Updating the zooplankton species list for the Belgian part of the North Sea

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ABSTRACT. Many marine species are threatened, and given the importance of biodiversity indices in the current European marine policy, taking stock of existing species and species diversity is crucial. Zooplankton form the basis of the pelagic food web, acting as staple food for fish larvae and adult pelagic fish, but are very susceptible to a changing climate. Inventorying zooplanktonic diversity is therefore important. Based on monthly sampling campaigns in 2009 and 2010, an update is provided on the zooplankton species list for the Belgian part of the North Sea. A total of 137 taxa are listed, some of which had rarely or never been observed in the area. This inventory revealed several species new to the Belgian marine species list: the calanoid copepod *Metridia lucens*, the cyclopoids *Oithona similis* and *Giardella callianassae*, the hydrozoans *Amphinema dinema* and *Eutima gracilis*, the mysid *Acanthomysis longicornis*, the polychaete worm *Tomopteris helgolandica*, the cladoceran *Penilia avirostris* and the monstrilloid copepod *Cymbasoma germanicum*. Additionally, we identified several males of *C. germanicum*, which have never been described before. Brief discussions are presented on spatial distribution and abundance of all taxa.

KEYWORDS. zooplankton, marine biodiversity, Belgian part of the North Sea, species list, faunal additions

INTRODUCTION

Biological diversity plays a crucial role in the way ecosystems function and in the many services they provide (VITOUSEK et al., 1997; LOREAU et al., 2001). Loss of marine biodiversity nationally, regionally and globally reduces the capacity of marine ecosystems to support the provision of goods and services, essential for human well-being (COCHRANE et al., 2010). Species lists are therefore indispensable fundamental tools to study species diversity and to calculate biodiversity indices in ecological studies.

The pelagic zone is the biggest habitat in the world, and also the biggest for Belgium (COSTELLO et al., 2010). Not only is it big, it is also ecologically very important, since the vast majority of fish species have a pelagic larval phase, including commercial fishes such as sole *Solea solea*, plaice *Pleuronectes platessa* and cod *Gadus morhua* (RUSSEL, 1976). These fish species must keep in step with their zooplanktonic food sources, for this is what their larvae eat. Furthermore, zooplanktonic organisms are very susceptible to a changing climate. The replacement of the cold water *Calanus finmarchicus* species assemblage in the North Sea by the warm water *C. helgolandicus*-dominated copepod assemblage with lower biomass and smaller species, is a textbook example of the severe consequences of a warming climate on marine ecosystems (RICHARDSON, 2008).

For the Belgian part of the North Sea (BPNS) very few historical lists of zooplankton species are available. The oldest known marine samples that contained zooplankton date from the early 20th century (Gilson collection, discussed in VAN LOEN & HOUZIAUX, 2002). However,

there was little to nothing published about the zooplanktonic species in these samples, as the main focus was on benthic organisms. VAN MEEL (1975) was the first to report zooplanktonic species lists from the Belgian part of the North Sea and adjacent waters.

More recent zooplanktonic research in Belgium has mainly focused on a limited number of species (e.g. VANDENDRIESSCHE et al., 2006; VAN HOEY, 2006), on diurnal zooplankton behavior (DARO, 1974) or on the interaction of calanoid copepods with the nuisance alga *Phaeocystis globosa* (SCHERFFEL, 1899) (e.g. GASPARINI et al., 2000; DARO et al., 2006; ROUSSEAU et al., 2006). In contrast, the zooplankton community structure and its dynamics in the Scheldt estuary have received considerably more attention (e.g. TACKX, 2002; MAES et al., 2002; APPELTANS et al., 2003; AZÉMAR et al., 2004; TACKX et al., 2005), but recent data on the marine part of the BPNS are extremely scarce. Considering climate change, the importance of biodiversity and the biogeographical changes in the distribution of planktonic species, an update of the zooplankton species list for the BPNS is certainly timely.

In 2010, the Flanders Marine Institute (VLIZ) compiled a species list for the Belgian marine waters (VANDEPITTE et al., 2010). For many zooplanktonic groups, the list is solely based on literature and therefore many species are geographically unverified. This study yields new and up-to-date information about the composition of zooplankton in the transitional region between the Atlantic Ocean and the North Sea and provides additional information for the Belgian Register of Marine Species (BeRMS) (VLIZ Belgian Marine Species consortium 2010).

MATERIALS AND METHODS

Sampling

Sampling was carried out monthly in 2009 and 2010 at ten monitoring stations in the BPNS positioned along a nearshore-midshore-offshore axis (Fig. 1). A WP2 net (200µm mesh size) fitted with flow meter (SMITH et al., 1968) was towed in an oblique haul from bottom to surface. Samples were fixed and preserved in a 4% formaldehyde solution.

Data are derived from a selection of 112 samples (53 nearshore, 30 midshore, 29 offshore), taken in salinity ranges from 29.9 – 35.0 PSU and temperature ranges from 2.0 – 20.9°C.

Species list

Using compound- and stereo-microscopes, taxa were identified to species level when possible, in order to attain the highest taxonomical resolution. The classification used is according to the World Register of Marine Species (WoRMS) (APPELTANS et al., 2011). Species that form an addition to the recently published Belgian Register of Marine Species (VANDEPITTE et al., 2010) are indicated with an asterisk (*) in Table 1. In addition, the different taxa have been subdivided according to their lifestyle; we distinguish between holoplanktonic (spend their entire life as plankton in the water column, *e.g.* calanoid copepods), meroplanktonic (spend a part of their life as plankters, e.g. decapod larvae) and tychoplanktonic taxa (are occasionally carried into the water column, e.g. species of Cumacea and Mysida).

RESULTS

Table 1 lists 137 taxa (101 identified to species level) found in the Belgian part of the North Sea in 2009 and 2010, of which 46 are considered holoplanktonic, 50 meroplanktonic and 41 tychoplanktonic. Four copepods, two hydrozoans, one mysid, one cladoceran and one polychaete have never been reported from the BPNS and are new for the Belgian Register of Marine Species. Additional information on densities and the spatial and temporal occurrence of these taxa in the BPNS is presented in Table 2 (appendix).

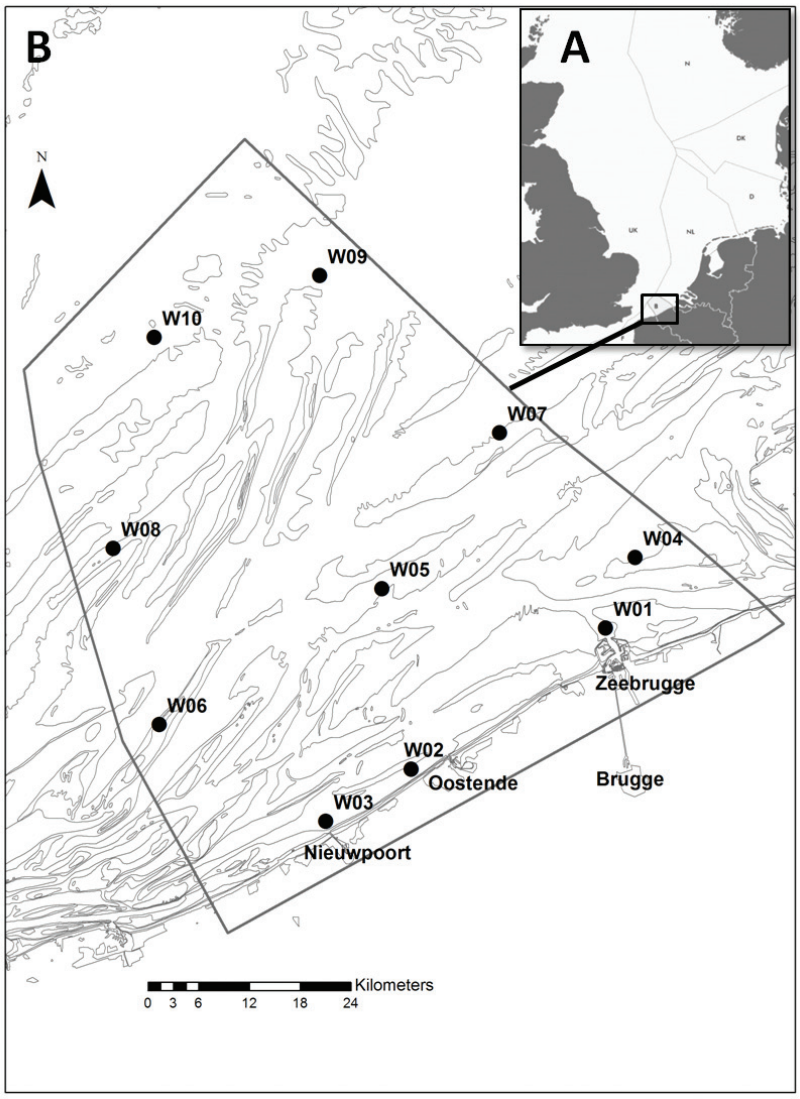


Fig. 1. – **A.** North Sea exclusive economic zones. **B.** Belgian part of the North Sea (BPNS) with ten stations (situated in nearshore W01-04-midshore W05-07-offshore areas W08-10) sampled monthly for zooplankton from January 2009 to December 2010.

DISCUSSION

This manuscript presents the first zooplanktonic inventory for the Belgian part of the North Sea in nearly forty years. Overall, 137 taxa were found in the net samples of which nine species (four copepods, two hydrozoans, one mysid, one cladoceran and one polychaete worm) were new to the Belgian Register of Marine Species (VANDEPITTE et al., 2010).

Species new for the BPNS

Cymbasoma germanicum is a rare monstrilloid species known only from a few female specimens collected at the Doggersbank, off Helgoland and Cuxhaven (RAZOULS et al., 2005-2011). We found 16 specimens, both males and females. The differences between *C. germanicum*, *C. rigidum* THOMPSON, 1888 and *C. zetlandicus* T. SCOTT, 1904 are subtle. A redescription of *C. germanicum*, including the description of the male, and comparison with its close relatives will be given elsewhere (FIERS & VAN GINDERDEUREN, in prep.).

Metridia lucens is a copepod most commonly found in the northern North Sea and northern

TABLE 1

List of holo-, mero- and tychoplanktonic taxa in the BPNS observed in the period 2009 - 2010. Species with asterisk (*) are new to the Belgian fauna (VANDEPITTE et al. 2010).

HOLOPLANKTON	
Protozoa **Dinoflagellata** **Order Noctilucales** Family Noctilucaceae *Noctiluca scintillans* (Macartney) KOFOID & SWEZY, 1921 **Cnidaria** **Scyphozoa** **Order Semaeostomeae** Family Cyaneidae *Cyanea lamarckii* PÉRON & LESUEUR, 1810 Family Pelagiidae *Chrysaora hysoscella* (LINNAEUS, 1767) Family Ulmaridae *Aurelia aurita* (LINNAEUS, 1758) **Order Rhizostomeae** Family Rhizostomatidae *Rhizostoma pulmo* (MACRI, 1778) **Cnidaria** **Hydrozoa** **Order Anthoathecata** Family Pandeidae *Amphinema dinema* (PÉRON & LESUEUR, 1810)* Family Bougainvilliidae *Nemopsis bachei* L. AGASSIZ, 1849 Family Margelopsidae *Margelopsis haeckeli* (HARTLAUB, 1897) Family Rathkeidae *Rathkea octopunctata* (M. SARS, 1835) Family Corynidae *Sarsia tubulosa* (M. SARS, 1835) **Order Leptothecata** Family Campanulariidae *Clytia hemisphaerica* (LINNAEUS, 1767) *Obelia* sp. Family Lovenellidae *Eucheilota maculata* HARTLAUB, 1894 Lovenellidae sp. Family Eirenidae *Eutima gracilis* (FORBES & GOODSIR, 1853)* *Eutonina indicans* (ROMANES, 1876)	**Ctenophora** **Order Beroida** Family Beroidae *Beroe gracilis* (KÜNNE, 1939) Family Bolinopsidae *Mnemiopsis leidyi* (A. AGASSIZ, 1865) Family Pleurobrachiidae *Pleurobrachia pileus* (O. F. MÜLLER, 1776) **Annelida** **Polychaeta** **Order Phyllodocida** Family Tomopteridae *Tomopteris (Johnstonella) helgolandica* (GREEFF, 1879)* **Arthropoda** **Crustacea** **Branchiopoda** **Order Diplostraca** Family Podonidae *Evadne nordmanni* LOVÉN, 1836 *Podon leuckartii* (G.O. SARS, 1862) Family Sididae *Penilia avirostris* DANA, 1849* **Arthropoda** **Crustacea** **Copepoda** **Order Calanoida** Family Acartiidae *Acartia* (*Acartiura*) *clausi* (GIESBRECHT, 1889) Family Calanidae *Calanus helgolandicus* (CLAUS, 1863) Family Candacidae *Candacia armata* (BOECK, 1872) Family Centropagidae *Centropages hamatus* (LILLJEBORG, 1853) *Centropages typicus* (KRØYER, 1849) *Isias clavipes* (BOECK, 1865) Family Pontellidae *Labidocera wollastoni* (LUBBOCK, 1857) Family Metridinae *Metridia lucens* (BOECK, 1865)*

Family Paracalanidae
Paracalanus parvus (Claus, 1863)
Family Clausocalanidae
Pseudocalanus elongatus (Boeck, 1865)
Family Temoridae
Temora longicornis (Müller O.F., 1785)

Order Cyclopoida
Family Corycaeidae
Corycaeus anglicus (Lubbock, 1857)
Family Cyclopinidae
Cyclopinoides littoralis (Brady, 1872)
Family Oithonidae
Oithona nana (Giesbrecht, 1893)
Oithona similis (Claus, 1866)*
Family Oncaeidae
Oncaea sp.

Order Harpacticoida
Family Euterpinidae
Euterpina acutifrons (Dana, 1847)

Order Monstrilloida
Family Monstrillidae
Cymbasoma germanicum (Timm, 1893)*

Arthropoda
Crustacea
Eucarida
Order Euphausiacea
Family Euphausiidae
Nyctiphanes couchii (Bell, 1853)

Arthropoda
Crustacea
Peracarida
Order Amphipoda
Family Hyperiidae
Hyperia galba (Montagu, 1815)

Chordata
Tunicata
Order Copelata
Family Oikopleuridae
Oikopleura (Vexillaria) dioica Fol, 1872

Chaetognatha
Order Aphragmophora
Family Sagittidae
Parasagitta elegans (Verrill, 1873)
Parasagitta setosa (Müller, 1847)

MEROPLANKTON

Mollusca
Bivalvia sp.
Gastropoda sp.
Order Pectinoida
Family Pectinidae
Pectinidae sp.
Order Euheterodonta
Family Pharidae
Ensis sp.
Order Myopsida
Family Loliginidae
Loligo sp.

Arthropoda
Crustacea
Copepoda
Family Clausidiidae
Giardella callianassae Canu, 1888*

Cirripedia
Cirripedia sp.

Order Decapoda
Anomura sp.
Brachyura sp.
Caridea sp.
Decapoda sp.
Family Callianassidae
Callianassa sp.
Family Crangonidae
Crangon crangon (Linnaeus, 1758)
Family Porcellanidae
Pisidia longicornis (Linnaeus, 1767)

Order Isopoda
Isopoda sp.

Order Tanaidacea
Family Tanaidae
Tanais dulongii (Audouin, 1826)

Cephalochordata
Order Amphioxiformes
Family Branchiostomidae
Branchiostoma lanceolatum (Pallas, 1774)

Echinodermata
Order Camarodonta
Family Parechinidae
Psammechinus miliaris (P.L.S. MÜLLER, 1771)

Order Forcipulatida
Family Asteriidae
Asterias rubens LINNAEUS, 1758

Order Ophiurida
Family Ophiotrichidae
Ophiothrix fragilis (ABILDGAARD, in O.F. MÜLLER, 1789)
Family Ophiuridae
Ophiura sp.

Order Spatangoida
Family Loveniidae
Echinocardium sp.
Bryozoa
Bryozoa sp.

Phoronida
Phoronida sp.

Chordata
Pisces
Pisces sp.
Order Perciformes
Family Ammodytidae
Ammodytidae sp.
Ammodytes marinus RAITT, 1934
Ammodytes tobianus LINNAEUS, 1758
Hyperoplus lanceolatus (LE SAUVAGE, 1824)
Family Callionymidae
Callionymus sp.
Family Trachinidae
Echiichthys vipera (CUVIER, 1829)
Trachinus draco (LINNAEUS, 1758)
Family Gobiidae
Gobiidae sp.
Pomatoschistus sp.
Family Carangidae
Trachurus trachurus (Linnaeus, 1758)

Order Pleuronectiformes
Family Bothidae
Arnoglossus laterna (WALBAUM, 1792)
Family Soleidae
Buglossidium luteum (RISSO, 1810)
Solea solea (LINNAEUS, 1758)
Family Pleuronectidae
Limanda limanda (LINNAEUS, 1758)
Pleuronectes platessa LINNAEUS, 1758

Order Clupeiformes
Family Clupeidae
Clupeidae sp.
Clupea harengus LINNAEUS, 1758
Sardina pilchardus (WALBAUM, 1792)
Sprattus sprattus (LINNAEUS, 1758)
Family Engraulidae
Engraulis encrasicolus (LINNAEUS, 1758)

Order Gadiformes
Family Gadidae
Merlangius merlangus (LINNAEUS, 1758)

Order Osmeriformes
Family Osmeridae
Osmerus eperlanus (LINNAEUS, 1758)

Order Syngnathiformes
Family Syngnathidae
Syngnathus rostellatus NILSSON, 1855

Order Scorpaeniformes
Family Triglidae
Triglidae sp.

TYCHOPLANKTON

Platyhelminthes
Platyhelminthes sp.

Nemertea
Nemertea sp.

Annelida
Oligochaeta sp.

Arthropoda
Arachnida
Acarina sp.

Arthropoda
Crustacea
Eucarida
Order Decapoda

Family Processidae
Processa modica WILLIAMSON, 1979

Peracarida
Order Amphipoda
Family Amphilochidae
Amphilochus neapolitanus DELLA VALLE, 1893
Family Calliopiidae
Apherusa bispinosa (BATE, 1857)
Apherusa ovalipes NORMAN & SCOTT, 1906
Family Atylidae
Atylus falcatus (METZGER, 1871)
Atylus swammerdami (MILNE-EDWARDS, 1830)
Family Pontopereiidae
Bathyporeia sp.
Family Corophiidae
Corophium sp.
Family Gammaridae
Gammarus crinicornis (STOCK, 1966)
Gammarus salinus (SPOONER, 1947)
Family Caprellidae
Caprella linearis (LINNAEUS, 1767)
Pariambus typicus (KRØYER, 1884)
Family Ischyroceridae
Jassa herdmani (WALKER, 1893)
Family Leucothoidae
Leucothoe incisa (ROBERTSON, 1892)
Family Megaluropidae
Megaluropus agilis (HOECK, 1889)
Family Microprotopidae
Microprotopus maculatus (NORMAN, 1867)
Family Microprotopidae
Orchomenella nana (KROYER, 1846)
Family Oedicerotidae
Pontocrates altamarinus (BATE & WESTWOOD, 1862)
Pontocrates arenarius (BATE, 1858)

Order Cumacea
Family Bodotriidae
Bodotria arenosa (GOODSIR, 1843)
Bodotria scorpioides (MONTAGU, 1804)
Family Diastylidae
Diastylis rathkei (KRØYER, 1841)
Family Pseudocumatidae
Pseudocuma sp.
Monopseudocuma gilsoni (GILSON, 1906)
Pseudocuma (Pseudocuma) longicorne (BATE, 1858)
Pseudocuma (Pseudocuma) simile G.O. SARS, 1900

Order Isopoda
Family Cirolanidae
Eurydice spinigera HANSEN, 1890

Order Mysida
Family Mysidae
Acanthomysis longicornis (MILNE-Edwards, 1837)*
Anchialina agilis (G.O. SARS, 1877)
Gastrosaccus sp.
Gastrosaccus sanctus (VAN BENEDEN, 1861)
Gastrosaccus spinifer (GOËS, 1864)
Mesopodopsis slabberi (VAN BENEDEN, 1861)
Schistomysis kervillei (G.O. SARS, 1885)
Schistomysis ornata (G.O. SARS, 1864)
Schistomysis spiritus (NORMAN, 1860)
Siriella armata (MILNE-EDWARDS, 1837)

Atlantic (FRASER, 1965; BARNARD et al., 2004). Its occurrence in the southern part of the North Sea appears to be rare: VAN MEEL (1975) detected the species in 1902-1910 samples. BRYLINSKI (2009) reported the find of a single male specimen in the Strait of Dover over a period of 30 years and FRANSZ (2000) emphasized the low abundance of the copepod among the zooplankton in the Dutch Part of the North Sea.

VAN MEEL (1975) considered *Oithona similis* as a species typical for the central part of the North Sea. In the southern part *O. similis* was reported near Gravelines (ANTAJAN, 2008) and in the Solent, English Channel (MUXAGATA & WILLIAMS, 2004). VAN MEEL (1975) however reported this species from a transect between Blankenberge (Belgium) and Orfordness (England), indicating that *O. similis* was found in the BPNS region.

Saphirella (SCOTT, 1894) morphs are now considered as the first copepodite stages (C1) of certain Clausiididae (BRYLINKSI, 2009). The adults of these pelagic larvae are parasitic species of Cyclopoida (RAZOULS et al., 2005-2011). BRYLINSKI (2009) identified *Saphirella* specimens in the French Channel corresponding to C1 of *Giardella callianassae*, a species never

reported from Belgian waters (VANDEPITTE et al., 2010). These copepodites of *Giardella* were also found in high numbers in our samples (Table 2).

The hydrozoan *Amphinema dinema* was collected by Gilson near Calais in 1905 (mentioned by VAN MEEL 1975). FRASER (1965) found *A. dinema* in the English Channel. Its presence off the Belgian coast was reported previously (LELOUP, 1952) but the species was omitted in the Belgian Register of Marine Species. The present study confirms its presence in the BPNS.

Eutima gracilis is a hydrozoan not mentioned from the North Sea or the English Channel by FRASER (1965) and VAN MEEL (1975), but it has been observed in English waters by others (RUSSEL, 1953; MEDIN, 2011). It appears to be restricted to European waters.

The mysid *Acanthomysis longicornis* has been observed in the vicinity of the BPNS. MEES et al. (1993) found it in the Westerschelde estuary close to the Belgian border, MÜLLER (2004) found it at Wimereux and ZIMMER (1933) as well reported *A. longicornis* from the southern North Sea.

Penilia avirostris is an abundant and widely distributed cladoceran in neritic tropical and subtropical waters, which has expanded north to temperate latitudes in the 20th century (ATIENZA et al., 2008). JOHNS et al. (2005) described how *P. avirostris* has increased in the North Sea since 1999, most probably due to warmer sea surface temperatures. The egg-carrying female found in this study proves that this species occurs and reproduces in the Belgian part of the North sea. *Evadne nordmanni* is a cladoceran not mentioned in the BeRMS (VANDEPITTE et al., 2010) and as such could be regarded as new for Belgian waters. However, VAN MEEL (1975) reports it present in high numbers in the BPNS in the early 20th century, indicating that this species has been found in the past.

Tomopteris (*Johnstonella*) *helgolandica* is the only holoplanktonic polychaete in the southern North Sea. It is known from Dutch waters, although rare (FRANSZ, 2000), and in the French Channel near Wimereux (DAUVIN et al., 2003).

Additional observations

The most abundant copepods (Table 2) were the calanoids *Acartia clausi*, *Temora longicornis*, *Paracalanus parvus*, *Centropages hamatus*, *Pseudocalanus elongatus* and the harpacticoid copepod *Euterpina acutifrons*. This corresponds with the observations by VAN MEEL (1975), DARO et al. (2006) and BRYLINSKI (2009).

In the North Sea, *Calanus finmarchicus* has shifted progressively northwards, while *C. helgolandicus* became more abundant and widely distributed in the 1980s (REID et al., 2003). In 2009-2010 only *C. helgolandicus* and not *C. finmarchicus* occurred in the samples taken in the BPNS, corresponding with the results of BRYLINSKI (2009) finding only the former species of *Calanus*. VAN MEEL (1975) on the other hand, mentions the calanoid *C. finmarchicus* attaining high densities in the southern North Sea in the '70s, while in the 19th century CANU (1892) reported only *C. finmarchicus* from the Boulonnais. SARS (1903) reported "*C. helgolandicus* has been recorded from the western coast of France by Dr. Canu", suggesting he did not agree with Canu's identification. This indicates that confusions exist in older literature between the two species *C. helgolandicus* and *C. finmarchicus*.

We investigated *Calanus* specimens from VAN MEEL (1975), sampled in the region of the BPNS in the early 20th century (stored in the RBINS collections in Brussels). They were *C. finmarchicus*, in contrast to the *C. helgolandicus* in our 2009 and 2010 samples.

In the present study, *C. helgolandicus* typically occurred around/on the offshore stations and was only occasionally caught nearshore. This copepod is known to reach high densities in the English Channel (BARNARD et al., 2004), and

TABLE 2

Average density (#m^{-3}), maximum density (#m^{-3}), and seasonal and spatial occurrence (near-mid-offshore) of all 137 taxa found in this study from January 2009 to December 2010. Spatial regimes (near-mid-offshore) with highest density are bold.

Taxon/Species	Average density	Maximum density	Seasonal occurrence	Spatial occurrence	Remarks
HOLOPLANKTON					
Dinoflagellata					
Noctiluca scintillans	1294,4	39806,3	Jul-Aug	off < near < **mid**	Summer species, but also very low densities found until October
Scyphozoa					
Aurelia aurita	< 0,1	< 0,1	Apr-Jul	off < mid < **near**	
Chrysaora hysoscella	< 0,1	< 0,1	Jun-Nov	off < mid < **near**	
Cyanea lamarckii	< 0,1	0,4	Mar-Jul	near < mid < **off**	
Rhizostoma pulmo	< 0,1	< 0,1	Sep-Nov	off < mid < **near**	
Hydrozoa					
*Amphinema dinema**	< 0,1	< 0,1			Six specimens found at W09: on 19/8/2009, 5/10/2009 and 11/8/2010
Clytia hemisphaerica	18,9	204,6	May-Dec	off < near < **mid**	
Eucheilota maculata	< 0,1	< 0,1			Two specimens found at W07 on 11/8/2010 and 6/9/2010
*Eutima gracilis**	< 0,1	0,84	Aug-Sept	near < **off**	Seventeen specimens found, almost all of them offshore
Eutonina indicans	< 0,1	< 0,1			Seen just once at station W02 on 7/4/2009
Lovenellidae sp.	< 0,1	0,5	Jul-Oct	mid < **off**	Twelve specimens found at W06, W08, W09 and W10
Margelopsis haeckeli	12,5	268,4	Apr-Oct	off < mid < **near**	
Nemopsis bachei	1,0	23,6	May-Sep	mid < **near**	Mainly found at station W01
Obelia sp.	2,2	104,4	Mar-Oct	off < mid < **near**	
Rathkea octopunctata	59,7	1402,2	Mar-Jun	**near**	Only found at station W01 and W02
Sarsia tubulosa	< 0,1	< 0,1	Mar-Oct		Five specimens found at stations W02, W07 and W09
Ctenophora					Peak in June,
Beroe gracilis	6,9	139,4	Apr-Dec	off < mid < **near**	Peak in October
Mnemiopsis leidyi	< 0,1	0,8	Sep-Dec	off < mid < **near**	Peak in spring (March-May)
Pleurobrachia pileus	1,6	79,3	All year	off < mid < **near**	
Polychaeta					
*Tomopteris helgolandica**	< 0,1	< 0,1			Two specimens at W09 (11/8/2010 and 6/9/2010) and W10 (6/7/2009)

Taxon/Species	Average density	Maximum density	Seasonal occurrence	Spatial occurrence	Remarks
Branchiopoda					
Evadne nordmanni	39,6	1085,2	Feb-Jul	off < mid < **near**	
*Penilia avirostris**	< 0,1	< 0,1			One specimen (female carrying eggs) found at W07 on 5/10/2009
Podon leuckartii	26,6	800,1	May-Oct	near < mid < **off**	
Copepoda					
Acartia clausi	753,6	3735,4	All year	near < mid < **off**	Highest densities in autumn and offshore
Calanus helgolandicus	5,0	96,7	All year	near < mid < **off**	Much lower densities in winter
Candacia armata	< 0,1	< 0,1			One adult individual was caught on 6/12/2010 at station W09
Centropages hamatus	265,3	4500,2	All year	off < near < **mid**	Highest densities in spring and summer
Centropages typicus	9,9	116,9	All year	off < near < **mid**	
Corycaeus anglicus	9,8	108,3	Aug-Feb	near < mid < **off**	
Cyclopinoides littoralis	11,9	118,1	All year	near < mid < **off**	
*Cymbasoma germanicum**	< 0,1	1,5	Jul-Sep	near < **mid**	16 specimens found (8 ♀, 1 copepodite and 7 ♂) at W01-05-06-07
Euterpina acutifrons	348,8	4250,0	Jul-Dec	off < mid < **near**	Very low numbers seen in Jan, Feb and May
Isias clavipes	4,4	46,7	Jun-Oct	off < near < **mid**	higher abundance at Westcoast (w03,w06) than at Eastcoast
Labidocera wollastoni	2,2	18,4	Aug-Sept	off < mid < **near**	
*Metridia lucens**	< 0,01	15,6			Only found at W02: 6 individuals on 18/10/2010 and 1 on 8/11/2010
Oithona nana	4,9	40,4	Jul-Oct	off < mid < **near**	
*Oithona similis**	20,6	283,0	Jul-Dec	off < mid < **near**	Much higher numbers at the coastal stations
Oncaea sp.	< 0,1	85,1			Only seen on 9/12/2009 at station W09
Paracalanus parvus	241,1	1663,0	All year	near < mid < **off**	Highest densities in summer and autumn
Pseudocalanus elongatus	17,1	540,5	All year	near < mid < **off**	Highest densities in spring and summer
Temora longicornis	713,6	7616,9	All year	off < near < **mid**	
Euphausiacea					
Nyctiphanes couchii	< 0,1	0,2	Jan-Feb	mid < **off**	In total 6 specimens were found
Amphipoda					
Hyperia galba	< 0,1	< 0,1			One specimen found at W01 on 11/6/2009 and 1 at W09 on 11/6/2010
Tunicata					
Oikopleura dioica	445,1	4153,8	All year	off < near < **mid**	Peak in spring (May-June)
Chaetognatha					
Parasagitta elegans	< 0,1	< 0,1			Only 1 specimen was found, on 11/6/2010 at station W02,
Parasagitta setosa	40,6	492,0	All year	near < mid < **off**	Densities much higher in summer than in other seasons

Taxon/Species	Average density	Maximum density	Seasonal occurrence	Spatial occurrence	Remarks
MEROPLANKTON					
Mollusca					
Bivalvia sp.	102,9	1753,2	Feb-Dec	off < mid < **near**	Veliger larvae and juvenile bivalvia
Pectinidae sp.	< 0,1	0,2			Found at 3 sites: W07 (8/9/2009), W09 (19/8/2009) and W10 (7/9/2009)
Ensis sp.	19,6	363,6	Mar-Oct	off < mid < **near**	*Ensis* spat, densities much higher nearshore (peak observed at W04).
Loligo sp.	< 0,1	< 0,1			One juvenile (1cm) found at W06 on 9/9/2009
Gastropoda sp.	5,1	65,0	May-Dec	near < off < **mid**	Juveniles, not identifiable
Copepoda					
Giardella callianassae *	104,2	1198,0	Jul-Dec	off < near < **mid**	Autumn species, peaking in October and November
Cirripedia					
Cirripedia sp.	115,4	987,6	All year	off < near < **mid**	Nauplius larvae and cyprid larvae
Decapoda					
Anomura sp.	< 0,1	0,4	Jul-Oct	near < mid < **off**	Zoea larvae, present in low densities
Brachyura sp.	6,1	73,2	All year	off < near < **mid**	Zoea larvae
Caridea sp.	6,4	45,2	All year	mid < near < **off**	Zoea larvae
Callianassa sp.	< 0,1	< 0,1			Three juvenile specimens caught on 11/8/2010 (W09) and 6/9/2010
Crangon crangon	0,3	3,1	May-Nov	off < mid < **near**	(W07), Zoea larvae, only counted when clearly identifiable, if not
Decapoda sp.	1,2	25,5	All year	near < off < **mid**	then record added to Caridea sp.
Pisidia longicornis	11,6	221,1	May-Oct	near < mid < **off**	Megalopa larvae, peak in numbers from Jul-Sep
					Zoea larvae, also 1 individual at W09 on 9/12/2009
Isopoda					
Isopoda sp.	1,8	21,6		near < off < **mid**	Microniscus larvae, found in Jan, Aug, Sep and Dec.
Tanaidacea					
Tanais dulongii	< 0,1	< 0,1			One specimen found on 15/7/2010 at W02
Cephalochordata					
Branchiostoma lanceolatum	1,1	11,3	Jul-Sep	mid < **off**	
Echinodermata					
Asterias rubens	30,5	592,5	Mar-Sep	off < mid < **near**	Bipinnaria and brachiolaria larvae
Echinocardium sp.	411,5	2881,5	May-Jul	off < near < **mid**	Echinopluteus larvae

