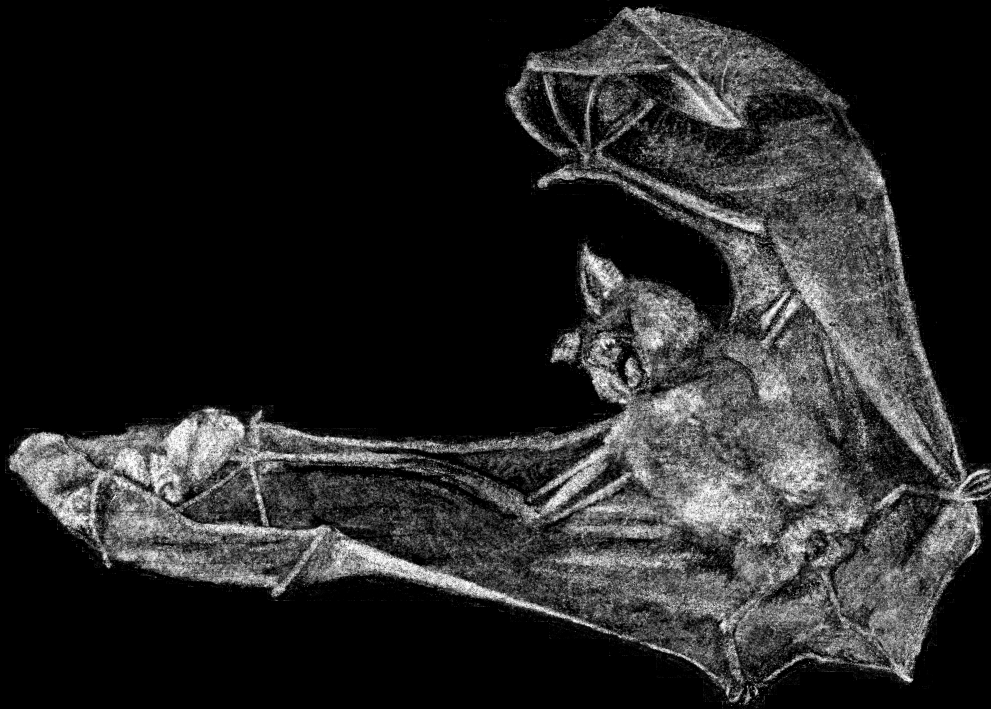# Foraging Ecology of *Rhinolophus euryale* Unveiled by DNA Metabarcoding

——— PhD Thesis by ———

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eman ta zabal zazu

Universidad del País Vasco   Euskal Herriko Unibertsitatea

Foraging Ecology of

Rhinolophus euryale

unveiled by DNA metabarcoding

A thesis submitted by Aitor Arrizabalaga Escudero to the University of the Basque Country for the degree of Doctor of Philosophy, under the supervision of Dr. Joxerra Aihartza Azurtza and Dr. Urtzi Goiti Ugarte.

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Amari, aitari, Jaioneri, amamari eta bereziki, aittitteri.
Zuon maitasun eta inspirazioagatik.

"Zoriak faboratzen dau prest dagoen gogoa"

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Laburpena

Saguzarren ekologia eta jokamoldea ulertzeko ezinbestekoa da beraien eta harrapakinen arteko elkarrekintza trofikoak identifikatzea. Nahiz eta beraien oinarrizko bazka-ekologia ezaguna izan (adb.: non eta zertaz bazkatzen diran), beraien bazka-nitxoaren inguruko zehaztasunak aztertzea metodologikoki guztiz mugatuta egon da. Testuinguru honetan, *DNA metabarcoding* delako teknika molekularrak saguzarren ekologian sakontzeko aukera berri bat eskaintzen du. Tesi honetan, *Rhinolophus euryale* eta *R. mehelyi* ferra-saguzarrak eredu hartuta, saguzarren bazka-ekologiaren inguruko hainbat galdera ekologiko zahar eta berri ebatzi dira *DNA metabarcoding*aren bidez.

Tesiaren lehenengo ikerketa-atalean paradigma ekologiko berri bat aurkezten da: *R. euryale* espeziearentzako harrapakin-eskuragarritasuna ez da bere ehiza-habitat egokien presentziarekin eta hauen kontserbazioarekin soilik bermatzen, hau da, hesi-bizi eta hostozabalen basoekin. Harrapakinek beraien bizi-fase desberdinak garatzeko beharrezkoak dituzten habitatak ere ezinbestekoak dira saguzarrentzat, nahiz eta habitat hauek ez dituzten bazkatzeko erabiltzen. Paradigma berri hau testatzeko, 56 *R. euryale*ren dieta espezie mailan zehaztu eta berau osatzen duten sits espezieen beldarren beharrizan ekologikoak bilatu dira bibliografian. Jandako espezie askoren larbek saguzarren ehiza-habitatak ez diren bestelako habitat batzuk behar dituzte beraien larba-fasea burutzeko, larreak eta belardiak esaterako. Gainera, larben beharrizan ekologikoak esangarriki aldatzen dira sasoitik sasoira. Beraz, *R. euryale*ren bazka-beharrizanak asetzeko ez dira nahikoa ehiza-habitat egokiak kontserbatzea paisai jakinean. Ehiza-habitatak sitsez hornitzeko beharrezkoak diren gainerako eremuak ere guztiz beharrezkoak dira. Azken hauetan egindako edozein eraldaketak eragin zuzena izan dezake *R. euryale*ren bazka-ekologian. Gure emaitzen arabera, saguzarrak kontserbatzeko beharrezko neurriek beraien harrapakinen fase desberdinak garatzeko beharrezko elementuak barneratu beharko lituzkete ere.

Sits-habitat-saguzar erlazioez gain, saguzarren bazka-ekologian eragina

duen beste aldagai bat antzeko espezieen presentzia da. Testuinguru honetan, antzeko animalia espezie askoren koexistentzia ahalbidetzen duen mekanismo garrantzitsua da bazka-baliagaien banaketa. Saguzarretan, ordea, ez da ondo ezagutzen. Tesiaren bigarren zati honetan *DNA metabarcoding*aren bidez ekologikoki oso antzekoak diren *R. euryale* eta *R. mehelyi*ren populazio sinpatriko baten dietaren osaketa eta gainezarpena aztertu dira. Gainera, populazio honen bestelako ezaugarri ekologikoak ezagunak dira aldez aurretik eginiko ikerketa-lan batean ikertu baitira. Emaitzei dagokienez, bi saguzar espezieen dieta zoriz esperoko litzakeena baino gehiago gainezartzen da, eta sits arrunt berdinetan oinarritua dago bereziki. Bestetik, behatutako dieta desberdintasunek saguzarrek sinpatrian duten habitat erabilera desberdintasuna islatzen dute. Aldez aurretik eginiko ikerlanetan populazio alopatriko eta sinpatrikoen artean behatutako habitat desberdintasunak eta lan honetan behatutako dietaren gainezarpen altua dela eta, bi ferra-saguzar espezie hauen koexistentzia bazka-baliagaien banaketan baino, habitaten banaketan oinarritua dagoela ondorioztatzen dugu. Lan honek ferra-saguzarren koexistentzia ahalbidetzeko nitxoaren dimentsio espazialak eta harrapakin espezie arruntek duten garrantzia azpimarratzen du.

Tesiaren azkenengo atalean, *R. euryale*ren bazka-nitxoaren malgutasuna eta harrapakinekin duen erlazio ebolutiboa aztertu dira. Bestetik, eskuragarri egon daitezkeen sitsen konposizio funtzionala aztertu da ere. Saguzarren ehiza-errentagarritasunaren ikuspuntutik, sitsek hainbat ezaugarri morfologiko, hegakera-modu, ihes- eta defentsa-mekanismo dituzte, baita saguzarren ultrasoinuak antzemateko gaitasuna ere. Ezaugarri guzti horien konbinazio desberdinek sitsen errentagarritasunean, eta beraz, saguzarren bazka-ekologian eragin desberdina izan dezaketela argudiatzen dugu, baita *R. euryale* bezalako sits-espezialista batentzako ere. *DNA metabarcoding*a eta *RLQ* eta *fourth-corner* tresna estatistikoen bidez, sitsen errentagarritasunarekin erlazionatuta egon daitezkeen ezaugarriak erabili dira adin, sexu, sasoi eta kolonia desberdinetako saguzarren dieta eta eskuragarri dauden sitsen konposizioa aztertzeko. Lortutako emaitzen arabera, kontsumitutako sitsen eta eskuragarri daudenen masa eta hegakera-moduarekin erlazionatutako ezaugarriak esangarriki

aldatzen dira sasoiaren arabera. Saguzar helduek hainbat sits-mota kontsumitu dituzte umatze-aurreko sasoian, sits lirain eta geldoak umatze sasoian eta energia askoko sits azkar eta maniobrakorrak umatze ostean. Emaitzek, sits-moten aldaketei aurre egiteko *R. euryale*ren malgutasun trofikoa adierazten dute. Beste alde batetik, saguzar gazteek helduekiko sits-mota esangarriki desberdinak kontsumitu dituzte maizago: espezie txiki, lirain eta geldoak. Desberdintasun hauek ale gazteen ehizarako esperientzia faltarekin egon daitezke erlazionatuta. Azkenik, sits-tinpanatuek dietaren gehiengoa osatu arren, arctiinoen taldeko tinpanatuak esperotakoa baino gutxiago kontsumitu dira. Emaitzek, arctiinoek *R. euryale* bezalako sitsetan espezializatutako saguzarrei ihes egiteko mekanismoak garatu dituztela iradokitzen dute. Ezaugarrietan oinarritutako dieta azterketek saguzarren bazka-ekologia eta erlazio ebolutiboak aztertzeko herraminta berritzailea eskaintzen dute, harrapakinen izen taxonomikoez haratago.

Oro har, sitsetan espezializatutako *R. euryale*ren bazka-ekologia "hesi-bizietan ehizatu eta sitsetaz elikatzen da" baino konplexuagoa dela erakusten du tesi honek. Ikerketa ekologikoek eta espezieen kontsernaziora bideratutakoek konplexutasun guzti hau hartu beharko lukete kontutan etorkizuneko lanetarako.

Resumen

La identificación de las relaciones tróficas entre los murciélagos y sus presas es esencial para entender su ecología y comportamiento. A pesar de que los aspectos básicos de la ecología de los murciélagos es conocida (p. ej. hábitats de forrajeo y presas principales), aspectos detallados sobre la ecología y evolución del nicho trófico no han sido posibles de investigar debido a limitaciones metodológicas. En este sentido, el denominado método *DNA metabarcoding* ofrece una solución alternativa para estudiar en detalle los hábitos alimenticios y ecología del forrajeo de murciélagos. En esta tesis se han elegido como especies modelo los especialistas en polillas *Rhinolophus euryale* y *R. mehelyi* para analizar una serie de paradigmas ecológicos.

En el primer estudio de esta tesis se presenta un nuevo marco teórico donde se argumenta que la disponibilidad de polillas para *R. euryale* no depende únicamente de la idoneidad y conservación de los hábitat de caza (esto es, setos vivos y bosques de hoja ancha), sino también de los hábitat donde los requerimientos ecológicos de los estadios larvales de las polillas se desarrollan. De hecho, los requerimientos de hábitat de imagos (fase volante) y larvas puede diferir considerablemente. En este primer estudio se ha examinado el grado en el que las presas de *R. euryale* se originan o dependen de hábitats no usados por el murciélago para cazar. Se determinó la dieta de 56 individuos mediante *DNA metabarcoding* y se buscaron los requerimientos de las fases larvarias de las presas en la literatura. Los datos revelaron que una gran parte de las plantas hospedadoras de las larvas de las polillas consumidas se dan en zonas que *R. euryale* no utiliza para cazar, es decir, en prados y pastizales. Además, el número de murciélagos consumiendo polillas originadas en lugares de caza descendió de la época de pre-cría a la de post-cría. Nuestros resultados muestran que *R. euryale* no solo depende de los hábitat donde caza, sino también de muchos otros que suministran de alimento las zonas de caza. Cualquier modificación de esos hábitat podría tener consecuencias importantes en la ecología del forrajeo de *R. euryale*. Con lo cual, las medidas de conservación no solo deberían de limitarse a incluir los hábitat utilizados por los murciélagos, sino todos aquellos requeridos

por el resto de etapas de vida de sus presas.

Más allá de las relaciones entre polillas-hábitats-murciélagos, la presencia de otras especies ecológicamente similares puede influir en la ecología del forrajeo de los murciélagos. En este sentido, el reparto de recursos alimenticios es un importante mecanismo que facilita la coexistencia de varias especies de animales. Aún así, este mecanismo no esta bien estudiado en murciélagos. En el segundo estudio de esta tesis se analizó la partición del nicho entre una población simpátrica de las especies hermanas *R. euryale* y *R. mehelyi,* para las que otros aspectos ecológicos fueron previamente estudiados. Mediante *DNA metabarcoding* se determinó la composición y solapamiento de sus dietas. Los resultados revelaron un solapamiento superior a lo esperado por azar debido al consumo de las mismas especies de polillas comunes. Además, las diferencias detectadas correspondían a especies ligadas a hábitats concretos, reflejando la segregación en el uso de hábitats anteriormente determinada para esta población simpátrica. Así, debido a las diferencias en uso de hábitat entre poblaciones alopátricas y simpátricas de *R. euryale* y *R. mehelyi*, y al alto grado de solapamiento alimenticio, se concluye que la coexistencia entre este par de especies hermanas esta principalmente mediado por la partición de la dimensión espacial del nicho, esto es, hábitats de caza. Este estudio subraya la importancia de la dimensión espacial y las especies de polillas comunes para la coexistencia estable de murciélagos de herradura altamente similares.

En el tercer estudio se utilizó una aproximación basada en las características de las polillas para analizar la flexibilidad trófica de *R. euryale*, así como la relación evolutiva con sus presas. También se analizó la disponibilidad funcional de polillas potencialmente disponibles a lo largo del tiempo y el espacio. Las polillas muestran una gran variedad de características que condicionan la rentabilidad de éstas para los murciélagos: masa, capacidades de vuelo, mecanismos evasivos y de defensa, así como la capacidad de percibir los ultrasonidos de los murciélagos. Argumentamos que la combinación de dichas características podría influir en su rentabilidad incluso para un especialista en polillas como *R. euryale*. Mediante *DNA metabarcoding* y los análisis estadísticos

de *RLQ* y *fourth-corner* se determinó la dieta funcional de *R. euryale*, así como la composición funcional de las polillas disponibles. Los resultados revelaron que la masa y las características relacionadas con la conducta de vuelo de las polillas cambió de manera significativa y similar a través del tiempo tanto en la dieta como en las especies disponibles. Los adultos de *R. euryale* pasaron de consumir varios tipos de polillas en la pre-cría, a polillas lentas y con bajo contenido energético en la época de cría, a polillas rápidas, evasivas pero con un alto contenido energético en la post-cría. Los resultados muestran que *R. euryale* es lo suficientemente flexible tróficamente para hacer frente a cambios estacionales en tipos de presas. Además, los juveniles consumieron especies más lentas y ligeras que los adultos. Éstos, en cambio, se alimentaron de especies más rápidas pero con superior contenido energético más frecuentemente. Estas diferencias están probablemente relacionadas a la inexperiencia en las habilidades de caza de los juveniles. Por último, la dieta de *R. euryale* está formada principalmente por especies timpanadas. Aunque parece que la subfamilia de las especies timpanadas Arctiine esta sub-representada en la dieta, lo que sugiere que este grupo podría haber desarrollado algún mecanismo para evitar ser cazada por especialistas como *R. euryale*. Creemos que esta novedosa aproximación basada en las características para el estudio de la dieta ofrece nuevos modos para analizar la dieta más allá de las descripciones taxonómicas.

En general, se concluye que la ecología del forrajeo del especialista *R. euryale* es mucho más compleja que "caza polillas en lindes de bosque". Estudios ecológicos y de conservación deberían considerar esta complejidad en futuras investigaciones.

Summary

The identification of the trophic relationships between bats and their insect prey is central to fully understanding their ecology and behavior. Although the basic aspects of their foraging ecology are known (e.g. where they forage, what they main prey are), fine-grained ecological and evolutionary aspects of their trophic niche have not been possible to address due to methodological limitations. In this context, DNA metabarcoding provides an alternative solution to further deepen the study of the dietary habits and foraging ecology of bats. We chose the moth-specialists *Rhinolophus euryale* and *R. mehelyi* horseshoe bats as model species to analyze the ecological paradigms presented in this thesis.

According to the first ecological framework analyzed in this thesis, moth availability for *R. euryale* may not only depend on the suitability of the foraging grounds where it forages (i.e. hedgerows and broadleaved forests), but also on habitats where the ecological requirements of the larval stages of moths are fulfilled. Indeed, moths shift in habitat requirements throughout their ontogeny; larvae and imagos differ in their ecological requirements. In this first study we test to what extent the prey of *R. euryale* originate either from the habitats where they are consumed, or from habitats outside the bat's foraging sites. We determined the diet of 56 *R. euryale* individuals by identifying their prey to the species level using DNA metabarcoding. Then, we searched for its prey's larval feeding requirements in the literature. We found that moths whose larval host plants occurred in non-foraging open grounds (i.e. pastures and meadows) were commonly observed in the diet of bats. More than 85% of the bats always preyed upon moths with larval requirements located in pastures and meadows. However, the number of bats preying upon moths likely originated in hedgerows and forests decreased from pre-breeding to post-breeding. Thus, our results confirmed that *R. euryale* does not only rely on the landscape elements where it hunts, namely hedgerows and broadleaved forests, but also on other source habitats that supply it with food, such as pastures and meadows. Any modification of those non-used prey-source habitats may have strong consequences on the foraging ecology of *R. euryale*. Therefore, conservation

measures should not be limited to merely protecting the foraging grounds of bats, but should also include the ecological requirements of their prey throughout their life stages.

In addition to the moth-habitat-bat relationship, the foraging ecology of a single bat species may also be influenced by the presence of other ecologically similar species. In this context, dietary niche partitioning is an important mechanism facilitating the coexistence of many animals. However, this mechanism is not well understood in bats. In the second study we analyzed the niche partitioning between sympatric populations of the sibling *R. euryale* and *R. mehelyi*. Using DNA metabarcoding, we measured the diet breath, composition and overlap of these populations, for which other aspects of their foraging ecology have been previously analyzed. Our results showed that interspecific diet overlap was higher than expected by chance due to the consumption of the same common moth species. Moreover, we also observed some small but significant dietary differences that corresponded to some habitat-specialist moths, reflecting the different use of habitats by *R. euryale* and *R. mehelyi*. Therefore, the spatial niche displacement measured for allopatric and sympatric populations of *R. euryale* and *R. mehelyi* in previous studies and the high dietary overlap reported in this study, led us to conclude that the coexistence of this pair of sibling bats is mainly mediated by the partitioning of the spatial niche dimension. This study highlights the relevance of the spatial dimension and common prey species for the coexistence of sibling horseshoe bats.

In the third study, we adopted a trait-based approach to analyze the degree of prey-specialization and adaptive flexibility of *R. euryale*, as well as the evolutionary relationship with its prey moths. We also analyzed the functional variability of the potentially available moth assemblage through time and space. Moths show a high diversity of functional traits that determine their profitability for bats: from body mass and flight capabilities, to combinations of evasive and defensive adaptations, including their ability to hear bats. We argued that the combinations of such traits could have different effects on their profitability even for moth-specialist predators such as *R. euryale*. Using DNA metabarcoding in

combination with RLQ and the fourth-corner analyses, we determined the functional diet of *R. euryale*, as well as the functional composition of the potentially available moth assemblage. Our results showed that traits related to energy content (i.e. mass) and flight performance (i.e. wing loading and maneuverability) changed significantly and similarly through seasons in both the diet and the moth assemblage in the study area. Adults of *R. euryale* shifted from pursuing and capturing varying moth types in the pre-breeding season, to mainly hunting slow fluttering moths with a low energy content in the breeding season, and fast and more evasive but energetically richer moths in the post-breeding season. These results showed that *R. euryale* is trophically flexible enough to take profit of seasonally variable moth types. Moreover, juvenile bats consumed lighter, more maneuverable but slower moth species more frequently than adults, which preyed more frequently upon heavier and faster species. This age-related diet difference is probably related to the naive hunting skills of young bats. Finally, we found that tympanate moths were the most frequently consumed species by *R. euryale*. However, tympanate arctiine species seemed to be under-represented in the diet, suggesting that these moths may have developed some effective level of protection against bats echolocating at high frequencies. We believe that the trait-based approach provides new insights into the way to study the diet of insectivorous bats beyond the taxonomic description.

The performed studies led us to conclude that the foraging ecology of the specialist bat *R. euryale* is much more complex than simply "foraging on moths in edge habitats." Ecological and conservation studies should consider this complexity for future research.

CHAPTER 1

General Introduction

Illustration by Maite Muro.

1.1. Foraging Ecology and Behavior: a general perspective

Food resources contribute directly to the growth and reproduction success of individual animals (i.e. fitness). Therefore, foraging, that is, obtaining food, is a fundamental task for all animals, which, in many cases, takes a considerable amount of their lifetime. For many animals foraging implies the need to search for, detect and recognize potential prey, decide whether to pursue or not, pursue, catch and finally consume prey (Stephens and Krebs, 1986). Additionally, all these actions are performed while they avoid being eaten by predators (Bednekoff, 2007). Consequently, the survival of individual animals is determined by the way in which they deal with each of these actions and respond to different ecological situations. Comprehensive knowledge of their relationship with prey species and habitats, with potential competitors, or their adaptive flexibility in response to prey fluctuations, that is, knowledge of their foraging ecology is required in order to understand their foraging behavior. This knowledge is particularly relevant in the era of the Anthropocene, in which landscapes, climate and distribution of species are being rapidly modified by humans (Voigt and Kingston, 2016).

1.2. Relationship between Foraging Habitats, Predators and Prey: New Insights for Species Conservation

Research on the foraging ecology of single species mainly focuses on *What* resources are consumed (i.e. prey species), and *Where* (i.e. foraging habitats) and *When* (e.g. day, night, season) those resources are consumed. In this context, researchers often consider the intensity at which animals select particular foraging habitats in order to assess the relevance of those habitats in their ecological niche (Manly et al., 2002). Obviously, those foraging habitats gather the prey species on which predators feed. Therefore, when designing conservation measures, it is generally assumed that protecting the foraging habitats guarantees the availability of prey. For instance, the hunting grounds of the European Lynx (*Lynx lynx;* Linaeus, 1758) are usually located in forested

habitats where their preferred prey (i.e. roe deer) needs to forage and shelter from predators at the same time (Belotti et al., 2013; Mysterud et al., 1999). Thus, the conservation plan for the European Lynx (Breitenmoser et al., 2000), prioritizes management of forests, both as foraging grounds for the lynx and as the main habitat for ungulates. Given that this is the case for many animal species, conservation research and guidelines are orientated towards protecting those habitats where predators and prey interact (e.g. Eurobats, 2014; Shuterland and Green, 2004).

Nevertheless, many animal species have complex life cycles and their ecological requirements change across their lifespan (Rudolf and Lafferty, 2011; Rudolf and Rasmussen, 2013). In this sense, the habitat requirements of predator and prey may differ considerably despite the fact that they trophically interact in particular habitats and moments. For instance, the leatherback turtle *Dermochelys coriacea* (Vandelli, 1761) forages on the medusa form of the jellyfish *Cyanea capillata* (Linnaeus, 1758) in the water column (Heaslip et al., 2012). However, the larvae of *C. capillata* are developed as polyps attached to the substrate on the benthos, in a completely different environment (Brewer, 1976). This implies that the source of medusas for turtles not only depends on the water column, but also on the benthos where polyps are developed. Nonetheless, this environment is not usually included as relevant for the foraging ecology of the leatherback turtle (Heaslip et al., 2012; Stewart et al., 2013), although they may be crucial for filling its foraging habitats with prey. Considering the large amount of animal species foraging on prey with complex life cycles such as insects, studies aiming to guarantee the conservation of prey for predators should also assess to which extent the foraging grounds of predators fulfill the lifelong ecological requirements of prey. However, to our knowledge no study has assessed the relevance of prey source habitats in the foraging ecology of predators.

1.3. Species Coexistence

In addition to the prey-habitat-predator relationship, there are many other processes involved in the foraging ecology of single species, such as those derived from the coexistence with ecologically similar species. The most well-known classic process is probably competition (Schoener, 1983; Dayan and Simberloff, 2005), and occurs when one species reduces the fitness of another as a consequence of exploiting the same resource (i.e. anything limited, used and diminished by organisms such as food, water, roosts). Closely related species, which usually have similar niches, compete more intensely than distantly related ones, limiting their chances for stable coexistence (Webb et al., 2002). In this framework, niche differences are relevant to facilitate the coexistence. However, the identification of niche differences does not necessarily mean that competition is the process behind these differences (e.g. in the use of habitats or food). In fact, there is ample evidence both in favor of and against the role of competition in shaping species coexistence in varying natural systems (e.g. reviewed in Mayfield and Levine, 2010). These evidences triggered the development of other hypotheses such as the neutral theory (Bell, 2000; Hubbell, 2001), which postulates that all species have identical fitness and it is only random variation in birth, death and dispersal that contribute to coexistence.

Nowadays, the coexistence of species is seen in the light of the new paradigm proposed by Chesson (2000). According to this paradigm, the coexistence of species is the result of interaction between niche differences and differences in competitive ability (i.e. fitness inequality). For instance, large differences in the competitive ability between sympatric species would require large niche differences for stable coexistence, such as high levels of resource partitioning (Adler et al., 2007; Chesson, 2000). Similarly, the coexistence of ecomorphologically similar and phylogenetically related species that have similar competitive abilities, should be facilitated by small niche differences. In a foraging context, this implies that similar and related species should show overlapping patterns of food and habitat use: small but sufficient differences that facilitate their stable coexistence. However, the identification of such small

differences in resource use can be methodologically challenging, particularly for small, elusive or nocturnal animal groups.

1.4. Functional Relationships between predator and prey: Novel Approach for Diet Analyses

Trophic relationships between predators and prey are a key element of the structure and functioning of natural communities. As mentioned above, these relationships are shaped by at least two basic requirements of both predators and prey: the need to efficiently exploit resources to grow and reproduce, and the need to avoid being eaten (Bednekoff, 2007; Stephens and Krebs 1986). In relation to the first requirement, efficient predators should forage in a way that maximizes the rate of net energy gain (Stephens and Krebs 1986). This implies that predators must be able to distinguish between prey types of varying profitability (i.e. energy gained per time unit of handling: detecting, pursuing, capturing and consuming) and adjust prey targets when prey availability changes, in order to ensure an average positive energy balance. Prey profitability results from a combination of traits (e.g. size, volume, hardness) that predators must be able to perceive and assess (Schnitzler, 1987), and it may vary with environmental conditions (e.g. prey abundance) or predator-specific requirements (e.g. breeding season) (Stephens and Krebs 1986). On the other hand, prey need to avoid predators (Bednekoff, 2007) and usually present a combination of varying morphological (e.g. camouflage), physiological (e.g. sensory, poisons) and behavioral (e.g. evasiveness) adaptations (i.e. traits) to avoid being eaten. These traits exert evolutionary pressures on predators, demanding more specialized foraging adaptations (Begon et al., 2006). These reciprocal selection pressures result in a co-evolutionary arms race where the development of attack capability in predators and of avoidance capability in prey continually escalates. Therefore, current morphological, physiological or behavioral traits of predators and prey are the result of past foraging selection pressures exerted on each other (i.e. adaptations; Danchin et al. 2008).

Consequently, not all prey are equally profitable for predators (Chai and Srygley, 1990; Spitz et al., 2014). Prey quality rather than taxonomical diversity or total abundance influences the foraging ecology of some top predator marine mammals (Spitz et al., 2012; 2014). It is challenging to identify the key prey-types (categorized by traits) that shape the trophic niche of species, especially for predators feeding on an overwhelming range of prey species (e.g. insectivores). Prey lists may vary temporally (e.g. due to changes in prey phenology; Kartzinel and Pringle, 2015), spatially (e.g. differing prey-assemblages across a predator's distribution area; Marciniak et al., 2007) or in relation to predator's intraspecific variation (e.g. sex, predatory experience; Beck et al., 2007). Moreover, predator-prey interactions may also vary due to structural (e.g. addition or removal of species, loss of habitats) and functional (Flynn et al., 2009) changes that many ecosystems face as a result of both anthropogenic and natural disturbances (Nel et al., 2014). Such disturbances are expected to intensify with global change (Grimm et al., 2013; Foley et al., 2005; Pereira et al., 2010). Hence, delimiting the trophic niches and adaptive flexibility of predators is of paramount significance in animal ecology, evolution and conservation. However, researchers face limitations when taxonomy-based traditional dietary approaches are implemented. These methodological limitations restrict the understanding of the foraging behavior of predators (e.g. opportunistic/selective) or the vulnerability of prey to predation (Green and Cote, 2014), and subsequently, the prediction of their foraging responses under varying situations (e.g. habitat disturbances).

Alternatively, trait-based dietary approaches enable to identify the key functional traits that define the prey-type and shape the trophic niche of predators beyond the mere taxonomical trophic relationships. These approaches open new insights into the structure and dynamics of complex predator-prey systems, and improve considerably researchers' ability to predict trophic relationships (e.g. diets) in disturbed or poorly researched areas. The innovative value and predictive potential of trait-based functional approaches have been recently tested for marine predator-prey systems (Green and Côté, 2014; Spitz et al. 2014), but they are yet to be applied to terrestrial systems.

1.5. The Foraging Ecology of Insectivorous Bats and the Advent of DNA Metabarcoding

Insectivorous bats (about 70% of extant bat species; ca 1300) and their arthropod prey (mostly insects) constitute a meaningful part of most terrestrial ecosystems of all continents except Antarctica (Kunz and Pierson, 1994). The evolution of flight and echolocation in bats has been crucial to exploiting the diverse group of nocturnal insects (Jones and Rydell, 2003). The high ecological diversity of insectivorous bats is related to many morphological, sensorial and behavioral adaptations tightly linked to flight and echolocation: from fast and large species using low-frequency echolocation calls adapted to forage on airborne insects in open spaces (e.g. *Tadarida teniotis;* Dietz et al., 2009), to slow and maneuverable small bats that rely on prey-generated sounds to capture them in clutter environments (e.g. *Myotis bechsteinii;* Dietz et al., 2009). These species-specific adaptations shape their trophic niche: what types of prey they are able to detect, pursue, capture and consume (e.g. Jones and Rydell, 2003; Swartz et al., 2003).

Most insectivorous bats seem to be considerably flexible in their diets (e.g. Clare et al., 2009; Jones and Rydell, 2003; Napal, 2011), whereas others have specialized in particular insect taxa such as mosquitoes, moths or beetles (e.g. *Pipistrellus nathusii*; Krüger et al., 2014, *Barbastella* sp.; Sierro and Arlettaz, 1997, *Myotis myotis*; Arlettaz, 1999, respectively). Diptera, Coleoptera and Lepidoptera are among the most relevant prey insect groups for bats, as well as other taxa that tend to swarm at night such as Ephemeroptera, Trichoptera and Hymenoptera (reviewed in Jones and Rydell, 2003). Research carried out on the foraging ecology of bats during the last 30 years has yielded extensive knowledge about their diets and habitat use (e.g. Jones and Rydell, 2003; Patterson et al., 2003; Whitaker et al., 2009). In this context, while the development of several techniques for habitat-use studies have enabled fine-grained assessments of their foraging grounds (e.g. small radio-transmitters for radio-tracking; Amelon et al., 2009), the study of their dietary habits have been mostly limited to morphological analyses of insect remains in bat feces (see

Whitaker et al., 2009). Bats thoroughly chew and digest their prey, and hence, the resolution of such analyses is usually restricted to the order or family level (Whitaker et al., 2009). Therefore, although the basic aspects of their foraging ecology are known (e.g. they prey on moths in edge habitats), it has not yet been possible to address fine-grained ecological and evolutionary aspects of the trophic niche in many insectivorous bat species, such as those mentioned in sections 1.2-1.4. In this sense, the fine-grained identification of the trophic relationships between bats and their insect prey is central to fully understand their ecology and behavior. It is therefore essential for the effective conservation management of ecosystems under severe threat in many regions of the planet due to land use change (Conrad et al., 2006; Fox et al., 2013; Voigt and Kingston, 2016).

DNA-based molecular approaches provide an alternative solution to further deepen the study of the dietary habits of bats (Whitaker et al., 2009). Since the first extensive molecular dietary approach by Clare et al. (2009), the development of arthropod-specific short primers for diet analyses (Zeale et a., 2011) and Next Generation Sequencing Technologies (see Pompanon et al., 2011) the cost and resolution of diet analyses have been substantially improved (but see limitations; Clare, 2014). Nowadays, it is methodologically possible to identify insect remains in bat feces at the species level. However, the success of this approach depends on two prerequisites: the validity of genetic markers as DNA barcodes (see below) and the availability of reference DNA barcode collections linked to known species for comparison.

The proposal of DNA barcodes for species level identification by Hebert et al. (2003a) marked a milestone in this field. A DNA barcode is a genetic marker that varies sufficiently among species but is conservative within species. In particular, Hebert et al. (2003a) proposed a region of the cytochrome c oxidase I mitochondrial gene (COI) as a robust marker for DNA barcoding of animals. Subsequently, it was validated for almost all the animal taxa (Hebert et al., 2003b), and adopted by the Consortium for the Barcode of Life (CBOL: www.barcodeoflife.org) as the universal marker for the identification of animal

specimens. Thus, COI enables species level identification of an "unknown" through comparison to reference databases (Meusnier et al. 2008), and offers an excellent tool for ecological studies (Valentini et al., 2009b).

Furthermore, the recent development of Next Generation Sequencing (NGS) technologies (also known as High-Throughput Sequencing; HTS) has enabled the simultaneous sequencing of multiple DNA samples, at a reduced time and lower cost than previous sequencing techniques (e.g. cloning and sequencing, Pompanon et al., 2011). NGS technologies, together with bioinformatic analysis and the use of DNA barcodes, have given way to the so-called DNA metabarcoding technique (Yu et al. 2012). This technique facilitates the identification of several species occurring in complex heterogeneous samples such as feces or soil (Valentini et al., 2009a, 2009b). It has led to a new frame in a variety of fields, including the study of trophic interactions in predator-prey systems and food-webs (Carreon-Martinez and Heath 2010; Clare, 2014; Pompanon et al., 2011), host-parasite systems (Hrcek et al. 2011), or ecosystem biomonitoring (Hajibabaei et al. 2011; Yu et al. 2012; Zhou et al. 2013). In comparison to traditional morphological approaches, DNA metabarcoding provides much finer taxonomical resolution to analyze complex heterogeneous samples. It offers the possibility to identify fragments hardly identifiable through morphology, it is less time-consuming, and the identification does not depend on the taxonomic skills of the researcher (Pompanon et al 2011). However, the success of DNA metabarcoding studies depends on the availability and completeness of reference sequence databases (Pompanon and Samadi 2015). For instance, COI reference sequences are completed unevenly for different taxonomic groups and skewed in relation to biodiversity research projects and geographical accessibility (see for instance the DNA barcode map in: www.boldsystem.org).

Although DNA metabarcoding is still developing as a methodology and needs considerable improvements, from DNA extraction steps to bioinformatic analyses (Clare, 2014), it is a suitable tool to accurately analyze the diet of bats at a resolution level and sample-size magnitude inconceivable with other

techniques. At the beginning of this thesis in 2012 few studies applied DNA metabarcoding to assess the diet of bats (Bohmann et al., 2011, Razgour et al., 2011). Shortly after, however, the suitability of this tool was effectively demonstrated, as illustrated by the number of researches published in this topic thereafter: Burgar et al., 2014; Clare et al., 2013, 2014; Emrich et al., 2014; Gonsalves et al., 2014; Hope et al., 2014; Krüger et al., 2014a/b; Mata et al., *In Press*; Sedlock et al., 2014; Vesterinen et al., 2013, 2016. Beyond the description of diets, the species level identification of prey items also enables the analysis of several ecological hypotheses hardly feasible before, for instance, the inference about foraging habitats of bats (Alberdi et al., 2012; Clare et al., 2011 Razgour et al., 2011) or gender-related functional differences (Mata et al., *In Press*). The high potential of DNA metabarcoding to study the foraging ecology of bats stimulated the development of this doctoral thesis, where new and old ecological paradigms are analyzed.

1.6. Model Species

In order to assess the ecological paradigms presented in sections 1.2-1.4, the horseshoe bats *Rhinolophus euryale* (Blasius, 1853) and its sibling and ecomorphologically almost identical *Rhinolophus mehelyi* (Matschie, 1901)(Family Rhinolophidae) have been chosen as model species. Several factors made them suitable to assess the above mentioned ecological questions using DNA metabarcoding:

First, their staple diet consists of moths (Goiti et al., 2008; Salsamendi et al., 2012a/b), which presents 4 methodological advantages:

i) Suitable PCR-primers for the amplification of short fragments of the COI barcode (Hebert et al., 2004) for Lepidoptera are available in the literature (Zeal et al., 2011, see Clarke et al., 2014).

ii) The potential bias introduced by the variation in DNA survival during digestion is minimized (i.e. Deagle and Tollit, 2006), as well as the potential bias related to the taxonomic preference of primers in the PCR amplification step for other taxa at the ordinal level (e.g. Clarke et al., 2014).

iii) European Lepidopteran species are well represented in reference COI barcode databases such as BOLD and NCBI (e.g. 1,170,186 specimens belonging to 101,193 species in BOLD -19/02/2016-), increasing the probability of identification of putative prey species.

iv) Extensive ecological and biological data for Lepidopterans is available in the literature and on the internet (e.g. Redondo et al., 2015; Robineau, 2007; Sterling and Parsons, 2012; Waring and Townsend, 2003; www.lepidoptera.eu), which enables the functional classification of prey species and the analysis of the diet beyond the taxonomy of prey species.

v) Efforts to measure availability are mainly focused on Lepidoptera, for which several capture methodologies and identification guides exist (e.g. Waring and Townsend, 2003).

In addition, the echolocation, morphology, foraging ecology and behavior of *R. euryale* and *R. mehelyi* have been well studied over the last 15 years (Aihartza et al., 2003; Goiti et al., 2003, 2004, 2006, 2008; Russo et al., 2001, 2002, 2005; Salsamendi et al., 2005; 2012a/b). This detailed ecological background in combination with the high taxonomic resolution of molecular tools opens the possibility to address the fine-grained ecological questions and aims presented in this thesis.

Moreover, evidence suggests that the High Duty Cycle (HDC) and Constant Frequency (CF) echolocation calls of horseshoe bats make them able to distinguish small differences among prey types (Kober and Schnitzler, 1990; Schnitzler, 1987; von der Emde and Schnitzler, 1990). Additionally, they have been reported to forage selectively according to environmental changes in prey profitability under laboratory conditions (Koselj et al., 2012), as well as in the wild (Jones, 1990). This allowed us to test functional relationships between horseshoe bats and their moth prey, since they are able to finely recognize, and hence, discriminate different prey types according to their profitability.

Furthermore, *R. euryale* and *R. mehelyi* are particularly sensitive to anthropogenic disturbances (e.g. population declines, light pollution, pesticides,

etc. Aihartza, 2001; Brosset et al., 1988; Horácek, 1984; Stebbings, 1988; Palmerin and Rodrigues, 1992). They are gregarious (large colonies localized in few roosts), and they are apparently habitat- and diet-specialists. This makes them potentially vulnerable to any landscape modifications. *R. euryale* and *R. mehelyi* are categorized as Near Threatened and Vulnerable by IUCN, respectively (Hutson et al., 2008a/b). Thus, the assessment of their fine-grained ecological requirements is essential to build effective conservation measures for these bats and other similar species.

1.7. Aims of the Thesis

In summary, the general aim of this PhD thesis is to gain a more detailed understanding of the foraging ecology of *Rhinolophus euryale*: the relationship with its insect prey and the environment that it inhabits, and how *R. euryale* interacts and partitions resources with the ecologically similar sibling *R. mehelyi* in sympatry. It also seeks to assess the trophic niche of the moth-specialist *R. euryale* and the functional relationships with its prey moths beyond taxonomy.

The specific objectives of the thesis are:

1- To analyze the link between the ecological requirements of the larval stage of prey species and the foraging habitats of *R. euryale* to: test whether its foraging habitats also cover the habitat requirements of the other life-stages of the consumed prey. Or whether the prey require sites outside the foraging range of *R. euryale* to complete their lifecycle.

2- To analyze the diet of two sympatric populations of sibling *R. euryale* and *R. mehelyi* at the species level, in order to test if they partition food resources, and understand which mechanisms facilitate their coexistence.

3- To analyze the trophic niche of *R. euryale* by linking prey's traits related to profitability and bats' intraspecific traits (i.e. sex, size and ontogeny) through diet and across a spatiotemporal gradient to:

> 3.1. - Determine the trophic niche flexibility of the moth-specialist *R. euryale*. Test the hypothesis that the main prey-type of a medium-sized and maneuverable bat is also characterized by being medium-sized and maneuverable.
>
> 3.2. - Identify the key traits of prey linked to the diet of bats.
>
> 3.3. - Identify and discuss the evolutionary relationship between moths and *R. euryale* in wild populations.

