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Biology and population genetics of *Arvicola scherman cantabriae* (Rodentia, Arvicolinae)

PhD Thesis

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Summary

The montane water vole *Arvicola scherman* occurs in mountainous areas of Europe, living in underground burrow systems located in grasslands and fruit orchards. This species feeds on the root system of plants, including fruit trees. Specifically, the subspecies *A. scherman cantabriae* is nowadays one of the main causes of economical loss in apple orchards of Asturias (northwestern Spain). An official control program in Spain considers all sustainable phytosanitary measures that can reduce population growth of this species. Since the pest condition of *A. scherman* depends on its biology and ecology, a deep knowledge of these aspects is needed to set up specific and suitable control strategies. Thus, the aim of this research is to obtain essential information on the reproductive biology and population genetics of this species in the agricultural landscape of Asturias.

More than 800 individuals of *A. scherman cantabriae* were gathered in apple orchards located at low altitude in Villaviciosa and Nava municipalities during two annual cycles (from February 2011 to January 2013). Sexual characteristics, body measurements and relative age class of each specimen were recorded. Body condition of females, which indicates energy provision, and the number of embryos of each one were also wrote down. Skeletal muscle samples of 137 specimens from ten demes were used to conduct a microsatellite-based analysis (12 microsatellite loci). These orchards are placed in a landscape conformed by a mosaic of small and different land-use plots, which was assessed in a vector based geographic information system and it was focused on soil-occupancy categories.

Pregnant females and young specimens were detected over the whole year, which mean that *A. scherman cantabriae* showed a continuous breeding pattern during the study period. Intra-annual changes in body mass and size of sexual organs of males did not affect significantly reproduction at a population scale. Thus, primary demands of these voles seem to be properly fulfilled during the whole year and hence energy budgets can be destined to cop continuous reproduction. To our knowledge, no other *A. scherman* population shows regularly this reproductive pattern. Females were able to produce a high number of litters per year (7.30) although litter size was relatively moderate (embryos/female: first year: 3.87; second year: 3.63). Each female was able to produce 28.25 pups per year. The reproductive potential showed by Cantabrian voles is,

to our knowledge, the highest one reported to date for this species; probably because the breeding season does not entail a critical factor in this area. A positive correlation between litter size and the body condition of the mother was observed. Therefore, the body condition of females seems to be one of the main factors involved in the variation of the reproductive potential in *A. scherman cantabriae*.

These studied demes showed relatively low level of genetic diversity (H_E = 0.621; H_O = 0.601; A_R = 4.42) probably due to both the inbreeding and genetic drift effects. Significant genetic differentiation appeared among demes, which revealed a strong pattern of significant isolation-by-distance both for Euclidean distances (r = 0.790) and effective distances (r = 0.780). The spatial autocorrelation analysis detected four genetic clusters or populations in this study area (120 km^2). Thus, this mosaic of different land-use plots decreases connectivity among suitable habitats even at local scale, in which *A. scherman* populations mainly depend on birth and death rates. An estuary and a four-lane road did not suppose a barrier for gene flow of this species. Less seasonal environment and highly patched landscape would suggest that this species does not show well marked multiannual fluctuations of density at large scale in this area.

Control strategies for *A. scherman cantabriae* at a regional scale can be discarded. The monitoring of each population, or management unit, will be essential to know the population dynamic and to establish coordinated control strategies. Preserving and promoting this patchy landscape would favour the presence of predators and hamper dispersion of this species. A continuous population control throughout the year would be advisable, using sustainable methods, such as traps, the installation of barriers and/or coordinated manipulation of habitat.

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Chapter 1. Introduction

1.1 Taxonomy and geographical distribution of the genus *Arvicola*

Arvicoline rodents are distributed along North America and Eurasia (Wilson & Reeder 2005) and occupy mainly meadows and bush areas, both in natural and human-modified habitats (O'Brien 1994; Palomo *et al*. 2007; MacDonald & Barrett 2008). Some arvicoline taxa have burrowing behaviour and live frequently underground or semi-underground, although surface movements under vegetation are common (Blanco 1998; MacDonald & Barrett 2008). The genus *Arvicola* Lacépède 1799 is a Palaearctic arvicoline group that show a noticeable phenetic and ecological plasticity, including both semiaquatic and fossorial representatives.

Since the 1970's two species have been traditionally considered within the genus *Arvicola*: the southwestern water vole, *Arvicola sapidus* Miller 1908, and the northern water vole, *Arvicola terrestris* (Linnaeus 1758) (Corbet 1978, Gromov & Polyakov 1992, Shenbrot & Krasnov 2005). The populations of the first species are always associated with water streams or marshes and their individuals are relatively homogeneous in morphology (Ventura 2012). *Arvicola terrestris* is highly polymorphic and shows two ecological forms: the semiaquatic form, that populates rivers, streams, lakes, marshes and wet areas both in lowlands and mountains; and the fossorial form that occurs in dry or mesic grasslands where they live in underground burrows (Meylan 1977). Nevertheless, Blasius (1857) had early noted that the name *amphibius* should have taxonomic priority over *terrestris*. Additionally, due to the fossorial specimens of *A. terrestris* are morphologically and ecologically differentiated from semiaquatic ones, Musser & Carleton (2005) recognised two species in their taxonomic approaching on this species, backed up by the previous literature on this issue (Zagorodniuk & Panteleyev 2000 and references therein). Thus, specimens with semiaquatic habits were attributed to *A. amphibius* (northern water vole), and those that live underground and have a fossorial behaviour were assigned to *A. scherman* (montane water vole). However, recent phylogenetic analyses do not support this taxonomical pattern, in which both morphotypes would be likely to be the extremes of a phenotypic continuum rather than two discrete morphotypes (Kryštufek *et al*. 2015). So, it has been proposed the use of *A. amphibius sensu lato* (*s.l.*) to assemble fossorial (*A. scherman*) and semiaquatic populations (*A. amphibius sensu stricto, s.s.*) (Kryštufek *et al*. 2015). Castiglia *et al*. (2016) also encountered this lack of genetic differentiation among nearby populations of fossorial and semiaquatic voles in the Italian peninsula, which in turn both ecotypes should be attributed to a new species, *Arvicola italicus*. Since this taxonomic problem is not fully resolved, the taxonomic pattern by Musser & Carleton (2005) has been followed in the present research.

The geographic distribution of *A. amphibius* includes a great part of Europe (excluding Spain and Portugal, midwest of France, Ireland, South and North of Norway, and South of Greece); through Caucasus, North of Turkey, North of Israel, Iran and Iraq; east through Siberia from Arctic Sea south to lake Biakal and northwestern China (Fig. 1.1) (Musser & Carleton 2005). Otherwise, *A. scherman* has a more restricted distribution, occurring in the main mountainous massifs of Europe, including northern Spain (Cantabrian region and the Pyrenees), northern Portugal, the Alps, the mountains of central Europe and the Carpathians (Kryštufek *et al*. 2015 and reference therein) (Fig. 1.1).

Fig. 1.1. Distribution of *A. amphibius* and *A. scherman*. Adapted from Kryštufek *et al*. (2015).

1.1.1 *Iberian subspecies of* Arvicola scherman

Two subspecies of *A. scherman* are currently recognised for the Iberian Peninsula (Ventura & Gosálbez 1990a; Musser & Carleton 2005): *A. scherman monticola*, which occurs in the Pyrenees, and *A. scherman cantabriae* (Fig. 1.2), distributed along the Cantabrian mountains, Sierra de los Ancares, the north of the provinces of Zamora and Guipuzcoa, and the northern tip of Portugal (Ramalhinho & Mathias 1988; Ventura 2007; Romero-Suances 2015; Fig. 1.3). Cantabrian specimens show significantly lower body size and smaller skull dimensions than those from Pyrenean populations (Ventura & Gosálbez 1990a; Ventura 1993). Both taxa also differ significantly in skull morphology (Ventura and Gosálbez 1990a). Additionally, divergences in non-metric cranial traits suggest the existence of a certain genetic differentiation between Pyrenean and Cantabrian montane water voles (Ventura and Sans-Fuentes 1997).

Fig. 1.2. Specimen of *Arvicola scherman cantabriae*

Fig. 1.3. Distribution of *A. scherman* subspecies in the Iberian Peninsula. Information obtained from Ventura (2007) and Romero-Suances (2015).

1.2 The montane water vole *Arvicola scherman*

1.2.1 Biology and ecology

The body mass of adult specimens of *A. scherman* ranges from 80 to 183 g and their body length from 12 to 18 cm (Quéré 2009, Ventura 1993). The tail (4-8 cm) and the ears (10-14 mm) are relatively small in size (Quéré 2009; Ventura 1993). This species lives preferably in fresh and wet soils with abundant vegetation, where it digs extensive burrow systems (Airoldi 1976). These underground networks consist of superficial galleries (less than 20 cm from the surface) aimed to food-obtaining, and deeper galleries (40-100 cm deep) used for nests and food storing chambers (Airoldi 1976; Airoldi & De Werra 1993). The daily activity occurs both during day and night and it is polyphasic, with six phases of activity and rest through 24 hours (Airoldi 1979). Fossorial water voles use the incisors to dig, then remove the earth with their paws, and finally push the excavated earth out with the head forming soil mounds in the surface (Airoldi *et al*. 1976).

The montane water vole lives in independent familiar burrow systems occupied by a couple of adults with their offspring (Morel 1981), although complex associations during the breeding season have been reported (Airoldi 1978; Morel 1981). The area occupied by a family group seldom exceeds 200 m^2 and both males and females are responsible for its maintenance and defence (Meylan 1977; Morel 1981; Quéré 2009). The reproductive activity of fossorial water vole populations studied to date significantly decreases in winter due to the unfavourable environmental conditions (Airoldi 1978; Morel 1981; Pascal 1981; Ventura & Gosálbez 1990b; Ventura *et al*. 1991). Even so, *A. scherman* shows high reproductive capabilities, since a female is able to have six litters per year, with a litter size of 2 to 9 embryos (Quéré 2009, Ventura & Gosálbez 1990c). This relatively-high population growth allows populations of *A. scherman* from France to reach averages of 500 individuals per hectare, and up to 1,000 voles/ha during population peaks (Delattre & Giraudoux 2009).

Dispersal and colonisation of new habitats are essential processes in *A. scherman* (Saucy 2002; Berthier *et al*. 2006; Foltête *et al*. 2016). The natal dispersal in this species is conducted by juvenile and subadult specimens above ground (Saucy & Schneiter 1998). These movements take place mostly during rainy nights probably due to: lower predation pressure (dark and rainy conditions decrease predator senses), predator overwhelming (a synchronous dispersal saturates predators), and more favourable conditions to burrow and successfully settle in wet soils (Saucy & Schneiter 1998; Saucy 2002). Otherwise, above-ground movements of adult specimens are uncommon (Saucy 2002) and can be associated with breeding dispersal (Le Galliard *et al*. 2012).

The achievement of successful dispersal and subsequent settlement supposes effective movements, nevertheless, landscape characteristics can influence this process in *A. scherman* (Fichet-Calvet *et al*. 2000; Foltête & Giraudoux 2012; Foltête *et al*. 2016). On the one hand, homogeneous landscapes, such as large meadow areas, allow connectivity among favourable habitats and hence effective movements in this species (Giraudoux *et al*. 1997; Fichet-Calvet *et al*. 2000; Foltête & Giraudoux 2012). On the other hand, heterogeneous landscapes can prevent colonisation of fossorial water voles directly by slowing their dispersion and indirectly by favouring predation (Giraudoux *et al*. 1997; Fichet-Calvet *et al*. 2000; Foltête *et al*. 2016). Connected favourable habitats lead to merged populations of *A. scherman* and a synchronization of their population densities (Giraudoux *et al*. 1997; Morilhat *et al*. 2008; Berthier *et al*. 2013). For example, fossorial water voles from France and Switzerland show well-marked multiannual fluctuations of density, commonly named cycles, every 5 to 9 years (Saucy 1988; Giraudoux *et al*. 1997; Fichet-Calvet *et al*. 2000). Consequently, *A. scherman* outbreaks spread as a wave at a regional scale with an estimated propagation speed of 7.4 km per year in France (Berthier *et al*. 2013). Individual dispersal of fossorial water voles is unlikely to reach more than a hundred of meters (Saucy 1988; Saucy & Schneiter 1994), which suggests that outbreak spreads would involve consecutive effective movements of these synchronous populations (Fichet-Calvet *et al*. 2000; Berthier *et al*. 2013).

Fossorial water voles consume both epigeic and hipogeic parts of plants (Kopp 1993), preferably roots, bulbs and tubers (Airoldi 1976) of dicotyledons and some Poaceae (Kopp 1988). The energy demand of this species is relatively high, and hence each specimen should daily ingest the equivalent of its body mass on food (Quéré 2009). The presence of *A. scherman* in grasslands and orchards may trigger important agricultural damages and hence serious economic losses in several countries, such as France (Delattre & Giraudoux 2009), Switzerland (Blant *et al*. 2009), Germany

(Walther *et al*. 2008) and Spain (Miñarro *et al*. 2012). As a consequence of its activity, vegetation is covered by earth mounds which interfere with farming practices (Meylan 1977). In the case of apple (*Malus domestica* Borkh) trees, fossorial water voles feed on roots and only a few specimens may lead to substantial losses (Meylan 1977; Walther *et al*. 2008). Furthermore, damage to cherry, plum and pear trees (Meylan 1977), but also damages to kiwi trees, annual crops (Braña 2001) and grass silages can occur (personal observation).

The increase of favourable habitats in the main mountainous areas of Europe in the last decades has increased water vole damages to agriculture (Halliez *et al*. 2015). In that sense, traditional land use of subsistence, frequent in these regions, suffered an abandonment in favour of farming specialization in livestock breeding, which requires large grazing areas and leads to a decrease of ploughed fields (Halliez *et al*. 2015 and references therein). Furthermore, meadow plots intended for mowing lead to even more favourable habitat for fossorial water voles than grasslands (pastures), especially while grass is maintained high (Morilhat *et al*. 2007). Thus, this change in land uses could be the reason for an unimpeded spread of the fossorial water vole populations along agricultural landscapes in eastern and central France and in western Switzerland (Giraudoux *et al*. 1997; Morilhat *et al*. 2008).

1.2.2 Population control

Since the 1970s, most of population control strategies aimed to reduce the spread of fossorial water voles involve rodenticides, frequently bromadiolone (Defaut *et al*. 2009). High persistence of this rodenticide in the field allows voles to continue feeding beyond the lethal dose (Sage *et al*. 2008), and toxic bait can remain in store chamber inside burrows, being available for survivors and colonizers for long periods (Giraudoux *et al*. 2006). Thus, large proportion of contaminated fossorial water voles can be available for predators (Coeurdassier *et al*. 2014). Most *A. scherman* specimens remain in their galleries when they fall ill, being only available for predators such as mustelids and foxes (Sage *et al*. 2008), but 38 % of them can die aboveground (Saucy *et al*. 2001) and hence be consumed by birds of prey or scavengers, such as crows, buzzards or owls (Sage *et al*. 2008; Montaz *et al*. 2014). Overall, this practice involves a high risk of primary and secondary poisoning, whereby chemical control in France is strictly regulated since 2005 according to a threshold of vole density (Coeurdassier *et al*. 2014).

In Spain, the integration of a set of phytosanitary measures belonging to an official control program would entail prevention and control of high densities of *A. scherman* in accordance with the article 15 of the Law 43/2002 of plant health (BOE 2008). Thus, managers and land owners with damages caused by fossorial water voles are bound to reduce their population densities (BOE 2008). This official program could consider a restricted use of rodenticides when vole population densities reach very high values (BOE 2008). However nowadays, rodenticides should comply with current legislation and not involve adverse environmental consequences, produce resistances in the target species, unnecessary pain or to be a threat to non-target animals and humans (Regulation 528/2012/UE 2012). Thus, all phytosanitary products, excepting aluminium phosphide (56%), which is restricted to specialized professionals (BOE 2010), have nowadays been excluded from the official registration of plant protection products of the Ministry of Agriculture and Fisheries, Food and Environment (Government of Spain) for controlling vole populations in crop fields (MAPAMA 2017).

The official control program in Spain recommends all of those sustainable phytosanitary measures that are able to slow down the population growth of fossorial water voles through the implementation of cultural measures, control of refuge areas, promotion of natural enemies or systematic trapping (BOE 2008). That is, the application of effective, specific and environmentally benign control practices is advisable (Jacob 2013) in attempt to establish an integrated management strategy of this pest (Couval & Truchetet 2014). In this way, snap traps (Topcat®, Supercat®, pincer traps; Supplemental fig. 1.1) are widely used by farmers and land managers against voles and they can be effective once the trapper is skilled (Fuelling *et al*. 2010; Defaut *et al*. 2009). The installation of barriers surrounding plots placed both above- and belowground together with self-service traps for terrestrial predators can be a suitable method to stop fossorial water vole colonisation (Fuelling *et al*. 2010; Walther & Fuelling 2010). A toolbox of actions aimed to modify *A. scherman* habitat was considered in France (Defaut *et al*. 2009). High grazing intensity, the removal of the protective grass cover or soil cultivation cause the destruction of vegetation shelter which in turn promotes predation pressure and dehydration (Morilhat *et al*. 2007 and references therein; Defaut *et al*. 2009). Likewise, farm machinery, cattle trampling or simulation of trampling by machinery can destroy burrow systems and food resources for fossorial water voles (Morilhat *et al*. 2007; Defaut *et al*. 2009). However, just a moderate mowing does not show negative consequences on vole densities (Morilhat *et al*. 2007). Moreover, it is known that dispersal specimens of *A. scherman* can use *Talpa europaea* galleries as first step of colonisation (Morilhat *et al*. 2007 and references therein). Thus, a less-use of fertilization would result in a decrease of invertebrates and less number of *T. europaea* galleries, which ultimately would lead to less colonisation chances for fossorial water voles (Morilhat *et al*. 2007). Ultimately, managers should update their perception of risk and hence estimate fossorial water vole abundance through the quantification of earth mounds (Giraudoux *et al*. 1995; Miñarro *et al*. 2012). To achieve this, signs of activity caused by *A. scherman* and *Talpa* spp. should be accurately differentiated in sympatric areas; a linear distribution of mounds, and the occurrence of "earth sausages" and earth paths are attributed to mole activity (Supplemental fig. 1.2) (Giraudoux *et al*. 1995; Miñarro *et al*. 2012).

Higher numbers of predators can also entail a reduction of the rodent pest population (e.g. Pelz 2003; Fuelling *et al*. 2010; Paz *et al*. 2013). Fossorial water voles may be predated by specialized predators, such as stoats (*Mustela erminea*), weasels (*Mustela nivalis*) or stone martens (*Martes foina*), which decrease vole number further low densities (Giraudoux *et al*. 1994; Delattre *et al*. 2009). But they may be also predated by unspecialized predators, such as foxes (*Vulpes vulpes*) (Weber & Aubry 1993) or barn owls (*Tyto alba*) (Nores 1989), which can switch to alternative preys when vole densities decrease (Giraudoux *et al*. 1994; Delattre *et al*. 2009). Closed landscapes, such as those with abundant wooded areas, generally favour the presence of predators (Giraudoux *et al*. 1994). Accordingly, Giraudoux and co-workers (1994) proposed three main topics in landscape management to control fossorial water voles: favouring mosaic patterns of grasslands and forest; maintaining the forest heterogeneity; and preserving and developing hedgerow networks. In this way, an increase of the landscape patchiness and the establishment of hedgerows might result useful to hamper fossorial water vole diffusion (Foltête & Giraudoux 2012; Foltête *et al*. 2016).

1.3 The montane water vole in Asturias

1.3.1 Presence and damage

The presence of fossorial water voles in Asturias (northwestern Spain) goes back to the Solutrense (Upper Paleolithic, about 22,000 years before present) as highlighted the dentitions of several specimens found in deposits of Caldas (Oviedo) (Laplana-Conesa *et al*. 2006), Balmori, Coberizas and La Riera (Llanes) (García-Ibaibarriaga *et al*. 2012). However, the current presence of populations of *A. terrestris* (read *A. scherman*) in this region was indicated by Heim de Balsac & de Beaufort in 1969 through the study of barn owl pellets from Gijón and Picos de Europa. Shortly after, two studies focused on the diet of this bird of prey revealed a wide distribution of *A. scherman* in Asturias (Braña 1974, Nores 1989). Through comparative analyses of skull morphology between specimens from Ribadesella (Asturias) and the Aran Valley (Lérida), Ventura & Gosálbez (1988) recognised an own subspecies (*A. scherman cantabriae*) for the Cantabrian region.

The agricultural activity developed since prehistory propelled the substitution of deciduous forests by meadows in Asturias (Díaz-Maroto 2009), whereas formerly natural meadows were restricted to elevated areas (Díaz-González & Fernández-Prieto 2005). This deforestation continues presently; just in 14 years (1998-2012) the total forest surface in Asturias has decreased by 15.5% (SADEI 2017). Consequently, the distribution area of *A. scherman* in Asturias could have increased in the past due to an expansion of its favourable habitats, as observed in France (see Halliez *et al*. 2015). Nowadays, in Asturias this species occurs mainly on meadows, grasslands and orchards (Fernández-Ceballos 2005), from the sea level to mountains (Noval 1981; Nores 1986), at least up to 1,600 m a.s.l. (Supplemental fig. 1.3, personal observations). Nevertheless, fossorial water voles seem to lack in several areas of Asturias with theoretically-suitable habitats (Heim de Balsac & de Beaufort 1969; Ventura & Gosálbez 1988; Ventura 2007).

Damages produced by the montane water vole on its preferential habitats are well described in Asturias since the 1970s (Noval 1976, 1981; Nores 1986; Braña 2001). From the end of the 1980s to date, population outbreaks of *A. scherman* have been recorded in several municipalities, such as Valdés, Tineo, Sariego or Villaviciosa (Supplemental fig. 1.4). These recent outbreaks, and hence subsequent agricultural damage, has stirred up several popular beliefs widely extended, such as the release of airborne fossorial water voles (Supplemental fig. 1.4). However, high population densities might be related with an increase of suitable habitats for *A. scherman cantabriae* in recent years. This fact could be due to a general increase of permanent grasslands, a reduction of ploughed fields or directly land abandonment in this region, perhaps not so intense as it has happened in most montane areas of Europe (Halliez *et al*. 2015). Even so, from 1962 to 2009, permanent grasslands in Asturias increased from 21.3% to 65.2%, whereas the total ploughed land decreased from 6.3% to 4.2% (INE 2012).

1.3.2 Apple farming in Asturias

Apple orchards are scattered along Asturias, although most plots intended to this crop are mainly located in centre-eastern area of the region (Fig. 1.4). Agricultural areas of this region are characterized by smooth hills and valleys and the landscape is featured by a mosaic of small agricultural plots separated by hedgerows and woodlands (Supplemental fig. 1.5). The climate is temperate hyperoceanic (Rivas-Martínez & Rivas-Sáenz 2015) with abundant rainfall spread along the year and benign temperatures with low risk of frost and snowfall. A dense grass coverage is established in orchards all the year (Miñarro 2012) driven by this climate and fertile soils (Díaz-González & Fernández-Prieto 2005).

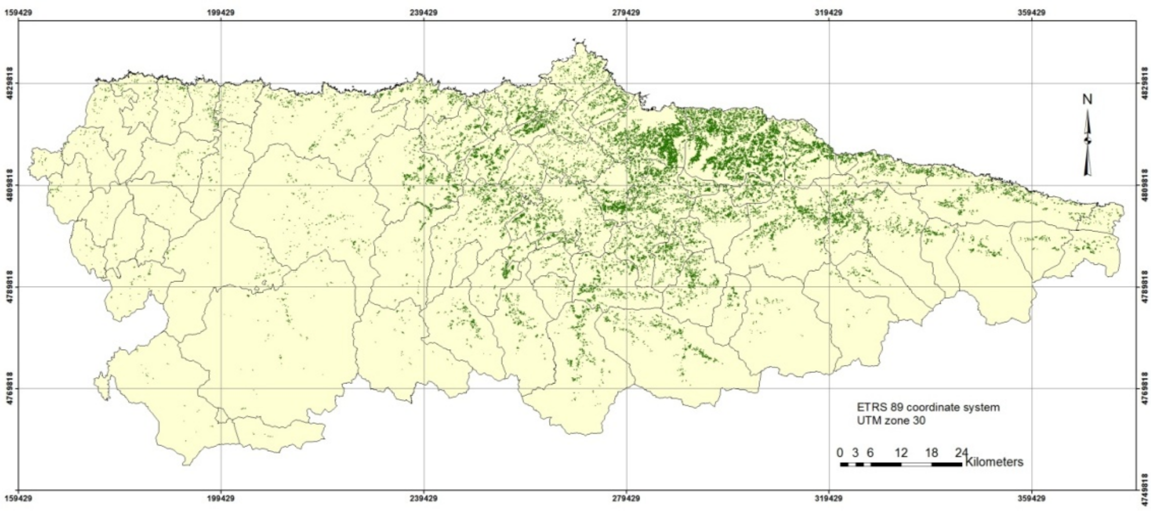


Fig. 1.4. Apple orchard (green spots) distribution in Asturias.

Traditionally, apple is cultivated in extensive orchards based mainly on large cider-apple trees with a tree-density of 100-250 trees/ha (Dapena *et al*. 2008). These trees have good anchorage and high branches which allow cattle grazing (mainly cows or sheep) without damaging trees (Dapena *et al*. 2008; Supplemental fig. 1.6). However, large trees are not precocious and their management ensues some disadvantages. Therefore, traditional apple orchards have been progressively substituted by semi-intensive ones since 1990s to improve productivity and facilitate management (Dapena *et al*. 2005). In this system trees are grown on semi-dwarfing rootstocks with a tree-density of 550-750 trees/ha, in which the irrigation and trunk support are unnecessary in most of cases (Dapena *et al*. 2005; Supplemental fig. 1.7). The sum of both types of apple orchards occupies 10,324 ha in Asturias (INDUROT 2010), a 32.4% of the total planted area intended to this crop in Spain (31,812 ha), whose national production reached 546,400 tons in 2013 (FAO 2013). Most of the apple production in Asturias is devoted to cider making, which involves 45 companies with an economical profit from 260,000 to 1.5 million € year^{-1} company^{-1} (Rubio 2012).

Local cider-apple cultivars are quite resistant to fungal diseases (e.g. scab, powdery mildew, European canker) and most harmful arthropods are kept at bay because of tolerant cultivars, biological control and specific treatments (Dapena *et al*. 2005). Moreover, the presence of the montane water vole and the Lusitanian pine vole, *Microtus lusitanicus* Gerbe (1879), in Asturian apple orchards (Supplemental fig. 1.8) has become the main cause of current economical loss (Supplemental fig. 1.4). Both species may cohabit in the same plots, although the negative relationship between their population densities suggest a competitive displacement probably for resources (Miñarro *et al*. 2012). The higher body mass of *A. scherman* suggests that this species may be a superior competitor (Miñarro *et al*. 2012). Fossorial water voles can injure substantially the root system (Fig 1.5a) and make the trees less productive (Meylan 1977; Walther *et al*. 2008) and more susceptible to falling down by winds or high fruit load due to deficient anchorage (Fig 1.5c). Furthermore, trees on semi-dwarfing rootstocks have significant lower resilience to vole damages than large trees, especially just after planting (Miñarro *et al*. 2012) (Fig 1.5b). Damages caused by fossorial water voles might reach 40,000 €. ha^{-1} year^{-1}, as reported in German apple orchards (Walther *et al*. 2008).

Fig. 1.5. Damages caused by *A. scherman* on apple trees in Asturias. Damages on root system (a, by M. Miñarro), damages on the root system of young trees (b), and a damaged large tree belonging to an extensive orchard (c).

The Asturian apple orchards are visited by several terrestrial predators, such as stoats, weasels or foxes; or avian predators such as barn owls, tawny owls (*Strix aluco*) or buzzards (*Buteo buteo*); all of them are able to feed on *A. scherman* (Giraudoux *et al*. 1994; Álvarez-Laó *et al*. 1996). Other mammals that could be find in these orchards are wild boars (*Sus scrofa*), roe deers (*Capreolus capreolus*), and small mammals such as *Talpa occidentalis* -which also lives in burrows and generates earth mounds on the ground- (Miñarro *et al*. 2012), Millet's shrew (*Sorex coronatus*), white-toothed shrew (*Crocidura russula*) and wood mice (*Apodemus sylvaticus*) (personal observations).

1.3.3 Current situation to face A. scherman

Until now, the control of fossorial water vole populations in Asturias has been achieved by placing rodenticides or snap traps at burrow entrances, without coordinate actions among farmers and according to the grower perception of threat. Since winter is thought to be the most unfavourable season for rodent reproduction in temperate-climate areas (e.g. MacDonald & Barrett 2008), it is generally accepted by farmers that fossorial water voles stopped reproduction in this season. Thus, the general strategy of rodenticide use and trapping is to increase both activities in winter, when the crop is less demanding in labour, in order to focus efforts on removing adults before the expected start of reproduction in spring.

In recent years, the Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA) of Asturias has conducted some studies to improve the scarce knowledge on this species in this agroecosystem and hence lay the groundwork for its control. These studies were focused on the identification and use of presence signs to estimate abundance of fossorial water voles in apple orchards (Miñarro *et al*. 2012), habitat preferences (Fernández-Ceballos 2005) or incidence of vole damage on different apple rootstocks (Fernández-Ceballos & Dapena 2007). Furthermore, some informative articles have been written to inform managers and landowners about new insights (Miñarro & Dapena 2010, 2011; Miñarro *et al*. 2013). Nevertheless, a deep knowledge of essential aspects of *A. scherman cantabriae* biology and ecology is required to enhance the implementation and success of sustainable control methods (see Delattre & Giraudoux 2009; Jacob 2013; Ranchelli *et al*. 2016).

1.4 Hypothesis and objectives

The reproductive characteristics of the montane water vole are known exclusively from a population of *A. scherman monticola* located above 900 m a.s.l. in the Spanish Pyrenees (Ventura & Gosálbez 1990b, c). Since environmental factors associated to altitude can affect reproduction in rodents (Zammuto & Millar 1985; Hille & Rödel 2014), important differences in the reproductive pattern between this Pyrenean population and *A. scherman cantabriae* populations located in apple orchards below 400 m a.s.l. in Asturias can be expected. Likewise, since the reproductive potential in water voles is related to the habitat characteristics (see Williams *et al*. 2014) and the body dimensions of the mother (Wieland 1973; Ventura & Gosálbez 1990b), this reproductive variable could differ noticeably between Pyrenean and Cantabrian populations.

The current land use is a key factor on the dispersal and colonisation of *A. scherman* (Duhamel *et al*. 2000; Halliez *et al*. 2015; Foltête *et al*. 2016). High proportion of interconnected optimal habitats allows an unimpeded population diffusion and hence it can lead to population outbreaks (Giraudoux *et al*. 1997; Fichet-Calvet *et al*. 2000; Berthier *et al*. 2013). However, heterogeneous landscapes, such as the Asturian one, could obstruct, at least in part, fossorial water vole movements (Foltête &

Giraudoux 2012; Foltête *et al*. 2016). Therefore, *A. scherman* populations might be structured at a smaller scale than in homogeneous landscapes due to a restriction of dispersal and colonisation processes. The study of the landscape effect on *A. scherman* dispersal can be achieved using microsatellite-based analyses, which provide suitable information on population genetics (e.g. Coulon *et al*. 2006; Driscoll 2007; Le Galliard *et al*. 2012).

The main goal of this research is to know the reproductive biology and population genetics of *A. scherman* in apple orchards immersed in the agricultural landscape of Asturias. This approach is intended to provide new and necessary insights about key aspects to go in depth on the pest role of this vole. Specific goals are the following:

1. To obtain detailed information on the reproductive cycle of *A. scherman* in apple orchards from Asturias (Chapter 2).