Taxon/Species	Average density	Maximum density	Seasonal occurrence	Spatial occurrence	Remarks
Ophiothrix fragilis	263,3	10861,3	May-Dec	near < mid < **off**	Ophiopluteus larvae
Ophiura sp.	62,1	1593,9	All year	off < mid < **near**	Ophiopluteus larvae
Psammechinus miliaris	4,6	58,5	May-Jul	off < near < **mid**	Echinopluteus larvae
Bryozoa					
Bryozoa sp.	18,2	230,9	All year	off < near < **mid**	Cyphonauta larvae of Bryozoa
Phoronida					
Phoronida sp.	< 0,1	< 0,1			Actinotrocha larvae, 3 at W02 (14/05/09) and 1 at W07 (10/6/2010)
Pisces					
Ammodytidae sp.	2,2	31,5	Jan-Jul	near < mid < **off**	Larvae, found at W09 on 11/3/2009 and 10/3/2010
Ammodytes marinus	< 0,1	7,2			Larvae, found at W03 on 17/2/2009 and at W08 on 26/1/2009
Ammodytes tobianus	< 0,1	< 0,1	Jan-Jul	near < mid < **off**	Larvae, no larvae were recorded in nearshore samples
Arnoglossus laterna	< 0,1	0,8	Jun-Aug	mid < **off**	Larvae, 1 individual found at W05 on 8/7/2009
Buglossidium luteum	< 0,1	< 0,1			Larvae
Callionymus sp.	0,2	1,6	May-Aug	mid < **off**	Larvae, too small to be identifiable to species level
Clupeidae sp.	0,3	2,9	Mar-Jul	near < off < **mid**	Larvae
Clupea harengus	1,1	16,8	Jan-May	near < mid < **off**	Larvae, 1 specimen at W09 on 19/8/2009
Echiichthys vipera	< 0,1	< 0,1			Larvae, only seen at station W01 and W02. Five specimens found
Engraulis encrasicolus	< 0,1	0,9	Jul-Aug	**near**	Larvae
Gobiidae sp.	0,4	13,1	Jun-Oct	off < mid < **near**	Larvae, 8 specimens could be identified with certainty
Hyperoplus lanceolatus	< 0,1	0,6			Larvae, not found in nearshore and midshore samples
Limanda limanda	< 0,1	0,87	Feb-May	**off**	Larvae, scarce
Merlangius merlangus	< 0,1	0,9	Apr-May	mid < **off**	Larvae, 1 specimen found at station W01 on 15/7/2010
Osmerus eperlanus	< 0,1	< 0,1			Larvae, too small to be identifiable to order/family level
Pisces sp.	11,7	221,1	All year	near < off < **mid**	Larvae, positively identified once on 17/2/2009 at station W06
Pleuronectes platessa	< 0,1	< 0,1			Larvae, too small to be identifiable to family level
Pleuronectiformes sp.	< 0,1	0,5	Jan-Sep	near < mid < **off**	Larvae, only counted when clearly identifiable, if not then added to
Pomatoschistus sp.	< 0,1	0,6	July-Dec	off < mid < **near**	Gobiidae
Sardina pilchardus	0,5	4,7	Jun-Jul	mid < **off**	Larvae, not recorded nearshore
Solea solea	< 0,1	0,6	May-Jul	mid < **off**	Larvae, found in low numbers, not found in nearshore samples
Sprattus sprattus	0,9	26,1	Apr-Jul	near < mid < **off**	Larvae
Syngnathus rostellatus	< 0,1	< 0,1			Larvae, 1 specimen found at station W01 on 10/8/2010
Trachinus draco	< 0,1	< 0,1			Larvae, 1 specimen at W09 on 14/7/2010
Trachurus trachurus	0,2	1,9	Jun-Sep	mid < **off**	Larvae, no larvae were recorded in nearshore samples
Triglidae sp.	< 0,1	< 0,1			Larvae, 4 specimens found at W05, W07 and W09 in July 2009

Taxon/Species	Average density	Maximum density	Seasonal occurrence	Spatial occurrence	Remarks
TYCHOPLANKTON					
Platyhelminthes					
Platyhelminthes sp.	3,3	63,8	Jun-Oct	near < **off**	
Nemertea					
Nemertea sp.	< 0,1	< 0,1			Found twice on 14/5/2009, at station W08 and W10
Annelida					
Oligochaeta sp.	< 0,1	0,9			Four specimens found at W01 on 26/1/2009
Arachnida					
Acarina sp.	< 0,1	< 0,1			Two specimens found, at station W02 (14/5/2009) and W07 (8/7/2009)
Decapoda					
Processa modica	< 0,1	< 0,1			Only 1 specimen found at station W09 on 14/7/2010
Amphipoda					
Amphilochus neapolitanus	< 0,1	< 0,1			Found once at station W06 (9/9/2009) and once at W07 (6/9/2010)
Apherusa bispinosa	< 0,1	< 0,1			Found once at station W05 on 26/1/2009
Apherusa ovalipes	0,2	4,6	All year	near < mid < **off**	
Atylus falcatus	< 0,1	< 0,1			Strikingly, only found once, at W01 (6/10/2009)
Atylus swammerdami	4,2	122,0	All year	near < off < **mid**	Very common in pelagic samples, caught at each station each month
Bathyporeia sp.	< 0,1	0,6	Jan-Sep	mid < off < **near**	Only juveniles were caught in pelagic samples
Corophium sp.	0,1	3,2	Jan-Jun	near < off < **mid**	
Gammarus crinicornis	< 0,1	< 0,1			Found once at W01 on 11/6/2009
Gammarus salinus	< 0,1	< 0,1			Two specimens found: 1 at W01 (11/6/2009) and 1 at W02 (9/3/2010)
Caprella linearis	< 0,1	< 0,1			Found once at W09 on 6/7/2009
Pariambus typicus	0,9	21,6	Aug-Sep	mid < **near**	
Jassa herdmani	< 0,1	< 0,1			Found once at W01 on 15/7/2010
Leucothoe incisa	< 0,1	< 0,1			Found once at W06 on 10/7/2009
Megaluropus agilis	0,9	31,6	All year	near < off < **mid**	
Microprotopus maculatus	0,1	6,1	Aug-Mar	off < mid < **near**	
Orchomenella nana	< 0,1	< 0,1			One found at W02 (6/10/2009), W03 (9/9/2009) and W06 (17/2/2009)
Pontocrates altamarinus	< 0,1	0,5	Feb-Dec	near < off < **mid**	
Pontocrates arenarius	< 0,1	0,2			Found once at W01 (7/12/2010) and once at W09 (9/11/2010)

Taxon/Species	Average density	Maximum density	Seasonal occurrence	Spatial occurrence	Remarks
Cumacea					
Bodotria arenosa	< 0,1	0,3			Five specimens found at W03, W06, W07 and W09
Bodotria scorpioides	< 0,1	< 0,1			One specimen found at W09 on 6/12/2010
Diastylis rathkei	< 0,1	0,6			Three found at W01 (8/9/2010) and W02 (12/5/2010 and 8/11/2010)
Monopseudocuma gilsoni	0,3	6,6			11 found at stations W02, W07 and W09 from February until March
Pseudocuma sp.	9,8	169,3			Juvenile *Pseudocuma* sp. Were sometimes found in very high densities
Pseudocuma longicorne	< 0,1	< 0,1			One specimen found at W09 on 13/05/2009
Pseudocuma simile	< 0,1	6,2			Seen at W07 (8/4/2009 and 11/8/2010) and W09 (11/3/2009)
Isopoda					
Eurydice spinigera	< 0,1	< 0,1			Only 1 specimen found at station W10 on 21/1/2009
Mysida					
*Acanthomysis longicornis**	< 0,1	0,5	Feb-Dec	**off**	10 specimens found in Feb, Sep and Dec, at stations W08, W09 and W10
Anchialina agilis	< 0,1	< 0,1			Found once at W09 on 16/2/2009
Gastrosaccus sp.	1,4	174,7	All year	mid < off < **near**	Many juvenile *Gastrosaccus* were observed
Gastrosaccus sanctus	0,2	2,4	Jan-Sep	near < **off**	
Gastrosaccus spinifer	0,2	14,4	All year	mid < near < **off**	
Mesopodopsis slabberi	1,3	43,3	All year	off < mid < **near**	
Schistomysis kervillei	0,8	32,8	All year	off < mid < **near**	
Schistomysis ornata	< 0,1	< 0,1			Found once at W03 on 14/5/2009
Schistomysis spiritus	0,8	69,0	All year	mid < **near**	Almost all specimens were caught nearshore
Siriella armata	< 0,1	0,6			Three specimens found at W07 (8/9/2009) and 1 at W09 (21/1/2009)

is often transported to the BPNS by prevailing marine currents conveying Atlantic water through the Channel towards the southern North Sea (HOWARTH, 2001).

Parasagitta elegans is a chaetognath from the Atlantic Ocean and the more boreal parts of the North Sea (FRASER, 1965). VAN MEEL (1975) described how the species sometimes occurred in the Channel when conveyed in Atlantic currents reaching the North Sea. The fact that we caught only one individual of *P. elegans* but many thousands of *P. setosa* suggests that it is (or has become) a very rare species. Although species discrimination in chaetognaths is difficult, the present study confirms the presence of *P. elegans* in the BPNS.

Nyctiphanes couchii is the only euphausid recorded in the present study. It occurs in high densities in the central and northern North Sea, straying into the BPNS, especially during the colder winter months (RUSSEL, 1935; VAN MEEL, 1975). It has previously been reported from Belgian waters by CATTRIJSSE & VINCX (2001) and LOCK et al. (2011).

The invasive ctenophore *Mnemiopsis leidyi* was first reported from the North Sea in Dutch coastal waters in August 2006 (HOLSTEIJN, 2002). Reports of autumn blooms of lobate ctenophores off the Dutch coast prior to the first *M. leidyi* sightings were previously attributed to *Bolinopsis infundibulum* (O.F. MÜLLER, 1779) (FAASSE & BAYHA, 2006). Whether *M. leidyi* was present along the Dutch coast before 2006 remains to be settled as the two ctenophores can easily be confused. *Bolinopsis infundibulum* is a cold-water species and considered rare along the Dutch coasts. It was only in August 2007 that *M. leidyi* was first seen in the BPNS, in the port of Zeebrugge (DUMOULIN, 2007). Because of its presence within the port, its introduction into Belgian waters is most probably related to ballast water transport in cargo ships, as was indicated for *M. leidyi* in the Black and Caspian Seas and in the Dutch part of the North Sea (VINOGRADOV et al., 1989; IVANOV et al., 2000; FAASSE & BAYHA, 2006). Today, only four years after the first sighting/observation in 2007, *M. leidyi* occurs all along the Belgian coastline, up to 27 km offshore at the Thornton wind park as well as in all ports. Sightings of adult individuals in the coldest winter months imply that the species can survive Belgian winters. The spatial and temporal distribution along the Belgian coastal zone of *M. leidyi* is separately described in VAN GINDERDEUREN et al. (subm.).

Another invasive coelenterate recorded in this study is the hydrozoan *Nemopsis bachei*. This species was caught along the entire coastline, most abundantly around the port of Zeebrugge, where it was discovered in 1996 (DUMOULIN, 1997).

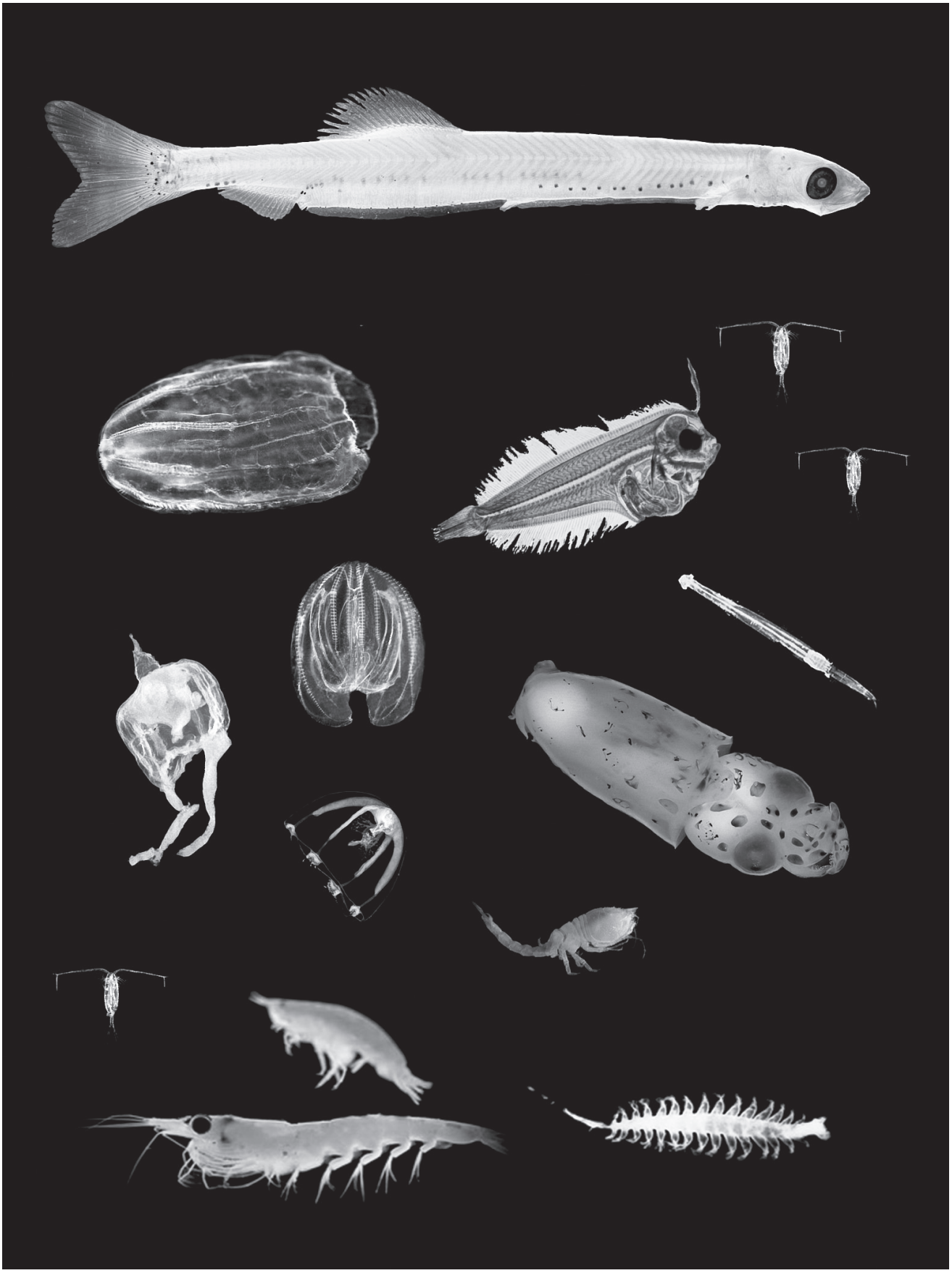
Cyanea lamarckii is the most frequently observed scyphozoan in this study. Its occurrence is in accordance with other jellyfish studies in the southern North Sea (BARZ & HIRCHE, 2007). In contrast to other species of Scyphozoa encountered, this jellyfish reached its highest densities offshore rather than nearshore (Table 2).

CONCLUSIONS

This study presents the first zooplanktonic inventory for the Belgian part of the North Sea in nearly 40 years. Among the 137 taxa encountered, nine are additions to the Belgian Register of Marine Species (BeRMS). We found 16 specimens of the very rare monstrilloid *Cymbasoma germanicum*, including several male specimens, which have not previously been described.

The calanoid copepod *Calanus finmarchicus* appears to have completely disappeared from the scene. The sole member of this genus in the collected samples is *C. helgolandicus*.

The distribution of the invasive coelenterates *Nemopsis bachei* and *Mnemiopsis leidyi* appears to have considerably expanded since their introduction in 1996 and 2007 respectively, as



they now occur along the entire Belgian coastline in well established populations.

This list contributes to the present-day knowledge of the total species richness in the southern North Sea and as such forms a valuable basis for ecological surveys.

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Fig. 2. – The pelagic is the biggest habitat in Belgium, in fact on a broader scale it is the biggest habitat in the world. Not only is it big, it is also very important. Almost all fish species - including all commercial fish we want on our plate such as sole *Solea solea*, place *Pleuronectes platessa* and cod *Gadus morhua* - have a pelagic larval phase. These fish species must keep in step with their planktonic food sources, for this is what their larvae eat. Therefore we must consider this as a possible "planktonic bottleneck". Combine this with the fact that the zooplankton is very susceptible to a changing climate (some species are moving north by 40km/y), due to shifts in sea water temperature, oceanic inflow and phytoplankton production and it becomes easy to understand why in many countries lots of minds are bent towards zooplanktonic research.
I hope that this illustration draws attention to the great zooplanktonic biodiversity in the Belgian part of the North Sea, portrayed in this manuscript.
From top to bottom and left to right: *Clupea harengus* larva; *Cymbasoma germanicum* female and male, *Arnoglossus laterna* larva, two *Calanus helgolandicus*, *Mnemiopsis leidyi*, *Sagitta setosa*, *Amphinema dinema*, *Nemopsis bachei*, *Loligo* sp. juvenile, *Diastylis rathkei*, *Aurelia aurita*, *Nyctiphanes couchii*, *Tomopteris helgolandica*.

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Observations on FGF immunoreactivity in the regenerating tail blastema, and in the limb and tail scars of lizard suggest that FGFs are required for regeneration

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ABSTRACT. Tail regeneration in lizards depends on the stimulation of growth factors, including Fibroblast Growth Factors (FGFs). Light and ultrastructural immunolocalization of FGFs was compared between the regenerating tail blastema and the limb where no regeneration occurs. A likely epithelial-mesenchymal transition occurs following amputation in both tail and limb and FGFs are present in the wound epidermis of both organs at 7-14 days post-amputation, and at lower intensity in mesenchymal cells of the blastema. Immunoreactivity for FGFs disappears in the limb wound epidermis after 14 days post-amputation and in the epithelium covering tails induced to form scars, whereas it remains in the apical tail epithelium. These observations suggest that scarring in the limb or the induced scarring in the tail correlate with the disappearance of FGFs. Basic FGF is concentrated in the incomplete basement membrane between the epidermis and the tail blastema where the essential signaling process that allows the continuous growth of the regenerative blastema may occur. The study suggests that the successful regeneration of lizard tail is dependent on the presence of FGFs in the wound epidermis, which are probably released into the blastema.

KEY WORDS. regenerating tail; scarring limb; Fibroblast Growth Factors; immunolocalization; ultrastructure

INTRODUCTION

Lizards can regenerate an amputated tail while they cannot regenerate an amputated limb (Marcucci, 1925, 1930; Barber, 1944; Bellairs & Bryant, 1985; Alibardi & Toni, 2005). Lizards represent a non-mammalian, amniote model for the analysis of tissue regeneration (Alibardi, 2010a, b) closer to mammalian models than the amphibian model (Mesher, 1996; Geraudie & Ferretti, 1998; Stocum, 2006; Carlson, 2007; Harty et al., 2003). These reptiles allow analysis of the factors that limit tissue regeneration, and the results can be compared with the formation of scar tissue in warm-blooded amniotes such as mammals (Alibardi, 2010a,b).

Microscopical studies on tail regeneration have shown that a regenerative blastema and a large mass consisting of cartilaginous, fat, muscle and nervous tissues are formed (Hughes & New, 1959; Simpson, 1965; Cox, 1969; Bellairs & Bryant, 1985; Alibardi & Sala, 1988). The blastema is the loose connective tissues formed above the stump from the accumulation of a mass of proliferating mesenchymal-like cells, which are covered by a regenerating or wound epidermis. The growth and progressive differentiation of cells within the blastema gives rise to the new tail. Conversely, in the limb, after an intense and lasting inflammatory response, no blastema is formed and the connective tissue forms scar tissue as it does in mammalian wounds (Barber, 1944; Zicka, 1969; Alibardi, 2010a,b).

In the tail the nervous tissues, especially the spinal cord and the central ependyma, are essential components of the regeneration process and directly or indirectly stimulate the regeneration of the other tissues (Simpson, 1970; Whimster, 1978; Alibardi, 2010b). Also, the presence of an apical wound epidermis with an incomplete basement membrane, separating epidermal cells from the underlying mesenchymal cells

of the blastema, is essential for the continuous regeneration of the tail (ALIBARDI, 1994a, b, 2010a, b). It is, however, unknown whether in this region an exchange of trophic material between epidermis and dermis takes place.

It has been speculated that, as in the case of amphibian limb and tail regeneration, a neurotrophic factor may be produced from the nervous tissue or the ependyma, or a signaling molecule/s from the wound epithelium may stimulate the regeneration and growth of the surrounding tissues (SIMPSON, 1970; ALIBARDI & MIOLO, 1990). In amphibians, among possible neurotrophic molecules, Fibroblast Growth Factors (FGFs, acidic or FGF1, and basic or FGF2) are the proteins that better mimic the action of trophic factor for regeneration of the limb (reviewed in GERAUDIE & FERRETTI, 1998). FGFs and their receptors are particularly localized in the wound epithelium (especially in the apical epidermal cup, AEC) and in the mesenchymal cells of the blastema in the newt or in the axolotl limbs (POULIN & CHIU, 1995; HAN et al., 2001; GIANPAOLI et al., 2003).

Recent immunocytochemical studies have shown that the regenerating spinal cord and nerves of lizards also contain relatively high levels of FGF1 and FGF2 (ALIBARDI & LOVIKU, 2009). This observation has extended the importance of FGFs as stimulator molecules for regeneration in reptiles, whose ancestors included the first amniotes that evolved during land adaptation. Therefore the process of regeneration in lizards represents an interesting model, closer to mammals than the amphibian model of regeneration, to analyze the factors limiting tissue regeneration in amniotes, including mammals (ALIBARDI, 2010b). Since regeneration in lizards is inhibited by wounding or cauterizing the regenerating tail, which then turns into a cicatrizing outgrowth, the study of the scarring process can reveal some differences in the expression of specific molecules, including growth factors. The cicatrization of the tail leads to the formation of a dense and irregular connective tissue, which replaces the normal mesenchymal connective tissue of the blastema. It is not known whether a detectable amount of FGFs is also present in the limb of lizards, and in which tissue/s the factor is expressed in higher levels. In particular, the details of FGF immunolocalization in the wound epidermis and in the mesenchyme of the blastema are not known in lizards, and in reptilian tissues in general.

Using immunofluorescence and ultrastructural immuno-gold cytochemistry, the present study compares the localization of FGFs, in the tail wound epidermis and blastema mesenchyme with FGF localization in the limb wound in order to detect a possible difference in the presence of these growth factors that can be correlated with regeneration (tail) and scarring (limb).

MATERIALS AND METHODS

The present study was conducted on a total of 61 adult lizards (*Podarcis sicula*) of both sexes, as detailed below. The animals were kept in a terrarium at 25-33°C with a photoperiod of 12-14 hours of light. The experimental procedures were in accordance with approved ethical protocols from the University of Bologna. The animals were kept a 4°C for 4-5 hours before amputation, following anesthesia using ethylic ether. Amputation was performed with a sharp razor blade at the 1/3 proximal of the rear limb, basically amputating most of the thigh or arm (about 1 mm or less was left as stump). At the same time also the tail was amputated at about 1/3 proximal, by twisting the tail to exploit the natural fracture planes present in the tail (autonomous planes). The animals were left at room temperature (22-27 °C) in cages over blotting paper for at least two days, with water available, to allow the stump surface to form a dry clot.

After two days the animals were returned to their previous cages at 25-33°C. The stump of the amputated tail or limb was collected at 2 days (n=4), 4 days (n=3), 6-7 days (n=4), 8-10 days (n=4), 12-14 days (n=3), 16-18 days (n=3),

22 days (n=3), and around 30 days (n=3). Other cicatrix outgrowths or short cones from the limbs (0.5-1 mm in length) were collected at 16 days (n=3), 22 days (n=4) and 35 days (n=5) after amputation. While the tail was regenerating by 8-18 days (2-10 mm), the limb appeared as a pale cicatrix at 22 and 35 days post-trauma.

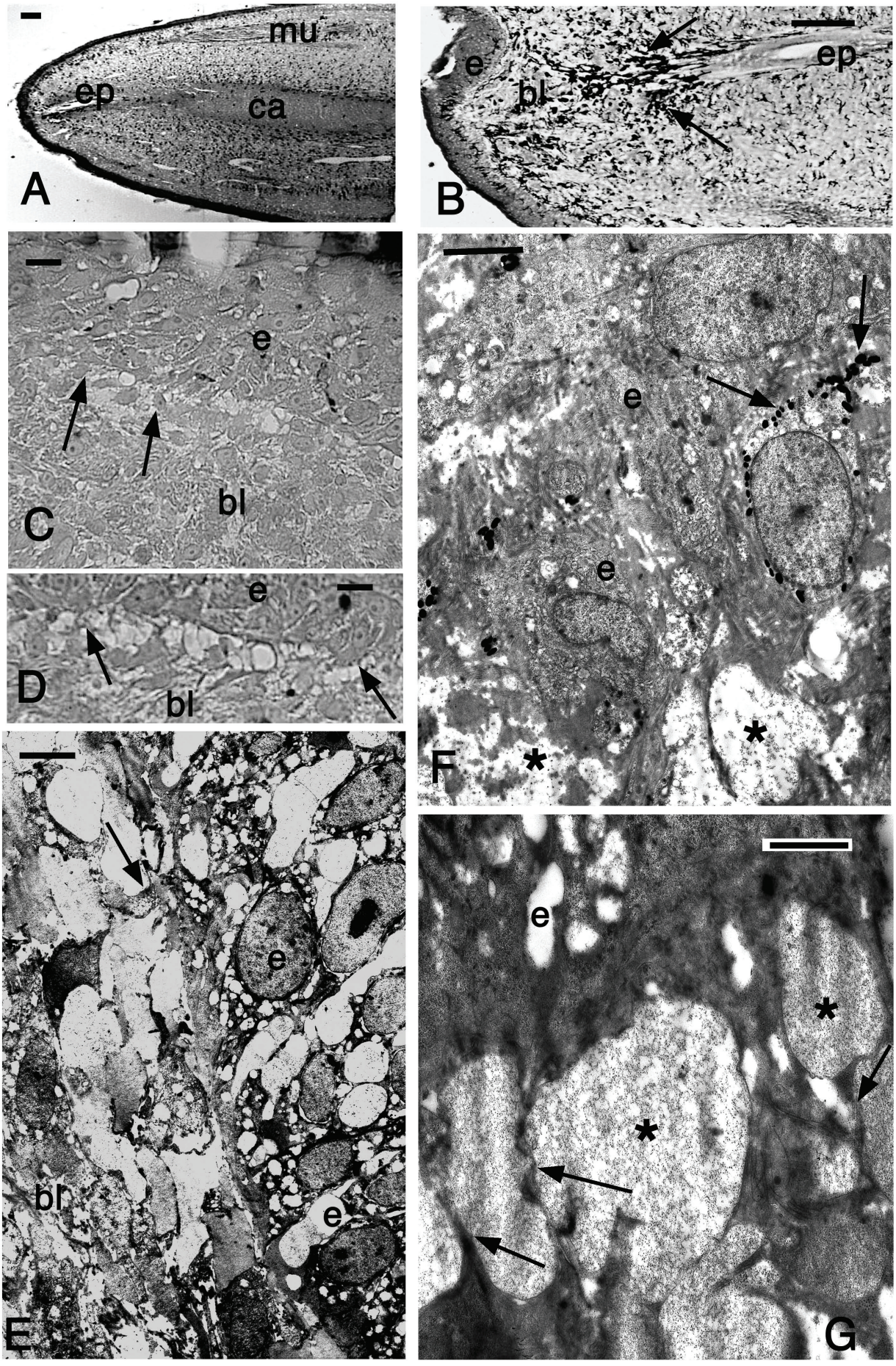
Other lizards (n=12), after tail amputation and formation of the blastema, underwent the removal of 2-3 mm of the apical part of the blastema, which lead in some cases to the inhibition of regeneration. This occurred by the formation of a short cicatrix stump that rapidly formed a scaled outgrowth in the 3 weeks following post-removal. From some lizards (n=10), the fibrous scar outgrowths (at about 16 days in two individuals, at 3 weeks in five individuals, and 5 weeks post-trauma in three individuals) were then collected and immediately fixed.

The normal blastema or regenerating cones and scarring outgrowths were halved with sharp scissors, and half of the organ was fixed with glutaraldehyde for morphological study, and the other half was fixed with Carnoy's fluid or paraformaldehyde for immunocytochemical study. Tissues were immediately fixed at 0-4°C in 2.5% glutaraldehyde in 0.12 M Phosphate buffer at pH 7.2 for 6-8 hours. These tissues were rinsed in the buffer, osmicated for two hours (2% OsO_4), dehydrated and embedded in the hydrophobic Durcupan Resin according to standard protocols. The other tissues were fixed at 0-4°C in freshly-made 4% paraformaldehyde in 0.1 M Phosphate buffer at pH 7.4 for 7-8 hours, rinsed in buffer, dehydrated in ethanol and embedded in the hydrophilic Bioacryl resin (SCALA et al., 1992). Finally, other tissues were fixed in Carnoy's fluid for 4-5 hours at 0-4°C, dehydrated in ethanol, and embedded in Bioacryl resin.

The embedded tissues were sectioned longitudinally using an ultramicrotome, and semithin sections (2-3 mm thick) and thin sections (70-90 nm thick) were collected. Semithin sections were stained in 1% toluidine blue for histological or immunohistochemical study (see details in Alibardi & Toni, 2005). Briefly, light microscopic immunocytochemistry was performed incubating the sections overnight at 0-4°C in the Fibroblast Growth Factor (FGF) antibodies diluted 1:200 in buffer (Tris 0.05 M at pH 7.6 containing 1% BSA). The FGF2 antibody (Sigma, F3393) was raised in rabbit against the 1-24 N-amino acid sequence of bovine bFGF. The anti FGF1 antibody was produced in rabbit injecting the entire sequence of the recombinant FGF1 (Sigma) (SCHULTZ et al., 1993). In control sections, the primary antibody was omitted. After being rinsed in buffer, the sections were incubated for 60 min at room temperature in a fluorescein-conjugated anti-rabbit antibody (Alexa 1:1000, or Sigma 1:100), rinsed in buffer, mounted in 10% glycerol, and observed under a fluorescence microscope equipped with a fluoroscein filter. Photographs were taken with a digital camera and computerised using Adobe Photoshop 5.0.

Thin sections of 30-80 nm thickness were collected on copper grids (those fixed in glutaraldehyde) or on nickel grids (those fixed in 4% paraformaldehyde). The sections on copper grids were routinely stained with uranyl acetate and lead citrate, rinsed, dried, and observed under CM-100 Philips and Hitachi-600 transmission electron microscopes.

