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CONTENTS

Cover photograp by the Laboratory of Oceanology (ULg): *Posidonia oceanica* meadow in the harbor of STARESO, Calvi Bay, Corsica; see paper by PÊTE D. *et al.*, page 59.

Local distribution patterns of harvestmen (Arachnida: Opiliones) in a Northern temperate Biosphere Reserve landscape: influence of orientation and soil richness

Izaskun Merino-Sáinz & Araceli Anadón*

Departamento de Biología de Organismos y Sistemas, Universidad de Oviedo, C/ Catedrático Rodrigo Uría s/n, 33071 Oviedo (Asturias, Spain)

* Corresponding author: Araceli Anadón, e-mail: aanadon@uniovi.es

ABSTRACT. The study at a local scale of the fauna in a natural mountain landscape provides insights regarding the patterns and the factors influencing distribution. We test if each type of natural forest and some open habitats in the Muniellos Biosphere Reserve have their own unique harvestmen assemblages. We further investigate the presence of groups of sites sharing harvestmen assemblages and the factors and indicator species involved. Nineteen sites with well-known phytosociological association were sampled during nine surveys from late 2001 to 2002 by means of three sampling protocols. The quality of the inventories was assessed via the corresponding species accumulation curves. The cluster analysis using the Bray Curtis similarity index showed the presence of two main distinct groups of sites. One group consisted of seven lower forest sites, while the second group contained samples from more open sites and lighter forests. IndVal analyses show the first group has six characteristic species and the second group has one. ANOSIM analyses revealed that the harvestmen community composition was significantly different between the two clusters. Orientation appears to be one main driver of harvestmen assemblages on Mount Muniellos: a clear distinction between the two clusters appears along the boundary of shady to sunny habitats. The vegetal associations that house the higher harvestmen species richness have the higher soil richness. Seven rare and infrequent species were found in forests with richer soil.

KEY WORDS: Arthropoda, vegetation, diversity, assemblages, Spain.

INTRODUCTION

There is a need to measure and describe natural and disturbed landscapes in order to relate distribution patterns to their causes and consequences (RICKLEFS 1987). The level of species diversity in a particular area represents a balance between regional processes, such as dispersal and species formation, and local processes, such as biotic interactions and stochasticity (RICKLEFS 1987, 2004; WIENS & DONOGHUE 2004).

Determining landscape patterns at small 'microlandscape' scales can potentially serve as a model for larger-scale landscape systems (MILNE 1988). One of the advantages is that measurements may be taken with a level of

detail that is difficult to attain at a broader scale. Specific results can provide evidence of the factors influencing distribution in addition to suggestions regarding the mechanisms through which patterns may arise.

CURTIS & MACHADO (2007) described the ecology of harvestmen focusing on spatial and temporal patterns in the occurrences of harvestmen species and the assemblages of species in natural environments. These can be described and compared using simple parameters such as species composition, species richness and the relative abundance of each species. So far, only one study on the Iberian Peninsula has followed this approach (RAMBLA 1985). Some recent papers on the ecology of Opiliones have tested the type of distribution of particular species

(LIPOVSEK et al. 1996; MITOV 1997), biotope preferences (STOL 2003, 2004), ecological profiles (MITOV & STOYANOV 2005), patterns of distribution (KOMPOSCH 2000; MUSTER 2001; ACOSTA & GUERRERO 2011), the study of natural reserves (ZINGERLE 1997, 1999) and faunistic similarity among different islands (TSURUSAKI et al. 2005) and forests (PINTO-DA-ROCHA & DA SILVA 2005), the relationship between elevation and harvestmen species richness (KOMPOSCH & GRUBER 1999; ALMEIDA-NETO et al. 2006), the role of fragmentation (BRAGAGNOLO et al. 2007) and the influence of grazing history in harvestmen biodiversity (PASCHETTA et al. 2013).

The Muniellos Biosphere Reserve in Asturias, Northern Spain, is mainly covered by forests and has barely been exposed to human influence due to its geographical isolation and rugged landscape. It may be considered "near-natural" (i.e. pristine) in the sense of PETERKEN (1993) and is therefore considered a site of special scientific interest. Sampling was carried out at nineteen sites of a well-known vegetation type at microscale resolution in order to elaborate the

Muniellos Inventory of Invertebrates (ANADÓN et al. 2002). As the basic data on harvestmen fauna are already known (MERINO SÁINZ & ANADÓN 2008, 2009) it is possible to study their spatial patterns. All the sites in the lower altitudes of Mount Muniellos are in close vicinity to each other, composing a mosaic within one square kilometer. So we studied their distribution at a local microscale level.

Here, we tested if each type of natural forest and some open habitats in the Muniellos Biosphere Reserve have their own unique harvestmen assemblages. We further investigated the presence of groups of sites sharing harvestmen assemblages and the factors and indicator species involved.

MATERIALS AND METHODS

Study area

The Muniellos Biosphere Reserve (Fig. 1) is situated in Cangas del Narcea (Asturias, North-
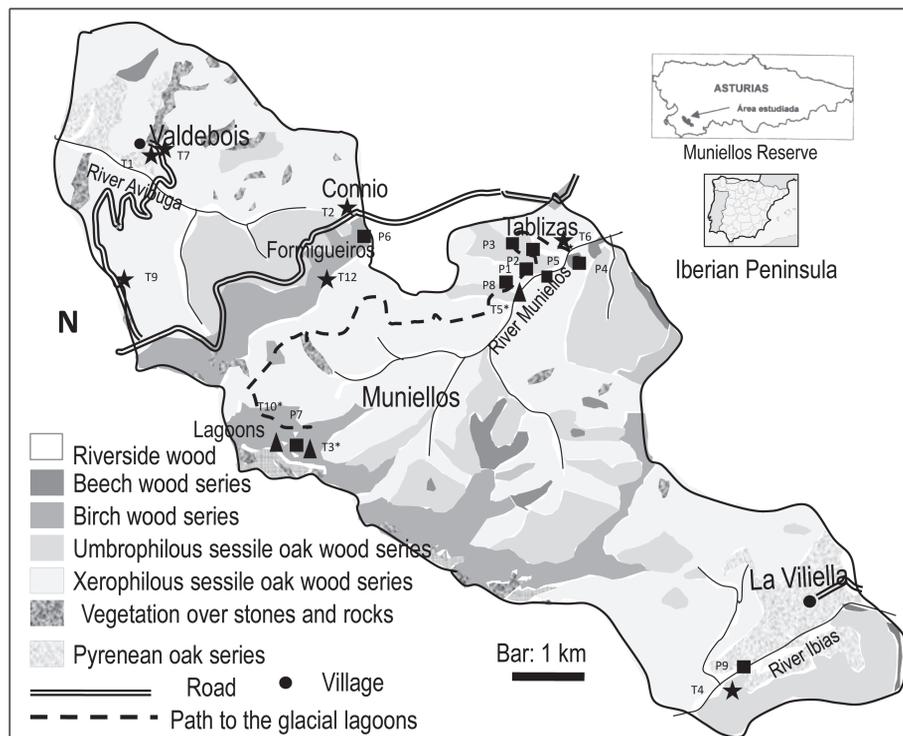

Fig. 1. – Map of Muniellos Biosphere Reserve with the sites studied. Main vegetation series are depicted in different levels of shading. Degraded areas of shrub and meadows are embedded within them. ■ = plot; ★ = transect; ▲ = transect with pitfall trap. Birch trees predominate at high altitudes, sessile oaks in lower areas.

Western Spain). It contains three drainage basins with acid Palaeozoic Variscan rocks and a very thin layer of soil. The basins surprisingly are locally named mountains. The climate of the reserve is temperate oceanic. Mount Muniellos has an upper humid ombrotype, steep slopes and three glacial lagoons and has been a protected area since 1964. Mount La Viliella and Mount Valdebois have a humid ombrotype, slighter slopes and each one contains a very small village. The climate belt is mainly montane (FERNÁNDEZ PRIETO & BUENO SÁNCHEZ 1996).

Phytogeographically, the reserve belongs to the Orocantabrian Province, Atlantic Superprovince, within the Eurosiberian Region (DÍAZ GONZÁLEZ & FERNÁNDEZ PRIETO 1994), on the border with the Mediterranean Region. Mature forests (Principado Asturias 2001) cover 67% of the reserve, with sessile oak (*Quercus petraea*) forests (2,900 ha) predominating at lower altitudes and birch (*Betula celtiberica*) forests (507 ha) at higher altitudes. Beech (*Fagus sylvatica*) forests in more humid areas, Pyrenean oak (*Quercus pyrenaica*) forests in warmer areas and two types of gallery forests complete the mature woodlands, while different types of shrubland occupy 18% of the surface. *Erica australis* subsp. a*ragonensis,* red heath shrubs, cover 9% of the reserve. Mixed forests including maples (*Acer pseudoplatanus*) and sessile oaks cover particularly small territories with richer soils originating from landslides.

Sampling sites and collecting methods

Eight plots and twelve transects were selected based on their vegetation type to study the invertebrates (OCHARAN et al. 2003) of the reserve (Fig. 1, Table 1). The sites were situated on a wide range of altitudes and included twelve forests, four shrublands, two grasslands and a peatbog. The nine sampling periods started at the following time periods: 10th November in 2000; 29th April, 18th June, 6th August, and 25th October in 2001; and 18th February, 16th April, 1st July and 26th September in 2002 (for

details, see ANADÓN et al. 2002). Each sampling period lasted two weeks and was carried out by at least five individuals with no specialization in harvestmen. Each individual used the same sampling method within all localities and periods.

Three sampling protocols were applied. Plots (P) of 50 m x 50 m were sampled by four active sampling methods, each method for one hour: capture with entomological net, vegetation sweeping with an entomological net, direct capture and beating; and by three additional passive methods: Malaise trap, seven pitfall traps and soil extraction by Berlese funnels. The protocol for each transect (T) included the first three aforementioned sampling methods for one hour. In addition, three transects (T*) were also sampled with pitfall traps. The pitfall traps had no bait, only water and sodium polyphosphate. They were active for two days in 2000 and five days in 2001 and 2002.

Data analyses

All specimens of harvestmen were identified and catalogued, along with their localities, date and sampling method (MERINO SÁINZ & ANADÓN 2008, 2009). This material is deposited in the BOS-Opi 1-493 and BOS-Opi 931 Arthropod Collections, Department of Biology of Organisms and Systems, University of Oviedo, Spain (MERINO-SÁINZ et al. 2013).

The diversity was studied as species richness and as true diversity, $^2D= 1/\lambda$ (HILL 1973; JOST 2007; TUOMISTO 2010) with $\lambda = \Sigma_{i=1}^{s} p_i^2$, pi being the proportional abundance of the ith species. True beta diversity is the quotient between the true gamma diversity of a data set and the average true alpha diversity of all the compositional units; here, the sampling sites: $^2D_{\beta} = {^2D_{\gamma}} / {^2\overline{D_{\alpha}}}.$

PRIMER V6 program (CLARKE & GORLEY 2006) was used to obtain species accumulation curves, hierarchical clustering (CLUSTER), multidimensional scaling (MDS), analysis of similarity (ANOSIM), and similarity percentage

TABLE 1

Characteristics of sampling sites, identified by their two abbreviations. Geographical UTM coordinates, slope (high 50-65%; medium < 50%), orientation and altitude (m above sea level).