4- To characterize and analyze the functional variability of the potentially available moth assemblages through time and space to: test if they fluctuate functionally as well as they do taxonomically.

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CHAPTER 2

General Material and Methods

2.1. Study Species

Rhinolophus euryale and *R. mehelyi* are morphologically and ecologically highly similar sibling horseshoe bat species (Csorba, 2003, Fig. 2.1). They are widespread throughout the Mediterranean region, where their distributions overlap extensively in many areas of Anatolia, North Africa and the Iberian, Apennine and Balkan Peninsulas (Csorba, 2003).

2.1.1. The Mediterranean horseshoe bat - *Rhinolophus euryale* (Blasius, 1853)

R. euryale is a medium-sized horseshoe bat with a forearm length of 44-51 mm and a mass of 7-16 gr (Dietz et al., 2009; Fig. 2.1). Regarding its echolocation system, *R. euryale* emits peak echolocation calls at a frequency of ca 104 kHz (Heller and von Helversen, 1989, Russo et al., 2001, 2007; Salsamendi et al., 2005). This species is characterized by short and broad wings, which allow it to perform a highly maneuverable flight suited to cluttered environments (Norberg and Rayner, 1987; Salsamendi et al., 2005). It is widespread throughout the Mediterranean region, from east to west, being present in Transcaucasia, Middle East, Turkmenistan, Anatolia, the Balkan, Apennine and Iberian Peninsulas, Morocco, Algeria and Tunisia, and also in Slovakia, Hungary, France and some Mediterranean islands (Csorba et al., 2003; Dietz et al., 2009; Ibañez, 1999). *R. euryale* is mostly a cave-dwelling gregarious species linked to karst topography, although it may also use human-made structures (Dietz et al., 2009; Uhrin et al., 2012). For foraging it selects semi-open or highly cluttered broadleaved arboreal habitats such as hedgerows, edges and forests (Aihartza et al., 2003; Goiti et al., 2006; 2008; Russo et al., 2002; Salsamendi et al., 2012b). The diet of *R. euryale* is mainly based on moths, although other prey may also be important in some seasons (Andreas et al., 2012; Goiti et al., 2004; Salsamendi et al., 2012b). It is listed as Near Threatened in the IUCN Red List (Hutson et al., 2008a).

Figure 2.1. *R. euryale* (left) and *R. mehelyi* (right). Picture courtesy of Jesús Nogueras.

2.1.2. Mehely's horseshoe bat - *Rhinolophus mehelyi* (Matschie, 1901)

R. mehelyi is also a medium-sized horseshoe bat with a forearm length of 47-55 mm and a mass of 10-18 gr (Fig. 2.1). Regarding its echolocation system *R. mehelyi* emits peak echolocation calls at a frequency of ca 107 kHz (Heller and von Helversen, 1989, Russo et al., 2001, 2007; Salsamendi et al., 2005). The species is characterized by longer and thinner wings than *R. euryale*, which allow a less maneuverable but faster flight, suited to more open environments (Norberg and Rayner, 1987; Salsamendi et al., 2005). The species is also distributed throughout the Mediterranean region, discontinuously, from east to west, being present in Transcaucasia, Middle East, Anatolia, the Balkan, Apennine and Iberian Peninsulas, Morocco and other parts of North Africa, as well as some Mediterranean islands (Csorba et al., 2003; Dietz et al., 2009). *R. mehelyi* is also a cave-dwelling gregarious species linked to karst topography, but may also use human-made structures (Dietz et al., 2009). For foraging it also selects semi-open and cluttered broadleaved habitats such as forest edges, dehesas and forests, as well as other open woody habitats (Russo et al., 2005; Salsamendi et al., 2012a,b). Moths are also the main prey of *R. mehelyi*, although other prey may also be important in some seasons or for yearling bats

(Salsamendi et al., 2008, 2012b). It is listed as Vulberable in the IUCN Red List (Hutson et al., 2008b).

2.2. Study Design

The first part of this study (Chapter 3) was carried out with the largest known population of *R. euryale*, which inhabit in Karrantza Valley, in the Basque Country (Northern Iberian Peninsula, Fig 2.2). For the second part (Chapter 4) two sympatric populations of *R. euryale* and *R. mehelyi* inhabiting in Sierra de Villuercas were chosen (Extremadura, Spain, Central-Western Iberian Peninsula, Fig 2.2). Finally, the third part (Chapter 5) was carried out with two populations of *R. euryale* inhabiting contrasting landscapes in the Basque Country: the aforementioned one in Karrantza Valley (the same as in part one, Western Biscay) and a second population located in Lea-Artibai Valley (Eastern Biscay, Fig 2.2). For parts one and three samples were collected in a single night in May, July and September of 2012, coinciding with pre-breeding, breeding and post-breeding seasons, respectively. For part two samples were collected during June-July (breeding season) of 2007 as part of the field-work of Egoitz Salsamendi's PhD thesis.

Having analyzed only three populations of *R. euryale* and one of *R. mehelyi*, we are aware of the limitations of studying few colonies (e.g. specific landscape, colony-related behavior, colony size; Whitaker et al., 2009). However, the trophic ecology and the foraging behavior of the studied colonies (Goiti et al., 2004, 2006, 2008; Salsamendi et al., 2012a/b) are similar to other colonies of *R. euryale* and *R. mehelyi* described elsewhere in Europe (Andreas et al., 2012; Koselj and Krystufek, 1999; Russo et al., 2002, 2005). Consequently, we believe that the observed results adequately represent the diet and the foraging ecology of other populations of *R. euryale* and *R. mehelyi*.

Figure 2.2. Location of the study areas: Chapter 4 was carried out in Karrantza Valley, Chapter 5 in Sierra de Villuercas and Chapter 6 in Karrantza and Lea-Artibai Valleys.

2.3. Study Areas

In Biscay, the two known *R. euryale* breeding colonies are located in Karrantza and Lea-Artibai valleys. Both colony roosts are complex limestone caves that are used as a hibernaculum during winter and for breeding from mid-April to mid-June (*own data*). Both caves are also used by other species throughout the year: *R. ferrumequinum*, *R. hipposideros*, *Myotis emarginatus* and *Miniopterus schreibersii*.

2.3.1. Karrantza Valley (Chapter 3 and 5)

Karrantza Valley is located in the westernmost part of the Basque Country (northern Iberian Peninsula: 30T 46968E, 478950N). It is a hilly valley with elevations of 200–855 ma.s.l., characterized by an Atlantic temperate oceanic climate. Rainfall occurs throughout the year (annual mean 1400mm). The predominant land use of the site is devoted to dairy cattle breeding, along with small *Pinus radiata* and *Eucalyptus globulus* plantations. Thus, the landscape (Fig. 2.3) consists of a mosaic of small meadows and pastures, surrounded by an important hedgerow network consisting mainly of *Salix atrocinerea, Corylus avellana, Rubus ulmifolia, Acer campestre, Quercus robur* and *Crataegus monogyna*, interspersed with tree plantations and deciduous and holm oak woodland patches. The deciduous woodlands consist mainly of *Quercus robur, Fraxinus excelsior, Castanea sativa* and *Corylus avellana*. A limestone mountain range borders the northwest part of the valley, which provides abundant natural cavities and dense *Q. ilex* woods with limestone outcrops.
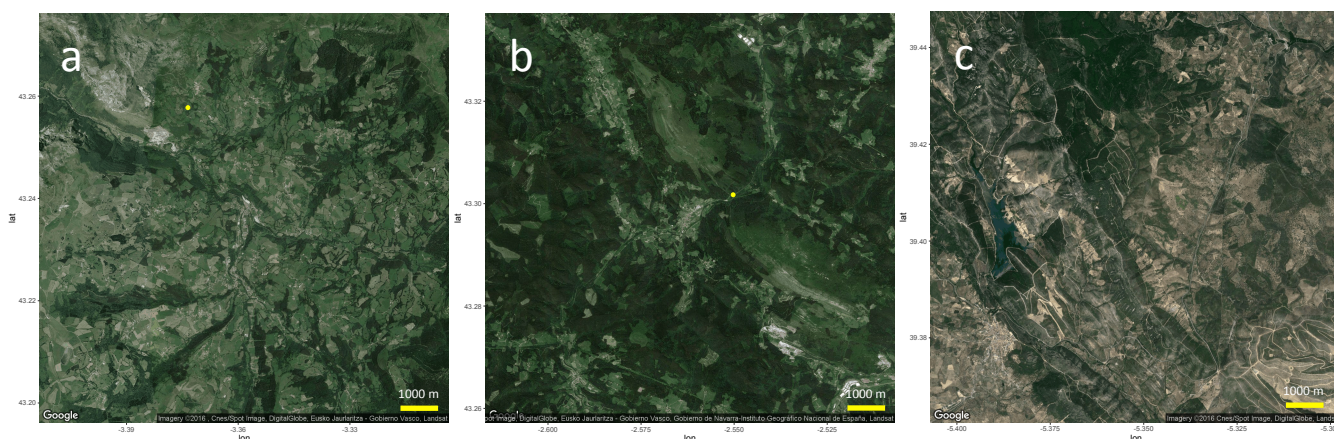
Figure 2.3. Study areas a) Karrantza Valley, b) Lea-Artibai Valley, c) Sierra de Villuercas mountain range. The location of the colony roost is shown by a yellow dot for Karrantza and Lea-Artibai Valleys.

2.3.2. Lea-Artibai Valley (Chapter 5)

Lea-Artibai Valley is located in the northern-central part of the Basque Country (northern Iberian Peninsula: 30T 53647E, 479442N). It is a hilly and steep valley with elevations ranging ca 40–700 ma.s.l., characterized by an

Atlantic temperate oceanic climate. As in Karrantza, rainfall occurs throughout the year (annual mean 1400mm). The landscape of Lea-Artibai Valley (Fig 2.4) is dominated by *Pinus radiata* plantations, along with small *Eucalyptus globulus* plantations. These plantations are interspersed with small farming patches, as well as with small deciduous and holm oak woodland patches. The composition of broadleaved forests is similar to that described for Karrantza. The landscape is also rich in limestone outcrops and natural cavities.

2.3.3. Sierra de las Villuercas (Chapter 4)

Sierra de las Villuercas mountain range is located in western Spain (western-central Iberian Peninsula: 30S 2924 4359). For more information see Salsamendi et al., 2012b. It is a mountainous area characterized by continental climate. The annual mean precipitation is 523 mm. The landscape of Sierra de Villuercas mountain range (Fig 2.5) is characterized by diverse habitats: large corn and rice crops, dehesas consisting of *Quercus rotundifolia* and *Q. suber*, pastures and meadows, olive plantations (*Olea europaea*), broadleaved forests consisting of *Q. pyrenaica* and *Castanea sativa*, riparian forests dominated by *Populus* sp. and *Alnus glutinosa*, and coniferous and eucaliptus plantations.

2.4. Fecal Samples: Bat Captures and Ethics Statements

Bats were captured with a 2 × 2 m harp trap (Tuttle, 1974), located in the entrance of the colony roosts from 00.30 a.m. onwards, as bats returned to the caves after foraging. Each captured bat was held individually in a clean cloth bag until it defecated (a maximum of 40-90 min). Bats were sexed and aged: juveniles were distinguished from adults by illumination of the cartilaginous epiphyseal plates in their phalanges (Anthony, 1988). The weight and forearm length of each bat individual were measured. Fecal material was collected for each individual bat and was frozen within 6 h from the moment of collection. Bats were immediately released into the cave after handling.

Capture and handling protocols followed published guidelines for the treatment of animals in research and teaching (Animal Behaviour Society, 2006; Sherwin, 2006). Captures and procedures in Chapters 4 and 6 were approved by the Ethics Committee at the University of the Basque Country (Ref. CEBA/219/2012/GARIN ATORRASAGASTI). Captures were performed under license from the Regional Council of Biscay, and met Basque legal requirements. The captures and procedures of Chapter 5 were approved by the Regional Council of Extremadura (license number: 0532041 PC 120), and met Spanish legal requirements.

2.5. Molecular Analyses - DNA metabarcoding

2.5.1. DNA extraction, PCR amplification, library construction and sequencing

The individual bat was considered as the sampling unit (Whitaker et al., 1996). 10-30 mg of feces per bat were used for DNA extraction with the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA), following the manufacturer`s instructions and Zeale et al. (2011).

A 157 bp length fragment of the mitochondrial DNA cytochrome *c* oxidase subunit I barcode region (COI) was PCR amplified from each DNA extract using modified (see below) ZBJ-ArtF1c and ZBJ-ArtR2c primers (Zeale et al., 2011). For the library preparation (i.e. library = uniquely tagged sample), each sample was tagged with a unique combination of Multiplex Identifier primers (MID; Binladen et al., 2007). These unique tags allowed us to bioinformatically separate and distinguish all the amplicons originated from each individual bat sample.

In this thesis two different Next Generation Sequencing (NGS) platforms have been used for deep sequencing DNA samples: Roche`s 454 Junior NGS platform (Chapter 3) and Ion Torrent sequencing platform (Chapter 4 and 5). The general procedure for PCR amplification and library preparation is similar

for both sequencing platforms. Thus, both procedures are described in this Chapter, but referring to differences intrinsic to each one.

2.5.1.a Library generation and sequencing with Roche`s 454 Junior NGS platform

The first decision related to the NGS platform was the number of samples that could be sequenced together in a run (i.e. a single sequencing round). This was due to the differences between the sequencing outputs (i.e. number of produced sequences) of different platforms. Additionally, we ignored the number of prey species consumed by single bat individuals (i.e. faecal samples). Therefore, although this step is not mentioned in the literature, it would determine the number of sequences that would be obtained for each sequenced faecal sample (e.g. a single bat consuming a single prey species or 50 bats with an average prey consumption of 10 species/bat). This step is particularly important for sequencing in NGS platforms with low sequencing outputs such as Roche's 454 Junior (ca 70,000 sequences). However, this problem virtually disappears in NGS platforms where the sequencing output is of millions, e.g. Ion Torrent, Illumina's MiSeq and HiSeq platforms.

Therefore, before the library preparation for Roche's 454 Junior NGS platform, the average number of prey species detected in the diet of bats in molecular studies was checked in the literature (Table 2.1). Then, considering the richness of prey taxa consumed by different bat species in varying geographic locations, we decided a maximum mean number of species that *R. euryale* would likely consume in a single night in the study area: 20 species/individual bat.

We also decided the average number of copies per species-sequence (i.e. sequence coverage) we should obtain to consider the sequencing result reliable: 40 copies/species/bat individual. Thus, we expected to obtain an average of 800 sequences per individual sample in a single sequencing run. Finally, we divided this number by the average sequencing output of Roche's 454 Junior (i.e. 70.000

at the moment of the study) in order to obtain the number of faecal samples that would be reasonable to sequence together in a single run: 87.5 faecal samples.

Table 2.1. Mean, minimum and maximum number of prey taxa (or haplotypes) consumed by bat species in different molecular dietary studies.

Bat Species	Mean prey/sample	Min-Max	Reference
Plecotus macrobullaris	2.75	1-8	Alberdi et al., 2012
Chaerephon pumilus	23.3	1-222	Bohmann et al., 2011
Mops condylurus	15.5	3-46	Bohmann et al., 2011
Lasiurus borealis	3.5	1-7	Clare et al., 2009
Plecotus austriacus	4.2	1-17	Razgour et al., 2011
Plecotus auritus	5.3	1-17	Razgour et al., 2011

In Chapter 3 the library preparation was performed in two consecutive PCR amplifications. For the first PCR amplification the ZBJ-ArtF/R primers were modified with a 454's binding site (17/16 bp Roche's Universal Junior Tail, Fig. 3.6). We obtained miniCOI amplicons with binding sites in both ends (Fig. 2.6). In the second PCR, we re-amplified the amplicons obtained for each faecal sample using a unique combination of MIDs (10 bp) modified with the complementary sequences of 454's binding sites and Roche's key (4 bp) and adaptor sequences (21 bp, Fig. 3.6). The final amplicon (ca 314 bp) for each faecal sample was tagged with a unique combination of MIDs.

The first PCR was performed in 25 μl PCR reaction using the Biotaq PCR kit (Bioline, www.bioline.com). Each reaction contained 16.6 μl deionised water, 2.5 μl Buffer10X, 1 μl $MgCl_2$ 50mM, 0.5 μl BSA 20mg/l, 0.5 μl DMSO, 0.25 μl dNTPs 25mM, 0.75 μl of each primer at 10 μM and 0.16 μl of BIOTAQ DNA polymerase and 2 μl sample DNA. The second PCR was performed in 20 μl PCR reaction using the PCR kit described above. Each reaction contained 2 μl Buffer10X, 1 μl $MgCl_2$, 0.2 μl dNTPs, 2.5 μl of each forward and reverse Multiplex Identifier at 20 μM, 0.2 μl BIOTAQ DNA polymerase and 1 μl DNA from the first PCR product. Thermocycler conditions were: 95°C – 15 min; 50 cycles of 95°C -

30 sec, 52°C – 30 sec, 72°C – 30 sec; 72°C – 10 min. All PCR products were visualized on a 2.5% agarose gel, running at 90V for 60 minutes.

PCR products were pooled into a single group at approximately equimolar ratios based on agarose gel band strength quantified by Quantity One 1-D Software (www.bio-rad.com). The pooled sample was divided into 4 subsamples and purified with the clean-up reaction developed by Rohland and Reich et al. (2012). After purification, the 4 subsamples were pooled together and quantified using Bioanalyzer (Bioanalyzer 2100, Agilent Technologies). The pooled sample was diluted 1:100 in TE-Tween buffer. Emulsion PCR (emPCR) was performed using Roche`s GS Junior Titanium emPCR kit (Lib-A) according to manufacturers instructions. The sample was deep sequenced on Roche`s 454 GS Junior using GS Junior Titanium Sequencing and GS Junior Titanium Pico TiterPlate Kits according to manufacturers instructions. Logistical support was provided by the Molecular Ecology Lab, Doñana Biological Station (CSIC, Seville, Spain).

2.5.1.b Library generation and sequencing with Ion Torrent platform

In the case of Ion Torrent platform, library preparation was performed in a single PCR reaction per faecal sample. The ZBJ-ArtF/R primers were directly extended at the 5' end by the MIDs (10 bp) and Ion Torrent platform's key (4 bp) and adaptor (26) sequences (Fig. 2.6).
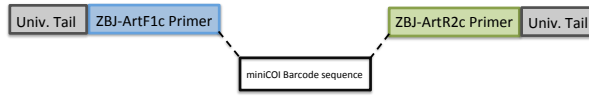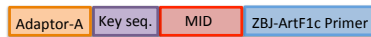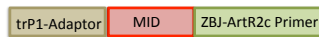
Figure 2.6. Primer designs and library preparation procedure used for the sequencings on a) Roche's 454 Junior - two PCR strategy: in the first PCR the target region miniCOI was amplified using modified ZBJ primers with a Roche's specific binding site (Universal Tail). In the second PCR, the amplicon of the first PCR was re-amplified using primers formed by the complementary of the specific binding sites, MIDs, key sequences and Roche's A and B primer sequences. And, b) Ion Torrent's chip 318 NGS platforms - one PCR strategy: the target region miniCOI was amplified using modified ZBJ primers with MID, key, A and trP1 adaptors.

We completed PCRs in a 20µl reaction contained 10 µL of Qiagen multiplex PCR (Qiagen CA) master mix, 6 µL of water, 1 µL of each 10 µM primer

and 2 μl of DNA. Thermocycler conditions were: 95°C – 15 min; 50 cycles of 95°C - 30 sec, 52°C – 30 sec, 72°C – 30 sec; 72°C – 10 min. We visualized each product on a 2% agarose pre-cast 96 well E-gel (Invitrogen, Life Technologies). We performed product size selection using the PCRClean DX kit (Aline Biosciences). We eluted the product in water and measured the concentration on the Qubit 2.0 spectrophotometer using a Qubit dsDNA HS Assay Kit (Invitrogen, Life Technologies). We normalized the products to 1 ng/μL prior to final library dilution. Sequencing was conducted on the Ion Torrent (Life Technologies, Applied Biosystems) sequencing platform using a 318 chip and following the manufacturers guidelines. Library preparation and sequencing were carried out in the Canadian Centre for DNA barcoding (CCDB, Ontario, Canada), with the assistance of Elizabeth L. Clare.

2.6. Bioinformatics

The analysis was performed following three main stages (for workflow illustration see Fig. 2.7):

(i) Quality control, sequence pre-processing and collapsing of identical sequences into a single sequence were performed using PRINSEQ 0.20.4 (Schmieder and Edwards, 2011), FASTX-Toolkit 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/index.html) and AdapterRemoval (Lindgreen, 2012).

(ii) Clustering of sequences into Molecular Operational Taxonomic Units (MOTU) was carried out with the QIIME pick_otu and uclust methods (Caporaso et al., 2010). Bioinformatic pipelines are attached in the Appendix: Supplementary Material S2.1.

(iii) The taxonomic assignment of each MOTU was performed by comparing the representative sequence of each MOTU against reference sequences in the NCBInr/nt reference database (http://www.ncbi.nlm.nih.gov/) using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Chapter 3) and the Barcode Of Life Database (BOLD; www.boldsystems.org/; Chapter 4 and 5), following the identification criteria of Clare et al. (2013) with some modifications.

Only MOTUs with a sequence similarity higher than 98% with known species from databases were kept. We only considered species known to occur in the Iberian Peninsula or the Atlantic region of France (Redondo et al., 2015; Robineau, 2007). Moreover, when more than one species known to occur in the study area matched the same MOTU, we compared by hand each of the reference sequences downloaded from BOLD or GenBank with the representative sequence of the MOTU to discard any possible matching error. Comparisons were carried out with the program GENEIOUS® v.8.1.7 (www.geneious.com). Then, we also checked the flight phenology of matched species to discard any improbable possibility (e.g. moth species flying in May, consumption occurring in September). Moreover, we checked the occurrence of any ambiguous putative species with the species list published for the study area (Chapter 4), or with our own moth collection formed by light-traps captures (Chapter 5). Any ambiguous matching result was discarded for further analysis.
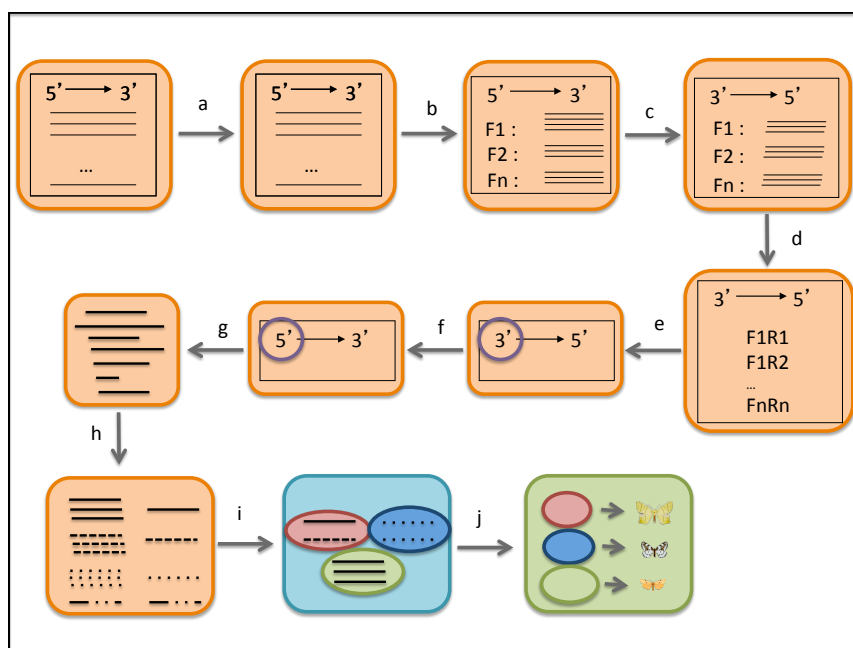
Figure 2.7. Illustration of the bioinformatic workflow. Step (i) in orange, step (ii) in blue and step (iii) in green. a) quality filtering of fastq file and conversion to fasta; b) grouping of sequences according to forward MIDs; c) reverse-complementary of sequences; d) grouping of sequences according to reverse MIDs; e) clipping the reverse primers, MID and adaptor sequences; f) clipping the forward primers, MID and adaptor sequences; g) filtering sequences by

length (156-158bp); h) collapsing identical sequences into unique sequences and removal of singletons*(see explanation below), i) MOTU analysis; j) identification of likely species by comparison to reference databases.

2.6.1. About MOTU clustering and sequence coverage thresholds: Where are the cutoffs?

Since different MOTU-building algorithms could produce varying results from the same data set (e.g. jMOTU or QIIME's pick_otu), and intra- and interspecific variation within the COI region of insect species can vary among consumed taxa, sequences were clustered into MOTUs at different (93–99%) similarity values (Clare et al., 2013). Then, after the taxonomic assignment of the MOTUs at each of the similarity values, we selected those MOTUs created at a similarity threshold where different taxa were not collapsed into the same MOTU—underestimation—nor did they split into more than one MOTU—overestimation.

Another ambiguous threshold used in the literature is the number of copies that a given sequence should have to be included in further analysis. For instance, singletons and doubletons (i.e. sequences with one or two copies, respectively) are automatically discarded in the literature due to their low probability of being "real" sequences (product of PCR or sequencing errors). But, what about tripletons or quadrupletons? Or, are the quadrupletons of a Roche's 454 Junior sequencing dataset, where the total sequencing output is 70,000 sequences, and of an Ion Torrent's chip 318 output, where the total output is > 3,500,000 sequences, equivalent? This is probably not the case.

In order to choose a conservative and standardized threshold among different sequencing platforms, we plotted the number of MOTUs clustered at different sequence-coverage values (1–100 copies/sequence) and the number of MOTUs obtained for each coverage value (Fig 2.8). We observed that there was very little or no apparent loss of identified MOTUs at different sequence-coverage values until the point where the number of MOTUs reach an asymptote.

However, the number of unknowns or rare results decreased with higher coverage values. Thus, we considered that a reasonable sequence-coverage cutoff was the value where the number of MOTUs reached an asymptote: 4 for Roche's 454 sequencing data (Chapter 3) and 10-15 for Ion Torrent's sequencing data (Chapter 4 and 5). Only MOTUs clustered with sequences containing more than 4 or 15 copies, respectively, were used for further analysis. We considered MOTUs built below these cutoffs as non-reliable, probably originating from sequencing errors that might introduce false positive taxa assignment. By taking this conservative approach we aimed to minimize potential noise from further ecological analysis, although we are aware we might be missing rare species.
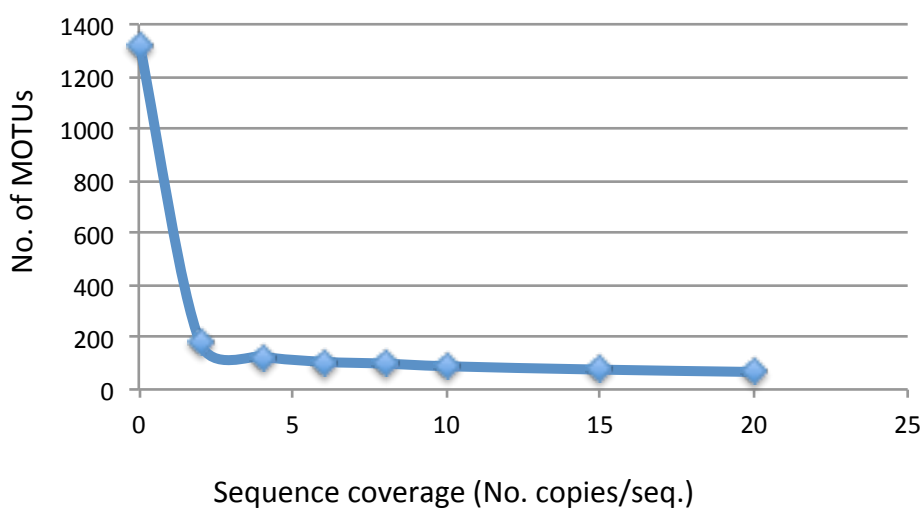
Figure 2.8. Graph showing the number of MOTUs (Y axis) clustered at different sequence coverage values (copies/sequence; X axis). Sequences were clustered into MOTUs at 97% similarity threshold. Illustrated data was obtained with Roche's 454 Junior sequencing platform.

2.7. Limitations and considerations of DNA metabarcoding

Each of the steps described above has some methodological problems that should be considered when applying DNA metabarcoding to diet analyses. These include, among others, DNA extraction bias, PCR inhibitions, PCR introduced errors, sequencing errors, the arbitrariness of bioinformatic filtering thresholds, the incompleteness of reference databases, etc. Moreover, the main

limitation of DNA metabarcoding for diet analyses is perhaps the impossibility to quantify DNA (Clare, 2014; Deagle et al., 2013; Elbrecht and Leese, 2015; Pompanon et al., 2012). The sequence number is not proportional to the biomass of consumed prey (reasons are reviewed in Clare, 2014). Thus, it is not possible to estimate the abundance or the volume of each of the consumed prey taxa in a single fecal sample. Dietary results are limited to interpreting presence/absence data. Therefore, we were limited to semi-quantitatively measuring the frequency of prey taxa across the analyzed samples. This should be considered when reading and interpreting the dietary results of this thesis.

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CHAPTER 3

Trophic Requirements Beyond Foraging Habitats: the Importance of Prey Source Habitats in Bat Conservation
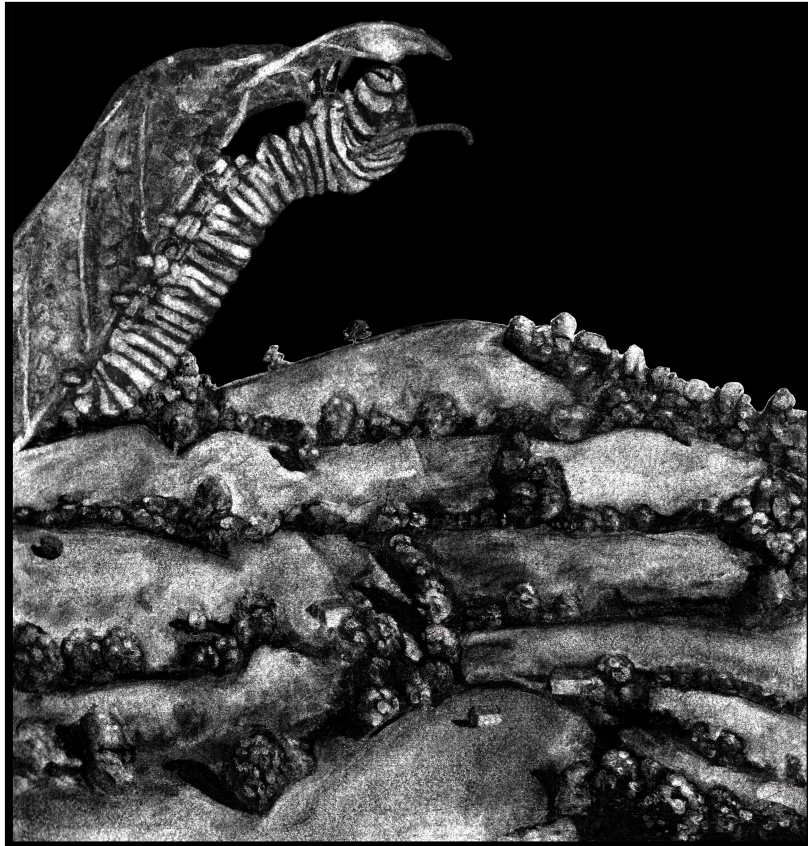
Illustration by Maite Muro.

An article on this Chapter was published in Biological Conservation:

Abstract

Conservation efforts for endangered animals commonly focus on the protection of foraging habitats, aiming to ensure sufficient food availability. However, the diet of many species is based on animals that undergo habitat shifts across ontogenetic life stages, yielding considerable differences between the lifelong habitat requirements of both predator and prey. Consequently, prey availability may not only depend on the suitability of the foraging grounds where predator and prey coincide, but also on habitats where the ecological requirements of the non-prey stages are fulfilled. In this study we test to what extent prey of the insectivorous bat *Rhinolophus euryale* originate either from the grounds where they are consumed, or in areas/habitats outside the bat's foraging sites. We analyzed the diet of *R. euryale*, by identifying its prey to the species level using DNA metabarcoding, and by searching for its prey's larval feeding requirements in the literature. We found that the larvae of the moth prey grow both inside and outside the grounds where they are hunted by the bats once the moths reach their adult stage. The importance of prey that originated from outside the bat's foraging grounds varied considerably across seasons. As a result, *R. euryale* does not only rely on the landscape elements where it hunts, but also on other source areas/habitats that supply it with food. This study shows that conservation measures that aim to address the foraging requirements of predatory species should not be limited to merely protecting their foraging grounds, but should also take into account the ecological requirements of their prey throughout their life stages.

Keywords

ontogenetic habitat shift, trophic requirements, predator-prey interactions, DNA metabarcoding, landscape

3.1. Introduction

Ensuring prey availability and suitable foraging areas are key factors in the successful conservation of endangered species (Sinclair et al., 2006). As such, they are two of the main topics addressed in conservation scientific studies (e.g. Agosta, 2002; Fenton, 1997; Shuterland and Green, 2004; Russo and Jones, 2003). It is generally assumed that by conserving foraging areas, prey availability is also ensured. However, this assumption is not adequate when the ecological needs of the prey exceed the foraging ground of the predator. Furthermore, predator-prey interactions and food web studies are commonly defined in terms of fixed communities, despite the temporal and spatial heterogeneity of trophic relationships (Miller and Rudolf, 2011; Polis and Strong, 1996). Niche shifts across ontogenetic life stages are commonplace in animals with complex life cycles (Rudolf and Lafferty, 2011; Rudolf and Rasmussen, 2013), so the lifelong habitat requirements of predators and prey may differ considerably despite the fact that they need to coincide in time and space (Ryall and Fahrig, 2006).

Holometabolous insects are one of the main exponents of ontogenetic habitat shifts, owing to the sheer difference in requirements of larvae and imagos (Gullan and Cranston, 2000; Miller and Rudolf, 2011). Holometabolous insects such as lepidopterans, coleopterans and dipterans are the main prey of many insectivore vertebrates at different stages of their life cycle, including, caterpillars for birds (Barbaro and Battisti, 2011; Busby and Sealy, 1979; Hogstad, 1988), moths for bats (Dietz et al., 2009), and both larvae and imago for rodents and lizards (Bellows et al., 1982; Brown et al., 2014). Consequently, insectivores' prey availability may not only depend on the suitability of the grounds where predators and their insect prey forage, but also on habitats and areas where the ecological requirements of the non-prey stages are fulfilled, i.e. the places and habitats where the larvae that will become prey at the adult stage develop. Any change in these habitats can alter population source-sink dynamics of the prey (Pulliam 1988; Schreiber and Rudolf, 2008). In addition, the predator-prey interactions could also be affected, leading to changes in ecosystem structure and processes (Rudolf and Rasmussen, 2013).

To date the ecological requirements of prey have not been investigated. This is most likely due to the lack of species-level information on the consumed prey, especially in the case of insectivorous and elusive animals. Visual analyses of stomach and fecal contents have seldom provided taxonomic resolution beyond the order or family level (Whitaker et al., 2009). However, the implementation of molecular tools for diet analysis has triggered an important step forward in the last few years (Clare, 2014; Pompanon et al., 2011). The species-level identification of prey items provided by molecular tools has allowed researchers to unveil ecological information hidden in the food items. For instance, Alberdi et al. (2012) inferred foraging habitats based on consumed species, Clare et al. (2013) used dietary information to assess the quality of aquatic habitats, and McCraken et al. (2012) reported bats foraging on and tracking pest moths on a regional scale. Accordingly, we are now able to broaden the scope of conservation studies, to go in more depth into prey-predator relationships, as well as to assess the finer ecological requirements of prey species.

Semi-natural landscapes, created by traditional land use and composed of grasslands, hedgerows and forest patches, are of paramount importance for the conservation of many elusive vertebrate and invertebrate species. These include birds, rodents, bats, butterflies and moths that interact as predators and prey (Dover and Sparks, 2000; Marshall and Moonen, 2002; Merckx et al., 2012; Millán de la Peña et al., 2003; Tscharntke et al., 2008; Slade et al., 2013). In particular, this mixture of vegetation structures enhances foraging opportunities for the Mediterranean Horseshoe bat (*Rhinolophus euryale*, Blasius 1853; Goiti et al., 2008; Hutson et al. 2008), a moth-specialist bat with declining populations throughout the Mediterranean Basin (Andreas et al., 2012; Hutson et al. 2008). Changes in agriculture and land use policies have led to the alteration of this landscape type (EEA, 2005), resulting, for example, in the decline of many bird and lepidopteran species (EEA 2005, 2013; SEO/BirdLife 2014; Söderström et al., 2001). We argue that predators such as the Mediterranean Horseshoe bat may not only lose foraging grounds (as well as nesting sites in birds) through

direct removal of hedgerows or woodland patches. They may also be affected by the transformation of *non-used* landscape elements that act as prey-source habitats that are essential for the other life-stages of their insect prey. As such, the extent to which a habitat- and prey-specialist predator is dependent on the habitat requirements of the non-prey stages of consumed prey has direct implications for conservation. For instance, conservation guidelines for *R. euryale*—and other bat species— have so far focused mainly on the conservation of their feeding and roosting areas (Eurobats, 2014), under the assumption that these portions of the landscape fulfill the functional needs of the species. However, the precise ecological requirements of the consumed prey throughout their entire life cycle, and thus the implications for the foraging requirements of *R. euryale*, remain unknown.

In particular, we aim to test whether the foraging habitats of an insectivorous bat also cover the habitat requirements of the other life-stages of the consumed prey. Or whether the prey require sites outside the foraging range of bats to complete their lifecycle, which should therefore be considered as part of the predators' foraging requirements (both spatial and ecological) in order to achieve effective conservation management. Considering the ontogenetic niche shift of insects, adult prey's flying behavior, and the high level of landscape heterogeneity where *R. euryale* inhabits, we predicted that the habitat needs of consumed prey are not fulfilled by the ecological characteristics found in the foraging grounds of bats. The entire landscape could be acting as a prey source, where the relevance of different habitats would temporally and spatially vary due to larvae-host plant specificity and phenology. This study aims to gain insight into the complex predator-prey relationships between bats and insects. It also advocates a global vision that encompasses elements beyond first-level relationships for the conservation of threatened species.

3.2. Material and Methods

3.2.1. Study Area, Sample Collection and Ethics Statement

Bats were captured in one of the largest known breeding colonies of *R. euryale* during May, July and September of 2012, coinciding with *R. euryale*'s pre-breeding, breeding and post-breeding seasons respectively. Details about the study area, bat-capture procedure and ethics statement are described in Chapter 2.

3.2.2. DNA extraction, PCR amplification, sequencing and bioinformatic analysis

DNA was extracted, PCR amplified and sequenced from 20 individual bat fecal samples per season. Details about the laboratory procedures and bioinformatics are explained in Chapter 2.

3.2.3. Foraging requirements of prey at larval stage

We searched for the host plants of the caterpillars of identified prey moth species in the HOSTS database (Natural History Museum, London; Robinson et al. 2010) and elsewhere (Robineau, 2007; Waring and Townsend, 2003; Sterling and Parsons, 2012). The same sources were used to compile information about the migratory and pest status of moth species. We then created a database with the feeding host plants of moth species present in the following vegetation types: deciduous woodland, hedgerow, forest edge, meadows, pastures, shrubland, holm oak forest and exotic plantations (www.sivim.info). Concurrently, since the feeding sites of *R. euryale* occur in deciduous woodlands, hedgerows and forest edges, we classed these vegetation types as *hunting grounds* (Goiti et al., 2008). The remaining vegetation types were classed as *non-hunting grounds.* Next, we classified the caterpillars of the moths observed in the bat's faeces as follows:

- Within hunting grounds: >60% of the caterpillar host plants appear in deciduous woodlands, hedgerows and forest edges in our study area.

- Non-hunting open grounds: >60% of the host plants appear in meadows, pastures, shrubland and other open areas.

- Non-hunting clutter grounds: >60% of the recorded host plants appear in holm oak forest and exotic plantations.

- Ubiquitous: where none of the previous criteria are fulfilled.

Host plants were classified into herbaceous (forbs and graminoids), shrub, broadleaved tree, coniferous tree, and non-plant category (e.g. fungi, mosses, insects, leaf-litter). A given feeding guild category was assigned if >60% of the host plants consumed by a given moth species corresponded to that category. If the previous criterion was not fulfilled with any plant category the caterpillar was classed as a generalist feeder.

3.2.4. Diet analysis

We compared the prey composition in *R. euryale*'s diet at the ordinal level between different seasons using the nonparametric Kruskal-Wallis test. Association between the observed dietary composition and seasons was tested by Pearson's Chi-squared test and visualized in an association plot using the package vcd for R 3.0.2 (Meyer et al., 2006, 2014; Zeileis et al., 2007). The significance level of the test was set at p<0.05. Statistical analyses were performed using R 3.0.2 (R Development Core Team. 2008).

The percentage of occurrence of each prey type in the diet of the studied bat population was calculated as the number of bats from which such prey type was identified, divided by the total number of bats examined, and multiplied by 100 (McAney et al., 1991; Whitaker, 2009).