The sections on nickel grids underwent immunogold labeling for FGF1 and FGF2 (as indicated above). Briefly, sections were incubated for 10 min in the Tris buffer containing 1% cold water fish gelatin to block non-specific binding sites, then the grids were incubated overnight at 0-4°C in the primary antibodies (FGF1 and FGF2 as above). Grids were again rinsed in the buffer, a 10 nm gold conjugated anti-rabbit secondary antibody was applied for one hour at room temperature, grids were rinsed in buffer and then in distilled water. Grids were stained for 6 min in 2% aqueous uranyl acetate, rinsed and observed with a CM-Philips 100 electron microscope operating at 80 kV.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RESULTS

Light microscopy and ultrastructure

The regenerating blastema of the conical new tail at 8-18 days of regeneration was surrounded by a multi-stratified (wound) epithelium, which produced a thin corneous layer (Fig. 1A, B). Close to the apical wound epithelium at the apex of the tail was located the ependymal ampulla, the foremost part of the central canal of the regenerating spinal cord, surrounded by a few growing axons and, more externally, by pigmented cells or melanophores (Fig. 1B).

Close analysis of the wound epidermis in the apical regions of the regenerative blastema at 7-16 days post-amputation showed that the boundary between epidermis and mesenchyme was often ill-defined (Fig. 1C, D). This histological aspect of the regenerating skin appeared clearly not just in tangentially-cut sections of the epidermal-dermal boundary but also in more central sections (perpendicularly-sectioned). It appeared that some keratinocytes were in continuity with mesenchymal cells without the presence of a basement membrane separating the epithelium from the mesenchyme.

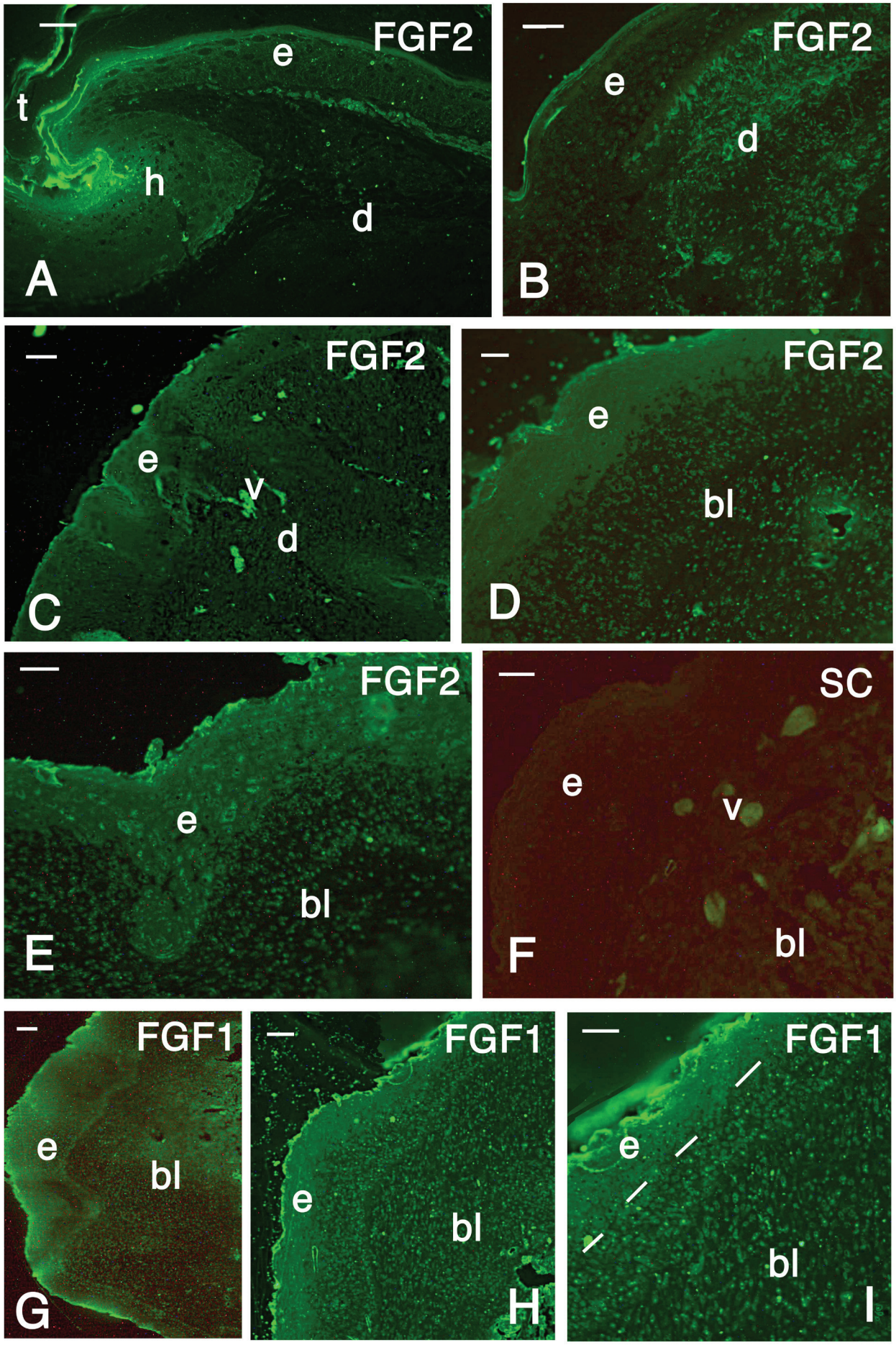
Ultrastructural observations in these areas at 7-14 days post-injury showed the presence of numerous pale spaces or vesicles within the wound epithelium and between the epithelium and the mesenchyme, while sparse cytoplasmic bridges disrupted the continuity of the epithelium (Fig. 1E). Detailed analysis of the pale spaces located among the basal keratinocyte elongation and mesenchymal cells of the blastema suggested that these structures were not extracellular or degenerating spaces among cells (Fig. 1F). Instead, at least in some cases, the pale spaces represented true sections of cytoplasmic blebs (stout elongations of the cytoplasm) from either epithelial or mesenchymal cells, and contained mainly free ribosomes and some flocculent, amorphous material (Fig. 1G). The cytoplasmic blebs were surrounded by a membrane or were enveloped by the cytoplasm of keratinocytes or mesenchymal cells present in these ill-defined, transitional zones (Fig. 1G).

Light immunocytochemistry

The immunofluorescence for FGF2 (bFGF) showed that the reactive epidermis of the wounded scales close to the blastema was evenly stained at 6-14 days post-amputation (Fig. 2A). In comparison, FGF2 immunofluorescence was absent or limited to the basal layer in the epidermis of normal, unwounded scales, where the (reactive) dermis was also immunofluorescent (Fig. 2B).

The stratified wound epidermis of the tail at 7-18 days post-amputation also showed a diffuse immunofluorescence in keratinocytes, a positive

Fig. 1. − Light microscopic (**A-D**) and electron microscopic (**E-G**) aspects of the regenerating tail blastema. **A**. coniform regenerating tail at 12-14 days outlined by the thick wound epithelium and containing an apical ependyma ampulla surrounded by the blastematic mesechyme. Bar: 50 mm. **B**. detail of the apical part of the regenerating tail showing numerous melanocytes (arrows) surrounding the ependymal ampulla and among blastema cells. Bar: 50 mm. **C**. detail of the apical wound epithelium at 7-8 days post-amputation. Some cells in the mesenchyme (arrows) seem in continuity with the epidermis. Bar: 10 mm. **D**. further detail of the wound epithelium at 7-8 days post-amputation showing two elongations (arrows) from epithelial cells into the underlying mesenchyme. Bar: 10 mm. **E**. Electron micrograph showing the interface between the wound epidermis and blastemal cells at 7-8 days post-amputation. An epithelial elongation contacting the mesenchyme is seen (arrow). The asterisks indicate numerous pale spaces in the mesenchyme beneath the epithelium, representing sections of cytoplasmic blebs derived from the epithelium. Bar: 2 mm. **F**. detail of the most basal cells of the wound epithelium, which are not separated by a basement membrane from the underlying mesenchyme (arrows indicate pale or degenerating spaces between keratinocyets and mesenchymal cells). Bar: 2.5 mm. **G**. further detail of cytoplasmic blebs (asterisks) surrounded by keratinocytes bridges (double arrows). Bar 1μm. **Abbreviations**: bl, blastema; c, regenerating cartilaginous axis (tubule); e, wound/regenerating epidermis; ep, ependymal ampulla; mu, regenerating muscles bundles.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fluorescence in the regenerating ependymal ampulla, but a lower immunoreactivity was instead present in mesenchymal cells of the blastema (Fig. 2C, D). FGF2-immunofluorescent keratinocytes were also seen in the forming epidermal pegs of regenerating scales (Fig. 2E). The controls showed no labeling in keratinocytes and a non-specific fluorescence was often observed in blood cells located within the vessels of the regenerating blastema (Fig. 2F).

The immunofluorescence for FGF1 (aFGF) showed a similar general localization to that of FGF2, but slightly more intense with the employed concentration of the antibodies in the wound epidermis, and it also showed similar localization in the mesenchymal cells and their nuclei in the regenerative blastema (Fig. 2G-I).

Detailed examination of different sections containing the apical wound epidermis stained for FGF2, of the regenerating tail at 7-14 days of regeneration (Fig. 3A, B), and of the limb at 7-8 and 12-14 days of regeneration (Fig. 3C-F), showed that the basal layers were not clearly distinct from the underlying mesenchyme. Therefore while some FGF-positive cells appeared confined within the epithelium (keratinocytes), other FGF2-positive cells were also present in the "frying" boundary between the epithelium and the mesenchyme. While the wound epidermis of the tail in the apical region maintained immunofluorescence for FGF2 at 18 days (elongating tail, see Fig. 3C) and longer, the immunofluorescence disappeared in the wound epithelium and connective tissue of the limb at 16, 22, and 35 days post-amputation (Fig. 3G, H).

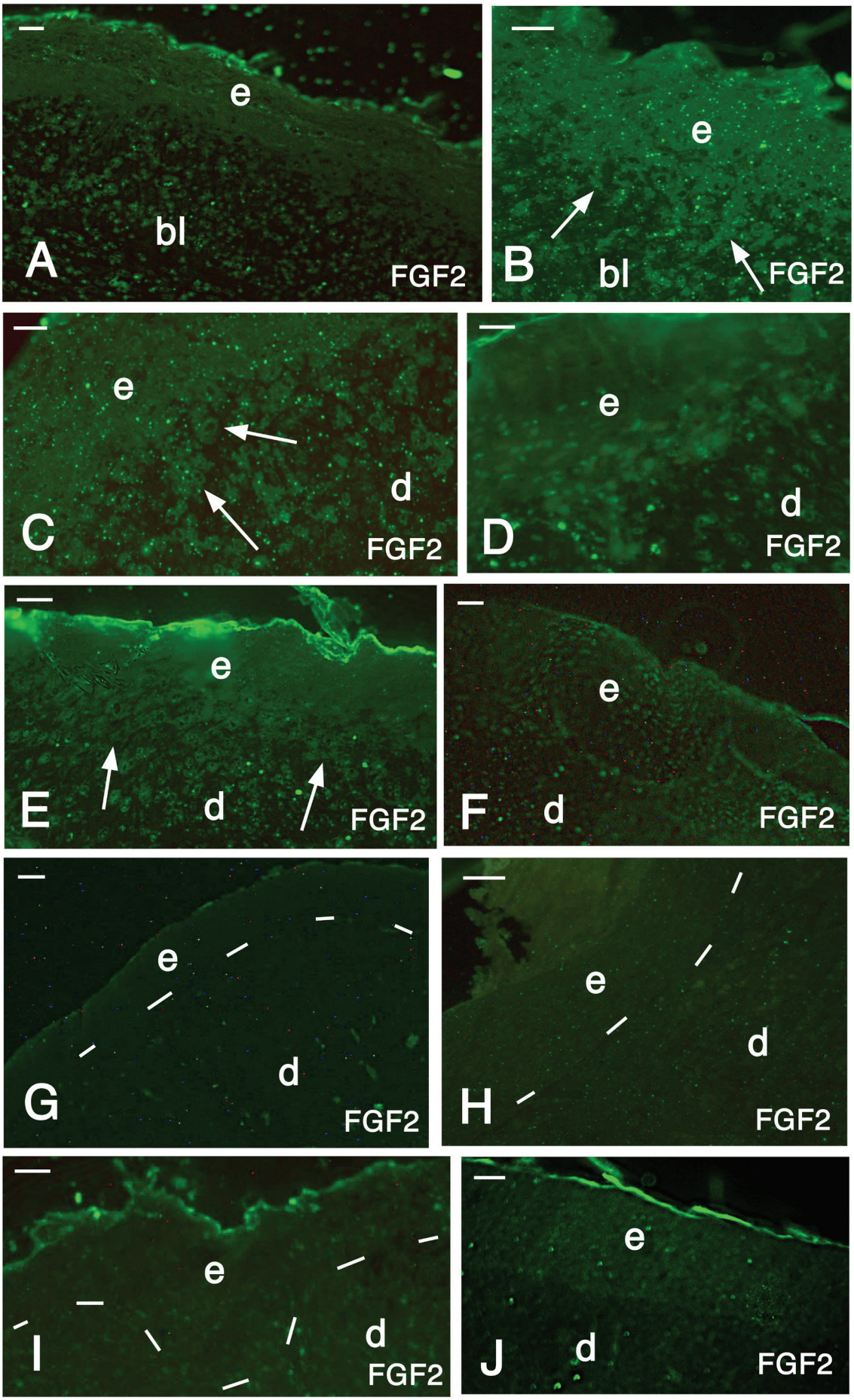
A similar lowering or a complete disappearance of the immunofluorescence, for FGF2 was noted in the epidermis of scarring tails at 16, 21, and 35 days post-injury (Fig. 3I, J). Controls sections were immunonegative (data not shown).

Detailed examination of sections that were immunoreacted for FGF1 (aFGF) showed a low to absent immunoreactivity in the normal epidermis and dermis (tail or limb) (data not shown). Immunofluorescence was instead observed in the wound epithelium of the tail and of the limb at 6-7 days post-amputation (Fig. 4A). At 12-14 and 25 days post-amputation the immunoreactivity for FGF1 disappeared in the limb while the epidermis became thinner and formed a thicker stratum corneum (Fig. 4B, C). A similar disappearance of FGF1 immunoreactivity was also noted in the epidermis and dermis of the scarring tail at 3 weeks post-amputation (fig. 4D). In mature scars of both limbs (35 days post-amputation) and tail (35 days post-amputation), the immunoreactivity for FGF1 in the epidermis was completely absent while the dense scar connective tissue appeared variably immunopositive for FGF1 (Fig. 4E, F). The controls from sections of tail and limb showed no immunoreactivity (Fig. 4G, H).

Ultrastructural immunocytochemistry in the tail blastema

The fine distribution of FGF2 in the apical wound epithelium showed that gold particles were diffusely distributed in the cytoplasm of all layers of the epidermis and among keratin bundles (Fig. 5A). We observed that nuclei

Fig. 2. − Immunofluorescence for FGF2 (A-E), control (F), and for FGF1 (G-I) in regenerating tail blastemas (12-14 days post-amputation). **A**. reactive scale proximal to the regenerating tail. Bar: 25 mm. **B**. normal scale lacking FGF2-immunoreactivity in the epidermis and immunolabeling in the dermis. Bar: 10 mm. **C**. apical blastema showing immunolabeling in the wound epidermis and little in the mesenchyme. Bar: 20 mm. **D**. immunolabeled wound epithelium and cells of the blastema. Bar: 20 mm. **E**. detail on epidermal bleb with immunofluorescent keratinocyets. Bar: 20 mm. **F**. immunonegative control where red blood cells inside blood vessels show a non-specific fluorescence. Bar: 20 mm. **G.** general view of blastema immunolabeled for FGF1. Bar: 20 mm. **H**. detail showing immunofluorescence in the wound epithelium and diffuse in the mesenchymal cells of the blastema. Bar: 20 mm. **I**. other detail of FGF1-labeling that also shows labeling in numerous nuclei of mesenchymal cells beneath the wound epidermis (dashes). Bar: 20 mm. **Abbreviations**: bl, mesenchymal blastema; d, dermis; e, epidermis; SC, serum control; v, blood vessel.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appeared less labeled, but quantification was not done. A higher concentration of gold particles was frequently observed along the wounding and incomplete plasma membrane contacting the mesenchyme (Fig. 5B). The labeling was mainly associated with the lamina lucida or with the lamina reticularis (non compacted parts of the basal lamina present beneath the lamina densa contacting the mesenchyme) but not specifically with the lamina densa (the dense component of the basement membrane). The latter was, however, discontinuous in the apical wound epidermis.

In control sections, both the cytoplasmic and basement membrane labeling was absent (Fig. 5C). FGF2 immunolabeling was also present in non-apical wound epithelium, although the labeling was even more diffuse in the cytoplasm and generally among keratin bundles of upper spinosus and pre-corneous keratinocyets (data not shown).

A lower, diffuse FGF2 immunolabeling was seen in the cytoplasm of mesenchymal cells of the blastema, including those apparently detaching from the epithelium (data not shown). A little labeling was often noted in the extracellular material associated with the plasma membrane (glycocalix) but little to no labeling was instead seen in the extracellular matrix of the blastema. Many endothelial cells of regenerating blood vessels in the blastema also contained a diffuse, cytoplasmic labeling (data not shown).

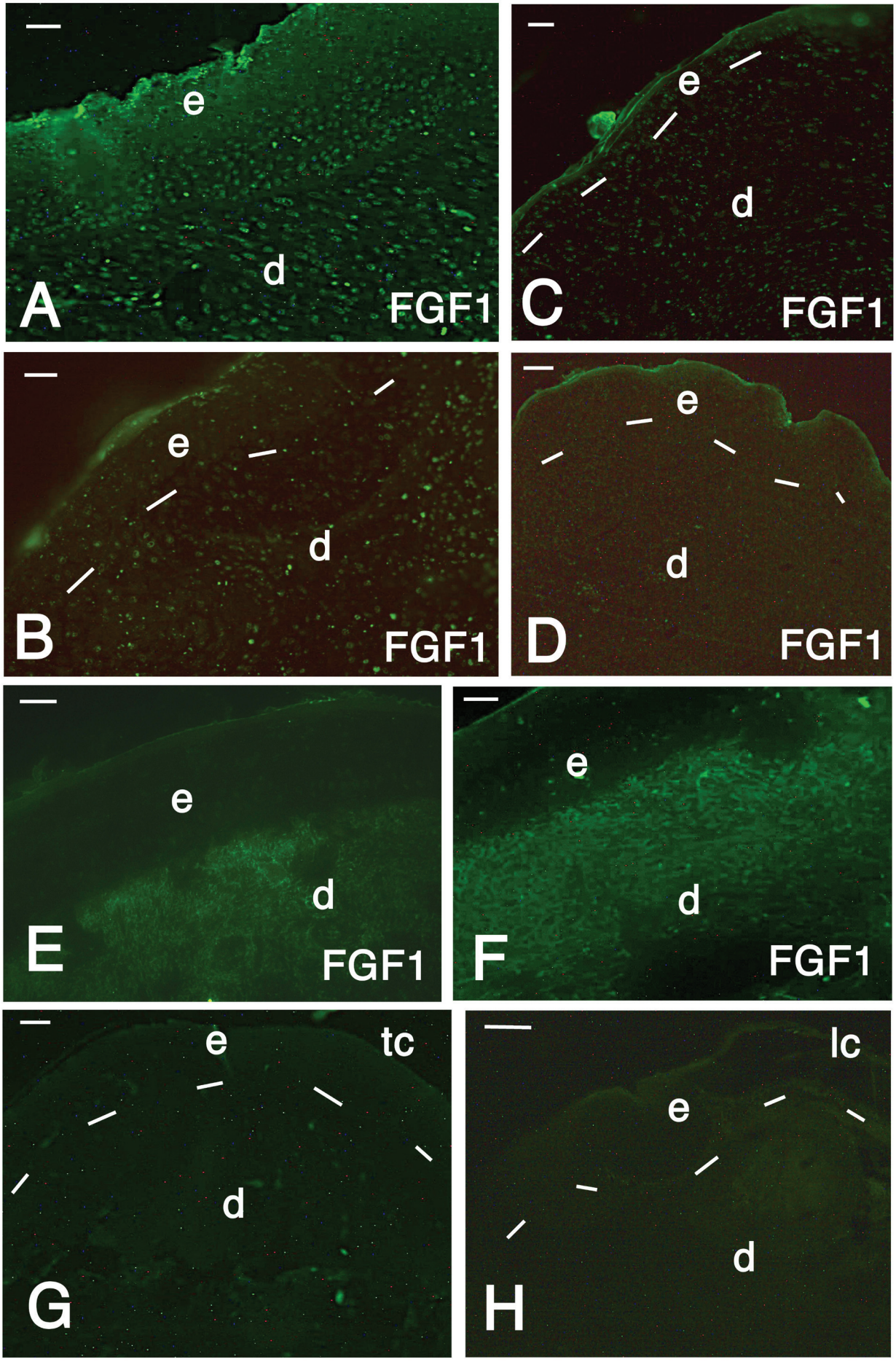
The immunogold labeling using the FGF1 antibody showed similar aspects to those observed with the FGF2 in the wound epithelium, but the nuclear labeling was often higher or similar to that present in the cytoplasm, in both epithelial and mesenchymal cells (data not shown). Also in mesenchymal cells the nuclear labeling often appeared prevalent over the cytoplasmic labeling, the latter was diffuse or more localized in the external cytoplasm of mesenchymal cells (Fig. 6A). The extracellular matrix of the cell surface (glycocalix) of blastema cells contained some gold particles that were virtually absent in the remaining extracellular matrix. The cytoplasm of endothelial cells of blood capillaries was also labeled for FGF1 (Fig. 6B). Control sections were immunonegative, as previously seen for keratinocyets of the wound epithelium.

DISCUSSION

Localization of FGF in regenerating versus non-regenerating organs

The present, qualitative observations on the regenerative blastema of the tail and early limb (12-14 days post-trauma) of the lizard *P. sicula* confirm previous immunocytochemical studies on the lizard *Lampropholis guichenoti* (Alibardi & Loviku, 2009). The study has further indicated that FGF immunoreactivity is only present in the basal layers of the normal epidermis (where cell proliferation occurs),

Fig. 3. − Immunofluorescence for FGF2 in the epidermis and mesenchyme of tail (A-B), limb (C-H), and scarring tail (I-J). **A**. at seven days the immunopositive regenerating epidermis shows an uneven boundary with the mesenchyme. Bar: 230 mm. **B**. detail of the epithelium-mesenchyme boundary at 12 days post-injury where epithelial cells appear in continuity with the mesenchyme (arrows). Bar: 20 mm. **C**. the apical wound epithelium of a tail at 16-18 days also appears in continuity with mesenchymal cells. Bar: 20 mm. **D**. the thick epithelium of a limb at 7 days post-injury appears in continuity with the mesenchyme. Bar: 20 mm. **E**. at 12-14 days the immunopositive limb epithelium appears more regular and separated from the mesenchyme. Bar: 20 mm. **F**. other limb epithelium at 12-14 days where immunolabeling is reduced. Bar: 20 mm. **G**. almost immunonegative limb epidermis at 16-18 days post-amputation. Bar: 20 mm. **H**. immunonegative epidermis and mesenchyme at 22 days post-injury. Bar: 20 mm. **I**. almost immunonegative epidermis in injured regenerating tail at 16 days post-amputation. Bar: 20 mm. **J**. apical epidermis of cicaticial tail with reduced or absent immunoreactivity. Bar: 20 mm. **Abbreviations**: bl, blastema (mesenchyme); d, mesenchymal cells of the dermis; e, wound/regenerating epithelium. Dashes underline the epidermis.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and in the wound epidermis of the tail and, initially, also in the stump of the limb, where cell proliferation and migration are active (SIMPSON, 1961; COX, 1968; ALIBARDI, 1994a, b; ALIBARDI & TONI, 2005). The immunolocalization of FGFs in proliferating endothelial cells of the capillaries also suggests these growth factors are implicated in cell division (ALIBARDI, 1993). Normal differentiated tissues (dermis, muscles, bone or cartilage, normal nerves, fat tissue etc) do not show immunoreactivity for FGFs, indicating that the factors are absent and not active in mature tissues where little cell proliferation occurs.

These new data on a reptilian species are in line with previous information on the localization and mitogenic effect of FGFs on tissue regeneration in amphibians (BOILLY et al., 2000; POULIN et al., 1995; HAN et al., 2001; GIAMPAOLI et al., 2003). The present observations further suggest that the wound epidermis of the regenerating tail in the lizard also produces FGFs. Conversely, the lack of FGF immunolocalization after 2 and more weeks from the amputation seems somehow to be connected with scarring in the limb and in the wounded tail. It is not known whether the rapid formation of a basement lamina in the limb or in the scarring tail may be consequent to the loss of FGF in the epidermis. Such a loss may prevent epidermal-dermal communication or exchange of trophic or signaling factors, and regeneration and growth would be halted.

While FGF2 remains at least in the apical proliferating epidermis of the tail, both in the naturally scarring limb wound epithelium and in that of scarring tails, it finally disappears, an indication that cell proliferation rapidly terminates in these tissues. Moreover, the epithelium forms a differentiated basement membrane and also a hard corneous layer like the corneous layer present in normal, mature scales. The potential to form an AEC containing FGF, which appears possible in the tail blastema, is therefore impeded in both the limb and in the scarring tail. In conclusion, the present study has shown that also in the lizard, an amniote with high regeneration ability in the tail, the higher levels of FGFs are present in regenerating tissues. Future studies should evaluate whether specific FGFs such as FGF8, FGF10, or FGF7, are present in regenerating vs non-regenerating lizard tissues.

Ultrastructural localization and epithelium-mesenchymal communication

The present ultrastructural study has also indicated that diffuse cytoplasmic and nuclear distributions of FGF1 and FGF2 are present in regenerating tissue, especially in keratinocytes of the wound epithelium more so than in blastema cells. FGF is also localized in endothelial cells of forming blood vessels. The study brings further evidence that an epithelial-mesenchymal transformation/transition (EMT) also occurs during tail and limb regeneration in lizards (ALIBARDI, 2010a,b). The EMT is a process that occurs during embryogenesis (epithelium into mesenchyme, see HAY, 1995), inflammatory reactions in various adult organs (KALLURI & NEILSON, 2003; IWANO et al., 2002), and in cancer (RADISKY, 2005; LEE et al., 2006; KLYMKOWSKY & SAVAGNER, 2009).

The immunolocalization at the ultrastructural level of FGFs in different cells of the lizard blastema has shown no specific organelle

Fig. 4. − Immunofluorescence for FGF1 in the limb (A-D), scarring tail (E-F), and in controls (G-H). **A**. regenerating skin with thick epidermis of a limb at 12 days post-amputation. Bar 20 mm. **B**. limb skin at 16-18 days with no immunofluoerence in either epidermis or dermis. Bar: 20 mm. **C**. immunonegative limb skin at 22 days post-amputation. Bar: 20 mm. **D**. limb scarred skin at 35 days post-amputation with reactive dense dermis. Bar: 20 mm. **E**. immunonegative tail scar skin in both epidermis and dermis (21 days post-amputation). Bar: 20 mm. **F**. other tail scar at 35 days post-amputation with immunofluorescent dermis. Bar 20 mm. **G**. serum control for the tail. Bar: 20 mm. **H**. serum control for the limb. Bar: 20 mm. **Abbreviations**: d, mesenchymal cells of the dermis; e, wound/regenerating epithelium; lc, serum control for the limb; tc, serum control for the tail.

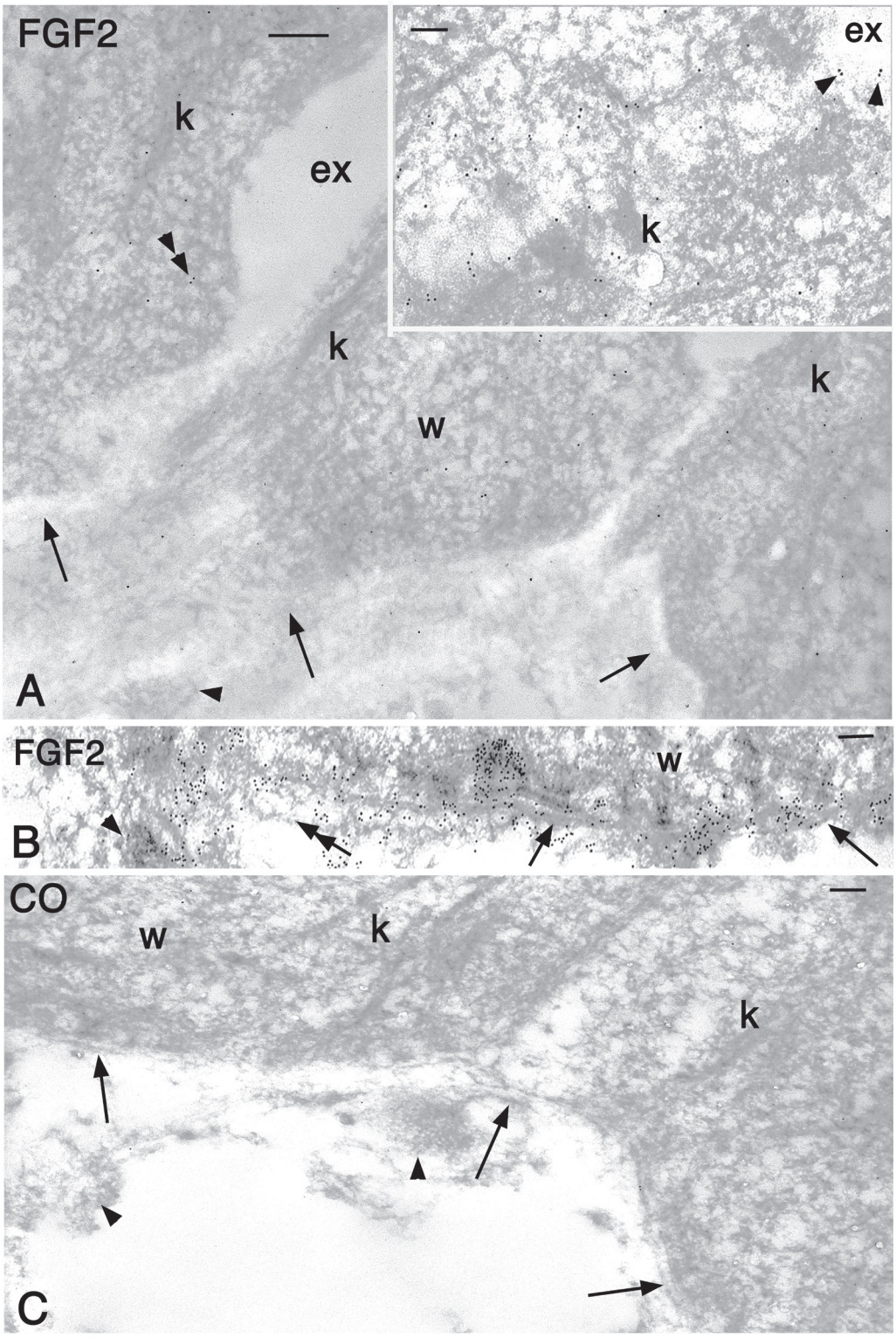

Fig. 5. − FGF2 immunogold-labeling of tail apical wound epithelium. **A**. detail of basal part of the wound epithelium with wavybasement membrane (arrows). The arrowhead indicates likely dermal cell process. The double arrowheads indicate the loose keratin network present in these cells. Bar: 200 nm. The inset (Bar: 100 nm) shows the diffuse labeling in the cytoplasm of a wound keratinocyte, and around vesicles (arrowheads). **B**. detail of intense labeling along the incomplete basement membrane (arrows) underlying apical wound keratinocytes. The arrowhead indicates some likely hemi-desmosomal material. Bar: 100 nm. **C**. immuno-negative control detail of the basal cytoplasm of wound epithelium cells with basement membrane (arrows). Arrowheads indicate amorphous extracellular material. The double arrow indicates the amorphous part of the basement membrane. Bar: 100 nm. **Abbreviations**: ex, extracellular space among keratinocytes; k, keratin bundle; w, wound epithelium.

distribution as this growth factor is synthesized and apparently released through a non conventional, ER- and Golgi-independent mechanism of cellular extrusion (NICKEL & SEEDORF, 2008). In the regenerating wound epithelium of lizard, keratinocytes probably produce an increased quantity of FGFs that may possibly relate to the EMT.

