.00001 | 0.00034 | **0.00016** |
| M5A[3] | "Mock" | Hainbat | 0.00016 | 0.00017 | 0.00007 | 0.00035 | **0.00020** |
| M5B[1] | "Mock" | Hainbat | 0.00016 | 0.00004 | 0.00008 | 0.00018 | **0.00010** |
| M5B[2] | "Mock" | Hainbat | 0.00016 | 0.00006 | 0.00024 | 0.00012 | **0.00014** |
| M6A[1] | "Mock" | Hainbat | 0.000032 | 0.000005 | 0.000032 | 0.000061 | **0.000033** |

**S3.2. Taula.** (jarraipena).

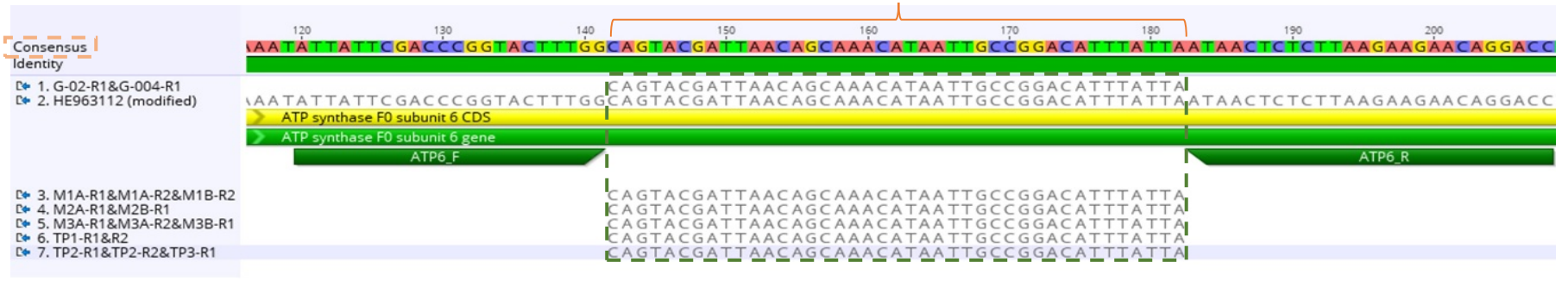
| | | | [DNA]"input"(ng/µL) | [DNA]qPCR(ng/µL) | | | |
|---|---|---|---|---|---|---|---|
| **Lagina** | **Mota** | **Espeziea** | **R123** | **R1** | **R2** | **R3** | **Mean** |
| M6A[2] | "Mock" | Hainbat | 0.000032 | - | 0.000100 | 0.000060 | **0.000082** |
| M6B[1] | "Mock" | Hainbat | 0.000032 | 0.000041 | 0.000041 | - | **0.000041** |
| M6B[2] | "Mock" | Hainbat | 0.000032 | - | 0.000020 | - | **0.000019** |
| 074rvA[1] | Gorotza | Rhi | 0.00016 | 0.00003 | 0.00002 | 0.00028 | **0.00011** |
| 074rvA[2] | Gorotza | Rhi | 0.00016 | 0.00011 | 0.00035 | 0.00014 | **0.00020** |
| 074rvA[3] | Gorotza | Rhi | 0.00016 | 0.00009 | 0.00032 | 0.00014 | **0.00018** |
| 074rvB[1] | Gorotza | Rhi | 0.00016 | 0.00004 | 0.00013 | 0.00031 | **0.00016** |
| 074rvB[2] | Gorotza | Rhi | 0.00016 | 0.00016 | 0.00044 | 0.00008 | **0.00023** |
| 074rvB[3] | Gorotza | Rhi | 0.00016 | 0.00007 | 0.00033 | 0.00006 | **0.00015** |
| 074haA[1] | Gorotza | Rhi | 0.00016 | 0.00007 | 0.00007 | 0.00026 | **0.00013** |
| 074haA[2] | Gorotza | Rhi | 0.00016 | 0.00009 | 0.00052 | 0.00024 | **0.00028** |
| 074haA[3] | Gorotza | Rhi | 0.00016 | 0.00007 | 0.00062 | 0.00020 | **0.00030** |
| 074haC[1] | Gorotza | Rhi | 0.00016 | 0.00012 | 0.00013 | 0.00027 | **0.00017** |
| 074haC[2] | Gorotza | Rhi | 0.00016 | 0.00006 | 0.00032 | 0.00019 | **0.00019** |
| 074haC[3] | Gorotza | Rhi | 0.00016 | 0.00004 | 0.00043 | 0.00027 | **0.00025** |
| 075pu[1] | Gorotza | Pku | 0.00016 | 0.00076 | 0.00200 | 0.00469 | **0.00248** |
| 075pu[2] | Gorotza | Pku | 0.00016 | 0.00054 | 0.00554 | 0.00276 | **0.00295** |
| 075pu[3] | Gorotza | Pku | 0.00016 | 0.00298 | 0.00113 | 0.00368 | **0.00260** |
| 0816ma[1] | Gorotza | Ppi | 0.00016 | 0.00007 | 0.00024 | 0.00044 | **0.00025** |
| 0816ma[2] | Gorotza | Ppi | 0.00016 | 0.00025 | 0.00002 | 0.00052 | **0.00026** |
| 0816ma[3] | Gorotza | Ppi | 0.00016 | 0.00017 | 0.00007 | 0.00058 | **0.00028** |
| 0913maD[1] | Gorotza | Mda | 0.00016 | 0.00006 | 0.00004 | 0.00028 | **0.00013** |
| 0913maD[2] | Gorotza | Mda | 0.00016 | 0.00013 | 0.00008 | 0.00055 | **0.00026** |
| 0913maD[3] | Gorotza | Mda | 0.00016 | 0.00027 | 0.00003 | 0.00038 | **0.00023** |
| 0927sa[1] | Gorotza | Ppi | 0.00016 | 0.00003 | 0.00005 | 0.00031 | **0.00013** |
| 0927sa[2] | Gorotza | Ppi | 0.00016 | 0.00001 | - | 0.00005 | **0.00003** |
| 0927sa[3] | Gorotza | Ppi | 0.00016 | 0.00001 | 0.00009 | - | **0.00005** |
| 074ha[1] | Gorotza | Rhi | 0.2 | 0.122 | 0.213 | 0.326 | **0.22** |