2. To determine the reproductive potential of *A. scherman* in apple orchards from Asturias (Chapter 3).

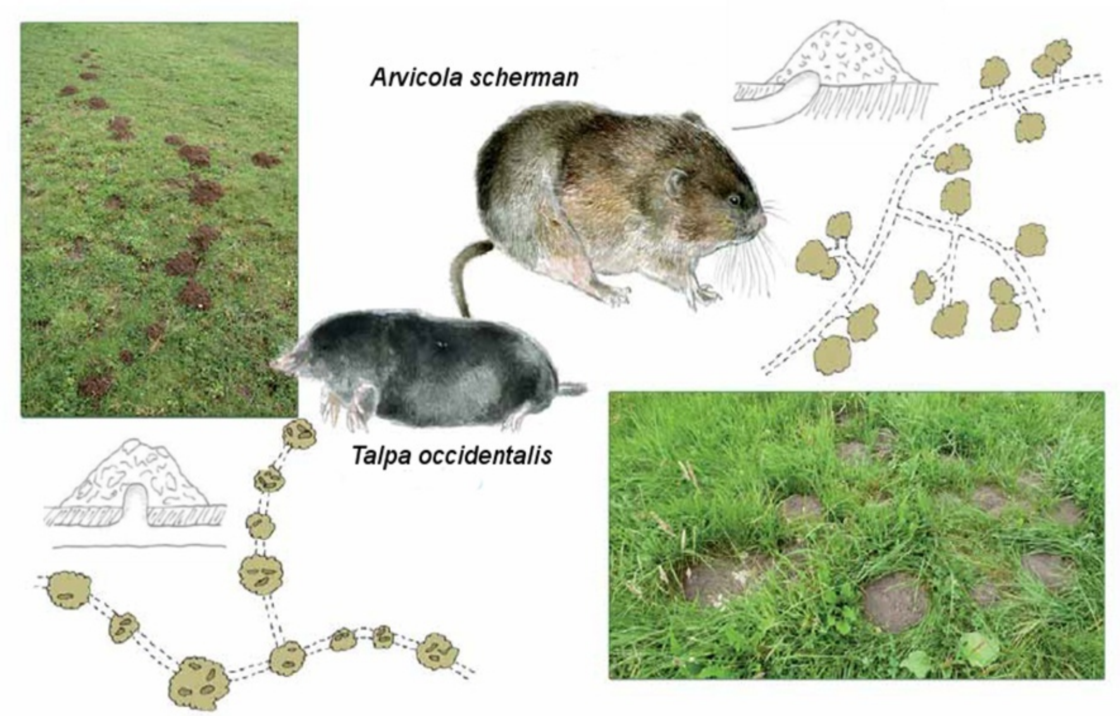
3. To highlight the possible differences in the reproductive pattern between Iberian subspecies of fossorial water voles (*A. scherman cantabriae* from Asturias and *A. scherman monticola* from Pyrenees) regarding the different environmental conditions and the different morphological characteristics (Chapter 2 and 3).

4. To assess the effect of the agricultural landscape of Asturias on the population genetics of *A. scherman* (Chapter 4), through two approaches: i) exploring the intra- and inter- levels of genetic diversity among several demes and assessing their loss of genetic diversity; and ii) revealing the gene flow and the spatial genetic structure of *A. scherman* in the main area of apple production in Asturias.

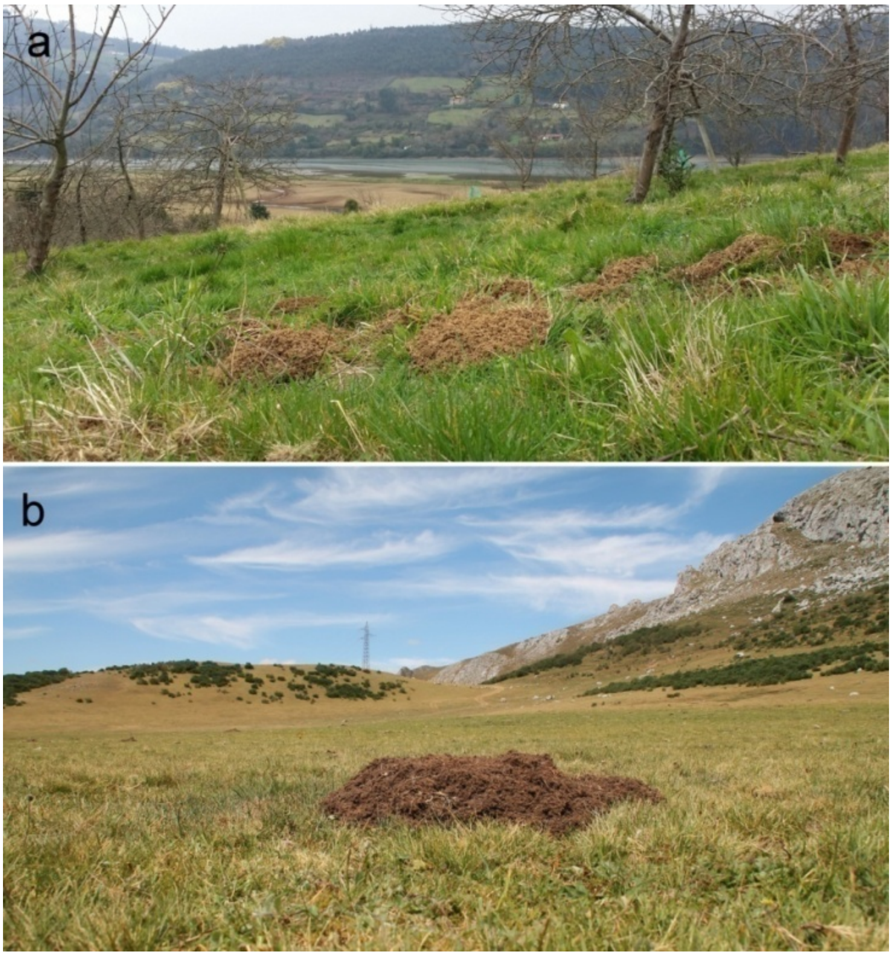
5. With the information obtained on the reproduction and genetic issues, a further aim is to propose sustainable control methods focused to decrease population densities of fossorial water voles, both in our study area and in others with similar environmental characteristics (Chapter 5).

1.5 Supplemental information

Supplemental fig. 1.1. Different snap traps for voles: Supercat®, pincer traps and Topcat® (by M. Miñarro). Instructions to use Supercat® and Topcat® traps: Find the vole gallery with a searching rod and cut a hole to access to the tunnel. Next, set the trap into the hole in such a way that the passage through the trap should correspond with the height and direction of the tunnel. Finally, cover the remains of the open hole with earth or grass and release the mechanism. (Lower figure taken from http://www.topcat.ch/Instruction-1_5.html).



Supplemental fig. 1.2. Surface signs of activity and burrow systems of *A. scherman* and the Iberian mole, *T. occidentalis* (Adapted from Miñarro *et al*. 2012).



Supplemental fig. 1.3. Surface signs of activity of *A. scherman* at 15 m a.s.l in Villaviciosa, (Asturias) (a) and at 1,600 m a.s.l. in Lena (Asturias) (b).

14 / Asturias

Los campesinos, que están desconcertados, han comprado una máquina en Suiza para luchar contra los animales

Una plaga de ratas arrasa las praderías de las vegas valdesanas de Barcia, Santiago y Otur

NOTICIA BOMBA

VIAJE GRATIS A LAS REBAJAS DEL CENTRO DE LA PIEL

TRIANGULO INFORMATICO

Area metropolitana

Roedores en paracaídas

EVELIO G. PALACIO

Los vecinos de Cabranes y Villaviciosa andan locos detrás de unos ratones que acaban con las pomaradas. Dicen que al perro flaco todo se le vuelven pulgas, y sólo faltaba que los malditos roedores destruyeran lo poco que queda en pie del campo. Más que perros, lo que necesitarían los vecinos de Villaviciosa son gatos, que por lo menos no cuestan tanto como el veneno ni meten tanto ruido como las escopetas con las que alguno se dedica a cazar las ratas-topo como si fueran liebres.

Lo curioso es que la invasión de los pomares aparece cuando por toda la zona rural asturiana proliferan las más inverosímiles historias de ratas y reptiles. La veda la abrió el diputado Fernando Angel Cuervo, al que se le ocurrió llevar a la Junta una culebra en un tarro para regalar a María Luisa Carcedo. Por aquel entonces corría el bulo, también por Villaviciosa, de que, por presiones de los ecologistas, se habían repoblado montes con víboras lanzadas desde una avioneta.

Aquello fue, nunca mejor dicho, una serpiente de verano. Pero la bola siguió rodando, y ahora los que tienen habilidades aeronáuticas son los ratones. Un vecino de Sariego decía el otro día que sabía de un ganadero que había encontrado un ratón con paracaídas. Como si fuera un personaje de Disney recién salido de la pantalla o un «paraca» de Alcantarilla (Murcia) en maniobras por el Norte.

El caso es que, por increíble que parezca, la broma corre de boca en boca como en la mejor época de la tradición oral. Todos hablan de oídas y es imposible hallar la fuente directa. Ahora la invasión de Villaviciosa y Cabranes acabará dándole carta de naturaleza y colocará esta trola en el olimpo de las leyendas.

L.N.E. 28 Abril. 1993

lne.es NOTICIAS Centro

Comarca de la SidraEl problema

Los cosecheros piden medidas para acabar con los topillos que arrasan las pomaradas

Los afectados reclaman la declaración de plaga I Los roedores atacan las raíces y han reducido a menos de la mitad los manzanos jóvenes

Villaviciosa / Nava / Sariego,

Mariola MENÉNDEZ

El problema, lejos de mejorar, ha empeorado. Hace un año por estas fechas que los fruticultores asturianos, principalmente dedicados al cultivo de manzanos, lanzaron la voz de alarma sobre los daños que venía causando desde hace unos nueve años la rata-topo -conocida científicamente como «Arvícola terrestris»- en sus pomaradas de la Comarca de la Sidra. Estos roedores se alimentan de raíces, lo que provoca que los árboles que atacan se sequen y mueran. Los cosecheros de manzana de sidra demandan de nuevo medidas eficaces urgentes para acabar con estos animales que arrasan con sus plantaciones.

Antonio Torres, con herramientas contra los topillos. mariola menéndez

Los topillos se multiplican por el buen tiempo y arrasan en Sariego

Los roedores desesperan a los dueños de las pomaradas: "Es muy difícil luchar contra ellos, se reproducen rápido"

Supplemental fig. 1.4. Some Asturian media press news related to population outbreaks and damages caused by *A. scherman* and *M. lusitanicus*.

Supplemental fig. 1.5. Agricultural landscape of Asturias. The topography is characterized by smooth hills and valleys, and land management leads to a mosaic of small agricultural plots separated by hedgerows and woodlands (Figure taken from http://www.laspain.com/fotos-de/asturias/boal/327.html).

Supplemental fig. 1.6. Extensive orchards based on large cider-apple trees in Asturias (a, b). Cattle grazing, mainly cows or sheep was frequent (b) (by M. Miñarro).

Supplemental fig. 1.7. Semiintensive apple orchard in Asturias.

Supplemental fig. 1.8. Specimens of *M. lusitanicus* (a) and *A. scherman* (b), and their correspondingly surface signs of activity (c and d) observed in apple orchards of Asturias.

Chapter 2. Continuous breeding of fossorial water voles in northwestern Spain: potential impact on apple orchards

Adapted from: Somoano A, Ventura J, Miñarro M (2017) Continuous breeding of fossorial water voles in northwestern Spain: potential impact on apple orchards. *Folia Zoologica*, **66**, 37-49.

2.1 Introduction

The montane water vole, *Arvicola scherman* (formerly fossorial form of *Arvicola terrestris*; for details see Musser & Carleton 2005, but see also Kryštufek *et al*. 2015) occurs in mountainous areas of southern and central Europe (Meylan 1977, Morel 1981), living underground and constructing extensive burrow systems in grasslands, pastures and orchards (Airoldi 1976). These fossorial water voles consume both epigeic and hipogeic parts of plants (Kopp 1993), and specifically in fruit crops they may feed on tree bark and roots injuring and even killing the trees (Meylan 1977, Walther *et al*. 2008). In the particular case of apple orchards, *A. scherman* caused damages valued between 50 and 40,000 € ha^{-1} $year^{-1}$ in Germany (Walther *et al*. 2008). Likewise, in Asturias (northwestern Spain), where apple is cultivated in around 10,000 ha for cider production, fossorial water voles are frequently responsible for noticeable economic losses (Miñarro *et al*. 2012, Somoano *et al*. 2016). In fact, *A. scherman* is considered one of the most harmful agricultural pests in several countries (e.g. Walther *et al*. 2008, Blant *et al*. 2009, Delattre & Giraudoux 2009), including Spain (Miñarro *et al*. 2012), where preventive actions to reduce their population densities have been recommended (BOE 2008).

The management of rodent pests requires effective, specific, environmentally benign, economically feasible and socially acceptable approaches (Jacob 2013). Thus, especially in organic farming, there is a need for environmentally sustainable strategies, avoiding the use of rodenticides which generate genetic resistance in target species and are a risk for non-target wildlife (Rattner *et al.* 2014). In France, for example, anticoagulant rodenticides have been routinely applied to control fossorial water vole populations since the 1990s (Defaut *et al*. 2009), with negative effects on predators (Coeurdassier *et al*. 2014) and scavengers (Montaz *et al*. 2014). Ultimately, a deep knowledge of relevant aspects on the pest biology and ecology are required for the implementation and success of sustainable control methods (Delattre & Giraudoux 2009, Jacob 2013, Ranchelli *et al*. 2016).

Two subspecies of *A. scherman* are currently recognised in Spain (see Ventura 2007): *A. scherman monticola*, which is found in the Pyrenees, and *A. scherman cantabriae*, which extends throughout the Cantabrian region (northwestern Iberian

Peninsula), from lowlands to mountains. These taxa are geographically isolated and show significant morphological differences (Ventura & Gosálbez 1990a, Ventura & Sans-Fuentes 1997). Although the reproduction pattern of the Pyrenean subspecies has been analysed in several studies (see Ventura & Gosálbez 1990b, c), that knowledge is scarce for Cantabrian populations. The breeding characteristics reported for *A. scherman monticola* correspond to populations from Pyrenean meadows located at about 900 m a.s.l., habitat very different from apple orchards below 400 m a.s.l. on the Asturian coast, where many populations of *A. scherman cantabriae* are established. Since altitude has an important moulder effect on reproduction in rodents (Bronson 1979, Murie *et al*. 1980, Zammuto & Millar 1985), even at small scale in the same area (Dunmire 1960, Hille & Rödel 2014), important differences in the reproduction between Pyrenean and Cantabrian *A. scherman* populations can be expected.

Arvicola scherman populations studied to date restrict their breeding to a specific favourable period (Airoldi 1978, Morel 1981, Pascal 1981, Ventura *et al*. 1991), in which the individuals synchronize the demands of reproduction according to favourable conditions. Temperature and rainfall determine the amount of available food which ultimately condition the reproductive physiology of rodents (Nelson *et al*. 1992, Demas & Nelson 1998, Bergallo & Magnusson 1999, Pierce *et al*. 2005, Medger *et al*. 2012). In this way, it is known that habitats with favourable environment allow rodent species to lengthen their reproductive period (Sicard & Fuminier 1996, Bergallo & Magnusson 1999, Trebatická *et al*. 2012). Thus, the length of the breeding season is defined by the seasonal variations of the environmental conditions (Smith & McGinnis 1968, Kriegsfeld *et al*. 2015), which might also occur in *A. scherman* populations even among temperate areas.

The main goal of this study was to generate for the first time detailed information on the reproduction cycle of *A. scherman* in Northwest of Spain, specifically in agricultural environments located at low altitudes. We also aimed to highlight reproductive differences between Cantabrian and Pyrenean populations of this species to provide robust information, which in turn can be useful for planning and improving efficient and sustainable population control strategies and to assess their effects accurately.

2.2 Material and Methods

2.2.1 Study site

The study was conducted in ten experimental and commercial apple orchards over two years (first year: February 2011-January 2012, second year: February 2012-January 2013). These orchards are separated from each other by 20.5 km as maximum and are located in two municipalities of Asturias (northwestern Spain): Villaviciosa (43°38′55″ N, 5°26′08″ W) and Nava (43°21′31″ N, 5°30′29″ W), which are separated by 15 km. Their surface ranged from 1 to 7.6 ha and their altitude from 3 to 270 m a.s.l. Apples in Asturias are mainly produced in an area characterized by an irregular topography of smooth hills and valleys, and distributed in a mosaic landscape of small agricultural plots separated by hedgerows and woodland.

The studied area has a temperate hyperoceanic climate (Rivas-Martínez & Rivas-Sáenz 2015) with abundant rainfall spread evenly along the year, and mild temperatures even in winter with low risk of frost and snowfall. Relatively high rainfall and fertile soils of Asturian meadows favour the establishment of an evergreen and dense grass coverage in orchards all the year around (Díaz-González & Fernández-Prieto 2005, Miñarro 2012). Meteorological data comprising monthly rainfall as well as temperatures and day-length in hours (Fig. 2.1) were registered for the area of Villaviciosa throughout the study period.

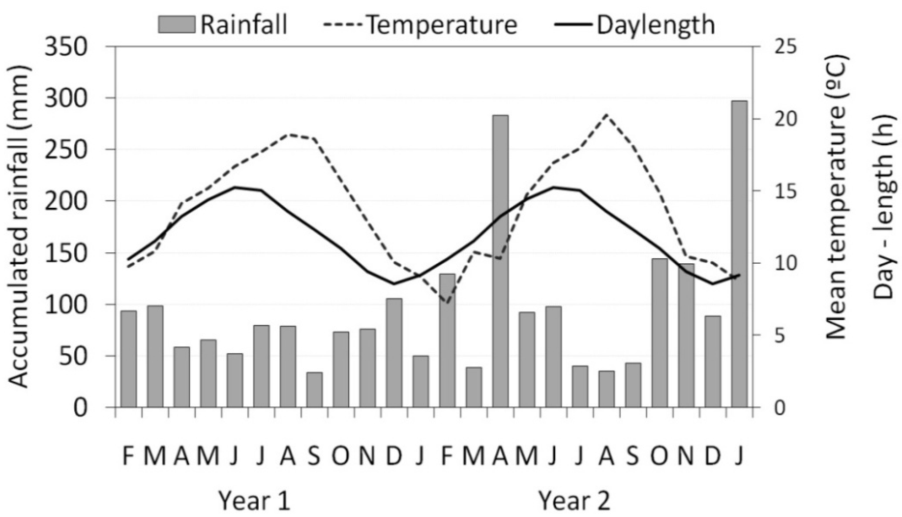


Fig.2.1. Climatic conditions in the study area. Variation of monthly temperature, rainfall and day-length in hours during the sampling period (Year 1: February 2011-January 2012; Year 2: February 2012-January 2013).

2.2.2 Specimens and data collection

A total of 823 voles (401 males, 422 females) were captured. Each month, one or more orchards, depending on orchard surface area and vole density, were visited for trapping. Voles caught in a single month were grouped and analysed together. Where possible, the same orchard was not visited in two consecutive months to avoid potential alterations on population patterns due to trappings. Voles were captured with snap traps (Topcat® Andermatt Biocontrol, Switzerland) placed in galleries. Traps were activated during the day and checked twice per day for maximum of five days. Each captured specimen was assigned to a particular burrow and thus voles caught in the same trap during the same trapping session were considered as living together. Shortly after capture, body mass (BM) was registered with a precision scale to the nearest 0.5 g and voles were cryopreserved at –20°C before necropsy (Supplemental fig. 2.1, 2.2). In the field work we have followed the recommendations of the Directive of the European Parliament and the Council on the Protection of Animals Used for Scientific Purposes (Directive 2010/63/UE 2010).

Head and body length (HBL) and carcass mass (CM) of each animal were taken. In males, the position of testes (abdominal or scrotal), maximum length (TL) and maximum width (TW) of left testis, length of the left branch of seminal vesicle (SVL), and cell content of testis and epididymis tail (Gosálbez 1987) were recorded (Supplemental fig. 2.3). Testicular volume (TV) was calculated as follows: $TV = 4/3\ \pi\ (TL/2)\ (TW/2)^2$. Length variables were measured using a digital calliper to the nearest 0.01 mm (see Ventura 1993). From these characteristics the following sexual maturity states were established: immature males, individuals lacking spermatids or spermatozoids in the testis; submature males, voles with few spermatozoids and spermatids in the testis and without or with a very scarce number of spermatozoids in the epididymis; and mature males, specimens with a large amount of spermatozoids in both structures (Supplemental fig.2.4) . Although spermatozoids were not quantified, the differences between mature and submature males was clear since, contrary to what happened in the latter, in mature individuals the presence of spermatozoids in epididymis and testis was homogeneous and high in the smears.

In females, the main criterion used to establish the maturity state was determined from the histological characteristics of the ovaries, which were removed and preserved in 70 % ethanol until the analysis was conducted. Four longitudinal histological sections (sections of 5 μm thick taken 150 μm apart) were performed on the left ovary. Sections were stained with hematoxylin-eosin (Supplemental fig. 2.5). Other sexual parameters considered in females were: status of the vulva (closed or open), degree of development and vascularisation of the uterus, and the presence of placental scars and/or embryos (Ventura & Gosálbez 1990b) (Supplemental fig. 2.6). The following sexual maturity states were established: immature females, specimens without *corpora lutea*, closed vulva, uterus poorly developed and vascularised, without placental scars; submature females, voles without *corpora lutea* but with secondary follicles and/or Graafian follicles in the ovary, open vulva, uterus poorly vascularised, without placental scars or embryos; active mature females, individuals with *corpora lutea*, uterus completely developed and well or scarcely vascularised, showing placental scars and/or embryos, and with open vulva; inactive mature females, specimens with *corpora lutea*, uterus completely developed and well or scarcely vascularised, with or without placental scars, without embryos and with closed vulva. The mass of embryos was subtracted from BM of pregnant females (Supplemental fig. 2.7) to assess the general pattern of BM variation during the study period. Moreover, pregnant females were discarded in determining BM differences between sexes and relative age classes (Table 2.1) in order to prevent considering the mass gain associated to pregnancy (Nazarova & Evsikov 2008).

A subsample of 611 specimens was classified into six classes of relative age (0-V). This subsample was formed by all immature and submature individuals, and a random selection of mature ones to balance their high number of captures. The assignation to each class was done according to the following criteria: moulting stage (Supplemental fig. 2.8), sturdiness of the mastoid and the condylar processes, the separation of the angular process from the ascending branch of the mandible (Supplemental fig. 2.9), and the values of the interorbital crests index (ICI), which relates the breadth of the interorbital crests and the rostral length (see Ventura 1992, Ventura & Gosálbez 1992 and references therein) (Supplemental fig. 2.10). The approximate age intervals corresponding to these age classes are the following (see Ventura & Gosálbez 1992 and references therein): class 0, 0-3 weeks; class I, 3-6 weeks; class II, 6-10 weeks; class III, 10-14 weeks; class IV, 14-30 weeks; class V, specimens older than 30 weeks (Supplemental fig. 2.11). Condylobasal length (CL) and length of upper diastema (LUD) were also measured. Cranial variables were taken only on undamaged skulls (Supplemental fig. 2.9).

2.2.3 Data analyses

To obtain values for BM and HBL to set sexual maturity at a population level, BM_{50} and HBL_{50} were calculated. This approach provides the values of BM and HBL at which 50% of specimens of the population are mature, and marks a cut-off value above which all individuals can be considered as adults (Pelikán 1972). BM_{50} and HBL_{50} were calculated using a probit regression model, which relates a binomial response variable (mature–non-mature) with an independent variable (BM and HBL) (Finney 1952, Pelikán 1972, Ventura & Gosálbez 1990b).

Significant differences in body and cranial measurements due to age class and sex were tested by a 2-way ANOVA test, followed by a Tukey's test to establish differences between age classes. Sexual differences in body and cranial measurements in each class were assessed by Student's t-tests. To determine the significance of intra-annual and inter-annual variations in BM and CM a 2-way ANOVA test was used. Intra-annual and inter-annual variation in SVL and TV were analysed by a 2-way ANCOVA test using BM as covariate. The relationships between SVL and TV, and BM and CM were determined by Pearson correlations. Partial correlations controlling for BM were used to assess the effect of temperature, rainfall and day-length on TV and SVL. Relationships between the monthly percentages of both pregnant and active mature females respect to the mean values of rainfall, temperature and day length were determined by Pearson correlations. The significance of intra- and inter-annual variations in the number of pregnant females was evaluated using Kruskal-Wallis test. Mann-Whitney U-test and Bonferroni adjustment for multiple tests (α = 0.05/number of pair-wise comparisons (6) = 0.0083) were used to determine differences between maturity classes. Differences in sex ratio were assessed by chi-square test. Statistical analyses were performed using SPSS 22.0 (IBM Corp. 2013).

2.3 Results

2.3.1 Body and skull size variation

In general, mean HBL and BM were similar for males and females, and increased progressively with relative age class (HBL: $F_{5, 605} = 548.01$, $p < 0.001$; BM: $F_{5, 494} = 370.91$, $p < 0.001$; Table 2.1). Only HBL was affected by the interaction of sex and age classes ($F_{5, 605} = 3.12$, $p < 0.01$): class I, males (mean = 100.9 mm), females (97.3 mm); class V, males (134.9 mm), females (137.6 mm). Cranial dimensions also varied significantly with age class (CL: $F_{5, 511} = 566.32$, $p < 0.001$; LUD: $F_{5, 539} = 573.22$, $p < 0.001$; ICI: $F_{5, 536} = 529.63$, $p < 0.001$) (Table 2.1). Significant differences between sexes were detected for CL ($F_{5, 511} = 8.00$, $p < 0.01$) and LUD ($F_{5, 539} = 11.61$, $p < 0.01$), with males showing significantly higher values than females in classes IV (CL: males = 32.5 mm, females= 32.1 mm; LUD: males = 11.8 mm, females = 11.6 mm) and V (CL: males =33.6 mm, females = 33.1 mm; LUD: males = 12.2 mm, females = 11.9 mm). Body mass and CM were correlated significantly in both sexes (males: r = 0.967, $p < 0.001$; females: r = 0.957, $p < 0.001$); since mean values for both variables showed similar inter-annual and intra-annual variation patterns (results concerning CM are not shown).

2.3.2 Reproduction in males

In our sample, males with a BM below 48 g were immature or submature, and above 77 g all males were mature (Fig. 2.2). Males with a HBL below 111 mm were immature or submature, and above 134 mm all males were mature. Submature specimens were detected above 25 g BM and 87 mm HBL. Males could be considered as mature with a BM (BM_{50}) over 64.9 g and a HBL (HBL_{50}) over 122.8 mm (Fig. 2.2).

All males belonging to class 0 were immature, and males of class I immature or, in a lower percentage, submature (Table 2.2). Most submature individuals corresponded to classes II (43.9%) and III (46.3%), although the youngest mature male corresponded to age class II. All class IV and V males were mature, although some specimens (16.5%) had few spermatozoids in testis and epididymis.

Table 2.1. Body and cranial measurements according to relative age classes of *A. scherman cantabriae*. (HBL: head and body length; BM: body mass; CL: condylobasal length; LUD: length of upper diastema; ICI: interorbital crests index; M: male; F: female. *p < 0.05; **p < 0.01; n.s.: not significant).

Variable	Age class	n males	n females	Mean ± SD	Range	Sex
HBL	0	8	9	88.7 ± 7.26	75.27 - 102.44	n.s.
	I	39	40	99.1 ± 8.13	75.33 - 120.67	* M > F
	II	50	44	110.8 ± 7.01	87.20 - 127.00	n.s.
	III	47	51	121.4 ± 6.41	96.00 - 138.22	n.s.
	IV	58	92	130.6 ± 5.88	115.87 - 144.48	n.s.
	V	75	92	136.4 ± 6.02	116.68 - 157.00	** M < F
BM	0	7	9	23.4 ± 4.39	15.00 - 30.00	n.s.
	I	37	39	35.9 ± 8.83	18.00 - 56.50	n.s.
	II	50	43	48.1 ± 9.41	22.00 - 73.00	n.s.
	III	46	47	62.4 ± 9.69	29.00 - 91.00	n.s.
	IV	58	46	76.3 ± 10.61	45.00 - 108.00	n.s.
	V	75	50	89.5 ± 12.84	47.00 - 124.00	n.s.
CL	0	4	5	24.2 ± 1.13	22.27 - 26.22	n.s.
	I	28	30	26.5 ± 1.01	24.05 - 28.39	n.s.
	II	35	37	28.8 ± 1.03	26.57 - 32.02	n.s.
	III	37	40	30.7 ± 1.04	28.09 - 33.06	n.s.
	IV	57	79	32.3 ± 0.99	29.65 - 34.82	* M > F
	V	73	86	33.3 ± 1.08	30.14 - 35.88	** M > F
LUD	0	6	8	8.1 ± 0.38	7.40 - 8.63	n.s.
	I	35	33	9.0 ± 0.51	7.63 - 10.05	n.s.
	II	39	40	10.1 ± 0.49	8.76 - 11.81	n.s.
	III	36	43	10.9 ± 0.51	9.72 - 12.15	n.s.
	IV	57	81	11.7 ± 0.48	10.53 - 12.90	** M > F
	V	75	86	12.1 ± 0.53	10.53 - 13.55	** M > F
ICI	0	6	8	0.2207 ± 0.0484	0.3161 - 0.1679	n.s.
	I	35	32	0.1753 ± 0.0412	0.2863 - 0.0929	n.s.
	II	37	41	0.1293 ± 0.0485	0.2781 - 0.0086	n.s.
	III	37	43	0.0674 ± 0.0309	0.1573 - 0.0089	n.s.
	IV	57	79	0.0248 ± 0.0221	0.2222 - 0.0000	n.s.
	V	74	87	0.0017 ± 0.0053	0.0342 - 0.0000	n.s.

Testicular volume was positively correlated with SVL (r = 0.805, $p < 0.001$). Mean values of TV and SVL for mature specimens with few spermatozoids did not differ significantly from the values corresponding to mature males in any month of both years (Mann-Whitney U-test with Bonferroni adjustment; $p > 0.0083$, in all cases).

Mature males with few spermatozoids were not detected in spring of the first year, and spring and summer of the second year (Fig 2.3).

Using BM as a covariate, TV and SVL in mature males showed significant intra-annual variations (TV: $F_{11, 283} = 3.19$, $p < 0.001$; SVL: $F_{11, 284} = 4.03$, $p < 0.001$; Fig. 2.4). Significant inter-annual differences were found in SVL ($F_{1,284} = 11.71$, $p < 0.01$) but not in TV ($F_{1,283} = 0.07$, $p = 0.787$; Fig. 2.4). A significant interaction was detected between month and year both in TV and SVL (TV: $F_{11,283} = 3.08$, $p < 0.01$; SVL: $F_{11, 284} = 3.85$, $p < 0.001$). Estimated marginal means of TV in mature males (Fig. 3) showed maximum values in spring of both years, and decreased till August (first year) or November (second year). Estimated marginal means of SVL (Fig. 2.4) did not show a clear inter-annual variation pattern. Partial correlations controlled by BM were significant between TV and day-length (r = 0.126, $p < 0.05$), whereas temperature (r = 0.006, $p = 0.920$) and rainfall (r = – 0.003, $p = 0.955$) did not show a significant relationship with TV. Partial correlation between SVL and day-length, and between the former variable and temperature were significant (r = 0.190, $p < 0.01$; r = 0.144, $p < 0.05$; respectively). Rainfall did not correlate significantly with SVL (r = – 0.051, $p = 0.390$).