Plot/transect	Valley name	Vegetation type	Phytosociological association	UTM	Slope	Orientation	Altitude
P9 (aldVi)	La Viliella	Alder gallery forest	*Valeriano pyrenaicae-Alnetum glutinosae*	29TPH9161	Flat	SE, sunny	540
T4 (oakVi)	La Viliella	Xerophilous sessile oak forest	*Linario triornithophorae-Quercetum petraeae*	29TPH9160	Flat	NE, shady	665
T7 (pasV)	Valdebois	Pasture	*Merendero-Cynosuretum cristati*	29TPH8268	High	S, sunny	660
T1 (brooV)	Valdebois	White broom shrubs	*Cytiso scoparii-Genistietum polygaliphyllae cysetosum multiflori*	29TPH8268	Medium	SW, sunny	700
T9 (heaV)	Valdebois	Red heath	*Daboecio cantabricae-Ericetum aragonensis*	29TPH8267	Medium	SW, sunny	1,050
T6 (mdw)	Tablizas	Meadow	*Merendero-Cynosuretum cristati*	29TPH8867	Flat	N-S, sunny	660
T5* (ashT)	Tablizas	Ash gallery forest	*Festuco gigantae-Fraxinetum excelsioris*	29TPH8866	Flat	N-S, shady	700
P5 (ashP)	Tablizas	Ash gallery forest	*Festuco gigantae-Fraxinetum excelsioris*	29TPH8867	Flat	N-S, shady	650
P4 (bee)	Tablizas	Beech forest	*Blechno spicanti-Fagetum sylvaticae*	29TPH8867	High	N, shady	720
P3 (uoak)	Tablizas	Ombrophilous sessile oak forest	*Luzulo henriquesii-Quercetum petraeae*	29TPH8867	High	NE, shady	830
P2 (moak)	Tablizas	Mixed forest: maples & sessile oaks	*Luzulo henriquesii-Aceretum pseudoplatani*	29TPH8867	High	NE, shady	850
P1 (xoak)	Tablizas	Xerophilous sessile oak forest	*Linario triornithophorae-Quercetum petraeae*	29TPH8867	High	SE, sunny	860
P8 (heaM)	Tablizas	Heath of red heather	*Daboecio cantabricae-Ericetum aragonensis*	29TPH8867	High	SE, sunny	900
P6 (birC)	Connio Pass	Birch wood	*Luzulo henriquesii-Betuletum celtibericae*	29TPH8568	Medium	NE, shady	1,280
T2 (gorC)	Connio Pass	Gorse with heather	*Halimio alyssoidis-Ulicetum cantabrici*	29TPH8568	Flat	SW, windy, sunny	1,320
T12 (birFo)	Connio Pass (anthill)	Open birch forest	*Luzulo henriquesii-Betuletum celtibericae*	29TPH8567	Flat	NE, sunny	1,450
P7 (birdLl)	Lagoons (La Isla)	Open birch tree forest	*Luzulo henriquesii-Betuletum celtibericae*	29TPH8464	Flat	N, sunny	1,340
T3* (pbog)	Lagoons	Peatbog with heather	*Calluno vulgaris-Sphagnetum capillifolii luzuletosum enriquesii*	29TPH8464	High	NE, shady	1,350
T10* (birLH)	Lagoons (Honda)	Open birch forest	*Luzulo henriquesii-Betuletum celtibericae*	29TPH8464	Flat	N, sunny	1,410

analysis (SIMPER). The species accumulation curves assess the quality of the inventory. The sampling dates (in the case of captures) were taken as measures of sampling effort and were randomized 999 times. The asymptotes of the curves were estimated fitting the Clench function to the smoothed curves by means of a Simplex and Quasi-Newton method (HORTAL et al. 2004) using the Statistica v6 program (StatSoft 2001). The function provides a good fit when R^2 approaches 1 (JIMÉNEZ-VALVERDE & HORTAL 2003). The asymptote of the curves, being the point where the slope reaches 0 (HORTAL et al. 2004), predicts the estimated species richness of each sufficiently well-sampled site. When the value of the final slope is lower than 0.1 and the percentage of collected species is over 70, the inventory is considered reliable and the community to be well sampled (HORTAL & LOBO 2005). Moreover, five non-parametric estimates of total species richness: Jacknife 2, Jacknife 1, Chao 1, Chao 2, and Bootstrap were obtained.

Although three different sampling protocols were applied, no sites and data were discarded a priori from the Basic Data table.

We conducted an ANalysis Of SIMilarity (ANOSIM) (CLARKE & GORLEY 2006) to test for significant differences in harvestmen assemblages between each pair of sites and between the two main clusters of sites based on a permutation test. To estimate beta diversity, the distance between two sites based on the Bray-Curtis coefficient of similarity was calculated on the square root transformed abundance data. Triangular matrices of the distances across sampling sites (according to their species assemblages) were used in the hierarchical clustering (CLUSTER), carried out with average group linkage, and in a non-metric multidimensional scaling (MDS), which represents the distances among the sites in a geometric space.

The similarity percentage analysis (SIMPER) identifies the species primarily providing the discrimination of similarity or dissimilarity between two observed sample clusters.

Specificity and fidelity of each harvestmen species within the groups of sites were explored via the indicator value index (IndVal) (DUFRÊNE & LEGENDRE 1997; DE CÁCERES & LEGENDRE 2009), which measure the association of a species for a given clustering of sites. Indicator species are defined as the most characteristic species for a cluster of sites and it is most frequent in this cluster and present in the majority of sites belonging to that cluster (DUFRÊNE & LEGENDRE 1997). Indicator species analyses were run using the package "indicspecies" 1.7.3 2014-07-10 (DE CÁCERES & JANSEN 2014) in R (R Development Core Team 2012).

Species richness in terms of vegetation was studied qualitatively (see CURTIS & MACHADO 2007), scoring the richness and abundance present in forested *versus* open areas and the different types of forests and their situation on the mountain: gallery, mountainside, sunny, shady, low, medium or high.

RESULTS

A total of 765 individual Opiliones were sampled in the Muniellos Biosphere Reserve, belonging to 19 different species, with a true diversity of 8.34 effective species (Table 2). Average number of species per site was 7 species. True β diversity is 8.34/4.0 = 2.09 compositional units in the dataset. The estimation of global species richness with non-parametric estimators ranged between 20.6 using Bootstrap (q = 0.92) and 24.9 using Jacknife 2 (Fig. 2).

Pitfall traps, sweeping, hand picking and beating resulted in 40%, 34.9%, 18.7% and 5.8% of the specimens.

The overall inventory is sufficiently reliable (Table 3).

However, the asymptotes at each particular site are generally far from the observed richness value, and suggest that <70% of the species were captured.

TABLE 2

Harvestmen species distribution in Muniellos Reserve. Abundance, true diversity 1/λ. P = plot; T = transect, T* = transects with pitfall trap.

Vegetation types	aldVi	ashP	mdw	pasV	oakVi	ashT	brooV	bee	uoak	moak	xoak	heaM	heaV	birC	gorC	birLl	pbog	birLH	birFo	Total	Sites
Plots and transects	P9	P5	T6	T7	T4	T*5	T1	P4	P3	P2	P1	P8	T9	P6	T2	P7	T*3	T*10	T12		
altitude m.a.s.l.	540	650	660	660	665	700	700	720	830	850	860	900	1050	1280	1320	1340	1350	1410	1450		
Paroligolophus agrestis	43	12			11	34		44	6	2				16		4			6	178	10
Oligolophus hansenii		7			2	4	1	4	7	3	2			13		7	6			56	11
Leiobunum blackwalli	19	10	3	1	7	25	3	6	11	2	3									90	11
Leiobunum rotundum	5	13	1		9	11	1	13	13	10										76	9
Ischyropsalis hispanica	3	2			1	1		8	3	3		1		2	1		1	1	1	27	10
Homalenotus laranderas	23	2	2			11		6	1	1	2	9								56	9
Trogulus nepaeformis	22	4			2	11	2	7	1	3										51	8
Phalangium opilio	11	2		2	3	1					28	15	2	5	21	5	11	2	1	109	14
Odiellus seoanei	6	1	1			1		2			2	5		4	1			3		25	9
Odiellus simplicipes	8	1			1	3	1				4	2		22	3		3	6		51	10
Nemastoma hankiewiczii	11	1				6		2												20	4
Dicranopalpus sp.								1	1											2	2
Anelasmocephalus cambridgei	2					2														4	2
Gyas titanus						3		2		2										7	3
Sabacon franzi								2								2				4	3
Nemastomella dentipatellae										2										2	1
Hadziana clavigera										2										2	1
Paramiopsalis sp.										1										1	1
Megabunus diadema										1										1	1
Total abundance	153	55	6	3	36	113	7	94	42	34	41	32	2	62	26	18	21	12	8	765	
Species richness	11	11	3	2	8	13	4	11	7	11	6	5	1	6	4	4	4	4	3	19	
True diversity ^2D_a, $1/\lambda$	6.50	6.14	2.57	1.80	4.80	5.75	3.27	3.82	4.57	6.96	2.05	3.05	1	4.03	1.50	3.45	2.64	2.88	1.68	8.34	

TABLE 3

Species richness (S): raw data and accumulation curves. N = sampling units; R^2 = curves coefficient of determination; ES = estimated species richness; %, S/ES = % species collected; p = final slope of the species accumulation curve (0 indicates a perfect inventory).

| Plots & transects	N	S	Abundance	R^2	ES	%S/ES	p
P1 xoak	20	5	39	0.997	6.38	78.4	0.05
P2 moak	16	11	33	0.999	18.84	58.4	0.28
P3 uoak	15	7	42	0.999	9.07	77.2	0.1
P4 bee	16	11	94	0.999	16.2	67.9	0.22
P5 ashP	14	11	55	0.999	17.69	62.2	0.298
P6 birC	13	6	62	0.989	7.3	82.2	0.07
P7 birLI	9	4	18	0.998	5.45	73.4	0.12
P8 heaM	16	5	32	0.996	6.8	73.5	0.08
P9 aldVi	17	11	153	0.989	13.5	81.5	0.1
T1 brooV	3	4	7	0.998	11.18	35.78	0.85
T2 gorC	5	4	26	0.999	7.33	54.57	0.36
T*3 pbog	8	4	21	0.999	5.25	76.19	0.12
T4 oakVi	12	8	36	0.998	11.9	67.23	0.21
T*5 ashT	21	13	113	0.998	17.06	76.2	0.15
T6 mdw	4	3	6	0.99	5.48	54.74	0.34
T*10 birLH	5	4	12	0.998	6.02	66.44	0.27
All plots	136	17	528	0.976	17.5	97.1	0.009
All transects	63	15	234	0.995	16.54	90.69	0.03
All plots & transects	199	19	762	0.956	19.46	97.64	0.006
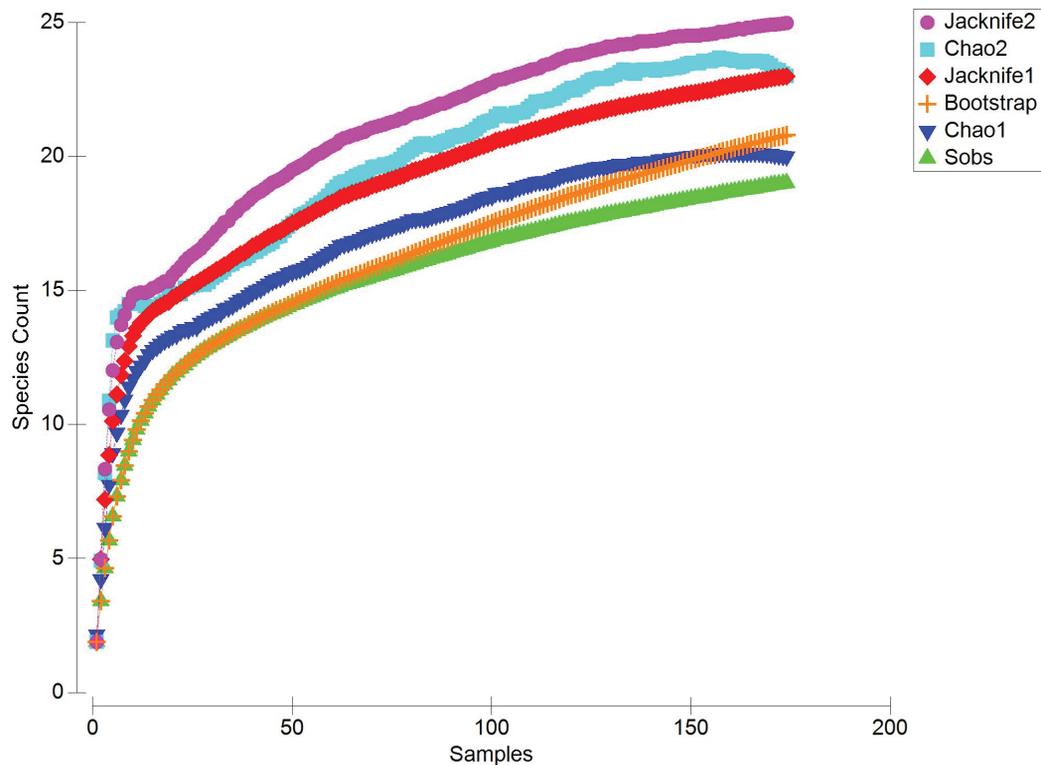
Fig. 2. – Species accumulation curve for observed (Sobs) Opiliones of all plots and transects together, and for 5 different non-parametric estimators of species richness: Bootstrap, Chao 1, Chao 2, Jacknife 1 and Jacknife 2. Sobs is closest to Chao 2 and Bootstrap estimator.

Cluster analyses, MDSs and ANOSIM and SIMPER of the sites

The cluster analysis of the sites based on their species composition returned two distinct groups (A1 and A2; Fig. 3). A1 includes seven low-altitude forest sites: ash gallery forest, alder gallery forest, beech forest, mixed forest of maples and sessile oaks, ombrophilous sessile oak forest, and "xerophilous" sessile oak forest of La Viliella. These forests are shady to different degrees and have higher harvestmen species richness (7-13 species/site), as well as higher average true alpha diversity 5.5 (3.82-6.96 effective species/site). Only the alder gallery forest is sunnier due to the width of the river.

Cluster A2 contained seven sites: the xerophilous sessile oak forest of Muniellos, two shrublands (heather and gorse) and one birch forest (subcluster A2.1) and two other birch forests and the peatbog (subcluster A2.2). All are higher-altitude sunny sites with 4-6 species richness with a lower average true diversity of 2.8 (1.50-4.03) species per site.

Clusters D1 and D2 contained pasture T7, heathland T9 and high birch forest T12. Cluster

C contained a meadow and a shrub with brooms. The meadow T6 in the core of the reserve appeared very poor in harvestmen and yielded very few specimens represented by only three species. All these sites of the last three clusters had in common few harvestmen specimens sampled and only 1-4 species.