3.3. Results

PCR amplicons were obtained from 19 of the 20 extracted individual bat fecal samples collected per season. One of the samples from the breeding season

was excluded from subsequent analysis because it contained sequences with less than 4 copies. Overall, we obtained 126 MOTUs from the 56 analyzed fecal samples: 64 MOTUs in the 19 samples from the Pre-breeding season, 59 MOTUs in the 18 samples from Breeding, and 35 MOTUs in the 19 samples from the Post-breeding season. The number of MOTUs per individual bat ranged between 1 and 21, with a mean value of 5.5 (SD ± 3.88).

3.3.1. Diet composition

In total, we identified 97 of the 126 MOTUs to the species level (77%), 13 to genus (10.3%), 2 to family (1.6%), and 2 to order (1.6%) (Appendix S3). The remaining 12 did not match any reference sequence (9.5%) and were classified as "unknown". Most of the MOTUs were classified as Lepidoptera (84.9%), only 4.0% and 1.6% were assigned to Neuroptera and Diptera respectively (Fig. S3.1 in supplementary material S3). The following species were assigned to more than one MOTU: *Thyatira batis*, *Alcis repandata*, *Melanthia procellata*, *Mythimna albipuncta*, *Mythimna unipuncta*, *Xestia c-nigrum* and *Pseudoips prasinana*.

The diet composition of *R. euryale* at the ordinal level did not differ between seasons (Kruskal-Wallis H=2, df=2, p=0.3). The diet consisted primarily of Lepidoptera, accounting for 85% of the total MOTUs for all seasons. The seasonal diet of *R. euryale* is summarized in the supplementary material S3 (Figure S3.1).

The majority of the identified lepidopteran species belonged to the families Geometridae and Noctuidae (Fig. S3.1 and Table S3.1, supplementary material). The highest percentage of occurrence values were reported for the geometrids *Alcis repandata*, *Cyclophora* sp., *Idaea* sp., *Peribatodes rhomboidaria*, *Petrophora chlorosata*, *Xanthorhoe ferrugata* and the noctuids *Agrotis exclamationis*, *Cosmia trapezina, Lycophotia porphyrea, Hoplodrina ambigua, Mythimna unipuncta, Ochropleura plecta, Photedes minima* and *Xestia c-nigrum.* Some families appeared only seasonally, such as Nolidae (*Pseudoips prasinana*) during the pre-breeding season, Crambidae in the breeding season and

Drepanidae (*Thyatira batis*) during both pre- and breeding seasons. The seasonal diet of *R. euryale* at the family level within Lepidoptera is summarized in the supplementary material S3 (Figure S3.1).

All consumed dipterans belonged to the family Tipulidae, and no dipterans were detected in the breeding season. Conversely, neuropterans mostly appeared during the breeding season, and all of them belonged to the families Chrysopidae or Hemerobiidae.

3.2. Foraging requirements of prey at larval stage

We were not able to associate 3 MOTUs from the breeding season to any host plant category due to the low resolution of the identification level (family and order level). In addition, one MOTU matched *Apatema apolusticum,* but we were unable to find any information about the species' ecology. These 4 MOTUs were detected in the feces of one single individual bat and were excluded from further analyses. One moth species consumed by one bat (*Thaumetopoea pytiocampa*) was classified under "Non-hunting clutter grounds" (Table S3.1, supplementary material), and was excluded from the Pearson's Chi square χ2 test.

Moths whose larval host plants occurred in non-foraging open grounds were commonly observed in the diet of the surveyed population, both as a whole and seasonally (Figure 3.1.b, Table S3.2). More than 85% of the bats preyed on moths with larvae that feed on herbaceous plants belonging to non-foraging open grounds (Figure 3.1). Furthermore, the number of bats preying on moths originating in vegetation units used by bats as foraging grounds was 84%, 72% and 42% for pre-breeding, breeding and post-breeding seasons respectively (Figure 3.1a). These moths were predominantly broadleaved tree feeders (Figure 3.1b). Half of the surveyed bats preyed on moths with ubiquitous caterpillars that grow on plants from both within and outside of *R. euryale*'s foraging grounds (ubiquitous larvae, Figure 3.1b) during the pre-breeding and breeding seasons, and only 2 bats (10%) in the post-breeding season. One bat in

the post-breeding season preyed on the one moth reported to feed solely on conifers (non-foraging clutter grounds): the Pine Processionary *Thaumetopoea pityocampa.*

a)

b)
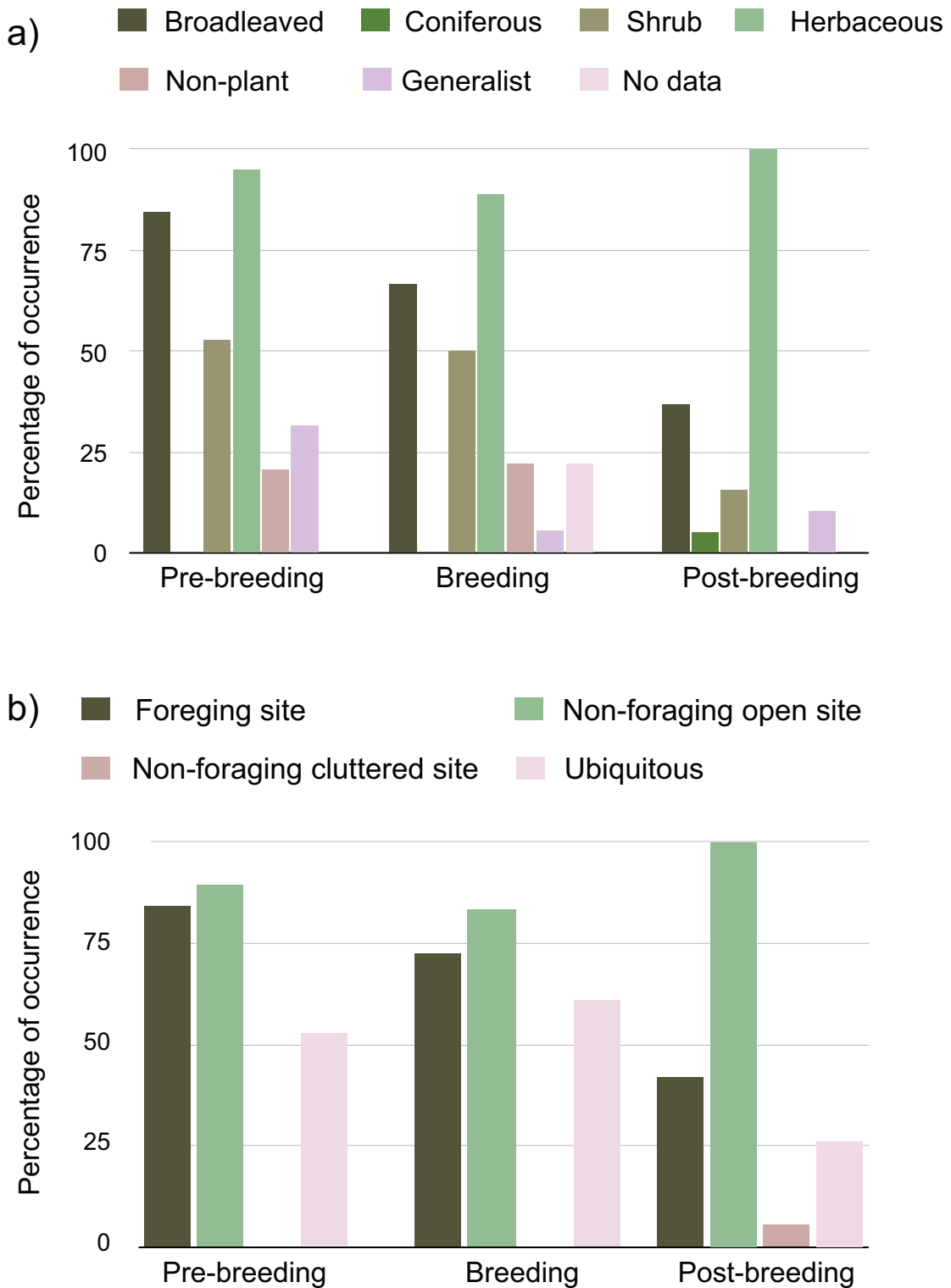
Figure 3.1. Percentage of occurrence of prey taxa (a) categorized in accordance to their host plant life-form, and (b) categorized in accordance to their host

plants location in relation to *R. euryale*'s foraging grounds during the bat's pre-breeding, breeding and post-breeding seasons.

There is a significant relation between season and both the feeding guild of the moth larvae (Chi square: χ^2 = 28.85; df = 8; p < 0.001; Figure 3.2b) and the host plant location in respect to *R. euryale*'s foraging grounds (Chi square: χ^2 = 21.53; df = 4; p < 0.001; Figure 3.2a). There was a significant decrease in the consumption of moths that originated from *R. euryale*'s foraging grounds from pre- to post-breeding seasons, and a corresponding opposite trend of moths feeding on herbaceous plants likely to be located in the bats' non-foraging grounds. This was caused mainly by a reduction in the consumption of broadleaved tree and shrub feeder moths. In the pre-breeding season bats consumed more moths with larval requirements linked to the bat's foraging grounds (broadleaved tree and shrub species) than would be expected if no association existed between moth classes and seasons. In the breeding season bats foraged slightly more than expected on moths linked to shrubs, whilst the opposite trend was observed in the post-breeding season. Few of the consumed prey were assigned as non-plant or generalist feeders, and a weak non-significant relation was observed between the consumption of generalist or ubiquitous moths and seasons (Figure 3.2).
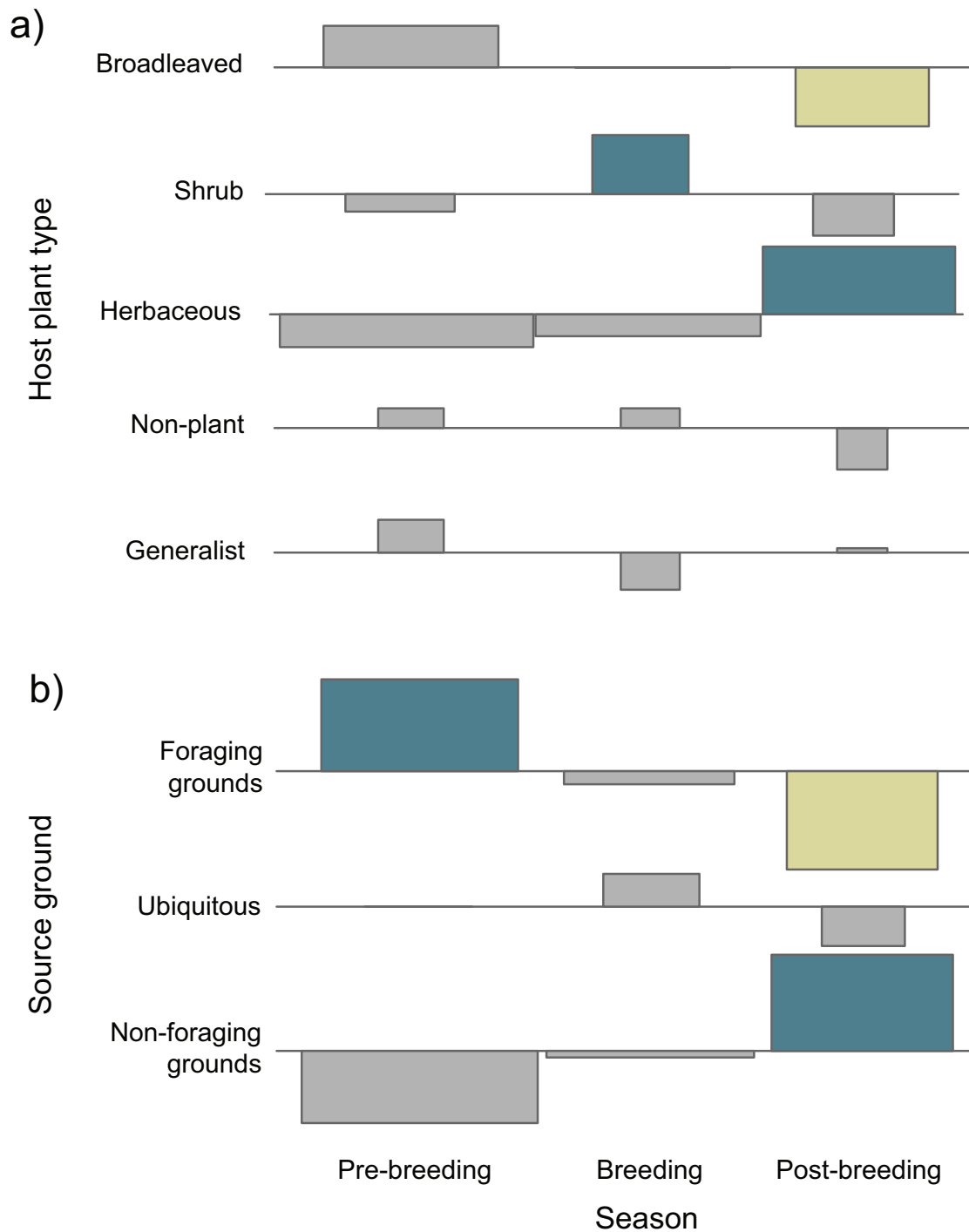
Figure 3.2. Association plots showing the standardized deviation of the observed consumption of moth prey items from those expected throughout seasons, and under the null hypothesis of independence. Each cell is represented by a box with height proportional to the standardized difference between observed and expected consumption counts (Pearson's Residuals, refer to Zeileis et al., 2007), and width proportional to the squared root of the expected consumption counts. The area of the box is proportional to the deviation between observed and expected counts. Colored boxes indicate a standardized significant deviation

from expected greater than 2 (Zeileis et al., 2007). Boxes above the horizontal line indicate greater than expected observed counts (in blue: significant deviation), whereas boxes below the line indicate lower than expected counts (in yellow: significant deviation). (a) Identified seasonal prey consumption according to the moth's caterpillar host plant life-form. (b) Identified seasonal prey consumption according to *R. euryale*'s foraging grounds.

3.4. Discussion

We found that the larval host plants of a significant proportion of the moth prey occur outside the habitats where the adults are hunted by bats. These findings highlight the relevance of taking into account the feeding and habitat requirements of prey through different life stages in order to fully understand the foraging requirements of bats and other predators consuming prey with complex life cycles. To the best of our knowledge, our study is the first to track down the prey consumed by an insectivorous bat to their source habitats. We believe our results provide relevant information for researchers and land managers working on the conservation of predators linked to species with ontogenetic niche shifts.

3.4.1. Diet composition

Lepidopterans matched 85% of the identified MOTUs, in line with results from other authors that relied on morphological diet analysis (Goiti et al., 2008, 2004; Mikova et al., 2013; Salsamendi et al., 2012), confirming that *R. euryale* is a moth specialist. Among the 11 moth families identified in our study, 7 belong to the so-called group of macro-moths (Waring and Townsend, 2003), and comprised more than 90% of the consumed moth species (Fig. S3.1 and Table S3.1, supplementary material). The most frequently consumed moths belong to the Noctuidae and Geometridae families. These two families are the largest in terms of number of species (Wating and Townsend, 2004), and probably the most common and abundant macro-moths in our study area. Similar findings

have been reported in other temperate regions (Schoeman and Jacobs, 2003; Wickramasinghe et al., 2004). Thus, the staple diet of *R. euryale* consists of medium-sized macro-moths.

3.4.2. Advantages and limitations of molecular diet analysis

This study has only been possible due to the high-level taxonomic identification that current molecular diet analysis techniques provide (Pompanon et al., 2011; Clare, 2014). Despite this, the novel molecular approach by no means provides a panacea for diet studies (discussed in: Bohmann et al., 2011; Boyer et al., 2013; Brown et al., 2012; Clare, 2014; Pompanon et al., 2011; Razgour et al., 2011). When dealing with threatened species and aiming to detect trophic interactions, special considerations should be taken in the analytical steps where false positive prey identifications or dietary over/underestimation might arise. In this regard, we adopted a conservative approach at the amplicon coverage threshold (likely related to false positive identifications) and MOTU clustering (related to dietary over/underestimation) steps. Although this method may exclude rare prey species from the analysis, such species most likely have little biological relevance in terms of the bats' energy-intake. We did not overcome dietary overestimation (Alberdi et al., 2012; Razgour et al., 2011). As Razgour et al. (2011) pointed out, the observed overestimation can be related to intraspecific polymorphisms in the 157 bp miniCOI (Valentini et al., 2009), taxonomic ambiguity among some Lepidoptera, or due to an incomplete reference database. Despite the need for further improvement in the analytical steps of molecular diet studies (Clare, 2014), results can be combined with existing biological information about the consumed insect prey. This combination enables a better understanding of the food web structure and dynamics, so that effective management guidelines can be proposed.

3.4.3. Foraging requirements of the predator and prey

R. euryale consumed moths that require ecosystem elements beyond the habitats where they are captured by the bat. It forages against clutter in

hedgerows and broadleaved forests, with no foraging activity being reported over pastures or meadows (Aihartza et al., 2003; Goiti et al., 2008; Russo et al., 2002, 2005; Salsamendi et al., 2012). However, prey consumed in the bat's foraging grounds depend on host plants that are found both in the bats' foraging and non-foraging habitats for their larval development. For instance, most of the consumed moths require plant species typically growing in pastures and meadows. A small number of these plant species might also grow in other woody habitats. However, in our study area the biomass of such plants in pastures and meadows is considerably larger than in any other woody habitat. We identified a total of 35 plant families that were likely to occur in non-foraging open habitats. Among them Asteraceae, Gramineae, Leguminosae and Polygonaceae accounted for 48% of the recorded larval host plants (Table S3.2). Consequently, a large number of moths consumed by *R. euryale* most likely emerged from outside the bat's foraging areas.

Therefore, a movement process occurs from the areas where the prey emerges from to the grounds where bats hunt them. Like bats, moths are flying animals that can move easily through the landscape from their emergence areas. These movements might vary considerably across taxa and time due to dispersal abilities, as well as the trophic needs and phenology of moths (Betzholtz and Franzen, 2011; Fuentes-Montemayor et al., 2012; Merckx et al., 2009; Murakami et al., 2007; Slade et al., 2013). This yields a prey input for bats that varies in ecological and spatial requirements through time. Our data suggest that such prey input dynamics occur in our study area. Moths with larval requirements linked to trees and shrubs (mainly geometrids) were more abundant in the diet of *R. euryale* during the pre- and breeding season. In contrast, during the post-breeding season the majority of the consumed moths relied on herbaceous plants from open habitats. Slade et al. (2013) showed that different functional groups of moths require different degrees of landscape connectivity. For instance, some taxa with herb-feeding larvae and herb-feeding or non-feeding adults move shorter distances than species feeding on trees and shrubs (Slade et al., 2013). Consequently, *R. euryale*, along with many bat and bird species preying on moths (Barbaro and Battisti, 2011; Dietz et al., 2009), relies on prey

with a varying degree of movement patterns and spatial and functional requirements of the landscape through time.

Moreover, some habitats outside the bat's foraging range may also be acting as prey sources, trophically linking *R. euryale* to distant areas, as nine of the prey moths are known to be either very mobile or migratory species (Table S3.1). These species are reported to fly large distances at the landscape, country or even continental scale (Chapman et al., 2010; Slade et al., 2013). This would imply that *R. euryale* does not only rely on the landscape elements within its home-range, but also on other distant areas that supply them with food. Some migratory food elements can be seasonally important for many predators, as has been reported for other bat species, such as *Nyctalus lasiopterus* that prey on migratory passerine birds (Ibañez et al., 2001). At an even larger geographical scale, linking different systems, the brown bear preys on salmon that returns from the sea (Hilderbrand et al., 2004). Since the level of convergence of prey source and predator's hunting areas is variable, the higher the diversity of the landscape, the higher the bat's chances of fulfilling its foraging requirements.

In addition, some moth species showed temporal peaks of consumption (>%20 occurrence values, Table S3.1), which may correspond to their sudden arrival or emergence in the bat's foraging grounds (e.g. mass-emerging species). Some authors reported bats consuming large amounts of mass-emerging insect species such as lepidopterans, coleopterans, trichopterans or ephemeropterans (Goiti et al., 2004; Clare et al., 2011; McCracken et al., 2012; Vesterinen et al., 2013). These *arrivals* may play an important role in the bat's energy intake and can vary in length and intensity, both among moth species and within the same species. Furthermore, some of the consumed taxa are potential crop pests (e.g. *Agrotis* sp. in crop plants and seedlings, *Autographa gamma* and *Mythimna unipuncta* in several crop plants such as hay and barley, *Thaumetopoea pityocampa* in pine plantations; Carter, 1984), suggesting that *R. euryale* could be an effective pest consumer if insect population booms were to occur, as has been postulated for other bat species (Cherico et al., 2014; McCracken et al., 2012).

Despite the fact that most of the consumed moths showed a variety of larval feeding requirements with a variety of potential source habitats, *R. euryale* probably encounters them in hedgerows, forest edges or isolated trees, regardless of the season (Goiti et al., 2008). Moths of different functional groups may be using such linear elements as landmarks for dispersal (Slade et al., 2013), or for other purposes such as shelter or protection from predators, forming prey-rich areas for bats. As McCraken et al. (2012) reported, bats are able to track and exploit local prey abundance. Similarly, *R. euryale* might identify and exploit such prey-rich spots. Therefore, our results raise interesting questions regarding the foraging ecology of *R. euryale*. For example, what is constraining *R. euryale* to forage in edge habitats? Are bats' echo-morphological characteristics limiting them to forage in specific habitats due to a better prey detectability and capturing effectiveness? Are bats selecting habitats richer in prey availability?

3.4.4. Implications for conservation

We are aware of the limitations of the study of a single bat colony (e.g. specific landscape, colony-related behavior, colony size; Whitaker et al., 2009). Nonetheless, the trophic ecology and the foraging behavior of the studied colony, researched thoroughly over a 12-year period (Goiti et al., 2003, 2004, 2006, 2008), is similar to other colonies of *R. euryale*. In these colonies, moths were the main prey, and wooded structures such as hedgerows, broadleaved forest and forest edges were identified as the specific foraging sites (Andreas et al., 2012; Koselj and Krystufek, 1999; Russo et al., 2002, 2005; Salsamendi et al., 2012). We believe that the observed results may represent the diet and the foraging behavior of other *R. euryale*'s population living in similar landscapes. Therefore, in landscapes with a high patchiness, non-foraging grounds may also be essential for providing the diversity and, likely, the abundance of prey needed to sustain rich hunting grounds.

Management guidelines and conservation recommendations for *R. euryale*, as for many other European rhinolophid and vespertilionid bat species, have focused on protecting their roosts and foraging grounds (e.g. Eurobats,

2014; Goiti et al., 2006, 2008; Schofield, 2008). *R. euryale* is highly dependent upon caves for roosting (Dietz et al., 2009), and woodland/hedgerow edge-structures are of paramount importance for its trophic ecology (Goiti et al., 2008; Salsamendi et al., 2012). We advocate the inclusion of a third element, namely the source habitats of its prey, as an essential factor to be taken into account to ensure the conservation of this and other similar threatened species (Hutson et al., 2008). Several bat species feed on prey with varying habitat requirements throughout their lifespan, such as *Plecotus* sp. on moths (Alberdi et al., 2012; Razgour et al., 2011), *Myotis myotis* on coleoptera (Arlettaz, 1996), *M. lucifugus* on prey emerging from water habitats (Clare et al., 2011), *Trachops cirrhous* on frogs (Ryan et al., 1982). Other species hunt migratory prey originating in source habitats beyond the bats' home-range, such as *Tadarida brasiliensis* on migratory pest moths (McCracken et al., 2012), and *N. lasiopterus* on migratory passerine birds (Ibañez et al., 2001). This new perspective identifies as a risk factor any intensification or change in the land use that alters the habitats required by the prey at any life-stage or lifespan moment, even when the hunting grounds of the bats remain untouched.

Similar to the majority of bats, many birds and small vertebrates in terrestrial systems (as well as many predators in other systems), are trophically linked to prey with complex life-cycles (e.g. in marine environments: predators foraging on species with pelagic or benthonic larvae or adults; for example the leatherback turtle on jellyfish). As our results show, predator and prey overlapped in a small proportion of their niche-space: in habitats where predation occurs. However, the conservation of these foraging habitats does not ensure that the trophic requirements of the prey are included, as prey might rely on a wider variety of landscape elements during their lifespan. Therefore, conservation efforts addressing the foraging requirements of a given species should not be limited to merely protecting its foraging grounds, but guidelines should also take into account the ecological requirements of prey throughout their lifecycle. Any changes to habitats required by the rest of the life-stages of prey could affect not only source-sink dynamics of prey populations (Pulliam 1988; Schreiber and Rudolf, 2008), but the predator-prey interactions as well,

leading to changes in ecosystem structure and processes (Rudolf and Rasmussen, 2013). When developing conservation measures for insectivorous species inhabiting mosaic-like heterogeneous landscapes, we advocate for a landscape-level management rather than focusing on the habitat-level. This is in line with what several studies suggested for different taxa inhabiting heterogeneous landscapes (e.g. Dover and Sparks, 2000; Fuentes-Montemayor et al., 2012; Law and Dickman, 1998; Marshall and Moonen, 2002).

In this particular case, the preservation of the traditional farmland landscape could be enough to ensure resource availability, because a bocage landscape (Baudry et al., 2000) provides all the functional and structural elements required by both prey and predator. These semi-natural landscapes are of paramount importance for the conservation of many vertebrate and also invertebrate species (Dover and Sparks, 2000; Marshall and Moonen, 2002; Merckx et al., 2012; Millán de la Peña et al., 2003; Tscharntke et al., 2008; Slade et al., 2013). However, European agricultural policies have led to the decline of hedgerows and grasslands (EEA 2005), which has resulted in the decline of many bird and lepidopteran species across Europe (EEA 2005, 2013; SEO/BirdLife 2014; Söderström et al., 2001). Similarly, the substitution of meadows by exotic tree monocultures (which is slowly taking place in the study area), or even pasture abandonment, are likely to affect moth taxonomical and functional diversity (Kadlec et al., 2009; Merckx et al., 2012; Pavlikova and Konvicka, 2011; Slade et al., 2013). They are therefore likely to directly affect prey availability for *R. euryale*, especially during the post-breeding season in late summer, when most of the prey's larval stages are strongly associated with grassland plant species.

In summary, our results show that the ecological requirements of *R. euryale* often go beyond the habitats where it interacts with its moth prey. These findings could be achieved because species-level identification of prey is now possible through DNA metabarcoding, alongside the extensive literature gathered about moths and their larvae. The combination of these two powerful resources open the door to a more in- depth study of the relationship between

bats' foraging grounds and their prey source, and to identify hitherto overlooked ecological requirements. Beyond the well-known motto "think globally, act locally", our findings suggest that when aiming to conserve predator species that inhabit heterogeneous landscapes and are linked to prey with ontogenetic habitat shifts, to succeed locally we will have to act on a broader scale, even regionally. This broader-scale approach must be taken into account for the development of effective management and conservation measures.

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CHAPTER 4

Unveiling the niche partitioning of sibling horseshoe bats by DNA metabarcoding

Illustration by Maite Muro.

Abstract

Niche partitioning is an important mechanism facilitating the coexistence of species. In a foraging context, partitioning may occur by differences at three main niche dimensions: dietary, spatial and temporal dimensions. Although several sympatric carnivore and herbivore species clearly partition trophic resources, this is not clear for insectivorous bats. In this study we used DNA metabarcoding to measure the diet breath, composition and overlap of sympatric populations of the sibling *R. euryale* and *R. mehelyi,* for which habitat use, hunting behavior, coarse-grained diet, morphology and echolocation call parameters had been previously and simultaneously analyzed. Their dietary niche dimensions overlapped considerably due to the consumption of the same common prey species. Although we observed some small but significant dietary differences, they corresponded to some habitat-specialist moths, reflecting the different use of space (i.e. habitat) by *R. euryale* and *R. mehelyi*. Based on our results and the spatial niche displacement directly measured for allopatric and sympatric populations of *R. euryale* and *R. mehelyi* in previous studies, the coexistence of this pair of sibling bats is mainly mediated by the partitioning of the spatial niche dimension. To our knowledge, this is the first study combining species-level diet analysis and homing-in radio-tracking data of the same population of sympatric sibling bats. Despite potential shortcomings, the high resolution of these techniques allowed us to scrutinise the differences between their foraging niches. This highlighted the relevance of the spatial dimension and common prey species for the coexistence of sibling horseshoe bats.

Keywords: *Sibling species, niche, coexistence, common prey species, spatial segregation*

4.1. Introduction

Foraging resource partitioning may occur by differences at three main niche dimensions in animals (Pianka, 1974): dietary dimension (prey), spatial dimension (foraging area) and temporal dimension (foraging time), creating a complex n-dimensional niche space. Many animal communities show patterns of niche structure, where they differ in parts of at least one dimension. These differences are often accompanied by phenotypic divergence (e.g. feeding-related organ morphology, physiology, behavior). For instance, carnivores belonging to several families across different geographic regions show a marked structure related to carnassial tooth length, associated with food processing (i.e. dietary dimension; Davies et al. 2007). Similarly, large mammalian herbivores in Africa show fine-grained dietary partitioning even within guild members (Kartzinel et al. 2015). In contrast, there is little evidence of structure in the dietary niche dimension of many coexisting and eco-morphologically similar taxa, especially small insectivorous animals such as birds and bats (Loyn, 2002; Schoeman and Jacobs, 2011).

In a foraging context, bats form structurally (i.e. specie number) and functionally (i.e. feeding behavior) relevant assemblages in many ecosystems (Altringham, 1996; Kunz et al., 2011), where several ecologically similar or even cryptic species coexist (Clare, 2011; Clare et al., 2011a). The detailed analysis of the foraging ecology and behavior of entire assemblages, or even guilds, is extremely difficult due to the number of species and the methodological limitations involved. Hence, the mechanisms allowing their coexistence are still a classic topic of debate among scientists (Patterson et al., 2003). Recent dietary analyses based on DNA metabarcoding have found low values of dietary resource partitioning between eco-morphologically similar, or simply sympatric insectivorous species (Krüger et al., 2014; Salinas-Ramos et al. 2015; Sedlock et al., 2014, but see Burgar et al., 2014), suggesting that food partitioning is not an important factor facilitating their coexistence. Yet, these and other studies suggest that the foraging resource partitioning between similar bats is mainly determined by the differentiation of the temporal (Emrich et al. 2011) or spatial

(Razgour et al., 2011) niche dimensions. However, the most accurate picture about evolutionary niche separation is shown by sympatric sibling species (Arlettaz, 1999), which might be seen as the simplified version of bat communities (Mayr, 1977). Sibling species are morphologically very similar and phylogenetically closely related species that share a recent common ancestor. Therefore, any morphological, physiological or behavioral interspecific difference between them is more likely to reflect an adaptation related to niche separation (Arlettaz, 1999). Studies partly or simultaneously analyzing the dietary, spatial and temporal niche dimensions of cryptic or sibling sympatric species such as *Myotis myotis* and *M. blythii* (Arlettaz et al., 1997, 1999), *Pipistrellus pipistrellus* and *P. pygmaeus* (Barlow, 1997; Davidson-Watts et al., 2006; Sattler et al., 2007), *Plecotus auritus* and *P. austriacus* (Razgour et al., 2011), *Scotophilus dinganii* and *S. mhlanganii* (Jacobs and Barclay, 2009) or *Rhinolophus euryale* and *R. mehelyi* (Salsamendi et al., 2012b) have reported varying levels of food and habitat partitioning, although the majority suggested the segregation of foraging habitats as the main mechanism allowing their coexistence. Yet, the mechanisms underlying the spatial (e.g. habitat) segregation are not always clear (Arlettaz, 1999; Razgour et al., 2011; but see Salsamendi et al. 2012b).

R. euryale and *R. mehelyi* are moth-specialist sibling species that diverge in their foraging habitats when occurring in sympatry (Russo et al. 2005; Salsamendi et al., 2012b), but do not in allopatric conditions (Goiti et al., 2008; Salsamendi et al., 2012a; Salsamendi et al., 2012b). There is weak evidence of either temporal or diet segregation. Thus, Salsamendi et al. (2012b) suggested that interspecific competitive interactions might be the source of such spatial segregation, as both species occupied narrower habitat niches in sympatric conditions, in accordance to subtle differences in wing-loading and wing shape. Moreover, both species mainly foraged on moths, but the lack of resolution of the diet analysis prevented Salsamendi et al. (2012b) from identifying any functional pattern of fine-grained diet segregation.

In this study we want to go further in depth to understand the resource partitioning among sibling bat species. Thus, we performed a fine-grained dietary analysis of *R. euryale* (Blasius, 1853) and *R. mehelyi* (Matschie, 1901) using DNA metabarcoding. We molecularly analyzed the diet of the bat populations that Salsamendi et al. (2005, 2012b) studied for the habitat use, hunting behavior, coarse-grained diet, morphology and echolocation call parameters simultaneously. Hence, due to the detailed ecological background previously gathered about these species, we believe our study-system offers an excellent case to identify any resource partitioning related to the segregation of the ecological niche. In fact, *R. euryale* and *R. mehelyi* do not largely differ in either echolocation characteristics or morphological parameters (Salsamendi et al. 2005), and hence, in either prey-size detection or prey-size handling. Both belong to the "narrow-space flutter-detecting" guild (Schnitzler et al. 2003). And, the importance of common and widespread moth species seem to have in the diet of moth-eating bats (e.g. Chapter 3; Razgour et al., 2011). We hypothesize that i) *R. euryale* and *R. mehelyi* will not largely differ in the species-level composition of their staple diet, ii) subtle differences in prey composition will reflect the spatial segregation of foraging habitats, and iii) the coexistence of these species will mainly be mediated by differences in the spatial niche, rather than by prey partitioning. Furthermore, we reviewed the existing literature related to resource partitioning between pairs of sibling or cryptic sympatric bat species and discussed the variety of potential mechanisms affecting the resource segregation patterns for different groups of bats.

4.2. Material and Methods

4.2.1. Sample collection

Bats were captured in the Sierra de Las Villuercas mountain range in Extremadura, Spain, in central-western Iberian Peninsula (UTM 30S 2924 4359), during the breeding season (June-July) of 2007. Further details about bat capture, handling permits and identification procedures are described in Chapter 2.

4.2.2. DNA extraction, PCR amplification, sequencing and bioinformatics

We extracted DNA from faecal samples from 37 and 34 individual bats of *R. euryale* and *R. mehelyi*, respectively, following the procedure described in Chapter 2. MOTUs were clustered with sequences containing more than 15 copies at a 94% similarity threshold as explained in Chapter 2. Identified macro-moth prey species were cross-checked with the species list obtained for the region of Extremadura (Novoa Perez et al., 2002).

4.2.3. Diet analysis

We compared the consumed prey species diversity (at MOTU level) between *R. euryale* and *R. merelyi* by calculating the interpolation and extrapolation curves of Hill numbers (or the effective number of species, see Chao et al., 2014) for Shannon diversity developed for unequal sampling effort and incidence data (presence/absence data; Chao et al. 2014). The 95% confidence intervals were obtained by a bootstrap method based on 500 replications. Analyses were performed using the iNEXT package for R (Chao et al. 2014; Hsieh et al. 2014).

To test whether the diet of bat individuals significantly differed by bat species at MOTU level, we conducted a distance-based redundancy analysis (db-

RDA) based multivariate anova, using a distance matrix as response and the species identify of individual bats as explanatory matrix (Borcard et al., 2011). Jaccard distance measure was used for the calculation of dissimilarities between diets of individuals. Analysis were performed using the capscale() function in the Vegan package (Oksanen et al. 2011) for the software R and following Borcard et al. (2011).

In combination with the db-RDA multivariate anova analysis, dietary niche overlap between both rhinolophid bats was measured based on Pianka's (1973) index (Krebs, 2014) at MOTU level at two scales: 1) based on all detected MOTUs; and, 2) based on the most frequently consumed taxa (excluding MOTUs consumed by just one individual as they may not constitute their staple diet and distort niche overlap results; Brown et al., 2013; Krebs, 2014). We used null models to test whether the observed niche overlap differs from what would be expected by chance. Analyses were performed with EcoSimR (Gotelli and Ellison, 2013). Null models were calculated based on the randomization algorithm RA3 provided by Gotelli and Ellison (2013) (based on Lawlor, 1980a; Winemiller and Pianka, 1990) and 10,000 simulated resource utilization matrices were generated to compare with observed resource utilization data.

Data on the wingspan length of identified lepidopteran prey species and their habitat associations were collected (Robineau, 2007; Waring and Townsend, 2003; Sterling and Parsons, 2012; www.lepidoptera.eu; www.lepiforum.de). Lepidopteran prey were classified according to 4 habitat types. This classification was based on the structural complexity of the foraging habitats used by the same colony of *R. euryale* and *R. mehelyi* reported by Salsamendi et al. (2012b), namely: 1) clutter: habitats with a high canopy perimeter and canopy cover values (e.g. broadleaved woodlands, riparian forest); 2) semi-open: forested open habitats (e.g. dehesas, forest clearings and edges); 3) open habitats (e.g. pastures, meadows, crops); and 4) generalist: species likely occurring in any habitat type. Differences between bats' dietary compositions and moths' habitat associations were analyzed by db-RDA based multivariate anova (Borcard et al., 2011). Similarly, moth species were classified

as large (>45mm wingspan), medium (44-21mm) and small (<20mm). Differences between bats' dietary compositions regarding moths' size were also analyzed by db-RDA based multivariate anova (Borcard et al., 2011). In both cases, Bray-Curtis distance measure was used for the calculation of dissimilarities between diets of individuals. The relationship between prey size and habitat was tested by Pearson's Chi-squared test (significance level set at 0.05). All statistical analyses were performed using R v.3.1.2 (R Core Team, 2014).

The percentage frequency of each prey type was calculated as the total counts of each prey MOTU (i.e. prey items), divided by the total prey counts and multiplied by 100 (McAney et al., 1991; Whitaker, 2009).

4.3. Results

PCR amplicons were obtained from 37 and 34 faecal samples of *R. euryale* and *R. mehelyi* respectively. We identified a total of 62 MOTUs for each bat species, of which 32 were consumed by both of them. These overlapping 32 MOTUs constituted more than 75% of consumed prey for both bat species -in terms of percentage of frequencies- whereas the remaining species-specific 30 MOTUs constituted less than 25% of the consumed prey (Fig. 4.1). We identified 63% of the MOTUs to species or genus level and 3.3% to family level (Supplementary Material Table S4.1). The remaining 33.7% (*R. euryale*'s 17 MOTUs and *R. mehelyi*'s 18 MOTUs) were classified as *unknown*, as their low similarity to reference sequences did not fulfil the set identification criteria (see details in Chapter 2). Two MOTUs were identified as belonging to the noctuid *Agrotis ipsilon,* indicating that this taxon was over-split in the MOTU identification. The following prey species were excluded from the habitat-level analysis, as we were not able to find any information related to their main habitat type: *Eurodachtha canigella* (Lecithoceridae), *Apamea arabs* (Noctuidae) and *Ephestia mistralella* (Pyralidae).

a) Diet overlap of consumed MOTU-list

b) *R. euryale* - exclusive and shared prey items (%F)   c) *R. mehelyi* - exclusive and shared prey items (%F)

■ *R. euryale* - exclusively consumed

■ shared by *R. euryale* and *R. mehelyi*

□ *R. mehelyi* - exclusively consumed
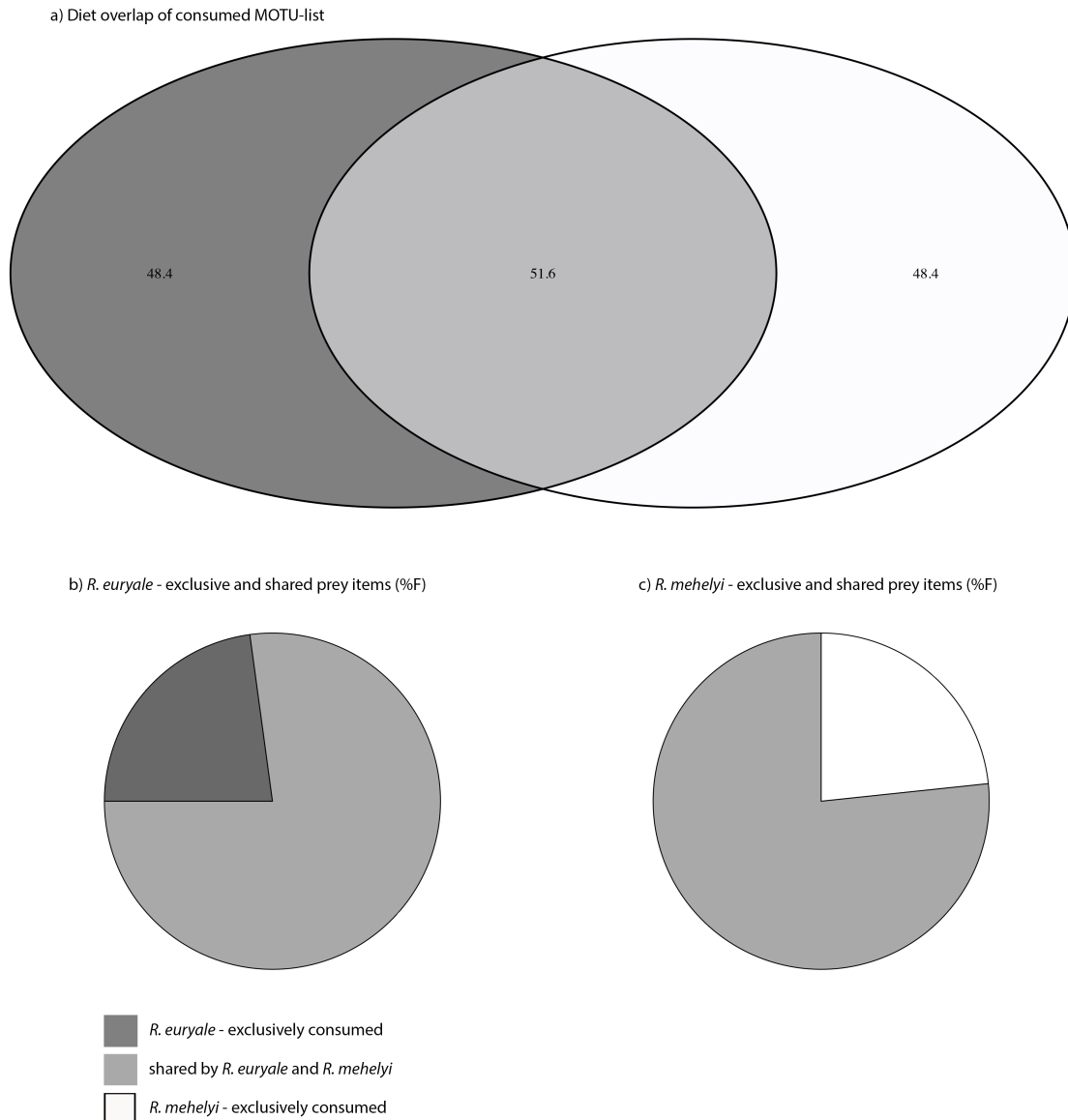
Figure 4.1. MOTUs exclusively consumed by *R. euryale* (dark grey), exclusively consumed by *R. mehelyi* (white) and consumed by both bat species (light grey). a) Venn diagram showing the overlapping proportion (%) of consumed MOTU list. b) and c) pie charts showing the percentage of frequency (%) of prey consumed by both bat species and the exclusively consumed ones: b) by *R. euryale*, and c) *R. mehelyi*.