The passage of FGF through the basement membrane is strongly suggested by the present TEM observations. Our study indicates that FGF2 accumulates along the immature basement membrane of the wound epithelium, and it is most likely released by the wound keratinocytes. The observed immuno-localization suggests that the continuous production of FGFs from regenerating keratinocytes can locally stimulate blastema cells to proliferate, as has been previously indicated for the blastema of amphibians (BOILLY et al., 2000; POULIN et al., 1995; HAN et al., 2001; GIAMPAOLI et al., 2003). The extrusion of FGF through the plasma membrane following a diffusion mechanism driven by the extracellular capture of FGF2 by heparan sulphate has been indicated as a characteristic of the extracellular release of FGF2 (NICKEL & SEEDORF, 2008).

A free cytoplasmic localization of FGF2 has been reported for fibroblasts (AKTAS & KAYTOU, 2000) and developing neurons but not in glial cells (JANET et al., 1987). Another study however found that FGF2 was present in both the cytoplasm and nuclei of astrocytes and in a few types of adult neurons (WOODWARD et al., 1992). Other ultrastructural studies on FGF1 localization have indicated that this growth factor is also present within stimulated adult neurons but not extracellularly (ELDE et al., 1991). Only in mastocytes is bFGF prevalently associated with secretory granules, and therefore in these cells FGF2 follows the classic secretory pathway (QU et al., 1998).

In blastema cells of the lizard tail, the diffuse immunolocalization of FGFs in the cytoplasm and glycocalix suggests that they may act in an autocrine manner on cell proliferation, as postulated for amphibian blastema. The nuclear localization of FGF1 has been reported also in previous work on fibroblasts (AKTAS & KAYTON, 2000), neurons (Janet et al., 1987; ELDE et al., 1991), and astrocytes (WOODWORTH et al., 1992). These results indicated that immunodetection of these molecules may vary in relation to the physiological or differentiative state of these cells.

In conclusion, the present morphological study indicates that a process of EMT is operating during early stages of tail and limb regeneration in lizards (an amniote model of regeneration, see ALIBARDI, 2010b), and that FGFs, especially FGF2, are involved in maintaining a growing front for tail regeneration, a process initially present but soon aborted in the scarring limb or tail. It is not known whether the observed FGF-immunoreactivity may also be due to more specific forms of FGFs (eg FGF8 or FGF10) or to a potential lizard KGF (FGF7), the typical growth factor for the epidermis and hairs in mammals (GUO et al., 1996; ANDREADIS et al., 2001).

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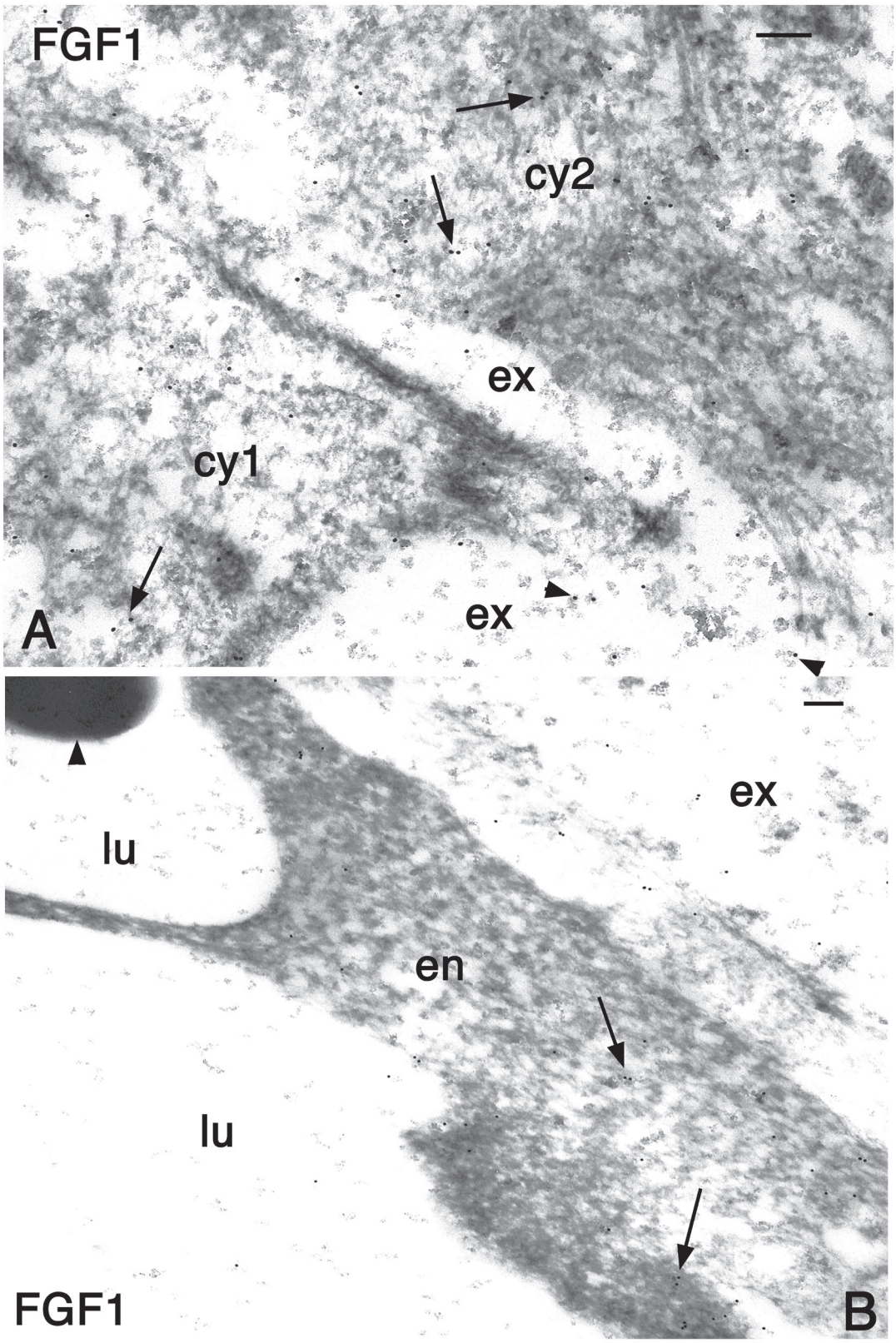

Fig. 6. − FGF1 immunogold-labeling of blastema (mesoderm, **A**) cells and endothelial cell (**B**). **A**. diffuse FGF1 immunolabeling (arrows) in two blastema cells with few gold particles present in the extracellular space close to the surface (arrowheads). Bar: 100 nm. **B.** endothelial cell with diffuse immunolabeling (arrows). The arrowhead indicates a red blood cell. Bar: 100 nm. **Abbreviations**: cy1 and cy2, cytoplasm of cell one and two; end, endothelial wall; ex, extracellular space; lu, lumen of the capillary.

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Distribution of anticoagulant resistance in the brown rat in Belgium

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ABSTRACT. Anticoagulant resistance is known as one of the major factors interfering with rodent control. Within this context we investigated the distribution of anticoagulant resistance in Flanders, northern Belgium. From 2003 to 2005, we tested 691 rats from different locations with blood clotting response tests for their susceptibility to the anticoagulant compounds warfarin, bromadiolone and difenacoum. Of these, 119 were also screened for a mutation in the VKORC1 gene that is suspected to be responsible for anticoagulant resistance. Warfarin resistant rats were found in the western and eastern parts of Flanders. The same distribution pattern was found for bromadiolone with the exception of the south-eastern area, where this form of resistance was largely absent. We detected difenacoum resistance in only six rats and did not observe any resistant rats in the central part of Flanders. Susceptible rats were found all over Flanders. Genetic analyses showed that anticoagulant resistance in Belgium was related to two different mutations in VKORC1, namely Y139F and L120Q. Our results indicate that rodent control should be regionally tailored to be most effective.

KEY WORDS. blood clotting response, rodent control, warfarin, bromadiolone, difenacoum, VKORC1

INTRODUCTION

Through the ages brown rats have been poisoned because of the damage they cause and the diseases they carry (MEEHAN, 1984; GRATZ, 2006; HEYMAN et al., 2009). Before the discovery of warfarin, rodent control was mostly achieved with acute poisons (BUCKLE, 1994a). With the introduction of warfarin and related anticoagulant compounds, also known as coumarins, a new class of rodenticides became available in the 1940s. The delayed action of anticoagulants does not cause bait-shyness and makes them particularly suitable for the control of a neophobic species such as the brown rat. Furthermore they are relatively safe, due to the existence of the antidote vitamin K1. As a result, rodent control became largely an issue of chemical intervention with less emphasis placed on sanitation and exclusion measures (FRANTZ & PADULA, 1998; PELZ et al., 2005). Since the 1950s, anticoagulants have been the most widely used rodenticides (MEEHAN, 1984).

Coumarins act as a vitamin K antagonist and block the vitamin K cycle in the liver, preventing the reduction of vitamin K epoxide to vitamin K by vitamin K epoxide reductase (VKOR). Vitamin K is an essential co-factor in the activation of several vitamin K-dependant coagulation factors through which it plays an important role in blood coagulation (OLDENBURG et al., 2008). When coumarins bind with VKOR, intoxication with anticoagulants will lead to a deficiency of vitamin K and coagulation factors, causing coagulation disorders such as spontaneous bleeding and eventually death. In resistant rats, VKOR is slightly modified and prevents a proper binding with the rodenticide, which thus fails to work (THIJSSEN, 1995). This mechanism is based on a single nucleotide polymorphism (SNP) in the VKORC1gene, which codes for the VKOR enzyme (ROST et al., 2004). At least eight different SNPs are related to anticoagulant resistance in the brown rat. In Belgium, as in France, the mutation known as TAT-139-TTT or Y139F is present (PELZ et al., 2005; GRANDEMANGE et al., 2010).

After the first discovery of warfarin-resistant rats in Scotland in 1958 (BOYLE, 1960) other foci of resistance arose in Wales and southern England in the 1960s (KERINS et al., 2001). On the European continent the first traces of resistance were found in Denmark in 1962 (LODAL, 2001). About ten years later it also occurred in Germany (PELZ, 1995) and in the meantime this trait developed in North Carolina USA (FRANTZ & PADULA, 1998). Furthermore, resistance to first-generation anticoagulants such as warfarin has also been found in France, Japan, Brazil, Portugal, Italy and Canada (MACNICOLL & GILL, 1987; GREAVES, 1994; PELZ et al., 2005).

Due to increasing warfarin resistance, the industry developed stronger second-generation products such as bromadiolone, difenacoum, brodifacoum and difethialone. These rodenticides were also based on the 4-hydroxycoumarin structure, but with increased lipophilicity and thus prolonged half-lives (ATTERBY et al., 2001). Unfortunately, these stronger anticoagulants present a greater risk of primary intoxication of non-target species and secondary intoxication of scavengers and predators (BRAKES & SMITH, 2005; HOARE & HARE, 2006).

Rodent pest management today depends on the anticoagulant rodenticides because of their outstanding efficiency and excellent safety profile. Monitoring for resistance is important if we are to understand the scope of its spread and to manage resistant rodent populations (BUCKLE, 2006). Resistance is defined by the European and Mediterranean Plant Protection Organization as follows; "Rodenticide-resistant rodents should be able to survive doses of rodenticide that would kill 'normal' or 'susceptible' conspecifics" (EPPO, 1995). GREAVES, (1994) describes anticoagulant resistance as a major loss of efficacy in practical conditions where the anticoagulant has been applied correctly, the loss of efficacy being due to the presence of a strain of rodent with a heritable and commensurately reduced sensitivity to the anticoagulant. In this study, resistance is based upon a positive blood clotting response (BCR) test result.

In Denmark, but also in Germany and the United Kingdom, scientists monitored the evolution and distribution of resistance (KERINS, 2001; LODAL, 2001; PELZ, 2001). In these countries, they observed that resistance expanded geographically and towards stronger active ingredients e.g. bromadiolone and difenacoum. In the United Kingdom brodifacoum resistance has also been reported (GILL et al., 1992). Resistance to different anticoagulants is known as cross resistance, and evolves often from first- to second-generation anticoagulants (also known as resistance hierarchy) (PELZ, 1995). This means that resistance to anticoagulants of higher potency will always be accompanied by resistance to compounds of lower potency. LUND (1984) mentioned anticoagulant resistance in house mice in Belgium, but no data concerning resistant rats were provided. The work reported here is the first documented study of the distribution of anticoagulant-resistant rats in Belgium. The aim of this study was to assess the presence of any resistant rats in Flanders and to study the extent of this resistance trait, both geographically as well as functionally. More specifically, we wanted to test whether resistance in brown rats was restricted to warfarin, or whether it extended to frequently-used second-generation anticoagulants like bromadiolone and difenacoum. In a later stage, we examined DNA samples to find out if mutations in the VKORC1 gene could explain our positive BCR results.

MATERIALS AND METHODS

Rats

Wild rats (*Rattus norvegicus*) were captured using live traps and caged individually in our laboratory for animal science. Once in the lab they received Carfil Quality maintenance rat food and fresh tap water. No extra menadione or vitamin K3 was administered. The rats were kept in the laboratory for at least three weeks before testing, in order to exclude rats in gestation and diseased or intoxicated rats.

The use of living rats in our study was approved by the local ethics committee of 'The Institute for Agricultural and Fisheries Research of the Flemish Government' and was in agreement with the legislation on laboratory animal science.

Blood clotting response test

To distinguish resistant from susceptible rats we applied a blood clotting response (BCR) test, involving the measurement of changes in coagulation time after the administration of a small dose of anticoagulant. For the detection of warfarin resistance, we used the BCR test described by MARTIN et al. (1979) and MACNICOLL & GILL (1993). BCR tests for bromadiolone and difenacoum resistance were carried out according to GILL et al. (1993, 1994). Briefly, the rats received a solution of anticoagulant (2ml/kg) by oral gavage. The concentration used for warfarin and difenacoum was 0.25% (dose 5mg/kg). For bromadiolone, male and female rats were given a solution of 0.05% (dose 1mg/kg) and 0.12% (dose 2.4mg/kg) respectively. After 24 hours for warfarin and 96 hours for bromadiolone and difenacoum, we took blood by means of a retro-orbital puncture and measured the prothrombin time (PT) (Coadata 501 coagulometer for whole blood, Helena capillary reagent rabbit brain thromboplastin). We converted the PT into the percentage coagulation activity (PCA) by means of a calibration curve based on a dilution series of a mixed blood sample of five Wistar rats for each sex. The cutoff point for warfarin resistance was a PCA of 17%, for bromadiolone and difenacoum this was a PCA of 10%. Warfarin-susceptible rats were euthanized after the experiment. Due to resistance hierarchy, we considered them as also bromadiolone and difenacoum susceptible. Warfarin-resistant rats were subsequently tested with bromadiolone and difenacoum. The minimum interval between warfarin and bromadiolone BCR tests was one week, while between bromadiolone and difenacoum tests it was six weeks. Anticoagulant administration and blood sampling were performed under isoflurane anaesthesia.

Genetic analysis

Rat DNA was extracted from tissue samples (tail tip) with the Qiagen tissue kit (Qiagen). PCR amplification of part of exon 3 of the VKORC1 gene was performed using the primers and conditions described in PELZ et al. (2005). The presence of a mutation in individual samples was analysed by temperature gradient capillary electrophoresis (TGCE) (CHOU et al., 2005) on a SCE9610 Genetic Analyzer (Spectrumedix Inc.) applying a 45-55 °C gradient with a ramp period of 24 minutes. Electropherograms were analysed with the Revelation 2.41 software (Spectrumedix). Samples showing a heteroduplex were considered as heterozygous mutants. Samples showing a homoduplex were further analyzed by mixing the test sample with a known homozygous wild type (WT) reference sample and repeating the TGCE analysis to distinguish between homozygous mutants and WT. In this way, homozygous WT animals could be discriminated from homozygous and heterozygous mutants.

Samples from animals carrying a mutation as revealed by TGCE were further analysed using an allele-specific amplification refractory mutation system (ARMS) PCR (YE et al., 2001) for the presence of the Y139F mutation, which has been previously detected in Flanders (PELZ et al., 2005). As the mutation Y139F is different from the one published (Y139C), the original inner ARMS primers were slightly modified: F-primer: 5'-TGATTTCTGCATTGTTTGCATCACCAC GTT-3' and R-primer: 5'-CAACATCAGGCCC GCATTGATGGAAT-3'. Amplification products were analyzed by agarose gel electrophoresis.

Some of the samples that were negative in the ARMS PCR were sequenced and revealed the presence of the L120Q mutation (PELZ et al., 2005). For this mutation, a new ARMS PCR was developed with inner primers F: 5'-TGGTGTCTGTCGCTGGTTCTCTGTAGC A-3' and R: 5'-ATACAGGACAAAGAACAGG ATCCAGGGCA-3'.

For routine analysis, the forward inner primers of the mutations Y139F and L120Q were labelled with an FAM-dye and a NED-dye, respectively, and analysed simultaneously on the SCE9610 sequencer. Results were analysed with the Genospectrum v3.0.0 software (Spectrumedix).

Statistical analysis

We used a Log-Linear analysis to test, per river catchment, whether more rats than expected were resistant to warfarin and subsequently to bromadiolone. The analysis was based on the Fisher exact test and the level of significance was corrected for multiple testing (Table 1).

To measure the level of agreement between the BCR test and the genetic analysis we calculated kappa. Kappa expresses the proportion of agreement beyond chance and is only valuable when the results of both tests are not significantly different and the prevalence is between 0.2 and 0.8 (Dohoo et al., 2003).

RESULTS

From 2003 to 2005 we tested 691 rats from different locations for warfarin resistance. Of these, 550 had a PCA less than 17% and 141 rats had a PCA above 17% and were respectively warfarin susceptible and resistant. Between the BCR tests for warfarin and bromadiolone resistance, 19 rats died. Consequently only 122 warfarin-resistant rats were tested with bromadiolone. Of these, 88 were also bromadiolone resistant. Between the bromadiolone and difenacoum BCR tests we lost 28 rats. Six bromadiolone resistant rats were also difenacoum resistant.

Because advances in genetic research on resistance took place after the onset of this study we were not able to test all our rats for mutations in VKORC1. We screened 26 susceptible and 93 resistant rats for the presence of a mutation in VKORC1. None of the 26 susceptibles carried a mutation in VKORC1 but 87 resistant rats did. Six rats that had a positive BCR result for warfarin resistance did not carry a mutation in VKORC1; their PCA varied between 17 and 27 %. All the bromadiolone and difenacoum resistant rats tested genetically carried a mutation. Two different mutations were found in exon 3. Mutation one showed an SNP in codon 139 where adenine was replaced by thymine (TAT-139-TTT), which resulted in a replacement of the amino acid tyrosine by phenylalanine in VKOR (Y139F). The second mutation was found in codon 120 where thymine was replaced by adenine (CTG-120-CAG) and the amino acid leucine was substituted by glutamine (L120Q).

The results of the BCR test and the mutation screening were not significantly different and the prevalence was between 0.2 and 0.8. With kappa=0.864, CI 95%: 0.758-0.969 higher than 0.8 both tests agreed almost perfectly (Dohoo et al., 2003).

The geographical distribution of resistant brown rats in Flanders was significantly different from random (Table 1, Fig. 1). We used the existing river catchments as geographical units. Anticoagulant resistance was found in three different regions. In the southeast region, which corresponds to the Demer river catchment, 26 rats were resistant to warfarin. Only four of these rats were bromadiolone resistant, resulting in significantly less bromadiolone resistance than expected (Table 1). One rat was also difenacoum resistant. Between bromadiolone and difenacoum BCR tests there was a significantly higher loss of rats; 14 out of 25 rats compared to 14 out of 97 rats for the other regions. All 26 warfarin-resistant rats were tested genetically and carried mutation L120Q.

In the west and in the east of Flanders, we saw that the majority of the warfarin resistant rats (91%) were also resistant to bromadiolone. Five of these bromadiolone resistant rats were resistant to difenacoum. The rats in this region carried mutation Y139F. In the central part the resistance trait was absent.

TABLE 1

Warfarin- and bromadiolone-resistant rats (by BCR test) per river catchment. 691 rats were tested with warfarin, of which 141 were warfarin resistant. Of these rats 122 animals were also tested with bromadiolone. The geographical distribution of warfarin- and bromadiolone-resistant brown rats in Flanders was significantly different from random. WS: warfarin susceptible, WR: warfarin resistant, BS: bromadiolone susceptible, BR: bromadiolone resistant, level: level of significance based on Fisher exact and corrected for multiple testing.

RIVER CATCHMENT	WS	WR	level	BS	BR	level
1 Yser	30	18	ns	5	11	ns
2 Bruges Polder	20	13	ns	0	11	ns
3 Ghent Canals	30	4	ns	2	2	ns
4 Lower Scheldt	145	6	<0.001	1	3	ns
5 Leie	47	32	<0.001	4	24	ns
6 Upper Scheldt	0	2	ns	0	1	ns
7 Dender	81	1	<0.001	0	1	ns
8 Dijle	38	0	<0.01	0	0	ns
9 Demer	61	26	ns	21	4	<0.001
10 Nete	49	0	<0.001	0	0	ns
11 Meuse Antwerp	7	0	ns	0	0	ns
12 Meuse Limburg	42	39	<0.001	1	31	<0.01
total	550	141		34	88	

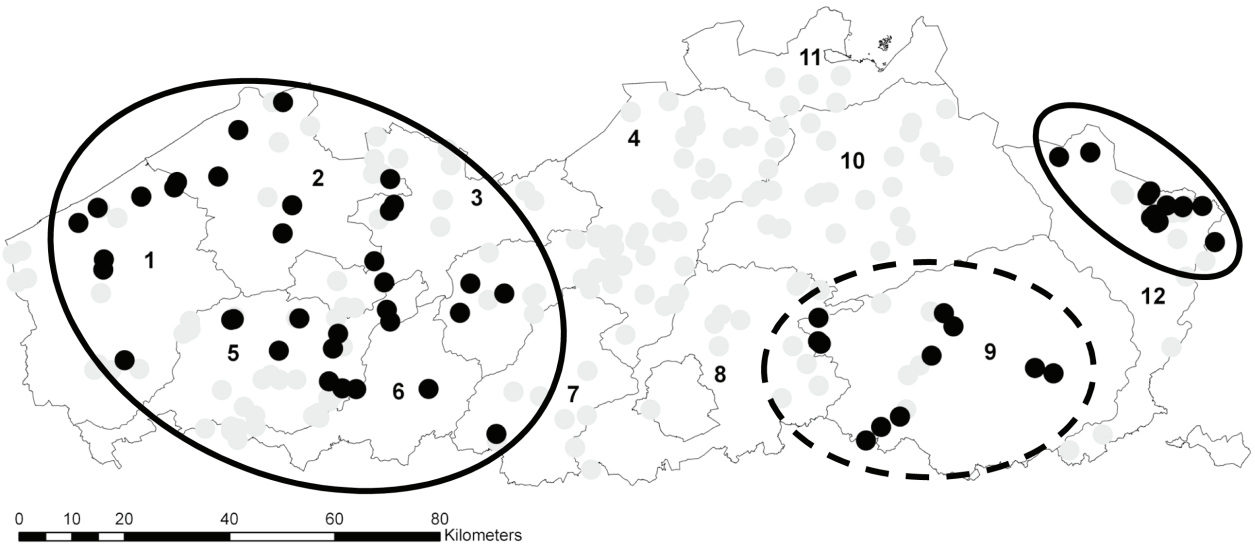

Fig. 1. − Three different areas with resistance were found in Flanders. In the west and east these areas were characterised by bromadiolone resistance (full line) and linked to mutation Y139F in VKORC1. In the south-east the area was characterised by warfarin resistance (broken line) and the presence of mutation L120Q. The grey dots represent locations where only susceptible rats were found. The black dots stand for locations where at least one resistant rat was caught. The numbers correspond with the river catchments in Table 1.

DISCUSSION

This study shows that resistance to anticoagulants also occurs in Flanders, with a clear distribution pattern caused by a different genetic background and resistance to different coumarins. In a small region such as Flanders, which is one of the most densely populated and urbanised areas in Europe (EEA 2007) and is characterized by a large amount of traffic along roads and watercourses enhancing rat migration, we expected less regional variation in anticoagulant resistance. We know that brown rats are widespread (STUYCK, 2003) and the availability and use of different types of anticoagulant rodenticides does not differ much between localities. Therefore we believe that neither increased rodent control intensity, due to higher rat densities, nor different use of poisons, is responsible for the different levels of resistance. Similarly, we can not explain the lack of resistance in the central part of Flanders; in Upper-Scheldt, Dender, Dijle and Nete river catchments, significantly fewer than expected resistant rats were recorded (Table 1). Data from other countries such as Denmark, Germany and the United Kingdom has shown that anticoagulant resistance expands (KERINS, 2001; LODAL, 2001; PELZ, 2001), both geographically and functionally, from first to second generation rodenticides. The situation observed in Flanders provides a unique opportunity to follow the trend of resistance in a resistance-free area, surrounded by areas with resistant rats.

In the west and east of Flanders, we found warfarin-resistant rats, most of which were also bromadiolone resistant. Only a few of these bromadiolone resistant rats were difenacoum resistant and this to a lesser degree than to bromadiolone. Therefore we believe that the resistance hierarchy in both areas was as follows: warfarin < bromadiolone < difenacoum. The same hierarchy pattern was previously found in Denmark and Germany (PELZ et al., 1995). The rats in these regions of Flanders carried mutation Y139F. This mutation is common in France where it also confers resistance to bromadiolone (GRANDEMANGE et al., 2009). More recently it was also found in Korea and in the UK (ROST et al., 2009; PRESCOTT et al., 2011), the latter in a place where applications of the anticoagulant rodenticide bromadiolone had been unsuccessful. The situation in France as well as in the UK is consistent with our findings.

In the Demer river catchment, the majority of the resistant rats were resistant only to warfarin, with only a few rats also testing bromadiolone resistant. Compared to other river catchments this difference was significant (Table 1). Additionally, the bromadiolone-resistant rats showed a BCR test result for bromadiolone close to the cutoff point, indicating a very low degree of resistance. We also noticed a major loss of rats between the bromadiolone and difenacoum BCR tests. Such a high mortality after a BCR test is exceptional as the doses used in these tests are considered as non-lethal (GILL et al., 1993). This observed mortality confirmed the presence of fewer bromadiolone resistant rats and resulted in a possible underestimation of difenacoum resistance. Moreover, the only difenacoum-resistant rat caught in the Demer river catchment showed a higher level of difenacoum resistance than bromadiolone resistance. The much lower degree of bromadiolone resistance and a possibly different resistance hierarchy, can be explained by the presence of another resistant strain in the Demer river catchment. All the resistant rats in the Demer carried mutation L120Q, a mutation initially found in the Berkshire and Hampshire strain in the UK (PELZ et al., 2005), and later, also in France (GRANDEMANGE et al., 2010). These strains in the UK are known for their difenacoum resistance (GILL et al., 1993), but in our study this mutation's contribution to anticoagulant resistance is mainly restricted to the first generation rodenticide, warfarin.

At the beginning of this study, we chose to work with BCR tests to evaluate anticoagulant resistance in Flanders. At that time, it was probably the best solution as the BCR tests then had replaced feeding tests for reasons of accuracy and animal welfare (KERINS et al.,

2001). Nowadays, often only genetic tests are used to evaluate the presence of resistance. It is not clear how closely positive BCR test results correlate with the definition of GREAVES (1994), which emphasises major loss of efficacy of the rodenticide in practical conditions. Although BCR tests provide no direct indication of the practical impact of the resistance observed (BUCKLE, 1994b), we now know that a positive BCR result has a high, positive predictive value for the presence of an SNP in the VKORC1 gene (PELZ et al., 2005), which certainly contributes to resistance. To assess the practical implications of anticoagulant resistance on rodent control using the BCR test, it is possible to work with a resistance factor based on the multiple of the discriminating dose as suggested by PRESCOTT et al. (2007). In our opinion, the major advantage of a BCR test remains the fact that it measures the effect caused by the rodenticide itself apart from the resistance mechanisms behind it. This means that changes in pharmacodynamics or pharmacokinetics (MARKUSSEN et al., 2008) that differ from changes in VKORC1 will also be detected. A disadvantage of the BCR test is that rats trapped in the wild could bias the BCR test result because of an earlier bait uptake in the field. Indeed, the half-life of second-generation anticoagulants in the liver can extend to 300 days (EPA 2007). The same bias also plays a role with subsequent testing of different compounds in the lab. At the beginning of our study, we found that an interval of three weeks between the bromadiolone and difenacoum BCR tests was not enough to normalise the PT within its normal range. As a result, we extended the interval up to six weeks for all the following tests to normalize the PT. However, this still did not guarantee the absence of any negative effects on the difenacoum BCR result. For these reasons, we believe that it is better not to test rats with more than one second generation anticoagulant, as this can lead to an underestimation of the resistance level. But more importantly, BCR tests should be part of each resistance screening as they indicate resistance independent of the mechanism behind it.

The BCR tests we used were based on the methods described by MARTIN et al. (1979), MACNICOLL & GILL (1993) and GILL et al. (1993, 1994). It was PRESCOTT et al. (2007) who re-evaluated these BCR tests and designed the standardised BCR (SBCR) test. This is a sensitive method designed to detect the slightest form of anticoagulant resistance. The discriminating dose was determined by using a group of susceptible rats. To predict the likely impact on field control, a resistance factor based on the multiple of the discriminating dose is used. The SBCR is based on the International Normalised Ratio (INR) and allows comparison of blood clotting data obtained by different thromboplastin reagents used in different labs. This is not possible with PCA values. As a consequence, the thresholds of PCA we used –17% for warfarin and PCA, 10% for bromadiolon and difenacoum, corresponding with INR values of about 3.5 and 7 respectively – probably do not match the thresholds used in the original BCR tests. The usual threshold for the SBCR test is an INR value of 5, and the time between administration of the anticoagulant and the blood sampling is reduced for all anticoagulants to 24h.

For the detection of warfarin resistance we used a discriminating dose of 5mg/kg versus 3.02 mg/kg for male and 4.26 mg/kg for female rats used in the SBCR test. A higher discriminating dose together with a lower threshold means that we probably underestimated warfarin resistance compared to the SBCR test. A correction for the different threshold alone means that about 12% of the rats that we regarded as susceptible should have been resistant under the SBCR test. This rather small difference in result between the BCR and the SBRC tests can be explained by the variation in our results. About 75% of the warfarin resistant rats have a PCA>30% or INR<2 and about 88% of the susceptible rats have a PCA<12% or INR>5. This means that about 15% of the rats that we tested were in the range of INR 2 – 5. For this minority of rats, it is not always possible to tell whether they are resistant or susceptible, an uncertainty which also exists when using the SBCR test, since the cut off for that test was arbitrarily defined (PRESCOTT

et al., 2007). This again shows that resistance to anticoagulants is not clear-cut. Based on our and previous results (PELZ et al., 2005), it seems that mutations in VKORC1, resulting in changes in pharmacodynamics, contribute more clearly to anticoagulant resistance as illustrated by the high kappa value we found. Since the mutations in VKORC1 are probably responsible for the variation in blood clotting between the groups of resistant and susceptible rats, variation within the groups and overlap between the groups could then be explained by changes in metabolism or pharmacokinetics (MARKUSSEN et al., 2008).

Comparison of our results for bromadiolone and difenacoum resistance in the light of the SBCR is complex. The major advantage of second-generation anticoagulants in rodent control is their prolonged half-life, which is dependent on changes in clearance and apparent volume of distribution (BRECKENRIDGE et al., 1985). As a result, the plasma concentration and, therefore, also the effect of the rodenticide, remain higher for a longer period. In the SBCR, the interval between administration and blood sampling is reduced from 96h to 24h, to exclude changes in clotting time by pharmacokinetically-based effects (PRESCOTT et al., 2007). By shortening the interval, however, the beneficial effect of the prolonged half-life of second-generation anticoagulants is untested, as is its benefit in the context of anticoagulant resistance. Furthermore, there are indications that changes in pharmacokinetics affect anticoagulant resistance in brown rats (MARKUSSEN et al., 2008). For the same reason HEIBERG (2009) did not shorten the time interval of her BCR tests.