| | | | [DNA]"input"(ng/µL) | [DNA]qPCR(ng/µL) | | | |
|---|---|---|---|---|---|---|---|
| **Lagina** | **Mota** | **Espeziea** | **R123** | **R1** | **R2** | **R3** | **Mean** |
| 074ha[2] | Gorotza | Rhi | 0.2 | 0.150 | 0.049 | 0.349 | **0.18** |
| 074ha[3] | Gorotza | Rhi | 0.2 | 0.199 | 0.052 | 0.187 | **0.15** |
| 074ha[1] | Gorotza | Rhi | 0.04 | 0.012 | 0.036 | 0.065 | **0.038** |
| 074ha[2] | Gorotza | Rhi | 0.04 | 0.026 | 0.008 | 0.064 | **0.033** |
| 074ha[1] | Gorotza | Rhi | 0.008 | 0.0029 | 0.0061 | 0.0131 | **0.007** |
| 074ha[2] | Gorotza | Rhi | 0.008 | 0.0104 | 0.0042 | 0.0104 | **0.008** |
| 074ha[3] | Gorotza | Rhi | 0.008 | 0.0069 | 0.0018 | 0.0110 | **0.007** |
| 074ha[1] | Gorotza | Rhi | 0.000032 | 0.000007 | 0.000014 | 0.000092 | **0.000038** |
| 074ha[2] | Gorotza | Rhi | 0.000032 | 0.000016 | 0.000028 | 0.000066 | **0.000037** |
| 074ha[3] | Gorotza | Rhi | 0.000032 | 0.000032 | 0.000015 | 0.000034 | **0.000027** |

**S3.2. Taula.** (jarraipena)




**S3.1. Irudia.** Laginketa-eremuen kokapen geografikoa eta entsegu kualitatiborako leku bakoitzean erabilitako lagin kopurua. Mcry= *Myotis crypticus*; Mda= *M. daubentonii*; Mem= *M. emarginatus*; Msc= *Miniopterus schreibersii*; Pku= *Pipistrellus kuhlii*; Ppi= *P. pipistrellus*; Reu= *Rhinolophus euryale*; Rhi= *R. hipposideros*; Rfe= *R. ferrumequinum*; Tte= *Tadarida teniotis*.




**S3.2. Irudia.** qPCR produktuen DNA sekuentziazioaren emaitzak: anplikoi guztietan *T. pityocampa* sekuentzia berdina aurkitu zen.