4.3.1. Diet breath, diversity and composition

Both bat species showed very similar diet diversity values at the MOTU level (Figure 4.2). Confidence intervals (95%) highly overlapped, implying the diversity of consumed prey did not differ significantly between species.
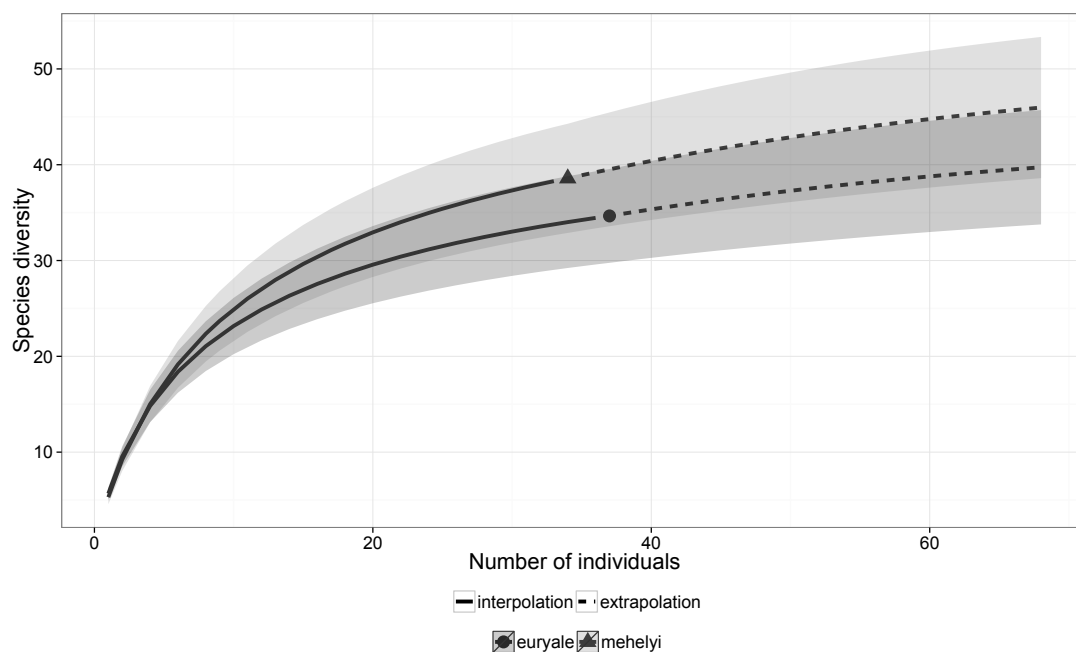
Figure 4.2. Comparison of sample-sized-based interpolation (solid line) and extrapolation (dashed line) curves with 95% confidence intervals for Hill numbers q = 1 (Shannon diversity). Curves were extrapolated to double the base sample size. Reference samples are indicated by solid dots.

The MOTUs, excluding the *unknown* (33.7%), were matched to 5 prey orders in the diet of *R. euryale*: Coleoptera, Diptera, Lepidoptera, Neuroptera and Orthoptera, and to 4 orders in the diet of *R. mehelyi*: the same except Orthoptera. However, in both cases most of the MOTUs belonged to the order Lepidoptera (55-58%). The rest of the orders constituted less than 14% of consumed species. A total of 15 lepidopteran families were identified, from which noctuids were the most consumed by both bat species (30-40%), of those identified. Species consumed by more than 5 individuals of both species were: the noctuids *Agrotis ipsilon*, *Agrotis* sp. (*A. segetum* or *A. trux*), *Calophasia platyptera*, *Peridroma saucia*, *Sesamia nonagroides* and *Pempelia palumbella*. The widely distributed

and common moth *Agrotis ipsilon* was by far the most frequent prey species in the diet of both bat populations.

4.3.2. Diet similarity and overlap

db-RDA based multivariate anova showed that the diet between both bat species was significantly different at MOTU level (Table 4.1). However, the identity of bat individuals (*R. euryale* or *R. mehelyi*) just explained 2.26% of the total variance observed in the diet, meaning that the intraspecific diet variability was very high. Since most of the prey species were equally associated to both bat species (supplementary material, Figure S4.1), diet alone cannot be used to reliably identify predator's species.

Table 4.1. Output diet models from the db-RDA based multivariate anova analysis at MOTU, moth-habitat and moth-size level. df=degrees of freedom, Ad. R^2=adjusted variation explained by the model, and N. Perm=number of permutations.

		df	Ad. R²(%)	F	Perm	p
MOTU	Model	1	2.26	2.54	999	0.002
	Residual	69				
Habitat	Model	1	6.9	4.31	999	0.002
	Residual	68				
Size	Model	1	3.22	2.65	999	0.031
	Residual	68				

Similarly, ecological modelling indicates that *R. euryale* and *R. mehelyi* showed a high degree of diet overlap at MOTU level, which was greater than expected by chance (O_{jk} = 0.83, p < 0.000). After excluding MOTUs only consumed by one bat individual ("rare species"), dietary niche overlap was still high and significantly greater than expected by chance (O_{jk} = 0.75, p < 0.0004).

There wass a significant relationship between bat species and the moths they consumed, classified according to their main habitats (Table 4.1). Although both bats mainly consumed generalist moths or moths related to semi-open habitats (supplementary material, Fig S4.1), differences between their diet partly arose from the consumption of habitat-specialist moths. The moths related to clutter habitats were mainly consumed by *R. euryale* (e.g. MOTU61; *Catocala nymphagoga*; clutter), and similarly, the moths related to open woody habitats were mainly consumed by *R. mehelyi* (e.g. MOTU40; *Mythimna loreyi/sicula.*; open).

The diet of both bat species comprised primarily medium sized moths, accounting for 88% of prey items of *R. euryale* and 74% of *R. mehelyi* (supplementary material, Figure S4.1-S4.2). Large moths were equally consumed by both bat species. Contrarily, *R. mehelyi* consumed more small moths than *R. euryale*.

There was no significant relation between the size of moths and their main habitat (Chi square: $\chi^2 = 6.97$; df = 6; p = 0.32). This means, for instance, that clutter habitat moths were not significantly larger than open habitat moths, or vice versa.

4.4. Discussion

We found little evidence of diet segregation between the sympatric sibling horseshoe bats *R. euryale* and *R. mehelyi*. As predicted, their dietary niche dimensions highly overlapped due to the consumption of the same common prey species. Although we observed some prey differences, these appear to be very habitat linked, mirroring the spatial segregation observed by radio-tracking data (Salsamendi et al., 2012b). Considering that *R. euryale* and *R. mehelyi* did not differ in the structural complexity of their foraging habitats in allopatry (Goiti et al., 2008; Salsamendi et al., 2012a), but that they substantially segregated them in sympatry (Salsamendi et al., 2012b), the high dietary overlap indicates that

the shift of the spatial niche dimension is the main mechanism facilitating the coexistence of this pair of sibling horseshoe bats. This is the first study combining species-level diet analysis and homing-in radio-tracking data of the same population of sibling bats. Despite potential shortcomings, the high resolution of these techniques allowed us to scrutinise the differences between the foraging niches of sympatric sibling bats. This highlighted the relevance of the spatial dimension for their coexistence.

4.4.1. Diet overlap

Although we reported small differences between the diet of *R. euryale* and *R. mehelyi* at the putative species level (i.e. MOTU), the overlap was considerable. Both bats showed almost identical values of dietary niche breadth and high levels of Pianka's niche overlap: their staple diet consisted of medium-sized Lepidoptera. The differences between their echolocation call structures (Russo et al., 2007; Salsamendi et al., 2005) and morphological parameters (Norberg and Rayner, 1987; Salsamendi et al., 2005) seem too subtle to enable access to different prey types (Jacobs et al. 2007; Houston et al., 2004; Schuchmann and Siemers, 2010). In fact, the high-duty-cycle (HDC) constant frequency (CF) calls of rhinolophid bats are adapted to detect and classify fluttering insects, like moths, in clutter (Schnitzler and Kalko, 2001), and therefore, sympatric species foraging in similar habitats will likely respond to the same type of prey (Schoeman and Jacobs, 2011). This is supported by the high level of diet overlap observed for several sympatric "narrow-space flutter-detecting" horseshoe bats (Jacobs et al. 2007; Jiang et al. 2008; Schoeman and Jacobs, 2011). Similar levels of diet overlap have been described for other pairs of similar bat species in sympatry (e.g. Bohmann et al. 2011; Krüger et al. 2014; Razgour et al. 2011).

Beyond potential phenotypic similarities constraining species-specific prey availabilities (Siemers and Güttinger, 2006), though, many molecular studies are showing the presence of common and widespread moth species in the diet of bats belonging to different foraging guilds. For instance, common moths such as the noctuids *Agrotis* spp., *Apamea* spp., *Noctua* spp., *Mythimna*

spp. or the geometrids *Idaea* spp. (Robineau 2007; Waring and Townsend, 2004) have been consumed by the following European bat species: the alpine long-eared bat *Plecotus macrobularis* that forages in supraforestal open-space areas (Alberdi et al., 2012), the aerial-hawking bat *P. austriacus* that forages in open and semi-open habitats (Razgour et al., 2011), the narrow-space flutter-detecting bats *R. euryale* and *R. mehelyi* that forage in and within edge habitats (present study; Chapter 4), the trawling bat *Myotis dasycneme* that mainly forages along water bodies (Krüger et al., 2014) or the behaviorally flexible *M. nattereri* that forages in clutter (Siemers and Shift, 2006) on both larvae and imago of common moths during winter (Hope et al. 2014). These observations indicate that habitat generalist moths or large moths (i.e. high energetic content) with high dispersal abilities (Chapman et al., 2010; Slade et al. 2013) are simultaneously exploited by ecomorphologically different bat species and may constitute important components of their diet, either seasonally or locally. Therefore, the alarming decline of those common and widespread moths in some areas (Conrad et al., 2006) is likely to have strong impacts on the foraging ecology and coexistence of sympatric bat species (Wickramasinghe et al., 2004). Consequently, bat management and conservation guidelines should integrate the conservation of those common key prey species.

4.4.2. Diet mirrors the spatial segregation of foraging habitats

In line with our predictions, sympatric *R. euryale* and *R. mehelyi* consumed habitat-specific moth species that mirrored the habitat segregation observed by radio-tracking data (Salsamendi et al. 2012b): moth species associated with clutter habitats were mostly (but not strictly) consumed by *R. euryale*, whereas species linked to open woody habitats were mainly (but not strictly) consumed by *R. mehelyi*. We also found small dietary differences related to prey's size: *R. mehelyi* consumed slightly higher proportion of smaller moths. Interestingly, there was no apparent relationship between the foraging habitats and the size classes of moths (i.e. smaller moths were not significantly related to open woody habitats). Prey-size related differences might be associated to subtle differences between the echolocation call and morphological characteristics of

both bats, since the higher call frequency and larger body size of *R. mehelyi* may facilitate the detection of smaller sized prey (i.e. prey detection hypothesis; Barclay and Brigham, 1991; Houston et al. 2004) and the manipulation of larger prey, respectively. However, the latter association can be excluded as both bats preyed similarly on larger moths. Moreover, wavelength differences between the peak echolocation call frequency between *R. euryale* and *R. mehelyi* (circa 104 kHz; 3.267 mm wavelength, and circa 107 kHz; 3.215 mm wavelength, respectively) seem to be too small (0.052mm) to allow any size-related prey partitioning (Jacobs et al. 2007; Houston et al., 2004; Schuchmann and Siemers, 2010).

On the other hand, it has been suggested that the larval host plant of moths influences the vertical stratification and diversity of adult moth assemblages in forests (Brehm, 2006; Hirao et al., 2008). Most of the larval host plants of the small moths consumed by *R. mehleyi* are herbaceous plants that can be found on the ground of grasslands or forests (Sterling and Parson, 2012; Ronineau, 2007; Olea et al., 1999). Therefore, adult phases of those micro-moths are more likely to occur near to the ground level. Precisely, *R. mehelyi* was often observed foraging close to the ground (30-50 cm; Gaisler, 2001; Salsamendi et al., 2012b), whereas *R. euryale* always foraged close to vegetation at the canopy level (Goiti et al., 2006; Russo et al., 2005; Salsamendi et al., 2012b). These observations indicate that both habitat and size-related prey differences might reflect the differential use of the 3-dimensional space by horseshoe bats, consistent with the behavioral flexibility and spatial segregation predicted for other sympatric rhinolophids (Kingston et al., 2000; Siemers and Ivanova, 2004).

Prey-habitat and prey-size related results should be cautiously considered due to methodological limitations, linked to the fact that our identifications indicate likely prey species (i.e. MOTUs), and several *unknown* species were excluded from the analysis. These *unknowns* could modify the observed differences obtained from the identified MOTUs. However, our results are not too skewed, since they matched with the habitat selection and behavioral patterns reported for the studied bat populations (Salsamendi et al., 2012b). As

in previous research (e.g. Razgour et al. 2011), our results also show that molecular diet studies are useful tools to infer differences in foraging behavior among sympatric species. However, such approaches are still constrained by the completeness of local reference sequence databases and the limited knowledge about prey's ecology.

4.4.3. Resource partitioning and coexistence of sibling bats

Although there is extensive literature about the foraging ecology of sympatric bat species (e.g. reviewed in Patterson et al., 2003), here we focus our discussion on pairs of sibling or cryptic sympatric bats. Any interspecific differences (i.e. behavioral, ecological, morphological or physiological) between them are more likely to reflect their adaptive value in the context of niche separation (Arlettaz, 1999, Jacobs and Barclay, 2009). The evidence gathered so far highlights that sibling or cryptic pairs of species can differ considerably in their dietary and spatial niche dimensions without apparently differing in their morphological or echolocation call parameters (Arlettaz, 1999; Davidson-Watts et al. 2006; Jacobs and Barclay, 2009). For instance, sympatric *Myotis myotis* and *M. blythii* differ significantly in their diets, which highly correlate with differences in their habitat selection patterns (Arlettaz, 1999). Authors suggested that diet and habitat partitioning were more an effect of species-specific differences in functional adaptations (e.g. behavioral) than of interspecific competition for food or hunting space (Arlettaz, 1999). Similar conclusions were obtained for the cryptic *Pipistrellus pipistrelly* and *P. pygmaeus* by Davidson-Watts et al. (2006). Razgour et al. (2011) observed that although the cryptic bats *Plecotus austriacus* and *P. auritus* highly overlapped in their diets, they differed in the consumption of habitat specialist moths, which, indeed, reflected differences in bats' specific foraging habitats. Instead of food selection driving spatial segregation, they suggested that bats were just consuming similar prey in their respective habitat types (Razgour et al., 2011). Contrarily, Jacobs and Barclay (2009) found that sibling *Scotophilus dinganii* and *S. mhlanganii* highly overlapped in their dietary, spatial and temporal niche dimensions, although they suggested that micro-habitat level differences could exist.

However, these bats significantly differed in their roosting niche dimension, which might result in the increase of their fitness (Jacobs and Barclay, 2009). In fact, small fitness differences (i.e. similar competitive abilities) might require small differences in resource partitioning to enable their coexistence (Adler et al., 2007; Chesson, 2000). Therefore, there is evidence of varying degrees of resource partitioning between different pairs of sibling bats, which suggests that diverse combinations of mechanisms allow the coexistence of entire guilds or communities of bats.

The main difference between our pair of sibling horseshoe bats and the other *Myotis*, *Plecotus*, *Pipistrellus* and *Scotophilus* pairs is that in the later there is no documented evidence for trophic niche shifts between allopatric and sympatric populations (Arlettaz et al., 1997, 1999; Razgour et al., 2011). No expansion of the foraging niche was reported for allopatric populations of *M. myotis* and *M. blythii* in relation to sympatric ones (Arlettaz et al., 1997). Hence, the observed resource partitioning is unlikely to be the result of active interspecific competition, at least in the case of *Myotis* pairs (Arlettaz et al., 1997). It remains unclear, however, which behavioral or ecological mechanisms underlie habitat segregation in these species pairs (Arlettaz, 1999; Davidson-Watts et al., 2006; Razgour et al., 2011). However, *R. euryale* and *R. mehelyi* clearly shifted in the spatial dimension of their trophic niche between allopatric and sympatric populations (Goiti et al., 2008; Russo et al., 2005; Salsamendi et al., 2012a/b), and they highly overlapped in the consumption of medium-sized moths in sympatry. Although there is no species-level diet information for allopatric populations of *R. mehelyi*, considering that the staple diet of *R. euryale* consisted of medium-sized moths in both sympatric (present study) and allopatric conditions (Chapter 4; Andreas et al., 2012), it is very likely that their dietary niche dimension remains almost unmodified in both conditions. Therefore, our results suggest that selection for the same prey-type drives the segregation of the foraging space in this pair of sibling horseshoe bats. This segregation probably contributes to reducing the interspecific competition that may arise when the density of their staple prey type is reduced, the density of both predator bats is too high, or their energetic requirements are increased.

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High dietary overlaps have also been reported for other horseshoe bat species (Jacobs et al. 2007; Jiang et al. 2008; Schoeman and Jacobs, 2011), and none of those studies excluded the possibility of bats differing in the use of foraging (micro) habitats. In contrast with the previously cited vespertilionid bats, which might show higher flexibility in echolocation characteristics (e.g. genus *Myotis*; Siemers and Schnitzler, 2004), HDC- CF bats are specialized to forage on fluttering insects in clutter habitats (Schnitzler and Kalko, 2001). Consequently, they are sensorially adapted to forage on very similar specific prey availabilities (see "prey conspicuousness" in Siemers and Güttinger, 2006). Contrarily, many horseshoe bats differ in their wing morphologies, and hence, in their flight performance (Dietz et al., 2006; Kingston et al., 2000; Norberg and Rayner, 1987; Salsamendi et al., 2005) or show very flexible hunting behaviors (Siemers and Ivanova, 2004). This suggests the relevance of the spatial partitioning in facilitating the coexistence of horseshoe bat species (Kingston et al., 2000).

Coexistence of species depends on the combination of both stabilizing processes (e.g. niche differences) and differences in average fitness (Adler et al., 2007; Brown, 1989; Chesson et al. 2000). In bats, the quantification of stabilizing processes such as resource partitioning or the differential responses to common environmental fluctuations is not exempt from methodological difficulties. In fact, little is known about the spatiotemporal structure and behavior of terrestrial prey insects, which might be one of the most important factors defining what bats are able to capture and eat (Jones and Rydell, 2003). Insect assemblages fluctuate through time and space (Lopez-Carretero et al., 2014) and bats' foraging activity and behavior are correlated with the fluctuations in abundance of key insects (i.e. Lepidoptera, Coleoptera and Diptera; Jones, 1990; Wickramasinghe et al., 2004). However, species-specific responses to these fluctuations might vary considerably among bat species (likely conditioned by their foraging flexibility), as has been observed for insectivorous birds (Murakami, 2002). For instance, the staple food of some aerial-hawking bats consists of predictable mass-emerging insects especially available at dusk

(Rydell et al., 1996), and might constitute punctually unlimited "food clouds" (e.g. swarming dipterans in ponds). Other insects, like moths, might be segregated within the 3-dimensional space (Hirao et al., 2008) in a less predictable way for aerial hawking or gleaning bats. The high spatiotemporal and behavioral diversity of bat-insect systems and the putative fitness differences among bats may imply that their coexistence is facilitated by a variety of combinations along a continuum between extreme stabilizing processes (i.e. extreme resource partitioning) and similar fitness (Cheeson, 2000). This would explain why varying degrees of niche dissimilarities have been reported among different pairs of sibling bat species.

In summary, our data highlight the relevance of common and widespread moth species in the diet of sympatric bat species. Moreover, subtle dietary differences revealed by molecular tools mirrored the spatial segregation of foraging habitats of bats observed by radio-tracking (Salsamendi et al., 2012b). Our study also highlights the potentiality of molecular tools to infer ecological patterns (as shown by e.g. Alberdi et al., 2012; Chapter 3; Clare et al., 2011; Razgour et al., 2011). However, this capability is restricted by the completeness of reference sequence databases and the limited knowledge about prey's ecology and behavior. This accentuates the need to simultaneously analyze the dynamics of insect assemblages potentially available for bats. Besides, our results also uphold that the displacement of the spatial niche dimension, rather than the dietary niche, seems to highly influence facilitating the coexistence of sibling horseshoe bats. Finally, effective guidelines for the conservation of sympatric similar horseshoe bats —and moth eating bats as a whole— should: 1) include the measures needed to protect common and widespread prey species, and 2) guarantee the structural diversity of foraging habitats.

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CHAPTER 5

Trait-based Diet Analysis of a Moth Specialist Horseshoe Bat: Are all Moths Alike for Them?
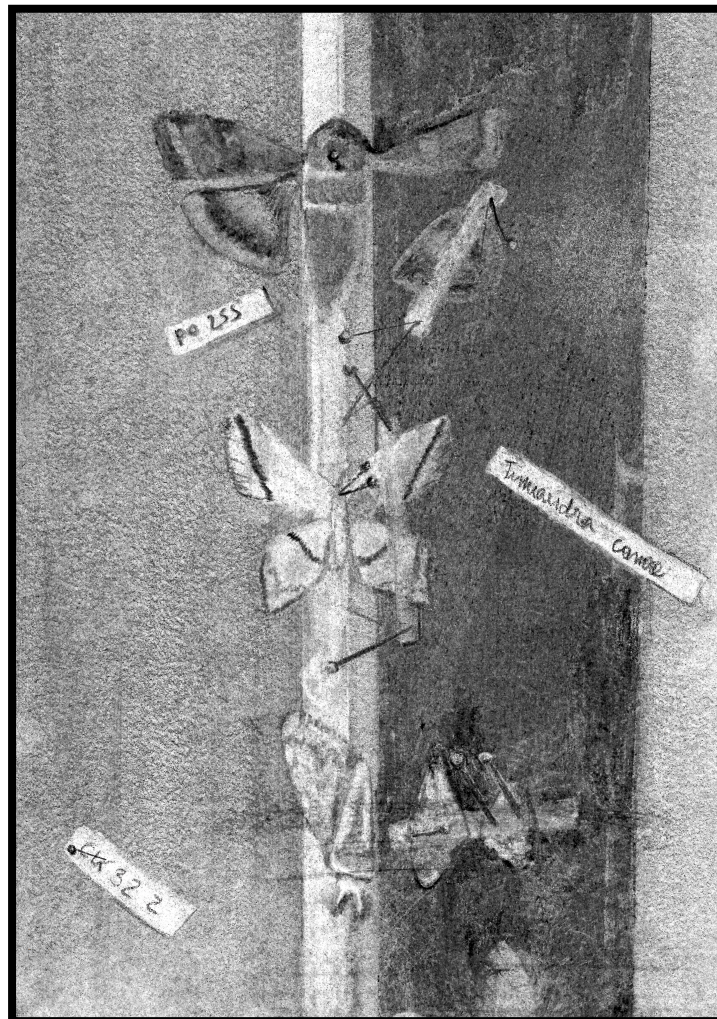
Abstract

Understanding the degree of prey-specialization and adaptive flexibility of moth specialist bats, as well as their evolutionary relationship with prey is pivotal to assessing their ability to adapt to varying environments. However, this is limited by taxonomy-based traditional diet analyses where the identification and interpretation of functional relationships are restricted due to the vast diversity of consumed prey species and the high diversity of evasive and defensive adaptations of moths. In this study we aimed to analyze the trophic flexibility of *R. euryale* and its evolutionary relationship with prey, by linking prey's functional traits (e.g. mass, wing-loading) and bats' intraspecific variables (i.e. sex, size and ontogeny) through diet and across a spatiotemporal gradient. Diet was analyzed using DNA metabarcoding in combination with RLQ and the fourth-corner analyses. We also aimed to analyze the functional variability of the potentially available moth assemblage through time and space. After determining the diet of 126 bat individuals and moths captured in 54 light-traps, our trait-based approach showed that seasonality greatly influenced the functional composition of bats' diet, as well as the potentially available moth assemblages: traits related to energy content (i.e. mass) and flight performance (i.e. wing loading and maneuverability) changed significantly and similarly in both cases. These results showed that *R. euryale* is trophically flexible enough to take advantage of seasonally variable moth types. Juvenile bats more frequently consumed lighter, more maneuverable but slower moth species than adults. These differences were probably related to the naive hunting skills of young bats. Arctiine moths seemed to be under-represented in the diet of *R. euryale*, suggesting that these moths developed some effective level of protection against highly specialized moth-eating bats. Our results showed that trait-based approaches open new insights to understanding the foraging ecology, evolutionary relationships and conservation of insectivorous bats.

Keywords: *foraging ecology*, *traits, moths, profitability, bats, arms-race.*

5.1. Introduction

Insectivorous bats and their arthropod prey (mostly insects) have undoubtedly influenced the evolution of each other's traits at least during the last 50 million years (Conner and Corcoran, 2012; Teeling et al. 2005), meaning that bats and insects are functionally linked. Several insects such as moths use a range of adaptations and behavioral responses in order to minimize the chance of being consumed by bats (Conner and Corcoran, 2012; Jantzen and Eisner, 2008; Rydell et al. 1995). Many bats, on the other hand, show species-specific counter-adaptations and strategies that facilitate effective foraging on them (Conner and Corcoran, 2012; Goerlitz et al., 2010). Bats are morphologically, ecologically and behaviorally very diverse, and their species-specific sensorial and morphological adaptations determine the types of prey they are able to detect, pursue, capture and consume (Jones and Rydell, 2003; Siemers and Güttinger, 2006; Swartz et al., 2003).

Some bats are specialized to prey upon few prey types such as the moth-specialists *Barbastella* sp., *Plecotus* sp., *Tadarida teniotis* or many *Rhinolophus* sp. (Razgour et al., 2012; Rydell and Arlettaz, 1994; Salsamendi et al., 2012b; Sierro and Arlettaz, 1997). Although their morphological, sensorial and behavioral foraging adaptations are considerably different, from large and fast aerial hawking *Tadarida teniotis* (Dietz et al., 2009) to the highly maneuverable and clutter specialist *Rhinolophus euryale* (Dietz et al., 2009), all of them mainly rely on moths. Specialized predators are particularly vulnerable to environmental variation (MacArthur and Pianka, 1966). Thus, understanding the degree of prey-specialization and adaptive flexibility of these bats, as well as their trophic relationship with prey, is pivotal in ecology and conservation. Firstly, in order to understand the evolutionary relationships shaping their foraging ecology, and secondly, to assess their ability to adapt to varying environments in anthropogenically modified landscapes (Voigt and Kingston, 2016). In this context, although extensive literature is available about bats' ecomorphological adaptations (e.g. Swartz et al., 2003), little is known about prey adaptations and their relationship with their predator bats.

In dietary studies, traditionally, these functional relationships are described considering the hearing-based evasive capacity of tympanate moths (Roeder 1962, 1975) and the bats' ability to approach them by echolocating either above or below the hearing capacity of moths, or by relaying on the passive listening of prey-generated sounds (Rydell et al., 1995). Moths, however, show a large number of combinations in evasive and defensive morphological, physiological and behavioral adaptations to avoid being hunted. And this makes the functional relationships between specialized bats and their moth prey considerably complex. In addition to the tympanate nature (Rydell et al. 1995), other adaptations include aposematism (Hristov and Conner, 2005), Batesian mimicry (Barber and Conner, 2007), sonar jamming (Corcoran et al., 2009), occasional and ground-hugging flight behavior of some earless species (Fullard and Napoleone, 2001; Rydell, 1998) or fast flight and large bodies (Rydell and Lancaster, 2000). Additionally, the normal flight of moths tends to be mostly erratic and, irrespective of their tympanate nature, moths are characterized by their evasiveness. As defined by Jantzen and Eisner (2008): the ability to fly quickly, at varying speed and direction, i.e. unpredictable. This evasiveness varies in relation to the flight speed and the relation between forewing and hindwing areas (Jantzen and Eisner, 2008). Therefore, we argued that the combinations of such adaptations (i.e. traits) have varying effects on the profitability of moths even for those specialized predators. All moths are not equally profitable for them, since profitability is dependent on the total energy gain after pursuing and capturing them, which is, in fact, related to prey's flight performance and avoidance mechanisms, as well as to the hunting skills of predators.

However, the analysis of the functional trophic relationships between specialist bats and moths might be limited by taxonomy-based traditional diet approaches, where the vast diversity of consumed prey species (e.g. Chapter 3-4) and the high diversity of evasive and defensive adaptations of moths restricts the assessment and interpretation of any meaningful relationship. Additionally, the foraging behavior of bats may vary at the intraspecific level due to differences

between the foraging requirements of sexes (e.g. high energy-demand for females during pregnancy and lactation, and for males during the mating season; Lintott et al., 2014; Dietz and Kalko, 2007) or the naivety in the hunting experience of first year fliers in comparison to that of adults (Adams, 1996). Moreover, all this needs to be observed in environments where moth species availability may vary seasonally or in relation to the location (Jones et al., 1990; Summerville et al., 2003). In this sense, trait-based dietary approaches may provide an innovative and effective method to analyze the trophic flexibility of bats (Spitz et al., 2014), which is key to assessing their ability to adapt to varying environments.

In this study we aimed to analyze for the first time a bat-moths predator-prey system linking prey's functional traits and bats' intraspecific variables (i.e. sex, size and ontogeny) through diet and across a spatiotemporal gradient. We selected as a model species the moth-specialist bat *Rhinolophus euryale* (see Chapter 1). Given its slow but highly maneuverable flight capacity suited to forage in clutter, its size and sensorial specialization to forage on moths, we tested the general hypothesis that the staple diet of *R. euryale* is composed of medium-sized maneuverable moths, irrespective of space, time or intraspecific variability. However, some punctual differences in prey types were expected to arise due to differing energy requirements among sexes or age of bats. Moreover, considering the spatiotemporal fluctuation of moth assemblages (Choi et al., 2011; Lopez-Carretero et al., 2014; Summerville et al., 2003), we hypothesized that moth assemblages would also fluctuate in the functional composition in our study area. Our specific aims were to i) determine the trophic niche flexibility of *R. euryale* both on taxonomic and functional grounds: assess how intraspecific and environmental variability influence bats' foraging ecology; ii) identify the key traits of prey linked to the diet of *R. euryale*; iii) characterize and analyze the functional variability of the potentially available moth assemblage through time and space; iv) identify and discuss the evolutionary arms-race relationship between *R. euryale* and moth prey in wild populations.

5.2. Material and Methods

5.2.1. Study Area and Bat Captures

The study took place in two contrasting landscapes located in the Atlantic region of the northern Iberian Peninsula: Karrantza and Lea-Artibai Valleys (Biscay, the Basque Country). Both valleys are representative of the countryside landscapes of the Atlantic region of the Iberian Peninsula, where other colonies of *R. euryale* occur. They are located at a linear distance of 66 km from each other, separated by the metropolitan area of Bilbao. More details are given in Chapter 2.

Bats and moths were captured during bats' pre-breeding (May), breeding (July) and post-breeding (September) seasons in 2012. Bat captures were conducted in a single night for each season and location in order to minimize disturbance: the 14th and 25th of May, the 3th and 9th of July and the 9th and 13th of September (in Karrantza and Lea-Artibai respectively, for more details see Chapter 2).

5.2.2. Diet analysis: DNA extraction, PCR amplification, sequencing and bioinformatics

A total of 126 fecal samples, with the following distribution, were used for the molecular analysis (gathered from Karrantza and Lea-Artibai respectively): 20 and 12 samples in May; 20 and 22 in July; and 40 (18 from adults + 22 from juveniles) and 12 (10 from adults + 2 from juveniles) in September. Detailed explanations of DNA extraction, PCR amplification, sample tagging and sequencing procedures are given in Chapter 2.

In order to discriminate PCR and/or sequencing artifacts from true biological sequences, we performed two PCR replicates per each DNA extract sample (Hope et al. 2014). Each replicate was tagged with a unique primer

combination in order to identify replicates bioinformatically (see Chapter 2). Sequencing was performed on an Ion Torrent platform (see Chapter 2).

Bioinformatic analyses were performed as explained in Chapter 2, but in this case only identical sequences present in both PCRs from the same extract were kept for further analysis. MOTUs were built and taxonomic assignments were carried out by comparing the representative sequence of each MOTU against BOLD, as explained in Chapter 2. MOTUs were classified as "unknown" when they didn't match any reference sequence or, when they did so but the matching sequence didn't belong to Iberian or French species. "Unknown" and family-level identified MOTUs were excluded from further analysis.

5.2.3. Bats: intraspecific and environmental traits

Morphological and physiological conditions may result in differences among individual bats foraging requirements (e.g. Adams, 1996; Mata et al., *In Press*). Besides, temporal and spatial variability may influence bats' diet (e.g. Chapter 3; Clare et al., 2013). Therefore, in our trait-based functional diet analysis we included *seasonality* and *location* as environmental traits, and *size* (measured as forearm length and body mass), *sex* and *age*, as bats' intraspecific traits (Table 5.1).

5.2.4. Moths: captures and identifications

Moths were captured using light traps (6W, UV light) two nights before and two nights after each bat capture session. Light-traps were located as evenly as possible within a 5 km radius from each of the colony roosts, and across the main 6 habitat types available for *R. euryale*: namely, broadleaved woodlands, holm oak forest, hedgerows, pine plantations, eucalyptus plantations and grasslands. Light-traps were activated at dusk for 4 hours coinciding with the first activity peak of both moths (Scalercio et al. 2008) and bats (Goiti et al. 2006). A total of 216 light-traps were evenly located at each defined habitat,

season and location. Moths were captured alive using a clothing-bag located inside the light-trap. They were frozen in the bags within 1-6 hours from capture.

Moth specimens were identified to species level whenever it was possible either visually or through DNA barcode analysis. Visual identification of moth specimens was carried out with the specialized assistance of Jordi Dandart (Barcelona Museum of Natural Science, Barcelona, Catalonia), Ibon de Olano (curator of the Lepidopteran collection of Araba Museum of Natural Science - ANZM-, Vitoria-Gasteiz, The Basque Country) or by field guides for macro- and micro moth identification: Robineau, 2007; Sterling and Parsons, 2012; Waring and Townsend, 2004. The identification of some moths was performed through amplification of their full COI barcode (648-bp) using LEP-F1/LEP-R1 primers designed by Hebert et al. (2004) for the Lepidoptera. All generated data are going to be uploaded to GenBank and BOLD (www.bodsystems.org). Note that some moth species belonging to the same or related genera were almost morphologically identical and difficult to identify at the species level, in particular when coloration patterns were deteriorated after manipulation or in light-traps. These specimens were classified into the following groups: *Eudonia*-complex (including *Eudonia sp.* and *Scoparia sp.*), and *ScopCabe*-complex (including *Scopula sp.*, *Cabera sp.* and *Lomographa sp.*). Unfortunately with some taxa neither morphological nor genetic identification was possible and they were excluded from further analysis.

5.2.5. Functional traits of moths

To characterize the traits of the moths consumed by *R. euryale*, we assigned the functional traits of moths captured in the field (1-5 specimens per species) to the species molecularly identified in the diet. Additionally, we crosschecked the morphological measures we obtained with those available in the bibliography or museums (specified below) in order to identify possible outliers. The functional traits of those species identified in the diet but not captured in the study area were measured from museum specimens (ANZM) or from scaled pictures provided by Thomas Merckx (TM's own specimens). We

assume that the functional traits of the measured specimens are representative of availability, and thus, of the moth individuals consumed by bats.

We defined functional traits of prey as those morphological and anatomical features that likely influence the profitability of prey-moths for *R. euryale*. We measured fresh body mass and forewing length as indicatives of energy content and prey-size, respectively. Wing loading was measured as an indicative of flight speed (Wing loading (N x m^{-2}) = weight x gravitational acceleration / wing area; Norberg and Rayner, 1987), and aspect-ratio of forewings (AR= 4 x forewing length^2/forewing area; Merckx and Van Dyck, 2006), traditionally used to measure the maneuver capacity in two-winged animals (e.g. birds), as an indicative of wing-shape and maneuver capacity. However, hindwings are key elements to perform the characteristic and highly maneuverable erratic flight of lepidopterans (Jantzen and Eisner, 2008). Thus, we defined the trait "maneuverability" as the ratio between hindwing and forewing areas (Maneuverability = hindwing-area/forewing-area), where higher values likely indicate higher maneuver capability (*sensu* Jantzen and Eisner, 2008). Many moth species —called "tympanate"— are able to detect approaching bats thanks to their ultrasound-sensitive hearing (Roeder, 1967; Fullard, 1982, 1987). We also included this ultrasound-hearing capability in moths as an additional functional trait, in order to assess its relationship with the rest of the traits and with *R. euryale*. We did not include other behavioral, physiological or flight-related traits such as reduced flight activity, toxicity, unpalatability, sonar jamming or aposematism into the analysis because the limited available documentation only refers to some few species of moths. However, they may likely affect bats hunting success. All functional traits included in the analysis are summarized in Table 5.1.

Table 5.1. Functional traits for moths, bats and environmental variables included in the RLQ and fourth-corner analyses.

Bat and Environmental traits	Data type	Categories/Codes	Prey traits	Data type	Categories/Codes
Body mass (gr)	Continuous	Bmass	Fresh mass (mg)	Continuous	Mass
Forearm length (mm)	Continuous	FL	Forewing length (mm)	Continuous	ForeWinLen
Sex	Categorical	male, female	Maneuverability	Continuous	Manouv
Season	Categorical	May, July, September	Aspect-ratio	Continuous	AspectRatio
Locality	Categorical	Karrantza, Aulesti	Wing-loading (N/m2)	Continuous	WingLoad
			Tympanate	Binary	Tympa.yes, Tympa.no

Fresh mass of moth specimens was measured after 10-15 minutes of defrosting on a precision balance (Pioner®, Ohaus™). As it was not possible to obtain fresh mass for some specimens, we alternatively measured dry mass and extrapolated fresh mass from it. For that we used 2 simple regression models for light (<40mg dry mass: $R^2 = 0.81$) and heavy (>40mg dry mass: $R^2 = 0.95$) moth specimens, built from 298 specimens for which both fresh and dry mass data was available, belonging to 35 species of 5 families: Crambidae, Drepanidae, Erebidae, Geometridae and Noctuidae. Scaled fore- and hindwing pictures were taken for 1-5 specimens per taxa (camera: Cannon EOS-450D; lens: 18-55mm). Wing parameters were measured digitally as illustrated in Figure S5.1 (ImageJ, version 1.46r: Abramoff et al. 2004). Tympanate nature of moths was checked in the literature (Roeder, 1967; Rydell and Lancaster, 2000; Rydell and Young, 2002; Spangler, 1988). The functional traits of the species not captured by light traps but identified in the diet of *R. euryale* were obtained from reference collection at ANZM, TM's own collection, and scaled pictures available in the Barcode of Life Data System (www.boldsystems.org).

Note that to perform the functional analysis of potentially available moth-assemblages we only used presence/absence data, in order to make them comparable with dietary results. Then, we randomly chose an even number of light-traps per season and location due to the uneven sample size of processed light-traps.