CONCLUSIONS

In Flanders, the degree of resistance to different anticoagulants used in rodent control showed a clear geographical distribution and was linked to the presence of two different mutations in VKORC1. One strain of rats in the west and the east of Flanders was characterised by its resistance to warfarin and bromadiolone apparently related to mutation Y139F in VKORC1. Another strain located in the southeast, a region corresponding with the Demer river basin, was mainly warfarin-resistant. Here, resistance was linked to mutation L120Q. In both strains, the first signs of difenacoum resistance had appeared. The central part of Flanders did not reveal any resistant rats and no mutations in VKORC1 were found.

Our results show that resistance monitoring should be an essential part of adaptive rodent management when confronted with rodent control failure caused by anticoagulant resistance.

We suggest that future research should focus on resistance monitoring not only in the central area of Flanders, where this trait is currently lacking but also in the resistant areas where we could determine possible changes in resistance prevalence and hierarchy. The BCR test and the detection of mutation in VKORC1 resulted in a similar outcome for warfarin resistance. Despite their close agreement, a combination of genetic and (S)BRC tests should be used, especially for second generation anticoagulants and as long as some of the mechanisms causing anticoagulant resistance remain unclear.

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Differences in field behavior between native gastropods and the fast-spreading invader *Arion lusitanicus* auct. non Mabille

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ABSTRACT. Dispersal is a crucial process for population exchange and expansion, and traits that facilitate dispersal may be positively selected during biological invasions. Here, we performed a basic study on differences in behavior between the slug *Arion lusitanicus* auct. non Mabille, 1868 (Gastropoda: Pulmonata), which is considered to be one of the 100 worst invasive species in Europe, and native gastropods. We assumed that the species is more active and less sensitive to otherwise aversive stimuli, and thus more likely to utilize novel environments. We quantified field densities and performed pitfall trap studies in 15 differently-structured habitats (urban, grassland, succession, riverine forest) in the floodplain of the LTER (Long Term Ecological Research) site 'Rhine-Main-Observatory' in Hesse, Germany. Here, *A. lusitanicus* was naturalized and scored 15 in terms of abundance rank, but was the dominant species in terms of trappability with the acidic Renner solution. A more detailed approach with a set of different baits showed that individuals of the invader were attracted to the acidic Renner solution, mustard oil, and garlic extract, all of which the native snails and slugs avoided. The results support the hypothesis that the invasive slug differs from other gastropods in its behavioral response to unusual, novel stimuli that may indicate some potential threat to other gastropod species. Future studies are needed to show if this behavior is related to personality traits such as exploration, boldness and risk-taking, and if it may have been positively selected in the context of the slug being passively spread in severely-transformed habitats such as gardens and greenhouses.

KEY WORDS. behavioral ecology, non-native organisms, dietary conservatism, foraging, trap efficiency

INTRODUCTION

Dispersal and colonization success are the drivers for gene flow and population dynamics in the metapopulation framework, and among the main issues in restoration ecology and invasion biology (Bowler & Benton, 2005). Dispersal creates a spatial response to environmental changes. An increase in dispersal ability can reduce losses otherwise associated with reaching distant resources. Indeed, it has been noted that individuals at the dispersal front differ from those of well-established or only slowly expanding populations in morphology (e.g. Phillips et al., 2006; Hassall et al., 2009) and in behavior (e.g. Alford et al., 2009). Similarly, individuals from invasive species differ from those of non-invasive species in their dispersal-related behavior (e.g. Schöpf Rehage & Sih, 2004; Cote et al., 2010).

Knowledge of behavioral differences between invasive and native species is thus crucial for a better understanding of the mechanisms underlying invasion success and to predict the spread of invasive species. However, studies on dispersal-related behavior have so far focused on fast-moving vertebrates or arthropods. Yet, there are several highly successful slow-moving invaders that may serve as models for the study of dispersal-related behavioral traits. For example, the Lusitanian slug *Arion lusitanicus* auct. non Mabille, 1868 (in some publications syn. *A. vulgaris* Moquin-Tandon, 1855) scores

among the 100 worst invasive pest species in Europe (DAISIE, 2010). The species probably originates from SW Europe, has been spread over large parts of the rest of Europe during the last decades (KOZŁOWSKI, 2007), and has also been introduced to the USA (DAISIE, 2010). It has most likely been repeatedly introduced by ornamental plant trade (e.g. SCHMID, 1970), and rapidly spread thereafter. It is a notorious feeding generalist (e.g., BRUELHEIDE & SCHEIDEL, 1999; BRINER & FRANK, 1998; KOZŁOWSKI, 2007), displays high life-time productivity (KOZŁOWSKI, 2007) and is capable of self-fertilization (e.g. HAGNELL et al., 2006), although this is not its dominant mode of reproduction (ENGELKE et al., 2011). While eggs and juveniles are the main targets of beetle predation in the invaded range, the predators are obviously ineffective in controlling slug abundance (e.g. HATTELAND, 2010; HATTELAND et al., 2010).

Arion lusitanicus occurs on disturbed grounds and even in severely modified areas such as cities, suburbs and agricultural areas. In contrast, large close-canopy beech-dominated forests are scarcely invaded by the species (KAPPES, 2006; KAPPES et al., 2009). Among the characteristics of anthropogenic disturbance are artificial habitat structures (e.g. soil sealing, artefacts made from plastic and/or metal), different and novel food sources (e.g. human food waste, garden waste, introduced ornamental plants, fruits and crops, faeces of different animals), chemical modifications (e.g. liming, fertilization, plant and crop protection) and increased microclimatic amplitudes.

We thus hypothesized that individuals of *A. lusitanicus*, in contrast to native species, do not strictly avoid physicochemical modifications or unusual substances that in some cases can be exploited as food. We performed a field study using pitfall traps with different baits that should be either attractive (beer: EDWARDS, 1991), neutral (water during a period with intermittent rain), or repellant (strong acids, isothiocyanates: e.g. KOHN, 1961 and references therein; SAHLEY, 1990; INOUE et al., 2004, 2006) to gastropods. In a novel approach, we assessed the behavior of *A. lusitanicus* against the background of all the gastropod species we found in different habitat types.

MATERIALS AND METHODS

Data collection

Pitfall traps are known not to measure the true abundances of species in the habitat (BAARS, 1979), but rather reflect behavioral differences between species (GERLACH et al., 2009). We thus used pitfall traps to compare the trappability of *A. lusitanicus* with that of other gastropod species. The opening of the pitfall traps was 5.5 cm in diameter and the traps were protected against rain with a transparent plastic roof.

In a first sampling campaign, we sampled four major habitat types, namely transformed open habitats close to urban areas (n = 4), extensively-used open grounds (herb stands and grasslands, n = 3), herb-rich successional habitats (n = 4), and floodplain forests (n = 4) along the Kinzig River in the area of the Long Term Ecological Research (LTER) site 'Rhine-Main-Observatory' (www.lter-d.ufz.de) in southern Hesse, Germany (Figure 1B). We additionally quantified gastropod densities in each of the 15 locations. To this end, we sampled vegetation, plant litter and soil from four plots per location covering 0.25 m² each. As the plots are rather small for larger species, we included gastropod species found within a buffer of 2 m from the plot. These additional species received a lower score according to their probability of crossing the plots (0.5 = alive, 0.1 = dead). Large individuals (> 2.5 cm) usually were quantified and released in the field; medium-sized and small slugs were sorted from the fresh substrate collections in the laboratory, and the remaining snails were finally sorted from the air-dried material under a magnifying lens. Some slugs, such as those from the *A. subfuscus* complex or those belonging to the genus *Deroceras*, were determined anatomically.

For this first sampling campaign, eight replicate pitfall traps per location were filled with 250 ml Renner solution (10 % glacial acetic acid, 20 % glycerin, 30 % ethanol, 40 % water). Gastropods usually do not respond to glycerin, but display a negative response to acids (KOHN, 1961, and references therein). Ethanol and ethyl acetate, the latter being the product of acetic acid and ethanol, are both adverse stimuli for *Helix pomatia* (VOSS, 2000). This solution thus allows the assessment of potential behavioral peculiarities, and the substances do not evaporate as quickly as, for example, mustard oil. Pitfall traps remained for three weeks in the field (late July to mid-August 2010) and were checked and recharged weekly.

In a second pitfall trap sampling campaign, we compared the responses to five different baits.

(1) Some pitfall traps were filled with Renner solution.
(2) In the same habitats, we also offered pitfall traps filled with Pilsner type beer (4.9% ethanol) as beer is attractive for snails and slugs (EDWARDS, 1991; SCHÜRSTEDT & GRUTTKE, 2000; MAZE, 2009).
(3) Mustard oil (allyl-isothiocyanate, AITC) is a pungent secondary metabolite of several crucifer plants including mustard and horseradish, but isothiocyanates also occur in garlic. Slugs are naturally averse to these substances (SAHLEY, 1990; INOUE et al., 2004, 2006). We prepared an 800 µM solution of AITC by dissolving 400 µl in 8 ml methanol and adding this solution to 5 liters of water.
(4) A garlic solution was prepared as a cold extract of 20 g of smashed garlic cloves in 5 liters of water.

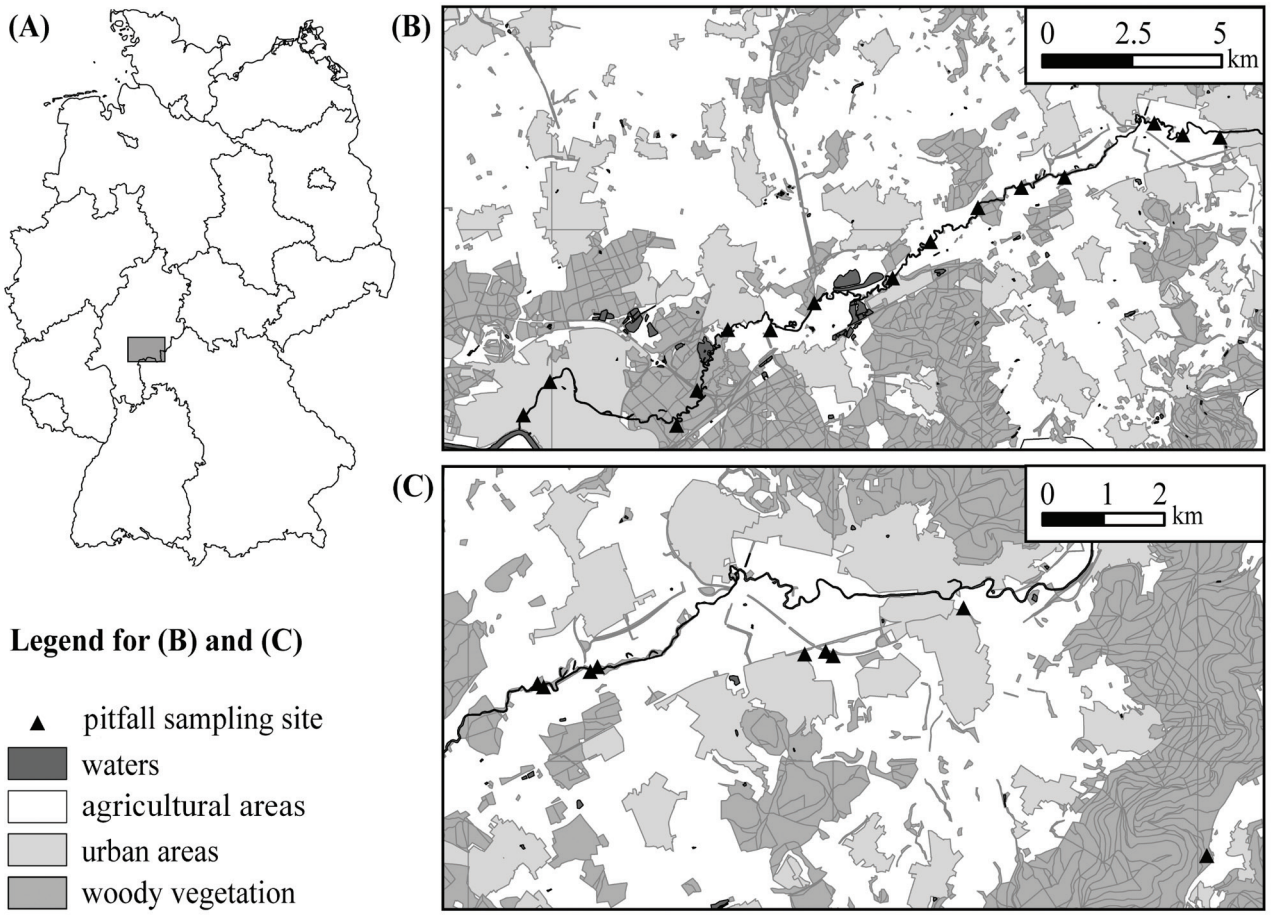

Fig. 1 − (A): Location of the survey area (shaded in grey) in the federal state of Hesse in Germany. (B): location of the pitfall traps in the sampling campaign with Renner solution. (C): location of the pitfall traps in the sampling campaign with the different baits. Where the pitfall trap locations were comparatively close in (C), contrasting habitat types were sampled.

(5) Water was considered to be neither deterrent nor attractive, because intermitted rainfall occurred throughout the study. Pitfall traps with water were thus used as a control for accidental drowning.

Two pitfall traps per bait type (250 ml) were placed in nine different locations (Fig. 1C) for five days during moist conditions in mid-August. The sampling was done in herb stands located along a railroad track (1x), at a drainage ditch (1x), between a poplar stand and a grain field (1x), around compost heaps in an allotment area (1x), in a small open floodplain close to a forest edge (1x), in a riparian willow stand (1x), along the edge between a grain field and grassland (2x) and between a grain field and a successional forest (1x). The traps were recharged after two days and removed after another three days. The catch from each interval was standardized to total numbers per bait (i.e., two traps) and 24 h.

Statistics

Trappability was calculated as the total number of individuals from the traps divided by the total number of individuals per m² from the field survey. High numbers thus indicate high attractiveness of the traps, whereas low numbers indicate avoidance behavior.

Abundance data were cubic root transformed. The data of the first sampling campaign were analyzed in a nested ANOVA with sites being nested in habitat types. Differences in the numbers of trapped *A. lusitanicus* between different habitat types were assessed using the Tukey HSD post-hoc test. Data from the second sampling campaign were analyzed in a one-way ANOVA with Tukey HSD post-hoc tests for differences between the efficiency of different bait types. Analyses were performed in JMP 4.0.

RESULTS

In the vegetation, leaf litter and soil surface samples taken in the 'Rhine-Main-Observatory', a total of 49 gastropod species were recorded. *A. lusitanicus* was ranked 15 in abundance (Table 1) but was the dominant species in the pitfall traps with Renner solution (n = 537 individuals, trap selectivity = 19.53). Density and trappability of *A. lusitanicus* were not correlated ($r = 0.25$, $F_{1,13} = 0.89$, $P = 0.36$).

Only a few other species were caught in the pitfall traps with Renner solution, and their trap selectivity was lower. All traps combined yielded five individuals of *Arion rufus* (total number from squares: 1.0 / trap selectivity = 5.00), one *Arion silvaticus* (6.0 / 0.17), one *Deroceras reticulatum* (32.0 / 0.03), one *Deroceras panormitanum* (1.0 / 1.00), one *Limax maximus* (1.5 / 0.67), one *Fruticicola fruticum* (18.6 / 0.05) and two subadults of the genus *Cepaea* (54.1 / 0.04 for the two *Cepaea* species combined).

The activity density of *A. lusitanicus* was highest in successional habitats with young woody plants and herb cover (Fig. 2). The nested ANOVA revealed that both the habitat type ($df = 3$, $F = 5.1$, $P < 0.001$) and the sampling location ($df = 11$, $F = 18.6$, $P < 0.001$) significantly influenced activity densities of *A. lusitanicus*.

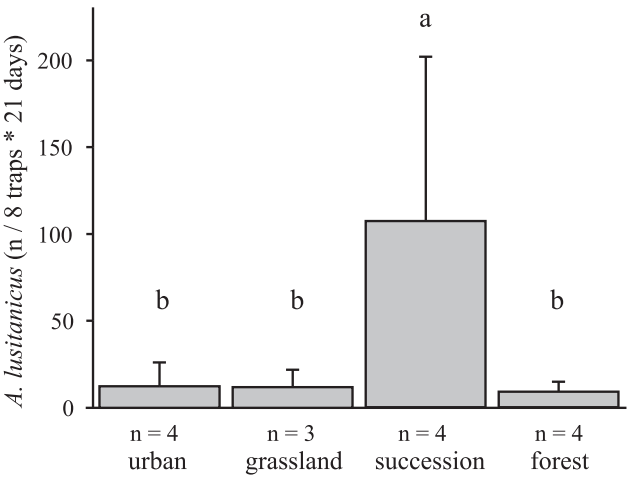

Fig. 2. − Differences between habitat classes in relation to the total catch of *Arion lusitanicus* (mean and standard deviation) in the eight pitfall traps (Renner solution) per location. Shared letters indicate a lack of significance for the Tukey HSD test in the nested ANOVA.

TABLE 1

Total numbers of individuals per 15 m² and average densities per m² of gastropod species of which more than 10 individuals were found in the 1 m² square survey in the 15 sites in the Rhine-Main-Observatory (compare Figure 1B).

species	total	mean±stdev. m^{-2}
Cochlicopa lubrica (O. F. Müller, 1774)	480.5	60.1±53.5
Alinda biplicata (Montagu, 1803)	231.3	28.9±34.4
Vertigo pygmaea (Draparnaud, 1801)	178.3	22.3±21.1
Discus rotundatus (O. F. Müller, 1774)	79.7	10.0±7.2
Punctum pygmaeum (Draparnaud, 1801)	63.2	7.9±8.4
Carychium tridentatum (Risso, 1826)	55.5	6.9±5.1
Acanthinula aculeata (O. F. Müller, 1774)	54.0	6.8±5.3
Aegopinella nitidula (Draparnaud, 1805)	53.7	6.7±4.5
Nesovitrea hammonis (Ström, 1765)	49.9	6.2±3.9
Cepaea hortensis (O. F. Müller, 1774)	39.1	4.9±2.8
Trochulus hispidus (Linnaeus, 1758)	36.9	4.6±2.9
Deroceras reticulatum (O. F. Müller, 1774)	32.0	4.0±1.5
Monachoides incarnatus (O. F. Müller, 1744)	30.0	3.8±4.0
Carychium minimum O. F. Müller, 1774	29.0	3.6±7.2
Arion lusitanicus auct. non Mabille, 1868	27.5	3.4±1.3
Succinella oblonga (Draparnaud, 1801)	24.9	3.1±4.0
Vallonia excentrica Sterki, 1893	22.7	2.8±2.7
Vitrina pellucida (O. F. Müller, 1774)	22.1	2.8±3.3
Vallonia costata (O. F. Müller, 1774)	20.9	2.6±3.4
Fruticicola fruticum (O. F. Müller, 1774)	18.6	2.3±2.3
Cepaea nemoralis (Linnaeus, 1758)	15.0	1.9±1.2
Vertigo pusilla O. F. Müller, 1774	13.0	1.6±3.4
Deroceras laeve (O. F. Müller, 1774)	12.5	1.6±0.9
Succinea putris (Linnaeus, 1758)	11.2	1.4±1.3

A. lusitanicus was found in traps with all bait types in the choice experiment of the second sampling campaign. Individuals of *A. lusitanicus* were present in all traps irrespective of the bait, but the species was less well trapped with AITC and had a significantly lower constancy in traps with water ($P < 0.05$, Fig. 3A, Table 2). Based on the number of individuals of *A. lusitanicus*, bait types were ranked as follows: water < AITC < garlic extract < Renner solution < beer (Fig. 3B). Native species were rarely trapped; the most frequent native species was *D. reticulatum* (Table 2). Native species only occurred in the AITC, water and beer traps (in increasing order of total catch, Fig. 3A).

DISCUSSION

Density and activity

Our study revealed that *A. lusitanicus* differed from native species in having a higher overall trappability and a positive response to otherwise adverse substances. High activity of individuals of a species can cause a higher share of the species in pitfall traps than would be expected from field densities (Baars, 1979), whereas a small movement range combined with specific microhabitat requirements may result in zero trappability, as we found for microsnails such as from the genera *Carychium*, *Punctum*, *Vertigo* and *Vallonia*.

TABLE 2

Gastropod catch from pitfall traps baited with beer, garlic solution, allyl-isothiocyanate (AITC) solution, Renner solution, and water in nine differently structured locations. Each bait type was offered in duplicate, thus mean and standard deviation are given per two traps and 24h.

species	beer	garlic	AITC	Renner	water
Arion lusitanicus auct. non MABILLE, 1868	10.37±5.03	1.05±1.08	0.31±0.30	2.23±2.17	0.08±0.13
Arion fuscus O.F. MÜLLER, 1774	0.02±0.07	-	-	-	-
Arion silvaticus LOHMANDER, 1937	0.04±0.13	-	-	-	-
Arion distinctus MABILLE, 1868	0.04±0.13	-	-	-	-
Arion intermedius (NORMAND, 1852)	0.09±0.20	-	-	-	-
Deroceras leave (O.F. MÜLLER, 1774)	0.19±0.22	-	-	-	0.02±0.07
Deroceras reticulatum (O.F. MÜLLER, 1774)	0.29±0.47	-	0.04±0.09	-	0.16±0.24
Succinea putris (LINNAEUS, 1758)	0.04±0.09	-	-	-	-
Eucobresia diaphana (DRAPARNAUD, 1805)	-	-	-	-	0.02±0.07
Fruticicola fruticum (O.F. MÜLLER, 1774)	0.02±0.07	-	-	-	-
Monachoides incarnatus (O. F. MÜLLER, 1774)	0.02±0.07	-	-	-	-
Helix pomatia LINNAEUS, 1758	-	-	0.02±0.07	-	-
Cepaea hortensis (O.F. MÜLLER, 1774)	-	-	0.02±0.07	-	-
Cepaea nemoralis (LINNAEUS, 1758)	0.04±0.13	-	-	-	-

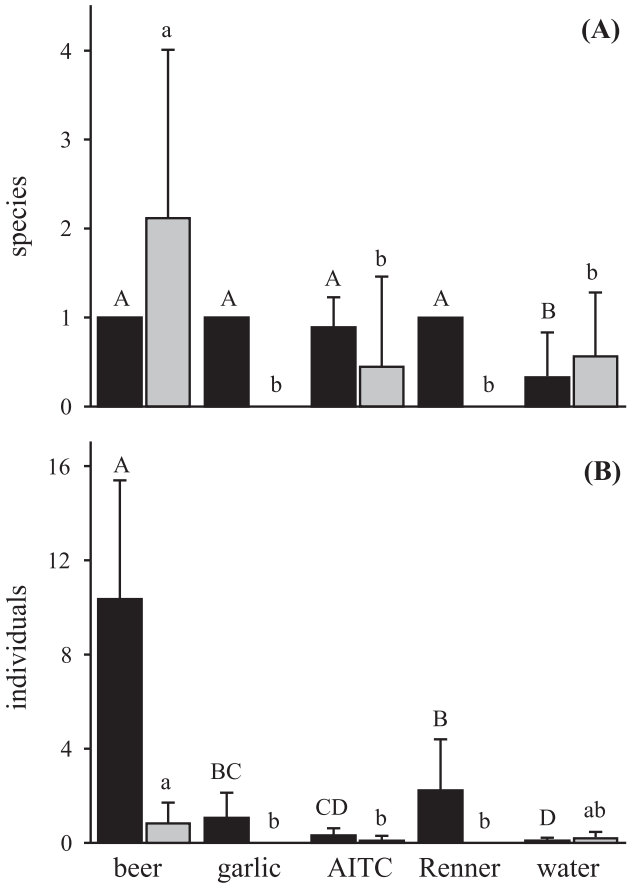

Fig. 3. − Mean and standard deviation of the total number of species (A) and number of individuals (B) trapped per bait type (two pitfall traps and 24 h) for all of the nine locations combined. Shared letters indicate a lack of significance in the Tukey HSD test. Black bars and capital letters refer to data of *Arion lusitanicus*, grey bars and small letters are for the native species.

Average field densities of *A. lusitanicus* in the habitats of the lower Kinzig valley were lower than, for example, those from herb-rich locations at the Lower River Rhine (KAPPES et al., 2007). Local densities can temporarily exceed 20 ind. m^{-2} under optimal shelter and food conditions (KOZŁOWSKI, 2007, and references therein). Unlike many other gastropods, the individuals of this species seem to be gregarious, and many individuals can be found sharing the same shelter. The activity of *A. lusitanicus* is comparatively high. Individuals were found to move on average 10.8 m per night (GRIMM & SCHAUMBERGER, 2002), although great individual plasticity in activity and home range size was observed (GRIMM & PAILL, 2001). Thus, within this comparatively active species, some individuals may be even more likely than others to be involved in local spread.

Response to baits

Behavior towards pitfall traps is known to be influenced by the liquid in the trap. Beer is highly attractive to gastropods (SMITH & BOSWELL, 1970; SCHÜRSTEDT & GRUTTKE, 2000). In our study, the highest number of gastropod species was caught in beer traps. However, individuals from other species were outnumbered by *A. lusitanicus*, probably because of differences in exploring the trap with its potential food source. Similarly, the behavior at different stages of encountering pitfall traps differs greatly between soil arthropod species that display different trappabilities (GERLACH et al., 2009).

In contrast to the positive response to beer, most gastropods are known to show negative reactions to acids (KOHN, 1961; VOSS, 2000) and isothiocyanates (SAHLEY, 1990; INOUE et al., 2004, 2006). Our study confirmed that most gastropods except *A. lusitanicus* avoid solutions with these substances. Perception of the pungent components of garlic is modulated through the thermosensitive TRP (transient receptor potential) family of ion channels (JORDT et al., 2004; BAUTISTA et al., 2005). TRP channels occur throughout the animal kingdom although the actual response to heat or cold depends on the taxon (e.g. VISWANATH et al., 2003). It has yet to be determined whether *A. lusitanicus* differs from the other gastropods in its perception of isothiocyanates.

Avoidance of acids reduces the risk of internal depletion of base cations and the associated reduction in fitness. Calcium salts, amongst others, are needed for shell growth and reproduction (WÄREBORN, 1979; TOMPA, 1976). Even though some species of the genus *Arion* have a strongly reduced internal shell, the egg shell of large *Arion* species, among them *A. lusitanicus*, is calcified or at least partially calcified (TOMPA, 1976). This lack of avoidance behavior towards acidic substances is in line with the observation that *A. lusitanicus* readily tests and feeds on plant species that contain oxalic acid such as the yellow wood sorrel (e.g. GRIMM et al., 1997) and the invasive giant knotweed (KAPPES et al., 2007). However, in our studies, attractiveness of the acidic Renner solution could not have been based on previous experience, as the habitats were dominated by *Urtica* stands, which typically do not provide strongly acidic food items. Instead, leaves of *Urtica dioica* are of neutral pH value (KAPPES et al., 2007).

Dispersal-related traits

Behavior can be discussed in terms of personality, that is, traits that are quite stable over time, that are heritable and that influence decisions of individuals within species (e.g. COTE et al., 2010). This can cause difficulties when describing the behavior of less-studied taxonomic groups or when comparing different taxa. Nevertheless, average levels of activity and exploration have, for example, been shown to differ between related invasive and non-invasive *Gambusia* shrimp species (SCHÖPF REHAGE & SIH, 2004). Exploratory behavior and novelty-seeking allow adaptation of the individual foraging strategy to spatio-temporal changes in food supplies (HARFMANN & PETREN, 2008; VAN OVERVELD & MATTHYSEN, 2010).

Among the risks of exploration is a higher mortality rate (e.g. STAMPS, 2007; BOON et al., 2008). Novel food items and situations can pose risks that often are overcome by dietary or behavioral conservatism; in our study this conservatism probably applied to most individuals of the active and abundant, but poorly trapped native *Deroceras reticulatum*. The high life-time productivity of *A. lusitanicus* with over 400 eggs laid by a single individual (KOZŁOWSKI, 2007) may be a strategy to compensate for a higher mortality and at the same time allow rapid population growth in newly colonized locations. STAMPS (2007) argued that selection for high individual growth rates would increase mean levels of risk-taking behavior across populations. Similarly, a high population growth rate may further encourage dispersal.

It is a much-debated question whether traits that favor dispersal or invasibility of their carriers were already common in populations in their native distribution range, or whether such traits are based on a rare genotype or even a single mutation that was positively selected for in the spreading process. *Arion lusitanicus*, for example, is very tolerant to, though not able to prevent, water loss (SLOTSBO et al., 2011). Our results, along with those of SLOTSBO et al. (2011) confirm that the species is less sensitive to otherwise aversive stimuli, and thus more likely to utilize novel environments and otherwise unusual dispersal routes. If a specific dispersal route is connected with some environmental stressors, for example high evaporation rates or chemical exposure, and some less sensitive genotypes can successfully overcome the stressors and colonize new areas, they will be selected for as long as the specific active or passive dispersal route persists, and in turn reinforce the use of the specific dispersal route.

We consequently expect that many more species, in which selection is against individuals taking dispersal-related risks under undisturbed conditions, may acquire (or loose) traits and become successful invaders under changing environmental conditions. Invasive pest slugs such as *A. lusitanicus* would be highly suitable organisms for testing this hypothesis and dispersal behavioral syndromes, which, according to COTE et al. (2010), include traits such as locomotor and feeding activity, boldness, exploration, sociability and aggressiveness. In suggesting this, we would like to stimulate more research on the biology and behavioral plasticity of *A. lusitanicus* in its original distribution range, in areas where the species is already well established, and at the recent dispersal front(s).

CONCLUSION

The invasive slug species *A. lusitanicus* differs from native gastropods in terms of its active behavior, combined with some insensitivity or inertness to stimuli that usually are adverse for gastropods. High reproductive output (a buffer against losses from mortality), combined with these dispersal-related traits, can facilitate colonization and thus the invasiveness of the species.

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Web-building spiders and blood-feeding flies as prey of the notch-eared bat (*Myotis emarginatus*)

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ABSTRACT. Conservation of the endangered notch-eared bat (*M. emarginatus*) requires a specific action plan based on precise ecological requirements of this species. The analysis of the diet of three colonies in southern Belgium revealed: (1) spatial and seasonal variations of the diet; (2) the consumption of web-building spiders (*Araneus diadematus*, *Araneus triguttatus*, *Cyclosa conica*, *Enoplognatha* sp., *Larinioides patagiatus*, *Neriene emphana*); (3) the predominance of blood-feeding dipterans in the diet (*Stomoxys calcitrans* and *Musca autumnalis*). Since the populations of these two ectoparasitic flies are sensitive to the use of antiparasitic drugs, these drugs should be used with caution by farmers and veterinarians in the vicinity of maternity colonies.

KEY WORDS: Food, spiders, *Stomoxys calcitrans*, *Musca autumnalis*, Geoffroy's bat.

INTRODUCTION

Bat populations are declining world-wide as a result of a growing number of factors, including habitat loss and fragmentation, disturbances to roosts, exposure to toxins and introduced predators (Racey, 1998). Relatively little attention has been devoted to the ecology of the notch-eared bat although this species is considered as endangered in Belgium and Luxembourg (Harbusch et al., 2002; Verkem et al., 2003; Lamotte, 2007; Kervyn et al., 2009). The habitats used by this species must be conserved in accordance with the Council Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora. The implementation of pertinent conservation measures requires information on its foraging habits.

Five studies have documented the food habits of the notch-eared bat in Europe (Bauerová, 1986; Krull et al., 1991; Beck, 1995; Steck & Brinkmann, 2006; Goiti et al., 2011). These studies pointed out the importance of spiders and flies in the diet of the notch-eared bat, but they failed to identify most of these arthropods to a specific level, which is, however, required in order to build a species-specific action plan for the conservation of this species.

The aims of this paper are (1) to describe in detail the diet of the notch-eared bat in southern Belgium, especially regarding spiders; (2) to point out intra-specific dietary differences in relation to the seasonal and geographical conditions; (3) to focus on implications of the diet for the conservation of this bat species.

MATERIALS AND METHODS

Three bat colonies were studied in southern Belgium (Figure 1). The first colony consists of about one hundred breeding females roosting in the church of Bolland, Province of Liège, a village situated in the Herve upland in a bocage landscape dominated by pastures and orchards. The second colony of about fifty breeding females occupies the attic of a private house

in Rochefort, Province of Namur, a small town in the Famenne region surrounded mainly by broad-leaved and coniferous forests, pastures and arable land. The third colony consists of circa thirty breeding females roosting in the church of Guirsch, Province of Luxembourg, a village situated in the Belgian Lorraine region in a landscape dominated mainly by broad-leaved and coniferous forests, and pastures.