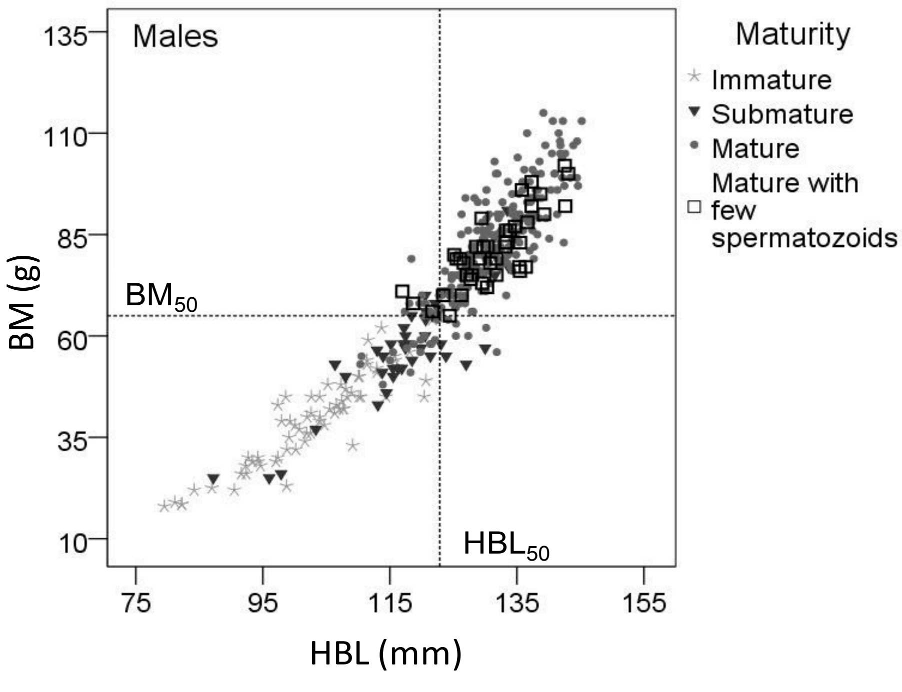


Fig. 2.2. Relationship between head and body length (HBL) and body mass (BM) according to sexual state. BM_{50} and HBL_{50} are the BM and HBL hypothetical values at which 50 % males of the population are matures and mark the limit above which all individuals can be considered as adults.

Table 2.2. Distribution (%) of captured specimens according to sex, sexual maturity state and age class.

			Age class					
Sex	Maturity	N	0	I	II	III	IV	V
Males	Immature	75	100	90.2	54.0	4.3	-	
	Submature	41	-	9.8	36.0	40.4	-	
	Mature	285	-	-	10.0	55.3	100	
Females	Immature	83	100	87.5	68.2	17.6	-	
	Submature	23	-	12.5	18.2	19.6	-	
	Mature	316	-	-	13.6	62.7	100	
	Total	823	18	81	95	98	531	

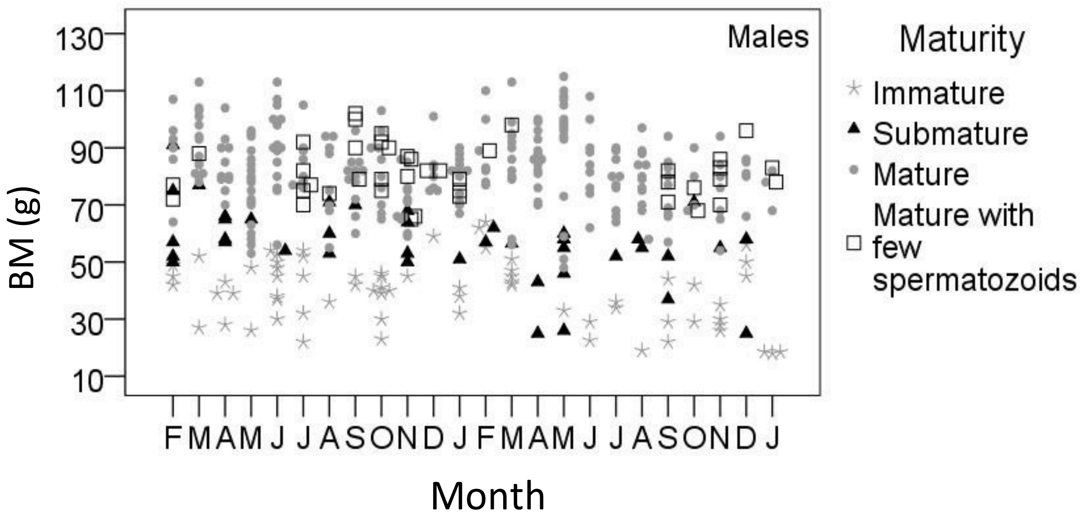


Fig. 2.3. Relationship between body mass (BM) and sexual maturity state of males in *A. scherman cantabriae* throughout the study period.

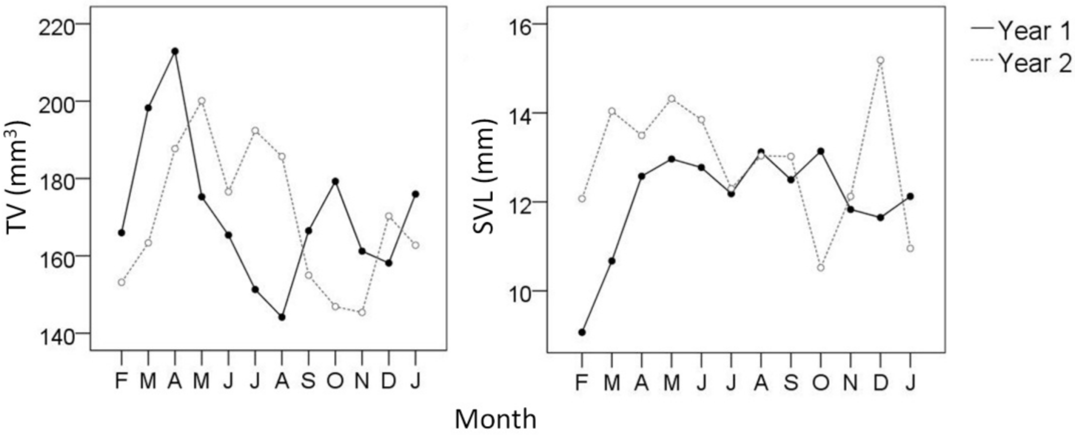


Fig. 2.4. Estimated marginal means of the testis volume (TV) and seminal vesicle length (SVL) using body mass as covariate in mature specimens of *A. scherman cantabriae* throughout the study period.

2.3.3 Reproduction in females

Females with a BM below 42 g were immature or submature, and all individuals above 80 g were mature (Fig. 2.5). Specimens with a HBL below 96 mm were immature or submature, and above 127.5 mm all individuals were mature. The submature specimens were detected with BMs above 29 g and HBLs of 96 mm. Females with BMs and HBLs higher than 59.9 g and above 121.9 mm, respectively, could be considered mature (BM_{50} and HBL_{50} respectively) (Fig. 2.5). In classes 0, I and II most females were immature (Table 2.2). The youngest mature female corresponded to class II. Submature females corresponded mostly to classes II and III, and all females of classes IV and V were mature.

Active mature females and/or pregnant females were found in all months of the study period (Fig. 2.6). No significant inter-annual (Kruskal-Wallis $H' = 0.104$, $p = 0.748$) and intra-annual (Kruskal-Wallis $H' = 18.341$, $p = 0.074$) differences in the number of pregnant females were found. These females appeared in all months except January 2013 (mature females: n = 8). Between April and November of the first year, pregnant females constituted more than 25 % of mature females. However, the occurrence of pregnant females in second year was more irregular (Fig. 2.6), with maximums in March (61.5 %) and November (58.3 %). Monthly percentages of both pregnant and mature active females were not significantly correlated with the monthly mean values of rainfall ($r = -0.380$, $p = 0.067$; $r = -0.326$, $p = 0.120$; respectively), temperature ($r = 0.215$, $p = 0.314$; $r = 0.322$, $p = 0.125$; respectively) or day length (r =

0.207, p = 0.331; r = 0.343, p = 0.101; respectively); likewise, there were neither significant correlations when the month before the month of capture was considered: rainfall (r = 0.220, p = 0.313; r = 0.415, p = 0.062; respectively), temperature (r = 0.160, p = 0.467; r = 0.012, p = 0.958; respectively), day length (r = 0.374, p = 0.079; r = 0.231, p = 0.288; respectively).

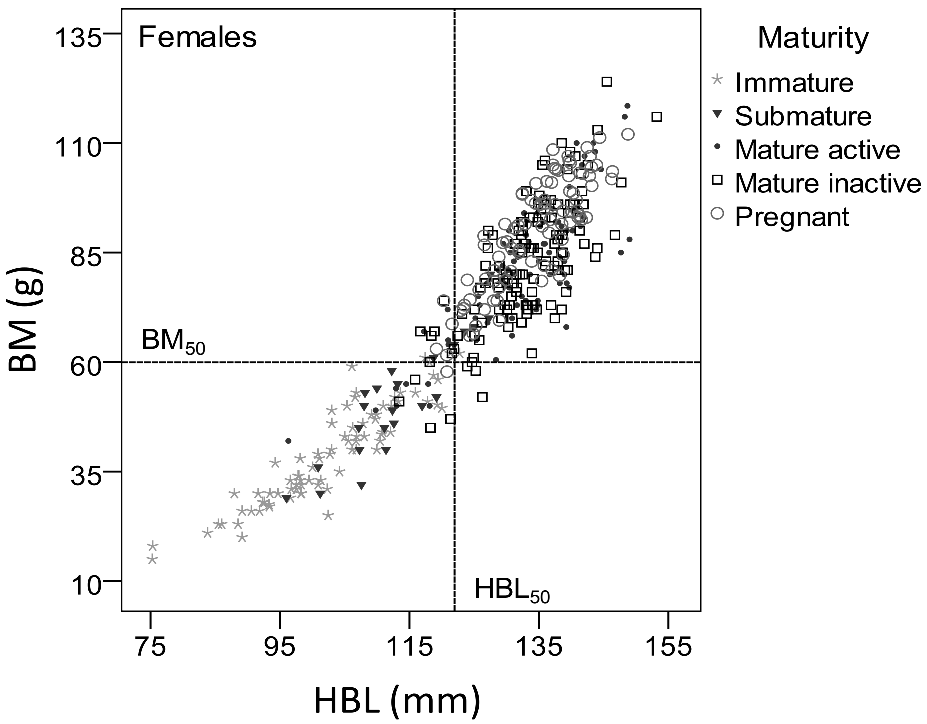


Fig. 2.5. Relationship between head and body length (HBL) and body mass (BM) according to sexual state. BM_{50} and HBL_{50} are the BM and HBL hypothetical values at which 50 % females of the population are matures and mark the limit above which all individuals can be considered as adults.

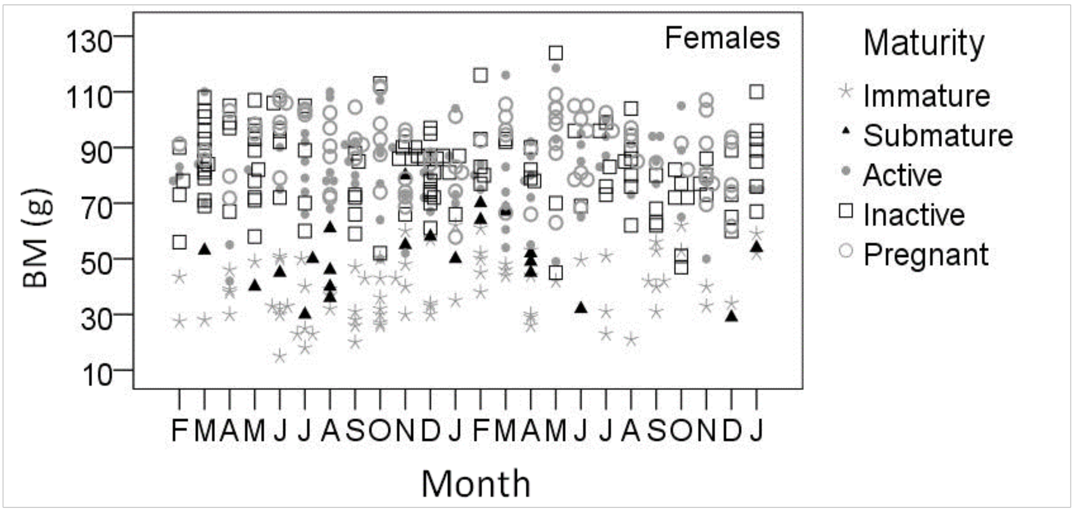


Fig. 2.6. Relationship between body mass (BM) and sexual maturity state of females in *A. scherman cantabriae* throughout the study period.

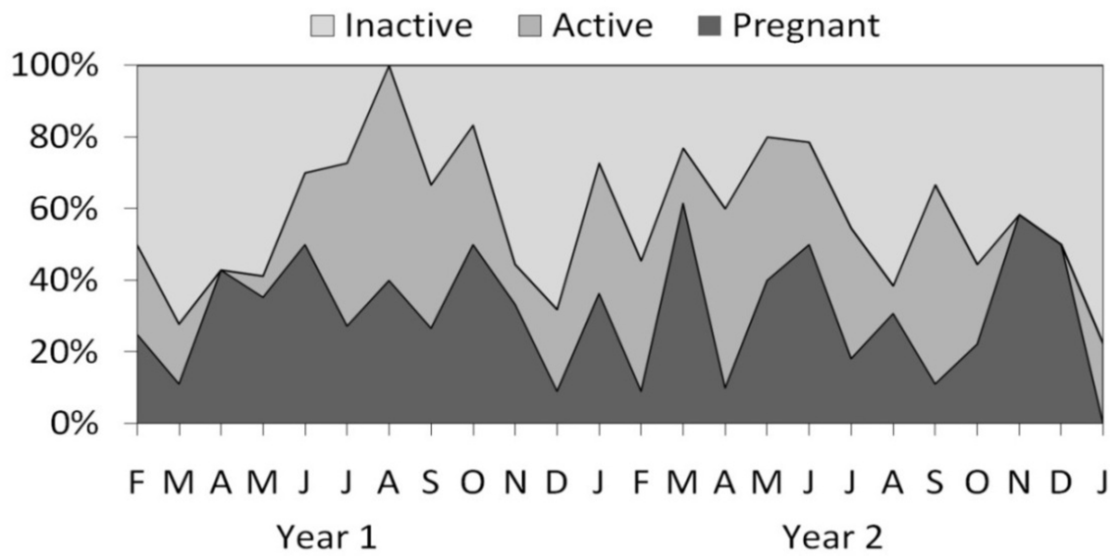


Fig.2.7. Variation of the population structure of mature females belonging to age classes IV and V according to sexual activity state during the study period.

2.3.4 Body mass variation

Mean variation of BM in mature male specimens belonging to classes IV and V (Fig. 2.8) showed significant intra- ($F_{11,253} = 4.64$, $p < 0.001$) and inter-annual differences ($F_{11,253} = 3.17$, $p < 0.01$). The BM means of these males were also affected by the month-year interaction ($F_{11,253} = 3.17$, $p < 0.01$). Conversely, mean variations in BM of adult females (classes IV and V, Fig. 2.8) were not significant neither intra- ($F_{11,}$

$_{266}$ = 1.67, p = 0.081) nor inter-annually ($F_{1, 266}$ = 1.07, p = 0.302). Mean BM of classes IV and V did not differ significantly between sexes (t = – 1 .85, d.f. = 517, p = 0.064). Mean BM values of both sexes showed relative higher values in June of the first year and May of the second year (Fig. 2.8).

2.3.5 Population structure

Total sex ratio (401 males, 423 females) was not significantly different from 0.5 (χ^2 = 0.43, p = 0.512). For each age class (class I: 0.97, class II: 1.14, class III: 0.88, classes IV-V: 0.93) the sex ratio was also not significantly different from that value (χ^2 test, p > 0.05 in all cases). The presence in all months of specimens from class 0 or I revealed that births occurred steadily (Fig 2.9), whereby, immature specimens of both sexes were captured continuously during both years (Fig. 2.10). Submature or mature specimens belonging to classes I, II and/or III were detected in all months, with maximums in May (72.7%), August (72.2%) and November (77.3%) of the first year and with a more regular presence between February and December of the second year (Fig. 2.10). The percentage of captured mature individuals of classes II and III decreased in winter of both years (Fig. 2.10). At least 45 % of captured voles in each month belonged to classes IV and V (Fig 2.9).

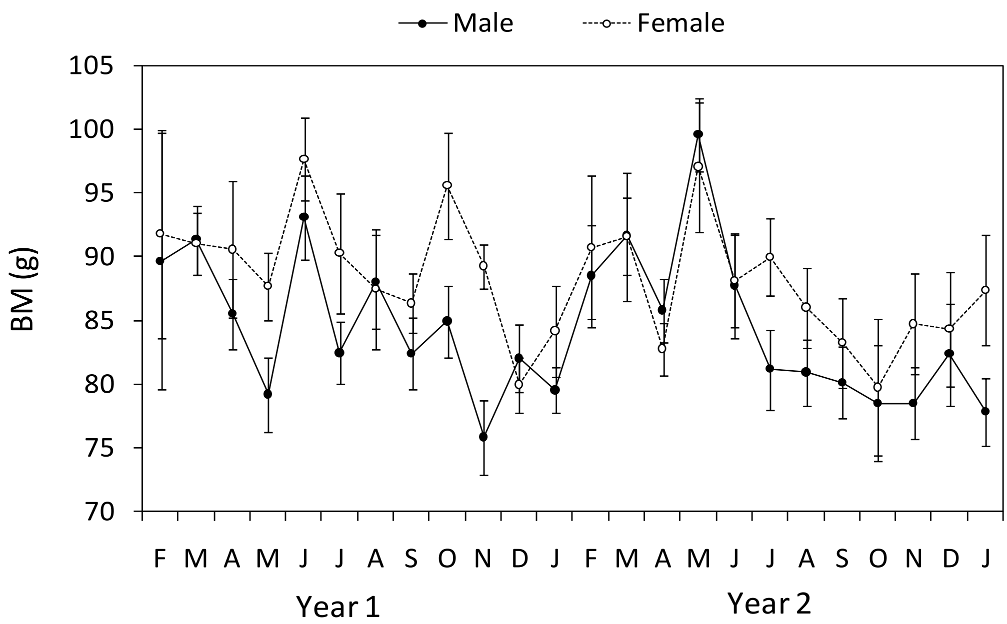


Fig 2.8. Monthly variation (mean ± SE) of male and female body mass (BM) in mature specimens belonging to age classes IV and V during the study period.

A total of 120 family groups were captured. Couples formed by adults individuals were observed in 42cases (35 %), 33.3% of them with pregnant females (n = 14). The number of captured couples with offspring was 59 (49.2 %), formed by one to five juveniles. The immature voles captured in each burrow mostly belonged to the same relative age class. Indeed, most immature specimens (93.6 %) corresponded to age class 0, I, and II, although some specimen of age class III were captured occasionally with mature couples (6.4 %). Occasionally, groups of more than two adults belonging to class IV and/or V were detected, mostly in May (42.1 %): one male-two females (n = 8), two males-one female (n = 6), two males-two females (n = 5). Of these associations, a pregnant female was observed in a group formed by two males-one female and in five groups of one male-two females. Furthermore, both females were pregnant in a group of one male-two females and in three groups of two males-two females.

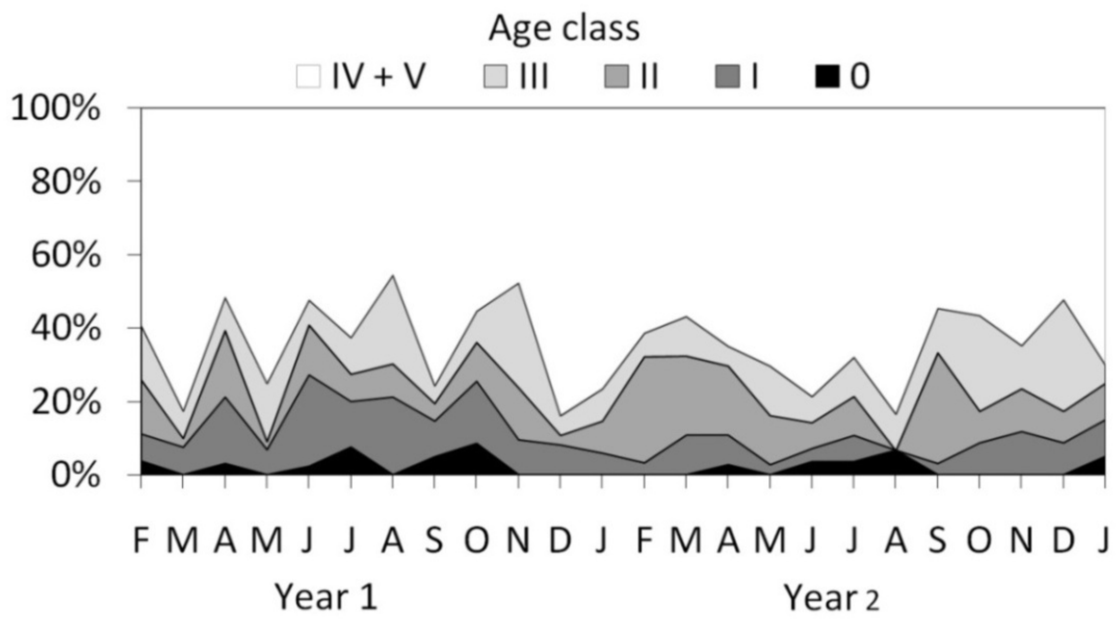


Fig. 2.9. Variation of the population structure according to relative age classes throughout the study period.

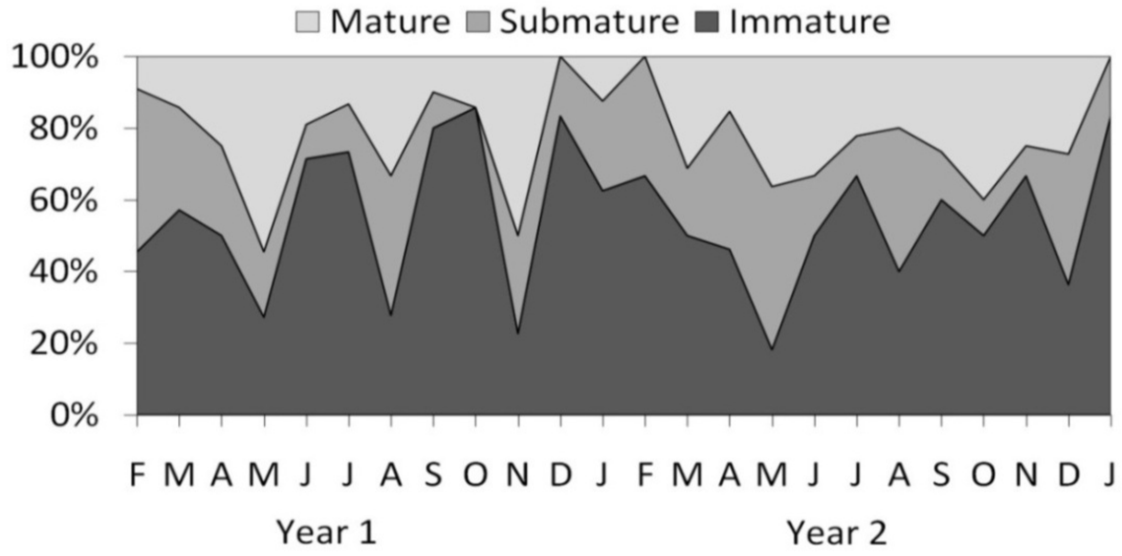


Fig. 2.10. Variation of the population structure of immature, submature and mature specimens belonging to age classes 0-III during the study period.

2.4 Discussion

Rodents should organize their activity and energy budgets according mainly to food availability for balancing the competing demands of reproduction (Hansen *et al*. 1999, Lima *et al*. 2001, Merritt *et al*. 2001, Solonen 2006). Thus, only when primary demands, such as thermoregulation and food obtaining, have been satisfied, energy remains can be used to cope reproduction (Bronson 1985, Trebatická *et al*. 2012), which is often limited to a part of the year in temperate areas (Hansen *et al*. 1999, Lima *et al*. 2001, Merritt *et al*. 2001, Solonen 2006). In this way, most studies on both semiaquatic and fossorial water vole populations have shown that reproduction activity ceases or significantly decreases in winter, which determines a lack or a scarce number of young specimens in early spring (Van Wijngaarden 1954, Kminiak 1968, Pelikán 1972, Wieland 1973, Airoldi 1978, Morel 1981, Pascal 1981, Evsikov *et al*. 1989, Ventura *et al*. 1991). However, our results show that *A. scherman* can breed continuously along the whole year in agricultural environments located at low altitudes in northwestern Spain. Winter reproduction has been previously reported in a population of *A. scherman* form the Jura mountains (Switzerland), but in a particular case of unusual benign temperatures during this season (Meylan & Airoldi 1975). Our study area has a temperate hyperoceanic climate (Rivas-Martínez & Rivas-Sáenz 2015), which ensues favourable environmental conditions the whole year. Interestingly, our results are concordant with those obtained in the Lusitanian pine vole (*Microtus lusitanicus*), another arvicoline apple pest that cohabits with fossorial water voles (Miñarro *et al*. 2012) and also breeds along the whole year in the study area (Miñarro *et al*. 2017). In fact, it has been found that environments with favourable conditions allow many populations of rodents to increase their reproductive effort (Murphy 1992, Koskela *et al*. 1998, Díaz & Alonso 2003) or even lengthen their reproduction throughout the whole year (Sicard & Fuminier 1996, Bergallo & Magnusson 1999).

Physiological receptors receive environmental signals and then interact with the neuroendocrine system to dictate reproductive behaviour throughout the secretion of sexual hormones, which control spermatogenesis and the ovarian cycle (Maeda *et al*. 1997). In this way, many rodent species may rely upon the photoperiod as a predictive cue in order to anticipate the onset and offset of optimal conditions for breeding (Nelson 1985, Bronson 1988, Steinlechner & Niklowitz 1992, Kerbeshian *et al*. 1994, Nelson *et al*. 1998, Gottreich *et al*. 2000). Thus, it has been found that in many species, males suffer a significant intra-annual fluctuation of the testis, epididymis and seminal vesicle masses associated with the day-length variation (e.g. Maeda *et al* . 1997, Gottreich *et al*. 2000, Pyter *et al*. 2005, Medger *et al*. 2012). Likewise, in *A. scherman monticola*, testis and seminal vesicle lengths correlated significantly with day-length (TL: $r = 0.798$, SVL: $r = 0.920$) and temperature (TL: $r = 0.639$, SVL: $r = 0.527$) (Ventura 1988). However, although photoperiod can be used as the main predictive cue for seasonal breeding, temperature and rainfall affect thermoregulatory costs and thus determine the amount of available food, which in turns can condition the reproductive physiology of rodents (Nelson *et al*. 1992, Demas & Nelson 1998, Bergallo & Magnusson 1999, Pierce *et al*. 2005, Medger *et al*. 2012). So, in our study area, mild temperatures and abundant rainfall allow fossorial water voles to overcome primary demands and also cope physiological costs of reproduction during the year. Whereby, although mature males of our population showed maximum mean values of the testis volume in spring, minimum averages of this variable appeared in different seasons in each studied year. Likewise, the variation of the mean of the seminal vesicle length showed a very irregular pattern during the study period, with few coincidences between years. Furthermore, in comparison with the results obtained for Pyrenean water voles, correlations between seminal vesicle and testis lengths with day-length, although significant, were clearly lower in our population. Overall, the lack of synchrony of the size variation of both sexual organs with seasonality in the Asturian population is probably the result of the continuous breeding along the whole year.

Body mass in both semiaquatic (Pelikán 1972, Evsikov *et al*. 1989, Zejda 1991) and fossorial water vole populations (Ventura 1988) can increase when sexual activity starts. Whereas our analyses revealed significant intra-annual variation in body mass in males of classes IV and V, the variation detected in females was not significant. The accumulation of reserve tissues before and, especially, after mating is an important adaptation for breeding in female water voles (Nazarova & Evsikov 2008). Thus, the continuous presence in our population of pregnant females along the study period could have masked a potential intra-annual variation of the body mass in adult females.

The onset of reproductive activity may be important to assess the rate at which population grows by recruitment. Sexual maturity in water voles starts when a male induces by mating the onset of oestrus in a preovulatory female (Yakovleva *et al*. 1997). Nevertheless, populations of arvicoline rodents in seasonal environments can perform a precocious reproduction if young are born at the beginning of the breeding period, whereas individuals born at other times can delay their sexual maturation (Zejda 1991 and references therein, Lambin & Yoccoz 2001). In this way, the range of the onset of sexual maturity in *A. scherman cantabriae* appears between classes II and III, which is concordant with the results obtained in *A. scherman monticola* (Ventura & Gosálbez 1990b). According to the body size differences between Pyrenean and Cantabrian fossorial water voles (see Ventura & Gosálbez 1990a,Ventura 1993), body mass at which each sex reach sexual maturity is comparatively smaller in *A. scherman cantabriae*.

Intersexual differences in skull dimensions of adult individuals (classes IV and V) of *A. scherman cantabriae* suggest a male-biased dimorphism, which has been reported in other arvicoline rodents, even among populations of the same species (Schulte-Hostedde 2007 and references therein). Nevertheless, the degree of sexual dimorphism in the body size and the relative size of testes are the key metric issues to predict the mating system in arvicoline species (Boonstra *et al*. 1993, Ostfeld & Heske 1993). In our sample, no significant differences neither in body mass nor in head and body length were found between adult males and females (classes IV and V). Moreover, in comparison with other arvicolines (see Heske & Ostfeld 1990), the mean value of the index between testis length and head and body length in adult individuals during the periods of maximal testis size was low (mean = 0.066, SD = 0.005). Furthermore, the sex ratio in our sample was balanced and our surveys in the field suggest that *A. scherman cantabriae* lives mainly in familiar burrow systems, which are independent from each other and are occupied by a couple of adults with their offspring. Overall, results obtained in the present study allow us to suggest that in our population monogamy is the rule, main mating system that has been reported for other fossorial water vole populations (Airoldi 1978, Morel 1981). Although the mating system in arvicolines can vary with population density (Waterman 2007 and references therein) and complex associations among individuals have been reported in other *A. scherman* populations (Airoldi 1978, Morel 1981), the scarce number of unusual associations found in the present study and the lack of parental assignments by genetic tests prevent us to report an alternative mating system for our population.

2.4.1 Implications for management

Apple orchards in Asturias are located in relatively small plots (70% are smaller than 2 ha) immersed in a mosaic landscape with high densities of semi-natural habitat (hedgerows and woodlands) and a topography with smooth hills and valleys, leading to a closed landscape. It has been hypothesized that such setting might modulate multiannual fluctuations in abundance of fossorial water voles directly by slowing dispersion and indirectly by the presence of specialist and generalist predators (Giraudoux *et al*. 1997, Fichet-Calvet *et al*. 2000, Foltête *et al*. 2009, Berthier *et al*. 2013). Anyway, although no studies on population dynamics has been conducted and thus there is not periodic data on vole density, some population outbreaks have been observed at a local scale in Asturias (Miñarro, pers. observ.). In such situations of tree-damage threat vole control is faced without coordinate actions among farmers and according to their perception of risk.

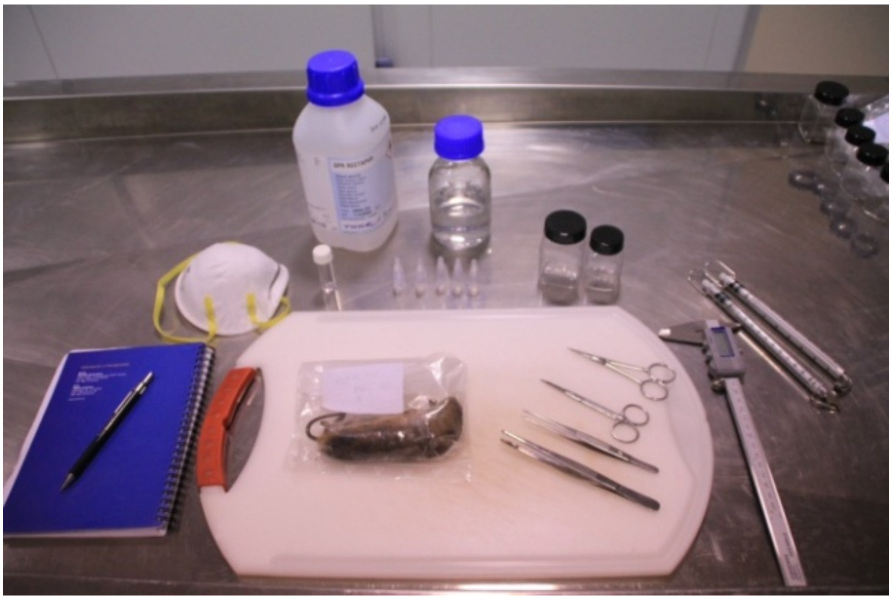
Population control of this species in Asturias has so far mainly been achieved by manually placing rodenticide baits or snap traps at burrow entrances. Until now, farmers increase control activity in winter in order to eliminate adults before the hypothetical breeding season, which was expected to start in spring. Thus, farmers would reduce population increase. However, our results show a continuous breeding regardless of season which suggests that population control could be performed along the whole year.