5.2.6. Statistical Analyses

The relationships between prey and bat traits (statistical unit: individual bats), as well as between the traits of potentially available moths and environmental variables (statistical unit: light traps), were tested by RLQ and the

fourth-corner analyses (Dray et al. 2014). Details about the analyses will be explained only for "prey-traits *vs* bat-traits" relationships, but the general structure is identical to test "available moth-traits *vs* environmental-variables" relationships. Moreover, RLQ and the fourth-corner analyses were performed independently, first, for all adult bats (excluding juveniles) across seasons and locations, and second, for adult and juvenile bats during the post-breeding season.

RLQ and the fourth-corner analyses are two complementary methods that describe the multivariate structure of the data and test the significance of bivariate associations between moths' and bats' traits respectively, and can be combined to summarize and test the main structure. These methods require three input matrices: R, L and Q. The first matrix (L: n x p) includes the presence or absence of the p moth species in the diet of n *R. euryale* individuals. The second matrix (Q: p x s) describes the p moth species of matrix L according to a set of s functional traits of moths (Table 5.1). The third matrix (R: m x n) describes the n *R. euryale* individuals according to a set of m traits of bats (Table 5.1). The RLQ and fourth-corner analyses were performed following the procedure recommended in Dray et al. (2014) with the package ade4 (Dray and Dufour, 2007) developed for the R software (R Core Team, 2014).

The RLQ analysis identifies the main associations between moths' and bats' traits through the bat's diet matrix L. It computes a s x m matrix that contains measures of the intensity of the link between moths' and bats' traits and summarizes the multivariate associations (Dray et al. 2014). Before applying the RLQ analysis, a separate ordination of each matrix is required in order to characterize the main structure of the diet of *R. euryale* (L), the traits of prey moth species (Q) and the traits of bat individuals (R). We ordered prey species and bat individuals by applying a Correspondence Analysis (CA) on the matrix L. As R and Q matrices contain both qualitative and quantitative variables, they were ordered by a Hill-Smith analysis (Hill and Smith, 1976). Then, the three analyses were combined by the RLQ analysis using the function rlq(). Graphical

outputs of the RLQ analysis were used to summarize the main relationships between bats' and moths' functional traits.

While the RLQ analysis extracts the main structure of the associations between moths' and bats' traits, the fourth-corner analysis explicitly tests the significance of bivariate associations between each moths' trait and each bats' one (Dray and Legendre, 2008). This approach may be prone to giving type I errors and false significance of bivariate associations, due to the fact that it considers variables measured on different statistical units and it performs great number of simultaneous bivariate tests (see details in Dray et al. 2014). Thus, we applied the sequential test proposed by ter Braak et al. (2012) for the control of type I errors (Model type = 6), we adjusted P values by the Benjamini & Hochberg (1995) correction, and the number of permutations was elevated to 49,999 using the function fourthcorner (). The significance level of the test was set at p <0.05.

We followed the approach suggested by Dray et al. (2014) in applying the fourth-corner analysis directly on the results of the RLQ analysis to summarize and test the significance of the associations between the RLQ axes and bats' and moths' functional traits using the function fourthcorner.rlq(). Type I error control, adjustment of P values and number of permutations were set as for the fourth-corner test. All analyses were performed using R v. 3.0.1 (R-Development Core Team 2013).

5.3. Results

5.3.1. Molecular analysis of diet

A total of 315 MOTUs were obtained from the 126 *R. euryale* faecal samples sequenced in the Ion Torrent platform. We identified 63.5% (200) of the MOTUs to species or genus level and 2.2% to family level. The remaining 34.3% were classified as "unknown". Lepidopterans accounted for 91% of the MOTUs

identified to species or genus level (182). The remaining MOTUs belong to Diptera (5%), Neuroptera (3%), Hymenoptera (0.5%) and Psocoptera (0.5%). After collapsing those MOTUs, we obtained a total of 168 lepidopteran taxa belonging to 16 families. The functional traits of 69.6% of those taxa were measured from individuals captured in the study area, 19.05% from specimens at the ANZM, 5.35% from BOLD system scaled pictures and 3.6% from Thomas Merckx's own collection. We could not get data of traits of the remaining 2.4%. For further analysis we only considered those taxa for which all the defined functional traits were measured: 137 taxa from 12 families (Supplementary material S5.1).

5.3.2. Moths: captured species and their functional traits

We successfully processed 14 light-traps from May, 35 from July and 12 from September in Karrantza, and 14 from July in Lea-Artibai. Thus, we characterized the moth availability in Karrantza Valley from May to September and only in July in Lea-Artibai Valley, comprising a total of 2,873 moth specimens belonging to at least 308 taxa of 18 families (Supplementary material S5.2). We completely characterized the traits of 290 species (Supplementary material Table S5.2). The ranges for the traits of the potentially available and consumed species are shown in Table 5.2. For RLQ and the fourth corner analyses all processed light-traps were used, except for Karrantza's in July, for which 14 light-traps were randomly selected.

Table 2.2. Minimum and maximum values measured for the traits of moth species identified in the light-traps (Availability) and in the diet of *R. euryale* (in Pre-Breeding, Breeding, Post-Breeding in Adults, and Post-Breeding in Juveniles).

Range	Availability		Pre-breeding		Breeding		Post-breeding - Adults		Post-breeding - Juveniles	
	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Mass (mg)	0.8	574.8	4.16	455.8	1.99	201.5	1.22	326.3	2.1	190.5
ForeWinLen (cm)	0.39	3.69	0.88	3.04	0.65	2.54	0.58	3.15	0.58	2.37
Manouv	0.44	1.45	0.62	1.21	0.6	1.35	0.6	1.35	0.6	1.35
AspectRatio	9.43	21.06	9.79	16.41	9.79	16.41	10.5	16.15	10.15	17.02
WingLoad (N/m2)	0.49	25.86	1.1	18.56	0.49	18.17	0.45	18.32	0.49	18.17

5.3.3. Functional Diet: Adults

The first two RLQ axes (83.40% and 13.72% of the total variance, respectively) summarize the relationship between adult bats' and prey's traits. The first axis (horizontal) identifies a gradient from high to low values of wing loading and mass (Fig. 5.1a). It identifies prey species with higher values of mass and wing loading (i.e. heavier and faster fliers) mainly consumed in the post-breeding season (Fig. 5.1b): namely noctuids such as *Xestia*, *Mythimna* or *Agrotis* spp (Fig. 5.1c). Contrarily, lighter and slower species were mainly consumed in the breeding season. Although it accounts for a small part of the variation, the first axis also identifies a sex-related trend of females to consume heavier and faster moths than males, consistently for all seasons and locations (Fig. 5.1d). The second RLQ axis outlines prey species with higher values of forewing aspect-ratio (i.e. narrower forewings) and higher maneuverability, mainly consumed in the breeding and post-breeding seasons. On the contrary, during the pre-breeding season bats mainly ate less maneuverable moths with longer forewings. This axis also identifies an opposed trend regarding the diet of both populations (i.e. Karrantza and Lea-Artibai), irrespective of seasons. In Karrantza, bats tended to eat more maneuverable and smaller moths, as opposed to the less maneuverable and larger moths consumed in Lea-Artibai. Non-tympanate moths were mainly consumed during breeding and post-breeding seasons, and mainly in Karrantza. As it has been highlighted so far, seasonality considerably contributed more than the other environmental and intraspecific bat variables to the variation found in the diet (Fig. 5.1b/d). Among consumed moths, outlines of the 4 major groups could be defined by their wing loading, mass, forewing length and maneuverability. The most evident and large group is composed of the majority of consumed moths and shows intermediate values for all the traits, irrespective of season and location (center; Figure 5.1c); the second group comprises heavy fast fliers (left); the third, small and maneuverable moths (down); and the forth, moths with long forewings (up).

Figure 5.1. Results of the first two axes of RLQ analysis for all adult bats: a) coefficients for traits, b) coefficients for bat traits, c) eigenvalues and scores of prey species, and d) eigenvalues and scores of individual bats. The "d" values give the grid size. Codes for prey species and bat individuals are available in supplementary material S5.1.

The fourth-corner analysis found some of the trends observed for the RLQ analysis to be correlated. Results for this test are provided in the supplementary material S5.3.

The combined RLQ and fourth-corner analysis reasserted the significant association between some traits and seasonality (Fig 5.2). The first RLQ axis (which explains 83.40% of the variance) is positively correlated with the breeding season and negatively with the post-breeding season. Regarding the moth-traits, mass and wing loading are negatively correlated with the first axis, indicating the same significant association previously detected by the RLQ and the fourth-corner analyses. Thus, bats significantly consumed lighter and slower moths in the breeding season (July), and heavier and faster fliers in the post-breeding season. There is a significant functional difference between the prey-

type   consumed   by   bats   in   relation   to   seasonality.
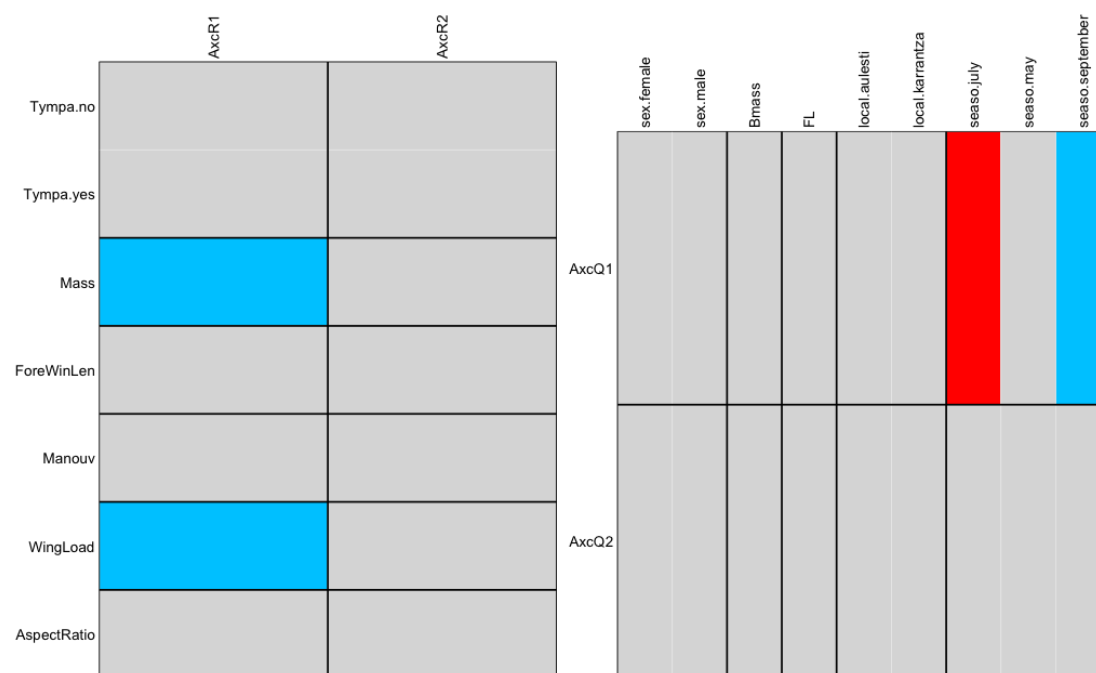
Figure 5.2. Combination of RLQ and fourth corner results for relationships of adult bats and prey traits. Left: Fourth-corner test between the first two RLQ axes for bat characteristics (AxR1/ AxR2) and prey traits. Right: Fourth-corner test between the first two RLQ axes for prey traits (AxQ1/ AxQ2) and bat characteristics. Red cells indicate positive significant associations, and blue cells negative ones (significance level: p < 0.05). Variables with non-significant association are shown in grey. Black lines separate different variables; white lines separate different modalities for categorical variables. *P* values were adjusted for multiple comparisons using the FDR procedure. Codes for traits and bat characteristics are available in supplementary material S5.1.

5.3.4. Functional Diet: juveniles vs adults

The first two RLQ axes explain 94.45% and 4.47% of the total variance, respectively. The first axis identifies a marked gradient related to age and body mass of bats in relation to the forewing-length, mass and wing loading of consumed moths (Fig. 5.3a/b). Adults (i.e. heavier bats) tended to eat heavier,

larger and faster flier moths (e.g. mainly noctuids: *Pheosia tremula*, *Catocala electa*, *Scoliopteryx libatrix*, *Mythimna unipuncta*, *Xestia baja*, *Xestia xanthographa*). Juveniles, on the contrary, tended to consume very maneuverable moths with thinner wings (i.e. higher aspect-ratio), lighter bodies and slower flight capacity (i.e. lower wing loading). These moths were mainly non-tympanate micro-moth species such as *Eccopisa effractella*, *Oncocera semirubella*, *Pleuroptya ruralis*, *Udea ferrugalis*, *Epinotia nisella*, *Agriphila inquinatella* (Fig. 5.3c). We observed a single exception: a juvenile male with a diet similar to that of adults (Jk17.m; Fig. 5.3d). The second RLQ axis identifies a sex-related trend, where males tended to consume more maneuverable non-tympanate moths, as opposed to the less maneuverable moths consumed by females. However, this second RLQ axis only accounted for less than 5% of the total variability.
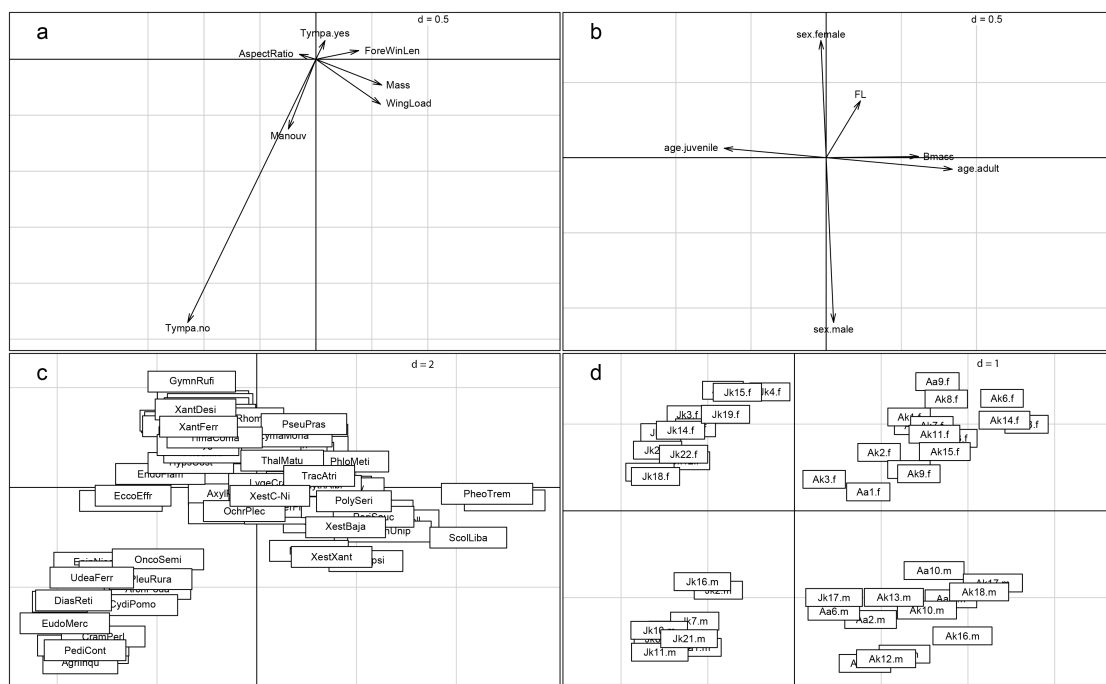
Figure 5.3. Results of the first two axes of RLQ analysis for adult and juveniles bats of post-breeding season: a) coefficients for prey traits, b) for bat traits, c) eigenvalues and scores of prey species, and d) of individual bats. The "d" values give the grid size. Codes for prey species and bat individuals are available in supplementary material S5.1.

The fourth-corner analysis found some of the trends observed for the RLQ analysis to be correlated. Results for this test are provided in the supplementary material S5.3.

The combined RLQ and fourth-corner analysis show that bats' age and biomass and moths' mass and wing loading were significantly associated to the first RLQ axis (Fig. 5.4). Adults (i.e. bats with higher body mass) were significantly consuming heavier and faster moths than juveniles, who were significantly eating lighter and slower moths. Thus, there was a significant functional difference between the prey-type consumed by bats in relation to their   ontogeny   and   body   mass.
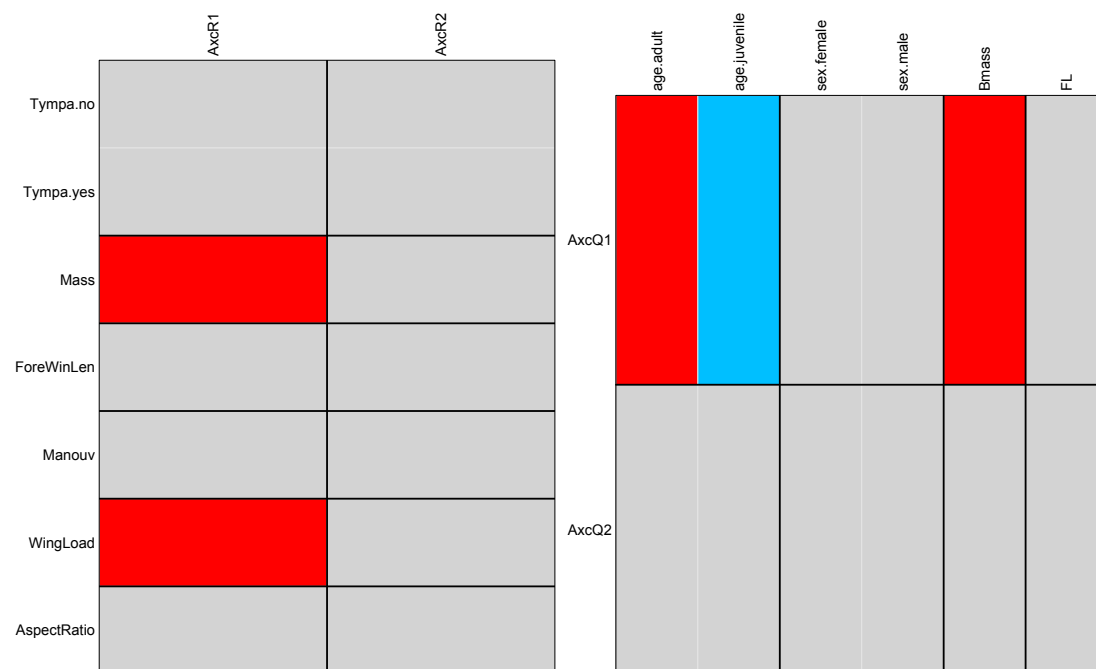
Figure 5.4. Combination of RLQ and fourth corner results for adult and juvenile bats of the post-breeding season and prey traits relationships. Left: Fourth-corner test between the first two RLQ axes for bat characteristics (AxR1/ AxR2) and prey traits. Right: Fourth-corner test between the first two RLQ axes for prey traits (AxQ1/ AxQ2) and bat characteristics. Red cells mark positive significant associations, and blue cells mark negative ones (significance level: p < 0.05). Variables with non-significant association are shown in grey. Black lines

separate different variables; white lines separate different modalities for categorical variables. *P* values were adjusted for multiple comparisons using the FDR procedure. Codes for traits and bat characteristics are available in supplementary material S5.1.

5.3.5. Potentially available functional moth-assemblage

The first two axes of the RLQ ordination account for 70.12% and 27.15% of the total co-inertia between seasons and moths' traits respectively. The first axis identifies, on one hand, tympanate species with higher mass and longer forewings (e.g. *Phalera bucephala*, *Pheosia tremula*; left part of Fig. 5.5a/c), mostly found in May (Fig. 5.5b). On the other hand, it identifies non-tympanate species with higher aspect-ratio (i.e. narrower forewings), low body mass and higher maneuverability, mainly found in Karrantza in July but also in September. These species are micro-moths of families Crambidae (*Agriphila inquinatella*, *Scoparia basistrigallis*), Pyralidae (*Oncocera semirubella*), Tortricidae (*Syndemis musculana, Zeiraphera isertana*), Yponomeutidae (*Yponomeuta plumbella*) and Tineidae (*Tinea* sp). The second RLQ axis identifies species with high values of both wing loading and maneuverability mainly found in September: Noctuidae (*Mythimna*, *Xestia* and *Noctua* species), Erebidae (*Phragmatobia fuliginosa*) and Lasiocampidae (*Malacosoma neustria*). It also identifies medium sized moths with both low values of mass and wing loading (i.e. slow and light fliers), mainly found in July in both valleys but especially in Lea-Artibai; they were mainly Geometridae such as *Eupithecia* and *Xanthorhoe* species, *Gymnoscelis rufifasciata*, *Lobophora halterata*. Many species showed intermediate values for the measured traits and were found across different seasons and locations. Overall, the RLQ analysis illustrates changes of the community of moths at both the taxonomical and functional level through seasons, and between locations within a single season.
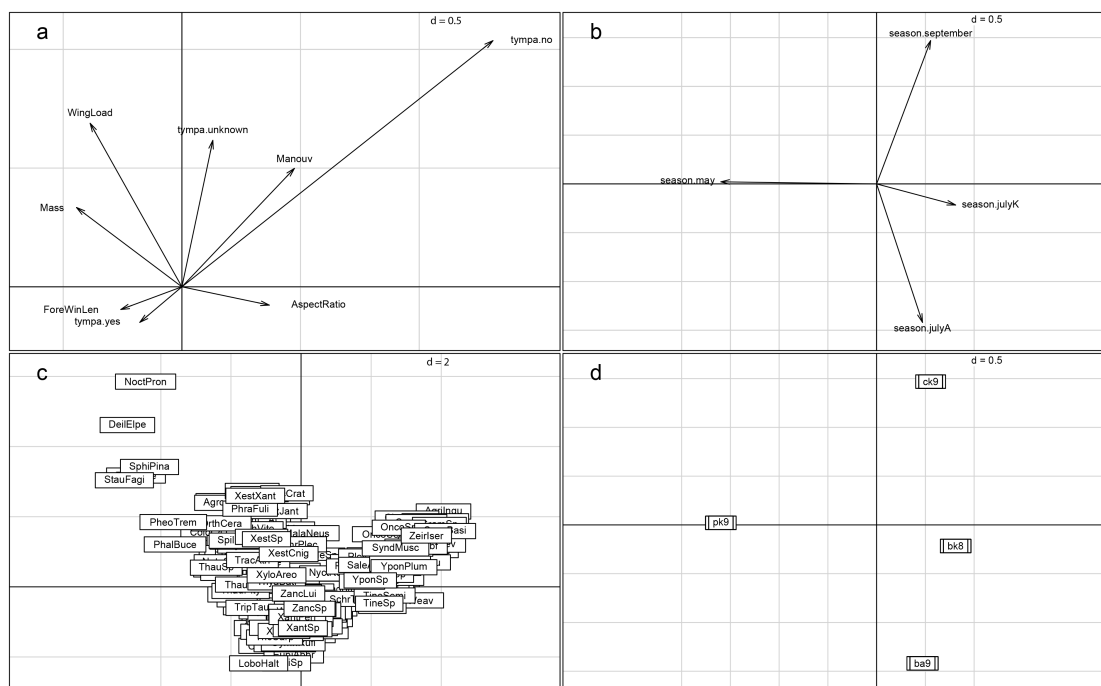
Figure 5.5. Results of the first two axes of RLQ analysis for the potentially available moth community: a) coefficients for moth traits, b) light-traps, c) eigenvalues and scores of moth species, and d) of light-traps. The "d" values give the grid size. Codes for prey species are available in supplementary material S5.1 and S5.2.

The fourth-corner analysis found some of the trends observed for the RLQ analysis to be correlated. Results for this test are provided in the supplementary material S5.3.

RLQ and the fourth-corner analyses combined found some correlations previously detected in both analyses (Fig. 5.6). Tympanate nature, mass, maneuverability and wing loading were significantly correlated with the first RLQ axis, as well as with May and July in Karrantza. In May we captured significantly more tympanate species characterized by heavier body, faster flight and lower maneuverability (i.e. larger species with a powerful and fast flight), contrary to what was observed in July in Karrantza (i.e. smaller species with slower flight and higher maneuverability). The second axis was positively correlated with September and negatively with Lea-Artibai in July. Associated traits are higher wing loading values for September (i.e. faster fliers) and the

opposite significant trend for Lea-Artibai in July (i.e. slow fliers and light bodied moths). Overall, our results show that the moth assemblage significantly changed both taxonomically and functionally through seasons and locations.
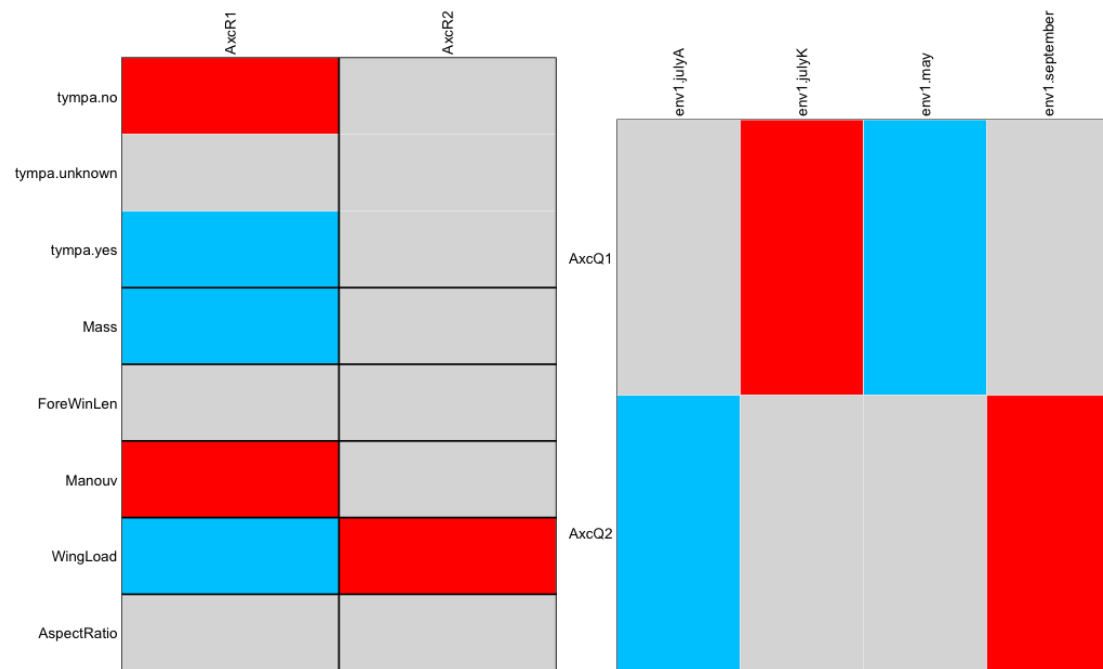
Figure 5.6. Combination of RLQ and fourth corner results for moths' traits and environmental variables. Left: Fourth-corner test between the first two RLQ axes for environmental variables (AxR1/ AxR2) and moth traits. Right: Fourth-corner test between the first two RLQ axes for moth traits (AxQ1/ AxQ2) and environmental variables. Red cells mark positive significant associations, and blue cells mark negative ones (significance level: $p < 0.05$). Variables with non-significant association are shown in grey. Black lines separate different variables; white lines separate different modalities for categorical variables. *P* values were adjusted for multiple comparisons using the FDR procedure. Codes for traits and environmental variables are available in supplementary material S5.1.

5.4. Discussion

To our knowledge, this is the first study investigating the functional foraging ecology of bats based on the combination of traits related to the profitability of prey. Our trait-based approach showed that the moth-specialist *R. euryale* consumed a wide range of moth types, the most widely consumed species presenting intermediate values for these traits. The potentially available moth assemblages functionally fluctuate across seasons, and this implies that moth-eating bats need to deal with such fluctuations. Accordingly, *R. euryale*'s functional diet significantly changed seasonally, shifting from small and maneuverable moths to heavy fast flyers or long winged ones. These results showed that *R. euryale* is trophically flexible enough to take profit of seasonally variable moth types. Both traditional and molecular approaches have hitherto limited the interpretation of bats' foraging ecology and their flexibility to study the species' richness and species' composition of their diets. We believe our functional approach provides a new viewpoint to assess the trophic niche of predators foraging on incredibly diverse and temporally variable prey taxa, as well as to analyze the evolutionary relationship between complex predator-prey systems such as those of bats and insects.

5.4.1. Trophic niche flexibility

Contrary to our predictions, *R. euryale* consumed a wide variety of moth types: from slow and maneuverable light moths to fast and heavy species. The echolocation system of *R. euryale*, as that of horseshoe bats in general, has been evolutionarily specialized to detect fluttering insects like moths in clutter (Lazure and Fenton, 2011; Schnitzler and Kalko, 2001). Correspondingly, this bat is morphologically adapted to perform the highly maneuverable and slow flight needed to forage in such clutter habitats (Norberg and Rayner 1987; Salsamendi et al. 2005). However, our results showed that *R. euryale* was not restricted to prey upon slow and maneuverable moths only, but consumed a variety of species significantly varying in "catchability", as defined by the combination of mass (i.e.

energetic content), wing loading (i.e. flight speed) and maneuverability (i.e. evasive flight capacity).

R. euryale hunted a range of species differing greatly in profitability, namely from the sphingid *Mimas tiliae* (mass: 455.8 mg, wing loading: 18.5 N m^{-2}, maneuverability: 0.62) to the pyralid *Salebriopsis albicilla* (mass: 8.6 mg, wing loa. ding: 4.17 N m^{-2}, maneuverability: 1.14). The former is a heavy and fast macro-moth likely flying fast in a regular path (i.e. predictable), whereas the later is a light micro-moth likely flying slowly but erratically. Considering that the consumption of each prey is the result of a series of actions that imply detection, decision-making, pursuing and capturing (Stephens and Krebs, 1986), a slow and maneuverable bat like *R. euryale* should likely approach those prey differently.

Apart from some extreme prey, the majority of consumed species were "standard" medium-sized moths such as Noctuidae, Geometridae and Erebidae. Yet, considerable differences in profitability may arise even among these "standard" moths. Consider, for instance, the widely consumed moths *Xestia c-nigrum* (Noctuidae) and *Idaea biselata* (Geometridae). Both are common and widespread species (Redondo et al., 2015; Robineau, 2007) that could be taken as representatives of the "typical" noctuid and geometrid moths. They do not differ greatly in forewing length (the trait traditionally used to assess moths' size; *X. c-nigrum*: 1.5 cm, *I. biselata*: 1.03 cm), but differ meaningfully in mass (71.4 to 5.2 mg, respectively), wing loading (9.1 to 1.5 N m^{-2}, respectively) and maneuverability (0.95 to 0.78, respectively). In summary, *X. c-nigrum* should be capable of performing faster changes in flight direction than *I. biselata*. This indicates that, even to approach the staple prey, *R. euryale* needs to deal with a diverse variety of moth types. Thus, the sole consideration of the forewing length seems to be functionally meaningless to assess the trophic niche of a single specialist predator. The reported trophic flexibility suggests that, to become a "moth-specialist", *R. euryale* had to achieve a wide range of adaptive hunting skills to successfully consume different types of moths.

In fact, the observed functional plasticity in diet may be related to the flexibility of prey-capture strategies reported for *R. euryale*, as well as for many other conspecific moth-eating horseshoe bats (Bontadina et al. 2002; Goiti et al., 2003; Neuweiler et al., 1987; Jones and Rayner, 1989; Russo et al., 2002; Siemers and Ivanova, 2004). *R. euryale* forages using different strategies such as continuous back and forth flights along edge structures and isolated trees, flying close to the canopy and diving into the foliage, and by perch hunting (Goiti et al., 2003; Russo et al., 2002), capturing moths by aerial hawking and vegetation-gleaning, or it may even be able to hunt by ground-gleaning (Siemers and Ivanova, 2004). The energy costs of such hunting strategies are very different and likely influence the bat's trophic niche (Voigt et al., 2010). Moreover, under laboratory conditions, flight cost affected the foraging energetics of *R. ferrumequinum* in foraging sessions with differing availabilities in prey profitability (Koselj et al., 2012). The flexibility of horseshoe bats in prey-capture techniques, the influence of flight energy costs in foraging energetics and the observed variation in the profitability of consumed species by *R. euryale* suggest that *R. euryale* may shift in the foraging strategies as prey types and abundance fluctuate in the environment. Probably balancing the cost of the foraging strategy and the energy gain, as reported for the conspecific *R. ferrumequinum* under laboratory conditions (Koselj et al., 2012). In this context, it would be interesting to functionally assess the foraging flexibility of other non-rhinolophid bat groups with frequency-modulated (FM) echolocation calls or those specialized in particular prey-capturing techniques (i.e. gleaning), in order to compare how different evolutionary strategies have shaped the trophic niche of bats.

5.4.2. Seasonality: moth assemblages and bats' diet

Trophic plasticity is an important adaptive feature of predators living in environments that vary either spatially or temporally (MacArthur and Pianka, 1966). It will determine the ability of animals to successfully adapt to rapid environmental changes. Most bats live in environments where the structure of habitats (e.g. plant phenology) and prey assemblages vary through time and location (e.g. López-Carretero et al., 2014; Summerville and Crist, 2003). In fact,

seasonality and locality greatly influence the diet composition of insectivorous bats (e.g. Clare et al., 2013; Goiti et al., 2008; Jones et al., 1990; Salinas-Ramos et al., 2015), or even the ecological requirements of prey species (Chapter 3). Little was hitherto known about the functional variability of prey, and its implications for bats' foraging ecology. Our light-trap captures showed that moth assemblages vary, not only taxonomically but functionally as well, across the spatiotemporal gradient within *R. euryale*'s foraging home-range. In particular, we observed a general transition from large and fast flier moths in May (i.e. pre-breeding) to slow fliers in July (i.e. breeding), and to both large fast fliers and small and highly maneuverable moths in September (i.e. post-breeding). Our results highlighted that mass, tympanate nature, maneuver capacity and wing loading were the key functional traits of moths varying across time and space. Our data provided the first insights about the qualitative spatiotemporal variation in prey profitability for moth eating bats.

Although we cannot accurately assess the changes in prey availability for *R. euryale* (Whitaker et al., 2009), our trait-based analyses identified that the bats foraged on significantly different moth types among seasons, where main differences were related to prey's energy content (i.e. mass) and flight performance (i.e. wing loading and maneuverability). We were not able to formally assess whether *R. euryale* preyed opportunistically or selectively upon moths, due to the limitations of DNA metabarcoding when quantifying prey consumption (Pompanon et al., 2012; Clare, 2014; Elbrecht and Leese, 2015). Nevertheless, the hypothetical relationship between prey availability and diet is worth discussing. Adult bats shifted from pursuing and capturing varying moth types in the pre-breeding season (i.e. May), to mainly hunting slow fluttering moths of low energy content in the breeding season (i.e. July), and fast and more evasive but energetically richer moths in the post-breeding season (i.e. September). The concordance of these trends with those observed in the potentially available moth assemblages suggests that bats foraged opportunistically at least in the pre-breeding (i.e. May) and breeding (i.e. July) seasons, likely following the functional qualitative fluctuations of prey. Adult moths usually have one or more short generations per year (i.e. voltinism;

Robineau, 2007). Considering the differences among the traits of many species, the taxonomic fluctuation in species and their generations might substantially influence the type of moths available for bats. For instance, *R. euryale* consumed the following moths according to their flying phenology: the noctuid *X. c-nigrum* is bivoltine with peak flight seasons in April-June and August-November, whereas the geometrid *I. biselata* is univoltine mainly flying in June-August (Robineau, 2007). Irrespective of the opportunistic or selective foraging behavior, our results highlight that seasonality greatly influences *R. euryale*'s functional trophic ecology beyond the locality or any other intraspecific variable.

5.4.3. Intraspecific functional differences

We expected to detect punctual intraspecific sexual differences in diet related to differing energetic requirements, especially during gestation or lactation for females (Kurta et al., 1989), and during mating season for males (post-breeding season; Dietz and Kalko, 2007). Although statistically significant differences were not observed between sexes or among individuals of different size, we observed a consistent tendency of females to forage more frequently on heavier and faster moths than males in all seasons and irrespective of the population. A similar difference —but statistically significant— has been reported between males and females of *Tadarida teniotis*, where the latter foraged on larger (i.e. higher energetic content) and migratory moths, opposed to males, likely reflecting gender segregation of foraging grounds (Mata et al., *In Press*). Sexual segregation of foraging habitats is widespread in bats (Senior et al., 2005), but untill the study of Mata et al. (*In Press*) no clear sexual segregation of diet has been described before. Lactating females of *R. euryale* travelled longer distances than males to foraging areas during the breeding season, even if they were using the same habitats as hunting grounds (Goiti et al., 2006). That was explained by the need of females to look for areas with smaller intraspecific competition, where they could get enough resources to fulfill their higher energy requirements (Goiti et al., 2006). However, the relationship between larger foraging ranges during the breeding season and the consistent tendency to consume larger and faster moths in all seasons, irrespective of the reproductive

condition is not clear. The tendency to consume heavier moths might reflect the higher energetic requirements of females during gestation (pre-breeding), lactation (breeding) and mating-season (post-breeding), where they should need to recover from depleted body reserves after the energetically demanding pre- and breeding seasons and start accumulating fat stores for hibernation (Kunz, 1998). Nevertheless, more data is needed to address any meaningful functional dietary differentiation between males and females in *R. euryale*.

In contrast, RLQ and the fourth corner analyses did identify significant differences between the main type of moths consumed by adults and juveniles in the post-breeding season. Adults consumed heavier and faster moth species more frequently, whereas juveniles mainly consumed lighter, slow and maneuverable micro-moths. Several studies on bats have reported that the diets of juveniles differ from that of adults: e.g. *Myotis lucifugus*, (Belwood and Fenton, 1976); *R. ferrumequinum*, (Jones, 1990; Ransome 1996); *Lasiurus cinereus* (Roselth et al., 1994); *Eptesicus fuscus* (Hamilton and Barclay, 1998); and *R. mehelyi* (Salsamendi et al., 2008). Some authors attributed ontogenetic diet and foraging habitat differences to the novelty of flight and echolocation of juvenile bats (Adams, 1996; 1997; Hamilton and Barclay, 1998; Rolseth et al., 1994). It is unlikely that the observed diet differences were related to a differential use of habitats, as juveniles use the same foraging habitat types as adults but closer to the roost (Goiti et al., 2006). However, prey profitability might differ between adults and juveniles due to differing skills in pursuing, capturing and handling prey (Hamilton and Barclay, 1998). Juveniles of *R. euryale* were significantly lighter than adult bats, but not significantly smaller, suggesting that their flight would be slower than that of adults (Adams, 1996). Thus, the hunting naivety of juveniles together with their slower flight performance would limit them to prey on the smaller and slower but more easily catchable moths, as the energetically richer but faster moths would be out of their reach. Adults, on the other hand, very likely selectively hunted the more profitable larger prey in the post-breeding season, while in the breeding season (when fast flier large moths were likely less abundant) they had to rely on preying upon smaller and slower moths,

most likely answering to availability.

5.4.4. Evolutionary arms-race relationship

The subsistence on moths requires a high degree of specialization (Rydell et al., 1995, Sierro and Arlettaz, 1997) because the majority of them are able to detect the echolocation calls emitted by most bat species and respond evasively (Roeder, 1967; Spangler, 1988; Rydell et al. 1995). *R. euryale* echolocates with peak frequency at ca 104 kHz (second harmonic; Dietz et al., 2009; Salsamendi et al., 2005), above the hearing capacity of most tympanate moths (Fullard, 1987), and hence, can presumably approach them without triggering any early evasive flight mechanisms. As predicted by the Allotonic Frequency Hypothesis (AFH; Fullard, 1988), its diet should be dominated by tympanate as well as non-tympanate moth species. In line with AFH, our results showed that the majority of consumed species were tympanate, namely Erebidae, Geometridae, Noctuidae and Pyralidae (Roeder, 1967; Spangler, 1988).

It is noteworthy that among tympanate taxa, the consumption of species of the subfamily Arctiinae (Erebidae) was apparently incidental, as we only detected two species in the diet of few bat individuals: *Diacrisia sannio* and *Spilosoma lutea*. However, we captured several species by light-traps, some of which were seasonally common: namely, *Eilema caniola, E. depressa, E. griseola, E. sororcula, Eilema sp., Euplagia quadripunctaria, Lithosia quadra, Miltochrista miniata, Phragmatobia fuliginosa, Spilosoma lutea,* and *S. lubricipeda*. Arctiine moths are most sensitive to frequencies between 30-50 kHz (Fullard, 1988), far below the peak frequency of echolocation calls of *R. euryale,* but close enough to their weaker first harmonic (ca 52 kHz; own data, Figure S5.2). Moreover, many of these moths are known to be toxic or produce anti-bat sounds (reviewed in Conner and Corcoran, 2012). Some species advertise their toxicity by producing ultrasonic "clicks" (aposematism; Hristov and Conner, 2005); other species mimic the anti-bat clicks produced by unpalatable species (Batesian mimicry; Barber and Conner, 2007), or use similar clicks to interfere with bats echolocation system and confuse them (sonar jamming; Corcoran et al., 2009). We are unaware of the batch of defensive mechanisms of the species identified in

our study area. However, the under-representation of arctiine moths in the diet of *R. euryale* suggests some effective level of protection developed against highly specialized moth-eating bats. This may explain why many arctiine species can afford to fly slowly and straight (Thomas Merckx's *personal observation*). Our results call for further research to assess the tuning of the hearing sensibility of these moths with the echolocation calls emitted by sympatric European bat species such as *R. euryale*. Similar to other moth species, some arctiine moths might have evolved ears sensitive to echolocation calls with peak frequencies higher than 60 kHz (Hofstede et al., 2013; Jacobs et al., 2008). Alternatively, they might be capable of detecting the weaker harmonics that many horseshoe bats emit at their hearing range (e.g. Jones and Rayner, 1989; Jones and Waters, 2000; Neuweiler et al., 1986).