Polythene sheets were placed on the attic floor, beneath the roosting bats, from the end of April to October 1999. Faecal pellets were collected every two weeks in Bolland and monthly in Rochefort, air-dried and stored in plastic bags. Due to access restriction, only one sample was collected in June 1999 in Guirsch. From these collections, pellets were taken at random, in order to reduce the number of pellets originating from the same individual. The sample size was determined *a posteriori* by examining the variation of prey proportions related to the number of analysed pellets (KERVYN, 1998). Large samples with a high diversity were analysed. This clearly indicated that after the analysis of approximately 10 droppings, inclusion of more droppings did not significantly alter the composition of the sample. A sample of 20 droppings allowed detection of all the identifiable taxa. Each faecal pellet was soaked in water on a microscope slide and teased apart under a binocular microscope using a pair of dissecting needles. Identification of insect pieces was facilitated by the general descriptions of SHIEL et al. (1997) WHITAKER (1988), MCANEY et al. (1991) and specialised documents (LOCKET & MILLIDGE, 1953; VAN EMDEN, 1954; D'ASSIS FONSECA, 1968; VAN HELSDINGEN, 1969; LECLERCQ, 1971; ROBERTS, 1985; SMITH, 1986; RANSY & BAERT, 1987; ROBERTS, 1987, 1998). Species of Araneae were only identified by genitalia (epigynes for females and palps for males), although the majority of the prey remains were legs or chelicerae. Insect fragments were also compared with specimens stored in the entomological collections of the Zoological Museum of Liège and the Royal Belgian Institute of Natural Sciences of Brussels. No attempt was made to accurately estimate the frequency of fragments or percentage volume of prey taxa within a dropping, because most fragments could not be attributed to any taxon. Moreover, the remains of a single prey are distributed among many droppings (ROBINSON & STEBBINGS, 1993). Results are expressed in relative frequency

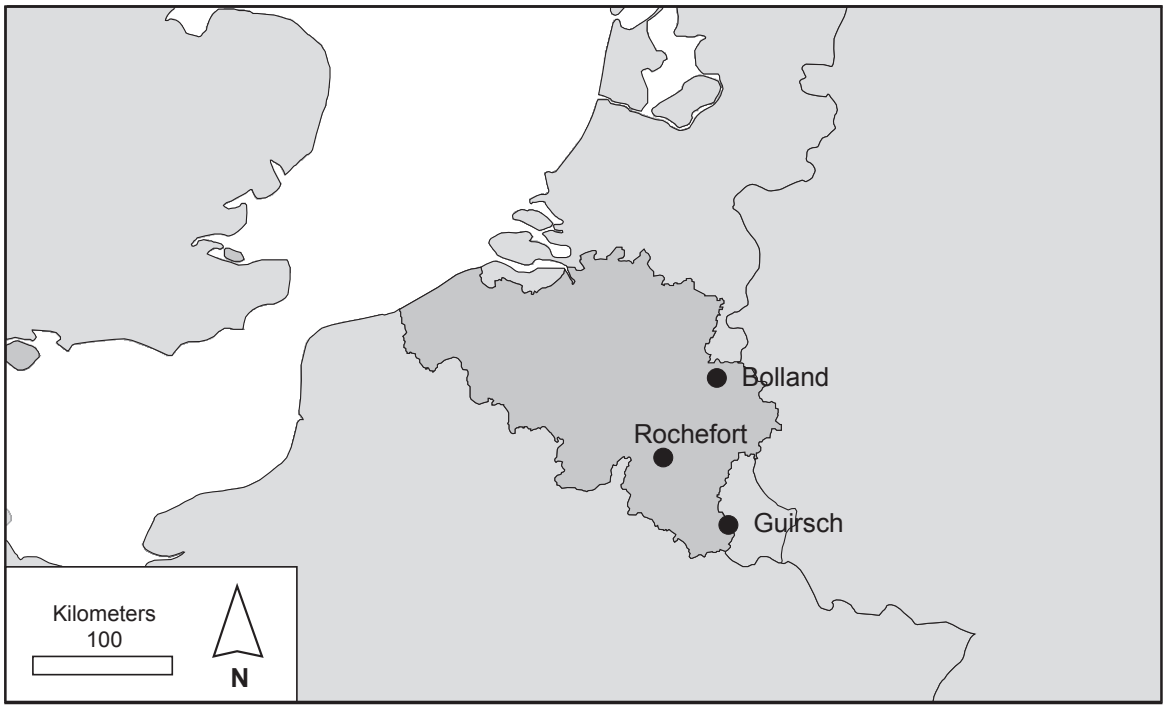

Figure 1 − Location of study sites in Belgium.

of occurrence, which represents the number of pellets containing the item among a sample of 20 pellets, divided by the total number of items. To detect possible variations, a goodness-of-fit test (SOKAL & ROHLF, 1981) was performed to compare the frequency distribution of prey items. A Newman-Keuls test was used to identify the origin of the variations.

RESULTS

Diet Composition

A total of 873 insect fragments were recorded from 320 droppings (Table 1). Of these, 788 items were identified and 85 were not. The mean number of prey taxa per dropping was 2.67 ± 1.44, with maximum of 9.0. Diptera accounted for the majority of identified prey, with a large proportion of stable fly (*Stomoxys calcitrans*) and face fly (*Musca autumnalis*) (Table 1 and Figure 2). These two species accounted for 53.4% of the prey in Bolland and 72.1% in the sample from Guirsch. However, the most striking difference between sites was the absence of stable and face flies in Rochefort. In contrast to this, Araneae – the second most important taxon – accounted for 29.4% in the diet from Rochefort, while this prey appeared in reduced proportion in Bolland (22.4%) and Guirsch (23.3%). The identified spiders in Bolland (7 items) were Araneidae with *Araneus diadematus* (n = 1), *Cyclosa conica* (n = 2), and *Theridiidae* (n = 3) with *Enoplognatha* sp. (cf. *ovata*) (n=1). In Rochefort (17 items), spiders were also mainly Araneidae, with *Araneus diadematus* (n = 1), *Araneus triguttatus* (n = 2), *Cyclosa conica* (n = 12), *Larinioides patagiatus* (n = 1) and *Linyphiidae* with *Neriene emphana* (n = 1). Other prey found in Bolland and Rochefort belong to the taxa Lepidoptera, Hymenoptera Apocrita – mainly Ichneumonidae, Coleoptera, Neuroptera, Thysanoptera and Psocoptera (Table 1).

Seasonal variations

Variation in consecutive samples was low (Figure 2). Samples were homogenous among sampling periods in Bolland (G-test global: G = 36.7; df = 30; ns) and Rochefort (G-test global: G = 17.88; df = 21; ns), but lepidopterans in Bolland were more numerous in May (G partial = 11.33; df = 5; $p < 0.05$).

DISCUSSION

Diet

Relevance and limitations of the method have been evaluated by several authors (RABINOWITZ & TUTTLE, 1982; KUNZ & WHITAKER, 1983; DICKMAN & HUANG, 1988; ROBINSON & STEBBINGS, 1993). Faecal analysis does not provide the exact composition of the ingested food. However, it allows an estimation of the food composition, especially for the most common prey items. Its use is valuable for seasonal or geographical comparisons of the diet. Results usually overestimate the proportion of large insects and of those prey items leaving easily identifiable pieces even after ingestion and digestive transit. Soft-bodied insects may be underrepresented.

Since notch-eared bats may forage up to 10 km from the roost (KRULL et al, 1991) and the transit time in bats is rapid (KOVTUN & ZHUKOVA, 1994), faeces collected in the roost may contain a higher proportion of insects caught near the roost (RABINOWITZ & TUTTLE, 1982).

This study provides, for the first time, species-level identification for the main prey of this bat in three Belgian colonies. Although the sampling period was limited to one single year, results may be considered as representative since intra-annual variation appears to be low. This gives a more comprehensive concept of the foraging behaviour and the foraging habitats of the notch-eared bat. The diet composition of these bats studied in Germany (KRULL et al., 1991; BECK, 1995; STECK & BRINKMANN, 2006) was also dominated by species of Diptera (*Muscidae*) and spiders, as we found in the colonies of Bolland

and Guirsch. The diet described by BAUEROVÁ (1986) and GOITI (2011) is somewhat similar to the food composition of the colony settled in Rochefort where spiders constitute a large part of the diet. The six species of spiders identified as prey items are all web-building spiders, usually found on bushes and trees (ROBERTS, 1995).

Foraging behaviour

A diet composed of spiders and nocturnally non-flying insects, such as muscids, supports the gleaning behaviour of the notch-eared bat, as predicted by NORBERG & RAYNER (1987) and observed by KRULL et al. (1991) and SCHUMM et al. (1991). The prey items identified here are shared in Europe with other foliage-gleaner or surface-gleaner bats such as *Plecotus auritus* and *Myotis nattereri* (BAUEROVÁ, 1982; GREGOR & BAUEROVÁ, 1987; SHIEL et al., 1991; SWIFT & RACEY, 2002; MOTTE, unpublished results). It is now well documented that the notch-eared bat forages in forests, in orchards or along forest edges (KRULL et al., 1991; BARATAUD, 1993; BRINKMANN et al., 2001; HUET et al., 2004; FLAQUER et al., 2008; ZAHN et al., 2010; GODIN, unpublished results), but this bat can

Table 1

Food composition of the notch-eared bat at three study sites.

Taxa	Bolland												
	99-05-13	99-05-29	99-06-12	99-06-26	99-07-12	99-07-24	99-08-08	99-08-23	99-09-05	99-09-18	99-10-29	Total	%
ARACHNIDA Araneae	15	10	6	11	12	9	11	8	13	18	12	125	22,4%
INSECTA Coleoptera	-	2	-	1	-	-	-	-	-	-	-	3	0,5%
INSECTA Coleoptera Carabidae	1	-	-	-	-	-	-	-	-	-	-	1	0,2%
INSECTA Coleoptera Chrysomeloidea Cerambycidae	-	-	-	-	-	-	-	-	-	-	-	-	0,0%
INSECTA Coleoptera Scarabeoidea Scarabeidae	-	1	-	1	-	1	-	-	1	1	-	5	0,9%
INSECTA Coleoptera Staphylinidae	1	-	-	-	-	-	-	-	-	-	-	1	0,2%
INSECTA Diptera	-	-	-	1	-	1	1	1	1	-	1	6	1,1%
INSECTA Diptera Anisopodidae	2	1	1	2	-	-	-	-	-	-	-	6	1,1%
INSECTA Diptera Calliphoridae	-	-	-	-	-	-	-	-	-	-	-	-	0,0%
INSECTA Diptera Chironomidae or Ceratopogonidae	-	-	-	-	-	-	-	-	-	-	-	-	0,0%
INSECTA Diptera Culicidae	-	-	-	-	-	-	-	-	-	-	-	-	0,0%
INSECTA Diptera Empididae	1	-	-	-	-	-	-	-	-	-	-	1	0,2%
INSECTA Diptera Muscidae	3	-	1	-	-	-	-	-	-	1	-	5	0,9%
INSECTA Diptera Muscidae *Musca autumnalis*	3	12	10	13	12	11	15	14	15	9	7	121	21,7%
INSECTA Diptera Muscidae *Stomoxys calcitrans*	8	12	18	14	20	19	19	19	18	15	15	177	31,7%
INSECTA Diptera Nematocera	-	-	-	-	1	-	-	-	-	-	-	1	0,2%
INSECTA Diptera Psychodidae	1	4	3	-	-	1	3	-	1	-	-	13	2,3%
INSECTA Diptera Scatophagidae	4	1	-	1	2	-	-	1	1	2	-	12	2,2%
INSECTA Diptera Syrphidae	-	-	-	1	-	-	-	-	-	-	-	1	0,2%
INSECTA Diptera Tipulidae	3	1	1	-	-	-	-	-	-	-	-	5	0,9%
INSECTA Hymenoptera Apocrita	-	1	-	-	-	-	-	-	-	-	1	2	0,4%
INSECTA Hymenoptera Apocrita Ichneumonidae	2	2	1	-	1	-	-	-	1	-	-	7	1,3%
INSECTA Lepidoptera imago	1	4	4	4	2	-	2	3	1	1	-	22	3,9%
INSECTA Lepidoptera larvae	5	2	-	-	-	-	-	-	-	-	-	7	1,3%
INSECTA Psocoptera	-	-	1	-	1	-	-	-	-	-	-	2	0,4%
INSECTA Neuroptera Chrysopidae	-	-	-	-	-	-	-	-	-	-	-	-	0,0%
INSECTA Neuroptera Hemerobiidae	2	-	-	-	-	-	-	-	-	-	-	2	0,4%
INSECTA Thysanoptera Thripidae	-	-	-	-	-	-	-	-	-	-	-	-	0,0%
Undetermined	2	1	3	5	3	3	4	5	1	3	3	33	5,9%
Total	54	54	49	54	54	45	55	51	53	50	39	558	100%

also opportunistically feed inside cowsheds (KRULL et al., 1991; SCHUMM et al., 1991; VERGOOSSEN & BUYS, 1997; BRINKMANN et al., 2001; DEKKER et al., 2008). A large consumption of spiders has seldom been documented in bats. It is known from only a few other gleaning-bat species worldwide: *Kerivoula papuensis* in Australia (SCHULZ, 2000) and *Myotis keeni* in Canada (BURLES et al., 2008).

Prey detection

Bats that capture animal prey from substrates often emit characteristic echolocation calls that are short-duration, frequency-modulated (FM) and broadband. Such calls do not seem effective for finding prey among cluttered backgrounds because echoes reflecting from the substrate mask the acoustic signature of prey (ARLETTAZ et al., 2001). Hence, like many other surface-gleaning bats, the notch-eared bat presumably detects its prey by listening for prey-generated sounds, in flight or sometimes from a perch (VERGOOSSEN & BUYS, 1997; BRINKMANN et al., 2001; DEKKER et al., 2008). Muscids are expected to be detected by their fluttering or buzzing noise, presumably initiated by the bat's flight movement near the substrate. Spiders are presumably captured on their web, since webs are often found among the notch-eared bat droppings, as a result of fur cleaning after foraging (BODIN et al., 2002). Spiders could be detected by echolocation or through the buzz generated by spider prey in the web.

Guirsch			Rochefort						Total	%	% of identified	Taxa
99-06-20	Total	%	99-05-22	99-06-20	99-08-22	99-09-21	Total	%				
10	10	23,3%	20	20	20	20	80	29,4%	215	24,6%	27,3%	Araneae
-	-	0,0%	-	1	-	-	1	0,4%	4	0,5%	0,5%	Coleoptera
-	-	0,0%	-	-	1	-	1	0,4%	2	0,2%	0,3%	Coleoptera Carabidae
-	-	0,0%	2	1	1	-	4	1,5%	4	0,5%	0,5%	Coleoptera Chrysomeloidea Cerambycidae
-	-	0,0%	1	-	1	-	2	0,7%	7	0,8%	0,9%	Coleoptera Scarabeoidea Scarabeidae
-	-	0,0%	-	-	-	-	-	0,0%	1	0,1%	0,1%	Coleoptera Staphylinidae
-	-	0,0%	4	7	2	6	19	7,0%	25	2,9%	3,2%	Diptera
-	-	0,0%	-	1	-	-	1	0,4%	7	0,8%	0,9%	Diptera Anisopodidae
-	-	0,0%	-	-	1	-	1	0,4%	1	0,1%	0,1%	Diptera Calliphoridae
-	-	0,0%	4	-	1	2	7	2,6%	7	0,8%	0,9%	Diptera Chironomidae or Ceratopogonidae
-	-	0,0%	-	-	1	-	1	0,4%	1	0,1%	0,1%	Diptera Culicidae
-	-	0,0%	-	1	-	1	2	0,7%	3	0,3%	0,4%	Diptera Empididae
-	-	0,0%	-	1	-	1	2	0,7%	7	0,8%	0,9%	Diptera Muscidae
15	15	34,9%	-	-	-	-	-	0,0%	136	15,6%	17,3%	Diptera Muscidae *Musca autumnalis*
16	16	37,2%	-	-	-	-	-	0,0%	193	22,1%	24,5%	Diptera Muscidae *Stomoxys calcitrans*
-	-	0,0%	-	-	-	-	-	0,0%	1	0,1%	0,1%	Diptera Nematocera
-	-	0,0%	-	-	-	3	3	1,1%	16	1,8%	2,0%	Diptera Psychodidae
-	-	0,0%	1	1	1	2	5	1,8%	17	1,9%	2,2%	Diptera Scatophagidae
-	-	0,0%	1	-	-	-	1	0,4%	2	0,2%	0,3%	Diptera Syrphidae
-	-	0,0%	5	4	3	-	12	4,4%	17	1,9%	2,2%	Diptera Tipulidae
-	-	0,0%	-	-	-	-	-	0,0%	2	0,2%	0,3%	Hymenoptera Apocrita
-	-	0,0%	7	4	1	4	16	5,9%	23	2,6%	2,9%	Hymenoptera Apocrita Ichneumonidae
2	2	4,7%	5	4	9	3	21	7,7%	45	5,2%	5,7%	Lepidoptera imago
-	-	0,0%	8	6	1	1	16	5,9%	23	2,6%	2,9%	Lepidoptera larvae
-	-	0,0%	-	-	-	-	-	0,0%	2	0,2%	0,3%	Psocoptera
-	-	0,0%	-	-	-	1	1	0,4%	1	0,1%	0,1%	Neuroptera Chrysopidae
-	-	0,0%	5	3	4	3	15	5,5%	17	1,9%	2,2%	Neuroptera Hemerobiidae
-	-	0,0%	-	2	1	6	9	3,3%	9	1,0%	1,1%	Thysanoptera Thripidae
0	0	0,0%	15	11	13	13	52	19,1%	85	9,7%	---	Undetermined
43	43	100%	78	67	61	66	272	100%	873	100%	100%	Total

Conservation

The importance of stable and face flies in the diet of this endangered bat raises an interesting conservation issue, since it provides a new example of human dependency among bats (Stebbings & Robinson, 1991). These flies are considered as pests and locally strongly controlled because of their impact on cattle health and related economic damage (Leclercq, 1971; Campbell et al., 2001; Rodríguez-Batista et al., 2005). Larvae of these prey species develop in decaying organic matter, such as horse and cow dung (Grabovac & Petric, 2003; Rodríguez-Batista et al., 2005), in cowsheds but also on pastures and orchards around cattle feeding sites of hay in round bales (Broce et al., 2005). As well as by sanitary measures in cowsheds, these insects are mainly destroyed by the application of antiparasitic drugs (Madsen et al., 1990; Mc Cracken, 1993). This practice should be banned or strongly reduced for the cattle around summer roosts of the notch-eared bat (Eurobats, 2010). This management measure is also recommended for the conservation of the serotine bat (Kervyn & Libois, 2008) and the endangered greater horseshoe bat (*Rhinolophus ferrumequinum*) (Ransome & Hutson, 2000), which regularly share roosts with the notch-eared bat. Both species feed on a key-prey, the dung beetle *Aphodius*, a non-target species also affected by antiparasitic treatments.

Conservation measures devoted to areas neighbouring notch-eared bat colonies should therefore take the problem of antiparasitic administration into account (Downs & Sanderson, 2010), particularly in Special Areas of Conservation of the Natura 2000 network, around forests and orchards but also in and around cowsheds. Amazingly, since cowsheds are a privileged foraging area, the presence of cattle within cowsheds in summertime seems to be of great importance to maintaining or restoring the local population of the notch-eared bat. The impact of livestock welfare regulations – imposing larger, cleaner and better ventilated cowsheds, in opposition to the ecological requirements of flies – would be interesting to analyse, since it presumably reduces the quality of this feeding area for notch-eared bats.

On the evolution of blood-suckling in bats

Many papers have presented hypotheses concerning the intermediate stages involved in the origin of the blood feeding strategies present in bats (Monteiro & Nogueira, 2011). The consumption of blood-feeding ectoparasites is considered as a first step in the development of blood feeding behaviour from the ancestral, insect-eating behaviour (Fenton, 1992; Baker, 2010). Our results on the diet of notch-eared bats illustrate the feasibility of this step in a vespertilionid bat. Further research would be worthwhile to test whether bats take significant advantage of ectoparasite meals.

CONCLUSIONS

Our results confirm that the diet of notch-eared bats (*Myotis emarginatus*) in southern Belgium is characterized by flies, but also spiders and other nocturnal non-flying insects. Local differences in diet composition can be explained by an opportunistic foraging behaviour. The importance of web-building spiders in the diet suggests that bats might be able to pluck spiders from their webs. These bats presumably detect their prey by the sounds they produce. The consumption of stable and face flies is congruent with the observations of individuals foraging within cowsheds. The present-day agricultural practice of eliminating flies with insecticides or transforming cowsheds may be hazardous for the survival of this bat. Thus, action plans designed for this Natura 2000 species should avoid such agricultural practices affecting the prey availability of this human-dependant bat.

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Variation in reproductive parameters of *Rhinella arenarum* (Hensel, 1867) (Anura: Bufonidae) between the reproductive and post-reproductive periods

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ABSTRACT. We compared reproductive parameters of *Rhinella arenarum* in a wetland of the Monte in Argentina during reproductive and post-reproductive seasons. Individuals were collected at random, monthly from November 2001 to October 2002. August through November was considered the reproductive period, and December through April was considered the post-reproductive period. Of the 116 adults, 75 were males and 41 were females. The reproductive parameters measured included body mass, fat body mass, number of mature ova, ova size, and testicular volume. There were significant differences between the reproductive and post-reproductive periods in females for body mass, fat body mass, and number of mature ova. Likewise, males also had significant differences between these periods for body mass and fat body mass. Apparently, *R. arenarum* has an opportunistic and continuous reproductive strategy. Entering dormancy with large fat bodies and testes in apparent spermatogenesis allows males to reproduce immediately after emerging in the spring. However, females have mature but fewer ova during this period, which is a reproductive feature shared by most temperate amphibians. Our data, and the primarily tropical distribution of *R. arenarum*, suggest that this species recently invaded the temperate region wherein males retained acyclic reproductive activity and females, owing to their higher reproductive costs, have evolved cyclic reproduction.

KEY WORDS: Argentina, Reproduction, *Rhinella arenarum*, San Juan, Seasonal variation.

INTRODUCTION

Amphibians exhibit a great diversity of reproductive patterns (Duellman & Trueb, 1986). Anurans that live in tropical areas, where temperatures do not show large seasonal fluctuations, have continuous reproductive patterns. By contrast, anurans of temperate and cold climates, where ambient temperatures show large variations, both daily and seasonal, breed discontinuously (Crump, 1974; Tsiora & kyriakopoulou-Sklavounou, 2001; Wells, 2007).

Lavilla & Rouges (1992) described the reproductive mode of *R. arenarum*, in which eggs are laid in gelatinous strings at the bottom of water bodies, where hatching and embryonic development also occur. Studies of reproductive parameters of a species allow us to better understand the reproductive modes and their ecological and evolutionary significance (Crump, 1974). Previous research has found a relationship between the size of females and fertility; thus it is expected that females of larger size have more eggs for each clutch than females of smaller size (Basso, 1990; Perotti, 1997; Peralta de Almeida-Prado & Uetanabaro, 2000; Díaz-Páez & Ortiz, 2001; Castellano et al., 2004; Sanabria et al., 2007a; Sanabria et al., 2007b).

Also, there are annual variations in the size of the fat body, which correlates with the functional

status of the gonads (MARTORI et al., 2005). DÍAZ-PÁEZ & ORTIZ (2001) found that in both sexes of *Pleurodema thaul* (LESSON, 1826) the increase in size of fat body is positively correlated with temperature, but not with rainfall. Furthermore, the size of the fat body is reduced (sometimes to zero grams) during the breeding season, as the stored fat is used as an energy source during and after winter aestivation. DUELLMAN & TRUEB (1986) suggested that the amount of energy devoted to reproduction depended on the season, age, and sex of the frog. Many amphibians accumulate energy reserves to survive long periods of dormancy (FITZPATRICK, 1976). Therefore, species with a short period of activity, in sites with little precipitation and short summer seasons, should partition the energy between reproduction and reserves (WELLS, 2007).

The distribution of energy for reproduction in a year can affect the amount of energy available for future growth or reproduction (RYAN et al., 1983). The aim of this investigation was to compare reproductive parameters (fat body mass, body mass, number of mature ova, size of ova, and testicular volume) between the reproductive and post-reproductive periods of *R. arenarum* in the arid region of San Juan, Argentina.

MATERIALS AND METHODS

The study area is located 25 km west of San Juan, Zonda Department (31.52716 S, 68.69580 W; Datum: WGS 84; elevation 724m). In winter, the wetland is reduced to two small bodies of water and in summer it increases in size until it becomes a large, flooded area (VICTORIA, 1999).

It is part of the Monte phytogeographical province (CABRERA, 1994) at 800 m asl, an arid region with an average annual temperature of 17.3ºC, annual average maximum of 25.7ºC, and annual mean minimum of 10.4ºC. Rainfall is concentrated in the summer with an annual average of 84 mm. Individuals were collected via haphazard monthly sampling from November 2001 to October 2002. The herpetological assemblage of this region is composed of 12 species, of which only three are anuran (*R. arenarum, Leptodactylus latrans* (STEFFEN, 1815)*, Pleurodema nebulosum* (BURMEISTER, 1861)) (SANABRIA & QUIROGA, 2010).

The months of August, September, October and November were considered the reproductive period, as they are the months during which amplexus and male songs are reported (SANABRIA et al., 2005). The months of December, January, February, March and April constitute the post-reproductive period in which the species generally is found foraging. The individuals were euthanized with an injection of 2.5ml of xylocaine 2% placed in the lymph sac, fixed with 10% formaldehyde and preserved in 70% alcohol.

All individuals were measured from snout to the cloaca (SVL) using digital calipers (Essex; China. Accuracy 0.01mm) and weighed with a digital scale (Denver; Boemia, NY, USA. accuracy 0.1 g).

In the laboratory, specimens were dissected and their gonads and fats bodies removed for further analysis. To determine the reproductive status of gravid females, the ovarian mass was weighed on a digital scale (Denver; Boemia, NY, USA. Accuracy 0.1g). In addition, we calculated the ovarian complement (number of mature ova) through a sample taken from the ovarian mass. The ova from a fraction of the ovarian mass were weighed and counted, and then we extrapolated the data to the total weight of the ovarian mass (CRUMP, 1974). We measured the diameter of mature ova with a binocular microscope (magnification 10X) and digital calipers. The criterion used to define mature ova was the degree of pigmentation. Immature ova resemble an undifferentiated mass where the ova has not begun to accumulate yolk in the cytoplasm, whereas in mature ova that are black in color, the yolk accumulation has begun to create an opaque and milky aspect, indicating the finalization of development (MARTORI et al., 2005).

To find the testicular volume of males, we measured the length and width of the testicles and calculated the volume using the spheroid formula (DUNHAM, 1983). In both sexes we extracted fat bodies and weighed them on a digital scale (Denver, Boemia, NY, USA. Accuracy 0.0001g). This method was used because the fat bodies have irregular form and are difficult to measure (VITT & OHMART, 1975). Means and standard errors were calculated for all data, and an ANCOVA was used to test differences between reproductive and post-reproductive periods using body weight and SVL as covariates.

RESULTS

We gathered data from 116 individuals of which 75 were males and 41 females. Females had an average fat body mass of 2.28 ± 0.41g, body mass of 155.9 ± 8.06g and SVL of 109.4 ± 1.6mm while males had an average fat body mass of 1.69 ± 0.19g, body mass of 100.7 ± 3.7g and SVL of 94.5 ± 1.1mm. Females exhibited larger size than males for all of the measured variables. Table 1 shows the variables of both sexes for the reproductive and post-reproductive periods.

TABLE 1

Variables of females (♀) and males (♂) in the reproductive and post-reproductive periods for fat body mass (FBM), body mass (BM), number of mature ova (NMO), size ovules (SO), and testicular volume (TV).

Variables	Periods	
	Reproductive	Post-reproductive
FBM ♀	0.48 ± 0.11	3.78 ± 0.57
BM ♀	138.56 ± 7.7	168.8 ± 12.2
NMO ♀	33219 ± 6304.6	25063.79 ± 2011.8
SO ♀	1.06 ± 0.09	1.16 ± 0.02
FBM ♂	0.85 ± 0.19	2.34 ± 0.27
BM ♂	90.09 ± 4.74	109.02 ± 5.21
TV ♂	80.34 ± 7.42	94.14 ± 7.83

Females of *R. arenarum* showed significant differences between the reproductive and post-reproductive periods for fat body mass (ANCOVA: $F_{1,37} = 21.68$; $P < 0.00004$; cov. = SVL) (Fig. 1), body mass (ANCOVA: $F_{1,37} = 3.80$; $P < 0.005$; cov. = SVL) (Fig. 2), and number of mature ova (ANCOVA: $F_{1,24} = 9.73$; $P < 0.004$; cov. = SVL) (Fig. 3). There were no significant differences in egg size (ANCOVA: $F_{1, 26} = 1.98$; $P > 0.17$; cov. = SVL).

Males also showed differences between the reproductive and post-reproductive periods

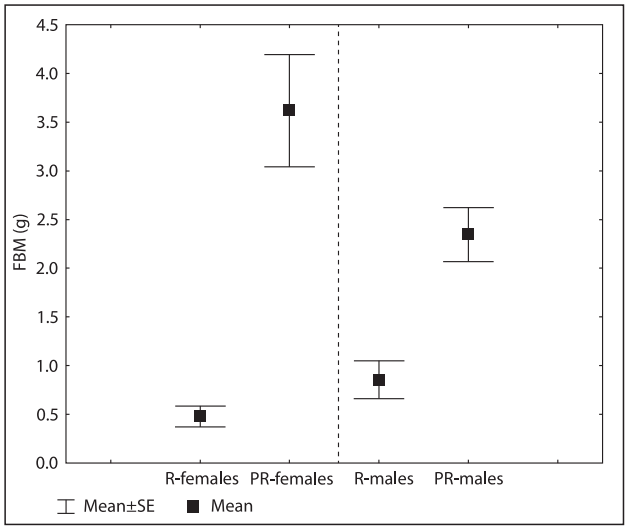

Fig. 1 − Variation in fat body mass (FBM) of the sexes between the reproductive (Rfemales, Rmales) and post-reproductive (PRfemales, PRmales) periods.

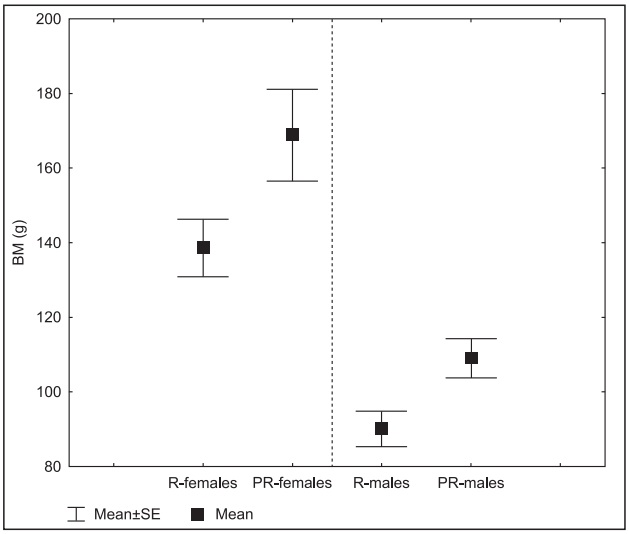

Fig. 2 − Variation in body mass (BM) of the sexes between the reproductive (Rfemales, Rmales) and post-reproductive (PRfemales, PRmales) periods.

for fat body mass (ANCOVA: $F_{1,72} = 21.17$; $P < 0.00002$; cov. = SVL) (Fig. 1) and body mass (ANCOVA: $F_{1,72} = 17.74$; $P < 0.00007$; cov. = SVL) (Fig. 2). There was no significant difference in testicular volume (ANCOVA: $F_{1,72} = 0.53$; $P > 0.46$ cov. = SVL).