This does not mean extending the use of rodenticides throughout the year but that control effort does not need to be seasonal as it used to be. It is well known that increasing the poison input in the agroecosystem enhances the risk for wildlife (Coeurdassier *et al*. 2014, Montaz *et al*. 2014). For that reason, sustainable control management strategies should preferably be taken into account instead of poisoning practices which are maintained over time.

As control measures of direct application, trapping beyond the death of the mature couple might lead to increase control effectiveness since some offspring could remain in the burrow. It is worthwhile to keep in mind that we found up to seven specimens in a burrow. On the other hand, dispersion in fossorial water voles is done aboveground by juvenile specimens (Saucy & Schneiter 1998, Saucy 2002). Installing fences can be useful to avoid invasions by dispersal specimens (see details in Walther & Fuelling 2010). Farmers should maintain these fences surrounding apple orchards continuously as young mature specimens appear throughout the year and could thus disperse at any time. Uncoordinated control actions by farmers involve potential colonisation movements of juveniles from source demes (without or scarce control) to nearby sink plots (with continuous removal of voles).

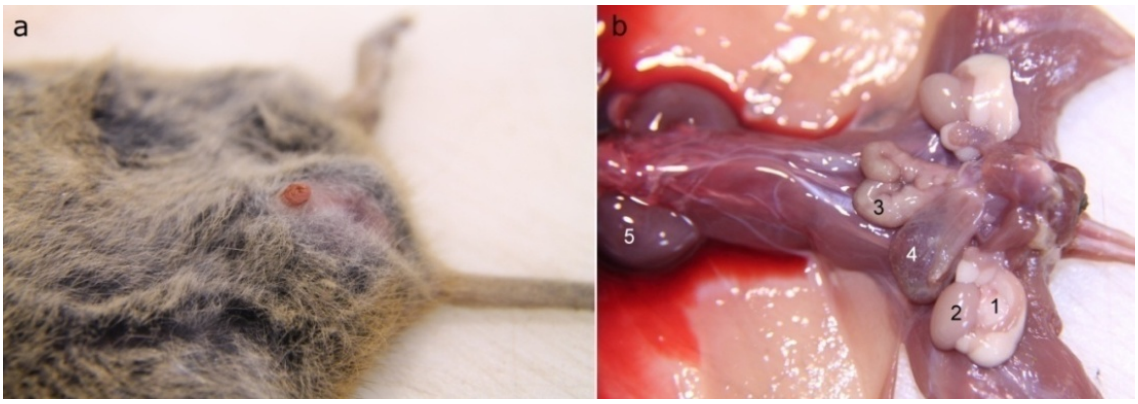
The enforcement of other sustainable control management strategies, such as frequent mowing (Morilhat *et al*. 2007, Jacob 2008), livestock grazing (Defaut *et al*. 2009) or the use of repellents (Fischer *et al*. 2013) can be suitable strategies to maintain fossorial water voles under physiological stress, which in turns might induce a decline in their population densities (Charbonnel *et al*. 2008). However, the effectiveness of these control strategies is frequently assessed by the relative abundance of voles (e.g. Morilhat *et al*. 2007, Jacob 2008, Delattre & Giraudoux 2009), without going into the underlying cause. The information obtained for this population can be used to assess direct consequences of control strategies on the reproductive biology of fossorial water voles, such as changes in population structure (Cerqueira *et al*. 2006), growth delaying (Stoddart 1971, Zejda 1991, Moorhouse *et al*. 2008) or delays in the onset of reproduction (Yakovleva *et al*. 1997).

2.5 Supplemental information

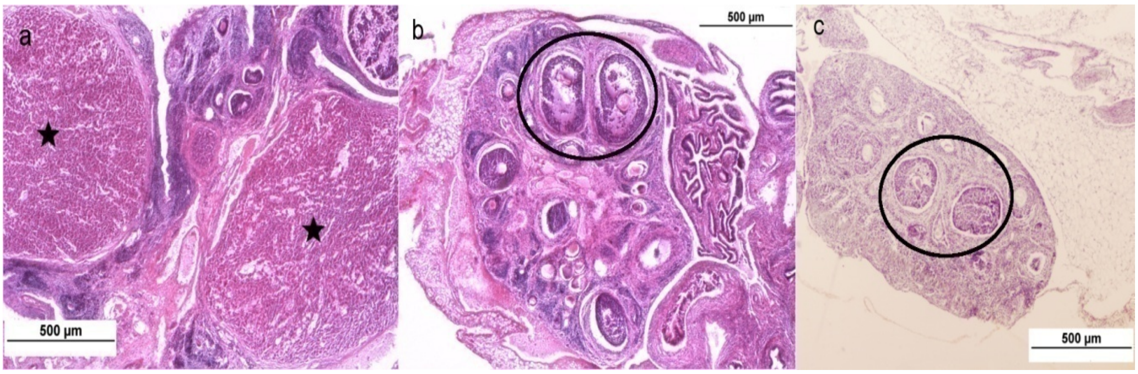


Supplemental fig. 2.1. Instrumental for necropsy. The necropsies were carried out in the necropsy room of the Animal Biotechnology Centre of SERIDA (Gijón, Asturias).

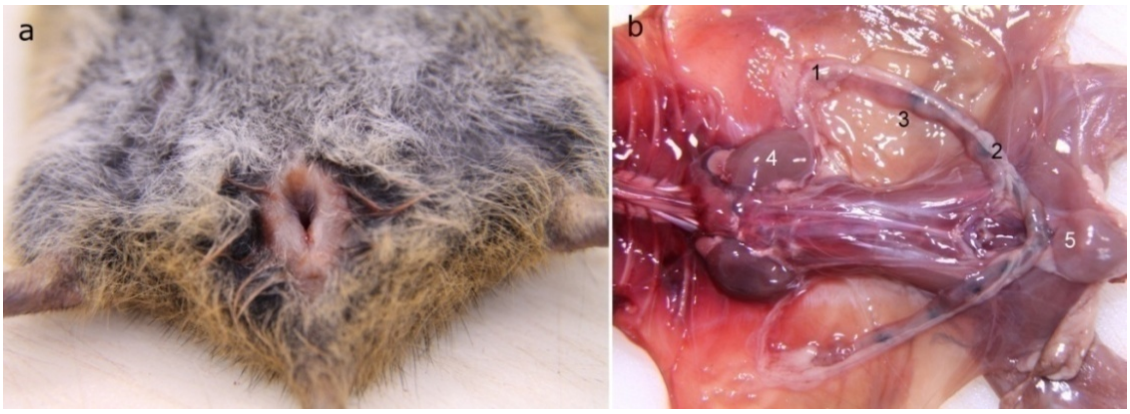
Supplemental fig. 2.2: Specimen of *A. scherman cantabriae* before necropsy (a), with the skin removed (b), and ventral (c) and dorsal (d) views of the removed skin.



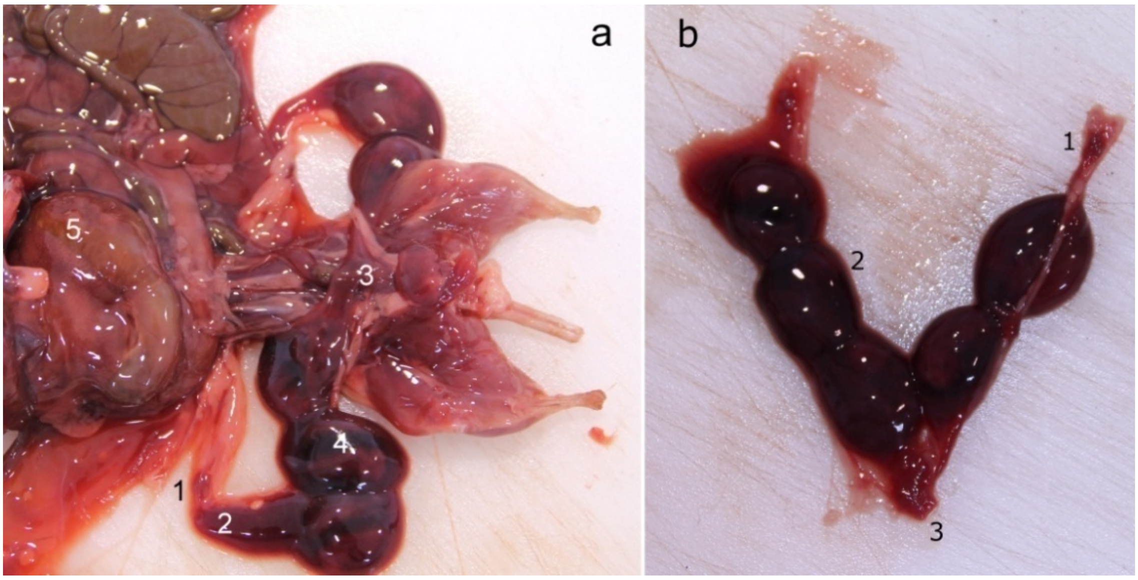
Supplemental fig. 2.3. External view of a mature male of *A. scherman cantabriae* with scrotal testis (a) and internal view after the necropsy (b) in which several organs are shown: epididymis (1), testicle (2), seminal vesicle (3), urinary bladder (4) and kidney (5).



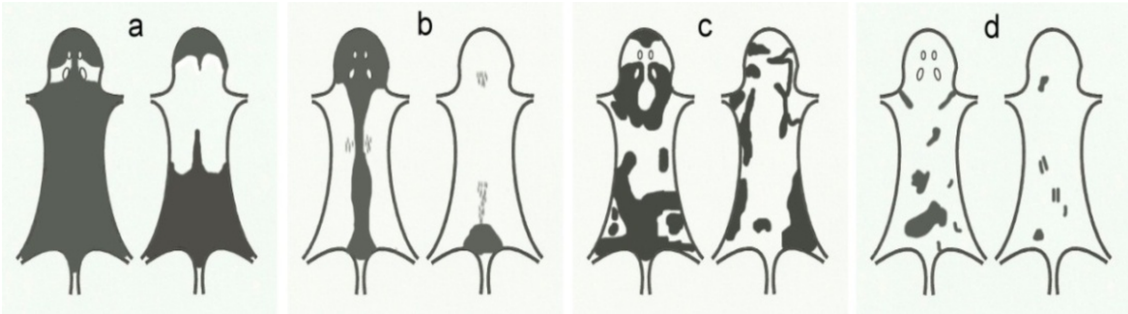
Supplemental fig. 2.5. Longitudinal histological sections from left ovaries of a mature (a), submature (b) and immature (c) females of *A. scherman cantabriae*. Sections were stained with hematoxylin-eosin. *Corpora lutea* (a), Graafian follicles (or advanced tertiary follicles) (b) and secondary follicles (or late primary follicles) (c) are indicated in each image respectively.



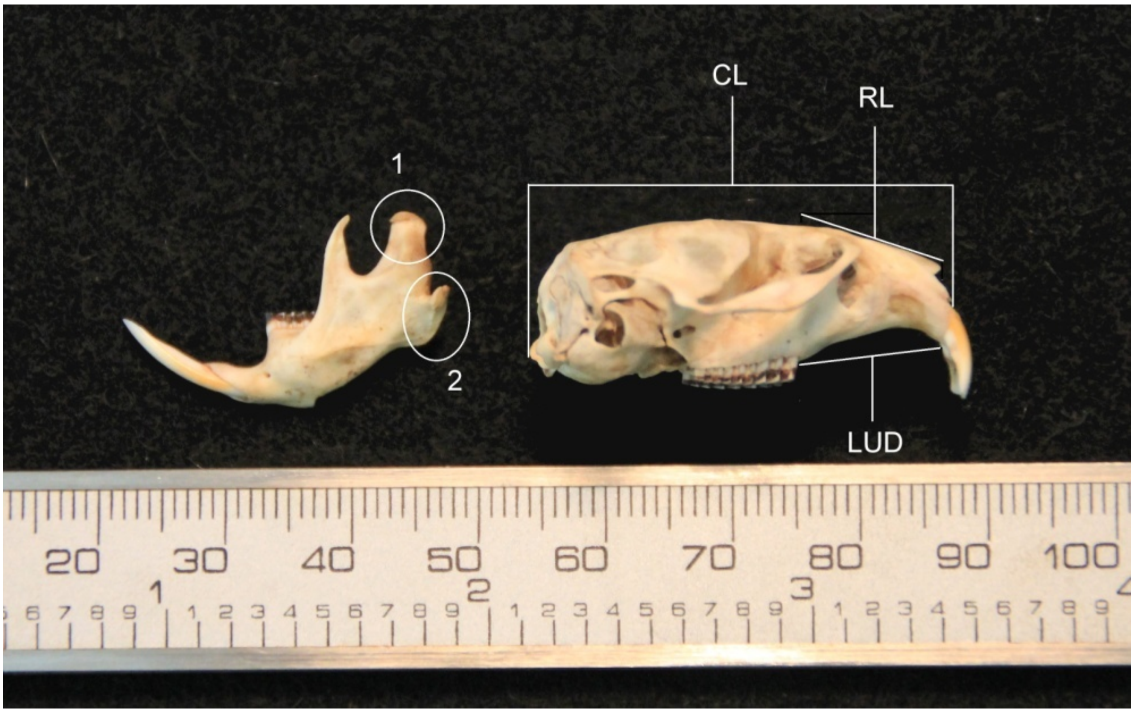
Supplemental fig. 2.6. External view of a mature female of *A. scherman cantabriae* with open vulva (a) and internal view after necropsy showing several organs: ovary (1), developed uterus (2), placental scar (3), kidney (4) and urinary bladder (5).



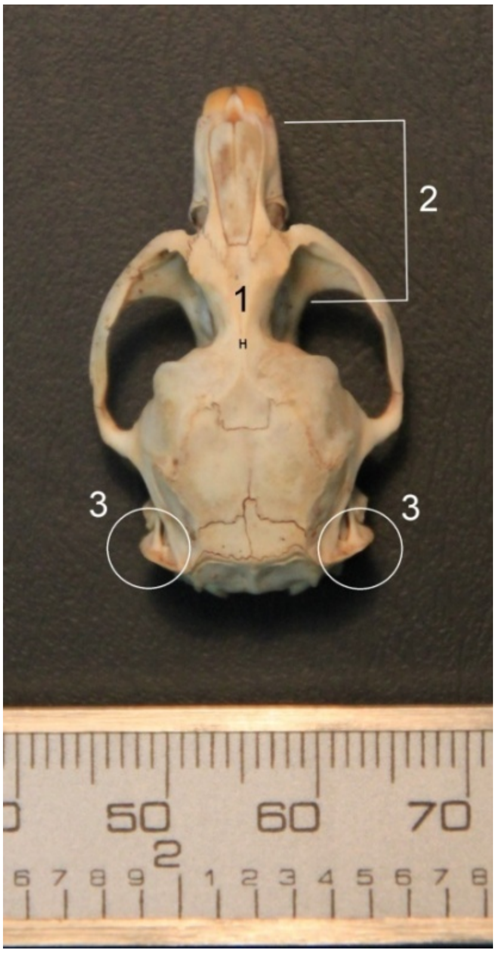
Supplemental fig. 2.7. Internal view of a pregnant female of *A. scherman cantabriae* (a) and its uteri with the embryos (b). Several organs are shown: ovary (1), uteri (2), vagina (3), embryo (4), stomach (5) and intestine (6).



Supplemental fig. 2.8. Melanic prints of *A. scherman cantabriae* according to successive moulting stages: first phase of first moult belonging to a specimen of age class I (a), second phase of first moult belonging to a specimen of age class II (b), irregular prints of second moult belonging to a specimen of age class IV (c), and spot prints typical of posterior moults in an specimen of age class V (d) (see Ventura 1988, 1992). The melanin is stored in the hair follicle during the first steps of fur development. Thus, these active follicles show melanic prints on the internal surface of skin until the melanin spread through the hair during its growth. Melanic prints loss intensity progressively with time. The lack of melanin means a completely constituted fur. Each moult is related with a specific melanic pattern.



Supplemental fig. 2.9. Skull and left mandible of an adult specimen of *A. scherman cantabriae*. The condylar process (1) and the separation of the angular process from the ascending branch (2) are shown in the mandible. Rostral length (RL), condylobasal length (CL) and length of upper diastema (LUD) are shown in the skull.

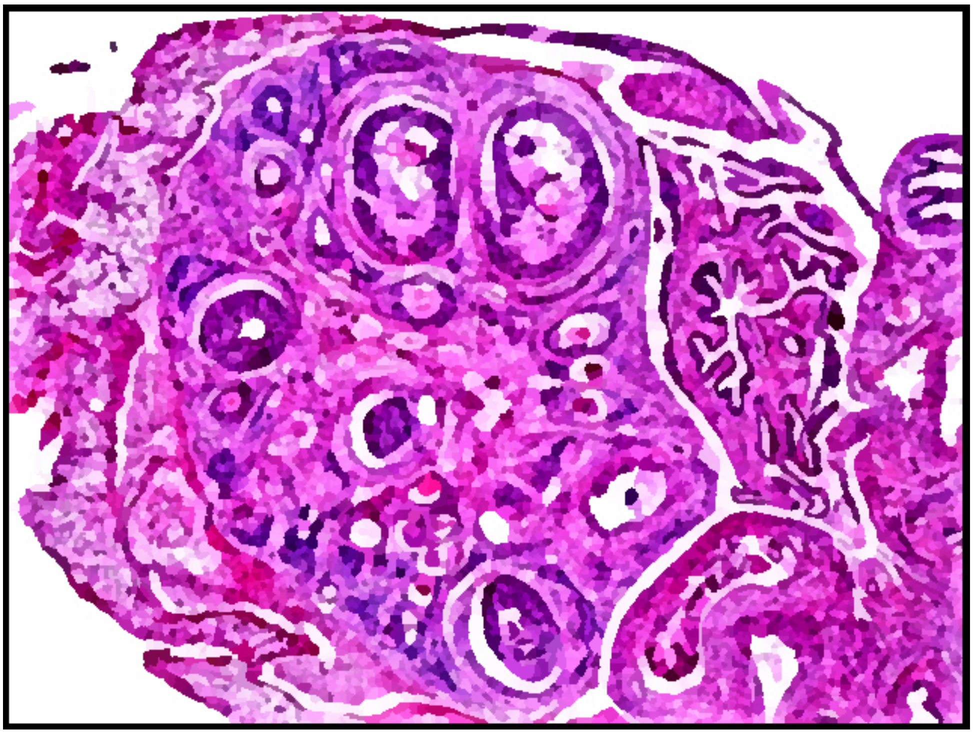


Supplemental fig. 2.10. Skull belonging of an adult specimen of *A. scherman cantabriae*. The breadth of the interorbital crests (1), rostral length (2) and sturdiness of the mastoid processes (3) are shown.

Supplemental fig. 2.11. Skulls of *A. scherman cantabriae* according to their relative age classes (Ventura & Gosálbez 1992, and references therein); 0 (0-3 weeks), I (3-6 weeks), II (6-10 weeks), III (10-14 weeks), IV (14-30 weeks) and V (older than 30 weeks).

Chapter 3. Reproductive potential of a vole pest (*Arvicola scherman*) in Spanish apple orchards

Adapted from: Somoano A, Miñarro M ,Ventura J (2016) Reproductive potential of a vole pest in Spanish apple orchards. *Spanish Journal of Agricultural Research*, **14**, e10xx. http://dx.doi.org/10.5424/sjar/2016144-9870.



3.1 Introduction

The montane water vole, *Arvicola scherman* (formerly fossorial form of *Arvicola terrestris*; for taxonomic consideration see Musser & Carleton 2005), is one of the most harmful rodent species in the European farmlands (Walther *et al*. 2008; Blant *et al*. 2009; Delattre & Giraudoux 2009; Miñarro *et al*. 2012). These voles live underground, dig extensive burrow systems and feed preferably on roots, bulbs and tubers (Airoldi 1976). In fruit crops, fossorial water voles feed on bark and roots and may kill the trees (Meylan 1977; Walther *et al*. 2008). Additionally, injuries to the root system make the trees less productive and more susceptible to falling down by winds or high fruit load due to deficient anchorage. Its occurrence in crops can cause important economical losses, being estimated in German orchards between 50 and 40,000 €/ha/year (Walther *et al*. 2008).

In the Iberian Peninsula two forms of this species are currently recognised (Ventura & Gosálbez 1990a; Musser & Carleton 2005): *A. scherman monticola*, which occurs in the Pyrenees, and *A. scherman cantabriae*, distributed along the Cantabrian region (northwestern Spain) and in the northern tip of Portugal. These taxa are geographically isolated and show significant morphological differences, being *cantabriae* 40% lighter (Ventura 1993). *Arvicola scherman* is officially considered a pest in Spain and preventive actions to reduce its population density have been recommended (BOE 2008). In Asturias (NW Spain) this species has become one of the main causes of economical loss in apple orchards (Miñarro *et al*. 2012), a crop that in 2010 occupied 10,324 ha in this region (INDUROT 2010) supposing the 32.4% of the total planted area intended to this crop in Spain (31,812 ha), whose national production, for example in 2013, reached 546,400 tons (FAO 2013).

The effect of seasonal environmental factors, such as the decrease of temperature or food availability, can vary with altitude, which in turn affects rodent reproduction at population scale (Dunmire 1960; Bronson 1979; Murie *et al*. 1980; Zammuto & Millar 1985; Hille & Rödel 2014). Until now, studies about reproduction of *A. scherman* populations have been conducted above 400 m a.s.l., where all of them showed a delimited breeding season (Airoldi 1978; Morel 1981; Pascal 1981; Ventura & Gosálbez 1990b; Ventura *et al*. 1991). However, populations located in apple orchards below 270 m a.s.l. in Asturias breed continuously throughout the year

(Somoano *et al*. 2017). Furthermore, it must be kept in mind that favourable habitats with plenty of food, such as crops, may allow rodents to invest their extra energy in increasing the reproductive effort (Doonan & Slade 1995; Koskela *et al*. 1998; Díaz & Alonso 2003; Eifler *et al*. 2003). Particularly, pregnant females of *A. scherman cantabriae* were detected over the whole year and, consequently, the recruitment of young specimens was continuous (Somoano *et al*. 2017). The optimal investment hypothesis predicts that litter size produced by a female should give the best reproductive success in a particular environment (Mappes *et al*. 1995). Therefore, reproductive potential among populations of *A. scherman* could vary through phenotypic plasticity or microevolution driven by the corresponding environmental conditions (Williams *et al*. 2014).

Both in semiaquatic (*A. amphibius*; see Musser & Carleton 2005) and fossorial water voles, the number of implanted embryos is positively correlated with the body dimensions of the mother (van Wijngaarden 1954; Pelikán 1972; Wieland 1973; Ventura & Gosálbez 1990c). In that sense, the reproductive potential is closely related to the body condition in semiaquatic water vole populations, which reflects energy provisions (Evsikov *et al*. 2008). Likewise, a good body condition in pregnant females leads to a higher number of ovulated oocytes and implanted embryos, and decreases the risk of pregnancy failure (Evsikov *et al*. 2008; Nazarova & Evsikov 2008; Yuzhik *et al*. 2015). Furthermore, mother body condition in *A. amphibius* has also effects on the reproductive success, life span, body mass (Nazarova & Evsikov 2008) and maturation of young (Yakovleva *et al*. 1997). Conversely, a worsening of the body condition might lead to lower fitness in females (Evsikov *et al*. 2008).

Management of rodent pests requires effective, specific, environmentally benign, economically feasible and socially acceptable approaches (Jacob 2013). Thus, and especially in organic farming, there is a need for environmentally sustainable strategies, avoiding the use of rodenticides which generate genetic resistance in target species and are a risk to non-target wildlife (Rattner *et al*. 2014). Anticoagulant rodenticides have been routinely applied in France to control fossorial water vole populations since the 1990s (Defaut *et al*. 2009) with negative effects on predators (Coeurdassier *et al*. 2014) and scavengers (Montaz *et al*. 2014). Enhancing the implementation and success of sustainable control methods require a deep knowledge of relevant aspects of the pest biology and ecology (Delattre & Giraudoux 2009; Jacob 2013; Ranchelli *et al*. 2016).

The reproductive characteristics of fossorial water voles in the Iberian Peninsula are known exclusively from studies on a population from the Aran Valley placed at about 900 m a.s.l. in the Spanish Pyrenees (Ventura & Gosálbez 1990b, c). Thus, regarding the lack of information on this subject on *A. scherman* from NW Spain and given the pest condition of the species in this geographic area, the main aim of this study was to determine the reproductive potential of fossorial water voles in the Cantabrian region (*A. scherman cantabriae*), specifically in apple orchards located at low altitude in Asturias. Since body size and the length of the breeding season differ substantially between Pyrenean and Asturian populations (Ventura & Gosálbez 1990a, 1990b; Somoano *et al*. 2017), differences in the reproduction potential between those populations can be expected. Thus, a further goal of the present research was to elucidate this question by comparing our results on *A. scherman cantabriae* with those previously reported for *A. scherman monticola.* The information obtained in this study could be useful to design effective control plans for fossorial water vole populations both in our study area and in others with similar environmental characteristics.

3.2 Material and Methods

3.2.1 Study site

The study was conducted in ten experimental and commercial apple orchards throughout two years (first year: February 2011-January 2012; second year: February 2012-January 2013). These orchards are separated from each other by 20.5 km as maximum and are located in two municipalities of the autonomous community of Asturias (northwestern Spain): Villaviciosa (43º 38’ N, 5º 26’ W) and Nava (43º 21’ N, 5º 30’ W). Their surface ranged from 1 to 7.6 ha and their altitude from 3 to 270 m a.s.l. Apples in Asturias are mainly produced in an area characterized by an irregular topography of smooth hills and valleys and distributed in a mosaic landscape of small agricultural plots separated by hedgerows and woodlands. The studied area has a temperate hyperoceanic climate (Rivas-Martínez & Rivas-Sáenz 2015) with abundant rainfall spread evenly along the year, and mild temperatures even in winter with low risk of frost and snowfall. The relatively high rainfall and fertile soils of Asturian meadows favor the establishment of evergreen and dense grass coverage in orchards all the year around (Díaz-González & Fernández-Prieto 2005; Miñarro 2012).

3.2.2 Specimen and data collection

A total of 422 female water voles were analysed. Each month, one or more orchards, depending on orchard surface and vole density, were visited for trapping. Individuals were captured with snap traps (Topcat® Andermatt Biocontrol, Switzerland) placed in galleries. Traps were activated during the day and checked twice per day for five days maximum. Shortly after capture the specimens were cryopreserved to -20°C until the necropsy. The recommendations of the Directive of the European Parliament and the Council on the Protection of Animals Used for Scientific Purposes (Directive 2010/63/UE 2010) was followed during the field work.

For each specimen, head and body length (HBL) and carcass mass (CM; body mass of the animal without neither the skin nor the thoracic and abdominal organs) were recorded using a digital calliper to the nearest 0.01 mm and a precision scale to the nearest 0.5 g, respectively. The body length of each embryo and the uterus of implantation were also registered. Litter size was evaluated according to the number of embryos implanted in uteri, discarding those showing macroscopic sign of resorption. Considering HBL range (1.4 - 38.1 mm) and mean value (12.5 mm) obtained in all embryos analysed, pregnant females whose embryos showed a HBL average lower than 6 mm were considered females in an early pregnancy state. The number of placental scars in each uterus was recorded.

The main criterion used to establish the sexual maturity state was the presence/absence of *corpora lutea*. Ovaries were extirpated and preserved in 70% ethanol until the analysis was conducted. Four longitudinal histological sections (sections of 5 µm thick separated between them 150 µm) were performed in the left ovary to obtain a whole representation of this organ. Sections were stained with hematoxylin-eosin. Other sexual parameters considered in each specimen were the following: development and vascularisation degree of the uteri, and presence and number of placental scars and/or embryos (Ventura & Gosálbez 1990c). The sexual maturity states were the following: immature, specimens without *corpora lutea*, uteri poorly developed and vascularised, without placental scars; mature, individuals with *corpora lutea*, uteri completely developed and well or scarcely vascularised, and showing placental scars and/or embryos.

Specimens were distributed into six classes of relative age (0-V) according to the following criteria (for details see Ventura & Gosálbez 1992): moulting stage, sturdiness of the mastoid process and the condylar process, the separation of angular process from the ascending branch of the mandible, and the values of the interorbital crests index, which relates the breadth of interorbital crests and the rostral length. The approximate age intervals corresponding to these age classes are the following (see Ventura & Gosálbez 1992 and references therein): class 0, 0-3 weeks; class I, 3-6 weeks; class II, 6-10 weeks; class III, 10-14 weeks; class IV, 14-30 weeks; class V, specimens older than 30 weeks. To assess differences on CM and body condition index (see below) between relative age classes, a random selection of non-pregnant females from relative age classes IV and V was used in an attempt to balance the number of analysed specimens in each age class.

3.2.3 Data analyses

The pregnancy frequency (F) gives information about how many times a female may become pregnant and was evaluated according to the method described by Emlen & Davis (1948): $F = (T/V)\cdot R$; where T = duration of the study (days), V = number of days in which pregnancy is visible (16 days: see Ventura & Gosálbez 1990b and references therein) and R = ratio of visible pregnant females in relation to the total number of matures ones.

Mann-Whitney U-tests were used to assess inter-annual differences in litter size and differences in embryo implantation in each uterus. Differences in litter size and the number of placental scars between relative age classes were assessed by Kruskal-Wallis tests. Then, Mann-Whitney U-tests with Bonferroni adjustment for multiple tests (α = 0.05/number of pair-wise comparisons) were used to establish differences between relative age classes.

The individual body condition index (BCI) was calculated as the difference between measured CM and the theoretically expected CM, which was obtained from the corresponding regressions against HBL after logarithmic transformation of the data of both variables (Evsikov *et al*. 2008). Females with visible signs of gestation were not taken into account to construct the regression equations. One-way ANOVA tests was used to assess differences in CM among relative age classes. This same test was also used to assess differences between years in BCI of mature non-pregnant and pregnant females. General linear models (GLM) were used to assess differences in BCI between years and relative age classes of non-pregnant and pregnant females. Tukey HSD tests were used to establish differences in BCI and CM between relative age classes. Changes in BCI of mature non-pregnant females along time were assessed by a Spearman correlation. This method was also used to determine relationships between pairs of the following variables: CM, BCI, litter size, number of placental scars and age class. Statistical analyses were performed with SPSS v22.0 (IBM Corp. 2013).

3.3 Results

3.3.1 Pregnant females

Out of the 422 females analysed, 101 females (32.0 % of total mature females; n = 316) were pregnant; 55of them were obtained during the first year and 46 during the second one (Table 3.1). Pregnant females were found in all months excepting January 2013. The youngest pregnant females corresponded to age class III and were detected in all seasons (Table 3.1).

3.3.2 Litter size

The total number of implanted embryos was 380, distributed in litters formed by 1-9 embryos (Table 3.2). The most frequent litter size was formed by four (35.6 %, n = 36) and three (33.7 %, n = 34) embryos, and the less frequent by nine (0.9 %, n = 1). The mean (± SE) number of implanted embryos per female was 3.76 (± 0.12). There were no inter-annual significant differences in the litter size (Fig. 3.1) (first year: 3.87 ± 0.18 (SE); second year: 3.63 ± 0.14 (SE); U = 1165.0; $p = 0.475$).

The mean number of embryos in the right uterus (2.11 ± 0.11) was significantly higher than the number in the left one (1.66 ± 0.10) (U = 3031.5; $p < 0.01$). Considering pregnancy states, females in early pregnancy state (n = 27) did not show significant differences in the average of embryos implanted in each uterus (right uterus: 1.92 ± 0.21; left uterus: 1.89 ± 0.21;U = 371.0; $p = 0.720$), whereas females in advanced pregnancy (n = 64) showed significant differences in this variable (right uterus: 2.16 ± 0.13 (SE); left uterus: 1.48 ± 0.11 (SE); U = 1260.0; $p < 0.001$).