On the other hand, many moth species are earless and do not apparently have any of the previously mentioned evasive or defensive mechanisms. Among these species two major groups can be outlined: large earless moths and small earless moths (Jones and Rydell, 2003), which might have evolved contrasting evasive mechanisms. The former (e.g. Lasiocampidae, Sphingidae) are significantly larger, have higher wing loadings and fly at higher body temperatures than the tympanate species (Rydell and Lancaster, 2000). This suggests that they are adapted for fast and erratic flight, an alternative adaptation to evade bat predation (Rydell and Lancaster, 2000). Similarly, Saturnidae is another family of large and earless moths, that, despite not being fast, their large size or their long hindwing tails might have evolved to avoid bats predation, either by size-constraints or by acoustic deflection facilitated by their long tails (e.g. luna moths, Barber et al., 2015). We occasionally captured some sphingids and lasiocampids by light traps, and no saturniid moth was ever captured. Moreover, we only detected one sphingid species in the diet of *R. euryale*. Considering the morphology and behavior of these moths, our observations suggest that large earless moths are not very abundant in the study area and are probably unprofitable —too large and/or too fast— for medium-sized and fluttering maneuverable bats such as *R. euryale*.

Finally, the majority of the remaining earless moths are small (e.g. Crambidae, Tortricidae). These families were considerably abundant in light-traps and were frequently consumed by *R. euryale,* especially by juveniles. Nevertheless, beyond their trophic relationship, it is noteworthy to mention that our trait-based approach identified that most of them have narrow forewings (i.e. high aspect-ratio) and proportionally very wide hindwings (i.e. high maneuver index). They seem to be considerably erratic flyers. In bats, high aspect-ratios are correlated with low maneuverability (Norberg and Rayner, 1987). However, our results showed that this might not be the case in small moths. The aerodynamics of insects, in particular of the smaller ones, is completely different to that of flying vertebrates (Norberg, 2002; Sane, 2003). This correlation should be considered in studies where aspect-ratio is used to infer flight-patterns in moths.

In summary, our prey-trait-based functional approach revealed a degree of trophic niche flexibility previously unidentified for a specialized moth predator: *R. euryale* hunted a wide variety of prey types across seasons or between ontogenetic stages, despite the fact that all prey were moths. Moreover, local moth assemblages significantly fluctuated both taxonomically and functionally across seasons. These findings could be achieved due to the high resolution level of DNA metabarcoding analysis for diet studies (Pompanon et al., 2012) and the development of RLQ and the fourth-corner methods to analyze the functional relationship between prey-traits and environmental/predator-characteristics (Dray et al., 2014). All prey are not equally profitable for predators (Spitz et al., 2014). Thus, the identification of key prey types for predators is the first step to successfully assessing their trophic niche. Trait-based approaches open new insights to understanding the foraging ecology, evolutionary relationships and conservation of animals (Green and Cote, 2014; Spitz et al., 2014), making them a powerful tool to identify and predict the spatiotemporal structure of complex predator-prey systems.

5.5. References

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CHAPTER 6

Concluding Remarks

This PhD thesis shows the research carried out on the foraging ecology of the horseshoe bat *Rhinolophus euryale*. It provides further knowledge: first, about the trophic relationship between *R. euryale* and the landscape it inhabits through its prey's ecological requirements; second, about the degree of food partitioning between *R. euryale* and its sibling *R. mehelyi*; and third, about the trophic niche flexibility of *R. euryale* regarding the functional characteristics of their main moth-prey.

6.1. Methodological observations

Before listing the main conclusions obtained in this PhD thesis, I will first make some methodological observations about DNA metabarcoding. Finally, I list the main conclusions obtained in this PhD thesis.

The results obtained in this thesis highlight that DNA metabarcoding is a highly useful tool to infer ecological patterns in bats. However, this technique is not exempt from problems when applying to the analysis of bat feces:

1.   The DNA extraction and PCR amplification of more than 250 faecal samples (some not included in this thesis) showed that the improper storage of feces in the field directly affect on the DNA extraction success (e.g. stored wet and in closed Eppendorf tubes, bad quality ethanol). The complete drying of feces and direct freezing seem to be the best preservation methods for a successful extraction and amplification.

2.   The feces of some bat species such as *Rhinolophus hipposideros* or some *R. mehelyi* showed signals of PCR inhibition with very low rates of successful amplification. This failure in DNA amplification could be due to chemical compounds inhibiting the PCR, such as phenolic or aromatic plant compounds attached to insect bodies (Juanele García *personal comment*). These observations should be taken into account in the context of DNA extraction success of bat diet studies.

3. The two-PCR strategy used in Chapter 3 for Roche's 454 library preparation gave several problems related to the amplification of intermediate amplicon types. These amplicons included sequences extended only in a single end, primer dimmers, etc., and they do not considerably differ in length from the target amplicon type. Therefore, they can only be detected by running the PCR products in electrophoresis gels for a long time, or by quantifying by bioanalyzer (an expensive option). If not detected and filtered, these amplicons are due to cause the improper equimoralization of samples prior to sequencing. We suggest avoiding the two-PCR strategy, or taking precautions in the filtering of intermediate amplicons.

4. The difference in orders of magnitude among the sequencing output of different NGS platforms, their intrinsic sequencing error, and the arbitrariness of filtering steps along the bioinformatic analysis make incomparable the ecological results originated from different platforms. The methodological approach used in this thesis to choose the sequence coverage and MOTU clustering thresholds seems to be useful in order to overcome these problems.

5. Additionally, the approach taken in Chapter 6 to only consider sequences appearing in two PCR replicates from each DNA sample enhances the confidence about the quality of sequences used for ecological analyses. However, like for previous observations, further experimental research using controlled DNA mixtures is needed to assess the effectiveness of all these methodological measures.

6.2. Concluding Remarks

1. The larval host plants of a significant proportion of the moth prey likely occur outside the hedgerows and broadleaved forests where the adult moths are hunted by *R. euryale*. The herbaceous plant families Asteraceae, Gramineae, Leguminosae and Polygonaceae accounted for 48% of recorded larval host plants. Consequently, a large number of prey moths likely emerge from meadows and pastures, that is, from outside the foraging habitats of *R. euryale*.

2. There is a seasonal fluctuation in the ecological requirements of consumed moths' larvae. Moth species with larval requirements linked to *R. euryale*'s foraging grounds are more abundant in their diet during the pre- and breeding seasons. In contrast, during the post-breeding season the majority of the consumed moths rely on herbaceous plants from open habitats for their development. Therefore, the higher the diversity of the landscape is, the higher the bats' chances of fulfilling their foraging requirements are.

3. Any change to the habitats required by the larval stages of *R. euryale*'s prey moths would directly affect prey availability for *R. euryale.* These habitats include both *R. euryale*'s hunting and non-hunting grounds. Karrantza Valley is dominated by a traditional farmland landscape where large hedgerow networks are interspersed with forests, pastures and meadows. Therefore, conservation measures for one of the largest colonies of *R. euryale* in the northern Iberian Peninsula should be focused on the landscape-level rather than on the habitat-level.

4. In Karrantza Valley *R. euryale* consumes common moths as well as some potential pest species such as *Agrotis* sp., *Autographa gamma*, *Mythimna unipuncta* and *Thaumetopoea pytiocampa*, suggesting that *R. euryale* could be and effective pest consumer. Assessing the intensity of such interaction deserves further research.

5. Sibling *R. euryale* and *R. mehelyi* are both specialized to forage on moths, and in sympatric populations their dietary niche dimensions highly overlap due to the consumption of the same moth species.

6. Most of those moths are middle-sized, common and widespread species and constitute important components of their diet, at least during the breeding season.

7. Management and conservation guidelines for sympatric *R. euryale* and *R. mehelyi* populations —and for other moth specialist bat species as well— should include the conservation of those common moth species.

8. There are also small but significant dietary differences between *R. euryale* and *R. mehelyi* regarding prey species and prey size. These differences are unlikely related to subtle morphological and echolocation call-related differences. Instead, dietary differences mirror the spatial segregation observed by radio-tracking data in previous studies. Moth species related to clutter habitats are mainly consumed by *R. euryale*, whereas species related to open woody habitats are mainly consumed by *R. mehelyi*.

9. The more frequent consumption of smaller moths by *R. mehelyi* is likely related to the vertical segregation of moth species in the space, rather than to differences in size-range detection capabilities.

10. There is no apparent niche displacement in the diet dimension from allopatric populations of *R. euryale* to sympatric populations of *R. euryale* and *R. mehelyi*. Based on the spatial niche displacement directly measured for allopatric and sympatric populations of *R. euryale* and *R. mehelyi* in previous studies, and the indirect evidence of such displacement inferred by molecular dietary results, the coexistence of sibling *R. euryale* and *R. mehelyi* is mainly mediated by the partitioning of the spatial niche dimension.

11. Potentially available moth assemblages significantly fluctuate both taxonomically and functionally through seasons. The traits of moths related to profitability for bats—namely mass, wing-loading, maneuverability and tympanate nature—vary seasonally. Therefore, moth profitability for bats inhabiting Karrantza and Lea-Artibai Valleys varies seasonally.

12. The type of moths consumed by *R. euryale* significantly change seasonally as well, irrespective of bats' sex, age or location. Prey moths shift from small and maneuverable species to heavy fast flyers or long winged ones. These results show that *R. euryale* is trophically flexible enough to take profit of seasonally variable moth types. The observed trophic plasticity could be related to the flexibility of prey-capture strategies reported for *R. euryale* in previous studies. *R. euryale* may shift in the foraging strategies as the profitability of prey types and abundance fluctuate in the environment; probably balancing the cost of the foraging strategy and the energy gain.

13. The moths' traits significantly linked with the diet of *R. euryale* are mass (surrogate of energy content), wing-loading (surrogate of flight speed) and maneuverability. The forewing length of moths does not seem to be functionally informative to assess the trophic niche flexibility of *R. euryale*, and by extension, of other insectivorous bats.

14. In the post-breeding season, juveniles of *R. euryale* prey upon light and slow moth species more frequently than adults. Juvenile bats are themselves significantly lighter than adults as well, and hence, probably slower. The hunting naivety of juveniles together with their slower flight performance would limit them to prey on the smaller but slower, and therefore more easily catchable moths, whereas the energetically richer but faster moths would be out of their reach. Adults, on the other hand, very likely hunt selectively the more profitable larger prey, as the ecological moth availability is the same for both age categories.

15. In line with the allotonic frequency hypothesis, the majority of consumed species are tympanate moths, namely Erebidae, Geometridae, Noctuidae and Pyralidae.

16. In contrast, the tympanate subfamily Arctiinae seems to be under-represented in the diet of *R. euryale*, a bat species supposedly echolocating above the hearing capacity of these moths. This data suggests some effective level of protection developed against this highly specialized moth-eating bat. We hypothesize two mechanisms, namely: 1) some arctiine moths might have evolved ears sensitive to echolocation calls higher than 60 kHz; 2) some arctiine moths might be capable of detecting the weaker harmonics that many horseshoe bats like *R. euryale* emit at their hearing range. Further research is needed to enlighten these issues.

APPENDIX - SUPPLEMENTARY MATERIAL

Supplementary Material - Chapter S2

S2.1. Bioinformatic pipelines for the analysis of NGS sequencing data.

```
# i) Step:

# 1. Quality filtering of sequences
# tool: fastq_quality_filter [-h] [-v] [-q N] [-p N] [-z] [-i INFILE] [-o OUTFILE]
#
# quality threshold = 20
#

/Users/bioinformatic_tools/bin/fastq_quality_filter -q 20 -p 90 -Q 33 -i
/Users/1_raw_data/diet.fastq -o /Users/2_qual_filtered/qual_filt_diet.fastq

# 2. fastq to fasta convertion
# tool: fastq_to_fasta

 /Users/bioinformatic_tools/bin/fastq_to_fasta -v -Q 33 -i
/Users/2_qual_filtered/qual_filt_diet.fastq -o /Users/3_fastq_to_fasta/qual_filtered_dieta

# 2.1. fasta width modification: one sequence in one line

/Users/bioinformatic_tools/bin/fasta_formatter -i
/Users/3_fastq_to_fasta/qual_filtered_diet -o /Users/3_width_formatter/qual_diet.fasta
-w 0


# 3. Splitting sequences into Forward MIDs
# tool: fastx_barcode_splitter.pl

cat /Users/3_width_formatter/qual_filtered_diet.fasta |
/Users/bioinformatic_tools/bin/fastx_barcode_splitter.pl --bcfile
/Users/MIDs/ForwardMID.txt --prefix /Users/4_individual_F_MID/ --suffix .fasta  --bol -
-mismatches 1

# 4. Reverse-Complementary of Forward-MID groups
# Tool: fastx_reverse_complement
# usage: fastx_reverse_complement [-h] [-r] [-z] [-v] [-i INFILE] [-o OUTFILE]

for i in {1..n}
do
        if [ -f "/Users/4_individual_F_MID/MID${i}F.fasta" ]
        then
 /Users/bioinformatic_tools/bin/fastx_reverse_complement -i
/Users/4_individual_F_MID/MID${i}F.fasta -o
/Users/5_RevCom_F_MID/MID${i}F_RevCom.fasta
        fi
done

# 5. Splitting the reverse-complemetary of F into R MIDS

for i in {1..n}
do
```

mkdir /Users/6_indivi_R_MID/${i}F_R

      if [-f "/Users/5_RevCom_F_MID/MID${i}F_RevCom.fasta"]
      then

echo "Splitting MIDs"
 cat /Users/5_RevCom_F_MID/MID${i}F_RevCom.fasta |
/Users/bioinformatic_tools/bin/fastx_barcode_splitter.pl --bcfile
/Users/MIDs/ReverseMID.txt --prefix /Users/6_indivi_R_MID/${i}F_R/MIDF${i}_ --
suffix .fasta --bol --mismatches 1
fi
done

6. cliping the end of sequences. Remember: we have the ReverseComplementary of the
sequences, so: 3' -- 5' . Here we are cliping the '5 end
#Forward Primer reverse complimented: CCAAAAATAAAATATAAWGTTCCAATATCT
#Tool: fastx_clipper
#fastx_clipper [-h] [-a ADAPTER] [-D] [-l N] [-n] [-d N] [-c] [-C] [-o] [-v] [-z] [-i INFILE] [-
o OUTFILE]
For example: 1F

/Users/bioinformatic_tools/bin/fastx_clipper -a
CCAAAAATAAAATATAAWGTTCCAATATCT -d 0 -c -n -v -i
/Users/6_indivi_R_MID/${i}F/MID1F_MID${i}R.fasta -o
/Users/7_clip_Forward/${i}F_R/MID1F_MID${i}R.fasta

7. Make Reverse Complementary of sequences
#Tool: fastx_reverse_complement
usage: fastx_reverse_complement [-h] [-r] [-z] [-v] [-i INFILE] [-o OUTFILE]

 /Users/bioinformatic_tools/bin/fastx_reverse_complement -i
/Users/7_clip_Forward/${i}F_R/MID${i}F_MID${j}R.fasta -o
/Users/8_RevCom/${i}F_R/MID${i}F_MID${j}R_RevCom.fasta

8. cliping the end of sequences: what we have now is 5' -- 3' . Here we are cliping the 3'
end
#Reverse Primer reverse-complimented: GGAGGATTTGGWAATTGATTAGTW
#Tool: fastx_clipper
#fastx_clipper [-h] [-a ADAPTER] [-D] [-l N] [-n] [-d N] [-c] [-C] [-o] [-v] [-z] [-i INFILE] [-
o OUTFILE]

 /Users/bioinformatic_tools/bin/fastx_clipper -a GGAGGATTTGGWAATTGATTAGTW -d
0 -c -n -v -i /Users/8_RevCom/${i}F_R/MID${i}F_MID${j}R_RevCom.fasta -o
/Users/9_allClipped/${i}F_R/MID${i}F_MID${j}R_clipped.fasta

9. Filtering sequences by length : only interested in 156-158 bp

#Tool: prinseq-lite -range_len

perl /Users/bioinformatic_tools/prinseq-lite-0.20.4/prinseq-lite.pl -verbose -fasta
/Users/9_allClipped/${i}F_R/MID${i}F_MID${j}R_clipped.fasta -verbose -range_len 156-
158 -out_good /Users/10_filtered/${i}F_R/MID${i}F_MID${j}R_filt

10. Width modification to 0 of renamed individual files
#Tool: fastx toolkit binaries Fasta formatter

 /Users/bioinformatic_tools/bin/fasta_formatter -i
/Users/10_filtered/${i}F_R/MID${i}F_MID${j}R_filt.fasta -o
/Users/11_widthModification/${i}F_R/MID${i}F_MID${j}R_width.fasta -w 0

11. Collapse sequences into unique haplotypes
#Tool: fastx_collapser -h
usage: fastx_collapser [-h] [-v] [-i INFILE] [-o OUTFILE]

 /Users/bioinformatic_tools/bin/fastx_collapser -v -i
/Users/11_widthModification/${i}F_R/MID${i}F_MID${j}R_width.fasta -o
/Users/12_collapse/${i}F_R/MID${i}F_MID${j}R_collap.fasta

Jaione Arrizabalaga's script: identical.pl
Only used in Chapter 5
usage: perl identicals.pl --dir1 /Users/... --dir2 /Users/...

perl /Users/bioinformatic_tools/identicals.pl --dir1 /Users/14_identicals/replicateI --
dir2 /Users/14_identicals/replicateII

ii) Step:
12. MOTU clustering in MacQIIME

macqiime

#separate MOTUs
        pick_otus.py -i /Users/12_collapse/pkReu1 -o /Users/13_MOTU/ -s 0.97 --
uclust_otu_id_prefix ${k}_pkReu1_MOTU_

#find representative sequences
        pick_rep_set.py -i /Users/13_MOTU/pkReu1.txt -f /Users/12_collapse/pkReu1 -
m most_abundant -o /Users/13_MOTU/pkReu1_motu.fasta

#print statistics
awk 'END{ print "Number of MOTUs," NR }' /Users/13_MOTU/sim97/pkReu1.txt >
/Users/13_MOTU/pkReu1_stats.csv
        echo "MOTU,Number of sequences" >> /Users/13_MOTU/pkReu1_stats.csv
        awk '{ print $1 "," NF-1 }' /Users/13_MOTU/sim97/pkReu1.txt >>
/Users/13_MOTU/pkReu1_stats.csv

##

Supplementary Material - Chapter S3

a)

b)
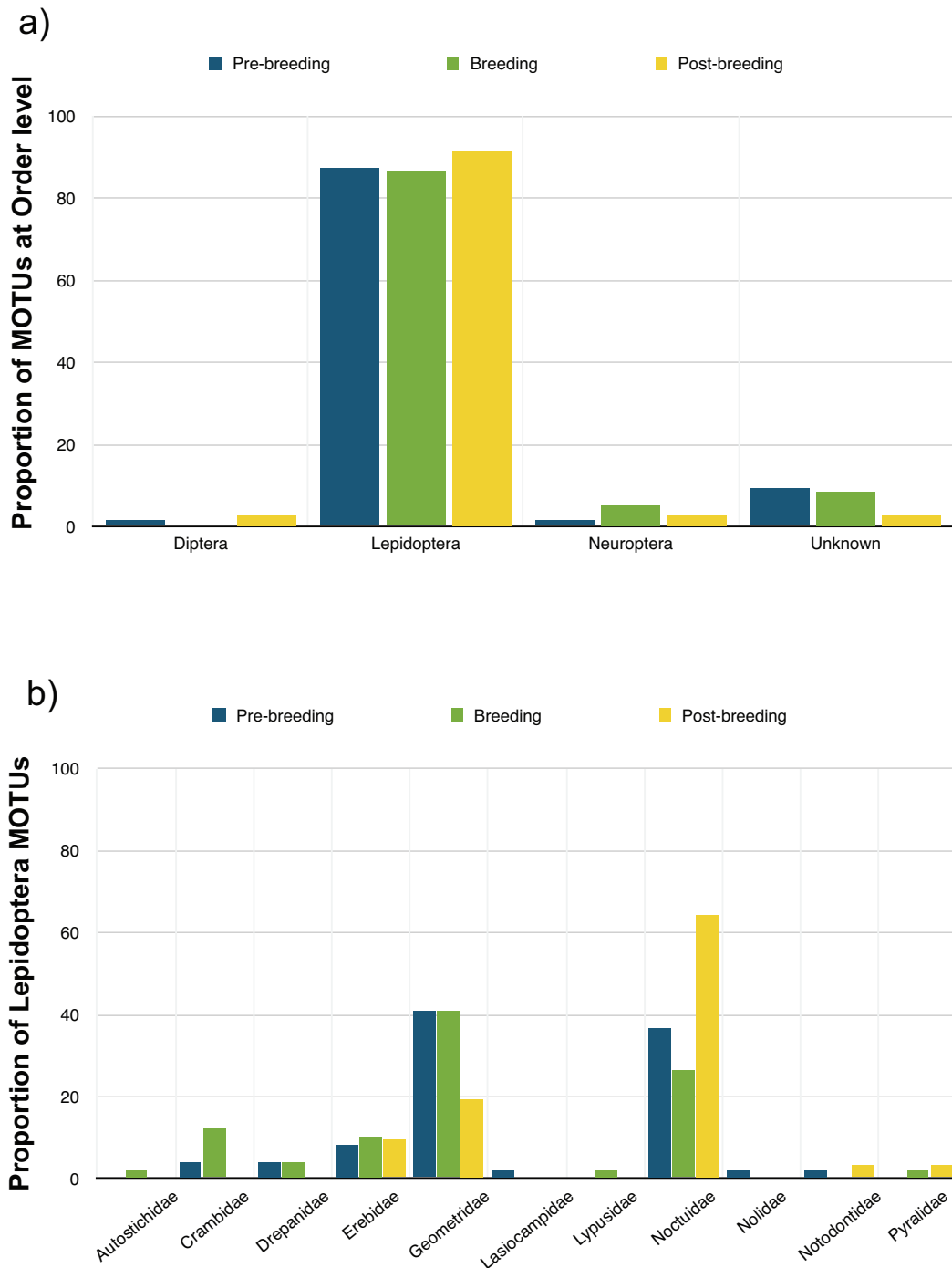
Figure S3.1. Seasonal diet of *R. euryale* at ordinal (a) and family level within Lepidoptera (b). Diet composition is presented as percentage frequency of

identified MOTU (%F) for the pre-breeding (blue bars, N = 19), breeding (green bars, N = 18) and post-breeding (yellow bars, N = 19) seasons.

Table S3.1. Prey taxa detected in the diet of *R. euryale*. Level refers to the confidence level of identification based on BLAST and taken from Clare et al. (2013), where level 1a = solid match to one or several species in a genus (100%); level 1b = good match, but could belong to a congener showing a higher sequence match (>98%); level 2 = match to more than one species belonging to different genera, only one of which is present in the sampling range (>98%); level 3 = match to several species of different genera within the same family or to reference sequences only identified to the family-level (>98%). Prey percentage of occurrence is given for pre-breeding (**PreB**), breeding (**B**) and post-breeding (**PostB**) seasons. **LF** indicates the larval feeding guild according to host plant life form: BT = Broadleaved Tree, C = coniferous, G = generalist, H = herbaceous, O = Other non-plant, S = shrub, - = No Data. **S** indicates the caterpillars' classification according to their host plant's likely occurrence in *R. euryale*'s foraging grounds: FS = Foraging Grounds, NFS-O = Non-Foraging Grounds -Open, NFS-C = Non-Foraging Grounds -Clutter, U = Ubiquitous. Potential migrant species are marked with **(M)**. MOTUs matching more than one species from which more than one of them might occur in the study area but are distinguishable due to flight phenology are marked with an *.

Order	Family	Genus/Species	Level	PreB(%)	Bree (%)	PostB(%)	LF	S
Diptera	limoniidae	*Limonia sp.*	1a	5,26				
		sp.	1a			5,26		
Lepidoptera	-	-	3		5,56		-	-
	-	-	3		5,56		-	-
	Autostichidae	*Apatema apolausticum*	1b		5,56		-	-
	Crambidae	*Crambus lathoniellus*	1b		11,11		H	U
		Crambus pascuella	2	10,53			H	NFS-O
		-	2		5,56		-	-
		Eudonia lacustrata	1b		5,56		O	NFS-O
		Eurrhypara hortulata	2		5,56		H	U
		Pleuroptya ruralis	1b		11,11		H	NFS-O
		Scoparia basistrigalis	2		16,67		O	U

Family	Species						
	Udea rubigalis	2	10,53			H	NFS-O
Drepanidae	*Ochropacha duplaris*	1b		5,56		BT	FS
	Thyatira batis	2	47,37	27,78		S	FS
	Watsonalla cultraria	1b	5,26			BT	FS
Erebidae	*Calliteara pudibunda*	1a		5,56		BT	FS
	Diacrisia sannio	1a		11,11		H	NFS-O
	Diaphora mendica	1b	5,26			H	NFS-O
	Hypena crassalis	1b		5,56		G	U
	Hypena proboscidalis	1b	5,26		10,53	H	NFS-O
	Lygephila craccae	2			5,26	H	NFS-O
	Lymantria monacha	1a		5,56		BT	FS
	Spilarctia luteum	1b	5,26	11,11		H	NFS-O
	Spilosoma lubricipeda	2	5,26			H	NFS-O
Geometridae	*Alcis repandata*	1b	42,11	5,56	10,53	BT	FS
	Asthena albulata	1b		5,56		BT	FS
	Cabera exanthemata	2	21,05	5,56		BT	FS
	Cabera pusaria	1b		11,11		BT	FS
	Cepphis advenaria	1b		5,56		BT	FS
	Cyclophora sp.	1b	26,32			BT	FS
	Cyclophora sp.	1b	5,26			BT	FS
	Cyclophora sp.	1b		5,56		BT	FS
	Cyclophora ruficiliaria	2	5,26			BT	FS
	Ectropis crepuscularia	1b	5,26			BT	FS
	Eulithis populata	2		5,56		BT	FS
	Hemithea aestivaria	1b		5,56		BT	FS
	Horisme radicaria	1b	15,79			O	FS
	Hypomecis punctinalis	1b	10,53			BT	FS

Family	Species						
	Idaea sp.	1b		11,11		H	NFS-O
	Idaea sp.				5,26	H	NFS-O
	Idaea biselata	1b		38,89	5,26	H	NFS-O
	Idaea degeneraria	2			5,26	H	NFS-O
	Idaea subsericeata	1b	5,26			H	NFS-O
	Lomaspilis marginata	1a		11,11		BT	FS
	Melanthia procellata	1b	15,79			O	FS
	Opisthograptis luteolata	1b	10,53	5,56		BT	FS
	Pachycnemia hippocastanaria	1a	5,26	11,11		S	NFS-O
	Paradarisa consonaria	1b	5,26	5,56		BT	FS
	Peribatodes rhomboidaria	1b	31,58	5,56	15,79	BT	FS
	Petrophora chlorosata	1a	31,58	33,33		H	U
	Plagodis pulveraria	1b	5,26			BT	FS
	Pseudoterpna	1b		11,11		S	NFS-O
	Scotopteryx luridata	2		5,56		S	NFS-O
	Selenia sp.	1b		5,56		BT	FS
	Selenia lunularia	1b	10,53			BT	FS
	Timandra comae	2			5,26	H	NFS-O
	Xanthorhoe designata	1b	5,26			H	NFS-O
	Xanthorhoe ferrugata	1b	21,05			H	NFS-O
	Xanthorhoe fluctuata	1b	5,26			H	NFS-O
	Xanthorhoe montanata	1b		11,11		H	NFS-O
Lasiocampidae	*Macrothylacia rubi*	1a	5,26			G	U
Lypusidae	*Pseudatemelia josephinae*	1b		5,56		O	FS
Noctuidae	*Abrostola sp.*	1b	15,79			H	NFS-O
	Acronicta rumicis	2	10,53		5,26	G	U
	Agrotis sp.	1b			21,05	H	NFS-O
	Agrotis exclamationis(M)	2	5,26	11,11	21,05	H	NFS-O

Species						
Agrotis ipsilon	2	5,26		15,79	H	NFS-O
Agrotis segetum	1b	5,26	5,56		H	NFS-O
Amphipyra pyramidea	1b			5,26	G	FS
Anaplectoides prasina	2		11,11	15,79	B T	FS
Apamea monoglypha	1b		5,56		H	NFS-O
Autographa gamma(M)	2		5,56	10,53	H	NFS-O
Axylia putris	1b		5,56	10,53	H	NFS-O
Charanyca trigrammica	2	5,26			H	NFS-O
Cosmia trapezina	1b		27,78		B T	FS
Craniophora ligustri	1b	5,26			B T	FS
Euplexia lucipara	2	5,26			G	U
Euxoa sp.	2			5,26	H	NFS-O
Hoplodrina ambigua	2	31,58	11,11	68,42	H	NFS-O
Lycophotia porphyrea	2		33,33	10,53	S	NFS-O
Macdunnoughia confusa(M)	2	10,53			H	NFS-O
Mythimna albipuncta(M)	1a	10,53			G	U
Mythimna unipuncta(M)	2	10,53		36,84	H	NFS-O
Mythimna vitellina(M)	1b	5,26		5,26	H	NFS-O
Ochropleura plecta	2	52,63	5,56	5,26	H	NFS-O
Orthosia gothica	2	5,26			G	FS
Peridroma saucia(M)	1b		11,11		H	NFS-O
Pheosia tremula	1b			5,26	B T	FS
Phlogophora meticulosa	1b	5,26		21,05	H	U
*Photedes minima**	3		44,44		H	NFS-O
Protodeltote pygarga	2	10,53			H	NFS-O
Thalpophila matura	1b			5,26	H	NFS-O
Trachea atriplicis(M)	2		16,67		H	NFS-O
Xestia baja	2			5,26	G	FS
Xestia c-nigrum(M)	2	21,05		21,05	H	NFS-O

Order	Family	Species						
		Xestia xanthographa	1b			5,26	H	NFS-O
		Zanclognatha tarsipennalis	2			5,26	S	FS
	Nolidae	*Pseudoips prasinana*	1b	21,05			B T	FS
	Notodontidae	*Drymonia sp.*	1b	5,26			B T	FS
		Thaumetopoea pityocampa	1a			5,26	C	NFS-C
	Pyralidae	*Acrobasis glaucella*	1b			5,26	B T	FS
		Eccopisa effractella	1b		5,56		B T	FS
Neuroptera	Chrysopidae	*Chrysoperla sp.*	1b		5,56		-	
		Chrysotropia ciliata	1b		16,67		-	
		Nineta flava	1b			5,26	-	
		Pseudomallada flavifrons	2		5,56		-	
	Hemerobiidae	*Wesmaelius sp.*	1b	10,53			-	

Table S3.2. Host plants and other food categories of moths' larvae identified in R. euryale's diet (Table S3.1). N = number of moth species reported to eat each host category.

	Family	species	N
Host plants	Aceraceae	*Acer*	4
		Acer campestre	2
		Acer platanoides	4
		Acer pseudoplatanus	1
	Alliaceae	*Allium ampeloprasum*	2
		Allium cepa	4
		Allium fistulosum	1
		Allium porrum	1
	Anacardiaceae	*Pistacia*	1
	Aquifoliaceae	*Ilex*	2
	Araceae	*Arum*	1
	Araliaceae	*Hedera*	5
	Asclepiadaceae	*Cynanchum vincetoxicum*	2
	Asparagaceae	*Asparagus*	3
		Asparagus setaceus	2
	Betulaceae	*Betula pubescens*	7
		Betulaceae	1
		Alnus	2
		Alnus glutinosa	6
		Betula	27
		Betula pendula	15
	Cannabaceae	*Humulus*	1
		Humulus lupulus	5
	Caprifoliaceae	*Sambucus*	1
		Sambucus racemosa	3
		Viburnum opulus	3
		Lonicera periclymenum	2
		Lonicera xylosteum	3
		Lonicera	4
		Lonicera caprifolium	1
	Caryophyllaceae	*Silene*	1
		Silene dioica	1
		Silene nutans	1
		Silene alba	1
		Caryophyllaceae	1
		Silene vulgaris	1
		Stellaria	3
		Stellaria media	3
	Chenopodiaceae	*Beta vulgaris*	11
		Chenopodium	3
		Chenopodium album	1
		Spinacia oleracea	8

Cistaceae	*Helianthemum nummularium*	2
Compositae	*Arctium tomentosum*	1
	Senecio	1
	Senecio nemorensis	1
	Artemisia vulgaris	3
	Senecio jacobaea	2
	Solidago	1
	Solidago virga-aurea	2
	Achillea	1
	Achillea millefolium	3
	Aster	1
	Calendula officinalis	3
	Carduus crispus	1
	Carduus nutans	1
	Carthamus tinctorius	1
	Centaurea jacea	1
	Centaurea phrygia	1
	Chrysanthemum	2
	Cichorium intybus	1
	Cirsium arvense	5
	Cirsium helenioides	1
	Cirsium palustre	1
	Cirsium vulgare	2
	Compositae	1
	Crepis	1
	Cynara cardunculus	1
	Cynara scolymus	4
	Helianthus annuus	4
	Hieracium	2
	Hieracium pilosella	2
	Hieracium umbellatum	5
	Lactuca	1
	Lactuca sativa	10
	Leontodon autumnalis	1
	Leucanthemum vulgare	1
	Matricaria	1
	Matricaria recutita	1
	Pulicaria	1
	Sonchus arvensis	2
	Sonchus asper	1
	Sonchus oleraceus	1
	Tagetes	1
	Tanacetum vulgare	3
	Taraxacum	13
	Taraxacum officinale	3
	Tussilago farfara	2

Convallariaceae	*Polygonatum odoratum*	1
Convolvulaceae	*Convolvulus*	3
	Convolvulus arvensis	1
	Calystegia	1
	Calystegia sepium	3
Cornaceae	*Cornus*	2
	Cornus sanguinea	2
Corylaceae	*Corylus*	9
	Corylus avellana	7
	Carpinus	1
Crassulaceae	*Umbilicus rupestris*	1
Cruciferae	*Alliaria petiolata*	1
	Brassica	3
	Brassica napus	3
	Brassica oleracea	10
	Brassica rapa	7
	Cruciferae	2
	Descurainia sophia	1
	Diplotaxis tenuifolia	1
	Erysimum	1
	Erysimum cheiranthoides	1
	Raphanus raphanistrum	2
Cucurbitaceae	*Citrullus lanatus*	1
	Cucumis sativus	2
	Cucurbita	1
	Cucurbita pepo	4
Cyperaceae	*Carex*	2
	Carex nigra	1
	Eleocharis palustris	1
Dennstaedtiaceae	*Pteridium aquilinum*	4
Dipsacaceae	*Knautia arvensis*	1
	Scabiosa	2
Ericaceae	*Vaccinium*	2
	Vaccinium myrtillus	16
	Erica	5
	Arctostaphylos	1
	Calluna	10
	Calluna vulgaris	10
	Rhododendron	3
Euphorbiaceae	*Euphorbia*	1
	Euphorbia polychroma	1
Fagaceae	*Quercus ilex*	3
	Castanea sativa	1
	Fagus	8
	Fagus sylvatica	4
	Quercus	25

		Quercus petraea	2
		Quercus robur	13
		Quercus rubra	1
	Filicopsida	sp	1
	Geraniaceae	*Geranium pratense*	1
		Geranium sanguineum	1
		Geranium silvaticum	1
		Pelargonium	1
	Gramineae	*Deschampsia*	1
		Deschampsia cespitosa	3
		Deschampsia flexuosa	2
		Calamagrostis	1
		Phleum	2
		Avena sativa	3
		Corynephorus	1
		Dactylis	2
		Dactylis glomerata	3
		Elytrigia repens	2
		Festuca	3
		Gramineae	24
		Hordeum vulgare	6
		Molinia	1
		Molinia caerulea	1
		Phleum pratense	1
		Phragmites australis	1
		Poa	3
		Secale cereale	4
		Sorghum	1
		Triticum	6
		Triticum aestivum	2
		Zea mays	9
	Grossulariaceae	*Ribes*	5
		Ribes rubrum	7
		Ribes uva-crispa	3
	Iridaceae	*Gladiolus*	2
		Gladiolus imbricatus	1
		Iris germanica	1
		Iris pseudacorus	1
		Iris sibirica	1
	Juglandaceae	*Juglans regia*	2
	Juncaceae	*Luzula*	2
		Luzula pilosa	2
	Labiatae	*Galeopsis*	3
		Galeopsis speciosa	2
		Galeopsis tetrahit	2
		Stachys sylvatica	1

		Labiatae	1
		Lamium	2
		Lamium album	1
		Lamium purpureum	1
		Marrubium	1
		Marrubium vulgare	1
		Melissa	1
		Mentha	2
		Mentha X piperita	2
		Stachys	1
		Thymus praecox	1
	Leguminosae	*Astragalus glycyphyllos*	1
		Astragalus	1
		Cicer arietinum	1
		Glycine max	4
		Lathyrus	2
		Lathyrus palustris	1
		Lathyrus pratensis	1
		Lotus	1
		Lupinus	3
		Lupinus angustifolius	1
		Medicago sativa	7
		Melilotus indica	1
		Phaseolus	3
		Pisum	1
		Pisum sativum	4
		Trifolium	8
		Trifolium hybridum	1
		Trifolium medium	1
		Trifolium pratense	2
		Trifolium repens	2
		Vicia	4
		Vicia cracca	3
		Vicia faba	2
		Vicia sylvatica	1
		Cytisus scoparius	1
		Ononis	1
		Ulex	3
		Ulex europaeus	1
	Liliaceae	*Lilium*	1
		Lilium martagon	1
	Linaceae	*Linum usitatissimum*	3
	Lythraceae	*Lythrum salicaria*	3
	Malvaceae	*Gossypium*	1
		Sida rhombifolia	1
	Mniaceae	*Mnium hornum*	1

	sp	2
Myricaceae	*Myrica gale*	1
Oleaceae	*Fraxinus*	4
	Fraxinus excelsior	4
	Ligustrum	2
	Ligustrum vulgare	2
Onagraceae	*Epilobium angustifolium*	7
Pedaliaceae	*Sesamum indicum*	1
Pinaceae	*Larix*	2
	Picea	1
	Picea abies	2
	Pinaceae	1
	Pinus	2
	Pinus sylvestris	1
Plantaginaceae	*Plantago*	7
	Plantago lanceolata	1
	Plantago major	8
	Plantago media	1
Polygonaceae	*Polygonum*	10
	Polygonum aviculare	5
	Polygonum calcatum	1
	Polygonum lapathifolium	3
	Polygonum sachalinense	1
	Polygonum undulatum	2
	Rumex	10
	Rumex acetosa	1
	Rumex acetosella	1
	Rumex crispus	8
	Rumex longifolius	1
Primulaceae	*Primula*	3
	Primula veris	2
	Lysimachia vulgaris	6
Ranunculaceae	*Aquilegia vulgaris*	1
	Ranunculus	2
	Ranunculus auricomus	1
	Anemone	3
	Ranunculus repens	1
	Clematis	5
	Clematis vitalba	2
Rhamnaceae	*Frangula alnus*	4
	Rhamnus cathartica	1
Rosaceae	*Filipendula ulmaria*	7
	Fragaria	5
	Fragaria vesca	1
	Geum urbanum	1
	Fragaria X ananassa	2

	Potentilla	2
	Potentilla palustris	2
	Rosa	9
	Rosaceae	3
	Sorbus torminalis	1
	Crataegus	10
	Crataegus intricata	1
	Crataegus laevigata	1
	Cydonia oblonga	1
	Malus pumila	18
	Malus sylvestris	2
	Prunus	6
	Prunus cerasus	3
	Prunus domestica	4
	Prunus padus	13
	Prunus persica	1
	Prunus spinosa	4
	Pyrus	3
	Pyrus communis	1
	Sorbus aucuparia	13
	Cotoneaster	1
	Rubus	14
	Rosa canina	1
	Spiraea X vanhouttei	1
Rubiaceae	*Galium*	8
	Galium boreale	1
	Galium mollugo	5
	Galium palustre	1
	Galium verum	8
Salicaceae	*Populus*	5
	Populus tremula	12
	Populus tremuloides	1
	Salix caprea	8
	Salix cinerea	3
	Salix purpurea	1
	Salix	25
	Salix aurita	5
	Salix repens	1
Saxifragaceae	*Saxifraga granulata*	1
Scrophulariaceae	*Digitalis*	1
	Antirrhinum majus	2
	Digitalis purpurea	1
	Linaria vulgaris	1
	Odontites verna	1
	Rhinanthus minor	1
Solanaceae	*Capsicum annuum*	2

		Lycopersicon	8
		Lycopersicon esculentum	3
		Nicotiana	6
		Petunia	1
		Solanum tuberosum	9
	Taxaceae	*Taxus*	1
	Tiliaceae	*Tilia*	6
		Tilia cordata	6
		Tilia platyphyllos	2
	Tropaeolaceae	*Tropaeolum majus*	2
	Ulmaceae	*Ulmus*	1
	Ulmaceae	*Ulmus glabra*	1
	Umbelliferae	*Angelica silvestris*	2
		Aegopodium podagraria	3
		Anthriscus	2
		Pimpinella	2
		Pimpinella saxifraga	1
		Apium graveolens	5
		Conium maculatum	1
		Daucus carota	5
		Heracleum sphondylium	1
		Petroselinum crispum	1
	Urticaceae	*Urtica*	10
		Urtica dioica	13
	Valerianaceae	*Valeriana*	1
		Valeriana officinalis	2
	Violaceae	*Viola*	1
		Viola canina	1
		Viola odorata	2
	Vitaceae	*Vitis*	8
	Woodsiaceae	*Athyrium filix-femina*	1
		Matteuccia struthiopteris	1
Other host	Fungi	*Fungi*	1
	leaf litter	-	2
	Insecta	*Bombus*	1
		Insecta	1
		Prays citri	1
		Pseudogonia rufifrons	1
		Vespa	1
	Lichen	*sp*	1
		Parmelia	1
		Peltigera	1

Supplementary Material - Chapter S4

Table S4.1. Prey identified in the diet of *R. euryale* (n=37) and *R. mehelyi* (n=34).