DISCUSSION

As do most anurans, *Rhinella arenarum* exhibits sexual dimorphism, with females larger than males (DUELLMAN & TRUEB, 1986; QUIROGA et al., 2004). Moreover, gender variation exists in the storage organs (fat bodies) with females storing more fat than males. According to SHINE (1979), females are larger than males in 90% of anuran species; this appears to be related to the ability of females to produce large numbers of ova (CRUMP, 1974; PEROTTI, 1994). There are other hypotheses, however, to explain larger female size, such as predation pressure on larger males because of the risks involved in territorial defense and mating (HOWARD, 1981; PERALTA DE ALMEIDA-PRADO & UETANABARO, 2000). However, both sexes show lower body mass during the reproductive period, which suggests that both males and females spend their energy in reproduction (BRATTSTROM, 1979).

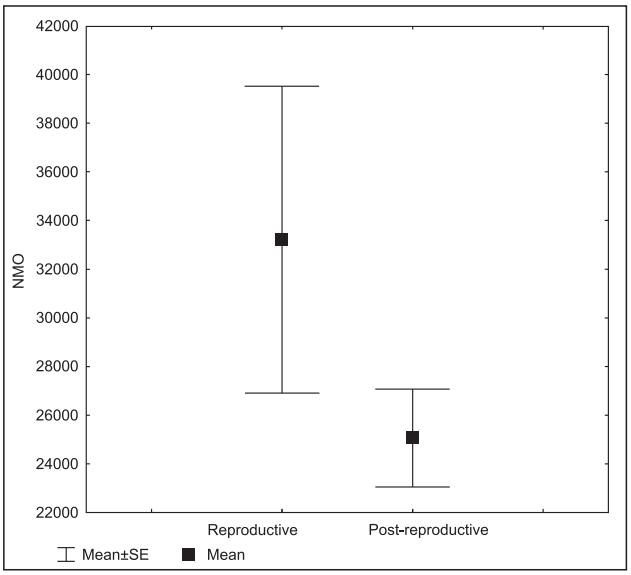

Fig. 3 − Differences in the number of mature ova (NMO) between the reproductive and post-reproductive periods.

Consequently, fat bodies also exhibit minimum size during the spawning period (DÍAZ-PÁEZ & ORTIZ, 2001).

Males have restrictions on their growth due to the energy demands during reproductive activity, where the major energy expenditure relates to sperm production, calls, and defense of breeding territory (DUELLMAN & TRUEB, 1986; WELLS, 2007; NAVAS et al., 2008). In addition, they consume only a small amount of food during the calling period (WOOLBRIGHT, 1989), and empty stomachs have been observed during this period (QUIROGA, unpubl. data).

On other hand, development of the fat bodies and increases in body mass have been observed during the post-reproductive period enabled by the large quantity of food ingested between the months of December and February (QUIROGA et al., 2009). Thus, *R. arenarum* can store enough energy and nutrients for the next period of hibernation and for reproduction during the following year (BRATTSTROM, 1979; WHITFORD, 2002). Energy acquisition in both sexes is an important factor in reproductive events, and thus gonadal function depends on the contribution of fat bodies (WELLS, 2007).

In females, the size of mature ova does not differ between the reproductive and post-reproductive periods. Presumably growth ceases once the ovum matures. However, the number of mature ova is significantly higher in the reproductive period. During this period, the ova are being deposited at any time (MARTORI et al., 2005). In contrast, during the post-reproductive period, the number of mature ova diminishes. It is likely these remaining mature ova will be deposited in the first clutches after aestivation, as observed for *Pleurodema thaul* by DÍAZ-PÁEZ & ORTIZ (2001). Also, SANABRIA et al. (2005) found that reproduction in *R. arenarum* begins in mid-August, coinciding with the end of winter hibernation. This strategy would allow the species to avoid or delay the predation of their eggs by invertebrate predators (HEYER et al., 1975).

Males of *R. arenarum* did not differ in testicular volume between the reproductive and post-reproductive periods. It is likely that during the activity period the testes are active and individuals potentially ready to breed (LAVILLA & ROUGE, 1992), as observed for *Rhinella fernandezae* by MARTORI et al. (2005). Thus, *R. arenarum* would exhibit continuous reproduction, agreeing with the observations of TSIORA & KYRIAKOPOULOU-SKLAVOUNOU (2001) for *Rana epeirotica*, where the weight of the testis was not related to spermatogenic activity, indicating potentially continuous spermatogenesis.

Males of *Rhinella arenarum***,** appear to exhibit a continuous, opportunistic reproductive strategy, as they maintain large fat bodies and mature sperm, allowing them to reproduce immediately after emerging in spring. By contrast, females have fewer mature ova during this period, reflecting a reproductive cycle shared by amphibians in temperate zones. Our reproductive data, and the primarily tropical distribution of *R. arenarum* (CEI, 1980), suggest that this species recently invaded the temperate region wherein males retained acyclic reproductive activity and females, owing to their higher reproductive costs, have evolved cyclic reproduction.

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The impact of sward height, forage quality and competitive conditions on foraging behaviour of free-ranging rabbits (*Oryctolagus cuniculus* L.)

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ABSTRACT. The habitat choice of the small hindgut fermenter, the European rabbit (*Oryctolagus cuniculus* L.), was studied in relation to sward height, forage quality, population size fluctuations and spatial distribution of burrows in a temperate grassland. In a multi-phase differential clipping experiment with alternating short and tall vegetation strips, rabbits tended to graze near the closest burrows in situations of equal vegetation heights, while a clear preference for short swards was found during summer (July). In this period, general crude protein content was significantly lower than in spring (April) and autumn (September), apparently leading to a potential forage quality deficit. The summer behavioural pattern with short sward preference coincided with the relatively higher crude protein content of short swards as compared to tall swards in this period, and with higher intraspecific competition, due to significantly larger numbers of animals present in summer. In autumn, rabbit densities decreased, while crude protein content of both short and tall vegetation increased to a higher, though not significantly different level, comparable with spring crude protein content. In those conditions, significant preference for low vegetation height could no longer be detected. Data suggest that selection for nutritive quality appears when intraspecific competition is high and nutritive quality remains under a certain threshold value. When, in autumn, competition decreases and nutritive quality increases again, short sward preference disappears. We conclude that short sward preference is primarily caused by the better forage quality of re-growth in periods of forage quality limitation, while this preference disappears when forage quality limitation no longer occurs.

KEY WORDS. rabbit, forage quality, vegetation height, crude protein.

INTRODUCTION

The optimal foraging theory states that herbivores maximize their net energy intake per unit time (MacArthur & Pianka, 1966; Stephens & Krebbs, 1986), which implies that foraging animals tend to select high quality forage as long as the energy gain exceeds feeding costs (e.g. searching and handling time and efforts, predation avoidance). While food intake rate by mammalian herbivores is predicted to increase asymptotically with food density (Lundberg, 1988; Lundberg & Astrom, 1990; Gross et al., 1993), food requirements can be expected to vary among mammal species with different body mass. Whereas larger herbivores may tolerate forage of low nutritional quality if available in sufficiently large quantities, the high metabolic rate and small digestive system of small grazers entails a need for higher-quality forage, albeit in smaller quantities (Demment & Vansoest, 1985; Olff et al., 2002).

Given the fact that fibre content of above-ground grassland vegetation increases - and nitrogen content decreases - during ageing (Pavlů et al., 2006), a grassland consisting of fully-grown, mature plant leaves is on average of lower nutritional quality than one consisting

of re-growing shoots with short swards. Hence, food intake rates by small herbivores are expected to be lower under high availability of low-quality food, and higher under low to intermediate availability of high-quality food (Type IV functional response) (DEKKER & VAN LANGEVELDE, 2007), as reflected by uni-modal, dome-shaped response curves (DURANT et al., 2003; VAN LANGEVELDE et al., 2008). Consequently, it is expected that when given the choice, small herbivores will prefer small to intermediate quantities of high-quality food instead of large quantities of low-quality food, although other factors, such as predation avoidance, interfere with feeding behaviour (BAKKER et al., 2005; LIMA & DILL, 1990).

The European rabbit (*Oryctolagus cuniculus*) is a medium-sized hindgut fermenter (DEMMENT & VANSOEST, 1985), which, due to its digestive system and medium-sized stature, relies on high quality, quickly digestible forage. While rabbits are expected to select foraging sites with short swards and high nutrient contents, other studies reveal contrasting patterns. For instance, strictly-controlled, experimental studies confirm a preference of rabbits for short swards (IASON et al., 2002; BAKKER & OLFF, 2003) while correlative field studies draw attention to the optimum grazing efficiency for swards of medium standing crop (VAN DE KOPPEL et al., 1996) or temperature-dependent habitat selection (VILLAFUERTE et al., 1993). Small refuge-living herbivores are also known to exhibit spatial foraging patterns determined by the location of their burrows (DEKKER, 2007). As the proportion of time spent on vigilance increases with distance from the nearest burrow, rabbits tend to graze in proximity to refuges until the food source is depleted (DEKKER et al., 2007).

To study if and to what extent, free-ranging small herbivores select for vegetation height or forage quality, we conducted a clipping experiment in a homogeneous grassland habitat in which the mammal herbivore community is dominated by European rabbits. To determine whether sward height preference could be attributed to structural or forage quality differences, we measured sward height in the field and analyzed standard forage-quality-determining variables (neutral detergent fibre, acid detergent fibre and acid detergent lignin, crude protein and mineral content (phosphorous and potassium)). The location of all rabbit holes near the study site was recorded in order to discriminate between habitat preference induced by habitat quality (sward height and nutritive content) and the proximity of burrows.

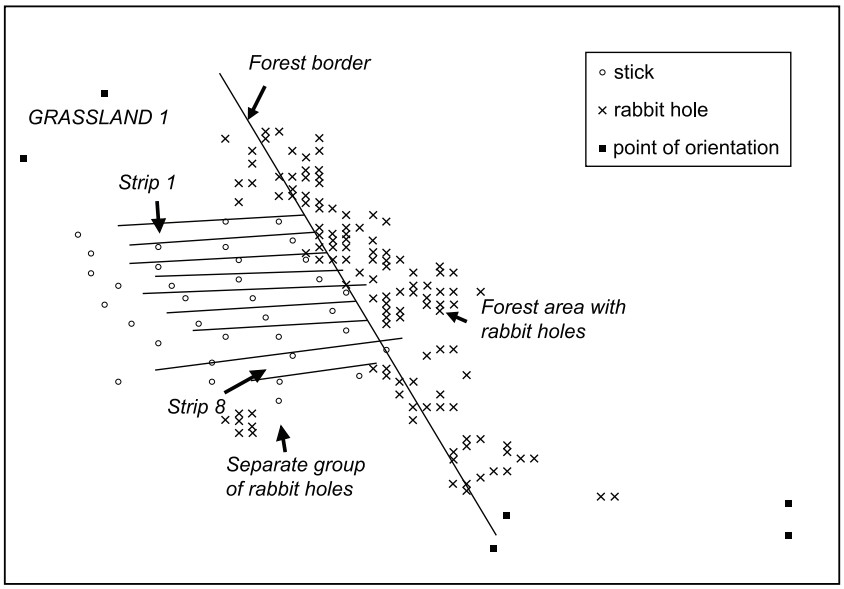
In this study we answer two main research questions:

1. Do free-ranging rabbits select for short or tall vegetation? If rabbits show a preference for a certain vegetation height, is this preference affected by seasonal changes in rabbit densities or forage quality or by the location of burrows?
2. What is the underlying mechanism for selecting short or tall vegetation? Do rabbits actively and in all circumstances select for high quality forage?

MATERIALS AND METHODS

Clipping experiments

We selected two flat, nearby, monotonous dry grasslands (500 m apart, separated from each other by two Poplar plantations. They are located in the Flemish Provincial Domain 'Puyenbroeck' (Wachtebeke, Belgium, 51°9'11"N, 3°52'43"E). Both were co-dominated by the grass species *Holcus lanatus* and *Agrostris capillaris* with *Cerastium fontanum, Ranunculus repens, Prunella vulgaris* and *Veronica chamaedrys* as constant dicotyledonous species. Both were bordered by plantations of *Populus* X *canadensis* under which rabbit burrows were concentrated. According to burrow distribution patterns, both Poplar stands were populated with different rabbit populations, ensuring foraging of both grassland stands by different rabbit populations. Within each grassland, a 72m x 30m study plot was delineated (Fig. 1a-c). Both plots were subsequently divided into eight strips of equal



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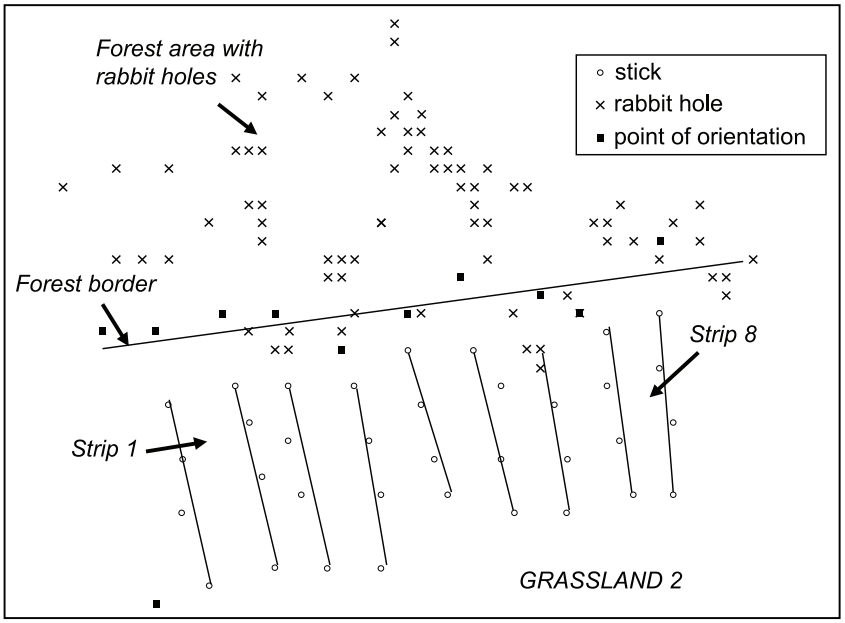

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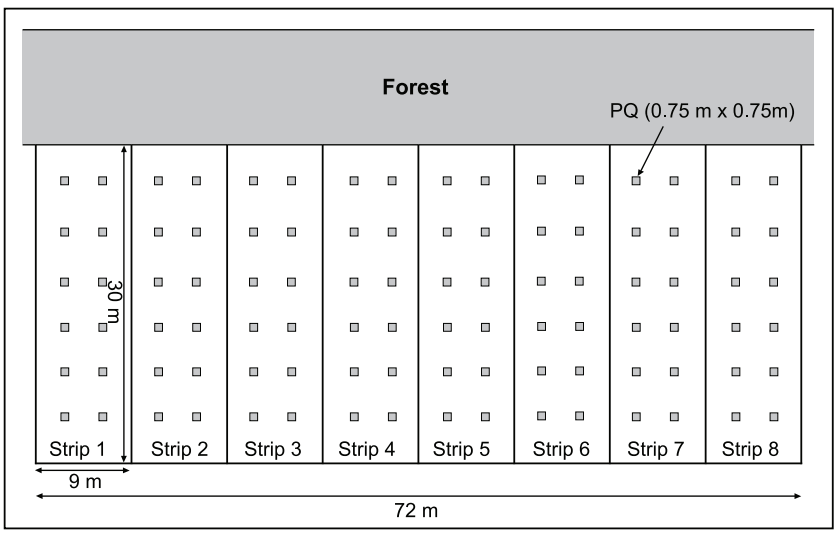

C

Fig. 1 – Schematic representation of experimental design. (A-B): both grassland sites with location of rabbit burrows (based on GPS coordinates), forest borders and grassland strips that were subjected to differential clipping treatments. (C): details of single grassland strip with indication of permanent quadrants for pellet counts. See text for details.

width and length (9x30m, numbered 1-8 in Fig. 1c), and twelve 75 x 75 cm permanent quadrates (PQs) were delineated in a systematic order within each strip (totalling 96 PQs per grassland). To structurally homogenize both plots all grassland strips were initially clipped at an equal height of 4.2 ± 2.61 cm (t_{-1}), which resulted in an average sward height of 6.6 ± 3.4 cm after 3 weeks of re-growth ('equal' (spring) treatment; t_0=25-26 April 2006). After 12 weeks, even numbered strips were clipped at equal height, while odd strips were left untouched (summer treatment; t_1=10-12 July 2006). After another 12 weeks, even strips were left untouched, and odd strips were clipped at equal height (autumn treatment; t_2=26-27 September 2006). Clipping can be considered as an extreme simulation of grazing, e.g. by other larger herbivores. Under the assumption that the latter have a general impact on sward height and consecutive re-growth of vegetation, conclusions drawn from this clipping experiment could give an estimate of possible facilitative or competitive interactions between both mammal herbivore types, although large herbivores give rise to structural diversity, which is absent in uniformly clipped fields.

Rabbit presence and burrow locations

Rabbit presence was estimated by the pellet counting method (Wood, 1988; Palomares, 2001; Bakker et al., 2005). Before the first general clipping event all rabbit pellets were removed from the PQs and immediately before both other clipping events, the total number of pellets per PQ was counted and then removed. Latitude-longitude coordinates of all rabbit burrows located in and within a range of 50 m around both grasslands were recorded using a Garmin GPS map76 (Fig. 1a and 1b) and distances between every PQ and the nearest rabbit burrow were calculated.

Vegetation parameters

At the start of the experiment (03/04/2006) and at the end of each experimental treatment (25/04/2006, 10/07/2006 and 26/09/2006 for respectively the equal spring, and the differential summer and autumn treatment), vegetation height was measured at the centre of each PQ as the height at which a disc (diameter 15 cm) with a central slot around a vertical ruler touched the vegetation first (combination of "drop disc method" and "sward stick method" described by Stewart et al., 2001). General vegetation composition of each site was measured in summer (July). Before each clipping event, vegetation samples were collected in the PQs in both grasslands and dried at 60°C to constant weight. Subsequently, the dried samples were milled through a 0.8 mm sieve and analysed using Near-Infrared Spectroscopy (NIRS) (Givens et al., 1997). The following measures of nutritive value were determined: concentrations of crude protein (%CP), neutral detergent fibre (%NDF), acid detergent fibre (%ADF), acid detergent lignin (%ADL), phosphorous (%P) and potassium (%K). A sub-sample of 10% of all vegetation samples was randomly selected for direct chemical analysis to fine-tune NIRS calibration lines; the latter are based on a wide range of grassland species of temperate grasslands. %N (needed to calculate %CP) of the samples was determined using the Kjeldahl technique (AOAC 1990), while cell wall components (%ADF) were determined using the method described by Van Soest *et al.* (1991). Cell wall components were analyzed using an ANKOM-220 fibre analyzer (ANKOM Technol. Corp., Fairport, NY) by sequentially adding neutral detergent (for %NDF), acid detergent (for %ADF) and 72% (wt/wt) sulphuric acid (for %ADL). Results of the chemical analysis were merely used for calibration purpose, while the results of the NIRS-analyses were used in the statistical analysis.

Statistical analysis

In the statistical analysis six treatments were distinguished: "short1" (t_{-1}, start of the experiment), "tall2" (t_0, equal spring treatment), "short3" and "tall3" (t_1, respectively the even and odd strips in the differential summer treatment),

TABLE 1

Average values and standard deviations for vegetation height, rabbit activity (number of pellets) and forage quality measures (CP, NDF, ADF, ADL, P and K). Different letters in the same column indicate significant differences between treatments.

Period	Treatment	Strip	Vegetation height (cm)	Pellet count (m^{-2})	%CP	%NDF	%ADF	%ADL	%P	%K
t_{-1}	Control	even = short1 odd = short1	4.3 ± 2.8 a 4.1 ± 2.4 a	- -	17.93 ± 4.49 c 19.23 ± 2.95 c	37.12 ± 2.72 a 35.02 ± 6.16 a	17.31 ± 1.04 a 16.64 ± 1.98 a	2.53 ± 0.89 a 3.11 ± 0.96 a	0.28 ± 0.05 c 0.3 ± 0.04 c	2.01 ± 0.25 b 2.14 ± 0.29 b
t_0	Equal spring treatment	even = tall2 odd = tall2	6.5 ± 3.3 ab 6.7 ± 3.6 ab	2.5 ± 7.2 a 3.6 ± 8.5 a	18.06 ± 3.16 c 17.43 ± 3.98 c	35.93 ± 4.89 a 38.07 ± 5.17 a	18.65 ± 1.52 a 19.71 ± 2.78 a	2.66 ± 1.31 a 3.32 ± 0.28 a	0.28 ± 0.03 c 0.28 ± 0.04 c	2.14 ± 0.16 b 2.11 ± 0.24 b
t_1	Differential summer treatment	even = short3 odd = tall3	8.8 ± 3.4 b 31.6 ± 21.1 d	18.6 ± 49.6 b 5.8 ± 12.9 a	12.73 ± 1.97 b 10.14 ± 0.69 a	41.52 ± 3.65 a 43.27 ± 13.77 a	22.83 ± 0.8 a 24.46 ± 6.07 a	3.79 ± 0.41 a 4.08 ± 0.87 a	0.22 ± 0.02 b 0.18 ± 0.01 a	1.91 ± 0.16 b 1.66 ± 0.1 a
t_2	Differential autumn treatment	even = tall4 odd = short4	15.2 ± 5.2 c 9.1 ± 2.3 b	2.1 ± 6.1 a 0.8 ± 3.8 a	17.59 ± 2.20 c 18.83 ± 2.09 c	39.2 ± 3.57 a 44.11 ± 2.34 a	22.37 ± 0.97 a 24.18 ± 1.85 a	4.15 ± 0.41 a 4.09 ± 0.92 a	0.25 ± 0.02 c 0.28 ± 0.03 c	2.16 ± 0.06 b 2.28 ± 0.13 b

and "tall4" and "short4" (t_2, respectively the even and odd strips in the differential autumn treatment).

Treatment effects of clipping regime on vegetation height were determined using analysis of variance (ANOVA) and Tukey HSD tests. Effects of clipping regime on rabbit densities were tested with linear mixed models with factors vegetation height, month and distance to the nearest rabbit hole as response variables, and factors grassland and strip as random effects. Furthermore, to test for treatment effects irrespective of distance to burrows, ANOVA and Tukey HSD tests were performed with rabbit densities and treatment as fixed factors. Effects of different clipping regimes on forage quality measures and vegetation height were tested with Wilcoxon rank sum tests. All statistical analyses were performed with R 2.13.0 (R Development Core Team, 2011).

RESULTS

Vegetation height

Average clipped vegetation height did not differ significantly either between even and odd strips in the control treatment or between the re-growth in even and odd strips in the equal spring treatment, indicating unbiased clipping methods and similar regeneration potential in even and odd strips (Fig. 2). In both differential treatment periods (even summer and odd autumn treatments) similar vegetation height was measured in clipped strips, whereas vegetation was significantly taller in unclipped than clipped strips. Average vegetation height in unclipped strips was significantly higher in summer than in autumn (Fig. 2 and Table 1).

Rabbit presence

During the equal treatment, the number of faecal pellets was negatively correlated with the distance to the nearest burrow ($p<0.05$), while a similar, albeit not significant, relationship was found in the differential treatment periods,. Furthermore, negative correlations between pellet numbers and both season and vegetation height ($p<0.05$) were detected in the differential treatment periods (Table 2).

The number of faecal pellets did not differ significantly between even and odd strips in the control treatment, while subsequent differential clipping treatments resulted in significant differences in pellet numbers. Significantly more droppings were found in the short strips in the

TABLE 2

Results of the linear mixed effects models with pellet counts as dependent variable, distance to nearest burrow, vegetation height and season as response variables, and grassland and strip as random effects, for equal and differential treatments. Significant interactions are in bold.

Treatment	Factor	Value	Std.Error	DF	t-value	p-value
Equal treatment	(Intercept)	1.4998529	0.4735357	175	3.167349	**0.0018**
	Distance nearest burrow	-0.0406124	0.0195366	175	-2.078788	**0.0391**
Differential treatment	(Intercept)	14.346682	2.7271512	365	5.260684	**0.0000**
	Distance nearest burrow	-0.041426	0.045371	365	-0.913049	0.3618
	Season	-3.139754	0.689741	365	-4.552077	**0.0000**
	Vegetation height	-0.055591	0.0246432	365	-2.255843	**0.0247**

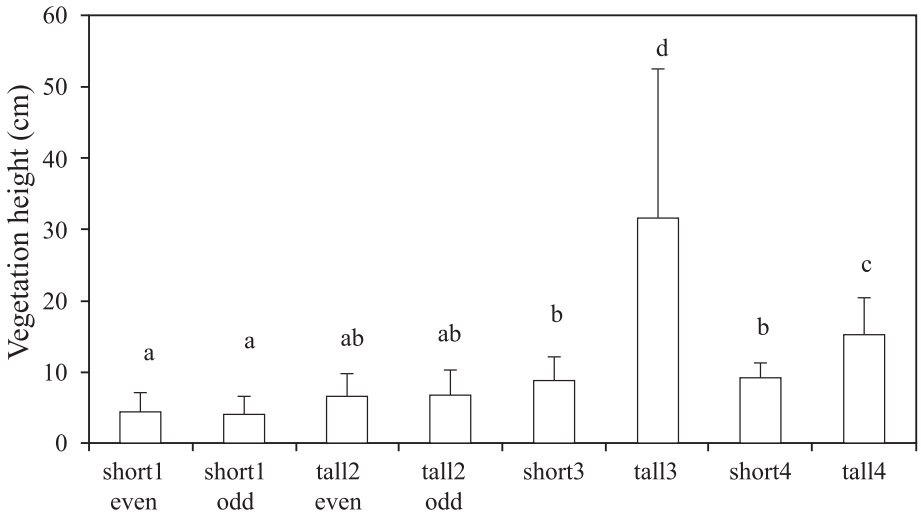

Fig. 2. – Comparison of vegetation height between treatments using ANOVA and Tukey's HSD tests. Different letters indicate significant differences between treatments (F= 104.84; p=0.0000).

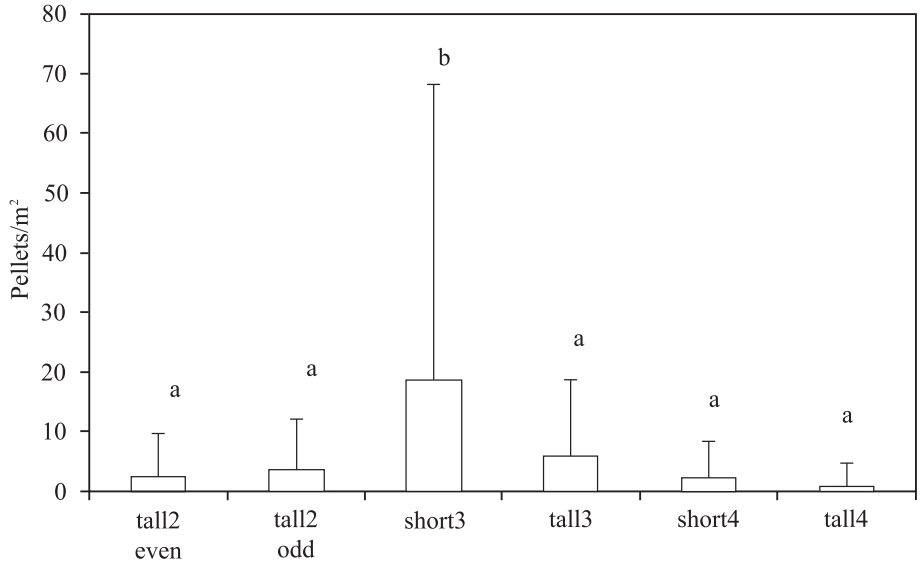

Fig. 3. – Comparison of pellet counts between treatments using ANOVA and Tukey's HSD tests. Different letters indicate significant differences between treatments (F= 8.9429; p=0.0000).

TABLE 3

Comparison of forage quality measures (CP, NDF, ADF, ADL, P and K) for different treatments and test periods (Wilcoxon rank sum test). Significance levels: NS: p>0.10, *: 0.10<p<0.05, **: 0.05<p<0.01, ***: p<0.01.

Period	Treatment	Strip	%CP	%NDF	%ADF	%ADL	%P	%K
t_{-1}	Original situation	short1: even - odd strips	NS	NS	NS	NS	NS	NS
t_0	Equal treatment	tall2: even - odd strips	NS	NS	NS	NS	NS	NS
t_1	Even treatment	short3 - tall3	**	NS	NS	NS	**	**
t_2	Odd treatment	short4 - tall4	NS	NS	NS	NS	NS	NS
t_1-t_2	Even vs. odd treatment	short3 - short4	**	NS	NS	NS	**	*
t_1-t_2	Even vs. odd treatment	tall3 - tall4	**	NS	NS	NS	**	**

differential summer treatment period, while a similar, though not significant trend was found in the differential autumn treatment period (Fig. 3 and Table 1). A peak in overall pellet numbers was found in summer (July) with in total 586 droppings, whereas a total of 146 droppings was counted in spring (April) and only 69 in autumn (September).

Forage quality

No significant differences were found between even and odd strips in both the control and autumn treatment in any of the forage quality variables, while significantly higher CP, P and K concentrations were found in the short sward strips during the summer treatment as compared with the long sward strips. As opposed to the

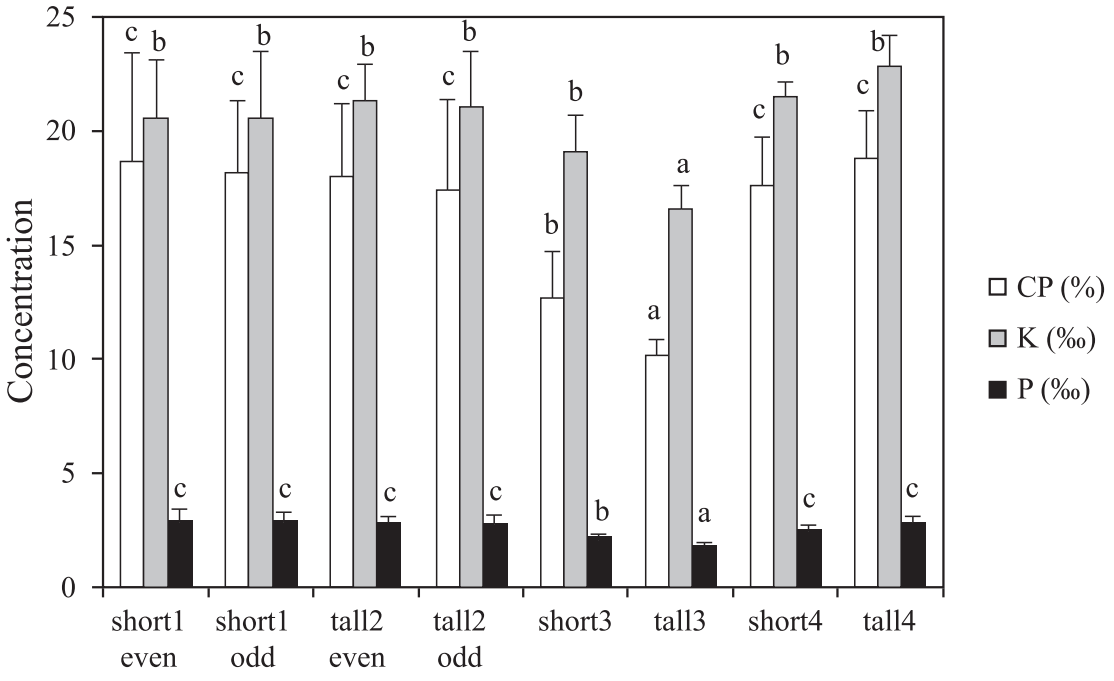

Fig. 4. – Comparison of forage quality concentration (crude protein, potassium and phosphorous) between treatments. Different letters indicate significant differences between treatments.

summer treatment, no significant differences in forage quality measures were found between tall and short vegetation after the autumn treatment. Additionally, when comparing both differential treatment periods, significantly higher concentrations for CP, P and K were found in general (short and tall combined) in autumn versus summer, indicating overall seasonal variations in these forage quality variables with a general forage quality dip in summer (Fig. 4 and Table 3).

DISCUSSION

Low versus tall sward preference

In line with preference patterns emerging from strictly-controlled laboratory and field experiments (Iason et al., 2002; Bakker & Olff, 2003), results from our field experiments support the hypothesis that free-ranging rabbits prefer low above taller vegetation when offered a direct choice. As control and treatment plots were reversed during consecutive experiments, preference for vegetation height was not confounded by possible site preference.