Macroscopic signs of embryonic resorption were observed in 11 females in advanced pregnancy (10.9%) (Table 3.2). Some resorption signs were observed in 12 embryos (3.1%), in litter sizes of three (n = 4), four (n = 4) or five embryos (n = 4). Moreover, there were 14 litters with unilateral implantations (14.1%); 9 of these litters were found in the right uterus (R: right, L: left; 4R-0L, n = 2; 3R-0L, n = 3; 2R-0L, n = 2; 1R-0L, n = 2) and 5 in the left one (0R-3L, n = 4; 0R-1L, n = 1).

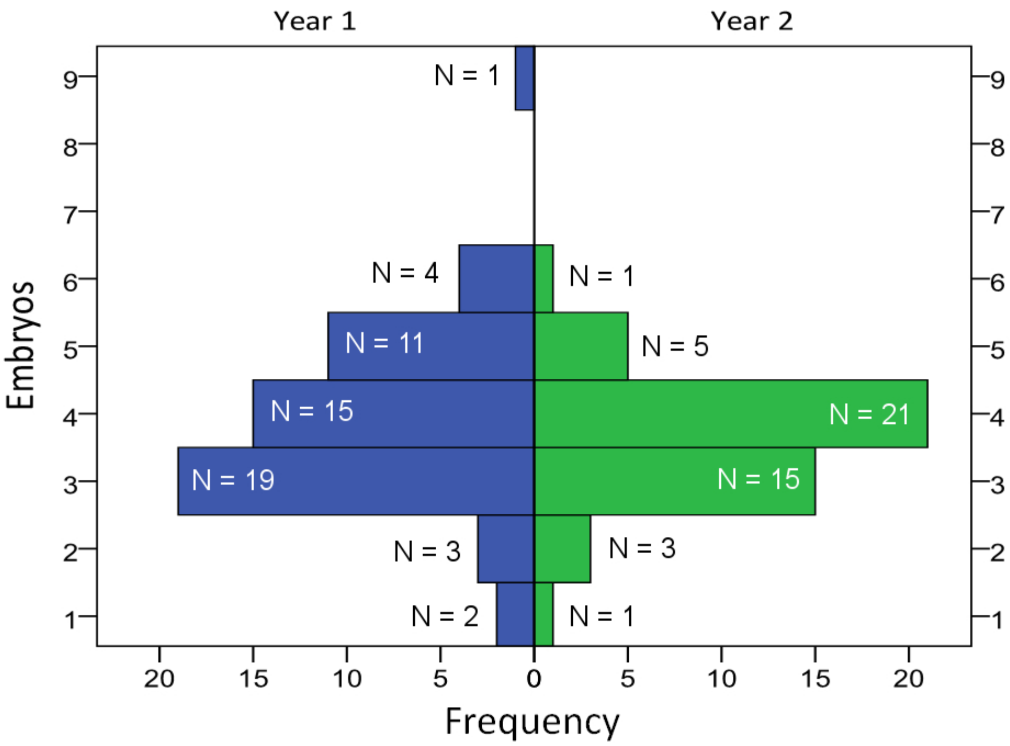


Fig. 3.1. Frequency of litter size per year in the sample of *A. scherman cantabriae* analysed

Litter size correlated positively with CM (r_s = 0.294; $p < 0.01$) (Fig. 3.2a) and increased with age (r_s = 0.217; $p < 0.05$), although it was not possible to establish differences between classes (H' = 4.881, $p = 0.087$) (Table 3.3). It is noteworthy that the only pregnant female with 9 embryos showed an extremely low CM value (-0.0294). So, we considered this individual as outlier and therefore it was discarded in the regression analysis. The number of placental scars per female ranged from 1 to 15, being four (13.8%) and three (12.0%) the most frequent values; this variable increased significantly with both relative age (r_s = 0.439; $p < 0.001$) (Table 3.3) and CM (r_s = 0.218; $p < 0.05$) (Fig. 3.2b).

Table 3.1. Number and percentage of pregnant females respect to the total number of mature females according to the relative age class and the month of capture in *A. scherman cantabriae.*

Year		Month	Age class			Total	N matures	% pregnants
			III	IV	V			
1st	2011	Feb		1	1	2	9	22.2
		Mar		1	1	2	19	10.5
		Apr	1	2	1	4	9	44.4
		May	3	4	2	9	20	45.0
		Jun	1	1	4	6	12	50.0
		Jul		1	2	3	13	23.1
		Aug	2	2	2	6	12	50.0
		Sep		3	1	4	16	25.0
		Oct		3	3	6	14	42.9
		Nov	3	2	1	6	15	40.0
		Dec		2		2	22	9.1
	2012	Jan	1	2	2	5	12	41.7
2nd		Feb			1	1	11	9.1
		Mar	1	2	2	5	13	38.5
		Apr	1	1		2	12	16.7
		May		4	4	8	14	57.1
		Jun	1	1	6	8	15	53.3
		Jul			2	2	11	18.2
		Aug		1	3	4	13	30.8
		Sep		1		1	10	10.0
		Oct		2		2	11	18.2
		Nov	1	5	2	8	14	57.1
		Dec	2	2	1	5	9	55.6
	2013	Jan				0	9	0
Total			17	43	41	101	315	32.1

Table 3.2. Litter size and number of individuals with resorbed embryos along the sampling period in *Arvicola scherman cantabriae.*

Year	Month	N	Litter size											Cases of resorbed embryos
			1	2	3	4	5	6	7	8	9	Mean	SE	
1st	Feb	2		1	1							2.50	0.50	
	Mar	2					2					5.00		
	Apr	4			1	1		2				4.75	0.75	
	May	9		1	1	4	2				1	4.44	0.65	1
	Jun	6		1	3	1	1					3.33	0.42	1
	Jul	3			1	1		1				4.33	0.88	
	Aug	6			3	2	1					3.67	0.33	1
	Sep	4	1			1	1	1				4.00	1.08	
	Oct	6			1	3	2					4.17	0.31	1
	Nov	6	1		4		1					3.00	0.52	1
	Dec	2				1	1					4.50	0.50	1
	Jan	5			4	1						3.20	0.20	1
2nd	Feb	1			1							3.00		
	Mar	5			1	1	3					4.40	0.40	1
	Apr	2				2						4.00		
	May	8			1	4	2	1				4.38	0.32	
	Jun	8		1	3	4						3.38	0.26	2
	Jul	2				2						4.00		
	Aug	4			2	2						3.50	0.29	
	Sep	1			1							3.00		
	Oct	2			1	1						3.50	0.50	
	Nov	8	1	1	2	4						3.13	0.40	
	Dec	5		1	3	1						3.00	0.32	1
	Jan	0												
	Total	101	3	6	34	36	16	5			1	3.76	0.12	11
	%	100	3.0	5.9	33.7	35.6	15.8	5.0			0.9			10.9

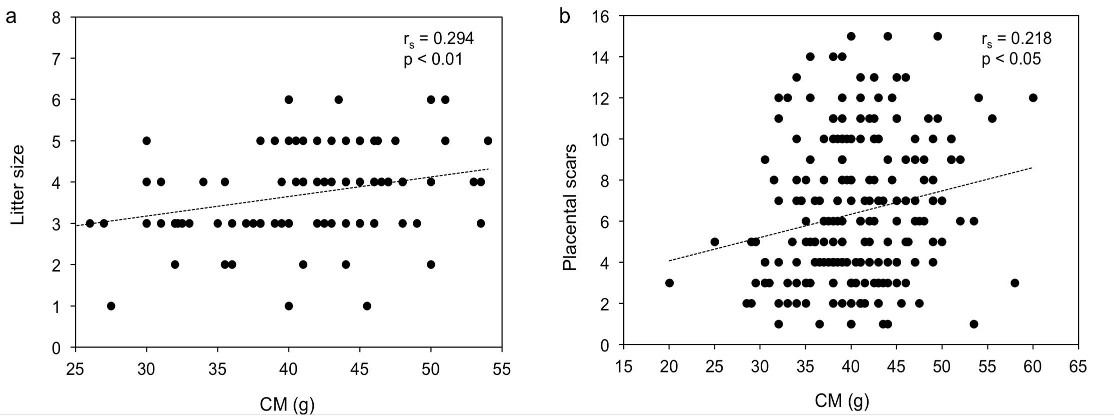


Fig. 3.2. Correlations between carcass mass (CM) and litter size (a), and CM and placental scars (b) in mature females of n *A. scherman cantabriae.*

Table 3.3. Carcass mass (CM) (g), litter size and number of placental scars according to relative age classes in *A. scherman cantabriae*. For each variable, significant differences between relative age classes are indicated by different letters.

Age class	N	CM			Litter size						Placental scars					
		Mean	SE		N	Mean	SE	Min	Max		N	Mean	SE	Min	Max	
III	51	31.2	1.1	a	17	3.3	0.2	1	5	a	13	3.5	0.6	1	9	a
IV	92	40.8	0.6	b	43	3.7	0.2	1	6	a	56	5.3	0.4	1	14	a
V	93	45.7	0.7	c	41	4.1	0.2	2	9	a	72	7.6	0.4	1	15	b

3.3.3 Potential fecundity

The ratio of visible pregnant females for the first year of the study was 0.32 (55 out of 173 mature females). Accordingly, the potential number of litters per mature female during the breeding season was 7.30 (F = (365/16)·0.32). The average litter size was 3.87 and the potential number of offspring by female was 28.25 (3.87·7.30). For the second year, the ratio of visible pregnant females was also 0.32 (46/142) and the potential number of litters was also 7.30. However, the average litter size was 3.63, and hence the potential number of offspring by female was slightly lower (26.50).

3.3.4 Body condition index

The BCI of non-pregnant females did not vary significantly with the relative age ($F_{5, 214}$ = 1.661; p = 0.145) neither between years ($F_{1, 214}$ = 0.057; p = 0.811). The result of the interaction of both factors showed that differences in BCI between age classes depend on the year ($F_{5, 214} = 2.364$; $p < 0.05$) (Table 3.4). Moreover, in pregnant females there were no significant differences in BCI between relative age classes ($F_{2, 94} = 2.582$; $p = 0.081$) neither between years ($F_{1, 94} = 0.193$; $p = 0.662$). Nevertheless, the interaction between relative age class and year in pregnant females showed that differences in BCI between age classes do not depend on the year ($F_{2, 94} = 0.444$; $p = 0.643$) (Table 3.4). A significant positive correlation between BCI and litter size was observed ($r_s = 0.230$; $p < 0.05$) (Fig. 3.3).

Table 3.4. Find the table in the next page. Body condition index (BCI) of non-pregnant and pregnant females for each age class and for mature females in *A. scherman cantabriae*. Significant differences in BCI between relative age classes for pregnant and non-pregnant females are indicated by different lower case letters. Significant differences in BCI between years in mature non-pregnant and pregnant females are indicated by different capital letters.

Age class	Year	Non-pregnant females						Pregnant females					
		N	Mean	SE	Min	Max		N	Mean	SE	Min	Max	
0	1st	7	-0.0584	0.0258	-0.1909	0.0251	a						
I		33	-0.0004	0.0120	-0.2124	0.1177	ab						
II		16	-0.0141	0.0113	-0.1181	0.0422	ab						
III		19	0.0157	0.0140	-0.1138	0.1367	b	11	0.0151	0.0109	-0.0448	0.0727	a
IV		20	0.0123	0.0099	-0.0546	0.0920	b	24	0.0268	0.0088	-0.0701	0.1054	a
V		20	0.0194	0.0115	-0.0995	0.1079	b	20	0.0144	0.0105	-0.1044	0.0896	a
0	2nd	2	0.0644	0.0133	0.0511	0.0776	a						
I		7	-0.0337	0.0133	-0.0995	0.0007	a						
II		28	-0.0222	0.0081	-0.1277	0.0945	a						
III		15	-0.0041	0.0099	-0.0922	0.0631	a	6	-0.0012	0.0090	-0.0240	0.0268	a
IV		28	-0.0141	0.0098	-0.1245	0.1568	a	19	0.0332	0.0115	-0.0582	0.1423	a
V		31	-0.0015	0.0103	-0.1577	0.0935	a	20	0.0124	0.0053	-0.0281	0.0508	a
Matures	1st	118	0.0106	0.0047	-0.1803	0.1615	A	55	0.0199	0.0058	-0.1044	0.1054	A
	2nd	96	-0.0068	0.0051	-0.1577	0.1568	B	46	0.0193	0.0058	-0.0582	0.1423	A

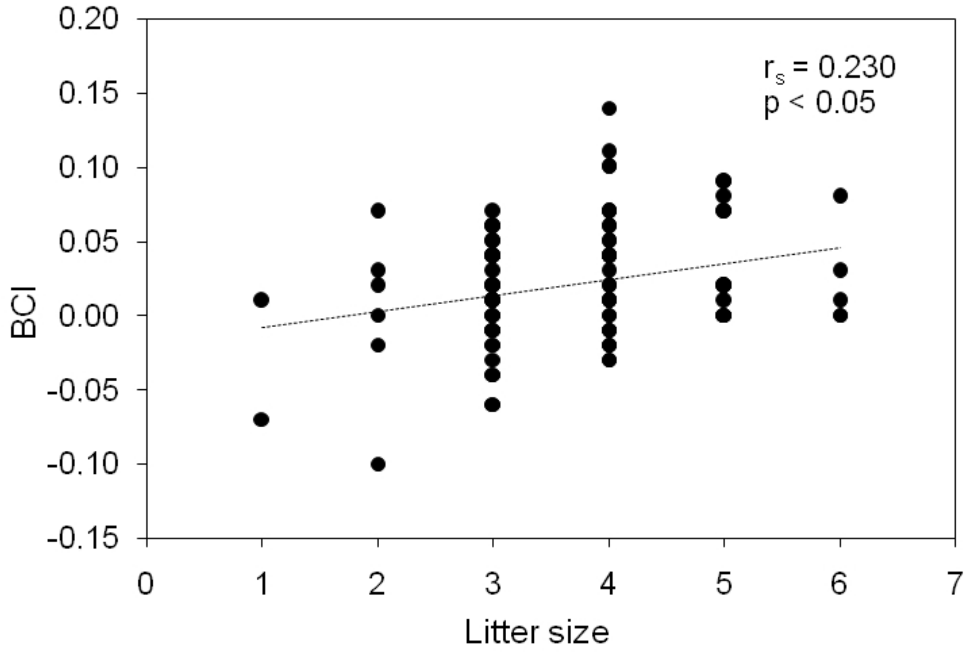


Fig. 3.3. Correlation between body condition index (BCI) and litter size in *A. scherman cantabriae.*

Considering only mature specimens, mean values of BCI of non-pregnant females were significantly higher in the first year than in the second one ($F_{1, 211} = 6.257$; $p < 0.05$); no significant inter-annual differences were found for pregnant females ($F_{1, 98} = 0.006$; $p = 0.938$) (Table 3.4). Moreover, BCI was significantly higher in pregnant females respect to non-pregnant ones in the second year ($F_{1, 137} = 9.726$; $p < 0.01$); whereas no significant differences between these groups were found in the first year ($F_{1, 172} = 1.380$; $p = 0.242$) (Table 3.4). In mature non-pregnant females BCI diminished along the sampling period ($r_s = -0.197$; $p < 0.05$) (Fig. 3.4).

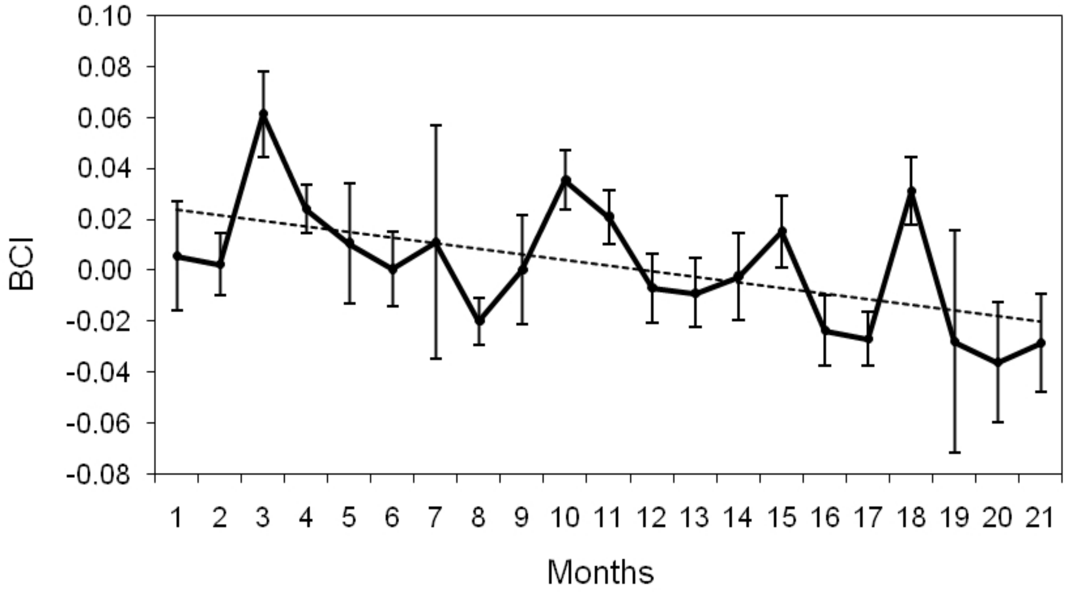


Fig. 3.4. Body condition index (BCI) (mean ± SE) of mature non-pregnant females in *A. scherman cantabriae* throughout the sampling period.

3.4 Discussion

General results of this study reveal that *A. scherman cantabriae* inhabiting Asturian apple orchards shows higher reproductive potential than Pyrenean fossorial water voles. Specifically, during the study period each female was able to produce 7.3 litters in a year, a value that to our knowledge is the highest one reported for *A. scherman* but also for *A. amphibius* (2.0 - 7.1 litters/year) (van Wijngaarden 1954; Tupikova & Švecov 1956; Hamar & Marin 1962; Kminiak 1968; Pelikán 1972; Wieland 1973; Ventura & Gosálbez 1990c). On the contrary, the average litter size we found in *A. scherman cantabriae* (3.76 embryos per female) was among the smallest values reported for both species (Perry 1943; van Wijngaarden 1954; Maksimov 1959; Kminiak 1968; Panteleyev 1968; Stoddart 1971; Pelikán 1972; Wieland 1973; Kratochvíl 1974; Blake 1982; Evsikov *et al*. 1989; Ventura & Gosálbez 1990c). As a result of these values, the potential number of offspring per female and year reached 28.25 in Cantabrian voles, which is higher than the value indicated for *A. scherman monticola* (23.5; Ventura & Gosálbez 1990c). Therefore, populations of *A. scherman* can show different reproductive potential in the Iberian Peninsula depending, at least partially, on the habitat, which might ultimately determine their role as agricultural pest.

The length of the breeding season is a critical factor in determining litter size in small mammals: reproduction can be extended throughout the year at lower elevations, whereas in higher elevations larger litters are needed to sustain an equal level of reproduction during a shorter breeding season (Dunmire 1960; Smith & McGinnis 1968; Spencer & Steinhoff 1968; Innes 1978; Tkadlec & Zejda 1998). Specifically in *Arvicola,* this pattern was found in semiaquatic water voles (Kratochvíl 1974). In that sense, apple orchards in Asturias are located in a temperate hyperoceanic climate (Rivas-Martínez & Rivas-Sáenz 2015), which implies benign weather condition that allows fossorial water voles to breed continuously along the year (Somoano *et al*. 2017), whereas the breeding season of *A. scherman* is limited to the period March-November in the Pyrenees (Ventura & Gosálbez 1990b). These differences between Spanish populations can be explained by an adaptation to seasonal changes of each geographic zone, as occur in other rodent species (Hansen *et al*. 1999; Solonen 2006). Although differences in litter size between the Cantabrian and Pyrenean populations can be related with an altitudinal effect, intrinsic factors could also be involved. For example, differences in ovulation rate between wild populations of the house mouse (*Mus musculus*) are mainly determined by body size, whereas the influence of extrinsic factors is low (Jacob *et al*. 2007). Therefore, a smaller litter size in the small-sized *A. scherman cantabriae* (Ventura & Gosálbez 1990a; Ventura 1993) cannot be discarded as an own characteristic of the subspecies. Additionally, we found in our sample that the body size of the mother was significantly correlated with the number of implanted embryos, result that is concordant with that reported for *A. scherman monticola* (Ventura & Gosálbez 1990c). Thus, an intra-population effect of the relationship between body size and litter size can be added to that found between these variables in an evolutionary context.

As expected and also as observed in *A. scherman monticola* (Ventura & Gosálbez 1990c), the number of placental scars increased with age in our population. Differences in the range of this variable between Cantabrian (1-15) and Pyrenean (2-20; Ventura & Gosálbez 1990c) *A. scherman* are probably due to differences, at a population level, in the relationship between the number of reproductive events and litter size, and also to differences in the persistence of placental scars.

Pregnant females with macroscopic signs of resorption were also found in other populations of both *A. scherman* and *A. amphibius* (van Wijngaarden 1954; Pelikán 1972; Wieland 1973; Ventura & Gosálbez 1990c). In our sample, females in early pregnancy had similar embryonic implantation in each uterus, which suggests a balanced ovulation in both ovaries. Nevertheless, a significantly larger number of embryos implanted in the right uterus was observed for the total pregnant females, which might be due to embryo resorption. Indeed, an unequal uteri-functioning has been well described formerly in mouse and rat (Wiebold & Becker 1987 and references therein). Moreover, Isakova *et al*. (2012) found in *A. amphibius* that an important percentage of embryos suffer a resorption in the uteri (16.7%) caused probably by mutation processes.

Body condition (relationship between the carcass mass and body length) indicates energy provision and plays an important role in the reproductive potential. Specifically, in *A. amphibius* it has been indicated that a good body condition in pregnant females leads to higher number of ovulated oocytes and implanted embryos, and decreases the risk of pregnancy failure (Evsikov *et al*. 2008; Nazarova & Evsikov 2008; Yuzhik *et al*. 2015). This seems to agree with our results in *A. scherman cantabriae* regarding the increase of litter size with the BCI of the mother. Although a clear correlation between the number of implanted embryos and the body mass of the mother has also been reported in both *A. scherman* and *A. amphibius* (Ventura & Gosálbez 1990c and references therein), our data suggest ultimately that the BCI is the key. Therefore, the body condition of the mother seems to be one of the main factors involved in the variation of the reproductive potential in Cantabrian voles.

The succession of monthly samplings during our two-year study involved a population control by removing specimens of both sexes from the burrow. Since the burrow system of fossorial water voles is generally inhabited by a breeding couple with their offspring (Airoldi 1976, 1978; Morel 1981; Somoano *et al*. 2017) and its maintenance and the storage of food are performed by the couple (Airoldi 1976), the removal of the male mate might suppose a handicap for the female. In this situation, the female might face the maintenance of the burrow and the acquisition of food by its own until de arrival of a new partner which in turns can induce a decrease of the body condition, as we observed in mature non-pregnant females along this study period.

Ultimately, this fact might have negative consequences on the reproductive success as it has been reported for several arvicoline species (Ostfeld 1985).

As conclusions, the reproductive potential of *A. scherman cantabriae* in areas of northwestern Spain located at low altitude and with a temperate hyperoceanic climate is relatively high in comparison with that of fossorial water voles from the Pyrenees. Specifically, mild temperatures and enough resource availability along the whole year in our study area allow fossorial water voles to breed continuously and to perform a greater number of reproductive events per year than *A. scherman monticola*. Conversely, the favourable environmental conditions in the Pyrenean meadows are restricted to a part of the year, what leads voles to concentrate its energy in fewer but larger litters. Therefore, the smaller litter size in *A. scherman cantabriae* seems to fit with a different reproductive pattern, in which the variation in this parameter within the population may be due, at least in part, to the body condition of the mother.

Chapter 4. Genetic diversity and spatial genetic structure of fossorial water voles in a patchy landscape

Somoano A, Bastos-Silveira C, Heckel G, Ventura V, Miñarro M

4.1 Introduction

A successful dispersal and posterior reproduction mean effective movements which ultimately involve gene flow across the landscape (Gauffre *et al*. 2008; Berthier *et al*. 2013; Craig *et al*. 2015). However, the degree to which a landscape allows population diffusion will be determined by connectivity and species residence in each type of habitat (With *et al*. 1997; Minor & Urban 2008; Galpern *et al*. 2011). This is especially true for agricultural landscapes, which suffer spatio-temporal variations (Gauffre *et al*. 2008). Furthermore, agricultural landscapes are frequently conformed by a patchwork of habitats of varying quality usually consisting in a mosaic of crops, abandoned plots and natural plots (Janova & Heroldova 2016). Thus, patch-dependent species, such as voles, would be influenced by the filtering effect of the matrix, the spatial and temporal scale of suitable habitats and their isolation (Ricketts 2001; Gilarranz & Bascompte 2012; Driscoll *et al*. 2013). So, population genetics of a vole species can depend on the environmental and landscape characteristics and land managements developed in each agroecosystem (Jacob & Hempel 2003; Marchi *et al*. 2013; Foltête *et al*. 2016). Studying the genetic diversity and population genetic structure of a species in different agricultural areas could provide suitable information to assess the influence of landscape on the gene flow and hence on dispersal movements (e.g. Schweizer *et al*. 2007; Le Galliard *et al.* 2011; Sutherland *et al*. 2014; Anderson *et al*. 2015).

The montane water vole *Arvicola scherman* (formerly fossorial form of *Arvicola terrestris*; for taxonomic considerations see Musser & Carleton 2005) occurs preferably in meadows, grasslands and orchards of the main mountainous areas of Europe (Kryštufek *et al*. 2015), where it digs extensive burrow systems (Airoldi 1976). These fossorial water voles usually disperse from 30 to 100 m (Saucy 1988), but their movements are strongly influenced by the current land use (Halliez *et al*. 2015), which highlights the importance of the connectivity among suitable habitat patches for population diffusion (Foltête *et al*. 2016). In this way, homogeneous landscapes, such as large grassland areas from France required for livestock breeding, supply many favourable habitats for this species and do not hamper its effective movements (Fichet-Calvet *et al*. 2000; Foltête & Giraudoux 2012; Halliez *et al*. 2015). This situation leads to merged populations of *A. scherman* whose population densities are synchronized (Giraudoux *et al*. 1997; Morilhat *et al*. 2008; Berthier *et al*. 2013). Fossorial water voles show multiannual fluctuations of density every 5 to 9 year periods in these large grassland areas (Saucy 1988; Giraudoux *et al*. 1997; Fichet-Calvet *et al*. 2000) and just suffer gene flow disruptions associated with sharp reliefs and population density levels at a regional scale (Berthier *et al*. 2005). Indeed, minimum levels of genetic diversity and spatial differentiation are associated with low density phases in this type of landscape, which is reverted during increasing density phases by the succession of unimpeded effective movements (Berthier *et al*. 2006).

On the other hand, effective movements of *A. scherman* might be hampered by agricultural landscapes characterized by heterogeneous mosaics, in which hedgerows, ploughed lands and wooded patches surround favourable habitats such as grasslands (Duhamel *et al*. 2000; Foltête *et al*. 2008; Morilhat *et al*. 2008). These patchy landscapes could prevent colonisation of fossorial water voles directly by slowing their dispersion and indirectly by favouring predation (Giraudoux *et al*. 1997; Fichet-Calvet *et al*. 2000; Foltête & Giraudoux 2012). Thus, an agro-ecological approach to diversify habitats at landscape scale by generating patches of different land-use and planting hedgerows has been recently developed in France to decrease *A. scherman* damages in grasslands (Foltête *et al*. 2016).

In Spain, farmers and land managers are bound to slow down the population growth of *A. scherman* in affected localities (BOE 2008). In fact, this species has become one of the main causes of economical loss in apple orchards of Asturias, in northwestern Spain (Miñarro *et al*. 2012). Indeed in this region, *A. scherman* shows continuous breeding (Somoano *et al*. 2017) and the highest reproductive potential reported for this species to date driven probably by favourable environmental conditions through the year (Somoano *et al*. 2016). The agricultural area of Asturias shows a highly-patched landscape named "bocage", typical of Atlantic coastal regions (Baudry *et al*. 2000). The land has been excessively parcelled due to historical reasons, and the subsistence and cash crop farming have not been completely abandoned as in other European areas affected by *A. scherman* (Halliez *et al*. 2015). This leads to closed landscapes characterized by a mosaic of small different land-use plots separated by hedgerows and woodlands. It is important to consider that landscapes with relatively high proportions of natural and semi-natural areas exhibit lower pest abundance or higher pest control in crop fields (Veres *et al*. 2010). Therefore, this agroecosystem can lead to low level of connectivity for *A. scherman*, which could be a potential framework to control population diffusion of this species (Giraudoux *et al*. 1994; Foltête *et al*. 2016).

A large proportion unfavourable habitats and scarce connectivity among habitat patches in the agricultural area of Asturias might hinder effective movements of fossorial water voles, and hence entailing disruptions of gene flow and the consequent loss of genetic diversity and population subdivision at local scale. Thus, an empirical study in this highly-patched landscape, aiming to assess genetic structure of *A. scherman* population, can uncover both the landscape features that affect gene flow and the effect of advisable field management of this species. This approach could be also useful to set up coordinated management actions to control its populations (Laikre *et al*. 2005; Brouat *et al*. 2007; Guivier *et al*. 2011). Indeed, the identification of demographically independent population units (managements units) inferred from high levels of genetic differentiation, provide suitable information for an independent management regime (Palsbøll *et al*. 2007) to establish population control strategies in this pest species (Piertney *et al*. 2016).

4.2 Material and Methods

4.2.1 Study area

The study was conducted in approximately 120 km^2 of an extensive agricultural landscape in the main area of apple production in Asturias (Fig. 4.1). Ten semi-intensive apple orchards, located in the municipalities of Villaviciosa (43º 38’ N, 5º 26’ W) and Nava (43º 21’ N, 5º 30’ W) were sampled. The orchard surface ranged from 1 to 7.6 ha and their altitude from 3 to 270 m a.s.l. This agricultural area shows a highly-patched landscape characterized by a mosaic of small agricultural plots separated by hedgerows, woodlands or shrubs areas (Fig. 4.1). These hedgerows and shrubs are constituted by holly, gorse, bramble, hazelnut or laurel, whereas deciduous trees, such as oaks and chestnuts, mainly conform the natural woodlots. Moreover, eucalyptus plantations are abundant in the surroundings of agricultural plots. Each plot can be intended for mowing, livestock, wood production, cropping or fruit production. Relatively high rainfall and fertile soils favour the establishment of an evergreen and dense grass coverage all the year around in meadows, grasslands and orchards. Human populations are scattered through the landscape conforming residential agricultural settlements (RASs), excepting the small-urban areas of each municipality. The relief of the area is generally smooth with moderate hills which rise 500 m a.s.l. An estuary and a four-lane highway (A-8) are present and separate some of these sampling sites in the north of the area (Fig. 4.1).

4.2.2 Specimen collection

From 11 to 18 voles were gathered per location, each one conformed by a single *A. scherman* colony or deme (see Results). These captures were carried out, from January 2011 to December 2012, as part of an initial demographic control of the populations through trapping with snap traps (Topcat® Andermatt Biocontrol, Switzerland) placed in galleries and checked twice per day for five days maximum. Shortly after capture, each specimen was cryopreserved to -20°C. A sample of skeletal muscle tissue was preserved in 70% ethanol at -20ºC. All specimens were sexed and classified as sexually immature or mature (see Somoano *et al*. 2017). The recommendations of the Directive of the European Parliament and the Council on the Protection of Animals Used for Scientific Purposes (Directive 2010/63/UE 2010) were considered in all procedures.

4.2.3 Landscape study

Patches were represented by polygons of soil occupancy, extracted from the Land Cover and Use Information System of Spain (SIOSE, Valcárcel *et al*. 2008). Soil-occupancy polygons were assessed in a vector based geographic information system (GIS) and simplified in nine categories: meadows, fruit orchards, grasslands, annual crops, shrubs, eucalyptus plantations, deciduous woodlots, buildings and roads, and river and water bodies (Fig 1). In accordance with the main farming activity developed in each RAS, each one of them was assigned to one of the former soil-occupancy categories.