MDS of Fig. 4 show the vegetation structure and the groups of sites. Forest sites are spread along the space and distributed in different clusters.

The similarity analyses (ANOSIM) between pairs of sites of the main clusters are summarized in Table 4. Differences were consistently found between sites belonging to the different clusters A1 and A2, but not between sites within one cluster. Hence, the assemblages in one cluster of sites differ from the assemblages in the other cluster of sites, but do not differ among themselves. Some exceptions can be found in P6 and P7, and P9. An ANOSIM test that evaluated all possible permutations (1716) between clusters A1 and A2, each with seven sites, revealed that the correlation in species composition within clusters equaled r = 0.766, which was significantly different from a random distribution (P < 0.001). These results prove the existence in Muniellos Biosphere Reserve of two major clusters of sites with different harvestmen assemblages. Similarity percentage analyses (SIMPER) (Table 5) gives the contribution of the species to internal similarity of the main clusters. The ANOSIM results table does not include T1, T6*, T7, T9 and T12 (with ≤ 8 specimens): no differences between them and any other site were detected.

The study of indicator species values (IndVal) of the groups of sites (Table 6) gave six indicator species for cluster A1 and one indicator species for cluster A2. The values of specificity and fidelity were very high. Cluster D1, D2 and C had no species associated. No species was simultaneously associated to two, three of four clusters of sites.
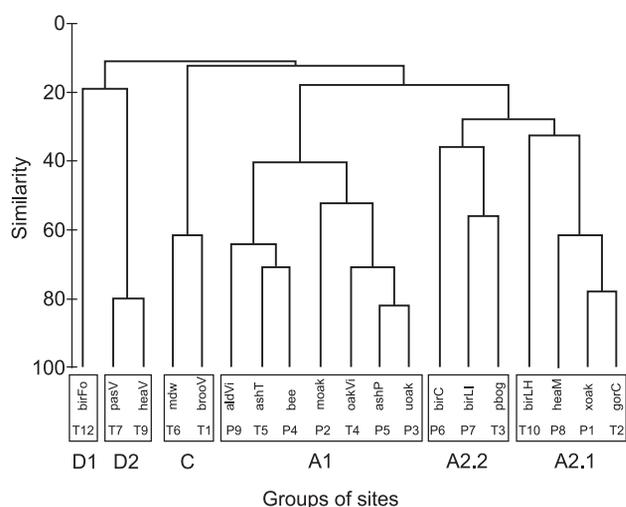

Fig. 3. – Cluster analysis of sites attending their harvestmen assemblages. Groups of sites A1 and A2 are supported by ANOSIM values and indicator species.

TABLE 4

ANOSIM analysis of differences in species composition: * = differences p≤ 0.05; ** = differences p≤ 0.01; *** = differences p≤ 0.001.

Cluster	Site		A1 P5	A1 P2	A1 P4	A1 P3	A1 P9	A2 P6	A2 P7	A2 P1	A2 P8	A2 T3*	A2 T*10
		Veg.	ashP	moak	bee	uoak	aldVi	birC	birLI	xoak	heaM	pbog	birLH
A1	P5	ashP	I	0	0	0	0	*	**	***	***	**	**
A1	P2	moak	0	I	0	0	0	**	*	***	***	*	***
A1	P4	bee	0	0	I	0	**	***	***	***	***	**	***
A1	P3	uoak	0	0	0	I	**	**	*	***	***	**	***
A1	P9	aldVi	0	0	**	**	I	*	***	***	**	**	**
A2	P6	birC	*	**	***	**	*	I	0	*	*	*	0
A2	P7	birLI	**	*	***	*	***	0	I	0	0	0	*
A2	P1	xoak	***	***	***	***	***	*	0	I	0	0	0
A2	P8	heaM	***	***	***	***	**	*	0	0	I	0	0
A2	T*10	birLH	**	***	***	***	**	0	*	0	0	0	I
A2	T2	gorC	***	***	***	***	**	0	*	0	0	*	0
A2	T*3	pbog	**	*	**	**	**	*	0	0	0	I	0
A1	T4	oakVi	0	0	0	0	0	0	*	**	**	**	0
A1	T*5	ashT	0	0	0	0	0	**	**	***	***	**	**

Four frequent species and eight rare species have low IndVal values and were not indicator species of the main groups of sites. The frequent species were *Oligolophus hansenii* (Kraepelin, 1896), *Nemastoma hankiewiczii* (Kulczynski, 1909), *Odiellus simplicipes* (Simon, 1879) and *Odiellus seoanei* (Simon, 1878). *O. simplicipes* is the actual name of *O. ruentalis* (Kraus, 1961). *O. seoanei* is the new identification of specimens previously attributed to *O. spinosus* (Bosc, 1792) (Merino Sáinz & Anadón 2008).

DISCUSSION

The first important result is that each phytosociological association does not have a specific harvestmen fauna: a different botanical
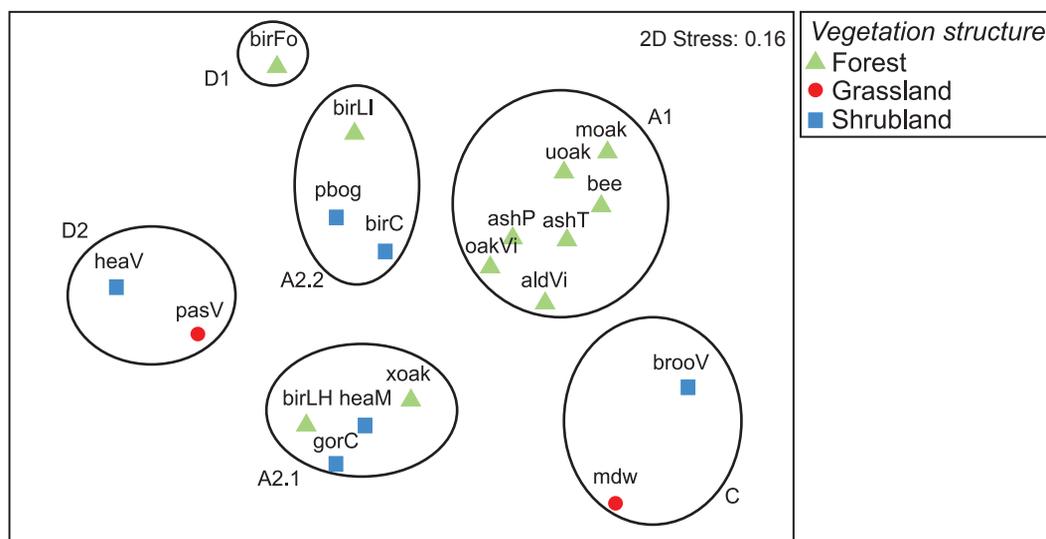

Fig. 4. – MDS of sites showing the vegetation structure and the groups of sites obtained with the cluster analysis.

TABLE 5

Species contribution to the internal similarity of the clusters of sites (SIMPER). Distribution: Hol = holarctic; Eur = European; IE = Iberian endemic.

Sites cluster	A1	A2.1	A2.2	Distrib
Average similarity	61.59	63.60	51.46	
Spp. contribution	%	%	%	
Leiobunum rotundum (Latreille, 1798)	21.01			Eur
Paroligolophus agrestis (Meade, 1855)	19.53		9.87	Hol
Leiobunum blackwalli Meade, 1861	16.81			Eur
Oligolophus hansenii (Kraepelin, 1896)	9.56		43.96	Eur
Homalenotus laranderas Grasshoff, 1959	9.16	8.06		IE
Trogulus nepaeformis (Scopoli, 1763)	7.8			Eur
Ischyropsalis hispanica Roewer, 1953	6.68			IE
Phalangium opilio Linnaeus, 1758		42.34	39.24	Hol
Odiellus simplicipes (Simon, 1879)		26.39		IE
Odiellus seoanei (Simon, 1878)		20.32		IE
Homalenotus laranderas Grasshoff, 1959		8.06		IE

characterization of the studied sites alone does not imply a differentiated harvestmen assemblage. The assemblages of forested areas are neither similar among them nor different to those of open habitats. Rather, there are two main clusters of sites, both including different forest sites: each cluster of sites shares different indicator species. Species richness and abundance vary according to the type of forest. Muniellos forests had 8.27±3.07 harvestmen species on average, while open habitats including different types of shrubland and a meadow had a much lower diversity of 4.0 ± 0.72. Abundance was highest in the gallery forests, the beech forest and the lowest altitude birch forest. Sessile oak forests and the mixed forest had medium abundances.

Curtis & Machado (2007) compiled data from different studies and showed that the average species richness of harvestmen in forested habitats is 2.8 times higher than in open habitats. They explain this on the basis of seasonal variations in abiotic factors in open habitats, mainly temperature and humidity, which may restrict the occurrence of many harvestmen species in this habitat, and the more complex structure of forested habitats, which may provide a greater diversity of suitable micro-habitats. The diversity of micro-habitats and food (Collembola and Acari) is also greater in forest habitats (see Mitov 2007).

Discontinuities: changes in harvestmen fauna and vegetation

Which factors are responsible for the variation? Orientation seems to have a decisive influence on harvestmen assemblages. An abrupt border was found between the two main clusters of sites. The abrupt change in southern *versus* northern orientation in the wedged valleys on Mount Muniellos results in a variation in xerophilous *versus* ombrophilous sessile oak forests, which was also reflected in the harvestmen fauna.

There is a border in the corner, between the shady (P2 and P3) and sunny (P8 and P1) slopes, along the path to the Mount Muniellos lagoons (Fig. 1). The vegetation changes abruptly there, though sessile oaks (*Quercus petraea*) cover P1, P2 and P3. The sessile oaks at P1 are shorter and sparser than at P3 and P2. The floristic composition of P1 is also substantially different from P2 and P3. Plot P1 and the heath P8 belong to the same series of vegetation (Fernández Prieto & Bueno Sánchez 1996); the xerophilous sessile oak forest series.

TABLE 6

Indicator species of cluster A1 and A2 with their specificity and fidelity values. *P* = significance level. Distribution: Hol = holarctic; Eur = European; IE = Iberian endemic.

Cluster A1	Specificity	Fidelity	Indicator Value	p	Distrib
Leiobunum rotundum (LATREILLE, 1798)	0.9737	1.000	0.987	0.001 ***	Eur
Leiobunum blackwalli MEADE, 1861	0.8889	1.000	0.943	0.001 ***	Eur
Paroligolophus agrestis (MEADE, 1855)	0.8539	1.000	0.924	0.001 ***	Hol
Trogulus nepaeformis (SCOPOLI, 1763)	0.9412	0.8571	0.898	0.001 ***	Eur
Homalenotus laranderas GRASSHOFF, 1959	0.7931	1.000	0.891	0.003 **	IE
Ischyropsalis hispanica ROEWER, 1953	0.7407	0.8571	0.797	0.017 *	IE
Cluster A2					
Phalangium opilio Linnaeus, 1758	0.7885	0.8571	0.822	0.015 *	Hol

The change in faunal composition in this border is supported by three different analyses: (a) the cluster analyses (Figs 3-4) separates the harvestmen assemblages of shady plots (P3 and P2, in A1) from those of sunny plots (P1 and P8, in A2); (b) the ANOSIM analyses (Table 4) yielded significant pairwise differences (***) between P3 and P2, relative to P1 and P8; and between the cluster A1 and A2; (c) the six indicator species of A1 are different from the only indicator species of A2. So the local hard boundary (FORMAN 2006) between the faunas must be located along the confluence of the sunny and the shady slopes.

The mixed forest of maples and sessile oaks (P2) and the ombrophilous sessile oak forest (P3) represent two different mature forests very close to each other belonging to cluster A1 with the same indicator species (Table 6).

Sites with a richer soil harboured a higher species richness. The mixed forest, the gallery forests, -ash tree forest and alder tree forest- and the beech forest, which all share a rich soil, showed the highest harvestmen species richness (11-13 species/site). Higher soil richness is indicated by the presence of the tree species ash, maple, lime (*Tilia platyphyllos*) and elm (*Ulmus glabra*), which are known to prefer rich soils. These sites also have higher harvestmen species richness and higher true diversity (Table 2). Those tree species grow at the bottom of valleys and over landslides, where there is high soil aeration and humidity, facilitating good decomposition of organic matter and producing mull humus (FERNÁNDEZ PRIETO & BUENO SÁNCHEZ 1996). The influence of soil factors on the species richness of scorpions has already been documented (POLIS 1990).

Gallery forests, due to their special position in the valleys, have a richer soil since they usually accumulate particulate matter and mineral nutrients carried overland by the surface flow of water (FORMAN 2006). They are especially important in nutrient-poor locations more typical of uplands, as is the case in Muniellos. Also the sampled beech forest was situated at lower altitudes in the Muniellos valley. The high species richness of the mixed forest is related to its richer soil over a landslide. This woodland constitutes an island of abundant maples surrounded by ombrophilous sessile oak forest, with poorer soils.