Level refers to the confidence level of identification (see Chapter 2).

MOTU	Order	Family	Genus/Species	Level	No. prey items	
					R. euryale	*R. mehelyi*
MOTU9	Coleoptera	Cerambycidae	*Arhopalus ferus*	1b	3	4
MOTU56	Diptera	Tachinidae	-	3	1	1
MOTU82		Anthomyiidae	*Hylemya sp.*	1a	0	1
MOTU87		Tachinidae	*Phryno vetula*	1a	1	0
MOTU90		Muscidae	*Neomyia cornicina*	1a	0	2
MOTU91			*Polietes lardarius*	1a	1	0
MOTU28		Calliphoridae	*Pollenia vagabunda*	1b	0	1
MOTU80	Lepidoptera	Blastobasidae	*Blastobasis phycidella*	1b	0	3
MOTU19		Cosmopterigidae	*Eteobalea intermediella*	1b	0	3
MOTU88		Crambidae	*Calamotropha paludella*	1b	1	1
MOTU59			*Metasia ophialis*	1b	1	0
MOTU68			*Ostrinia nubilalis*	2	1	3
MOTU66			*Udea ferrugalis*	2	0	1
MOTU74		Erebidae	*Catocala nymphaea*	2	2	0
MOTU61			*Catocala nymphagoga*	2	25	11
MOTU67			*Lygephila lusoria*	1b	2	0
MOTU43		Gelechiidae	*Teleiopsis sp.*	2	2	10
MOTU57		Geometridae	*Aplasta ononaria*	1b	2	0
MOTU76			*Camptogramma bilineata*	1a	4	1
MOTU48			*Ennomos quercaria*	2	7	1
MOTU18			*Gymnoscelis rufifasciata*	2	2	0
MOTU52			*Idaea ochrata/rufaria*	1b	2	0
MOTU31			*Pachycnemia hippocastanaria*	2	1	1
MOTU8			*Rhoptria asperaria*	1b	1	2
MOTU34		Lasiocampidae	*Malacosoma neustria*	1b	0	1
MOTU13		Lecithoceridae	*Eurodachtha canigella*	1b	0	1
MOTU42		Noctuidae	*Acronicta rumicis*	2	1	0
MOTU1			*Agrotis segetum/trux*	1b	12	9
MOTU35/55			*Agrotis ipsilon*	2	29	32
MOTU45			*Amphipyra tragopoginis*	1a	1	0
MOTU41			*Apamea monoglypha/sicula*	2	1	0
MOTU69			*Apamea arabs*	1b	2	0
MOTU3			*Autographa gamma/pulchrina*	1a	5	1
MOTU49			*Calophasia platyptera*	1a	11	5
MOTU33			*Cosmia trapezina*	1b	8	0
MOTU44			*Heliothis incarnata*	1b	0	1
MOTU78			*Mythimna albipuncta*	2	2	2
MOTU16			*Mythimna vitellina*	1a	0	2
MOTU26			*Peridroma saucia*	1b	6	8

MOTU5			*Polyphaenis sericata*	2	3	3
MOTU29			*Sesamia nonagrioides*	2	14	10
MOTU40			*Mythimna loreyi/sicula*	2	3	9
MOTU75		Nolidae	*Meganola strigula*	1a	1	0
MOTU81		Praydidae	*Prays fraxinella*	1b	1	1
MOTU85		Pterophoridae	*Crombrugghia laetus*	1a	0	2
MOTU86			*Emmelina monodactyla*	1a	0	1
MOTU30			*Stenoptilia zophodactyla*	1a	0	1
MOTU12		Pyralidae	*Pempelia palumbella*	3	5	7
MOTU6			*Ephestia mistralella*	1b	1	0
MOTU92			*Phycita sp.*	1a	2	0
MOTU58		Tortricidae	*Archips xylosteana*	2	0	2
MOTU37			*Cnephasia sp.*	1a	2	1
MOTU27			*Cnephasia sp.*	1b	1	1
MOTU22		Yponomeutidae	*Zelleria oleastrella*	1a	0	2
MOTU60	Neuroptera	Hemerobiidae	*Wesmaelius nervosus*	2	0	2
MOTU84		Chrysopidae	*Chrysoperla sp.*	1a	7	1
MOTU46			*Cunctochrysa albolineata*	1b	1	0
MOTU36			*Pseudomallada flavifrons*	1b	4	0
MOTU20		Myrmeleontidae	*Distoleon tetragrammicus*	1b	1	2
MOTU51	Orthoptera	Tettigoniidae	*Tessellana tessellata*	1a	1	0
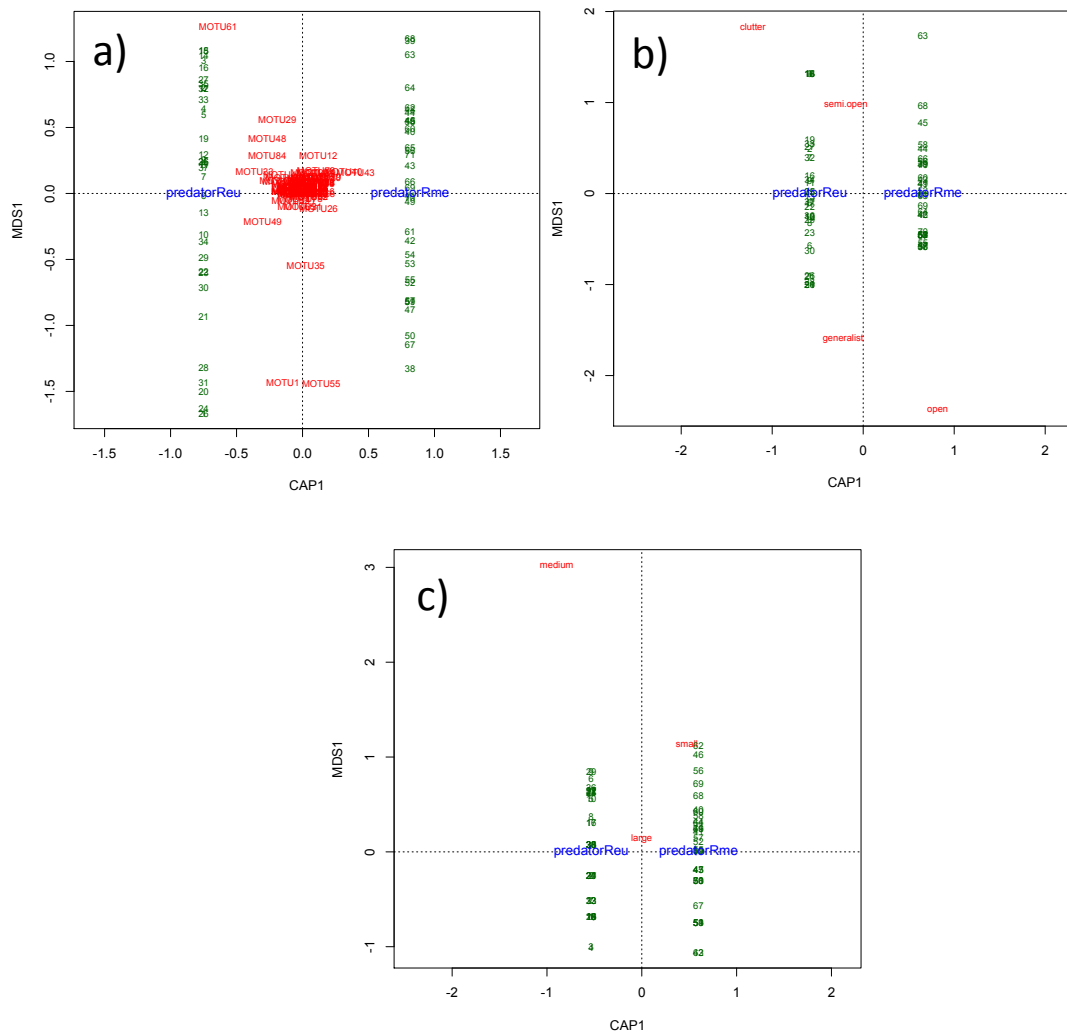
Figure S4.1. db-RDA based multivariate anova plot of dietary composition at a) MOTU, b) prey-habitat (clutter, semi-open, open, generalist) and c) prey size (large, medium, small) levels. X axis shows the constrained ordination of the variance by the explanatory variable "predator" (*R. euryale* or *R. mehelyi*) visualized by a Canonical Analysis of Principal coordinates (CAP). Y axis shows the first dimension of the unconstrained variance visualized by a Non-Metric Multidimensional Scaling (MDS). Prey MOTUs are shown in red, bat individuals in green, centroids of the explanatory variable "predator" in blue (Reu = *R. euryale*; Rme = *R. mehelyi*).
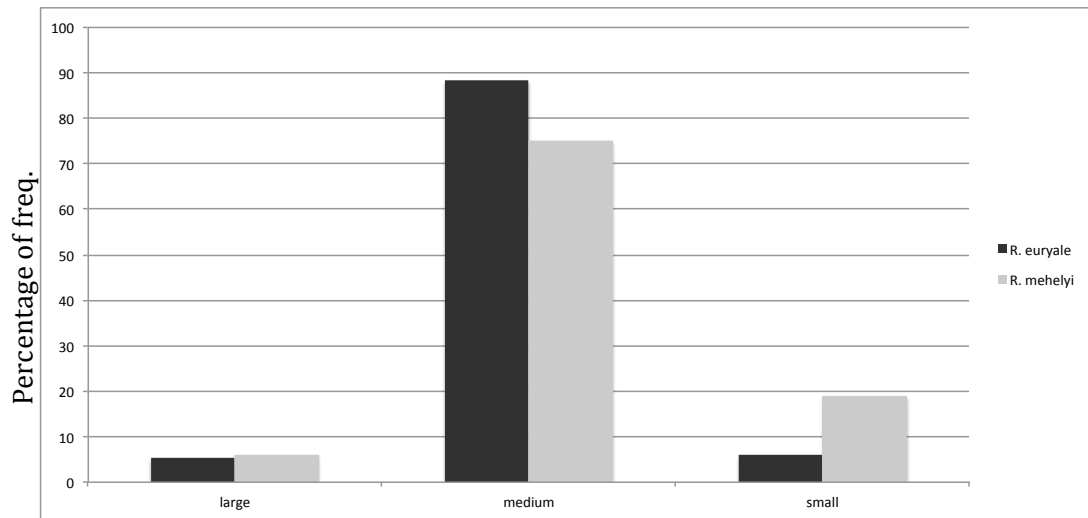
Figure S4.2. Percentage of frequency of moths identified in the diet of *R. euryale* (black) and *R. mehelyi* (grey) according to size.

Supplementary Material - Chapter S5

Figure S5.1. Illustration of wing anatomy and trait measurement procedure of moths. Forewing length was measured from the body-wing joining point to the tip of the wing (blue line). Forewing width, from the tip of the wing to the most distal point of the trailing edge (green line). Area of wings was measured by outlining the edge of both forewing and hindwings, and measuring the area therein.

S5.1. Information regarding the traits of the moth taxa identified in the diet of *R. euryale*: S5.1.1. Codes, description and units for moths' traits; S5.1.2. Codes, description and units for bats' traits; S5.1.3. Traits dataset of moth species included in the RLQ and fourth corner analyses (Q matrix); S5.1.4. Taxonomic information of all identified moth species (included and not included in the analyses), reference to abbreviations and number of analyzed specimens.

Table S5.1.1. Trait codes, description and units.

Moth Traits	Description
Mass	Fresh mass in grames (mgr)
ForeWinLen	Forewing length in cm
ForeWinArea	Forewing area in cm2
HindWinArea	Hindwing area in cm2
Manouv	Manouvrability = HindWinArea / ForeWinArea
WingLoad	Wing Loading (N/m2) = Mass(kg)*g / ForeWinArea (m2)
AspectRatio	Aspect Ratio = 4*(ForeWinLen)2 / ForeWinArea

Table S5.1.2. Codes, description and units for bats' traits.

Bat Traits	Description
Individual bat code - season	p = pre-breeding (May); b = breeeding (July); c = post-breeding(September)
Individual bat code - locality	K = Karrantza Valley; A or Aulesti = Lea-Artibai Valley
Age	sexual maturity of individuals, whether adult or juvenile
Sex	male or female
Mass (gr)	mass of individuals in grames
FL (mm)	forearm length of individuals in millimeters
Locality	locality where captures were performed: Karrantza Valley - Lea-Artibai Valley
Season	season of individual bat captures: May; July, September

Table S5.1.3. Traits dataset of moth species included in the RLQ and fourth corner analyses (Q matrix).

Abbreviation	Tympanate	Mass	ForeWinLen	ForeWinArea	HindWinArea	Manouv	WingLoad	AspectRatio
AbroTrip	yes	93.005	1.524	0.831	0.655	0.788	10.979	11.175
AcroPorp	yes	1.222	1.011	0.265	0.315	1.189	0.452	15.428
AcroRumi	yes	70.604	1.631	0.868	0.804	0.926	7.977	12.249
AediLeuc	yes	130.803	1.754	1.132	1.012	0.893	11.332	10.868
AgriInqu	no	13.200	1.176	0.343	0.445	1.298	3.781	16.152
AgroBigr	yes	165.040	1.831	1.181	1.017	0.861	13.709	11.350
AgroExcl	yes	134.460	1.418	0.726	0.620	0.855	18.179	11.091

AgroIpsi	yes	173.101	1.883	1.071	1.135	1.059	15.852	13.239
AgroNemo	no	4.400	0.929	0.241	0.194	0.805	1.791	14.324
AgroPuta	yes	96.872	1.517	0.876	0.818	0.933	10.848	10.501
AgroSege	yes	119.279	1.628	0.833	0.790	0.948	14.047	12.727
AlciRepa	yes	29.000	1.913	1.262	1.018	0.806	2.255	11.602
AnanHort	no	22.125	1.367	0.586	0.493	0.841	3.704	12.752
AnanLanc	no	10.250	1.403	0.512	0.413	0.807	1.964	15.378
AnapPras	yes	170.924	2.437	1.883	1.601	0.850	8.906	12.615
AngePrun	yes	35.160	2.183	1.737	1.572	0.905	1.985	10.976
ApamEpom	yes	110.895	1.967	1.270	0.950	0.748	8.566	12.190
ApamMono	yes	144.172	2.144	1.431	1.139	0.796	9.884	12.843
ApamScol	yes	79.100	1.500	0.755	0.593	0.785	10.278	11.921
AutoGamm	yes	117.777	1.850	1.158	1.071	0.925	9.980	11.826
AxylPutr	yes	41.320	1.367	0.547	0.549	1.004	7.416	13.679
CabeExan	yes	12.900	1.535	0.937	0.864	0.922	1.351	10.059
CabePusa	yes	21.150	1.414	0.746	0.607	0.814	2.781	10.721
CallPudi	yes	201.541	2.080	1.475	1.205	0.817	13.401	11.730
CampMarg	yes	31.480	1.785	1.077	0.907	0.842	2.866	11.835
CaraClav	yes	46.283	1.385	0.641	0.692	1.080	7.083	11.970
CaraMorp	yes	42.230	1.381	0.673	0.606	0.900	6.156	11.335
CataRubi	yes	15.349	1.321	0.607	0.464	0.764	2.481	11.499
CatoElec	yes	326.370	3.157	3.692	3.046	0.825	8.673	10.797
CeppAdve	yes	14.812	1.271	0.636	0.506	0.796	2.285	10.160
CeraRubr	yes	79.722	1.355	0.666	0.639	0.959	11.743	11.027
CharTrig	yes	119.048	1.607	0.980	0.896	0.914	11.917	10.541
CherFimb	yes	83.477	1.389	0.724	0.675	0.932	11.311	10.659
CherMult	yes	48.191	1.422	0.740	0.725	0.980	6.389	10.930
ChryCulm	no	13.867	1.008	0.305	0.354	1.161	4.460	13.325
ColoCory	yes	151.146	1.659	0.976	0.717	0.734	15.192	11.273
CosmOcel	yes	7.242	1.115	0.449	0.309	0.688	1.582	11.076
CosmTrap	yes	40.672	1.429	0.693	0.640	0.923	5.757	11.778
CramLath	no	4.900	0.913	0.234	0.253	1.081	2.054	14.249
CramPasc	no	11.633	1.104	0.303	0.369	1.215	3.763	16.077
CranLigu	yes	116.268	1.656	1.048	0.872	0.832	10.884	10.467
CrocDard	yes	84.967	2.029	1.517	1.211	0.798	5.495	10.855
CyclPunc	yes	11.700	1.573	0.821	0.651	0.793	1.398	12.055
DiacChry	yes	126.505	1.709	0.989	0.804	0.813	12.548	11.813
DiacSann	yes	61.423	2.088	1.671	1.671	1.000	3.605	10.435
DyssTrun	yes	20.800	1.514	0.798	0.636	0.797	2.557	11.490
EccoEffr	yes	4.200	0.658	0.117	0.127	1.085	3.522	14.802
EcliSila	yes	17.018	1.617	0.925	0.643	0.695	1.805	11.307
EctrCrep	yes	18.280	1.633	0.938	0.757	0.807	1.911	11.372
ElapVenu	yes	5.220	0.873	0.261	0.236	0.906	1.964	11.685
EndoFlam	yes	7.838	0.938	0.256	0.256	1.000	3.004	13.748
EpinNise	no	2.650	0.586	0.106	0.088	0.825	2.453	12.958
EpioRepa	yes	3.600	1.456	0.709	0.588	0.830	0.498	11.966

EudoLacu	no	3.100	0.791	0.179	0.191	1.067	1.699	13.982
EudoMerc	no	3.400	0.794	0.168	0.190	1.131	1.991	15.055
EuplLuci	yes	50.020	1.380	0.644	0.505	0.785	7.625	11.838
GeomPapi	yes	86.314	2.540	2.407	2.096	0.871	3.518	10.726
GymnRufi	yes	4.143	0.897	0.267	0.160	0.600	1.524	12.069
HabrPyri	yes	71.438	1.810	1.132	0.891	0.788	6.194	11.585
HadePerp	yes	83.894	1.324	0.607	0.475	0.783	13.558	11.552
HemiAest	yes	16.837	1.461	0.758	0.628	0.829	2.178	11.261
HermGris	yes	5.480	1.163	0.424	0.342	0.805	1.267	12.757
HermTars	yes	11.500	1.452	0.753	0.657	0.872	1.499	11.203
HermTars	yes	18.925	1.273	0.581	0.481	0.827	3.195	11.157
HoplAmbi	yes	75.350	1.404	0.657	0.588	0.895	11.259	12.006
HoriRadi	yes	17.435	1.456	0.749	0.524	0.700	2.284	11.321
HoriTers	yes	27.448	1.645	1.028	0.745	0.725	2.619	10.529
HydrFurc	yes	40.867	1.724	1.087	0.899	0.827	3.688	10.933
HylaFasc	yes	28.438	1.743	1.048	0.855	0.816	2.662	11.593
HypeCras	yes	22.400	1.336	0.729	0.689	0.945	3.014	9.794
HypeProb	yes	20.400	1.834	1.125	1.069	0.950	1.779	11.953
HypoPunc	yes	22.850	2.164	1.520	1.252	0.824	1.475	12.323
IdaeAver	yes	17.417	1.426	0.628	0.444	0.707	2.722	12.948
IdaeBise	yes	5.220	1.040	0.334	0.263	0.788	1.534	12.957
IdaeDege	yes	14.510	1.252	0.470	0.325	0.692	3.027	13.333
IdaeEuge	yes	10.461	1.199	0.493	0.353	0.716	2.082	11.664
IdaeSubs	yes	7.958	1.186	0.414	0.338	0.816	1.886	13.590
JodiLact	yes	4.977	1.145	0.443	0.387	0.874	1.102	11.838
LampOtre	yes	9.925	1.195	0.540	0.399	0.739	1.803	10.578
LomaMarg	yes	5.650	1.169	0.453	0.370	0.817	1.225	12.085
LomoTeme	yes	17.185	1.321	0.626	0.499	0.798	2.694	11.162
LycoEryt	yes	65.155	1.438	0.718	0.656	0.913	8.897	11.510
LycoPorp	yes	65.655	1.378	0.641	0.590	0.920	10.048	11.850
LygeCrac	yes	69.867	1.732	1.013	0.961	0.948	6.764	11.837
LymaMona	yes	60.540	2.058	1.525	1.228	0.805	3.895	11.110
MacdConf	yes	56.535	1.616	0.926	0.887	0.958	5.989	11.281
MecyAsin	no	11.725	1.296	0.462	0.417	0.903	2.492	14.555
MelaProc	yes	21.945	1.572	0.892	0.591	0.663	2.414	11.086
MenoAbru	yes	14.700	1.523	0.822	0.679	0.826	1.755	11.287
MimaTili	no	455.876	3.046	2.409	1.512	0.628	18.564	15.406
MythAlbi	yes	103.586	1.545	0.808	0.657	0.814	12.577	11.817
MythFerr	yes	163.328	1.701	0.998	0.908	0.910	16.055	11.597
MythUnip	yes	190.563	2.020	1.238	1.152	0.931	15.100	13.184
MythVite	yes	130.390	1.777	0.907	0.803	0.885	14.108	13.936
NolaConf	yes	12.130	1.008	0.351	0.278	0.792	3.390	11.579
OchrPlec	yes	50.209	1.236	0.523	0.530	1.015	9.425	11.689
OligStri	yes	17.400	1.211	0.505	0.418	0.828	3.378	11.610
OncoSemi	yes	22.760	1.241	0.389	0.526	1.352	5.743	15.834
OpisLute	yes	11.200	1.595	0.919	0.789	0.859	1.196	11.073

OrthGoth	yes	82.587	1.620	0.826	0.588	0.712	9.812	12.714
PachHipp	yes	16.200	1.474	0.621	0.536	0.863	2.559	14.001
ParaCons	yes	44.913	1.854	1.198	0.917	0.765	3.679	11.475
ParaPand	no	10.103	1.187	0.407	0.368	0.904	2.435	13.847
PeriAlch	yes	5.573	0.868	0.297	0.211	0.712	1.844	10.158
PeriRhom	yes	22.440	1.902	1.191	0.945	0.794	1.849	12.152
PeriSauc	yes	176.112	2.129	1.398	1.300	0.930	12.358	12.969
PetrChlo	yes	17.600	1.544	0.821	0.699	0.851	2.104	11.620
PheoTrem	yes	289.639	2.487	1.788	1.228	0.687	15.896	13.835
PhloMeti	yes	137.621	2.234	1.313	1.016	0.774	10.282	15.204
PhotMini	yes	16.779	1.187	0.493	0.436	0.885	3.340	11.438
PlagPulv	yes	33.290	1.755	1.118	0.877	0.784	2.920	11.020
PleuRura	no	24.440	1.502	0.666	0.606	0.909	3.598	13.541
ProtPyga	yes	12.980	1.069	0.415	0.353	0.851	3.070	11.016
PseuCoro	yes	32.992	1.425	0.741	0.646	0.873	4.370	10.972
PseuPras	yes	74.212	1.562	0.872	0.552	0.634	8.352	11.191
PtilCapu	yes	128.913	1.997	1.393	1.021	0.733	9.082	11.450
PyraAura	no	1.997	0.683	0.143	0.133	0.930	1.370	13.049
RheuUndu	yes	28.164	1.703	1.130	0.848	0.750	2.445	10.266
SaleAlbi	yes	8.600	0.848	0.202	0.231	1.144	4.177	14.240
ScolLiba	yes	270.648	2.028	1.449	1.183	0.816	18.323	11.351
ScopAmbi	no	4.860	0.883	0.190	0.226	1.189	2.509	16.415
ScopNigr	yes	21.667	1.439	0.687	0.551	0.802	3.094	12.057
SeleDent	yes	37.444	1.855	1.224	1.084	0.886	3.002	11.251
SeleLunu	yes	63.032	1.773	1.164	1.043	0.896	5.312	10.803
SpilLute	yes	87.968	1.699	0.991	0.865	0.872	8.708	11.657
ThalMatu	yes	71.300	1.799	1.121	0.956	0.853	6.240	11.548
ThyaBati	yes	66.132	1.781	1.050	0.909	0.866	6.181	12.088
TracAtri	yes	106.392	1.790	1.050	0.870	0.829	9.940	12.201
UdeaFerr	no	4.166	0.903	0.228	0.212	0.928	1.792	14.295
XantDesi	yes	6.100	1.193	0.477	0.354	0.742	1.255	11.935
XantFerr	yes	8.456	1.125	0.481	0.383	0.796	1.725	10.525
XantFluc	yes	9.567	1.376	0.683	0.546	0.799	1.374	11.089
XantMont	yes	13.620	1.484	0.838	0.644	0.768	1.594	10.512
XestBaja	yes	148.877	1.913	1.233	1.223	0.992	11.845	11.872
XestC-Ni	yes	71.391	1.578	0.770	0.739	0.959	9.095	12.942
XestXant	yes	128.800	1.517	0.832	0.880	1.058	15.187	11.064
ZancLuna	yes	17.016	1.334	0.587	0.522	0.891	2.846	12.141

Table S5.1.4. Taxonomic information of all moth species identified in the diet (included and not included in the analyses), reference to abbreviations, number of analyzed specimens for traits and the source of the specimens: no data (ND),

own data, Museum of Natural Science of Araba (ANZM), BOLD systems (BOLD) and Thomas Merckx's own collection (TM).

Family	Genus	Species name	Abbreviation	n_speci.	trait_source
Autostichidae	Apatema	apolausticum	ApatApol	0	ND
Crambidae	Agriphila	geniculea	AgriGeni	1	ANZM
	Agriphila	inquinatella	AgriInqu	1	own data
	Agrotera	nemoralis	AgroNemo	3	own data
	Anania	lancealis*	AnanLanc	1	own data
	Anania	hortulata	AnanHort	4	own data
	Chrysoteuchia	culmella	ChryCulm	3	own data
	Crambus	perlella	CramPerl	4	own data
	Crambus	lathoniellus	CramLath	3	own data
	Crambus	pascuella	CramPasc	3	own data
	Diasemia	reticularis	DiasReti	2	own data
	Eudonia	lacustrata	EudoLacu	1	own data
	Eudonia	mercurella	EudoMerc	1	own data
	Mecyna	asinalis	MecyAsin	5	own data
	Paratalanta	pandalis	ParaPand	2	own data
	Pediasia	contaminella	PediCont	1	BOLD
	Pleuroptya	ruralis	PleuRura	5	own data
	Pyrausta	aurata	PyraAura	1	own data
	Scoparia	ambigualis*	ScopAmbi	5	own data
	Udea	ferrugalis	UdeaFerr	3	own data
Drepanidae	Habrosyne	pyritoides	HabrPyri	5	own data
	Thyatira	batis	ThyaBati	4	own data
Elachistidae	Elachista	bifasciella	ElacBifa	1	own data
Erebidae	Calliteara	pudibunda	CallPudi	3	own data
	Catocala	electa	CatoElec	3	ANZM
	Diacrisia	sannio	DiacSann	4	ANZM
	Dysgonia	algira	DysgAlgi	1	own data
	Herminia	grisealis	HermGris	5	own data
	Herminia	tarsicrinalis	HermTars	3	own data
	Herminia	tarsipennalis	HermTars	4	own data + BOLD
	Hypena	crassalis	HypeCras	1	own data
	Hypena	proboscidalis	HypeProb	1	own data
	Laspeyria	flexula	LaspFlex	2	own data
	Lygephila	craccae	LygeCrac	3	own data
	Lygephila	pastinum	LygePast	1	own data
	Lymantria	monacha	LymaMona	5	own data
	Scoliopteryx	libatrix	ScolLiba	4	own data
	Spilosoma	lutea	SpilLute	5	own data
	Zanclognatha	lunalis	ZancLuna	4	own data
Geometridae	Alcis	repandata	AlciRepa	3	own data
	Angerona	prunaria	AngePrun	5	own data
	Cabera	exanthemata	CabeExan	1	own data

Cabera	pusaria	CabePusa	3	own data
Campaea	honoraria	CampHono	2	own data
Campaea	margaritaria	CampMarg	5	own data
Camptogramma	bilineata	CampBili	3	own data
Catarhoe	rubidata	CataRubi	1	own data
Cepphis	advenaria	CeppAdve	1	ANZM
Cosmorhoe	ocellata	CosmOcel	1	own data
Crocallis	dardoinaria	CrocDard	1	own data
Cyclophora	punctaria*	CyclPunc	1	ANZM + TM
Dysstroma	truncata*	DyssTrun	1	own data
Ecliptopera	silaceata	EcliSila	1	own data
Ectropis	crepuscularia	EctrCrep	5	own data
Epione	repandaria	EpioRepa	3	own data+BOLD
Geometra	papilionaria	GeomPapi	3	ANZM + TM
Gymnoscelis	rufifasciata	GymnRufi	2	own data+BOLD
Hemithea	aestivaria	HemiAest	5	own data
Horisme	radicaria	HoriRadi	1	ANZM
Horisme	tersata	HoriTers	1	ANZM + TM
Hydriomena	furcata	HydrFurc	3	own data
Hylaea	fasciaria	HylaFasc	5	own data
Hypomecis	punctinalis	HypoPunc	4	own data
Idaea	aversata	IdaeAver	5	own data
Idaea	biselata	IdaeBise	5	own data
Idaea	degeneraria	IdaeDege	5	own data
Idaea	dimidiata	IdaeDimi	1	own data
Idaea	eugeniata	IdaeEuge	2	own data
Idaea	subsericeata	IdaeSubs	1	own data
Jodis	lactearia	JodiLact	2	own data
Lampropteryx	otregiata	LampOtre	4	own data
Lomaspilis	marginata	LomaMarg	4	own data
Lomographa	temerata	LomoTeme	3	own data
Melanthia	procellata	MelaProc	3	own data
Menophra	abruptaria	MenoAbru	3	own data
Opisthograptis	luteolata	OpisLute	5	own data
Pachycnemia	hippocastanaria	PachHipp	3	own data
Paradarisa	consonaria	ParaCons	2	own data
Peribatodes	rhomboidaria	PeriRhom	5	own data
Perizoma	alchemillata	PeriAlch	4	own data
Petrophora	chlorosata	PetrChlo	5	own data
Plagodis	pulveraria	PlagPulv	4	own data
Pseudoterpna	coronilleria	PseuCoro	2	own data
Rheumaptera	undulata*	RheuUndu	1	ANZM
Scopula	floslactata	ScopFlos	2	BOLD*
Scopula	nigropunctata	ScopNigr	1	ANZM
Selenia	dentaria	SeleDent	5	own data
Selenia	lunularia	SeleLunu	1	own data

	Timandra	comae	TimaComa	3	own data
	Xanthorhoe	designata	XantDesi	1	own data
	Xanthorhoe	ferrugata	XantFerr	4	own data
	Xanthorhoe	fluctuata	XantFluc	1	ANZM
	Xanthorhoe	montanata	XantMont	1	ANZM
Glyphipterigidae	Acrolepiopsis	vesperella	AcroVesp	0	ND
Lysupidae	Pseudatemelia	flavifrontella	PseuFlav	1	BOLD
Noctuidae	Abrostola	triplasia	AbroTrip		ANZM
	Acronicta	rumicis	AcroRumi	3	own data
	Aedia	leucomelas	AediLeuc	0	ANZM
	Agrotis	bigramma*	AgroBigr	5	own data
	Agrotis	segetum*	AgroSege	1	own data
	Agrotis	exclamationis	AgroExcl	5	own data
	Agrotis	ipsilon	AgroIpsi	4	own data
	Agrotis	puta	AgroPuta	2	own data
	Anaplectoides	prasina	AnapPras	3	ANZM
	Apamea	monoglypha*	ApamMono	2	own data
	Apamea	epomidion	ApamEpom	3	ANZM
	Apamea	scolopacina	ApamScol	1	own data
	Autographa	gamma*	AutoGamm	0	ANZM + TM
	Axylia	putris	AxylPutr	5	own data
	Caradrina	clavipalpis	CaraClav	1	ANZM + TM
	Caradrina	morpheus	CaraMorp	1	ANZM + TM
	Cerastis	rubricosa	CeraRubr	1	own data
	Charanyca	trigrammica	CharTrig	1	ANZM
	Chersotis	fimbriola	CherFimb	1	ANZM
	Chersotis	multangula	CherMult	1	ANZM
	Colocasia	coryli	ColoCory	2	own data
	Cosmia	trapezina	CosmTrap	2	own data
	Craniophora	ligustri	CranLigu	1	ANZM
	Diachrysia	chrysitis	DiacChry	1	own data
	Elaphria	venustula	ElapVenu	5	own data
	Eremohadena	halimi	EremHali	0	ND
	Euplexia	lucipara	EuplLuci	5	own data
	Hadena	perplexa	HadePerp	1	ANZM
	Hoplodrina	ambigua	HoplAmbi	4	own data
	Lycophotia	erythrina	LycoEryt	5	own data
	Lycophotia	porphyrea	LycoPorp	3	own data
	Macdunnoughia	confusa	MacdConf	1	ANZM
	Mythimna	ferrago	MythFerr	1	ANZM
	Mythimna	albipuncta	MythAlbi	3	own data
	Mythimna	unipuncta	MythUnip	1	ANZM
	Mythimna	vitellina	MythVite	5	own data
	Noctua	janthina	NoctJant	2	own data
	Ochropleura	plecta*	OchrPlec	5	own data
	Oligia	strigilis*	OligStri	4	own data+BOLD

	Orthosia	gothica	OrthGoth	3	own data
	Peridroma	saucia	PeriSauc	1	ANZM
	Phlogophora	meticulosa	PhloMeti	1	own data
	Photedes	minima	PhotMini	4	own data
	Polia	hepatica	PoliHepa	1	BOLD
	Polyphaenis	sericata	PolySeri	1	ANZM
	Protodeltote	pygarga	ProtPyga	5	own data
	Thalpophila	matura	ThalMatu	1	own data
	Trachea	atriplicis	TracAtri	5	own data
	Xanthodes	albago	XantAlba	0	ND
	Xestia	baja	XestBaja	1	ANZM
	Xestia	c-nigrum	XestC-Ni	5	own data
	Xestia	xanthographa	XestXant	1	own data + TM
Nolidae	Nola	confusalis	NolaConf	1	ANZM
	Pseudoips	prasinana	PseuPras	3	own data
Notodontidae	Pheosia	tremula	PheoTrem	2	own data
	Ptilodon	capucina	PtilCapu	2	own data
Pterophoridae	Cnaemidophorus	rhododactyla	CnaeRhod	1	ANZM
Pyralidae	Acrobasis	porphyrella*	AcroPorp	1	ANZM
	Eccopisa	effractella	EccoEffr	1	own data
	Endotricha	flammealis	EndoFlam	1	ANZM
	Galleria	mellonella	GallMell	1	ANZM
	Hypsopygia	costalis	HypsCost	3	ANZM
	Oncocera	semirubella	OncoSemi	5	own data
	Phycitodes	binaevella	PhycBina	1	BOLD
	Salebriopsis	albicilla	SaleAlbi	1	own data
Sphingidae	Mimas	tiliae	MimaTili	4	BOLD
Tortricidae	Ancylis	badiana	AncyBadi	1	own data
	Archips	podana	ArchPoda	1	ANZM
	Celypha	lacunana	CelyLacu	3	BOLD
	Celypha	striana	CelyStri	1	BOLD
	Cydia	pomonella*	CydiPomo	1	ANZM
	Epinotia	nisella	EpinNise	2	own data
Ypsolophidae	Ypsolopha	nemorella	YpsoNemo	1	BOLD

* Notes of species identification. The following species were not possible to identify at 100% confidence in diet: Anania (lancealis*, crocealis, testacealis); Scoparia (ambigualis*, basistrigalis); Cyclophora (punctaria*, suppunctaria, quercimontaria); Dysstroma (truncata*, citrata); Rheumaptera (undulata*, hastata); Agrotis (bigramma*, crassa); A. (segetum*, trux); Apamea (monoglypha*, sicula); Autographa (gamma*, pulchrina); Ochropleura (plecta*, leucogaster); Oligia (strigilis*, latruncula); Acrobasis (porphyrella*, glaucella); Cydia (pomonella*, fagiglandana). The most likely species was assigned based on species captured in the study area and ecological characteristics (see Chapter 2).

S5.2. Information regarding the traits of the moth taxa captured by light-traps in the study area: S5.2.1. Traits dataset of moth species included in the RLQ and fourth corner analyses; S5.2.2. Taxonomic information of all identified moth species in light-traps (included and not included in the analyses), reference to abbreviations and presence in diet. For those species not present in the diet, just on specimen was measured for trait.