No overall correlations between forage quality traits and vegetation height were found. Although in the first differential summer clipping experiment forage quality measures for crude protein, potassium and phosphorous content were significantly higher in short swards, no significant forage quality differences between cutting treatments were found in the second differential autumn experiment. Nonetheless, a positive correlation between crude protein content and cutting frequency has been demonstrated by many authors. In temperate grasslands in France, Pontes et al. (2007) found a positive correlation between crude protein content and cutting frequency. A sequential clipping trial in an Icelandic hayfield, Fox et al. (1998) caused elevated protein content of *Phleum pratense* leaves from 7-13%. Other studies also demonstrated that grazing or clipping resulted in vegetation re-growth with higher crude protein content (Mayhew & Houston, 1999; Pavlů et al., 2006; Li et al., 2010 for *Poa pratensis*).

Forage quality threshold value as sward selection criterion

Apart from cutting or grazing effects (e.g. Pavlů et al., 2006), crude protein content in grasslands also tends to fluctuate with seasons. In a sown pasture vegetation (including both grasses and herbs) differences appeared but without a clear seasonal pattern (Pavlů et al., 2006), while a selection of grasses cultivated in monocultures, showed a significantly lower %CP in spring than in summer and autumn, the last two seasons not significantly different from one another (Pontes et al., 2007). Also in Puyenbroeck, seasonal fluctuations in crude protein concentration were measured, with the highest concentrations at the end of the growing season for both short and tall stands. Seasonal patterns were also found by Peitz et al. (1997) for cottontail rabbits in Oklahoma where summer forage quality was extremely low in all essential amino acids for all life processes whereas winter diets were probably adequate for maintenance and growth. In contrast with the findings of Pontes et al. (2007), in our experiment crude protein concentrations of the vegetation showed a significant dip in summer, when average values for %CP ranged between 12.73% for short swards and 10.14% for tall swards. Worth mentioning is that Pontes et al. (2007) only dealt with grass species, while in our experiment dicotyledonous plant species were an important vegetation component, among which *Veronica chamaedrys* and *Prunella vulgaris* were the most prominent. Both may have far slower re-growth response to clipping than the grass species involved (*Holcus lanatus* and *Agrostis capillaris*).

Taking into consideration that de Blas & Mateos (1998) recommend forage with a crude protein content of 15.3-18.4 % for meat rabbits, we hypothesize that crude protein levels in summer are around or even below a certain forage quality threshold, forcing the wild rabbits to select for the best quality levels. Hence, we assume that rabbits are attracted to the nutritionally more attractive short grasslands. This could be true for %CP, but also for %K and/or %P, but we found no

literature references to underpin this for the latter two variables. A laboratory experiment (SOMERS et al., 2008), has already shown that rabbits are able to differentiate between forage of different quality (expressed in protein content), which supports the hypothesis that also in the present study, rabbits actively selected sites with a higher forage quality when forage quality in general was low. It is interesting to mention that forage quality levels of the cultivated grasses used in the feeding trial of SOMERS et al. (2008) were similar to the summer %CP levels of vegetation at our study site (i.e. 10.18 ± 0.30 versus 13.46 ± 0.30 for both low and high quality food in the feeding trial while 10.14 versus 12.72 for both tall and short swards in summer in the present field experiment). Consequently, as the feeding experiment of SOMERS et al. (2008) revealed that domesticated rabbits actively choose for the more protein-rich option, we can assume that this is also the case for the wild rabbits in the presently discussed field experiment. As a response to seasonal and spatial variation in forage quality small herbivores tend to actively select plants or vegetation patches with a high nutritional content (HOLMES, 1991; DRENT & VAN DER WAL, 1999; MARTINS et al., 2002; BAKKER et al., 2005). MARTINS et al. (2002) illustrated the diversity of the diet of wild European rabbits with a seasonal shift in preferred foraging habitats according to forage availability. When combining both seasonal and spatial variation in our field experiment, crude protein levels in summer were significantly lower than in spring and autumn, but between short and tall strips it was still significantly higher in the first, which could drive rabbits to these short strips due to the slightly higher forage quality. This hypothesis is supported by the fact that in contrast with the equal treatment, during both differential clipping treatments no correlation was found between distance to burrows and defecation area, indicating that the availability of patches with a higher forage quality results in higher foraging efforts. In other studies rabbits also tended to forage further from their refuges once high quality food sources near the burrows became depleted (DEKKER, 2007).

Population dynamics

According to the pellet counts, rabbit numbers in summer were four times higher than in spring and even eight times higher than in autumn. This high summer level of rabbit population size can be explained by the breeding season rather than by migration from other territories, as the population was significantly reduced in autumn. This pattern corresponds with the high juvenile mortality found in other studies, e.g. VON HOLST et al. (2002) found a low survival of juveniles after weaning due to starvation, diseases and predation (11.4% for males vs. 15.6% for females). Also, at the start of the reproductive season, more does are reproducing and litter size tends to be higher than at the end of the breeding season (VON HOLST et al., 2002), which could explain the observed population size in summer. Nonetheless, the importance of other population regulating factors, such as migration, predation, diseases and seasonal variability in habitat preference, remains unknown. Irrespective of the cause of the fluctuation in population size, the combination of high population pressure and low nutritional quality in the summer period can at least partly explain the enhanced preference for short sward, having slightly higher nutritional value than the tall sward in this period. Also, due to the nutritionally unfavourable conditions in summer, intraspecific competition might lead to higher juvenile mortality and hence lower animal densities in autumn.

Sward height preferences caused by factors other than forage quality

However, rabbits may also prefer short vegetation for reasons other than forage quality, such as higher visibility of predators in more open vegetation (IASON et al., 2002; see also KOTLER & BLAUSTEIN, 1995). On the other hand, the location of burrows is more restrictive for rabbit movement patterns in short grasslands as short vegetation provides less protection against predators than tall swards (LOMBARDI et al., 2003). Earlier field experiments confirmed that rabbits are sensitive to perceived predation risk

(Bakker et al., 2005; Dekker, 2007), although such risk did not alter the spatial distribution of grazing individuals but rather resulted in shifts in foraging time versus vigilance. In other studies, sward height selection varied according to day/night activity patterns and temperature, with preference for dense vegetation during warm summer days (Villafuerte et al., 1993; Lombardi et al., 2003). Rueda et al. (2008) found season- and age-dependent habitat preferences in Central Spain, where adult rabbits preferred low volume swards in summer while juvenile distribution was dictated by the location of the warrens. Also, the selection for open vegetation may result from higher foraging efficiency, due to lower handling time, in low-open compared to tall-dense vegetation (van de Koppel et al., 1996), especially in summer when resource quality is low.

CONCLUSIONS

Although the selection for short sward cannot unambiguously be attributed to its better forage quality, our data nonetheless suggest that selection for nutritive quality appears when intraspecific competition is high and nutritive quality remains under a certain threshold value; in the experiment both factors coincide during summer (July). When, in autumn (September), competition decreases and nutritive quality increases again, short sward preference largely disappears. We conclude that short sward preference is primarily caused by the better forage quality of re-growth in periods of forage quality limitation, while this preference largely disappears when forage quality limitation no longer occurs.

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SHORT NOTES

Distribution of crayfish (Decapoda, Astacoidea) in Flanders (Belgium): an update

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There are thought to be approximately 600 crayfish species worldwide, which can be subdivided into two superfamilies: the Astacoidea, of which all species occur in the northern hemisphere, and the Parastacoidea, which have only been recorded in the southern hemisphere (1, 2). The Astacoidea can be subdivided into two families: Astacidae and Cambaridae. Crayfish constitute an important link in the food web since most species are keystone consumers of macroinvertebrates, detritus and macrophytes in lotic and lentic waters, and in turn serve as prey for several species including birds, fish and otter (3). It has been estimated that between one-third and one-half of the world's indigenous crayfish species are threatened with population decline or extinction (4). Non-indigenous crayfish introduced intentionally for astaciculture or unintentionally as unused bait or unwanted aquarium pets constitute the main threats to indigenous crayfish (5, 6, 7). In addition to displacement of indigenous crayfish species by non-indigenous species through competition, the crayfish plague (*Aphanomyces astaci*) has had a devastating effect on indigenous crayfish in Europe (8).

Because of their high commercial value, the cultivation of non-indigenous crayfish species increased enormously during the end of the 20th century in Europe, resulting in numerous introductions in a semi-natural environment (9). The introduction of crayfish in nurseries was very successful and several species were able to build up viable populations (8). This is mainly due to the robust nature of these freshwater crustaceans, coupled with fast individual and population growth, high fecundity and omnivorous behaviour (10). Non-indigenous crayfish can have an ecological as well as economic impact. They have the potential to reduce biodiversity and may also cause direct economic damage by reducing recruitment of commercially valuable fish stocks or by weakening dykes causing flooding danger (3, 7). In addition, non-indigenous crayfish may induce drastic habitat changes, altering the natural habitat and in this way, causing the decline of aquatic populations (11).

In Belgium, five crayfish species have been recorded, the indigenous *Astacus astacus* (Linnaeus, 1758) and four non-indigenous species: *Astacus leptodactylus* Eschscholtz, 1823, *Orconectes limosus* (Rafinesque, 1817), *Pacifastacus leniusculus* (Dana, 1852) and *Procambarus clarkii* (Girard, 1852) (5, 12, 13). *A. astacus* is threatened in Europe and faces extinction (5). Therefore, this species is classified on the IUCN red list as vulnerable (14). Gérard (1986) was the first to give an overview on the distribution of crayfish in Belgium based

on a survey that was carried out by the Station de Recherches Forestières et Hydrobiologiques of Groenendaal between 1982 and 1985 (15). Since then, the Walloon region especially (southern part of Belgium) has been investigated and some research regarding the current distribution of crayfish has been published (8, 16, 17). However, for Flanders (northern part of Belgium), little recent information is available on the presence and distribution of indigenous and non-indigenous crayfish. It is important to know the distribution and gain insight into the ecological effects of non-indigenous crayfish on aquatic communities. Careful mapping revealing patterns in crayfish diversity across regions and habitats is an important first step in this process. In this study, we present an overview of the current distribution of crayfish in Flanders based on existing databases supplemented with intensive field sampling.

Information on the distribution of crayfish in Flanders was retrieved from the database of the Flemish Environment Agency (VMM), which has monitored the water quality in Flanders since 1989. As a consequence, a large collection of more than 10,000 biological samples is currently available. Biological monitoring of macroinvertebrates took place by means of hand netting or use of artificial substrates as described by Gabriels et al. (2010) (18) and more than 2,500 samples containing Crustacea were identified to species level. Analysis of these samples revealed important information regarding the occurrence of crayfish in Flanders. Additional information was retrieved from the collections of the Royal Belgian Institute of Natural Sciences (RBINS). Field sampling (at predetermined locations as well as casual observations) carried out by the Laboratory of Environmental Toxicology and Aquatic Ecology (Ghent University) and the Research Institute for Nature and Forest (INBO) yielded additional information on the occurrence of the different crayfish species. Recent samplings of crayfish were performed at several locations where crayfish could be expected, from October 2010 to May 2011 by means of single fyke nets (0.25m diameter and a length of 0.50m), specifically designed to catch crayfish. The length (from rostrum to the end of the telson, accuracy=1 mm) and wet weight (Kern 440-53, accuracy=1 g) of all individuals were measured. The numbers of males and females (including gravid females) were recorded when possible.

In total, four non-indigenous crayfish species were found during the recent sampling campaign. In Flanders, the only indigenous crayfish, *Astacus astacus*, was recorded for the last time in 1945 in Lanaken (collection RBINS). *A. astacus* is a species that prefers clean running waters or ponds with well-oxygenated water (15). In Wallonia, *A. astacus* is still present in 41 stagnant water sites and six small streams, although its numbers have continued to decline since the 1990s due to the crayfish plague and competition with non-indigenous crayfish species (8). A decrease in water quality and habitat deterioration in combination with the crayfish plague and competition with non-indigenous crayfish species are probably the main causes of the extinction of *A. astacus* in Flanders.

Astacus leptodactylus, originating from East-Europe was introduced for the first time in Belgium in the 1970s and was first recorded in Flanders in 1986 (15). *A. leptodactylus* was originally introduced to replace stocks of indigenous crayfish, but it also seemed to be vulnerable to the crayfish plague and consequently did not fulfil the expected yield (19). Currently, the species occurs at six scattered locations in Flanders (Fig. 1): three ponds, one small stream and two canals. It has habitat preferences similar to *A. astacus* (15), but has a competitive advantage over the indigenous species (20) and is thought to be outcompeting the remaining populations of *A. astacus* in the southern parts of Belgium (8).

Pacifastacus leniusculus was introduced for the first time into Flanders in 1979 (14) and was recorded at three locations in Flanders before 1990 (Fig. 1). During recent sampling, the species was only found in one pond near Hasselt at low densities. Although this species is known to be

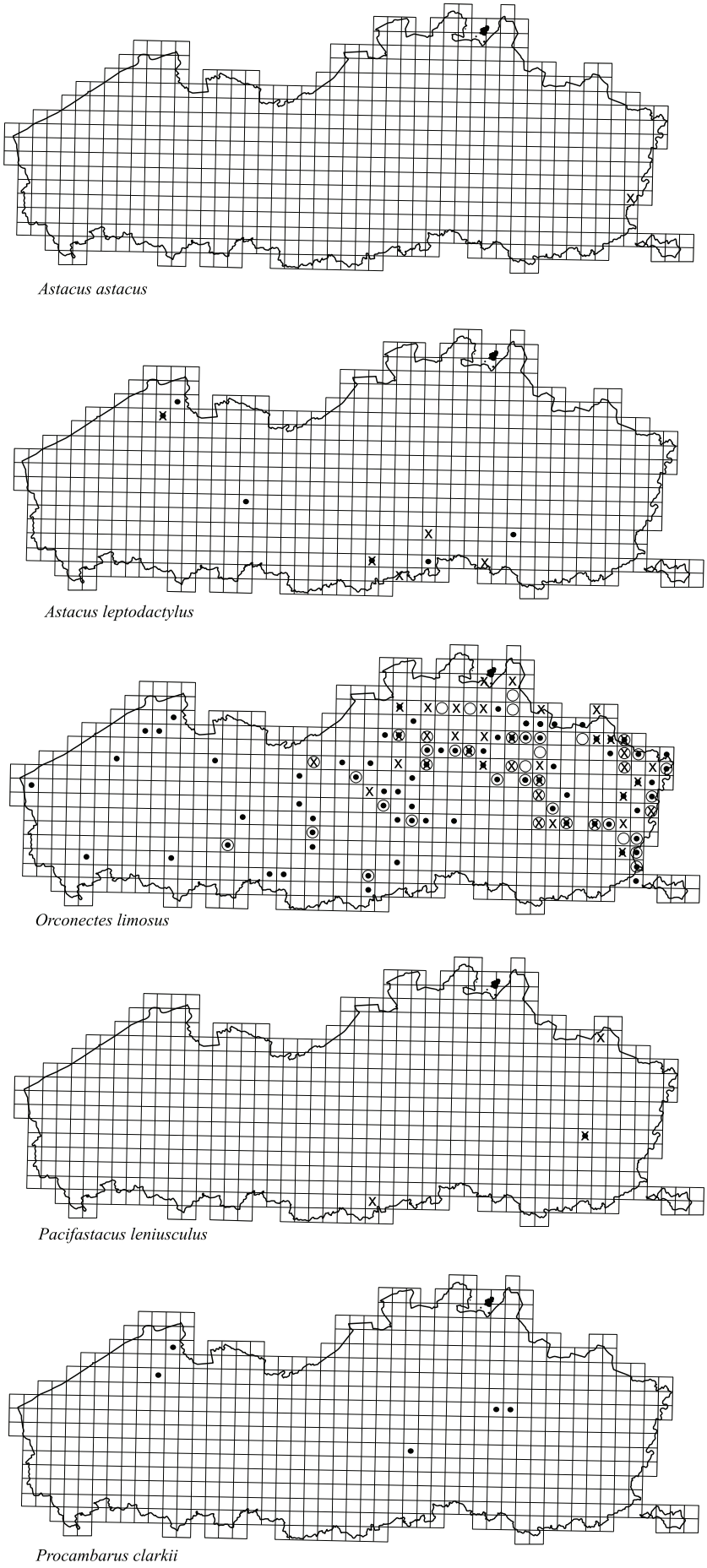

Fig. 1. − Distribution of the crayfish species in Flanders before 1990 (cross), from 1990 to 1999 (circle) and since 2000 (black dot) on a 5*5 km UTM grid.

successful and widespread throughout Europe (8), it seems to have a restricted distribution in Flanders. *P. leniusculus* also has similar habitat preferences to *A. astacus* (21). Despite the reported co-occurrence (22), *P. leniusculus* is able to outcompete the indigenous species (21). *P. leniusculus* attains a similar size, but grows faster, has earlier sexual maturity, produces larger clutches and is resistant to the crayfish plague (23).

The third non-indigenous species present in Flanders is *Orconectes limosus*, which was found for the first time in Flanders in 1977 (24). This very successful species is widely distributed, occurs in all types of aquatic systems (canals, rivers, brooks and ponds) and is the most common crayfish species in Flanders (Fig. 1). This species started its colonisation in the eastern part of Flanders where it rapidly invaded large watercourses (13). Since the 1990s *O. limosus* has spread to the West of Flanders (Fig. 1) with an average speed of 10 km per year. The average cumulative increase in its distribution area since 1977, measured as the number of 5*5 km UTM grid cells per year, was 12 grid cells per year. *O. limosus* appears not to be as sensitive to land use changes and human activities as the indigenous crayfish species (25). Moreover, it can withstand habitats unfavourable to indigenous species, such as soft substrates, turbid and muddy waters, polluted canals and organically-enriched ponds and lakes (26).

The most recently introduced species, *Procambarus clarkii*, was discovered in a pond near Zammel in 2008 (12). Currently, the species is reported at four other locations: a pond near Laakdal, not far from its first observation, a pond near Mechelen and several canals with slow running water near Bruges (Fig. 1). In one of these canals, the Damse Vaart, there is expected to be a large population of *P. clarkii* since the species is frequently caught and reported by fishermen. This might indicate that the species is in full expansion. Besides dispersal by human activities, rapid, active dispersal of the species may occur because it can spread over land and is thus not dependent on the aquatic environment for its dispersal (12). *P. clarkii* may become the next dominant species of crayfish, since it has been shown to outcompete several other crayfish species (27). It is known to contribute to biodiversity loss and habitat degradation in several freshwater systems of south central Europe (7) and is therefore also expected to have a negative impact on aquatic communities in Flanders.

Comparing the length and weight of the different species, *P. clarkii* is the largest, but individuals of the same size weigh less compared to *A. leptodactylus* (Fig. 2). The latter is often cultured due to its relatively large size, high weight and its high economic value (19). *O. limosus* is the smallest of these crayfish species. No individuals heavier than 60 g were found (Fig. 2). Good correlation between the size and the weight of the different species was observed (Fig. 2). With the exception of *Pacifastacus leniusculus*, we found large populations of all species, containing males, (gravid) females and juveniles (Table 1). During the catch in spring, more than 70% of *O. limosus* females carried eggs, whereas only 30% of *A. leptodactylus* females were gravid (Table 1).

Environmental impact and invasion stage were assessed for each species based on an environmental impact assessment protocol (ISEIA) and the geographic distribution of each species in Flanders (28). *O. limosus* was categorised as A3, indicating that the species has a high environmental impact (black list) and is widespread in Flanders. *Pacifastacus leniusculus* and *Procambarus clarkii* were assessed as species with possible high environmental impacts, but with isolated populations and consequently were categorised as black list species (A1). However, as *Pacifastacus leniusculus* only occurred at one location in Flanders its overall impact can be minimized. *A. leptodactylus* has a medium environmental impact, which is reversible and only some isolated populations occur in Flanders; this species has, therefore, been put on the watch list (B1). Our risk analysis of crayfish species is

comparable with previous results of an invasive species screening tool applied to crayfish in Italy (29). The top three species with the highest impact (*O. limosus*, *P. leniusculus* and *Procambarus clarkii*) were also encountered in Flanders and classified as 'black list species'. More detailed research and monitoring is needed in order to assess their impact on local communities and ecosystem functioning in Flanders.

This update on the current distribution of crayfish in Flanders clearly shows that the indigenous species *A. astacus* is extinct in Flanders and that meanwhile several non-indigenous species have now established good populations. Moreover, we hypothesize that *P. clarkii* has the potential to become the next dominant crayfish species in Flanders since it is rapidly expanding its range. A good overview of the distribution of the various species is vital to conservation efforts. The habitats of remaining indigenous populations in Belgium urgently need protection and appropriate management as sanctuary sites. In addition, our faunistic data can be helpful in identifying regions where *A. astacus* could be reintroduced. In order to reduce propagule pressure as a result of intentional introductions, it is important to build awareness among the public on the dangers related to the introduction of non-indigenous crayfish.

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TABLE 1

Overview of the crayfish species found in Flanders during recent samplings of several locations from October 2010 to May 2011, with indication of the female:male ratio, the percentage of gravid females and the average size with standard deviation.

Species	Origin	female:male	% gravid females	Av. size ± SD (mm)
Astacus leptodactylus (N=58)	East Europe	1:2	30	92±12
Orconectes limosus (N=64)	North America	1.5:1	76	86±10
Pacifastacus leniusculus (N=1)	North America	-	-	104
Procambarus clarkii (N=38)	North America	-	-	111±9

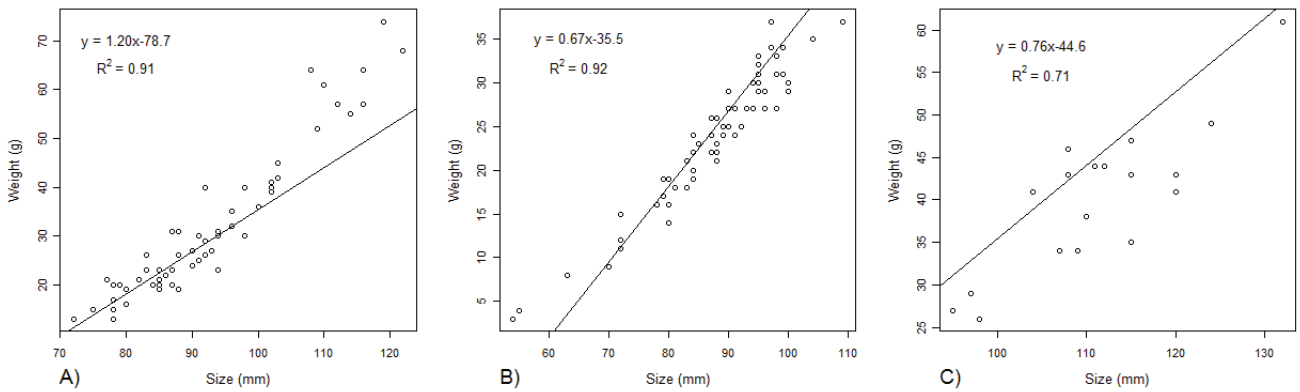

Fig. 2. − Relationship between size and weight of the crayfish species. (A): *Astacus leptodactylus*. (B): *Orconectes limosus*. (C): *Procambarus clarkii*.

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First record of the pelagic fish species blue whiting *Micromesistius poutassou* in the Belgian part of the North Sea

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The blue whiting *Micromesistius poutassou* (Risso, 1827) is a member of the cod family (Gadidae) and occurs in the western Mediterranean Sea and in the North Atlantic, ranging from Morocco to Spitsbergen in the east, and from Maine (US) towards southern Greenland in the west (1). It reaches lengths up to 50 cm and can weigh up to 830 g (1, 2). Blue whitings are bathypelagic oceanodromous fish that occupy depth ranges from 150-3000 m, but are mostly found at 300-400 m (3, 4).

These fish prey on small crustaceans but large individuals also forage on small fish and cephalopods. Blue whiting stocks are the target of the largest fishery in the Atlantic (5); the meat is sold both fresh and frozen, and is also processed as oil and fishmeal (1). Annual European landings fluctuate around 500, 000 tonnes (6).

In 2009 and 2010, monthly fish tracks using an otter trawl were carried out at ten monitoring stations (Fig. 1) in the Belgian part of the North Sea (BPNS). The trawl net with a 4*2 m diameter opening was dragged over the seabed for 30min at 3-4 mph.

On 16/7/2010 a whiting measuring 22 cm was caught near Nieuwpoort at station W03 (N 51°10'10", E 2°42'50") in very shallow waters (7 m depth). The fish had a partly digested adult brown shrimp *Crangon crangon* (Linnaeus, 1758) in its stomach, indicating that it had been feeding recently.

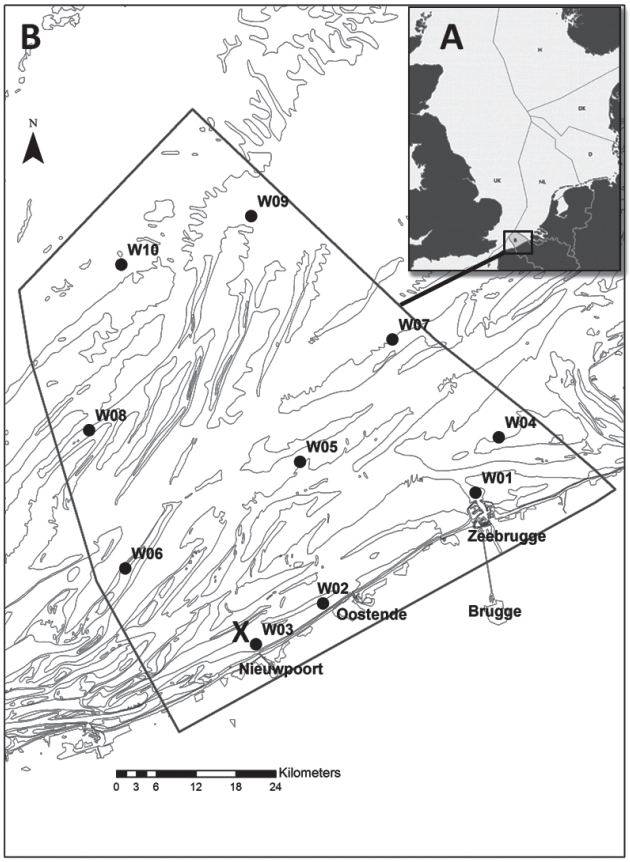

Fig. 1. − (A): North sea exclusive economic zones. (B): Belgian part of the North Sea (BPNS) with ten sampling stations sampled monthly in 2009 and 2010 for pelagic fish. The cross (X) indicates where the blue whiting was caught.

Photographs 1 and 2 of this individual show that the three dorsal fins are widely spaced and that the interspace between the second and third fin is larger than the base length of the first dorsal fin. Also obvious is that the mouth and gill cavities are black and that the lower jaw is somewhat protruding. The eyes are very big. These morphological features are characteristic for the blue whiting (6, 7). PAWSON (8) states that a blue whiting measuring 22cm is most likely three years old. Some blue whiting reach maturity in their third year, but recruitment to the breeding stock is not complete until most fish are 7-8 years old (8).

In order to validate the morphological identification, part of the caudal fin was cut for DNA analysis. This DNA was used as a template for the amplification of part of the mitochondrial cytochrome b gene in a PCR reaction with two in-house-developed primers UCYTB152BF (GGSGCWACTGTNATYACWAA) and UCYT B271BR (TANGCRAANAGRAARTAYCAYT CNGG). Amplified PCR products were sequenced on a capillary sequencer (ABI 3730XL). Subsequently, fragment analysis (364bp) was conducted. Positions showing two peaks were coded as degenerated. Sequence identity was evaluated performing an NCBI-BLASTN search against the GenBank database.

The sequence of the caught specimen showed a best hit with a similarity and maximum identification of 98-100% and a query coverage of 100% with 18 specimens of blue whiting present in the GenBank.

There are currently 121 fish species known to be present in the BPNS, of which 18 are considered vagrants. Six more species have probably gone extinct in Belgian marine waters (9). The last new fish species to be added was the Atlantic croaker *Micropogonias undulatus* (LINNAEUS, 1766), a non-indigenous species that was first observed in the BPNS in 1998. It probably reached our waters by ballast water transport (9). The Belgian Register of Marine Species BERMS (consultation date 18/7/2011) states that there are no registered sightings of blue whiting in the BPNS (9). This makes sense: blue whitings usually live in much deeper waters and were long considered rare in the shallow southern North Sea and English Channel (10). DE GROOT (11) reports a blue whiting caught in the Dutch part of the North Sea and BLACKER (12) described an influx of *M. poutassou* in the English Channel towards the southern North Sea in 1979-1980, which he attributed to an unusual intrusion of water from the south and west. On 27/1/1980 he caught a blue whiting in the close vicinity of the BPNS. MULLER (13) as well mentions blue whiting being caught in the Channel, at Wimereux (northern France). PERRY et al. (14) report that the southern boundary of the blue whiting distribution in the North Sea has shifted north by 816 km between 1978 and 2001. The authors state that this pelagic fish may retract completely from the North Sea by 2050.

These sightings indicate that blue whitings have wandered close to Belgian waters in the past and that a warming climate is likely to push blue whiting stocks further north.

Fig. 2. − blue whiting, caught at station W03 (Nieuwpoort) on 16/7/2010.

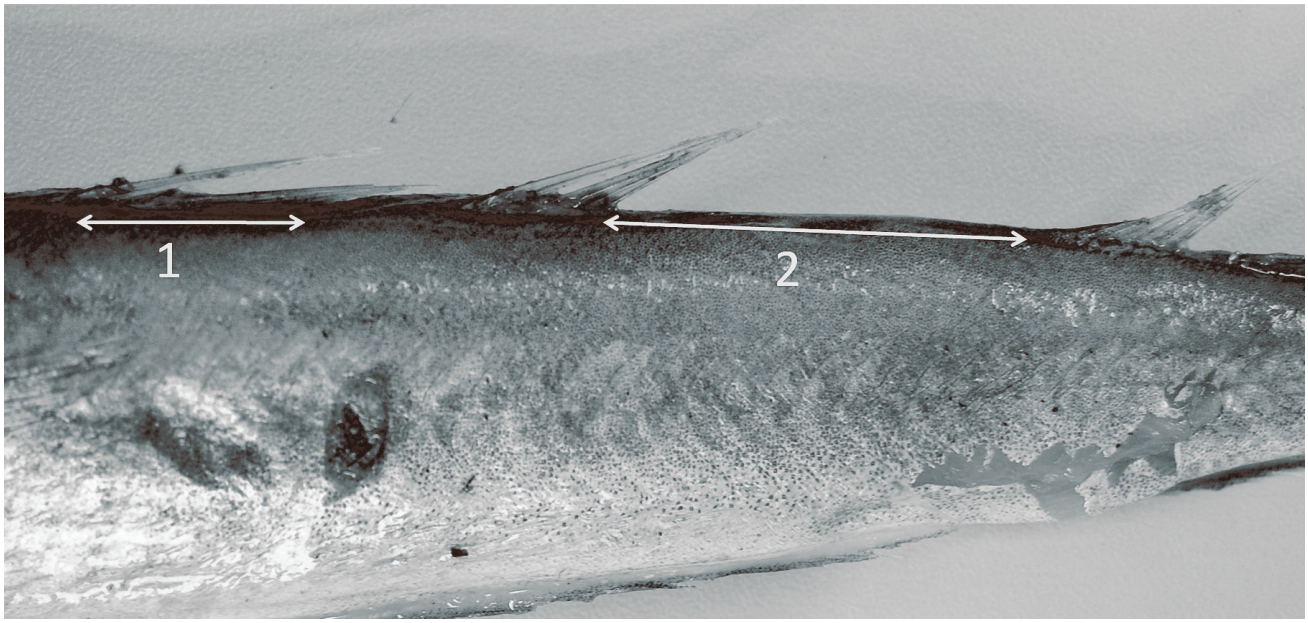

Fig. 3. − blue whiting dorsal fins are widely separated and the interspace between the second and third dorsal fin (2) is bigger than the base of the first dorsal fin (1).

Consequently, this manuscript describes the first reported sighting of blue whiting in Belgian waters, thereby adding this species to the Belgian marine species list.

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