A cost-distance model (friction/resistance layer) was developed according to the suitability of the patches to be used as habitats and by their permeability to move successfully through them (Adriaensen *et al*. 2003; Sawyer *et al*. 2011 and references therein). Each cell of the layer (5 m^2) is geo-referenced and provides a cost value (CV) attributed to each soil occupancy category. In this way, the cost values assigned to occupancy categories were provided by the studies of Foltête & Giraudoux (2012) and

Foltête *et al*. (2016) on *A. scherman* populations from France. All these soil-occupancy categories are present both in France and northwestern Spain and this approach is in accordance with the results of habitat preference of *A. scherman* in Asturias (Fernández-Ceballos 2005). Thus, the cost values were assigned to each soil occupancy category based on the assumption that a greater cost value implies less connectivity and *vice versa*: Cost value = 1: meadows and grasslands; Cost value = 25: annual crops and shrubs/hedgerows; Cost value = 50: buildings and roads; Cost value = 1,000: deciduous woodlots and river and water bodies. Apart from those, fruit orchards and eucalyptus plantations are quite frequent in this study area, and according to vole density records carried out by Fernández-Ceballos (2005), their CV have been assigned to 1 and 1,000 respectively.

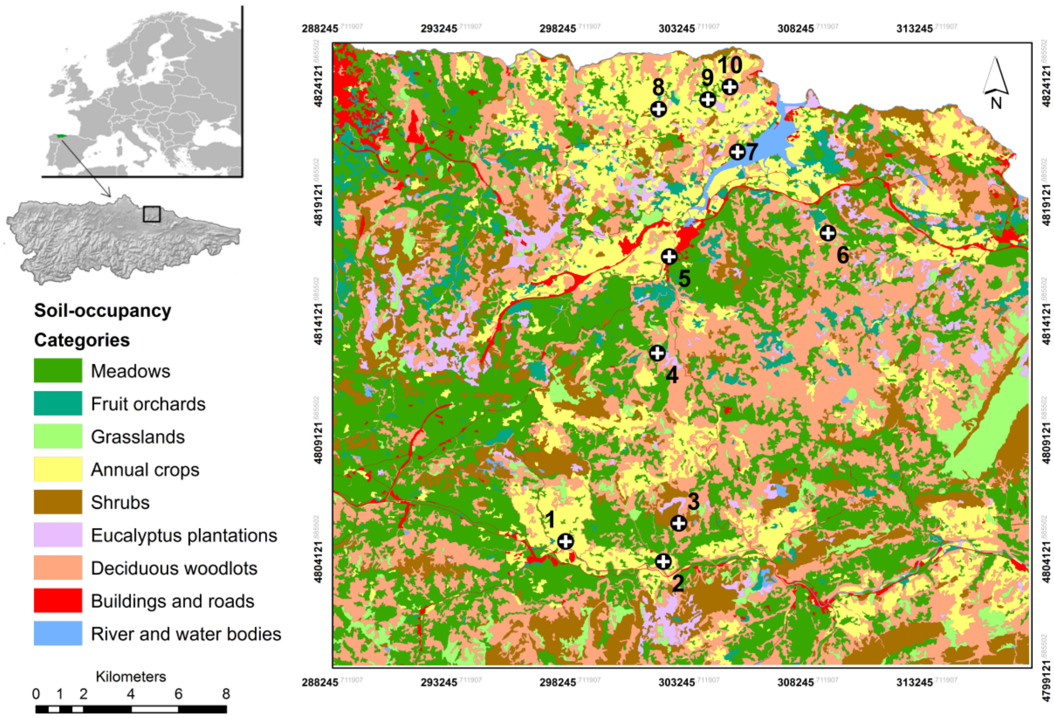


Fig. 4.1. Study area in Asturias (northwestern Spain). Map of soil occupancy (Land Cover and Use Information System of Spain, SIOSE). The sampled orchards are indicated by numbers: 1, Vegadali; 2, Ceceda; 3, Fresnadiello; 4, Poreño; 5, Serida; 6, Priesca; 7, Rozada; 8, Oles; 9, Teleña; 10, Marina.

Least-cost paths between pairs of sampling plots were calculated through an algorithm based on the accumulative cost to reach cell N_i plus the average cost to move through cell N_i and cell N_{i+1}, in which the algorithm considers eight neighbour cells and allows diagonal movements (ESRI 1996; Adriaensen *et al*. 2003). No maximum cost value was set up in this landscape to allow quantitative comparisons in all cases. The suitability of least-cost distances based on demographic results of this species allows to assess their correspondent effective distances (Foltête *et al*. 2016). Furthermore, a digital elevation model (DEM) (gridded at scale of 5 m) was included to calculate these distances regarding the slope of the terrain. Thus, our effective distances were calculated regarding landscape connectivity and terrain characteristics. Finally, to compare quantitatively the total cost of each cost-path among plots, a resistance index (*RI*) was developed:

$$RI = \frac{\sum_{i=1}^{n}(m_i CV_i)}{1{,}000}$$

where CV_i is the value of the cost for each category i, m_i is the total length (in meters) of the crossed patches, and n is the number of cost-value categories (four in this study).

The area of suitable habitats (meadows, orchards and grasslands) to the total land area (SH/TL) was calculated regarding the Ratio of Optimal to Marginal Patch Area (ROMPA) hypothesis (Giraudoux *et al*. 1997; Duhamel *et al*. 2000; Fichet-Calvet *et al*. 2000). The area between each pair of sampling plots was considered resulting of two circular buffers intersected, where their radius was the Euclidean distance between both orchards and its origin the centre of the plot. Spatial analysis and GIS data management were conducted with ArcGis 10.3 (ESRI 1996).

4.2.4 Microsatellite genotyping

Genomic DNA was extracted from skeletal muscle samples using GeneMatrix Tissue Purification Kit (EURx). DNA was analysed by PCR-amplification of 12 microsatellite loci in two multiplex panels: panel 1: AV3, AV8, AV11, AV15, AT2 and AT24; and panel 2: AV12, AV14, AV13P, AT9, AT13 and AT22 (Stewart *et al*. 1998; Berthier *et al*. 2004, 2005). AV13 reverse microsatellite primer was labelled with a "Pig-tail" to increase the accuracy of genotyping. Forward primers were labelled using FAM, NED, PET and VIC fluorocromes. Genotyping was conducted in 10 µL reactions. Each reaction contained 1 µL primer mix, 5 µL GoTaq Green Master Mix (containing *Taq*-polymerase, $MgCl_2$, dNTPs and PCR buffer) and 1.5 µL DNA (50 ng/µL). The PCR profile had an initial denaturation step at 93ºC for 2 minutes, then thirty cycles starting with 30 seconds at 91ºC, 30 seconds at 57ºC and 30 seconds at

74ºC. The PCR ended with an elongation phase at 74ºC for 10 minutes, and then the PCR products were stored at 4ºC. Fragment separation was carried out on an ABI 3100 sequencer and the genotypes were scored using GENEMAPER software 3.7 (Applied Biosystems) against the internal LIZ 500 size standard.

4.2.5 Population genetic and statistical analyses

The software CONVERT 1.3 (Glaubitz 2004) was used to prepare the input files for microsatellite data analyses. Expected (H_E) and observed (H_O) heterozygositiy levels (Nei 1987) were calculated with the program GENETIX 4.05 (Belkhir *et al*. 1996). The number of alleles (N_A), allelic richness corrected for minimum sample size (A_R), inbreeding coefficients (F_{IS}) for each population and fixation index (F_{ST}) for each pair of populations and overall were calculated using the software FSTAT 2.9 (Goudet 1995). Deviation from Hardy-Weinberg equilibrium (HWE) after sequential Bonferroni correction (signification level/pairwise comparisons) for each locus and overall loci, and linkage disequilibrium (LD) between all pairs of loci were tested within each population using exact tests based on Markov chain algorithm implemented in the software GENEPOP 4.2.2 (Raymond and Rousset 1995).

The relation between genetic similarity and geographic distances was assessed by the program Isolation by Distance Web Service (IBDWS) version 3.23 (Jensen *et al.* 2005). We determined the correlation between pairwise genetic distances (F_{ST}) with the Euclidean distance and effective distances. Mantel tests were performed using 10,000 iterations as implemented in IBDWS. The significance of the relationship was assessed by regressing pairwise decimal logarithm of geographic distances against the corresponding genetic distances using Reduced Major Axis (RMA) regression (Bohonak 2002). To evaluate the recent migration between populations we used the software GENECLASS 2.0 (Piry *et al*. 2004), following the setting recommended for not all source populations sampled (direct likelihood L_home). The probability for an individual to be a resident was assessed using Monte Carlo resampling procedure of Paetkau *et al.* (2004) and simulating 10,000 individuals resampling, with the frequencies-based method. The probability of type I error was set to $P < 0.01$, and default frequency of missing alleles to 0.01.

Spearman correlations were used to assess the relationship between A_R, H_O or F_{IS} and the percentage of individuals belonging to each maturity status in each population. Pearson correlation was used to assess the relationship between the ratio of suitable habitat to the total land area (SH/TL), resistance index values (*RI*), effective distances and the fixation index (F_{ST}) of *A. scherman* demes. All correlations were performed with SPSS v22.0 (IBM Corp. 2013).

4.2.6 Identification of genetic units

Genetic disruption within the studied area was assessed using non-spatial and spatial methods. Genetic structure was examined through a model-based Bayesian clustering method implemented in STRUCTURE 2.3.4 (Pritchard *et al*. 2000). This software considers multilocus genotypes and attempts to minimize LD and HW disequilibrium by estimating the number of populations (*K*) based on individual data. STRUCTURE was run with ten repetitions of 500,000 Markov Chain Monte Carlo (MCMC) iterations following a burn-in period length of 100,000 steps, the admixture model, correlated allele frequencies and no prior population information. The Evanno method (Evanno *et al*. 2005) was used to estimate the most likely number of clusters (*K* = 1 to 16) from the rate of change in log probabilities between each *K* evaluated. GENELAND 4.0.5 (Guillot *et al*. 2005) was used to determine the genetic spatial structure in the study area considering the spatial coordinates of the apple orchards where voles were captured. The most probable number of genetic clusters (*K*) was inferred by performing 500,000 MCMC iterations (thinning = 1,000) with a maximum rate of Poisson process fixed to 100. We allowed *K* to vary between 1 and 10 populations. The maximum rate of the Poisson process was fixed at 100, the maximum number of nuclei in the Poisson-Voronoi tessellation at 300, and the potential error for spatial coordinates was specified as 0.01. The MCMC was run 10 times to check the results and determine the most consensual *K*. Inferences from STRUCTURE were based on genetic data alone under the assumption that all clustering solutions are equally likely, whereas GENELAND incorporated spatial organization information for genotyped individuals (Gauffre *et al*. 2008; Guivier *et al*. 2011). Indeed, GENELAND generates a map of population ranges, in accordance with a model in which populations tend to be structured in spatially distinct areas (Guillot *et al*. 2005).

4.3 Results

4.3.1 Population genetic analyses

A total of 137 specimens (71 males, 66 females) were successfully genotyped at 12 microsatellite loci (Table 4.1). The number of alleles per locus ranged from 3 to 18. The observed heterozygosity per locus varied from 0.326 to 0.804, with an average value of 0.602, whereas the expected heterocygosity ranged from 0.497 to 0.871 with an average value of 0.732 (Table 4.1). All but one (AT22) loci conformed to HWE after Bonferroni correction when all populations were analyzed together (Table 4.1). Nevertheless, departure from HWE was also detected when all loci were considered together (Table 4.1). The loci AV13P and AV14 showed significant linkage disequilibrium across all populations ($P < 0.001$).

The allelic richness corrected for the minimum sample size for each deme ranged from 3.55 to 5.49, with an average value of 4.42 (Table 4.2). The levels of observed heterozigosity per deme ranged from 0.483 to 0.679, with an average value of 0.601, whereas the expected heterozigosity ranged from 0.564 to 0.698, with an average value of 0.621 (Table 4.2). All but one deme (*5*) conformed to HWE after Bonferroni correction (Table 4.2). Deme *8* showed significant heterozigosity deficiency associated with very high inbreeding coefficient (F_{IS}). There were no significant correlations between A_R, H_O or F_{IS} values and the percentage of immature or mature individuals in each deme ($P > 0.05$ in all cases).

The software GENECLASS revealed the presence of seven specimens identified as first-generation immigrants in the studied demes. Immigrants were detected in deme *1* (1), deme *2* (1), deme *5* (1), deme *6* (2), deme *7* (1) and deme *8* (1). The immigrant specimen from deme *2* was home-assigned migrant, implying that was genetically closer to its population of capture than other sampled population. Demes *6* and *7* received immigrants from demes genetically closer to demes *9* and *10*. Demes *1*, *5* and *8* received immigrants genetically different from any sampled deme. Two migrant specimens were sexually immatures (1 male, 1 female), another was a submature male, and the other four specimens were mature, one male and three non-pregnant females.

Table 4.1.Descriptive statistics of 12 microsatellite loci tested in ten *A. scherman* populations from apple orchards of Asturias when all specimens were pooled together. Number of alleles (N_A), size range of microsatellite alleles, observed heterocygosity (H_O), expected heterozygosity (H_E), Hardy-Weinberg equilibrium exact test (HWE P value) are shown. Significant probabilities after sequential Bonferroni correction are indicated in bold.

Locus	N	N_A	Range	H_O	H_E	HWE *P* value
AV3	137	8	124 - 164	0.529	0.578	0.093
AV8	137	13	296 - 354	0.616	0.860	0.001
AV11	137	9	362 - 394	0.725	0.763	0.289
AV12	137	9	172 - 204	0.609	0.759	0.064
AV13P	137	10	120 - 164	0.796	0.854	0.828
AV14	137	9	198 - 236	0.804	0.852	0.951
AV15	137	10	184 - 216	0.642	0.815	0.042
AT2	137	18	175 - 227	0.667	0.871	0.039
AT9	137	4	285 - 296	0.326	0.497	0.995
AT13	137	3	143 - 148	0.369	0.531	0.906
AT22	137	8	198 - 232	0.558	0.693	**<0.001**
AT24	137	10	127 - 156	0.580	0.709	0.487
All loci	137	9.3		0.602	0.732	**<0.001**

Table 4.2. Sampled orchard/deme, geographic coordinates of apple orchards, orchard area (ha), (N) number of specimens of *A. scherman* genotyped at 12 microsatellite loci, number of alleles (N_A), allelic richness corrected for minimum sample size (A_R), observed heterocygosity (H_O), expected heterozygosity (H_E), inbreeding coefficient (F_{IS}), Hardy-Weinberg equilibrium exact test (HWE P value); significant probabilities after sequential Bonferroni correction are indicated in bold.

Orchrard / deme	UTMx	UTMy	Area (ha)	N	N_A	A_R	H_O	H_E	F_{IS}	HWE *P* value
1- Vegadali	298049	4804471	2.31	18	6.08	5.34	0.657	0.698	0.060	0.001
2- Ceceda	302152	4803618	5.95	14	4.92	4.60	0.613	0.639	0.042	0.014
3- Fresnadiello	302809	4805229	2.84	14	5.83	5.49	0.673	0.697	0.037	0.697
4- Poreño	301923	4812449	7.58	11	4.58	4.58	0.614	0.619	0.010	0.989
5- Serida	302393	4816492	7.26	12	4.75	4.58	0.483	0.573	0.162	**<0.001**
6- Priesca	309079	4817418	2.03	14	3.67	3.55	0.613	0.569	-0.081	0.708
7- Rozada	305277	4820835	2.99	14	4.17	3.97	0.539	0.564	0.045	0.235
8- Oles	301922	4822568	4.55	15	4.25	4.01	0.506	0.611	0.177	0.007
9- Teleña	304030	4823030	1.13	14	4.08	3.99	0.679	0.632	-0.076	0.441
10- Marina	304955	4823559	1.00	11	4.08	4.08	0.629	0.608	-0.036	0.322

4.3.2 Genetic structure analyses

The genetic differentiation between all pairs of demes showed a F_{ST} range between 0.004 and 0.271 (Table 4.3), being 0.157 (± 0.009) the average (± SE) level of genetic differentiation among demes. The results of Mantel tests on the matrices of genetic and both geographic distances showed significant isolation-by-distance (IBD) patterns for both Euclidean distances (r = 0.790; P = 0.0001) and effective distances (r = 0.780; P = 0.0001) (Fig. 4.2). Genetic similarities were found among individuals belonging to demes separated up to 2.59 km for Euclidean distances and 3.84 km for effective distances (Table 4.3). However, it was possible to observe genetic differentiation between demes at 2.18 km of Euclidean distance and 2.53 km of effective distance (Table 4.3). Likewise regarding effective distances, demes with moderate genetic differentiation (F_{ST} = 0.10 - 0.15) could be observed from 3.90 to 32.34 km of distance, whereas demes with high genetic differentiation (F_{ST} = 0.15 - 0.25) were observed from 7.3 to 42.43 km of distance. Finally, a very high genetic differentiation ($F_{ST} > 0.25$) was observed at 17.95 of Euclidean distance and 32.32 km of effective distance between *A. scherman* demes (Table 4.3).

Table 4.3. Pairwise F_{ST} values (upper semimatrix) and pairwise Euclidean / effective distances (lower semimatrix, in km) for the studied *A. scherman* demes from Asturias. Significant F_{ST} values after correction for multiple comparisons (P < 0.001) and their correspondent geographic distances are indicated in bold.

	1	2	3	4	5	6	7	8	9	10
1- Vegadali		**0.116**	**0.112**	**0.156**	**0.166**	**0.203**	**0.271**	**0.187**	**0.211**	**0.253**
2- Ceceda	**4.21 / 6.63**		**0.067**	**0.113**	**0.178**	**0.236**	**0.262**	**0.189**	**0.210**	**0.250**
3- Fresnadiello	**4.94 / 9.07**	**1.87 / 2.60**		**0.102**	**0.146**	**0.186**	**0.198**	**0.145**	**0.164**	**0.189**
4- Poreño	**8.78 / 28.51**	**8.82 / 32.34**	**7.20 / 19.38**		**0.109**	**0.199**	**0.210**	**0.153**	**0.170**	**0.222**
5- Serida	**12.81 / 27.71**	**12.81 / 31.55**	**11.27 / 18.59**	**4.09 / 5.60**		**0.182**	0.166	**0.129**	**0.153**	**0.196**
6- Priesca	**17.04 / 38.39**	**15.50 / 42.23**	**13.74 / 29.27**	**8.66 / 16.29**	**6.70 / 12.01**		0.126	**0.119**	**0.104**	**0.136**
7- Rozada	**17.95 / 32.32**	**17.57 / 36.16**	**15.76 / 27.43**	**9.12 / 12.68**	5.25 / 7.30	5.19 / 14.34		0.084	0.049	0.055
8- Oles	**18.69 / 33.86**	**18.92 / 37.70**	**17.47 / 28.97**	**10.15 / 14.22**	**6.14 / 8.84**	**8.89 / 17.06**	3.83 / 5.84		0.067	**0.118**
9- Teleña	**19.58 / 34.26**	**19.54 / 38.09**	**17.88 / 29.37**	**10.89 / 14.62**	**6.80 / 9.23**	**7.62 / 17.45**	2.59 / 3.84	2.18 / 2.53		0.004
10- Marina	**20.48 / 35.63**	**20.29 / 39.47**	**18.63 / 30.75**	**11.70 / 15.99**	**7.64 / 10.61**	**7.57 / 18.83**	**2.95 / 5.21**	**3.39 / 3.90**	1.08 / 1.50	

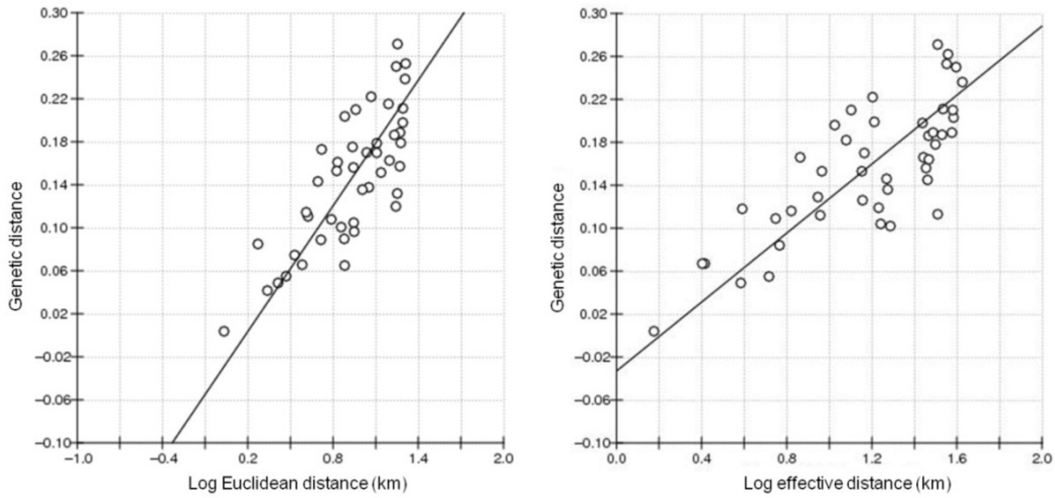


Fig. 4.2. Isolation-by-distance analyses for *A. scherman* demes from Asturias. Reduced major axis regression line of 12 microsatellite genotype data as estimated in IBDWS. The graphs show genetic distance (F_{ST}) versus logarithmic Euclidean distance (km) (r = 0.790; P = 0.0001; slope = 0.195; intercept = -0.036) or logarithmic effective distances (km) (r = 0.780; P = 0.0001; slope = 0.161; intercept = -0.033) for all possible pairwise combinations among the ten demes.

The Bayesian analysis implemented in the software GENELAND inferred the presence of four distinct genetic clusters (K = 4) (Fig. 4.3). The *blue* cluster was formed by demes *2* and *3*, located in the southeast of the study area. The *red* cluster*,* in the southwest, consisted only of specimens from deme *1*. Further north, the *orange* cluster was formed by demes *4* and *5*. Finally, *green* cluster consisted of demes *6*, *7*, *8*, *9* and *10*. All demes excepting deme *6* from *green* cluster were located on the left side of the estuary and the highway (Fig. 4.3). This last clustering harboured all demes located to the north of the study area. The genetic differentiation detected between pairs of clusters were all significant after correction for multiple comparisons (P < 0.0083): *a* and *b*: F_{ST} = 0.098; *a* and *c:* F_{ST} = 0.094; *a* and *d*: F_{ST} = 0.159; *b* and *c:* F_{ST} = 0.141; *c* and *d*: F_{ST} = 0.117.

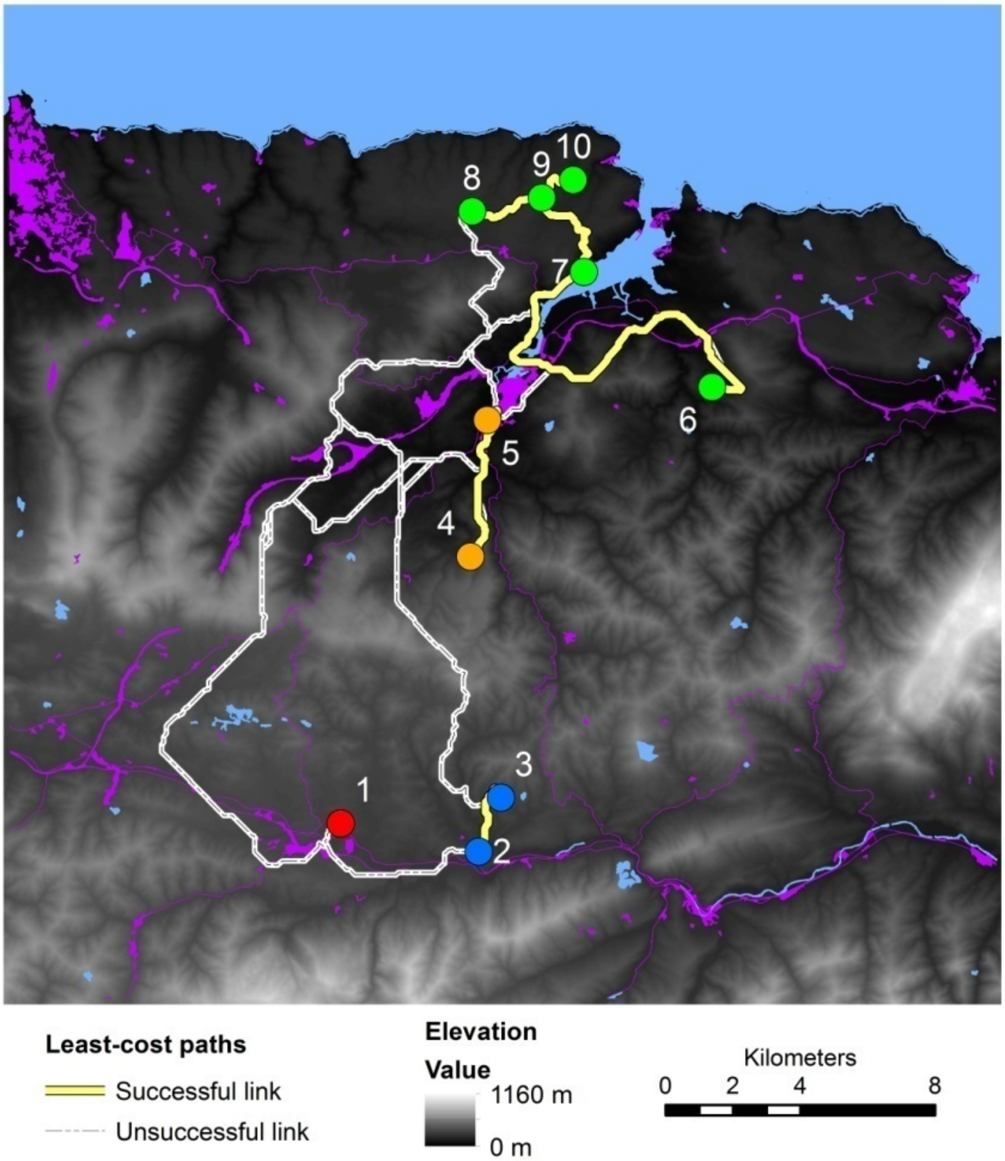


Fig. 4.3. Study area for the genetic structuring: *Arvicola scherman* demes were coloured by the estimated population kinship based on the mode of posterior probabilities (GENELAND): clusters *red*, *blue*, *orange* and *green* are shown. The main reliefs are shown throughout a digital elevation model, and builds and roads are represented in purple. Yellow lines indicate successful least-cost paths and white and black discontinuity lines indicate unsuccessful least-cost paths among demes. The sampled demes are indicated by numbers: 1, Vegadali; 2, Ceceda; 3, Fresnadiello; 4, Poreñoa; 5, Serida; 6, Priesca; 7, Rozada; 8, Oles; 9, Teleña; 10, Marina.

GENELAND results were not completely concordant with those obtained for the STRUCTURE analysis. This last software strongly suggested three genetic clusters (K = 3) (Fig. 4.4) while the estimated logarithm of likelihood for these data was low for other K values. This affected to the orange cluster which was clustered together with demes *2* and *3* to conform a unique genetic unit (Fig. 4.4). Moreover, the deme *5* seems to show an intermediate kinship between *green* and *blue* clusters regarding STRUCTURE results (Fig. 4.4). Anyway, STRUCTURE analysis was consistent with the rest of GENELAND results, in agreement with the establishment of the genetic clusters located at southwest (*red*) and at north (*green*) of the study area.

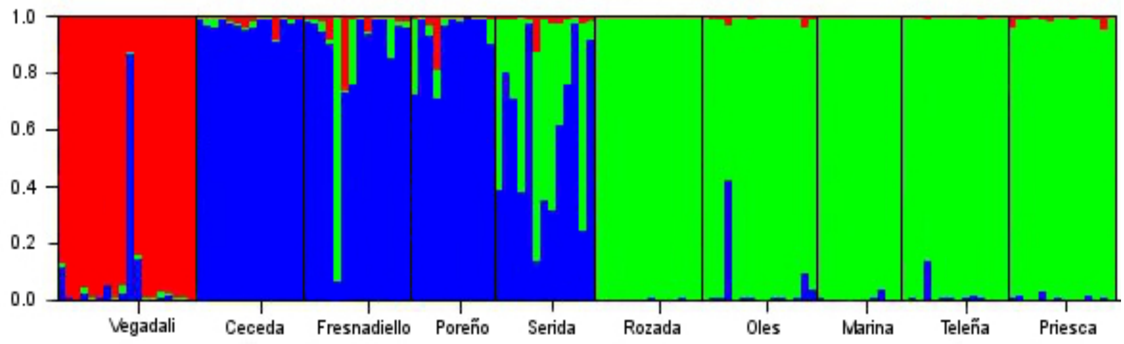


Fig. 4.4. Individual kinship coefficients inferred from Bayesian inference of genetic structure within STRUCTURE across ten demes of *A. scherman* from Asturias (northwestern Spain) for the most likely number of genetic groups (K = 3), which are defined by three colours. Black lines show assumed demes and each single vertical line represents an individual. Standard admixture model was used including sampling locations as prior information.

4.3.3 Landscape study

Along the studied landscape, the ratio of suitable habitat to the total land area (SH/TL) among deme pairs ranged between 53.4 to 8.0 %, with an average value (± SD) of 31.6 (± 10.0 %) (Table 4.4). A negative correlation between SH/TL and effective distances among demes was observed (r = -0.382; $p < 0.05$). However, there was no correlation between SH/TL and the resistance index values (*RI*) (r = 0.191; $P = 0.208$). Furthermore, in contrast to expectations, a significant correlation between the ratio SH/TL and the fixation index (F_{ST}) of *A. scherman* demes (r = 0.428; $P < 0.01$) was found.

According to the high patchiness of this landscape, the average (± SD) number of patches crossed in each least-cost path was 38.2 (± 18.8) and ranged between 6 to 68 patches regarding all paths. The number of these patches increased with the length of the effective distance ($r_s = 0.880$, $P < 0.001$), as expected. All but eight least-cost paths ran through suitable habitats at least the 70% of the total surface traversed, and none of the paths ran less than 50% by suitable habitats. The effective distances supposed an average (± SD) increase of 57.9 (± 14.1%) regarding Euclidean distances (Table 4.3).

Table 4.4. Resistance index values (*RI*) for each least-cost path between pairs of sampling plots (upper semimatrix) and values of SH/TL between pairs of sampling plots (lower semimatrix) in Asturias.

	1	2	3	4	5	6	7	8	9	10
1- Vegadali		29.78	55.19	51.65	51.63	97.86	147.12	153.04	151.53	153.07
2- Ceceda	45.18		20.83	56.04	66.58	100.17	150.29	159.26	151.47	156.99
3- Fresnadiello	37.75	53.41		70.16	82.03	130.59	161.71	170.02	172.63	167.56
4- Poreño	38.22	33.59	29.97		25.06	60.51	112.01	115.81	113.97	119.09
5- Serida	39.11	39.53	38.64	48.98		53.48	100.30	105.32	105.34	107.46
6- Priesca	35.80	36.59	36.19	33.16	36.14		153.30	132.14	156.17	159.73
7- Rozada	37.16	36.37	34.20	31.15	30.41	35.48		65.05	39.65	39.85
8- Oles	37.80	36.75	34.68	32.24	17.89	29.36	12.50		27.50	32.00
9- Teleña	37.80	36.52	33.94	30.88	21.75	27.56	8.67	11.39		6.67
10- Marina	37.28	35.89	34.16	28.67	22.91	26.73	8.04	10.20	20.49	

The *RI* value for each least-cost path was positively correlated with the length of the path (r = 0.632, $P < 0.001$) (Fig 4.5). Moreover, the *RI* values and the fixation index (F_{ST}) showed a significant correlation (r = 0.624, $P < 0.001$) for all pairs of demes. However, paths between plots belonged to *orange* and *blue* clusters showed lower *RI* value than expected given their effective distance (Fig 4.5) and their genetic isolation showed among their demes (Table 4.3). On the other hand, *RI* values observed between the deme *6* and several demes from *green* cluster (*7*, *9*, *10*) were higher than expected given their correspondently effective distances (Fig 4.5) and their relatively lower genetic differentiation (F_{ST}) (Table 4.3).