The mixed forest P2 hosts four endemic rare species *Sabacon franzi* (ROEWER, 1953), *Nemastomella dentipatellae* (DRESCO, 1967), *Paramiopsalis* sp. and *Hadziana clavigera* (SIMON, 1879), all endemic to the north of the Iberian Peninsula. *H. clavigera* is the actual name of *Peltonychia clavigera* (SIMON, 1879) (KURY & MENDES 2007). The presence of stenotopic taxa at P2 noticeably increases the species richness of this site with respect to P3.

Three European species considered to be rare in this landscape were found in the ash tree forest: *Anelasmocephalus cambridgei* (WESTWOOD, 1874), *Gyas titanus* (SIMON, 1879), and *Dicranopalpus* sp. Another European species *Megabunus diadema* (FABRICIUS, 1779) was found over the highest sampled site in an open birch forest.

Comparison with other faunas

The harvestmen fauna of Muniellos has six species in common with the fauna of San Juan de la Peña in the Pyrenees Mountains (RAMBLA 1985), where eleven species have been found. There, *Oligolophus hansenii* is the most abundant species. In the Pyrenees *Quercus ilex* forest and *Quercus faginea* forest, both typical of the Mediterranean climate, have fewer species than the other forests and their dominant species differ. In Muniellos, the species richness was higher (6-13) at low and medium altitude woodlands, maximal (10-13) in gallery, mixed and beech forests; xerophilous sessile oak forests as well as the birch forests (which grow at higher altitude) have medium (6-8) species richness; fewer species, ≤ 5, were found in open high-altitude birch forests (above 1,340 m) and in all open habitats (Table 2). In Muniellos *O. hansenii* was present in most of the forests and it was not an indicator species of any cluster.

Studies of some heath-gorse shrublands (ROSA GARCÍA et al. 2009a, b) in Illano (Asturias), 40 km north of Muniellos, have found nine species also present in Muniellos. Thus, there is a basic pool of species in the area, though with different relative frequencies in the two territories.

MITOV & STOYANOV (2005) studied and modelled ecological profiles of harvestmen species on the Vitosha Mountain, Bulgaria, and concluded that altitude contributes the most to explaining the ecological requirements of harvestmen, followed by soil type, vegetation belt (both presenting a very similar structure to that of the altitude zone) and exposure.

Vegetation belt, habitat type, soil type and light conditions are more strongly associated with the second ordination axis. The similar patterns of soil type, altitude zone and vegetation belt are due to strong interdependence between these factors (MITOV & STOYANOV 2005). Geographical exposition and soil richness in Vitosha were hence important, as it was found in Muniellos. The aforementioned study found two main groups of species: species with regional-wide distribution and species virtually restricted to low-altitude areas. In Muniellos the frequent species are either holarctic, European or Iberian endemic, and rare species are European, or Iberian endemic.

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Development of a fish-based index combining data from different types of fishing gear.
A case study of reservoirs in Flanders (Belgium)

Jan Breine[1,*], Gerlinde Van Thuyne[1] & Luc De Bruyn[2]

[1] Research Institute for Nature and Forestry, Duboislaan 14, B-1560 Groenendaal, Belgium. E-mails: jan.breine@inbo.be, gerlinde.vanthuyne@inbo.be

[2] Research Institute for Nature and Forestry, Kliniekstraat 25, B-1070 Brussels, Belgium and Evolutionary Ecology, Department of Biology, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerpen, Belgium. E-mail: luc.de-bruyn@inbo.be

* Corresponding author: jan.breine@inbo.be

ABSTRACT. Fish assemblages in reservoirs and lakes are mainly assessed by multiple sampling gear. The challenge exists in how to combine all the data from the different types of gear to develop a fish-based index. In this paper, we describe a novel approach to this challenge in reservoirs in Flanders. The developed approach can also be used for natural lakes in the same eco-region and for any combination of fishing methods. In a first step, we defined a reference list of fish species occurring in man-made Flemish reservoirs. To compile this reference list, we adapted the reference for Dutch lakes with recent data from freshwater reservoirs in Flanders. This reference list contains guild-specific information needed to define metrics. To pre-classify the reservoirs, a habitat status for each reservoir was set using abiotic parameters (pressures). Fish gear-dependent metrics were selected according to their response to these pressures. Threshold values for metrics were determined based on the species reference list and occasionally on the calculated metric values. The ecological quality ratios derived from the index calculation were validated with an independent set of data. The developed index proved to successfully assess the ecological status of the reservoirs in Flanders.

KEY WORDS: fish reference list, fish-based index, modelling, monitoring, European Water Framework Directive.

INTRODUCTION

The most effective way to define the ecological status of lakes and reservoirs is to assess their vegetation and fauna (LYCHE-SOLHEIM et al., 2013). Advantages of biological monitoring are well known and this is one of the reasons phytoplankton, macrophytes, benthic invertebrates and fish are suggested by the European Water Framework Directive (WFD) as biological quality elements to assess the integrity of lakes and reservoirs (EU WATER FRAMEWORK DIRECTIVE, 2000). In Europe, fish-based indices became important bio-assessment tools since the implementation of this directive. Some researchers in Europe assessed the suitability of fish communities in lakes and reservoirs to indicate anthropogenic deterioration (e.g. APPELBERG et al., 2000; CAROL et al., 2006; GARCIA et al., 2006). As a consequence, fish-based indices were developed to assess the ecological quality of lakes (BELPAIRE et al., 2000; HOLMGREN et al., 2007; BECK & HATCH, 2009; WIŚNIEWOLSKI & PRUS, 2009; LAUNOIS et al., 2011a; ARGILLIER et al., 2013) and reservoirs (CATALAN & VENTURA, 2003). A fish-based index is a multimetric procedure to assess the biotic integrity of aquatic ecosystems (KARR, 1981). A metric is a variable assessing an ecological attribute of a community that is sensitive to human impact and reacts unambiguously to impact changes (BREINE et al., 2010). Unfortunately, the majority of lake indices have been based on standardised procedures with stratified multi-mesh gillnet fishing only (CEN, 2005). Another difficulty with the earlier fish-based indices concerned the heterogeneity of the

survey methods. Some indices were developed using different fishing techniques without considering the gear specificity (e.g. BELPAIRE et al., 2000; BACKX et al., 2008). These indices have to be used with caution. Indeed CHOW-FRASER et al. (2006) observed that, although electric fishing and fyke netting each caught 60%-75% of the species present in a wetland, particular species and dominant functional groups tended to be gear specific. Still, metric responses to stress can be developed but patterns of response to particular anthropogenic pressures are unique to gear type (CHOW-FRASER et al., 2006). It is hence important to develop an index combining gear-specific metrics as it is the only effective ecological status assessment method integrating ecological, functional and structural aspects of aquatic systems.

Another crucial step in the development of a fish-based index is the realisation of a reference fish assemblage. Many lakes in Europe were identified as artificial or heavily modified water bodies (HMWB), the latter because their nature has changed fundamentally as a result of physical anthropogenic alterations. According to Article 4(3) of the WFD the principal environmental objective for HMWB and artificial water bodies, such as reservoirs, is to obtain a "good ecological potential" (GEP) instead of a "good ecological status" as required for natural systems. Similarly, the reference situation in HMWB is referred to as "maximal ecological potential" (MEP) instead of a "pristine status" (EU WATER FRAMEWORK DIRECTIVE, 2000). According to WFD, the MEP biological conditions should reflect the biological conditions associated with the closest comparable natural water body type at reference conditions as far as possible, given the MEP's hydromorphological and associated physical and chemical conditions. For an HMWB to be classified as attaining GEP status no more than slight changes in the values of the relevant biological quality elements may be observed as compared to their values at MEP. GEP thus represents a state in which the ecological potential of a water body is falling only slightly short of the maximum it could achieve without significant

adverse effects on the wider environment or on the relevant water use or uses (CWD, 2012). As a result the species list is the same for both MEP and GEP and they only differ in threshold values of the selected metrics. The biological potential can be defined once the hydromorphological, physical and chemical potentials are described.

As mentioned by LAUNOIS et al. (2011a) problems can arise in establishing a reference condition due to the lack of pristine lakes. Hence, we provide a reference condition approach that can be used for any kind of water type.

In this study we describe a new approach to develop a fish-based index combining data obtained from different types of fishing gear. As a case study we used data from reservoirs in Flanders. The proposed methodology is straightforward and can be used with any kind of data and water types.

MATERIALS AND METHODS

Study area

The study area comprised 26 reservoirs located in Flanders (13.521 km²) (Fig. 1). They were selected because they are incorporated into the Flemish freshwater fish-monitoring network. Only some reservoirs are connected to a river (river fed, see Table A, annex).

The surface area of the 26 reservoirs varies between 0.14 and 99 ha with an average depth ranging from 0.5 to 18.5 m (Table A, annex). According to criteria described by LEWIS (1983), all reservoirs could be considered as polymictic. In addition, nine reservoirs were selected for validation purposes (Fig. 1). Pressure values were calculated as the sum of scores for industry, agriculture activity (any including ploughing activities, grassland,…) and development constructions (number of houses); the investigated adjacent area extended 100 m inland from the banks as most reservoirs have no catchment or only small brooks feeding into

them. Data were recorded in the field or via Google Earth when data were missing. Industry (presence of industrial activities e.g. factories) was scored as present (1) or not (0). Thresholds for agriculture activity and development were: 1 if less than 10% of the area is used; 2= ≤30-≥10%; 3= ≤50->30%; and 4 if more than 50 % is used. We also assessed the natural state score of the banks: 1 = 100% natural, 2 = 25% or less of the bank surface is reinforced (concrete, stones etc.), 3 = between 25 and 50% is reinforced, and 4 = more than 50% is unnatural. The total pressure was obtained by summing all pressure scores and can vary between 3 and 13. A pressure class (status) was defined as follows: good or high = 3; moderate = 4; poor >4 and ≤8 and bad >9. Presence of trees was assessed as a predictor, recorded as percentage of area coverage and scored as follow: 4 (no trees); 3 = ≤ 10%; 2= > 10 ≤ 50% and 1= more than 50% of the area covered with trees.

Fish data

All field work was performed by trained fish biologists and technicians using the protocol described in BELPAIRE et al. (2000). Surveys occurred in autumn between 1996 and 2005 (development data) and between 2006 and 2012 (independent validation data). Fish assemblage data were obtained by electric fishing from a boat with two hand-held anodes, using a 5 kW generator with an adjustable output voltage of 300 to 500 V and a pulse frequency of 480 Hz. We surveyed on average 266 m (range: 25-2100 m; average width 2.5 m) long shore transects per ha with electric gear. The variability in effort is due to the fact that no standardised method was defined before the year 2000. At least four paired-fyke nets (90 cm diameter and 22 m long) were placed per reservoir for two successive days (48h) with, on average, one paired-fyke net per hectare (Table A, annex). Fish data recorded
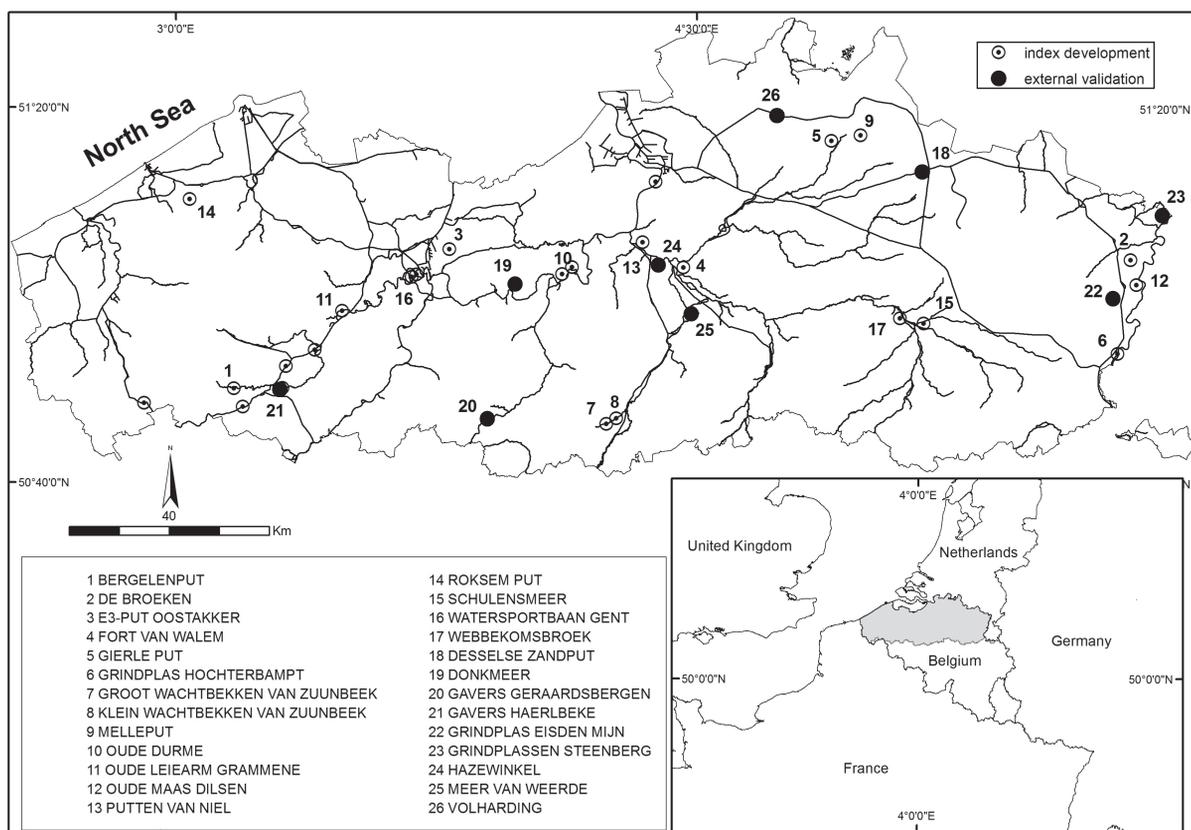

Fig. 1 – Overview of assessed reservoirs (1996-2005) and reservoirs used for the external validation (2006-2011) in Flanders, Belgium.

include species-specific fish densities, individual total lengths (TL, nearest 0.1 cm) and wet weights (nearest 0.1 g).