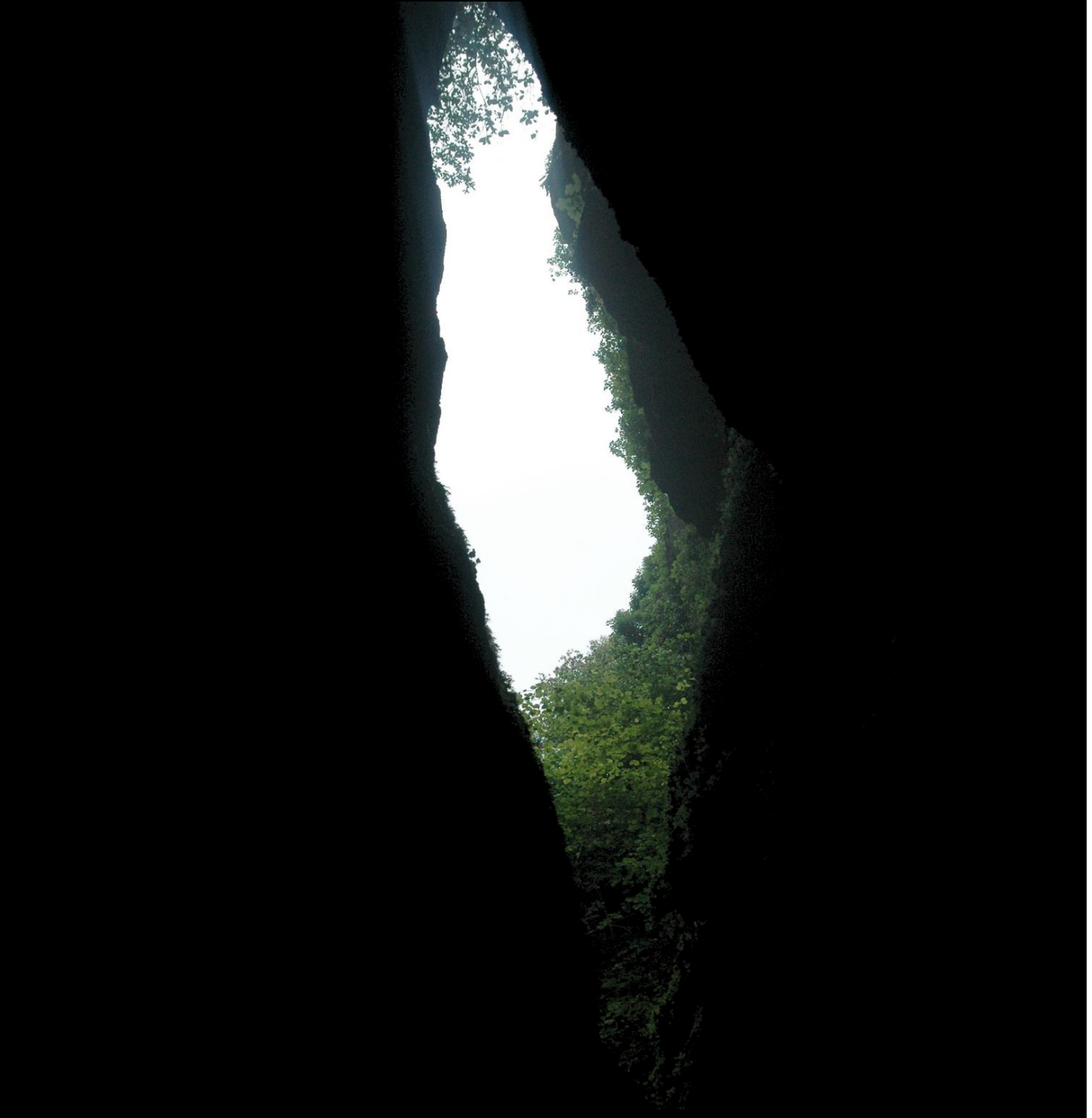
**S3.3 Irudia.** cPCR produktuen anplifikazio-bandak

Unai Baroja Ibáñez de Maeztu, 2021

# Unveiling the Ecosystem Services of Insectivorous Bats through a Comprehensive Approach

Saguzar Intsektiboroen Zerbitzu Ekosistemikoak argitara ekartzeko hurbilketa integrala

Unai Baroja Ibáñez de Maeztu, 2021



*Because there is still light at the end of the tunnel*