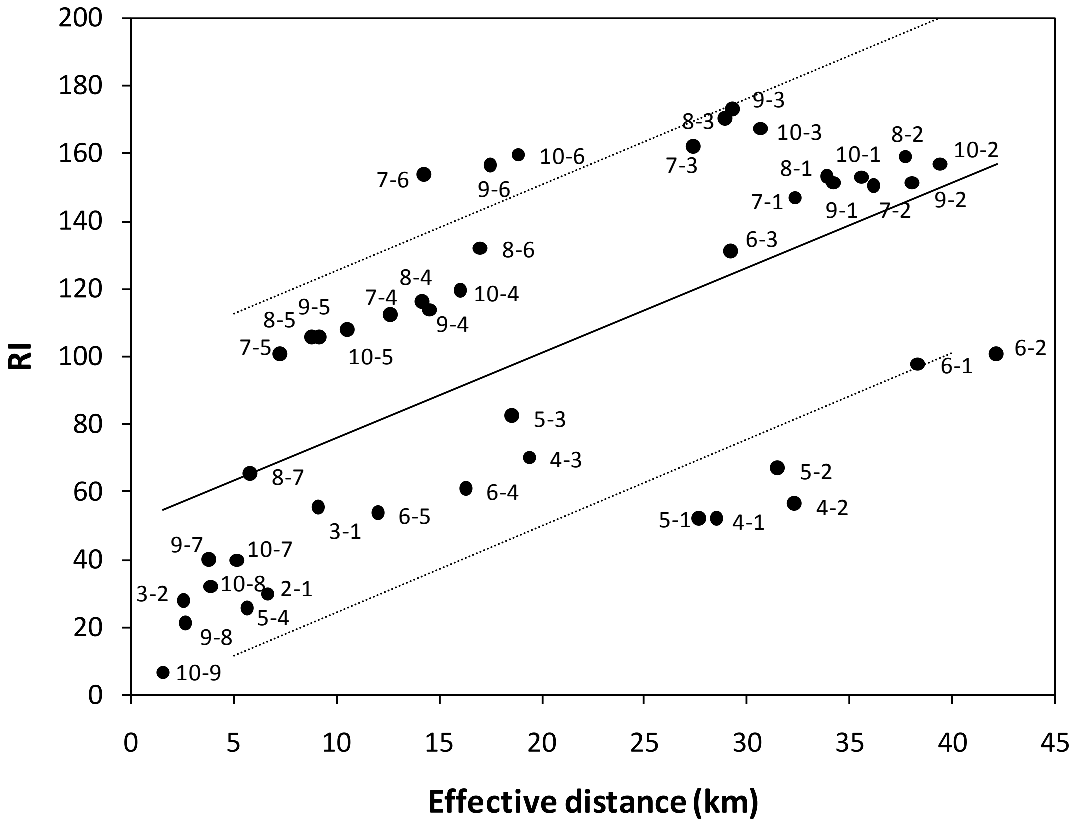


Fig. 4.5. Relation between effective distances (km) versus resistance index (*RI*) (r = 0.632, p < 0.001) for all possible pairwise combinations among ten *A. scherman* demes (black points) from Asturias (northwestern Spain): 1, Vegadali; 2, Ceceda; 3, Fresnadiello; 4, Poreño; 5, Serida; 6, Priesca; 7, Rozada; 8, Oles; 9, Teleña; 10, Marina. Continuous black line and both dashed black lines show the regression equation and the graphic representation of standard deviation respectively.

4.4 Discussion

General results revealed that the agricultural landscape of Asturias strongly influences *A. scherman* population genetics. These results provide genetic evidence of scarce communication among nearby demes, and hence a significant restriction on dispersal and colonisation of surrounding habitats. This fact confirms that a mosaic of different land-use plots decreases connectivity among suitable habitats even at local scale (120 km^2), and its maintenance over time would be advisable to obstruct *A. scherman* population diffusion.

4.4.1 Genetic diversity

The studied demes showed relatively low level of genetic diversity when considering H_E, H_O or A_R which probably highlights that effective movements of *A. scherman* are hampered enough to induce loss of genetic variability. So, departure from HWE was observed when all loci were considered together, which is consistent with a decrease in gene flow and hence genetic drift in this area. Both adults and juveniles can be detected at the same time along the whole year in these *A. scherman* demes driven by the continuous overlapping of generations (Somoano *et al*. 2017). This fact contributes also to deviate these loci from HWE, as formerly reported for northern water vole populations (*Arvicola amphibius*; Stewart *et al*. 1999; Aars *et al*. 2006; Melis *et al*. 2013). Conversely, this loss of diversity was not related with the absence of genetic signs of deviation from HWE for all but one of demes, as has been also occurred in other populations of this species (Berthier *et al*. 2005). Enough number of specimens could be able to avoid excessive loss of genetic diversity in *A. scherman* demes during low population densities (Berthier *et al*. 2005, 2006). The linkage disequilibrium, as occurred between two loci among these demes, has been formerly reported in northern water vole populations also featured by local structure and strong genetic drift, and could be due to genetically differentiated demes (Stewart *et al*. 1999).

The relatively lower number of first generation migrants detected and the positive inbreeding coefficient values (F_{IS}) observed in most demes suggest that immigration does not suppose an important source of new alleles. This highlights that population growth is more likely to take place by recruitment than by migration. A low-quality habitat could lead to a reduction of births, an increase of deaths (Krebs 1999), and an increase of the variance in reproductive fitness, which ultimately would trigger a loss of genetic variability (Aars *et al*. 2006). However, favourable environmental conditions permit high reproductive outcome of *A. scherman* in apple orchards (Somoano *et al*. 2016), which suggests that this loss of genetic variability is due to a restricted dispersal rather than the quality of the habitat. Since the level of observed heterozygosity in a given time is an indicator of its past and present demography (Hartl & Clark 1997; Gauffre *et al*. 2008), our results also suggest that these demes showed moderate/small effective population size (N_e) at that time. In this way, the surface of most apple orchards in Asturias rarely exceed of 3 ha (INDUROT 2010), that might lead to colonies of relatively small size, which in turn could send out less migrants, decreasing contribution to the connectivity (Hanski & Ovaskainen 2003). Additionally, connectivity among *A. scherman* demes can be reduced if these favourable patches are isolated (Foltête & Giraudoux 2012).

The level of genetic diversity observed in this study was considerably lower than the observed in previous microsatellite-based genetic studies conducted on *A. scherman* population located in open landscapes, characterized mainly by meadows and grasslands (Berthier *et al*. 2005) (mean values Asturias vs France: H_E = 0.621 vs. 0.824; H_O = 0.601 vs. 0.801; A_R = 4.42 vs. 8.34). Indeed, the genetic diversity observed in Asturias was even lower than that observed during a low density phase of *A. scherman* on a French homogeneous landscape (mean values: H_E = 0.799; H_O = 0.749; A_R = 7.94), in which the vole population was patchily structured (Berthier *et al*. 2006). Genetic studies conducted in populations of common voles, *Microtus arvalis*, located in heterogeneous but intensive agricultural landscapes, showed also higher level of observed heterozygosity (mean values: H_O = 0.770, northwestern Switzerland, Schweizer *et al*. 2007; H_O = 0.850, central-western France, Gauffre *et al*. 2008) than those here obtained for *A. scherman*. Although these previous agricultural systems are dominated by intensive annual crops which would prevent the establishment of *Microtus arvalis* (Jacob & Hempel 2003), those type of crops would not entail impassable barriers for this species (Gauffre *et al*. 2008), and the existence of green crops, fallows and field margins in intensive agriculture enhance *M. arvalis* colonisations (Jareño *et al*. 2015; Rodríguez-Pastor *et al*. 2016). Nevertheless, *M. arvalis* has a polygynous mating system in which breeding dispersal conducted by territorial males is a key aspect that can result in high genetic transfer between demes (González-Esteban & Villate 2007).

Continuous populations of northern water voles from UK and Finland also showed higher levels of genetic diversity (mean values: H_O = 0.762, A_R = 7.10, Aars *et al*. 2006) than those reported for Asturian voles. It is worth noting that water vole populations occupy water tributaries and their populations typically consist of small and discrete demes (Aars *et al*. 2006; Centeno-Cuadros *et al*. 2011). Furthermore, northern water voles have undergone a rapid decline over the last years in UK due to changes in habitat and predation by American mink (*Mustela vison*) (e.g. Rushton *et al*. 2000). Even so, subdivided populations of this species located in Scotland can retain similar (mean values: H_O = 0.661, A_R = 4.25, Steward *et al*. 1999) or higher levels of genetic diversity (mean values: H_O = 0.750, A_R = 8.93, Telfer *et al*. 2003; H_O = 0.749, A_R = 7.40, Aars *et al*. 2006) than Asturian *A. scherman* demes. Likewise, southwestern water vole, *Arvicola sapidus*, whose demes frequently experiment extinction-recolonisation events in southwestern Spain, also showed higher levels of genetic diversity than *A. scherman* in Asturias (mean values: H_O = 0.739, A_R = 5.11, Centeno-Cuadros *et al*. 2011). This fact might be due to juveniles of water voles can disperse long-distances (range 159 – 1,800 m) through using a "stepping-stone" process until settled down (Fisher *et al*. 2009; Centeno-Cuadros *et al*. 2011). On the contrary, northern water voles inhabiting on islands off the coast of Scotland and Norway showed lower genetic diversity (mean values: H_O = 0.410, A_R = 3.10, Telfer *et al*. 2003; H_E = 0.313, H_O = 0.471, A_R = 1.79, Melis *et al*. 2013) than those observed in our study, because of islands entail a strong disadvantageous scheme for water vole movements.

4.4.2 Spatial genetic structure

According to Sawyer *et al*. (2011), gene flow estimates are suitable to determine maximum effective distance under a given cost scheme. In that sense, maximum effective distance, and hence gene flow, occurred between demes located at 14.34 km in this study area. The F_{ST} values encountered here triggered the strongest pattern of isolation by distance to date for both Euclidean and effective distances in *A. scherman* populations. The IBD patterns reported for this species at large scale (5,000 km^2) in open grasslands from France showed considerably lower values for Euclidean distances (r = 0.570, slope: 0.013) and landscape distances (r = 0.510; slope: 0.010, similar to effective distances) (Berthier *et al*. 2005) than those here reported (Euclidean distance: r = 0.790, slope = 0.195; effective distances: r = 0.780, slope = 0.161). Accordingly, the overall F_{ST} value during a low density phase in *A. scherman* populations from France was also considerably lower (F_{ST} = 0.037, Berthier *et al*. 2006) than in our case (F_{ST} = 0.153). In this way, regarding *M. arvalis* in heterogeneous landscapes with intensive crops, the gene flow was scarcely restricted among populations separated by few km (2.5 km between sampling sites as maximum, mean value: F_{ST} = 0.028, Schweizer *et al*. 2007) or even by tens of km (500 km^2, F_{ST} = 0.002, Gauffre *et al*. 2008). Consequently, the IBD patterns of common voles on this type of agroecosystem were slightly significant (Gauffre *et al*. 2008) or absent (Schweizer *et al*. 2007). Moreover, studies on water vole populations in patchy habitats obtained lower (*A. sapidus*: F_{ST} = 0.072, Centeno-Cuadros *et al*. 2011) or similar levels of genetic differentiation (*A. amphibius*: F_{ST} = 0.140, Steward *et al*. 1999; F_{ST} = 0.170, Telfer *et al*. 2003; Aars *et al*. 2006) than in our case. Otherwise as expected, studies on insular northern water vole populations located showed higher genetic differentiation (F_{ST} = 0.370, Telfer *et al*. 2003; F_{ST} = 0.396, Melis *et al*. 2013) than that observed in the present study.

The spatial genetic structure showed by *A. scherman* in our study area arose as a result of population subdivision at local scale, in contrast to a homogeneous landscape, where gene disruption was mainly associated with population density at large scale and the genetic isolation is only significant during low density phases (Berthier *et al*. 2005). Nevertheless, both Bayesian methods differed in the number of genetic clusters that can be inferred. The software GENELAND uses spatial information concerning the origin of the samples in contrast to STRUCTURE (Gauffre *et al*. 2008; Guivier *et al*. 2011), and it is able to detect borders between inferred populations which coincides very well with putative barriers, while this information is not included in the algorithm itself (Coulon *et al*. 2006). Likewise, GENELAND is able to detect newly founded populations which have been modify by recent landscape changes even being far from drift-gene flow equilibrium (Coulon *et al*. 2006). Our results indicate that GENELAND approach can be suitable in our framework, identifying demographically independent populations and their spatial scale, as in other recent studies conducted on species located also in heterogeneous landscapes (Coulon *et al*. 2006; Gauffre *et al*. 2008; Basto *et al*. 2016). Thus, *A. scherman* demes were genetically structured in four clusters in an area of 120 km^2 in the main apple production zone of Asturias. However, STRUCTURE informed on a possible intermediate-genetic kinship of deme *5* (Serida) between cluster *green* (demes 6, 7, 8, 9, 10) and *orange* (demes 4, 5) that was corroborated by a not-significant F_{ST} value between demes *5* and *7* (Rozada). Several demographic control practices were likely conducted on deme *5* in the past, whereby, probably a mixture of descendants resulting from dispersal specimens belonging to nearby areas over time conform the current structure of this deme.

Previous studies reported that connectivity among suitable habitats can be considered as a key-factor in fossorial water vole diffusion (Foltête & Giraudoux 2012; Halliez *et al*. 2015; Foltête *et al*. 2016). The patchiness complexity of this landscape, and hence its low connectivity, was highlighted via the relationship between the ratio SH/TL and effective distances, resistance index (*RI*) or genetic differentiation (F_{ST}), which they turned out to be negatively correlated, not-correlated and positively correlated, respectively. Furthermore, the average number of different patches crossed in each least-cost path was relatively high in order to obtain minimum *RI* values. So, effective distances considerably lengthened regarding Euclidean ones. Therefore, these results showed that an increase of the surface of suitable habitats among *A. scherman* demes was not necessarily related with an increase of the gene flow, and it emphasized the importance of the degree of connectivity, as observed in other species located also in heterogeneous landscapes (e.g. Galpern *et al*. 2011; Royle *et al*. 2013; Garrido-Garduño *et al*. 2015).

Both the estuary and the four-lane highway might suppose a barrier between demes located on both sides as it was reported for fossorial water voles (Foltête & Giraudoux 2012) and also for other rodent species (Gerlach & Musolf 2000; Conrey & Mills 2001; Rico *et al*. 2007). However, effective distances and *RI* values could be overestimated as showed F_{ST} values. Estuary permeability could be higher when the tide is low (distance between shores: min. 100 m; max. 1.3 km), and roadside verges can act as habitats and corridors for rodent species (Redon *et al*. 2015), as corroborated several earth mounds of fossorial water voles observed in grassy margins (personal observations). This type of roads could not act as barrier for *A. scherman* as was observed in other vole species (Gauffre *et al*. 2008; Grilo *et al*. 2016). Moreover, intermediate genetic kinship between nearby demes of both sides of the small-urban area of Villaviciosa could show that this area may be low permeable but not impassable, being dependent on the connectivity among remnant patches (Baker *et al*. 2003).

The landscape approach conducted here was not able to explain genetic differences among some demes. Effective distances, *RI* values and the SH/TL ratio between demes of *red* and *blue* clusters did not show a reason of their genetic differentiation. The presence of small and isolated suitable habitats between both clusters could explain the lack of gene flow. This could occur because of least-cost modelling cannot fully incorporate quality, size or importance of individual source patches (Sawyer *et al*. 2011). Thus, further studies based on a circuit-theory taking multiple paths into account could be able to provide useful insight on matrix permeability (Foltête *et al*. 2016), and therefore, reveal underlying causes of gene flow disruptions. On the other hand, effective distances were larger than expected between demes of *orange* and *blue* clusters given their correspondently genetic differences, in order to avoid woody areas in uplands.

The population structure of *A. scherman* in this patchy landscape might resemble a patchy population, where the individuals of each population can have theoretically high levels of dispersal between demes and either no extinction by patch selection takes place (see Driscoll 2007). Indeed, Berthier *et al*. (2006) hypothesized that this kind of population structuring might be possible after a demographic crash of *A. scherman*, in which demes can suffer genetic drift as a consequence of the reduction of their population size and their genetic isolation. However, our findings suggest that stochastic processes of patch occupancy (Hanski & Ovaskainen 2003), together with low permeability of the matrix (Ricketts 2001; Prevedello & Vieira 2010), can cause this population structuring in Asturian *A. scherman* demes. The overall ratio of suitable habitat to the total land area (SH/TL) here observed could be associated with a medium ROMPA value, which is related with lengthy low density phases in agreement with slight fluctuations and sometimes high density populations (Giraudoux *et al*. 1997; Fichet-Calvet *et al*. 2000). Moreover, a lack of gene flow prompts independent demographic populations, whose fluctuations in abundance could occur asynchronously (see Berthier *et al*. 2013). Therefore, population outbreaks of *A. scherman* could occur at different time among nearby areas (see Saucy 1988; Giraudoux *et al*. 1997; Fichet-Calvet *et al*. 2000).

4.4.3. Management implications

To date, population control of fossorial water voles in Asturias has so far mainly been achieved without coordinate actions and according to the perception of risk damage in each plot. These uncoordinated control actions could involve potential colonisation movements of juveniles from source demes (without or scarce control) to nearby plots. Regarding the results of the present study, each genetic cluster could be considered as a management unit, where their individuals are considered a genetically homogeneous group (see Laikre *et al*. 2005; Piertney *et al*. 2016) and which depends mainly on local birth and death rates (see Palsbøll *et al*. 2006). Thus, the identification of four local management units in our study area encourages the establishment of coordinated actions, with independent management regime in each one (Palsbøll *et al*. 2007). These clusters occupied only few tenths of km^2 in this area, unlike what occurs in homogeneous landscapes where *A. scherman* is also considered a pest (Berthier *et al*. 2005, 2013). Likewise, our results entail an advantage for facing population control strategies of this species in this area, given that reasonable efforts are needed to carry out population management practices in independent populations units (Piertney *et al*. 2016). Moreover, it is quite likely that, if density cycles of *A. scherman* occur in this area, these would take place over a management-unit scale rather than at regional scale as occur in France (Giraudoux *et al*. 1997; Fichet-Calvet *et al*. 2000). Monitoring demographically these genetic clusters over time might be useful (Schwartz *et al*. 2007) to advise farmers about the possible risk of vole outbreaks. Thus, looking for fresh signs of *A. scherman* activity would be necessary to estimate vole abundance and to update perception of risk (Giraudoux *et al*. 1995; Miñarro *et al*. 2012) in each management unit. Ultimately, control strategies for this species at a regional scale can be discarded, which in fact would be logistically impracticable and probably less effective than a smaller scale approach.

Chapter 5. General discussion

5.1 Discussion

The main aim of this research was to obtain detailed information on the biology and population genetics of *A. scherman* in an agricultural area of Asturias. This goal has been achieved through the analyses of more than 800 individuals of *A. scherman cantabriae* gathered from apple orchards during two annual cycles, to obtain a structured sample.

Detailed information obtained from reproductive organs of both sexes together with the assignment of relative age classes allowed us to determine the reproductive cycle of this subspecies in suitable habitats in a temperate-hyperoceanic climate zone. This approach showed us the continuous presence of young specimens and sexually active individuals of both sexes the year around, which highlighted that *A. scherman* can breed continuously in apple orchards located at relatively low altitude in northern Asturias. Thus, primary demands of this species are properly fulfilled and hence energy budgets can be focused to cope reproduction throughout the whole year in this environment (see Merritt *et al*. 2001; Solonen 2006; Trebatická *et al*. 2012). This reproductive pattern could be considered uncommon among rodents inhabiting temperate areas (Kerbeshian *et al*. 1994, Nelson *et al*. 1998, Gottreich *et al*. 2000). Indeed, no other fossorial water vole population has been able to breed regularly during winter (Meylan & Airoldi 1975; Morel 1981; Pascal 1981; Ventura *et al*. 1991). Habitats influenced by a more continental climate, such as meadows from Pyrenees where *A. scherman* populations occur, suffer marked seasonally changes. The winter in those environments is usually cold with high probabilities of frost and snow. Therefore, energy reserves of fossorial water voles inhabiting those environments probably are intended for food obtaining to maintain homeostasis instead to cope reproduction (Solonen 2006).

Mature males of these Cantabrian populations did not show an important spermatogenesis decrease in any month of the year. Indeed, intra-annual fluctuation of sexual organs of these males did not affect significantly reproduction at a population scale. The food availability and thermoregulation of these voles are not significantly compromised in winter. So, a day-length decrease would not mark the end of favourable environmental conditions in this area, and hence the reproductive activity of *A.*

scherman cantabriae seems to be influenced only slightly by photoperiod signals (see Maeda *et al*. 1997; Medger *et al*. 2012).

Mature females of *A. scherman cantabriae* are able to ovulate continuously in this area, producing in average 7.3 litters per year. This fact implies a short resting period between breedings, given the time lasting of gestation (about 21 days, Quéré 2009) and weaning (around three weeks since birth, Ventura & Gosálbez 1992). Since the length of the breeding season does not suppose a critical factor in determining litter size in this environment, a lower number of embryos per female in *A. scherman cantabriae* (average value: 3.76 embryos per female) in comparison with *A. scherman monticola* (average value: 4.48 embryos per female; Ventura & Gosálbez 1990c) could allow Asturian populations to increase the life span of their offspring and hence obtaining high reproductive success (see Mappes *et al*. 1995). Therefore, populations of *A. scherman* can show different reproductive potential according to the habitat in the Iberian Peninsula, which ultimately determines their population growth by recruitment. In this way, the potential number of offspring per female and year was significantly higher in our populations (28.25) than in the Pyrenean one (23.5; Ventura & Gosálbez 1990c).

The average litter size of *A. scherman cantabriae* is expected to entail a good reproductive outcome, if not the optimal, for this environment (see Mappes *et al*. 1995). Nevertheless, the relationship between the mass and body length (body condition) of the mother seems to be a key factor regarding the variation of the reproductive potential in *A. scherman cantabriae*. Thus, a good body condition in pregnant females indicates energy provision and would lead to higher reproductive outcome in Cantabrian fossorial water voles.

Through the genomic DNA extraction of 137 specimens and their subsequent microsatellite-based analyses, we were able to assess the genetic diversity and also to accurately define the gene-flow pattern among ten demes of fossorial water voles located in this patchy agricultural area of Asturias. Since apple orchards at low altitude allow *A. scherman cantabriae* to have high reproductive outcome, a loss of genetic variability is expected to be due to a restricted dispersal rather than the quality of the habitat (Krebs 1999; Aars *et al*. 2006). Results obtained in this research revealed the effect of this agricultural landscape on *A. scherman* populations diffusion.

The genetic diversity found in *A. scherman cantabriae* was relatively low probably as a result of the genetic drift and the inbreeding effect. This level of genetic variability was considerably lower than that obtained in populations of *A. scherman* located in homogeneous landscapes that allow connectivity among favourable habitats and hence effective movements (Berthier *et al*. 2005, 2006, 2013). Moreover, the diversity loss observed in *A. scherman* demes located in the Asturian patchy landscape was considerably greater than that reported in *M. arvalis* populations located in heterogeneous landscapes conformed mainly by annual crops (Schweizer *et al*. 2007; Gauffre *et al*. 2008). In that sense, the combined effect of a mosaic of small and different land-use plots together with hedgerows and woodlot borders entails an unsuitable framework for the genetic exchange among fossorial water vole demes. That is, *A. scherman* specimens are not able to successfully achieve dispersion and/or posterior reproduction in colonised habitats in this agricultural area, which ultimately reflects a reduction in the gene flow. We can conclude that population growth is more likely to occur by recruitment in these *A. scherman* demes, while immigration would not suppose an important source of new specimens. Furthermore, the relatively small size of these suitable habitats (INDUROT 2010) probably allows just a moderate/small deme size of fossorial water voles, and hence this may reduce even more the contribution to the connectivity among demes due to the shortage of dispersal specimens (see Hanski & Ovaskainen 2003).

Genetic differences among these *A. scherman* demes ultimately triggered the strongest pattern of isolation by distance observed to date in this species (Berthier *et al*. 2005). These demes were genetically structured in three or four populations -depending on the analysis- in an agricultural area of 120 km^2. This genetic structuring at local scale considerably differs from that observed in homogeneous landscape in France, where genetic isolation is only significant during low density phases and at a larger scale (Berthier *et al*. 2005, 2006). An increase of suitable habitats in this studied area was not directly related with an increase of the gene flow among these *A. scherman* demes. Thus, the landscape complexity and its connectivity among suitable habitats strongly determine the diffusion of this species in this agroecosystem. Stochastic processes of patch occupancy (Hanski & Ovaskainen 2003) together with low permeability to movement of the matrix (Ricketts 2001; Prevedello & Vieira 2010) could determine the population structure of *A. scherman* in the Asturian landscape. Moreover our results indicate that both the estuary and the four-lane highway located in the north of the study area, do not suppose barriers to gene flow for *A. scherman*. In this way, the low tide would facilitate the estuary crossing and roadside verges can even be used by this species as habitats and corridors (Redon *et al*. 2014).

Despite no population density studies were conducted over time on *A. scherman cantabriae* in this area, the ratio of suitable habitats to the total land area in this landscape would lead to lengthy low-density phases, slight fluctuations and sometimes high density populations of fossorial water voles (see Giraudoux *et al*. 1997; Fichet-Calvet *et al*. 2000). These fluctuation in abundance could occur asynchronously in each one of these independent demographic populations (see Palsbøll *et al*. 2007), at a scale of tens of km^2. Moreover, the climate of this agricultural area entails a less seasonal environment, which in turn could drive more stable population dynamics (Tkadlec & Zejda 1998). So, the opportunistic exploitation of resources in relatively mild winters by *A. scherman cantabriae* could entail that these population outbreaks occur with unclear periodicity (Blanco 1998 and references therein). Otherwise, multiannual fluctuations of density in populations structured at large scale in seasonal environments are well established every 5 to 9 years (Saucy 1988; Giraudoux *et al*. 1997; Fichet-Calvet *et al*. 2000). Multiannual cycles in fossorial water voles could follow a gradient according to oceanic influence, which their occurrence might increase from less- to more-seasonal environments in Europe, as it has been reported in *M. arvalis* (Jacob 2014 and references therein).

5.2 Demographic control measures of *A. scherman* in Asturias

The strategy to control populations of a rodent pest should be designed for each specific situation because of its pest status depends on its own biology and ecology and on its interaction with the agroecosystem (Leirs 2003). The subspecies *A. scherman cantabriae* shows the highest population growth by recruitment found for fossorial water voles to date, highlighting that the potential crop-damage caused by this species could be greater in Asturias than in other agricultural areas, as the Massif Central (France), the Jura Mountains (France and Switzerland) or in the Pyrenees (Spain). Thus, encouraged by the Ministry of Agriculture and Fisheries, Food and Environment (Government of Spain) (BOE 2008), we propose reasonable and sustainable control methods focused to control *A. scherman* populations in attempt to establish a suitable management strategy of this pest in Asturias.

Some successful control methods has encouraged land managers to apply them elsewhere regardless the specific conditions where they were used. In that sense, the most used approach to manage vole populations have relied on direct reduction of densities using rodenticide baits (O'Brien 1994 and references therein; Default *et al.* 2009; Jacob 2014). Obviously, the continuous use of rodenticide baits to control this continuous-breeding vole supposes the input of high amounts of poison in the agroecosystem, which would imply higher exposure to non-target animals (Coeurdassier *et al.* 2014; Montaz *et al.* 2014) and might generate resistance against rodenticides in fossorial water voles (Ishizuka *et al.* 2008; Vein *et al.* 2011). The European legislation is increasingly restrictive in the use of rodenticides baits, so, only sustainable strategies are nowadays encouraged for a continuous population control of voles along time (Regulation 528/2012/UE 2012).

Since apple orchards can be considered an adequate framework for trapping given their relatively small size in Asturias (see Salmon & Gorenze 2002), the use of snap traps can be effective once trapper is skilled (Fuelling *et al.* 2010; Defaut *et al.* 2009). Traps are recommended to be maintained until the removal of all specimens from the burrow. Nevertheless, these practices might be also useful even it has not been removed all of them. The physiological stress induced to remnant females through a continuous trapping increases indirectly effectiveness of trapping. This fact could suppose an incentive while trapper is not skilled enough and/or over large crop plots, where traps could be labour-intensive (O'Brien 1994). Anyway, an appropriate identification of surface signs of activity of *A. scherman* is essential for avoiding undesirable trapping of the mole *T. occidentalis* (Miñarro *et al.* 2012).

Suitable practices, such as trapping or habitat manipulation -intensive grazing, cattle trampling, ploughing or herbicide application- should be coordinated among land managers to reduce potential colonization movements of juveniles from source demes (without or scarce control) to nearby sink plots (with continuous removal of voles). Non-ploughed fields are especially vulnerable because intact burrow systems remain and a protective grass coverage can be established (Witmer *et al.* 2009). Given that the land has been excessively parcelled in this region, coordinated actions to control fossorial water voles could imply collaboration of many land owners and hence it might entail organizational troubles. Nevertheless, coordinated actions could be established in each independent population (or management unit; Piertney *et al*. 2016) which occupies only few tenths of km^2 in this area.

The relatively small surface of most of apple orchards in Asturias (INDUROT 2010) and their high economic value might entail an acceptable framework to set up barriers surrounding plots placed both above- and below-ground together with self-service traps to avoid colonization by fossorial water voles (see Fuelling *et al*. 2010; Walther & Fuelling 2010). However, it could be not always practical to enclose a whole orchard, so, vole-trapping-fences might be used to draw protective lines between several crop plots and identified vole demes (Fuelling *et al*. 2010). Land managers should maintain these fences permanently as dispersal specimens can colonize new habitats at any time of the year. Nevertheless, it would be advisable to test the suitability of these barriers in Asturian apple orchards, and also economically quantify their installation and maintenance.

Secondary metabolites of some rootstocks could reduce apple tree attractiveness by fossorial water voles (Pelz 2003). A study conducted in the SERIDA showed that the rootstocks MM.106, PI.80 and M.7 suffered more damages than MM.111 and Franco rootstocks, which seemed to be less palatable (Fernández-Ceballos & Dapena 2007). Although there is not yet an in-depth study based on the metabolite compound of each rootstock, planting apple trees with MM.111 or Franco rootstocks in threatened plots might be advisable since they are less resilient to damage caused by *A. scherman*. Moreover, the root structure could be also an important characteristic to consider, as larger root dimensions entail more resilience. In that sense, vigorous rootstocks, as Franco which has tap root, could be recommended in apple orchards where fossorial water voles are present.

Increasing the presence of avian and terrestrial predators in apple orchards, such as stoats, weasels, foxes, barn owls, tawny owls or buzzards, might help to reduce *A. scherman* densities. Such predation impact on this species would depend on its population size and predator characteristics (Giraudoux *et al*. 1997). Specialized predators, such as mustelids, feed on *A. scherman* beyond the declining of its densities, destabilizing their populations; whereas unspecialized predators, such as fox and barn owl, can switch to alternative preys when *A. scherman* densities decline, stabilizing vole populations (Giraudoux *et al*. 1997). Thus, this agricultural land-use structure should be maintained and promoted in Asturias in order to favour the presence of both type of predators. Additionally, nest boxes and artificial perches to increase the use of cropped areas by birds of prey have proved to be an environmentally friendly and cheap control technique to reduce *M. arvalis* densities at larger scale in Mediterranean agricultural landscapes in Spain (Paz *et al*. 2013). The use of hay bales or other types of protective cover can be also recommended to increase de presence in orchards by mammalian predators (Witmer *et al*. 2009 and references therein).

Other population control strategies have been scarcely tested at large scale or could suppose unsuitable methods given their way of acting. Fertility control can be conducted throughout using steroids, agonists and antagonists of gonadotropin-releasing hormone, immunocontraceptive vaccines, natural plant extracts and chemicals (Tran & Hinds 2013, and references therein). However, species with continuous breeding might poses technical difficulties and important economical investment, and also non-target species can be affected by these compounds (Witmer *et al*. 2009). Some odour substances can diffuse widely within the burrow system of fossorial water voles being able to induce aversion or stress response, such the case of plant extracts as black pepper oil, Chinese geranium oil and onion (Fischer *et al*. 2013). Moreover, the odour of predators, such as mustelids, triggers a fear response in water voles, as in other arvicoline rodents as an innate anti-predation behaviour (Barreto & MacDonald 1999). The identification and use of effective repellents could help to reduce damage to crops, however, the challenge remains to translate this efficacy to a field application (Witmer *et al*. 2009).