Data are available from the Fish Information Database (VIS databank: http://vis.milieuinfo. be).

Species reference list

We adapted the reference species list described by BACKX et al. (2008) for the Dutch lakes with Flemish data from surveys for the period 1996-2005. We omitted species from the MEP/GEP list even if they previously occurred in a particular reservoir when: 1) fish are locally or regionally extirpated or 2) a reservoir or lake is not their preferred habitat (RAMM, 1990).

Exotic species were defined according to VERREYCKEN et al. (2007). The classification of species as 'native' and 'non-indigenous' was based on historical and archaeological records. All exotic species were omitted from the list as many authors (e.g. KARR, 1981; BELPAIRE et al., 2000) consider these as indicators of disturbance. Exceptions are pike-perch (*Sander lucioperca*, Linnaeus, 1758), common carp (*Cyprinus carpio*, Linnaeus, 1758) and Prussian carp (*Carassius gibelio*, Bloch, 1782) as they can be considered as naturalised. Moreover, pike-perch has a high oxygen demand (MARSHALL, 1977; FAO, 1984, 1989); hence, the species' presence is an indicator for good water quality.

Index development

Fish were attributed to guilds based on a literature review (BREINE et al., 2004, 2005). Species were categorised according to their tolerance for oxygen deficiency and habitat structure degradation such as shoreline bank modifications. Tolerance scores for oxygen deficiency and structural habitat modifications, from 1 (tolerant) to 5 (intolerant), were given to each species based on information from

BELPAIRE et al. (2000) and BREINE et al. (2007). Ecologically-relevant candidate metrics were selected from literature (BELPAIRE et al., 2000; JEPPESEN et al., 2000; MEHNER et al., 2004; GARCIA et al., 2006; JAARSMA, 2007; LAUNOIS et al., 2011b). For each reservoir, gear-specific metric values were calculated using reference species only (BREINE et al., 2010). To correct for differences in sampling effort, catch per unit effort (CPUE) was used i.e. survey data were standardised to catch results per m² (electric fishing) and catch per fyke day (number of fish per fyke per day).

Statistical analyses

To retrieve less-skewed distribution, percentage metrics were square-root transformed and count metrics were log-transformed (logx+1) (LAUNOIS et al., 2011b). Diversity metrics were not transformed.

First the correlation among pressure scores was assessed (measure of association, p (Fisher)) to avoid co-linearity. Pearson correlation was applied to assess correlation between reservoir depth and reservoir surface (log x+1) transformed values.

The response of metrics to pressures (log transformed values to meet requirements of linear models) and predictors (depth, surface, trees) was analysed with linear mixed regression models. As some locations were sampled several times we added locality and year as random effects. We started with a full model including all pressures and predictors. We applied a stepwise backward selection until only significant terms remained. Normality assumptions were assessed with residual plots. To define the goodness-of-fit, the marginal and conditional R^2 values for each fitted model were calculated as described by NAKAGAWA & SCHIELZETH (2013). Only the metric response to pressures was decisive for the selection (R^2 conditional>35%). Redundancy of responsive metrics was analysed with a Pearson correlation. To choose among the correlated

metrics (c ≥0.7; p ≤0.001), the one with the best fitted model was taken. Secondly, among the less correlated metrics (c <0.7 and ≥0.5; p ≤0.05), the one that least correlated with other metrics was selected.

The statistical software used was R.2.15.2 packages lme4, nlme and MuMIn (R Development Core Team, 2012).

Threshold value determination for the selected metrics was based on the reference list and followed Breine et al. (2010). Once the GEP was defined the other integrity classes were defined by applying trisection with GEP values.

For the relative percentage metrics (Mpi metrics) the GEP is the ratio of the number of the species included in a particular Mpi metric over the total number of species in the reference list (Breine et al, 2010).

For metrics assessing number of species 60% of the reference number was taken as the GEP status threshold value, while this was 80% for the metric tolerance value.

The average value from the highest impacted sites was used to define the minimum percentage weight of benthivorous species (BenWei) and the bream (*Abramis brama*, Linnaeus, 1758) and roach (*Rutilus rutilus*, Linnaeus, 1758) associated metric (AbrRut).

The sum of the metric scores obtained with each method gave the index of biotic integrity (IBI) score for a particular reservoir. To comply with the WFD, this score was transformed to an ecological quality ratio (EQR) calculated as a value between 0 and 1: EQR = (IBI -lowest IBI possible)/(maximum IBI possible - lowest IBI possible). The EQR for the MEP status is 1 under which four integrity classes are defined: GEP (lower threshold value 0.75), moderate (0.5), poor (0.25) and bad (<0.25). The transformation to equal interval classes was obtained using the following formula for each integrity interval (piecewise transformation):

$$T\ EQR = LV\ T\ EQR + (O\ EQR - LV\ O\ EQR)/ (UV\ O\ EQR - LV\ O\ EQR)*0.25$$

O and T stand for original and transformed EQR value, UV and LV (upper and lower value of integrity class). When, during one campaign, more than one site was assessed within one reservoir, data obtained with the same method were summed and transformed to catch per unit effort (i.e. per m² or per fyke day) to calculate the final EQR for the reservoir. Selected metrics were graphically screened with boxplots to assess the response to pressure. Allowing a class difference of one unit (see Breine et al., 2007, 2010), indices were validated by comparing the integrity class obtained per reservoir with its assessed pressure status. We assessed data of reservoirs used for the index development and an independent set of data consisting of fish data from nine reservoirs not included in the index development (surveys in 2006-2012). Finally a comparison was performed between the EQR values obtained with the old (Belpaire et al., 2000) and new indices (Pearson correlation, boxplot). To allow comparison, the old EQR values for each fishing sample within one year in a particular reservoir were averaged.

RESULTS

The selected reservoirs have different morphological characteristics and are subjected to different degrees of pressures (Table A, annex). The scores of the pressure assessment ranged between 4 and 8 (moderate and poor status). None of the assessed reservoirs seemed to have a good or high habitat-status (pressure score = 3).

In total 28 fish species were caught in reservoirs between 1996 and 2005. Eel (*Anguilla anguilla*, Linnaeus, 1758) and perch (*Perca fluviatilis,* Linnaeus, 1758) were the most frequently caught species with fyke nets and electric fishing. Perch and ruffe (*Gymnocephalus cernua*, Linnaeus, 1758) constituted the highest number of individuals caught with fyke nets, while roach and perch were most abundant during electric

TABLE 1

Candidate metrics with their predicted response to increasing disturbance.

Candidate metrics	Abbreviation	Metric type	Category	Predicted response to disturbances
# benthic species	MnsBen	species (count)	species composition and richness	↓
# invertivorous species	MnsInv	species (count)	trophic composition	↓
# local species	MnsLoc	species (count)	species composition and richness	↓
# omnivorous species	MnsOmn	species (count)	trophic composition	→
# piscivorous species	MnsPis	species (count)	trophic composition	↓
# species	MnsTot	species (count)	species composition and richness	↓
Percentage benthic individuals	MpiBen	relative percentage individuals	species composition and richness	↓
Percentage invertivorous individuals	MpiInv	relative percentage individuals	trophic composition	↓
Percentage omnivores	MpiOmn	relative percentage individuals	trophic composition	→
Percentage piscivores	MpiPis	relative percentage individuals	trophic composition	↓
Percentage recruitment	ManRec	relative percentage individuals	age structure	↓
Percentage specialised spawners	MpiSpa	relative percentage individuals	species composition and richness	↓
Shannon–Wiener diversity index	ManSha	diversity	species composition	↓
Tolerance value	ManTol	sum of values	species composition and richness	→
Total biomass per effort	ManBio	sum of biomass	abundance	↓
Percentage weight of *Abramis brama* and *Rutilus rutilus*	AbrRut	relative percentage individuals	abundance	↓
Median weight of *Abramis brama*, *Perca fluviatilis* and *Rutilus rutilus*	MedWei	median biomass	abundance	↙↗
Benthivore species (% weight)	BenWei	relative percentage weight	trophic composition	←
Sander lucioperca (% weight)	SanLuc	relative percentage weight	abundance	→
Perca fluviatilis (% weight)	PerFlu	relative percentage weight	abundance	←
Abramis brama (% weight)	AbrBra	relative percentage weight	abundance	→
Obligate species	OblSpe	species (count)	species composition and richness	←

fishing (Table B, annex). Twenty-one species were selected to occur in the reference (MEP/GEP) list, and guilds were attributed to the species included in this list (Table C, annex). A total of 22 candidate metrics were selected (Table 1).

The measure of association analyses allowed the selection of uncorrelated pressure variables to be used in the model. Only agricultural and industrial activities were correlated (V= 0.7; p= 0.003). Agricultural activities were selected as they affect water quality by the use of fertilisers and pesticides and because of their effects on soil erosion. Reservoir surface and depth were not correlated (Pearson c= 0.159; p= 0.382) and could be included in the model. The linear mixed model results are given in Table 2. For electric fishing data, seven metrics showed a significant relationship with the pressures and four with one of the descriptors. For the fyke net data, five candidate metrics showed a significant relationship with one pressure and six with one or two of the descriptors. Metrics that were not fitted by the model were omitted. Correlations between fitted metrics are given in Table 3.

To assess the ecological status with electric survey catches, two of the seven significant variables were selected (Table 4), more specifically 'relative percentage of specialised spawners' (individuals) (MpiSpa) and the 'relative percentage of invertivorous individuals' (MpiInv). For the fyke net data, four metrics were selected out of five possible candidates. These included the 'number of piscivorous species' (MnsPis), 'relative percentage of omnivorous individuals' (MpiOmn), 'relative weight percentage of benthivore species' (BenWei) and 'tolerance value' (ManTol). The response of the selected metrics to environmental pressures (pre-classification) is illustrated with boxplots showing how metric distribution changes along the pre-classification score (Fig. 2). Only one metric (MpiOmn) did not react well to increasing pressure. Compared to the other selected metrics the absolute values for its goodness-of-fit of

the model (R² marginal and conditional) were smaller (Table 2).

We considered 21 species in the reference list to be attributed to the selected metrics (Table C, annex). Below we give a short description of how the MEP/GEP for the six selected metrics was defined:

- Percentage specialised spawners (MpiSpa) (electric data)

There were six species involved: pike (*Esox lucius* LINNAEUS, 1758), gudgeon (*Gobio gobio* LINNAEUS, 1758), burbot (*Lota lota* LINNAEUS, 1758), ruffe, rudd (*Scardinius erythrophthalmus* LINNAEUS, 1758) and tench (*Tinca tinca* LINNAEUS, 1758). The relative species frequency in the reference condition (all 21 reference species present) equalled 28.5% (6/21)*100) and was taken as GEP. This metric was independent from depth and surface area (Table 2).

- Percentage of invertivorous individuals (MpiInv) (electric data)

Only three species were assessed: perch (<13 cm total length, PERSSON, 1983), ruffe and gudgeon. The maximum relative species frequency was 14.2% ((3/21)*100). This value was taken as the GEP status. The metric was depth-dependent.

- Number of piscivorous species (MnsPis) (fyke data)

Five species were assessed: burbot, wels catfish (*Silurus glanis*, LINNAEUS, 1758), pike-perch, perch (≥ 13cm total length, KOTTELAT & FREYHOF, 2007) and pike. MEP status was obtained when five piscivorous species were caught. For the GEP status three of these species were needed (60%). Indeed, according to the WFD, GEP tallies with slight changes in the values of the relevant biological quality elements as compared to the values found at maximum ecological potential (EU WATER FRAMEWORK DIRECTIVE, 2000). This metric was independent from depth and surface.