Table S5.2.1. Traits dataset of moth species included in the RLQ and fourth corner analyses (Q matrix).

	tympanate	Mass	ForeWinLen	ForeWinArea	HindWinArea	Manouv	WingLoad	AspectRatio
AbraGros	yes	18.925	1.983	1.381	1.244	0.901	1.344	11.390
AbroTrip	yes	93.005	1.524	0.831	0.655	0.788	10.979	11.175
AcleSp	no	6.766	0.998	0.357	0.333	0.933	1.859	11.160
AcroRumi	yes	70.604	1.631	0.868	0.804	0.926	7.977	12.249
AcroSp	yes	124.096	1.605	0.853	0.759	0.890	14.272	12.080
AgapZoeg	no	9.500	0.901	0.233	0.206	0.884	4.000	13.936
AgonOcel	unknown	9.388	1.013	0.280	0.262	0.936	3.289	14.660
AgriInqu	no	13.200	1.176	0.343	0.445	1.298	3.781	16.152
AgroBigr	yes	165.040	1.831	1.181	1.017	0.861	13.709	11.350
AgroCras	yes	193.000	1.922	1.245	1.195	0.960	15.207	11.869
AgroExcl	yes	134.460	1.418	0.726	0.620	0.855	18.179	11.091
AgroIpsi	yes	173.101	1.883	1.071	1.135	1.059	15.852	13.239
AgroNemo	no	4.400	0.929	0.241	0.194	0.805	1.791	14.324
AgroPuta	yes	96.872	1.517	0.876	0.818	0.933	10.848	10.501
AgroSege	yes	119.279	1.628	0.833	0.790	0.948	14.047	12.727
AgroSp	yes	75.500	1.700	0.900	0.795	0.883	8.230	12.844
AlciRepa	yes	29.000	1.913	1.262	1.018	0.806	2.255	11.602
AmphTrag	yes	103.100	1.555	0.860	0.785	0.913	11.761	11.247
AnanHort	no	22.125	1.367	0.586	0.493	0.841	3.704	12.752
AnanLanc	no	10.250	1.403	0.512	0.413	0.807	1.964	15.378
AnanTerr	no	4.300	1.133	0.365	0.288	0.789	1.156	14.068
AncyAcha	no	2.100	0.724	0.157	0.162	1.032	1.312	13.355
AngePrun	yes	35.160	2.183	1.737	1.572	0.905	1.985	10.976
ApamMono	yes	144.172	2.144	1.431	1.139	0.796	9.884	12.843
ApamScol	yes	79.100	1.500	0.755	0.593	0.785	10.278	11.921
ApamSp	yes	118.300	1.971	1.279	0.966	0.755	9.074	12.150
AphoSoci	yes	32.038	1.474	0.617	0.666	1.079	5.094	14.085
AtypPulm	yes	36.500	1.174	0.530	0.399	0.753	6.756	10.402
AxylPutr	yes	41.320	1.367	0.547	0.549	1.004	7.416	13.679

BryoTerr	unknown	2.700	0.632	0.096	0.069	0.719	2.759	16.643
BupaPini	yes	13.400	1.868	1.226	0.997	0.813	1.072	11.385
CabeExan	yes	12.900	1.535	0.937	0.864	0.922	1.351	10.059
CabePusa	yes	21.150	1.414	0.746	0.607	0.814	2.781	10.721
CabeSp	yes	10.700	1.434	0.753	0.653	0.867	1.394	10.924
CallJuve	yes	31.700	1.594	0.828	0.685	0.827	3.756	12.275
CallPuri	yes	201.541	2.080	1.475	1.205	0.817	13.401	11.730
CallSp	yes	44.200	1.449	0.755	0.619	0.820	5.743	11.124
CampBili	yes	18.800	1.357	0.725	0.588	0.810	2.544	10.152
CampHono	yes	65.655	2.377	2.055	1.689	0.822	3.134	10.993
CampMarg	yes	31.480	1.785	1.077	0.907	0.842	2.866	11.835
CampSp	yes	23.800	1.436	0.790	0.633	0.801	2.955	10.441
CataRubi	yes	15.349	1.321	0.607	0.464	0.764	2.481	11.499
CatoNymp	yes	85.700	1.905	1.322	1.048	0.793	6.359	10.980
CeraRubr	yes	79.722	1.355	0.666	0.639	0.959	11.743	11.027
CharGlau	yes	26.316	1.761	1.030	0.848	0.823	2.506	12.043
CharObsc	yes	47.800	1.720	1.104	0.927	0.840	4.247	10.719
CharTrig	yes	119.048	1.607	0.980	0.896	0.914	11.917	10.541
ChloSite	yes	15.349	1.437	0.766	0.628	0.820	1.966	10.783
ChloVata	yes	5.200	0.912	0.342	0.188	0.550	1.492	9.728
ChryCulm	no	13.867	1.008	0.305	0.354	1.161	4.460	13.325
CiliGlau	yes	10.800	1.271	0.596	0.441	0.740	1.778	10.842
CleoCinc	yes	34.660	1.828	1.099	0.893	0.813	3.094	12.162
ClepSp	no	1.300	0.664	0.151	0.119	0.788	0.845	11.679
CoenToph	yes	21.100	1.387	0.712	0.531	0.746	2.907	10.808
ColoCory	yes	151.146	1.659	0.976	0.717	0.734	15.192	11.273
ColoSp	yes	8.800	1.190	0.544	0.354	0.651	1.587	10.413
CosmTrap	yes	40.672	1.429	0.693	0.640	0.923	5.757	11.778
CramLath	no	4.900	0.913	0.234	0.253	1.081	2.054	14.249
CramPasc	no	11.633	1.104	0.303	0.369	1.215	3.763	16.077
CramPerl	no	16.425	1.131	0.335	0.384	1.147	4.813	15.292
CramSp	no	11.200	1.042	0.251	0.294	1.171	4.377	17.303
CrocElin	yes	56.100	1.899	1.325	1.077	0.813	4.154	10.887
CyclAnnu	yes	7.719	1.150	0.494	0.368	0.745	1.533	10.709
CyclPend	yes	8.196	1.221	0.497	0.377	0.759	1.618	11.999
CyclPunc	yes	11.700	1.573	0.821	0.651	0.793	1.398	12.055
CyclPupp	yes	17.100	1.285	0.660	0.548	0.830	2.542	10.007
CyclRufi	yes	9.200	1.192	0.482	0.376	0.780	1.872	11.791
CyclSp	yes	18.448	1.475	0.729	0.565	0.775	2.483	11.938
CydiFagi	no	4.400	0.629	0.136	0.120	0.882	3.174	11.637
CydiSple	no	9.600	0.893	0.243	0.204	0.840	3.876	13.127
DeilElpe	no	574.869	3.208	2.629	1.486	0.565	21.451	15.658
DeilRibe	yes	55.641	2.257	1.603	1.221	0.762	3.405	12.711
DiacChry	yes	126.505	1.709	0.989	0.804	0.813	12.548	11.813
DiarRubi	yes	85.500	1.486	0.748	0.711	0.951	11.213	11.809
DiarSp	yes	49.800	1.602	0.896	0.763	0.852	5.452	11.457

DiasReti	no	5.216	0.891	0.187	0.193	1.032	2.744	17.027
Drym	yes	135.100	1.735	1.012	0.689	0.681	13.096	11.898
DrymQuer	yes	144.100	1.767	1.035	0.707	0.683	13.658	12.067
DysgAlgi	yes	78.053	2.054	1.445	1.217	0.842	5.299	11.679
DyssTrun	yes	20.800	1.514	0.798	0.636	0.797	2.557	11.490
EanaCane	no	6.100	1.005	0.264	0.214	0.811	2.267	15.303
EariClor	yes	7.004	0.956	0.304	0.272	0.895	2.260	12.025
EccoEffr	yes	4.200	0.658	0.117	0.127	1.085	3.522	14.802
EcliSila	yes	17.018	1.617	0.925	0.643	0.695	1.805	11.307
EcpyRubi	no	5.600	1.043	0.300	0.287	0.957	1.831	14.505
EctrCrep	yes	18.280	1.633	0.938	0.757	0.807	1.911	11.372
EctrSp	yes	29.892	1.842	1.161	0.900	0.775	2.526	11.690
EileCani	yes	18.686	1.548	0.612	0.800	1.307	2.995	15.662
EileDepr	yes	32.276	1.630	0.606	0.705	1.163	5.225	17.537
EileGris	yes	27.400	1.742	0.738	0.838	1.136	3.642	16.448
EileSoro	yes	11.500	1.243	0.427	0.455	1.066	2.642	14.474
EileSp	yes	49.700	1.554	0.685	0.738	1.077	7.118	14.102
ElacGang	unknown	0.800	0.399	0.034	0.015	0.441	2.308	18.730
ElapVenu	yes	5.220	0.873	0.261	0.236	0.906	1.964	11.685
EndoFlam	yes	7.838	0.938	0.256	0.256	1.000	3.004	13.748
EnnoAlni	yes	123.300	1.801	1.196	1.090	0.911	10.113	10.848
EpilLino	yes	94.300	1.696	0.945	1.034	1.094	9.789	12.175
EpinNise	no	2.650	0.586	0.106	0.088	0.825	2.453	12.958
EpinTene	no	3.100	0.596	0.100	0.082	0.820	3.041	14.209
EpioRepa	yes	3.600	1.456	0.709	0.588	0.830	0.498	11.966
EpirAlte	yes	17.600	1.304	0.667	0.511	0.766	2.589	10.197
EthmFune	unknown	4.620	0.880	0.187	0.180	0.963	2.424	16.565
EthmQuad	unknown	2.200	0.707	0.116	0.087	0.750	1.861	17.236
EucoSp	no	3.100	0.730	0.160	0.144	0.900	1.901	13.323
EudoDelu	no	1.300	0.776	0.166	0.118	0.711	0.768	14.510
EudoLacu	no	3.100	0.791	0.179	0.191	1.067	1.699	13.982
EudoMerc	no	3.400	0.794	0.168	0.190	1.131	1.991	15.055
EupiAbbr	yes	4.143	1.047	0.329	0.178	0.541	1.235	13.328
EupiExpa	yes	9.700	1.145	0.419	0.241	0.575	2.271	12.516
EupiHawo	yes	1.997	0.759	0.201	0.136	0.677	0.975	11.464
EupiSp	yes	2.236	0.813	0.207	0.096	0.464	1.059	12.772
EupiSubf	yes	5.600	0.943	0.302	0.174	0.576	1.819	11.778
EuplLuci	yes	50.020	1.380	0.644	0.505	0.785	7.625	11.838
EuplQuad	yes	186.000	2.867	2.749	2.709	0.985	6.638	11.960
EuprSimi	yes	73.800	2.138	1.620	1.292	0.798	4.469	11.287
EverPall	no	13.200	1.170	0.470	0.386	0.821	2.755	11.650
GeomPapi	yes	86.314	2.540	2.407	2.096	0.871	3.518	10.726
GymnRufi	yes	4.143	0.897	0.267	0.160	0.600	1.524	12.069
HabrPyri	yes	71.438	1.810	1.132	0.891	0.788	6.194	11.585
HarpForf	unknown	8.900	1.262	0.423	0.296	0.700	2.064	15.060
HemiAest	yes	16.837	1.461	0.758	0.628	0.829	2.178	11.261

HermGris	yes	5.480	1.163	0.424	0.342	0.805	1.267	12.757
HermSp	yes	10.600	1.123	0.403	0.348	0.864	2.580	12.517
HermTars	yes	18.925	1.273	0.581	0.481	0.827	3.195	11.157
HomoSinu	yes	7.100	0.856	0.147	0.179	1.218	4.738	19.938
HoplAmbi	yes	75.350	1.404	0.657	0.588	0.895	11.259	12.006
HoplBlan	yes	75.669	1.390	0.668	0.644	0.964	11.112	11.569
HoplHesp	yes	57.787	1.338	0.611	0.550	0.900	9.278	11.720
HoplOcto	yes	64.400	1.389	0.650	0.608	0.935	9.719	11.873
HoplSp	yes	60.700	1.300	0.591	0.608	1.029	10.076	11.438
HydrFurc	yes	40.867	1.724	1.087	0.899	0.827	3.688	10.933
HydrSp	yes	14.800	1.569	0.944	0.792	0.839	1.538	10.431
HylaFasc	yes	28.438	1.743	1.048	0.855	0.816	2.662	11.593
HypeCras	yes	22.400	1.336	0.729	0.689	0.945	3.014	9.794
HypeProb	yes	20.400	1.834	1.125	1.069	0.950	1.779	11.953
HypoPunc	yes	22.850	2.164	1.520	1.252	0.824	1.475	12.323
HypoRobo	yes	41.600	2.600	2.204	1.705	0.774	1.852	12.269
HypoSp	yes	53.734	2.316	1.675	1.308	0.781	3.147	12.809
IdaeAver	yes	17.417	1.426	0.628	0.444	0.707	2.722	12.948
IdaeBise	yes	5.220	1.040	0.334	0.263	0.788	1.534	12.957
IdaeDege	yes	14.510	1.252	0.470	0.325	0.692	3.027	13.333
IdaeDimi	yes	5.097	0.896	0.292	0.225	0.771	1.712	10.997
IdaeDist	yes	2.600	1.023	0.314	0.238	0.758	0.812	13.332
IdaeEuge	yes	10.461	1.199	0.493	0.353	0.716	2.082	11.664
IdaeSp	yes	6.800	1.017	0.340	0.282	0.829	1.962	12.168
IdaeStra	yes	17.200	1.274	0.505	0.333	0.659	3.341	12.856
IdaeSubs	yes	7.958	1.186	0.414	0.338	0.816	1.886	13.590
JodiSlac	yes	4.977	1.145	0.443	0.387	0.874	1.102	11.838
LacaSp	yes	130.300	1.726	0.896	0.762	0.850	14.266	13.299
LampOtre	yes	9.925	1.195	0.540	0.399	0.739	1.803	10.578
LasiTrif	no	149.500	1.916	1.456	1.332	0.915	10.073	10.085
LaspFlex	yes	17.000	1.156	0.442	0.314	0.710	3.773	12.094
LeucPutr	yes	84.600	1.603	0.758	0.544	0.718	10.949	13.560
LigdAdus	yes	9.627	1.268	0.564	0.463	0.821	1.674	11.403
LithQuad	yes	27.746	2.014	1.050	1.010	0.962	2.592	15.452
LoboHalt	yes	8.673	1.330	0.638	0.290	0.455	1.334	11.090
LomaMarg	yes	5.650	1.169	0.453	0.370	0.817	1.225	12.085
LomoBima	yes	13.203	1.376	0.625	0.452	0.723	2.072	12.118
LomoSp	yes	8.900	1.556	0.821	0.640	0.780	1.063	11.796
LomoTeme	yes	17.185	1.321	0.626	0.499	0.798	2.694	11.162
LyciHirt	yes	124.096	1.935	1.288	1.016	0.789	9.452	11.628
LycoEryt	yes	65.155	1.438	0.718	0.656	0.913	8.897	11.510
LycoPorp	yes	65.655	1.378	0.641	0.590	0.920	10.048	11.850
LygeCrac	yes	69.867	1.732	1.013	0.961	0.948	6.764	11.837
LygePast	yes	44.800	1.945	1.290	1.100	0.853	3.407	11.730
LymaMona	yes	60.540	2.058	1.525	1.228	0.805	3.895	11.110
MacaAlte	yes	9.627	1.410	0.647	0.561	0.867	1.460	12.291

MacaLitu	yes	16.000	1.422	0.627	0.521	0.831	2.503	12.900
MacaNota	yes	9.627	1.410	0.647	0.561	0.867	1.460	12.291
MalaNeus	no	64.200	1.413	0.846	0.689	0.814	7.444	9.440
MecyAsin	no	11.725	1.296	0.462	0.417	0.903	2.492	14.555
MegaAlbu	yes	10.300	1.028	0.388	0.346	0.892	2.604	10.895
MegaStri	yes	11.900	0.961	0.318	0.249	0.783	3.671	11.617
MelaPers	yes	86.159	1.863	1.056	0.813	0.770	8.004	13.147
MelaProc	yes	21.945	1.572	0.892	0.591	0.663	2.414	11.086
MenoAbru	yes	14.700	1.523	0.822	0.679	0.826	1.755	11.287
MesaSeca	yes	47.200	1.363	0.602	0.553	0.919	7.692	12.344
MesaSp	yes	58.400	1.286	0.582	0.552	0.948	9.844	11.366
MesoAlbi	yes	22.501	1.583	0.984	0.764	0.776	2.243	10.187
MiltMini	yes	17.800	1.430	0.674	0.523	0.776	2.591	12.136
MomaAlpi	yes	66.609	1.672	1.030	0.716	0.695	6.344	10.857
MonoWeav	unknown	0.900	0.602	0.070	0.059	0.843	1.261	20.709
MythAlbi	yes	103.586	1.545	0.808	0.657	0.814	12.577	11.817
MythImpu	yes	53.257	1.450	0.669	0.596	0.891	7.809	12.571
MythL_Al	yes	122.700	1.644	0.802	0.730	0.910	15.009	13.480
MythSp	yes	47.297	1.499	0.733	0.673	0.918	6.330	12.262
MythVite	yes	130.390	1.777	0.907	0.803	0.885	14.108	13.936
NoctJant	yes	119.450	1.730	0.973	1.085	1.115	12.043	12.304
NoctJant	yes	152.999	1.774	1.061	1.178	1.110	14.146	11.865
NoctPron	yes	462.700	2.323	1.755	1.966	1.120	25.864	12.299
NolaSp	yes	4.143	0.874	0.240	0.174	0.725	1.693	12.731
NotoSp	no	3.428	0.756	0.169	0.173	1.024	1.990	13.527
NotoSp	yes	183.014	2.434	1.828	1.389	0.760	9.821	12.964
NyctReva	yes	17.600	1.058	0.352	0.360	1.023	4.905	12.720
OchrDupl	yes	10.600	1.363	0.650	0.503	0.774	1.600	11.432
OchrPlec	yes	50.209	1.236	0.523	0.530	1.015	9.425	11.689
OchrSp	yes	19.200	1.234	0.538	0.499	0.928	3.501	11.322
OecoSp	unknown	2.700	0.527	0.074	0.037	0.500	3.579	15.012
OligSp	yes	17.400	1.211	0.505	0.418	0.828	3.378	11.610
OncoSemi	yes	22.760	1.241	0.389	0.526	1.352	5.743	15.834
OncoSp	yes	16.300	1.154	0.352	0.511	1.452	4.543	15.133
OpisLute	yes	11.200	1.595	0.919	0.789	0.859	1.196	11.073
OrthCera	yes	133.916	1.510	0.834	0.666	0.799	15.752	10.936
OrthGoth	yes	82.587	1.620	0.826	0.588	0.712	9.812	12.714
OrthInce	yes	90.927	1.749	1.019	0.732	0.718	8.754	12.008
OrthSp	yes	38.237	1.512	0.696	0.573	0.823	5.389	13.139
OuraSamb	yes	115.018	2.555	2.493	2.311	0.927	4.526	10.474
PachHipp	yes	16.200	1.474	0.621	0.536	0.863	2.559	14.001
PandHepa	no	6.200	0.854	0.273	0.237	0.868	2.228	10.686
PandSp	no	12.249	0.995	0.346	0.273	0.789	3.473	11.445
ParaCons	yes	44.913	1.854	1.198	0.917	0.765	3.679	11.475
ParaPand	no	10.103	1.187	0.407	0.368	0.904	2.435	13.847
PareSimi	yes	11.400	1.307	0.548	0.375	0.684	2.041	12.469

PeriAlch	yes	5.573	0.868	0.297	0.211	0.712	1.844	10.158
PeriAnce	yes	424.241	2.767	2.130	1.582	0.743	19.539	14.378
PeriRhom	yes	22.440	1.902	1.191	0.945	0.794	1.849	12.152
PeriSp	yes	21.000	1.645	0.946	0.744	0.786	2.178	11.442
PetrChlo	yes	17.600	1.544	0.821	0.699	0.851	2.104	11.620
PhalBuce	yes	253.500	2.417	2.031	1.380	0.679	12.244	11.505
PheoTrem	yes	289.639	2.487	1.788	1.228	0.687	15.896	13.835
PhotMini	yes	16.779	1.187	0.493	0.436	0.885	3.340	11.438
PhotSp	yes	15.349	1.070	0.366	0.273	0.746	4.114	12.513
PhraFuli	yes	119.800	1.479	0.810	0.801	0.989	14.509	10.802
PlagDola	yes	20.594	1.541	0.806	0.661	0.820	2.507	11.785
PlagPulv	yes	33.290	1.755	1.118	0.877	0.784	2.920	11.020
PleuRura	no	24.440	1.502	0.666	0.606	0.909	3.598	13.541
PolyDubi	yes	130.000	1.883	1.248	0.875	0.701	10.219	11.364
ProtPyga	yes	12.980	1.069	0.415	0.353	0.851	3.070	11.016
PseuCoro	yes	32.992	1.425	0.741	0.646	0.873	4.370	10.972
PseuPras	yes	74.212	1.562	0.872	0.552	0.634	8.352	11.191
PterPalp	yes	101.100	1.939	1.235	1.053	0.853	8.031	12.177
PtilCapu	yes	128.913	1.997	1.393	1.021	0.733	9.082	11.450
PyraDesp	no	1.997	0.683	0.143	0.133	0.930	1.370	13.049
PyraSp	yes	9.700	1.305	0.402	0.485	1.206	2.367	16.946
RhodSacr	yes	5.335	1.182	0.462	0.359	0.777	1.133	12.096
RhopNaev	no	2.200	0.589	0.088	0.093	1.057	2.453	15.769
RivuSeri	yes	5.000	0.993	0.338	0.289	0.855	1.451	11.669
RusiFerr	yes	58.264	1.628	0.951	0.781	0.821	6.010	11.148
SaleAlbi	yes	8.600	0.848	0.202	0.231	1.144	4.177	14.240
SchrTaen	yes	6.050	0.925	0.231	0.220	0.952	2.569	14.816
ScopBasi	no	4.860	0.883	0.190	0.226	1.189	2.509	16.415
ScopCabe	yes	9.300	1.794	1.214	0.955	0.787	0.752	10.604
ScopCari	yes	16.064	1.354	0.652	0.556	0.853	2.417	11.247
ScopNigr	yes	21.667	1.439	0.687	0.551	0.802	3.094	12.057
ScopSp	yes	7.100	1.201	0.567	0.442	0.780	1.228	10.176
ScopSubf	no	5.900	1.028	0.287	0.307	1.070	2.017	14.729
SeleDent	yes	37.444	1.855	1.224	1.084	0.886	3.002	11.251
SeleTetr	yes	28.100	1.587	0.917	0.771	0.841	3.006	10.986
SphiMaur	no	298.810	3.699	2.896	1.669	0.576	10.122	18.899
SphiPina	no	527.800	3.632	3.363	1.963	0.584	15.396	15.690
SpilLubr	yes	135.583	1.786	1.112	0.941	0.846	11.961	11.474
SpilLute	yes	87.968	1.699	0.991	0.865	0.872	8.708	11.657
StauFagi	yes	419.424	2.640	2.096	1.434	0.684	19.630	13.301
SyndMusc	no	5.335	0.802	0.200	0.198	0.990	2.617	12.864
TeleSp	unknown	3.200	0.645	0.079	0.054	0.684	3.974	21.065
ThalMatu	yes	71.300	1.799	1.121	0.956	0.853	6.240	11.548
ThauPity	yes	96.200	1.843	1.055	0.682	0.646	8.945	12.878
ThauProc	yes	56.400	1.286	0.701	0.503	0.718	7.893	9.437
ThauSp	yes	123.400	1.705	1.042	0.676	0.649	11.618	11.159

TherObel	yes	9.627	1.419	0.600	0.469	0.782	1.574	13.424
ThyaBati	yes	66.132	1.781	1.050	0.909	0.866	6.181	12.088
TimaComa	yes	18.845	1.542	0.762	0.654	0.857	2.425	12.476
TineSemi	unknown	1.300	0.700	0.110	0.094	0.855	1.159	17.818
TineSp	unknown	3.189	0.824	0.141	0.108	0.766	2.219	19.262
TortSp	no	3.600	0.741	0.185	0.136	0.735	1.909	11.872
TracAtri	yes	106.392	1.790	1.050	0.870	0.829	9.940	12.201
TricCarp	yes	20.832	1.477	0.714	0.433	0.606	2.862	12.221
TricCrat	no	121.502	1.566	0.950	0.815	0.858	12.547	10.326
TripTaut	yes	65.417	2.118	1.695	1.298	0.766	3.786	10.586
TrisEmor	yes	18.000	1.294	0.586	0.387	0.660	3.013	11.430
UdeaFerr	no	4.166	0.903	0.228	0.212	0.928	1.792	14.295
WatsBina	yes	14.700	1.263	0.538	0.450	0.836	2.680	11.860
WatsSp	yes	19.500	1.494	0.787	0.650	0.826	2.431	11.345
WatsUnci	yes	11.400	1.506	0.774	0.641	0.828	1.445	11.721
XantBiri	yes	4.300	1.071	0.481	0.340	0.707	0.877	9.539
XantDesi	yes	6.100	1.193	0.477	0.354	0.742	1.255	11.935
XantFerr	yes	8.456	1.125	0.481	0.383	0.796	1.725	10.525
XantSp	yes	10.300	1.608	0.817	0.639	0.782	1.237	12.659
XestCnig	yes	71.391	1.578	0.770	0.739	0.959	9.095	12.942
XestDitr	yes	122.000	1.852	1.128	1.094	0.970	10.610	12.163
XestSp	yes	117.800	1.862	1.077	1.035	0.961	10.730	12.877
XestStig	yes	194.686	1.933	1.200	1.108	0.923	15.916	12.455
XestXant	yes	128.800	1.517	0.832	0.880	1.058	15.187	11.064
XyloAreo	yes	70.662	1.637	0.787	0.639	0.812	8.808	13.620
YponPlum	unknown	3.300	0.778	0.132	0.137	1.038	2.453	18.342
YponSp	unknown	7.719	0.975	0.218	0.192	0.881	3.474	17.443
ZancLui	yes	30.600	1.525	0.805	0.731	0.908	3.729	11.556
ZancLuna	yes	17.016	1.334	0.587	0.522	0.891	2.846	12.141
ZancSp	yes	14.156	1.353	0.606	0.524	0.865	2.292	12.083
ZeirIser	no	4.100	0.681	0.122	0.133	1.090	3.297	15.205

Table S5.2.2. Taxonomic information of all identified moth species in light-traps (included and not included in the analyses), reference to abbreviations and presence in diet. For those species not present in the diet, just on specimen was measured for trait.

family	species	Abbreviation	In Diet
Crambidae	Agriphila inquinatella	AgriInqu	yes
	Agrotera nemoralis	AgroNemo	yes
	Anania hortulata	AnanHort	yes
	Anania lancealis	AnanLanc	yes

	Anania terrealis	AnanTerr	no
	Chrysoteuchia culmella	ChryCulm	yes
	Crambus lathoniellus	CramLath	yes
	Crambus pascuella	CramPasc	yes
	Crambus perlella	CramPerl	yes
	Crambus sp	CramSp	yes
	Diasemia reticularis	DiasReti	yes
	Ecpyrrhorrhoe rubiginalis	EcpyRubi	no
	Eudonia/scoparia	EudoScop	yes
	Eudonia delunella	EudoDelu	no
	Eudonia lacustrata	EudoLacu	yes
	Eudonia mercurella	EudoMerc	yes
	Evergestis pallidalis	EverPall	no
	Mecyna asinalis	MecyAsin	yes
	Paratalanta pandalis	ParaPand	yes
	Paratalanta sp	ParaSp	yes
	Pleuroptya ruralis	PleuRura	yes
	Pyrausta despicata	PyraDesp	yes
	Scoparia basistrigallis/ambigualis	ScopBasi	yes
	Scoparia subfusca	ScopSubf	no
	Udea ferrugalis	UdeaFerr	yes
	Udea sp	UdeaSp	no
Depressariidae	Agonopterix ocellana	AgonOcel	no
Drepanidae	Cilix glaucata	CiliGlau	no
	Habrosyne pyritoides	HabrPyri	yes
	Ochropacha duplaris	OchrDupl	no
	Thyatira batis	ThyaBati	yes
	Watsonalla binaria	WatsBina	no
	Watsonalla sp	WatsSp	no
	Watsonalla uncinula	WatsUnci	no
Elachistidae	Elachista gangabella	ElacGang	no
	Ethmia funerella	EthmFune	no
	Ethmia quadrillella/funerella	EthmQuad	no
Erebidae	Calliteara puribunda	CallPuri	yes
	Dysgonia algira	DysgAlgi	yes
	Eilema caniola	EileCani	no
	Eilema depressa	EileDepr	no
	Eilema griseola	EileGris	no
	Eilema sororcula	EileSoro	no
	Eilema sp	EileSp	no
	Euplagia quadripunctaria	EuplQuad	no
	Euproctis similis	EuprSimi	no
	Herminia grisealis	HermGris	yes
	Herminia sp	HermSp	no
	Herminia tarsicrinalis	HermTars	yes

	Hypena crassalis	HypeCras	yes
	Hypena proboscidalis	HypeProb	yes
	Hypena sp	HypeSp	no
	Laspeyria flexula	LaspFlex	yes
	Lithosia quadra	LithQuad	no
	Lygephila craccae	LygeCrac	yes
	Lygephila pastium	LygePast	yes
	Lymantria monacha	LymaMona	yes
	Miltochrista miniata	MiltMini	no
	Phragmatobia fuliginosa	PhraFuli	no
	Rivula sericealis	RivuSeri	no
	Schrankia taenialis	SchrTaen	no
	Spilisoma luteum	SpilLute	yes
	Spilosoma lubricipeda	SpilLubr	no
	Trisateles emortualis	TrisEmor	no
	Zanclognatha lui	ZancLui	no
	Zanclognatha lunalis	ZancLuna	yes
	Zanclognatha sp	ZancSp	no
Gelechiidae	Bryotropha terrella	BryoTerr	no
	Dichomeris sp	DichSp	no
	Teleiopsis sp	TeleSp	no
Geometridae	Abraxas grossulariata	AbraGros	no
	Alcis repandata	AlciRepa	yes
	Angerona prunaria	AngePrun	yes
	Bupalus piniaria	BupaPini	no
	Cabera exanthemata	CabeExan	yes
	Cabera pusaria	CabePusa	yes
	Cabera sp	CabeSp	no
	Campaea honoraria	CampHono	yes
	Campaea margaritaria	CampMarg	yes
	Camptogramma bilineata	CampBili	yes
	Camptogramma sp	CampSp	no
	Catarhoe rubidata	CataRubi	yes
	Charissa glaucinaria	CharGlau	no
	Charissa obscurata	CharObsc	no
	Chloroclysta siterata	ChloSite	no
	Chloroclystis v_ata	ChloVata	no
	Cleora cinctaria	CleoCinc	no
	Coenotephria tophaceata	CoenToph	no
	Colostygia sp	ColoSp	no
	Crocallis elinguaria	CrocElin	no
	Cyclophora annularia	CyclAnnu	no
	Cyclophora pendularia	CyclPend	no
	Cyclophora punctaria	CyclPunc	yes
	Cyclophora puppillaria	CyclPupp	no

Cyclophora ruficiliaria	CyclRufi	no
Cyclophora sp	CyclSp	no
Deileptenia ribeata	DeilRibe	no
Dysstroma truncata	DyssTrun	yes
Ecliptopera silaceata	EcliSila	yes
Ectropis crepuscularia	EctrCrep	yes
Ectropis sp	EctrSp	no
Ennomos alniaria	EnnoAlni	no
Epione repandaria/vespertaria	EpioRepa	yes
Epirrhoe alternata	EpirAlte	no
Eupithecia abbreviata	EupiAbbr	no
Eupithecia expallidata	EupiExpa	no
Eupithecia haworthiata	EupiHawo	no
Eupithecia sp	EupiSp	no
Eupithecia subfuscata	EupiSubf	no
Geometra papilionaria	GeomPapi	yes
Gymnoscelis rufifasciata	GymnRufi	yes
Hemithea aestivaria	HemiAest	yes
Hydriomena furcata	HydrFurc	yes
Hydriomena sp	HydrSp	no
Hylaea fasciaria	HylaFasc	yes
Hypomecis punctinalis	HypoPunc	yes
Hypomecis roboraria	HypoRobo	no
Hypomecis sp	HypoSp	no
Idaea aversata	IdaeAver	yes
Idaea biselata	IdaeBise	yes
Idaea degeneraria	IdaeDege	yes
Idaea dimidiata	IdaeDimi	yes
Idaea distinctaria	IdaeDist	no
Idaea eugeniata	IdaeEuge	yes
Idaea sp	IdaeSp	no
Idaea straminata	IdaeStra	no
Idaea subsericeata	IdaeSubs	yes
Jodi slactearia	JodiSlac	yes
Lampropteryx otregiata	LampOtre	yes
Ligdia adustata	LigdAdus	no
Lobophora halterata	LoboHalt	no
Lomaspilis marginata	LomaMarg	yes
Lomographa bimaculata	LomoBima	no
Lomographa sp	LomoSp	no
Lomographa temerata	LomoTeme	yes
Lycia hirtaria	LyciHirt	no
Macaria alternata	MacaAlte	no
Macaria liturata	MacaLitu	no
Macaria notata/alternata	MacaNota	no

	Melanthia procellata	MelaProc	yes
	Menophra abruptaria	MenoAbru	yes
	Mesoleuca albicillata	MesoAlbi	no
	Opisthograptis luteolata	OpisLute	yes
	Ourapteryx sambucaria	OuraSamb	no
	Pachycnemia hippocastanaria	PachHipp	yes
	Paradarisa consonaria	ParaCons	yes
	Parectropis similaria	PareSimi	no
	Peribatodes perversaria	PeriPerv	no
	Peribatodes rhomboidaria	PeriRhom	yes
	Peribatodes sp	PeriSp	no
	Perizoma alchemillata	PeriAlch	yes
	Perizoma sp	PeriSp	no
	Petrophora chlorosata	PetrChlo	yes
	Plagodis dolabraria	PlagDola	no
	Plagodis pulveraria	PlagPulv	yes
	Pseudoterpna coronillaria	PseuCoro	yes
	Rhodometra sacraria	RhodSacr	no
	Scopula/cabera sp	ScopCabe	no
	Scopula caricaria	ScopCari	no
	Scopula nigropunctata	ScopNigr	yes
	Scopula sp	ScopSp	no
	Selenia dentaria	SeleDent	yes
	Selenia tetralunaria	SeleTetr	no
	Thera obeliscata	TherObel	no
	Timandra comae	TimaComa	yes
	Trichopteryx carpinata	TricCarp	no
	Triphosa tauteli	TripTaut	no
	Xanthorhoe biriviata	XantBiri	no
	Xanthorhoe designata	XantDesi	yes
	Xanthorhoe ferrugata	XantFerr	yes
	Xanthorhoe sp	XantSp	no
Lasiocampidae	Lasiocampa trifolii	LasiTrif	no
	Malacosoma neustria	MalaNeus	no
	Trichiura crataegi	TricCrat	no
Noctuidae	Abrostola triplasia	AbroTrip	yes
	Acronicta rumicis	AcroRumi	yes
	Acronicta sp	AcroSp	no
	Agrotis bigramma	AgroBigr	yes
	Agrotis crassa	AgroCras	no
	Agrotis exclamationis	AgroExcl	yes
	Agrotis ipsilon	AgroIpsi	yes
	Agrotis puta	AgroPuta	yes
	Agrotis segetum	AgroSege	yes
	Agrotis sp	AgroSp	no

Amphipyra tragopoginis	AmphTrag	no
Apamea monoglypha	ApamMono	yes
Apamea scolopacina	ApamScol	yes
Apamea sp	ApamSp	no
Atypha pulmonaris	AtypPulm	no
Axylia putris	AxylPutr	yes
Callopistria juventina	CallJuve	no
Callopistria sp	CallSp	no
Catocala nymphagoga	CatoNymp	no
Cerastis rubricosa	CeraRubr	yes
Charanyca trigrammica	CharTrig	yes
Colocasia coryli	ColoCory	yes
Cosmia trapezina	CosmTrap	yes
Diachrysia chrysitis	DiacChry	yes
Diarsia brunnea	DiarBrun	no
Diarsia rubi	DiarRubi	no
Diarsia sp	DiarSp	no
Elaphria venustula	ElapVenu	yes
Epilecta linogrisea	EpilLino	no
Euplexia lucipara	EuplLuci	yes
Hoplodrina ambigua	HoplAmbi	yes
Hoplodrina blanda	HoplBlan	no
Hoplodrina hesperica	HoplHesp	no
Hoplodrina octogenaria	HoplOcto	no
Hoplodrina sp	HoplSp	no
Lacanobia sp	LacaSp	no
Leucania putrescens	LeucPutr	no
Lycophotia erythrina	LycoEryt	yes
Lycophotia porphyrea	LycoPorp	yes
Melanchra persicariae	MelaPers	no
Mesapamea secalis	MesaSeca	no
Mesapamea sp	MesaSp	no
Moma alpium	MomaAlpi	no
Mythimna albipuncta	MythAlbi	yes
Mythimna impura	MythImpu	no
Mythimna l_album	MythL_Al	no
Mythimna sp	MythSp	no
Mythimna vitellina	MythVite	yes
Noctua janthe	NoctJant	no
Noctua janthina/janthe	NoctJant	yes
Noctua pronuba	NoctPron	no
Ochropleura leucogaster	OchrLeuc	no
Ochropleura plecta	OchrPlec	yes
Ochropleura sp	OchrSp	no
Oligia sp	OligSp	no

	Orthosia cerasi	OrthCera	no
	Orthosia gothica	OrthGoth	yes
	Orthosia incerta	OrthInce	no
	Orthosia sp	OrthSp	no
	Photedes minima	PhotMini	yes
	Photedes sp	PhotSp	no
	Polymixis dubia	PolyDubi	no
	Protodeltote pygarga	ProtPyga	yes
	Rusina ferruginea	RusiFerr	no
	Thalpophila matura	ThalMatu	yes
	Trachea atriplicis	TracAtri	yes
	Xestia c_nigrum	XestCnig	yes
	Xestia ditrapezium	XestDitr	no
	Xestia sp	XestSp	no
	Xestia stigmatica	XestStig	no
	Xestia xanthographa	XestXant	yes
	Xylocampa areola	XyloAreo	no
Nolidae	Earias clorana	EariClor	no
	Meganola albula	MegaAlbu	no
	Meganola strigula	MegaStri	no
	Nola sp	NolaSp	no
	Nycteola revayana	NyctReva	no
	Pseudoips prasinana	PseuPras	yes
Notodontidae	Drymonia querna	DrymQuer	no
	Drymonia sp	Drym	no
	Notodontidae sp	NotoSp	no
	Peridea anceps	PeriAnce	no
	Phalera bucephala	PhalBuce	no
	Pheosia tremula	PheoTrem	yes
	Pterostoma palpina	PterPalp	no
	Ptilodon capucina	PtilCapu	yes
	Stauropus fagi	StauFagi	no
	Thaumetopoea pityocampa	ThauPity	no
	Thaumetopoea processionea	ThauProc	no
	Thaumetopoea sp	ThauSp	no
Oecophoridae	Harpella forficella	HarpForf	no
	Oecophoridae sp	OecoSp	no
Pyralidae	Aphomia sociella	AphoSoci	no
	Eccopisa effractella	EccoEffr	yes
	Endotricha flamealis	EndoFlam	yes
	Homoeosoma sinuella	HomoSinu	no
	Oncocera semirubella	OncoSemi	yes
	Oncocera sp	OncoSp	no
	Pyralidae sp	PyraSp	no
	Salebriopsis albicilla	SaleAlbi	yes

Sphingidae	Deilephila elpenor	DeilElpe	no
	Sphinx mauronum	SphiMaur	no
	Sphinx pinastri	SphiPina	no
Tineidae	Monopis weaverella/laevigella	MonoWeav	no
	Tinea semifulvella	TineSemi	no
	Tinea sp	TineSp	no
Tortricidae	Acleris sp	AcleSp	no
	Agapeta zoegana	AgapZoeg	no
	Ancylis achatana * badiana	AncyAcha	yes
	Clepsis sp	ClepSp	no
	Cydia fagiglandana	CydiFagi	no
	Cydia splendana	CydiSple	no
	Eana canescana	EanaCane	no
	Epinotia nisella	EpinNise	yes
	Epinotia tenerana	EpinTene	no
	Eucosma sp	EucoSp	no
	Notocelia sp	NotoSp	no
	Pandemis heparana	PandHepa	no
	Pandemis sp	PandSp	no
	Rhopobota naevana	RhopNaev	no
	Syndemis musculana	SyndMusc	no
	Tortricidae sp	TortSp	no
	Zeiraphera isertana	ZeirIser	no
Yponomeutidae	Yponomeuta plumbella	YponPlum	no
	Yponomeuta sp	YponSp	no

S5.3. Results of the fourth-corner analyses

Potentially available functional moth-assemblage

The fourth-corner analysis (models 2 and 4 combined) found May negatively correlated with non-tympanated species (p=0.042) and maneuverability (p=0.008), and positively with mass (p=0.025). July was negatively associated with wing loading (p=0.008). September was positively correlated with maneuverability (p=0.008).

Functional Diet: Adults

Among the 63 possible associations, the fourth-corner analysis found 4 to be significant. There was a negative correlation between the breeding season and both mass (p=0.0009) and wing loading (p=0.0006), meaning that lighter and slower moths were consumed in July, whereas an opposed correlation was detected for the post-breeding season —bats significantly consumed more heavier and fast flier moths.

Functional Diet: juveniles vs adults

Among the 42 possible associations, we found 8 to be significant: adult bats have heavier bodies (p=0.001) and were positively associated with heavier moths (p=0.0004), longer forewings (p=0.03) and higher wing-loading values (p=0.001); contrarily, juveniles, significantly lighter in body mass (0.001), consumed lighter moths (p=0.0004) with shorter forewings (p=0.03) and slower flight (i.e. lower wing-loading values; p=0.001).
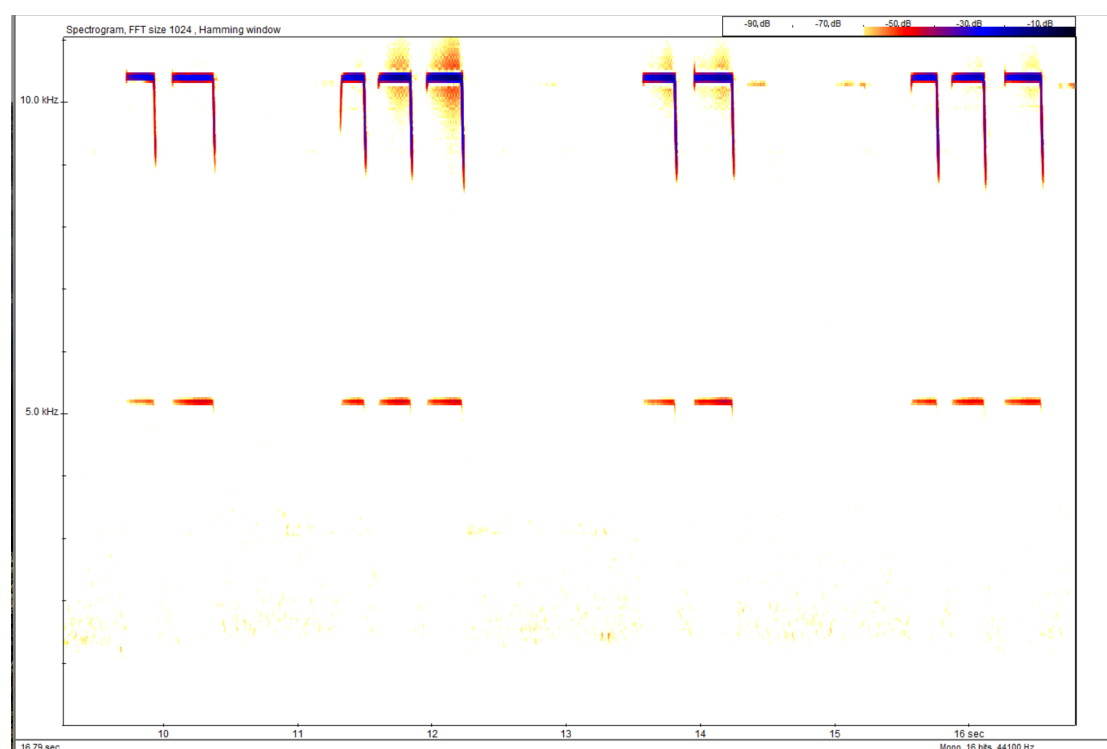

Figure S5.2. First (ca 52 kHz) and second (ca 104 kHz) constant frequency harmonics of *R. euryale* recorded in x10 time expansion by Joxerra Aihartza in the field.

The Mediterranean Horseshoe Bat – *Rhinolophus euryale*