5.3 Further research

The information obtained on the reproductive and ecological characteristics of *A. scherman cantabriae* in this agroecosystem entailed new issues which should be studied in depth. For example, the identification of other agricultural areas where *A. scherman cantabriae* is present could allow to be alert on potential damages to crops and update the pest status of this species at regional scale. So, what is the current distribution of *A. scherman* in Asturias? This subject may be approached through a wide field study focused on the identification of surface indices and/or direct captures of this species, or at least by a study based on an estimation model to obtain its potential distribution from presence data (Phillips & Dudík 2008; Romero-Suances 2015). It would be advisable to assess the environmental and landscape features and historical reasons which determine the distribution of fossorial water voles in this region. This ecological information might be also useful to forecast *A. scherman* colonisation processes of susceptible agricultural areas in Asturias.

Population control strategies should be focus on keeping *A. scherman* populations at low-densities, although it would be important to identify population growth phases to intensify, at that time, control actions. Thus, does *A. scherman cantabriae* show multiannual fluctuations of density? if so, how often outbreaks take place? The monitoring of this pest species is of utmost importance (see O'Brien 1994 and references therein), in which land managers should update their perception of risk via fossorial water vole abundance (Delattre & Giraudoux 2009; Miñarro *et al*. 2012). A participatory research may be advisable in which land managers send regularly population density data to research centres (SERIDA, Plant Health Service of Asturian Government) and collaborating companies for a global analysis. Thus, a research group can be set up with the aim to advise farmers about the risk of vole outbreaks, in the same way as the warning system FREDON acts in France (Delattre & Giraudoux 2009). Fossorial water vole abundance in apple orchards can be assessed through the account of activity signs in a 100-m transect, in which every 10 m the collaborator stops and looks for earth mounds in a 2-m strip between tree-rows (see Miñarro *et al*. 2012). Likewise, the same method can be used to assess also the abundance of the other vole pest, *M. lusitanicus* (Miñarro *et al*. 2017), but in this case through the presence of small burrow openings in the soil (Miñarro *et al*. 2012). Moreover, although we can hypothesize that these demographic cycles in *A. scherman cantabriae* could be independent in each population, the gathering of field data would be strongly recommended.

The demographic monitoring maintained over time would provide essential information about what kind of demographic cycle show *A. scherman cantabriae* in this landscape (see Giraudoux *et al*. 1997; Fichet-Calvet *et al*. 2000) and whether outbreaks occur with synchrony among different populations. Nevertheless, which are the factors that influence the amplitude of these multiannual fluctuation of density? On one hand, the abundance of voles in each year can be dependent on the current annual growth rate (first-order cycles), but can be influenced also by the density of the former year (second-order cycles) (Barraquand *et al*. 2014), in which population density and population growth rate show a negative feedback (e.g Reed & Slade 2008). On the other hand, several extrinsic factors, such as food availability and quality (Krebs 1999), pathogens (Cavanagh *et al*. 2004) and parasites (Cerqueira *et al*. 2007) can affect their mortality and fitness. Since both intrinsic and extrinsic factors could be involve in fossorial water vole density cycles, a necropsy study of specimens gathered along monitoring time should be conducted for this aim.

Sustainable practices maintaining *A. scherman* populations under physiological stress can be suitable practices to reduce the subsequent damage to crops (Charbonnel *et al*. 2008). However, which of them cause a significative stressful effect and which are their physiological consequences? The effectiveness of these practices can be accurately tested through the analyses of the corticosteroid concentration in blood or faeces, which highlights the physiological status of *A. scherman cantabriae* (Harper & Austad 2000; Ylönen *et al*. 2006). It would be recommendable to achieve a chronic-stress situation in which fossorial water voles undergo the suppression of reproductive and immune functions (Harper & Austad 2000). Since it was obtained important information on *A. scherman cantabriae* reproduction, direct consequences of control strategies can be hereinafter assessed on the reproductive biology of this species instead on just its relative abundance as usual (e.g. Morilhat *et al*. 2007, Jacob 2008, Delattre & Giraudoux 2009).

Moreover, the maintenance along time of each *A. scherman cantabriae* deme could depend on the land-use of surrounding plots and their characteristics. So, what is the influence of this agricultural landscape on fossorial water vole demes at fine scale? A combined approach of necropsies and a microsatellite-based analysis conducted along the time together with a landscape study at small scale could reveal the evolution of population structure and genetic diversity of each deme according to the proportion of natural surroundings and potential barriers. Thus, a deme whose habitat is surrounded by well structured hedgerows and/or nearby woodlands could be strongly dependent on its own population growth by recruitment and this could determine its population dynamics.

A negative relation between *A. scherman cantabriae* and *M. lusitanicus* densities has been observed in apple orchards (Miñarro *et al*. 2012), in which both species presumably compete for resources. So, what is the habitat use pattern of each species? A competitive displacement could entail a spatial segregation, in favour of fossorial water voles because of the body size (Miñarro *et al*. 2012). This habitat segregation facilitates coexistence between these competitive species, although other facts can also trigger this segregation (Pita *et al*. 2010). Moreover, their habitat use could occur a fine scale and showing seasonal preferences (Pita *et al*. 2011). The influence of habitat-manipulation practices may also influence this selection of habitat, and after population control periods, a possible alternative use of empty niches by these species could take place. Thus, a habitat selection study of both species taking into account a multiple spatial scale and recording population densities might supply information of habitat preferences and species interaction.

The social organization of *A. scherman cantabriae* was briefly assessed in this study through the assignment of each captured specimen to a specific burrow. Going in depth on this issue and on soil occupancy by this species could be advisable through a capture-mark-recapture method, especially in arvicoline species which can show different mating system (Soriguer 1986; Waterman 2007; Pérez-Aranda 2009). The use of passive integrated transponder (PIT) tags injected subcutaneously between shoulder blades allows an assessment of these subjects in a suitable manner (Harper & Batzli 1996). This technique can also permit an accurate monitoring over time of familiar groups by the assessment of growth rates and survivorship of each member. Moreover, since effective movements of *A. scherman cantabriae* seem to be severally hampered in this landscape, the radiotracking of dispersal individuals could yield empirical information and dispersal movements at fine or even at large scale, and the role of predators in this agroecosystem (Saucy 1994).

Chapter 6. Conclusions

1. Favourable environmental conditions of the main area of apple production in Asturias allow *A. scherman cantabriae* to breed continuously along the whole year. Consequently, the presence of young specimens and sexually active individuals of both sexes in all months highlight that this environment supplies all those biotic and abiotic requirements by this species to achieve this continuous reproductive pattern.

2. A continuous reproduction allows *A. scherman cantabriae* to produce a high number of litters per year, although with a relatively moderate litter size. This fact could allow this species to obtain high reproductive success as the breeding season is not restricted and hence does no suppose a critical factor.

3. The variation of the reproductive potential in *Arvicola scherman cantabriae* manly depends on body condition of the mother and thus on its physiological state.

4. The potential number of embryos per female and year was substantially higher in *A. scherman cantabriae* than in the Pyrenean subspecies *A. scherman monticola*. This is probably due to differences in the length of the breeding season and in life histories. Both subspecies reach sexual maturity at the same relative age class and monogamy seems to be the main mating system, which suggest an independency of both processes regarding environmental conditions.

5. The patchy landscape of the main area of apple production in Asturias, conformed by a mosaic of small and different land-use plots together with hedgerows and woodlot borders, entails an unsuitable scheme for *A. scherman cantabriae* to successfully achieve dispersion and/or posterior reproduction in colonised habitats. Thus, the genetic exchange among demes of this species is hampered and ultimately leads to a reduction in the gene flow.

6. The genetic diversity of *A. scherman cantabriae* encountered in this patchy landscape is relatively low, probably because the scarce arrival of new alleles do not hinder the loss of genetic variability due to inbreeding and genetic drift. This level of genetic variability is considerably lower than that obtained in the same species located in homogeneous agricultural landscapes conformed mainly by suitable habitats. Relatively low number of migrants and positive inbreeding coefficients suggest that population growth is more likely to take place by recruitment than by migration.

7. Genetic differences among nearby demes trigger a strong pattern of isolation by distance, which in fact, is the strongest one reported to date for this species and for populations of water voles located in mainland. In this way, genetic clustering revealed population subdivision on this species at local scale.

8. Population control strategies for *A. scherman cantabriae* are advisable to be maintained along the whole year. Coordinated practices at a regional scale can be discarded, which in fact would be logistically impracticable and probably less effective than a smaller scale approach. The monitoring of each management unit will be essential to know the population dynamic and to establish coordinated population control strategies. Preserving and promoting this patchy landscape would favour the presence of predators and hamper dispersal of this species.

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Appendix: Resumen

Introducción

El género *Arvicola* Lacépède 1799 es un grupo de roedores arvicolinos con distribución palaeártica que muestra una plasticidad fenotípica y ecológica notable. La especie *Arvicola terrestris*, es muy polimórfica y muestra dos formas ecológicas: una forma semiacuática, que habita áreas húmedas; y una forma excavadora, que habita praderas donde construye madrigueras subterráneas. Dado que ambas formas ecológicas se diferencian también morfológicamente, Musser y Carleton (Musser GG, Carleton MC (2005) *Arvicola* Lacépède, 1799; *Arvicola amphibius* (Linnaeus, 1758); Arvicola scherman (Shaw, 1801). In: Mammal Species of the World. A Taxonomic and Geographic Reference, 3rd ed (eds Wilson DE, Reeder DM), pp. 963-966. Johns Hopkins University Press, Baltimore, USA) reconocen dos especies en su aproximación taxonómica. De esta manera, los ejemplares semiacuáticos se asignan a la especie *A. amphibius* (rata de agua norteña), mientras que los ejemplares subterráneos se asignan a *A. scherman* (rata topera). Recientes análisis filogenéticos no apoyan este patrón taxonómico, y sugieren que ambos morfotipos son los extremos de un fenotipo continuo en lugar de dos morfotipos discretos. No obstante, dado que este problema taxonómico aún no está totalmente resuelto, en este trabajo se ha optado por seguir el patrón taxonómico planteado por Musser & Carleton (2005).

La rata topera está presente en los principales macizos montañosos de Europa, incluyendo el norte de España (Cordillera Cantábrica y Pirineos), el norte de Portugal, los Alpes, las montañas de Europa central y los Cárpatos. En la península ibérica se reconocen dos subespecies: *A. scherman monticola*, localizada en los Pirineos, y *A. scherman cantabriae*, distribuida por la cordillera cantábrica. Esta última subespecie presenta menor tamaño y dimensiones craneales que la primera, que podrían reflejar cierta divergencia genética entre ambos taxones.

La actividad reproductiva de las poblaciones *A. scherman* estudiadas hasta la fecha decae significativamente en invierno, debido a las condiciones ambientales desfavorables de esta estación. Aun así, esta especie es capaz de gestar seis camadas al año, cada una compuesta por entre 2 y 9 embriones. La dispersión y la colonización son procesos esenciales en esta especie. Dicha dispersión es efectuada en superficie mayoritariamente por ejemplares juveniles, y generalmente tiene lugar durante las noches de lluvia con el objetivo de evitar la depredación y facilitar el establecimiento en suelos blandos y húmedos. No obstante, las características del paisaje pueden influir significativamente en estos movimientos. Los paisajes heterogéneos pueden dificultar la colonización por *A. scherman*, directamente al frenar su dispersión e indirectamente promoviendo la depredación. Por el contrario, una alta conectividad entre hábitats favorece que esta especie experimente fluctuaciones plurianuales de densidad bien marcadas, como las observadas a escala regional en Francia y Suiza.

La rata topera consume preferiblemente raíces, bulbos y tubérculos de dicotiledóneas y algunas poáceas. Debido a su elevada demanda energética y a su presencia en praderas, huertos y plantaciones de frutales, *A. scherman* causa importantes pérdidas económicas en países como Alemania, Francia, Suiza y España. Las densidades poblaciones de esta especie han sido históricamente controladas mediante la utilización de rodenticidas. Sin embargo, esta práctica conlleva serios riesgos medioambientales, por lo que hoy en día su uso está restringido considerablemente. En España, el Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente (MAPAMA) obliga a los agricultores afectados por *A. scherman* a controlar sus densidades poblacionales mediante el uso de prácticas fitosanitarias sostenibles, como son la implementación de medidas culturales, el control de áreas de refugio, la promoción de enemigos naturales o la captura sistemática.

En Asturias (noroeste de España), la subespecie *A. scherman cantabriae* habita principalmente prados, pastos y cultivos desde el nivel del mar hasta las montañas. Se han constatado daños producidos por esta especie en sus hábitats preferentes desde 1970 en esta región. Sin embargo, en los últimos años se han observado varias explosiones demográficas. Este hecho podría relacionarse con un aumento general de prados permanentes. Las plantaciones de manzano son uno de los cultivos más afectados por esta especie en Asturias. La rata topera se alimenta de las raíces y de la corteza de estos árboles, convirtiéndose en uno de los principales factores limitantes en este cultivo y, por lo tanto, en la causa de importantes pérdidas económicas. De manera tradicional, la manzana se cultiva en extensas plantaciones con en árboles de gran vigor que permiten un aprovechamiento mixto con ganado. Sin embargo, estas plantaciones se han sustituido progresivamente por plantaciones semi-extensivas con árboles menos vigorosos, cuyas raíces son más sensibles a los daños causados por la rata topera. La mayoría de las parcelas destinadas a al manzano se encuentran en el centro-oriente de la región, cuyo paisaje se caracteriza por una orografía irregular no muy acentuada y por un mosaico de pequeñas parcelas agrícolas destinadas a diferentes usos separadas por setos y arboles. El clima es templado hiperoceánico con precipitaciones frecuentes y temperaturas benignas todo el año, que permite el establecimiento de una cobertura vegetal densa durante todo el año.

En la actualidad, el control de la población de *A. scherman cantabriae* en Asturias se afronta mediante la utilización de rodenticidas o trampas de golpe en las entradas de las madrigueras, sin acciones coordinadas entre agricultores y según su percepción de riesgo. El Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA) de Asturias ha llevado a cabo algunos estudios con el fin de mejorar el escaso conocimiento de la rata topera en este agroecosistema, y así sentar las bases para su control poblacional. No obstante, para mejorar la implementación y el éxito de posibles métodos de control sostenibles es necesario un profundo conocimiento de aspectos relacionados con la biología y la ecología de *A. scherman cantabriae* en el área agrícola del centro-oriente asturiano.

Planteamiento y objetivos

Las características reproductivas de la rata topera en España son conocidas exclusivamente para una población de *A. scherman monticola* situada por encima de 900 m.s.n.m. en los Pirineos. Dado que los factores ambientales asociados a la altitud pueden afectar a la reproducción en roedores, es probable que se observen diferencias en el patrón reproductivo entre esta población pirenaica y poblaciones de *A. scherman cantabriae* localizadas en plantaciones de manzano por debajo de 400 m.s.n.m. Del mismo modo, dado que el potencial reproductivo de *Arvicola* está relacionado con las características del hábitat y las dimensiones corporales de la madre, esta variable reproductiva podría diferir notablemente entre las poblaciones pirenaicas y cantábricas.

El uso actual de la tierra es un factor clave en la dispersión y colonización de *A. scherman*. Una alta proporción de hábitats adecuados interconectados entre sí permite una difusión poblacional sin obstáculos y, por lo tanto, puede dar lugar a explosiones demográficas sincronizadas. Sin embargo, paisajes heterogéneos como el asturiano, podrían obstruir los movimientos de las ratas toperas debido a una restricción de los procesos de dispersión y colonización. Por lo tanto, las poblaciones de *A. scherman* en esta zona agrícola podrían estructurarse a una escala local. El efecto que ejerce el paisaje sobre la dispersión de *A. scherman* se puede evaluar mediante análisis genéticos basados en microsatélites, los cuales proporcionan información adecuada sobre la genética poblacional.

El principal objetivo de esta investigación es conocer la biología reproductiva y la genética poblacional de *A. scherman* en plantaciones de manzano ubicadas en el paisaje agrícola de Asturias. De esta manera, se pretende obtener información sobre aspectos clave de esta especie plaga y mejorar su gestión. Los objetivos específicos son los siguientes:

1. Obtener información detallada sobre el ciclo reproductivo de *A. scherman* en plantaciones de manzano de Asturias (capítulo 2).

2. Determinar el potencial reproductivo de *A. scherman* en plantaciones de manzano de Asturias (Capítulo 3).

3. Indicar las posibles diferencias en el patrón reproductivo de las subespecies ibéricas de *A. scherman* (*A. scherman cantabriae* de Asturias y *A. scherman monticola* de los Pirineos) (capítulos 2 y 3).

4. Evaluar el efecto del paisaje agrícola de Asturias sobre la genética poblacional de *A. scherman* (Capítulo 4), a través de dos enfoques: i) explorar los niveles de diversidad genética de varios *demes* (conjunto de individuos que habitan una parcela) de rata topera y evaluar su pérdida de diversidad genética; ii) y revelar el flujo génico y la estructura genética espacial de *A. scherman* en la principal área de producción de manzana de Asturias.

5. Con la información obtenida sobre la reproducción y la genética poblacional, otro objetivo es proponer métodos de control sostenibles enfocados a disminuir la densidad poblacional de las poblaciones de rata topera, tanto en nuestra área de estudio como en otras con características ambientales similares (Capítulo 5).

Resultados y discusión

Para llevar a cabo el estudio se realizó la necropsia de más de 800 especímenes de *A. scherman cantabriae* capturados a lo largo de dos ciclos anuales en plantaciones de manzano localizadas en el centro-oriente asturiano. Se obtuvo información relevante sobre la biología reproductiva de esta especie, y se recogieron muestras de tejido muscular esquelético para realizar los ensayos genéticos. La información obtenida de los órganos reproductores de ambos sexos junto con la asignación de clases de edad relativa nos permitió determinar el ciclo reproductivo y la estructura poblacional de *A. scherman cantabriae*. Se observó la presencia continua de ejemplares jóvenes, y ejemplares sexualmente activos de ambos sexos durante todo el año. Los cambios intra-anuales en la masa corporal, el volumen testicular y la longitud de la vesícula seminal de los machos no afectaron significativamente la reproducción a una escala poblacional. Se concluye que la especie puede reproducirse continuamente en hábitats adecuados situados a altitudes relativamente bajas en el noroeste de España, donde un clima templado-hiperoceánico satisface todos los requisitos bióticos y abióticos necesarios. Ninguna otra población de *A. scherman* ha sido capaz de reproducirse según este patrón. Hábitats influenciados por un clima más continental, como son los prados pirenaicos donde se encuentran algunas poblaciones de *A. scherman monticola*, son susceptibles de sufrir cambios estacionales acusados, y por lo tanto la reproducción en invierno es poco probable.

Este patrón reproductivo permite que cada hembra madura de *A. scherman cantabriae* sea capaz de gestar 7.3 camadas al año. El tamaño de la camada en la subespecie cantábrica no se ve afectado por una limitación de la temporada de cría. Por contra, camadas más grandes distribuidas en menos gestaciones permiten a *A. scherman monticola* obtener un potencial reproductivo adaptado a una estacionalidad más acusada. Dado que el tamaño de la camada producido por una hembra debe dar el mejor éxito reproductivo en un ambiente particular, el número relativamente bajo de embriones por hembra en *A. scherman cantabriae* (primer año: 3.87 embriones/hembra; segundo año: 3.63 embriones/hembra) podría estar relacionado con un incremento de la supervivencia de la camada. En última instancia, el número potencial de crías por hembra y año en *A. scherman cantabriae* fue considerablemente mayor (primer año: 28.25; segundo año: 26.50) que el observado en *A. scherman monticola* (23.5).

No obstante, nuestros resultados mostraron que el tamaño de la camada aumenta con la condición corporal de la madre. La relación entre la masa y la longitud corporal parece ser uno de los principales factores relacionados con la variación del potencial reproductivo en esta subespecie. Una buena condición corporal en hembras preñadas podría conducir a un mayor número de ovocitos ovulados, embriones implantados y a una disminución del riesgo de fracaso de la gestación. En este sentido, un menor tamaño de camada en *A. scherman cantabriae* podría también estar relacionado con un menor tamaño corporal en relación a *A. scherman monticola*. Por otra parte, se constató una disminución progresiva de la condición corporal en hembras maduras no preñadas a lo largo de este período de estudio que, en última instancia, podría tener consecuencias negativas sobre el éxito reproductivo de estas hembras. La prospección continua de ejemplares en las plantaciones de estudio pudo implicar un aumento del estrés fisiológico de los ejemplares remanentes, quizás derivado de la eliminación de uno de los miembros de la pareja.

En ambas subespecies ibéricas el inicio de la actividad reproductiva tiene lugar a la misma edad relativa (aproximadamente seis semanas). Esta ausencia de diferencias podría estar relacionada con una regulación intrínseca ajena a factores ambientales, o bien dichos factores son lo suficientemente estables como para no afectar de manera diferente la maduración sexual de los ejemplares de *A. scherman cantabriae* nacidos en diferentes épocas del año. Sin embargo, como era de esperar dadas las diferencias corporales entre subespecies, la masa corporal a la cual cada sexo alcanza la madurez sexual es comparativamente menor en *A. scherman cantabriae*. Por otra parte, la monogamia es el principal sistema de apareamiento en *A. scherman cantabriae*, al igual que en otras poblaciones de rata topera. Esta subespecie mantiene grupos familiares a lo largo del tiempo formados por un par de adultos con su descendencia hasta la madurez sexual.

Los análisis genéticos se realizaron a partir del ADN genómico extraído de 137 especímenes de *A. scherman cantabriae* (71 machos, 66 hembras) pertenecientes a 10 *demes* localizados en los concejos de Villaviciosa y Nava. Cada muestra se analizó mediante la amplificación por PCR de 12 loci microsatélites divididos en dos paneles multiplex. Tras evaluar los genotipos resultantes, pudimos obtener información sobre la diversidad genética, el patrón de flujo genético y la estructura genética poblacional de esta especie en este paisaje agrícola. Se desarrolló un modelo de resistencias a la dispersión según la habitabilidad y la permeabilidad de los parches para esta especie, donde los polígonos de ocupación del suelo fueron evaluados en un sistema de información geográfica (SIG). Se obtuvieron las distancias efectivas entre *demes*, teniendo en cuenta la distancia geográfica de mínimo coste y la topografía. Se calculó la proporción de hábitats favorables respecto al total entre pares de puntos de muestreo o *demes*.

Los resultados de la heterozigosidad media esperada (H_E = 0.732), la heterozigosidad media observada (H_O = 0.602) y la riqueza alélica (A_R = 4.42) mostraron que la diversidad genética de *A. scherman cantabriae* es relativamente baja en esta zona de estudio. Dado que las plantaciones de manzano permiten a *A. scherman cantabriae* tener un alto potencial reproductivo, la pérdida de variabilidad genética es probable que se deba a una dispersión restringida en lugar de la calidad del hábitat. Este nivel de variabilidad genética fue considerablemente menor que el obtenido en la misma especie en un paisaje homogéneo que permite la conectividad entre hábitats favorables (valores medios: H_E = 0.824, H_O = 0.801), incluso durante una fase de baja densidad poblacional (valores medios: H_E = 0.799, H_O = 0.749). La pérdida de diversidad observada en los *demes* de *A. scherman* ubicados en este paisaje parcheado fue considerablemente mayor a la observada en poblaciones de *M. arvalis* localizadas en paisajes heterogéneos de cultivos anuales. En ese sentido, el efecto combinado de un mosaico de parcelas pequeñas y diferentes usos del suelo junto con setos y bordes arbolados implica un marco inadecuado para el intercambio genético entre *demes* de *A. scherman.* En este caso es probable que la pérdida y fijación de alelos sea debida a de la deriva genética y el efecto de la endogamia. El número relativamente pequeño de inmigrantes de primera generación detectados y los valores positivos de coeficiente de endogamia (F_{IS}) observados en la mayoría de los *demes*, sugieren que la inmigración no supone una fuente importante de nuevos alelos. Esto indica que el crecimiento de la población es más probable que tenga lugar mediante reclutamiento que por migración. Podemos concluir que *A. scherman* no es capaz de lograr con éxito la dispersión y/o posterior reproducción en los hábitats colonizados, que en última instancia se traduce en una reducción en el flujo génico. Además, estos hábitats favorables en Asturias suelen tener una superficie relativamente pequeña y probablemente albergan un tamaño poblacional relativamente pequeño, lo que puede reducir aún más la contribución a la conectividad entre *demes*.

La diferenciación genética (F_{ST}) entre pares de *demes* varió entre 0.004 y 0.271, siendo 0.157 el nivel medio. Los resultados de las pruebas de Mantel, que relaciona las distancias genéticas frente a las geográficas, mostraron patrones significativos de aislamiento por distancia (IBD) tanto para las distancias euclidianas ($r = 0.790$, $P = 0.0001$) como para las distancias efectivas ($r = 0.780$, $P = 0.0001$). Dichas diferencias genéticas entre estos *demes* desencadenaron el patrón de aislamiento por distancia más acusado hasta la fecha para la especie *A. scherman* y para las poblaciones de *A. amphibius* no insulares. En este caso, un aumento de hábitats adecuados entre *demes* no estuvo directamente relacionado con un aumento del flujo genético en *A. scherman*, probablemente debido a la complejidad del paisaje y la escasa conectividad entre estos hábitats. La máxima distancia efectiva, y por lo tanto el flujo génico, se produjo entre *demes* distanciados hasta 14.34 km en esta área de estudio. Se observó una subdivisión poblacional donde estos *demes* de *A. scherman* se estructuraron en cuatro grupos genéticos o poblaciones a una escala de 120 km^2. Esta estructura genética difiere considerablemente de la encontrada para esta misma especie en paisajes homogéneos franceses, donde la diferenciación genética se asocia principalmente con cambios en la densidad poblacional a gran escala y el aislamiento genético sólo es significativo durante las fases de baja densidad. Procesos estocásticos de ocupación del hábitat junto con una baja permeabilidad de la matriz, pudieron determinar la estructura de la población de *A. scherman* en este paisaje parcheado. Tanto el estuario como la autopista de cuatro carriles ubicada en el norte del área de estudio no supusieron barreras al flujo genético para *A. scherman*. La marea baja facilitaría el cruce del estuario por esta especie y los bordes de la carretera pueden ser utilizados como hábitats y corredores.

El control poblacional de un roedor perjudicial debe diseñarse para cada situación específica debido a que su condición de plaga depende de su propia biología y ecología, y de su interacción con el agroecosistema. En este sentido, la subespecie *A. scherman cantabriae* en Asturias muestra el mayor crecimiento poblacional por reclutamiento observado en esta especie, por lo que sus daños en esta región podrían ser más acusados que en otras zonas donde la especie también se considera plaga. La rata topera se reproduce de forma continua en esta área, por lo que sería recomendable que su control poblacional tuviese lugar durante todo el año. Obviamente, el uso de rodenticidas supondría una amenaza para el agroecosistema. El control poblacional de *A. scherman* en Asturias debe lograrse mediante métodos razonables y sostenibles, evitando el uso de rodenticidas.

Cada uno de los grupo genéticos detectados en este estudio depende principalmente de las tasas locales de nacimientos y muertes, y podrían gestionarse como unidades de manejo independientes. De esta forma, las estrategias de control para esta especie a escala regional pueden descartarse, lo que de hecho sería logísticamente impracticable y probablemente menos eficaz que un enfoque a menor escala. Las estrategias de control de la población deben centrarse en mantener las poblaciones de *A. scherman* en densidades bajas, aunque sería importante identificar fases de crecimiento poblacional para intensificar las acciones. Una investigación participativa puede ser aconsejable, en la cual los administradores de tierras tomen y envíen regularmente datos sobre la densidad poblacional de *A. scherman* al SERIDA para un análisis global.

Las trampas han sido utilizadas frecuentemente en esta región, y actualmente sigue siendo una práctica aceptable. Esta práctica se puede realizar a lo largo de todo el año y, dado el relativo pequeño tamaño de las plantaciones de manzano en Asturias, es un método con una eficacia aceptable. Prácticas sostenibles, como el trampeo continuo, el pastoreo intensivo o el arado, podrían mantener a las poblaciones de *A. scherman* bajo estrés fisiológico, y por lo tanto disminuir su potencial reproductivo. El uso de los portainjertos MM.111 o Franco puede ser recomendable dado que son menos apetecibles que otros portainjertos, y además el segundo desarrolla un sistema radicular más resiliente a los daños producidos por *A. scherman*. La instalación de barreras, situadas por encima y por debajo del suelo, rodeando a las parcelas junto con la instalación de trampas de autoservicio para depredadores terrestres podría ser un método adecuado para evitar colonizaciones. Aumentar la presencia de depredadores en las plantaciones de manzano también puede implicar una reducción de la población de *A. scherman*. En este sentido, es recomendable conservar este paisaje parcheado, mediante la preservación y el establecimiento de setos y áreas boscosas entre parcelas agrícolas, ya que favorecen la presencia tanto de depredadores específicos (armiños y comadrejas) como de depredadores generalistas (zorros, lechuzas, búhos o águilas).

Conclusiones

Según los resultados que se han obtenido es este estudio, se concluye lo siguiente sobre la biología y la genética poblacional de *A. scherman cantabriae*:

1. Las condiciones ambientales favorables de la zona agrícola de Asturias permiten a *A. scherman cantabriae* mostrar una reproducción continua. La presencia de ejemplares

jóvenes e individuos sexualmente activos de ambos sexos durante todo el año pone de relieve que este medio aporta todos los requisitos bióticos y abióticos necesarios por esta especie para lograr este patrón reproductivo continuo.

2. Una reproducción continua permite a *A. scherman cantabriae* producir un elevado número de camadas al año (7.30), aunque con un tamaño de camada relativamente moderado (valor promedio: 3.76). La temporada de cría no está restringida y no supone un factor crítico, por lo que permite a esta especie obtener un alto éxito reproductivo. Este potencial reproductivo es el más alto reportado para esta especie.

3. La condición corporal de la madre, y por lo tanto su estado fisiológico, parece ser el principal factor de variación del potencial reproductivo en *Arvicola scherman cantabriae*.

4. El número potencial de embriones por hembra y año (valor medio: 27.4) fue sustancialmente mayor en *A. scherman cantabriae* que en *A. scherman monticola* (23.5), lo que se debe probablemente a diferencias en la duración de la temporada de cría y razones históricas. Independientemente de las condiciones ambientales, ambas subespecies alcanzan la madurez sexual en la misma clase de edad relativa y la monogamia parece ser el principal sistema de apareamiento.

5. El paisaje de esta zona agrícola, conformado por un mosaico de pequeñas parcelas con diferentes usos del suelo junto con setos y bordes arbolados, implica un esquema inadecuado para que *A. scherman cantabriae* logre la dispersión y/o reproducción posterior en hábitats colonizados. Por lo tanto, el intercambio genético entre los *demes* de esta especie se ve obstaculizado y en última instancia conduce a una reducción en el flujo de genes.

6. La diversidad genética de *A. scherman cantabriae* observada en este paisaje irregular es relativamente baja, probablemente porque la escasa llegada de nuevos alelos no impide la pérdida de variabilidad genética debido a la endogamia y la deriva génica. Este nivel de variabilidad genética es considerablemente más bajo que el observado para la misma especie en paisajes agrícolas homogéneos, conformados principalmente por hábitats adecuados. El número relativamente bajo de migrantes y los coeficientes de endogamia positivos sugieren que el crecimiento de la población es más probable que ocurra por reclutamiento que por migración.

7. Las diferencias genéticas entre áreas cercanas desencadenan un fuerte patrón de aislamiento por distancia, el cual es el más fuerte registrado hasta la fecha para esta especie. De esta manera, el agrupamiento genético reveló la subdivisión poblacional de esta especie a escala local.

8. Es recomendable mantener las estrategias de control poblacional de *A. scherman cantabriae* durante todo el año. Las prácticas coordinadas a escala regional pueden ser descartadas, lo que de hecho sería logísticamente impracticable y probablemente menos eficaz que un enfoque a menor escala. El seguimiento de cada unidad de gestión será esencial para conocer la dinámica de la población y establecer estrategias de control coordinadas. Preservar y promover este paisaje irregular favorecería la presencia de depredadores y dificultaría la dispersión de esta especie.