TABLE 2

Reaction of metrics with uncorrelated pressures in reservoirs. The linear mixed model (lmer) assessed how far uncorrelated descriptors and pressures scores (Surlake: reservoir surface; Depth: average depth of reservoir; Dev: percentage of construction; Agr: percentage of agriculture activities; Tree: percentage of trees: Nat: percentage of natural banks) described metrics (log (L) or square root (SR) transformed (metric abbreviations are explained in Table 1).

model <-lmer(metric ~ Lake surface + Development + Depth + Natural banks + Agriculture + Trees +(1|reservoir) + (1|year))						
Metrics (E)	Selected model	p value variable 1	p value variable 2	p value variable 3	R² Mar	R² Cond
LMnsInv	0.460-0.048Tree	0.0154			0.244	0.528
SRMpiSpa	3.177+0.125Nat-0.612Tree	0.0044	0.0244		0.193	0.363
SRManRec	5.786+0.597Agr	0.0485			0.085	0.136
SRMpiOmn	8.384-0.181Depth	0.0008			0.277	0.404
SRMpiPis	4.576+0.193Depth-1.243Tree+0.979Nat	0.0060	0.0234	0.0472	0.264	0.583
SRMpiInv	4.869-1.272Tree+0.144Depth+1.012Nat	0.0101	0.0135	0.0323	0.209	0.523
SRAbrRut	0.3444-0.183Depth	0.0155			0.212	0.360
SRBenWei	1.196-1.775Agr-0.741Dev	0.0002	0.0181		0.254	0.275
SRSanLuc	0.259-0.101Depth+0.426Nat	0.0370	0.0940		0.083	0.168
SRPerFlu	0.346+0.033Surlake+0.124Depth+0.659Dev	0.0038	0.0041	0.0116	0.274	0.282
LManTol	0.622+0.005Depth	0.0717			0.091	0.276
Metrics (F)	Selected model	p value variable 1	p value variable 2	p value variable 3	R² Mar	R² Cond
LMnsTot	0.503+0.18Tree-0.016Depth	0.0007	0.0042		0.358	0.741
LManBio	2.5-0.576Tree-0.031Depth-0.006Surlake	0.0001	0.0040	0.0060	0.165	0.310
LMnsPis	0223+0.056Nat	0.0450			0.139	0.539
SRMpiSpa	1.901+0.187Tree-0.351Surlake-0.401Nat	0.0005	0.0006	0.0020	0.145	0.579
SRMpiOmn	2.021+1.268Agr+1.352Tree	0.0004	0.0024		0.281	0.390
SRMpiPis	3.979-0.316Depth-0.116Surlake	0.0098	0.0341		0.296	0.523
SRMpiInv	6.482-1.591Tree+0.034Surlake	0.0168	0.0495		0.221	0.532
SRAbrRut	-0.196+1.322Tree	0.0090			0.257	0.644
SRBenWei	-0.647+1.219Agr+1.288Tree	0.0036	0.0184		0.296	0.502
SRSanLuc	-0.453+0.889Tree	0.0310			0.167	0.468
LManTol	0.599-0.044Dev+0.068Tree	0.0150	0.0220		0.268	0.539

● Percentage of omnivorous individuals (MpiOmn) (fyke nets)

The omnivorous species included three-spined stickleback (*Gasterosteus aculeatus*, Linnaeus, 1758), eel, tench, bream, Prussian carp, common carp, ide (*Leuciscus idus*, Linnaeus, 1758), ninespine stickleback (*Pungitius pungitius,* Linnaeus, 1758), roach and rudd. The maximum relative species frequency was 47.6% ((10/21)*100), which was taken as the threshold between bad and poor status. A minimum weight percentage (7.9%) was defined by expert

judgment whereby the MEP/GEP threshold (15.9%) was divided by two. This metric was independent from depth and surface.

● Benthivore species (BenWei, % relative weight) (fyke nets)

The benthivorous species considered were bream, white bream (*Blicca bjoerkna*, Linnaeus, 1758), common carp, ruffe and tench. The average value for all surveys (n=197) was 18.1% and the average value for sites in a poor status was 42.0% representing the threshold between

bad and poor. A minimum weight percentage (7%) was defined as a minimum of benthivores that should be present, whereby the MEP/GEP threshold (14%) was divided by two. This metric was independent from depth and surface.

● Tolerance value (ManTol) (fyke nets)

If all reference species are present in one reservoir, then the maximal tolerance value of 50 was obtained, which is the sum of all tolerance values. The GEP status was obtained when 17 species were present (80%). The tolerance value of the 17 most frequently caught species was 40. This value was taken as the lower threshold for the GEP status. This metric was independent from depth and surface.

● Index scoring: EQR

Within one reservoir, data from different surveys within one year were grouped per method, giving one index value for each method. The sum of the metric scores obtained with each method gave the IBI score for a particular site. The maximum sum of the IBI scores is 5.2 as only two metrics have a MEP threshold value. The minimum possible sum of the IBI scores is 1.2 (6*0.2). This score was transformed to an EQR calculated as a value between 0 and 1. The appreciation of the status was defined by the EQR value (see Table 4).

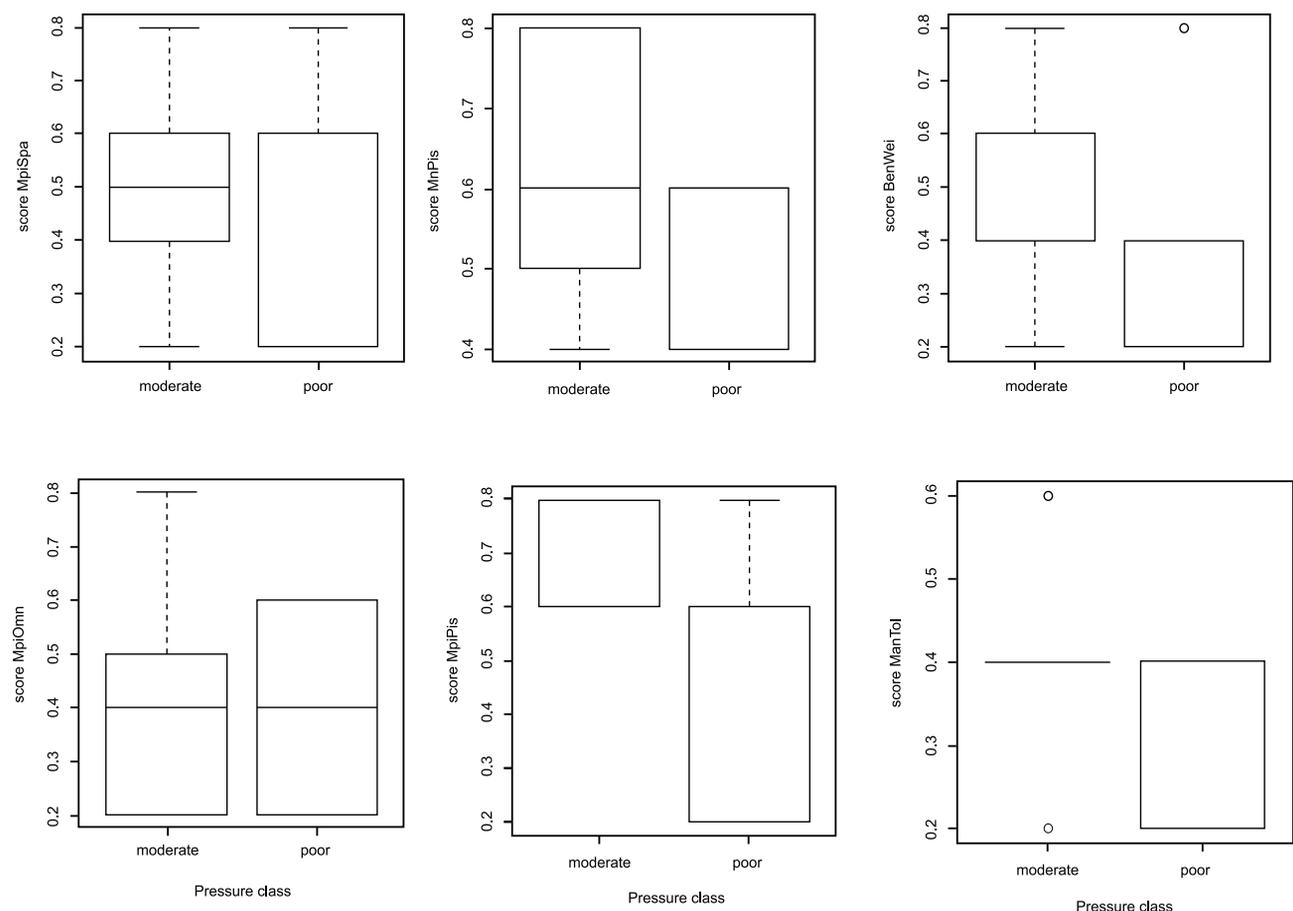Internal validation was performed using data of 17 reservoirs. We calculated the final EQR

Fig. 2 – Graphical screening of the scores of selected metrics as a function of the pre-classification of the reservoirs (Pressure class) by boxplots (for abbreviation of the metrics, see Table 1); bolt line = median, hinges = 25th and 75th percentiles, whiskers = range.

TABLE 3

Pearson coefficient (c) and significance (**p ≤0.001; * p≤0.05) for correlation analysis of model fitted metrics with electric and fyke data (abbreviations, see Table 1).

Electric	MnsInv	MpiSpa	ManRec	MpiOmn	MpiPis	MpiInv	AbrRut	BenWei	PerFlu	SanLuc
MpiSpa	0.0481	1								
ManRec	0.0965	0.2788*	1							
MpiOmn	-0.1699	0.0205*	-0.1964*	1						
MpiPis	0.2766*	0.2166*	0.1864*	-0.7123**	1					
MpiInv	0.3700**	0.1153	0.2349*	-0.7051**	0.9266**	1				
AbrRut	0.0334	-0.2274*	0.2003*	0.2955*	-0.1937*	-0.0756	1			
BenWei	0.1654	0.2111	0.1412	0.0456	-0.1139	-0.0401	0.018	1		
PerFlu	0.1182	-0.0756	0.2642*	-0.5314**	0.6938**	0.6993**	0.0561	-0.2327*	1	
SanLuc	0.1363	-0.0243	-0.1897*	0.0976	-0.0158	-0.0602	-0.0003	-0.0673	-0.2387*	1
ManTol	0.1330	0.4280*	-0.0267	-0.0716	0.1904*	0.0941	-0.2247*	0.0290	0.03782	-0.3105*
Fykes	**MnsTot**	**ManBio**	**MnsPis**	**MpiSpa**	**MpiOmn**	**MpiPis**	**MpiInv**	**AbrRut**	**BenWei**	**SanLuc**
ManBio	0.8138**	1								
MnsPis	0.5750**	0.4796**	1							
MpiSpa	0.2657**	0.1303*	-0.0404	1						
MpiOmn	0.4891**	0.6088**	0.1635	0.2625**	1					
MpiPis	-0.2866**	-0.2991**	0.5132**	-0.3236**	-0.5729**	1				
MpiInv	0.0878	0.0391	0.3967**	-0.1591*	-0.2003*	0.5904**	1			
AbrRut	0.5928**	0.3390*	0.2711**	0.0900	0.3486**	-0.1780*	-0.0672	1		
BenWei	0.5391**	0.4457**	0.1795	0.1892*	0.4060**	-0.3292**	-0.1071	0.4883**	1	
SanLuc	0.2201**	0.1935*	0.3908**	-0.1974*	-0.0400	0.2814**	-0.2182*	0.1029	0.0575	1
ManTol	0.3506**	0.4505**	0.3795**	0.2322*	0.4002**	0.1821*	0.5469**	0.0405	0.1603	-0.1484*

and compared its appreciation (i.e. integrity class) with the pressure status. One reservoir reached the GEP status, one had a bad status, six obtained a poor status, and nine had a moderate status (Table 5). Thirteen reservoirs had the same EQR appreciation as the pressure status (pressure class). Three reservoirs scored too high, i.e. the EQR was higher than the pressure status (one class difference). One reservoir scored too low two class differences).

For the external validation of the EQR of nine reservoirs (independent data), a high correspondence was found between the EQR appreciation and the attributed pressure status. Only one reservoir scored differently.

The Pearson correlation between the averaged EQR values (n=26) obtained with the initial index from BELPAIRE et al. (2000) and the

new EQR values did not show a significant correlation (c= 0.108; p= 0.598). With the old index, 14 reservoirs obtained an ecological status that diverged one class from the pressure status, and one reservoir diverged two classes (Table 5). The new index assessed the same reservoirs more accurately: only five showed a difference of one class. The new index also seemed to better separate the different pressure classes (Fig. 3).

DISCUSSION

Reference list

Species in the reference list are similar to those described for the Netherlands (BACKX et al., 2008). However, we did not include the European weatherfish (*Misgurnus fossilis* LINNAEUS, 1758) and spined loach (*Cobitis*

TABLE 4

Selected metrics for reservoirs and their threshold values for the metric and EQR-scores (abbreviations, see Table 1).

	MEP	GEP	Moderate	Poor	Bad
Electric data					
metric - score	1	0.8	0.6	0.4	0.2
MpiSpa (%)		< 28.5 ≥ 21.4	≥28.5 & < 21.4≥ 14.2	< 14.2 ≥ 7.1	< 7.1
MpiInv (%)		< 28.9 ≥ 14.2	≥ 28.9 & < 14.2 ≥ 9.4	< 9.4 ≥ 4.7	< 4.7
Fyke net data					
metric - score	1	0.8	0.6	0.4	0.2
MpiOmn (%)		< 15.9 ≥7.9	< 31.7 ≥ 15.9 & <7.9	< 47.6 ≥ 31.7	≥ 47.6
MnsPis (#)	5	<5 ≥ 3	2	1	0
BenWei (% weight)		< 14 ≥ 7	< 28.0 ≥ 14.0 & <7	< 42.0 ≥ 28.0	≥ 42.0
ManTol	50	<50 ≥40	<40 ≥27	<27 ≥13	<13
EQR	1	< 1 ≥ 0.75	< 0.75 ≥ 0.50	< 0.50 ≥ 0.25	< 0.25
Appreciation	MEP	GEP	Moderate	Poor	Bad

taenia LINNAEUS, 1758) as these do not (or rarely) occur in our reservoirs. Rheophilic species were omitted as they do not occur naturally in standing waters. We did not include alien species in our list. Unlike the observations by VANDEKERKHOVE et al. (2013), their presence was not always an indication of malfunctioning of the ecosystem as some of our alien species have relative high quality demands. In addition, some alien species only reside for a short time;

e.g. the brown bullhead (*Ameiurus nebulosus* LESUEUR, 1819) is disappearing from Flemish reservoirs (Schulensmeer, authors' observations between 1998-2011). Other species remaining for decades in our waters, e.g. pike-perch, are considered as naturalised. Only species occurring in the reference list were considered to assess the ecological quality of the reservoir. This approach is similar to the one used in BREINE et al. (2010).
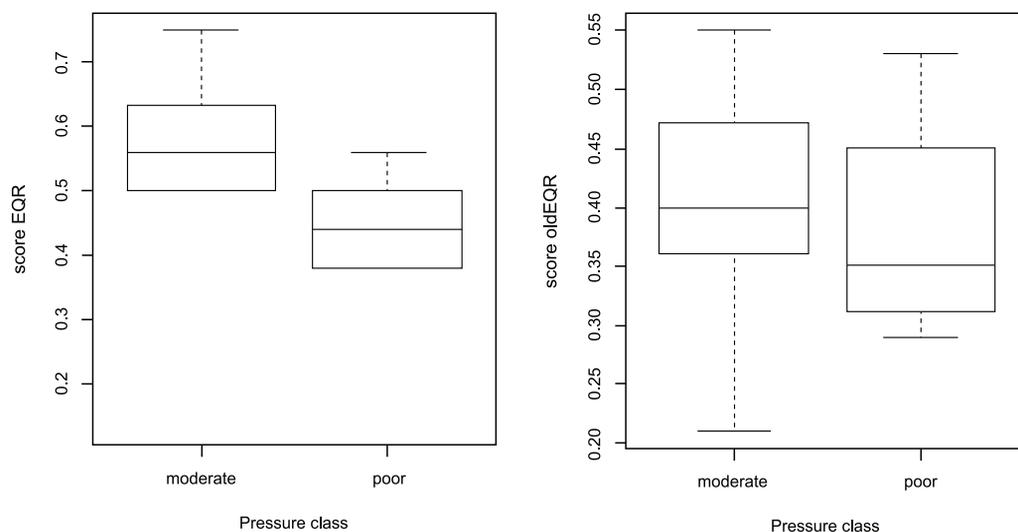

Fig. 3 – Boxplots showing the EQR value variation of the new and old index in the different pressure classes; bolt line = median, hinges = 25th and 75th percentiles, whiskers = range.

TABLE 5

Validation for reservoirs with internal data (1997-2005) and external independent data (2006-2011); for each reservoir metric scores, EQR and old EQR values (BELPAIRE et al., 2000), integrity and pressure class and class difference are given (abbreviations, see Table 1).

Reservoir (internal)	Year	MpiSpa	MpiPis	MnsPis	MpiOmn	BenWei	ManTol	EQR	Integrity class	Pressure class	Class difference	old EQR	Integrity class (old)
Bergelenput	2001	0.6	0.6	0.4	0.6	0.2	0.2	0.63	moderate	moderate	0	0.36	poor
De Broeken	2004	0.4	0.6	0.6	0.2	0.6	0.4	0.50	moderate	moderate	0	0.47	poor
E3-put Oostakker	2003	0.2	0.6	0.4	0.8	0.2	0.2	0.44	poor	poor	0	0.48	poor
Fort van Wallem	2003	0.2	0.6	0.4	0.6	0.4	0.2	0.38	poor	poor	0	0.43	poor
Gierle put	2001	0.6	0.6	0.6	0.2	0.2	0.2	0.44	poor	poor	0	0.45	poor
Grindplas Hochterbampt	2005	0.6	0.6	0.8	0.6	0.4	0.4	0.63	moderate	moderate	0	0.43	poor
Groot wachtbekken van Zuunbeek	2002	0.2	0.2	0.4	0.2	0.2	0.4	0.13	bad	poor	-1	0.45	poor
Klein wachtbekken Zuunbeek	2002	0.8	0.2	0.6	0.2	0.2	0.4	0.38	poor	poor	0	0.31	poor
Melleput	2003	0.6	0.6	0.6	0.6	0.2	0.4	0.56	moderate	poor	1	0.33	poor
Oude Durme	2005	0.4	0.8	0.6	0.2	0.8	0.6	0.69	moderate	moderate	0	0.41	poor
Oude Leiearm Grammene	2005	0.6	0.6	0.6	0.4	0.2	0.4	0.50	moderate	moderate	0	0.54	moderate
Oude Maas Dilsen	2002	0.4	0.8	0.8	0.6	0.6	0.4	0.75	GEP	moderate	1	0.55	moderate
Putten van Niel	1997	0.4	0.8	0.6	0.6	0.2	0.4	0.56	moderate	poor	1	0.53	moderate
Roksem put	2003	0.2	0.2	0.6	0.6	0.8	0.2	0.44	poor	poor	0	0.29	poor
Schulensmeer	1998	0.2	0.6	0.8	0.4	0.6	0.4	0.56	moderate	moderate	0	0.54	moderate
Watersportbaan Gent	2002	0.2	0.8	0.6	0.2	0.2	0.4	0.38	poor	poor	0	0.34	poor
Webbekomsbroek	1997	0.8	0.8	0.4	0.4	0.2	0.4	0.56	moderate	moderate	0	0.35	poor

Reservoir (external)	Year	MpiSpa	MpiPis	MnsPis	MpiOmn	BenWei	ManTol	EQR	Integrity class	Pressure class	Class difference	old EQR	Integrity class (old)
Desselse Zandput	2011	0.2	0.4	0.6	0.6	0.2	0.4	0.38	poor	poor	0	0.36	poor
Donkmeer	2008	0.4	0.6	0.8	0.4	0.2	0.6	0.56	moderate	moderate	0	0.39	poor
Gavers Geraardsbergen	2006	0.4	0.6	0.8	0.6	0.2	0.4	0.56	moderate	poor	1	0.3	poor
Gavers Harelbeke	2009	0.8	0.6	0.4	0.6	0.2	0.2	0.50	moderate	moderate	0	0.33	poor
Grindplas Eisden Mijn	2012	0.6	0.6	0.6	0.8	0.2	0.2	0.56	moderate	moderate	0	0.21	bad
Grindplassen Steenberg	2012	0.2	0.6	0.8	0.4	0.8	0.4	0.63	moderate	moderate	0	0.37	poor
Hazewinkel	2007	0.8	0.6	0.6	0.2	0.2	0.4	0.50	moderate	moderate	0	0.46	poor
Meer van Weerde	2008	0.2	0.6	0.6	0.6	0.6	0.4	0.56	moderate	moderate	0	0.39	poor
Volharding	2011	0.6	0.2	0.6	0.2	0.8	0.2	0.44	poor	poor	0	0.31	poor

Pre-classification

Similar to the approach explained by BREINE et al. (2007, 2010) and QUATAERT et al. (2011), the pre-classification is a device to rank reservoirs in a reasonable way with respect to anthropogenic pressures enabling the construction of a biotic index. The thresholds for the pre-classification attribution were based on expert judgement. However, the main point was not to have an absolute expression of the quality, but to have a good ranking with respect to human impact. The pre-classification of reservoirs based on abiotic variables is an important issue. It was used to make a first selection among the candidate metrics and for the external validation. The combination of scores expressing the pressures classified the reservoirs. Land cover percentages were also used by DRAKE & PEREIRA (2002). One of the largest factors contributing to impairment, namely non-point source pollution, is commonly associated with land-use modification (e.g., agriculture, urbanization) leading to eutrophication of surface waters (WANG et al., 2001; DODDS et al., 2009). As we focused on direct impacts from the neighbourhood, a zone of 100 m surrounding the reservoir was appropriate. Pre-classifying the reservoirs with presence absence data only (0 or 1) reduced the ranking efficiency as all reservoirs got the same score. Modelling with raw pressure values (log transformed) did not provide better results. Only for electric data, two metrics produced significant results: the metric 'relative percentage weight of perch' (PerFlu~0,7563+0,0314Surlake+0,1182Depth+0,0459Dev, R^2conditional=0.253) and 'the relative percentage weight of pike-perch' (SanLuc~1,9371+0,064Agr, R^2=0.294). Fyke data did not produce significant results.

We acknowledge that some important parameters were missing in our assessment, e.g. total phosphorus and total nitrogen (LAUNOIS et al., 2011b). Total phosphorus is an important parameter to assess the eutrophication of lakes and reservoirs (WETZEL, 1983). Nitrogen increases with human activities but is too variable to be a robust parameter (MOSS et al.,

2003). We used agricultural activity as a proxy for these parameters as measurements were only made in some reservoirs. The selected pressure parameters are known to have a negative impact on fish assemblages (DRAKE & PEREIRA, 2002; BACKX et al., 2008; LAUNOIS et al., 2011b, ARGILLIER et al., 2013).

Fishing methods

A single method underestimates the species richness (JACKSON & HARVEY, 1997). For lakes and reservoirs no single type of fishing gear is sufficient to survey all habitat types or to sample all fish species (WHITTIER, 1999; BONAR et al., 2009; KUBEČKA et al., 2009). The need to use multiple types of gear is a result of habitat heterogeneity, and the differences in habitat use of the associated species in lakes and reservoirs (FISCHER, 2012). In lakes and reservoirs fish can be pelagic, demersal or benthic. Therefore, adapted techniques should be used to assess the presence of the fish occurring in the water column or dwelling near the bottom. In deep reservoirs or lakes, electric fishing cannot be used nor can fykes easily be placed in vegetated habitats. As a consequence, data collected with multiple methods allows greater reliability in interpretations using information on lentic fish assemblages. By using different methods, we can retrieve a more accurate picture of the fish assemblage and therefore the ecological status of a reservoir (or lake) can be more precisely assessed. Multi-mesh gillnets are not used in Flanders as this method results in high fish mortality. Electric fishing in the littoral zone and fyke nets on the bottom are effective for sampling in lakes (JENNINGS et al., 1999). The sampling effort for each method should be such that adding an additional unit effort should not substantially increase species number or change proportional abundances. The fish protocol currently used in reservoirs in Flanders (1 fyke/ha overnight for two successive days with a minimum of 4 and a maximum of 20 per reservoir combined with electric fishing along 250 m long shore transects

per ha) has proven to fulfil this requirement (see also BELPAIRE et al., 2000).

Metric selection

The list of candidate metrics was based on literature. These metrics were chosen for their known reaction to human induced pressures and because they assess complementary aspects of the ecological functioning of the lakes. No explicit metric assessing alien species was included. The rationale is that if there is a significant pressure by alien species, this will be detected by other metrics.

The modelling approach allowed a first selection of metrics based on their sensitivity for one or more pressures. Only species occurring in the reference lists were considered for the calculation of the metric value. If only one fishing method had been used, the index would have consisted of less metrics. As a consequence, some effects of human disturbance would not have been assessed. Here, electric data metrics were sensitive to changes of the banks, while fyke net metrics also assessed impacts from agriculture and development. Our models showed that habitat quality (natural state of bank) and agricultural activities were major pressures explaining changes in fish assemblages. This corresponds with observations by LAUNOIS et al. (2011b) where habitat alterations and eutrophication in lakes seem to have a prominent effect on fish assemblages. European fish-based indices for lakes assess primarily eutrophication (RITTERBUSCH et al., 2011). To avoid over-fitting, a Pearson correlation was applied for examining redundancy among metrics. This method has also been applied by other authors (e.g. MINNS et al., 1994; MCCORMICK et al., 2001). Graphical screening of the selected metrics as a function of the pre-classification of the reservoirs by boxplots also showed that for nearly all metrics a clear gradient was seen (Fig. 2). The metric 'percentage of omnivores' was retained, though it did not seem to separate the pressure classes well. We considered that a less optimal metric can sometimes give invaluable information in

combination with other metrics (BREINE et al., 2007).

Rejected metrics fitted by the model

For electric fishing, the metrics assessing the 'invertivorous species' (MnsInv), 'percentage of omnivores' (MpiOmn), the 'relative combined weight of bream and roach' (AbrRut) and the 'tolerance values' (ManTol) were rejected as they only reacted to descriptors (deforestation or depth, Table 2). R^2 for the metric 'percentage of species that recruit' (ManRec) was small and its reaction to pressure was opposite to what was expected. Metrics assessing the 'relative percentage weight of perch' (PerFlu), 'benthivore species' (BenWei) and 'percentage weight of pike-perch' (SanLuc) were fitted but did not show the expected response.

For metrics assessing fyke net data, we rejected all metrics reacting to descriptors only. These included 'total number of species' (MnsTot), 'total biomass' (ManBio), 'percentage of piscivores' (MpiPis) and 'invertivores' (MpiInv), the 'relative combined weight of bream' (AbrRut) and the 'percentage weight of pike-perch' (SanLuc). The metric 'specialised spawners' (MpiSpa) was not selected as it decreased with increasing habitat quality, which was rather unexpected.

Properties of the selected metrics

- Percentage specialised spawners (MpiSpa) (electric data)

This metric was previously proposed by DIDIER (1997) and BREINE et al. (2004). It includes species having specific demands for spawning, and nest builders. As such, it assesses degradation of the spawning habitat. Due to degradation, fish will not spawn successfully and this will be reflected by the absence of one or more year classes or eventually lead to the extinction of one or more species (NICOLA et al., 1996; GASSNER et al., 2003). Extremely high values indicate a

disturbance (moderate status). In our study this metric reacted to the natural state of the banks. This metric scored 61.1% of the reservoirs in agreement with the pre-classification (Fig. 2).

- Percentage of invertivorous individuals (MpiInv) (electric data)

This metric is often integrated in an IBI (HUGHES & OBERDORFF, 1999). The invertivorous level decreases with degradation (BELPAIRE et al., 2000). In French lakes, this metric did not show a response with pressure because the assessed species were overall tolerant to degraded lake conditions (LAUNOIS et al. ,2011b). Here, we did not consider overall tolerant species, i.e. perch (<13 cm), ruffe and gudgeon. In our study this metric reacted to the natural state of the banks. Here, 83.3% of the reservoirs were correctly scored (Fig. 2).

- Number of piscivorous species (MnsPis) (fyke data)

The top of the food chain is represented by predators. This constitutes the piscivorous level, which also is sensitive to degradation (SHIELDS et al., 1995; MILLER et al., 1988; STEEDMAN, 1988). The presence of trophic specialists is very sensitive to increasing pressure and is often integrated in an IBI (HUGHES & OBERDORFF, 1999). This metric decreases in value as human impact increases (BACKX et al., 2008; BELPAIRE et al., 2000; LAUNOIS et al., 2011a). With our data this metric was sensitive to changes in habitat. A total of 55.5% of reservoirs were scored correctly using this metric.

- Benthivore species (BenWei, % weight) (fyke nets)

High values of the metric indicate unspecific degradation including eutrophication (RITTER-BUSCH et al., 2011). It measures the abundance of littoral and some pelagic species. Benthivore species are also used in the assessment systems of Germany, Lithuania, The Netherlands and Poland (RITTERBUSCH et al., 2011). In our study, the metric reacted to agricultural activities and scored 83.3% of the reservoirs correctly.

- Percentage of omnivorous individuals (MpiOmn) (fyke nets)

Increasing abundances of omnivores are observed in eutrophic, constructionally-modified lakes; and with increasing pressures (HICKMAN & MCDONOUGH 1996; WHITTIER 1999; DRAKE & PEREIRA, 2002). As eutrophication increases, the consequent higher primary production will lead to a higher total fish biomass (BELPAIRE et al., 2000). The absence of chemical pollution in Flemish reservoirs can explain why, in our study the metric is not bi-directional. The metric reacted to agricultural activities. Only 44.4% of the reservoirs were correctly classified.

- Tolerance value (ManTol) (fyke nets)

It is a good indication of human impact as lower values correspond with higher habitat degradation. The metric was selected as it reacts to construction. Using this metric 61.1% of the reservoirs were scored correctly. The mean tolerance value was also used in BELPAIRE et al. (2000) to assess the quality of standing waters in Flanders. It has also been used to assess ecosystem conditions in the US (NOVOTNY et al., 2005; MEADOR & CARLISE, 2007).

Metric scoring

Four metrics contain species that were rarely (crucian Carp (*Carassius carassius* Linnaeus, 1758), gudgeon, ide and ninespine stickleback) or never (burbot and wels catfish) caught. These species were still included as they are not extirpated, and because lakes or reservoirs are their preferred habitat. Including these species in the assessment did not affect the attribution of thresholds for the metric scores because 60%, for metrics assessing number of species, was taken as GEP. To compensate for gear specificity, the threshold values for metric 'tolerance value' were defined using the 17 most frequently caught species (80%). For the 'number of piscivorous species', three out of five species was scored as 'good'. Thresholds for the 'relative percentage of specialised spawners' and 'piscivores' were based on the

reference list independently from the catch results. Boxplots showed that, although overlaps exists, these metrics efficiently separated the different pressure classes. No adjustment for surface or depth was needed for the selected metrics (Table 2). Only MpiInv (electric fishing, Table 2) seemed to be influenced by depth, but in the model it had a small coefficient and scores were therefore attributed independently of the depth. Several methodologies were applied to determine metric scoring criteria whereby reference sites play a major role (BECK & HATCH, 2009). In the absence of reference conditions, minimally disturbed sites are sometimes used to select optimal metric scores and score classes are determined by dividing the total metric range into three or five equal portions (assuming a linear behaviour of the metrics) (BREINE et al., 2010). The Dutch fish-based index for lakes also uses the developed reference as a benchmark for the metric scoring (BACKX et al., 2008). Other member states use type-specific near reference sites to score the selected metrics (e.g. BELPAIRE et al, 2000; GASSNER et al., 2003; LAUNOIS et al., 2011a). Adapting this species list was the best option as the reservoirs in this study are impacted (moderate in the best case). Dividing the metric values into equal parts is a widely applied approach for indices (GOFFAUX et al., 2001). Due to data limitation, no other approach (linear regression or modelling) could be applied here to define metric thresholds. Only on two occasions was expert judgment used to define threshold values between the poor and bad status. For the 'percentage of omnivorous individuals' (MpiOmn) expert judgement (dividing MEP/ GEP threshold) was used only to define the minimum weight. A similar approach indicated the minimum weight for the metric assessing the 'benthivore species' (BenWei, % relative weight). The importance of this threshold is much less than the boundary between good and moderate. Indeed, according to the WFD, no actions have to be undertaken when a good status is reached. In a lower status (moderate, poor and bad) however, actions to improve the ecological status are needed.

The index score

In our study, data from different methodologies was assessed with different metrics as suggested by JENNINGS et al. (1999) and BECK & HATCH (2009). The index score was obtained by the sum of the individual gear-specific metrics. This value was then transformed to an EQR and an appreciation was attributed. Integrity classes have equal distance intervals. To define tendencies, of under- or over-estimation, we allowed a one-class difference between the habitat status (pre-classification) and the EQR as was done by GOFFAUX et al. (2001) and BREINE et al. (2004, 2011). Our validation showed that the newly-developed index was able to distinguish between different degrees of degradation within the pre-classified reservoirs. We consider the new index as an improvement as the EQR corresponded generally better with the attributed pressure status compared to the old index (Fig. 3). The first index for standing waters in Flanders (BELPAIRE et al., 2000) assessed reservoirs by combining fish results obtained from different fishing strategies without considering the gear specificity of these methods. In addition, the approach used now seems to be more robust as less expert judgement was used.

CONCLUSIONS

We developed a multi-metric index for reservoirs taking into consideration the different standardised sampling methodologies. The main aim was to present an approach that could be applied with any given set of data. The selected metrics are relevant allowing for an appropriate assessment of anthropogenic impacts on the fish communities. We also ensured that the metrics assess different aspects of the ecological functions of reservoirs for fishes, and that they are not redundant. The reference list provides a realistic goal i.e. presence of reference species corresponds to a good ecological potential. Finally the index is a clear communication tool for environmental managers, politicians and other target groups.

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TABLE A

Overview of surveys in reservoirs (1996-2005). Surface area, average depth, River fed with indication of stream system (IJ: IJzer; S: Schelde; M: Maas) and origin. The lower the scores for the pressure the better. Total number of fish caught with electric fishing (MnsTotE) and fyke nets (MnsTotF). The number of individuals (MnIind) and biomass (ManBio) expressed per m² for electric fishing (E) or per fyke day for fyke net catches (F) (i.e. the number of fykes multiplied by the days) *: Oude Leiearm Wevelgem consists of two reservoirs and Putten van Niel is a combination of five pits. Blanks= no data.

RESERVOIRS (YEAR OF SURVEY)	SURFACE AREA (HA)	AVERAGE DEPTH (M)	RIVER FED	ORIGIN	PRESSURE VALUE	ELECTRIC SURVEYS	FYKE DAYS	MNSTOT E	MNSTOT F	MNIIND E (/M²)	MNIIND F (/FYKE DAY)	MANBIO E (G/M2)	MANBIO F (G/FYKE DAY)
Bergelenput (2001)	8.0	5.0	N	sand extraction	4	1	14	9	5	0.23	371.36	26.57	3422.7
Blaarmeersen (2004)	17.0	13.5	N	man made	5		30		2		0.5		45.02
De Broeken (2004)	1.7	1.5	N	peat extraction	4	1	12	6	6	0.17	14.08	17.99	4071.0
E3-put Oostakker (2003)	2.5	2.8	Y (S)	sand extraction	6	4	10	10	7	0.42	39.3	29.94	909.1
Fort van Wallem (2003)	3.0	2.1	N	man made	6	5	16	11	9	0.31	13.56	24.07	1381.18
Galgenweel (2005)	47.2	6.0	Y (S)	dyke breach	5		40		10		32.55		2383.67
Gavers Harelbeke (2005)	53.0	10.0	Y (S)	sand extraction	5		36		5		24.22		1573.36
Gierle put (2001)	7.5	10.0	N	sand extraction	6	1	2	4	4	0.11	42.5	1.59	608.1
Grindplas Hochterbampt (2005)	14.0	18.5	N	gravel extraction	6	8	28	13	11	0.15	55.36	11.22	7789.69
Groot wachtbekken van Zuunbeek (1996 & 2002)	4.0	0.6	Y (S)	man made	8	1	24	12	12	31.46	16.59	98.88	9935.44
Grote Roggeman (1999)	4.3	0.5	N	bank creation	8	1		10		16.07		233.63	
Klein wachtbekken Zuunbeek (1996 & 2002)	2.0	0.6	Y (S)	man made	8	1	14	9	9	0.34	25.64	31.67	6134.08
Meer van Weerde (1998)	14.0	3.5	N	sand extraction	5	2	12	14	9	0.28	34.6	12.42	1064.0
Melleput (1996 & 2003)	25.0	11.0	N	sand extraction	5	9	90	10	8	0.08	2.59	0.72	202.8
Oude Durme (2005)	16.0	1.8	Y (S)	dyke creation	4	20	30	18	16	0.18	32.37	12.03	2424.15
Oude Leie Bavikhove (1998)	3.6	3.5	N	dyke creation	5	3		10		0.62		15.66	
Oude Leiearm Grammene (2005)	18.4	2.5	N	dyke creation	4	11	16	19	12	0.19	47.81	19.48	1553.43
Oude Leiearm Wevelgem* (1997)	3.2	2.0	N	dyke creation	5	5		11		0.26		18.56	
Oude Maas Dilsen (2002)	8.4	1.5	Y (M)	dyke creation	4	2	26	14	11	0.18	52.5	8.08	1989.59
Palingbeek (1999)	3.0	2.0	Y (IJ)	man made	5	3		10		0.28		57.15	
Putten van Niel* (1997)	7.94	10.0	Y (S)	clay extraction	5	10	20	11	10	0.3	20.35	15.26	962.16
Roksem put (2003)	40.0	7.0	N	sand extraction	5	4	40	3	5	0.02	6.4	3.47	630.3
Schulensmeer (1998)	89.0	4.2	Y (S)	sand extraction	4	13	16	17	13	1.2	69.56	24.34	4605.75
Sisput (2001)	0.14	3.0	Y (S)	dyke creation	5	2		6		0.15		7.96	
Watersportbaan Gent (2002)	22.0	2.5	Y (S)	man made	8	10	20	9	11	0.05	36.9	3.18	6692.0
Webbekomsbroek (1997 & 2004)	2.0	1.0	Y (S)	man made	4	4	24	16	10	1.19	56.71	26.18	1839.09

TABLE B

Recent fish data for reservoirs in Flanders (autumn 1996-2005). # is the number of catches per species grouped over all surveys. Frequency is the catch frequency in the reservoirs (#/campaigns). # ind. gives the number of individuals caught in the reservoirs. Last column indicates if the species is a MEP/GEP species. Fyke days equals the number of fykes multiplied by the days they were standing; n gives the number of electric surveys.

Scientific name	Fykes (520 fyke days)		Electric fishing (n=112)		MEP/ GEP
	frequency	# ind.	frequency	# ind.	
Abramis brama (LINNAEUS, 1758)	32.47	1918	36.60	116	X
Alburnus alburnus (LINNAEUS, 1758)	0.43	1	1.31	2	
Ameiurus nebulosus (LESUEUR, 1819)	6.93	487	5.88	343	
Anguilla anguilla (LINNAEUS, 1758)	80.95	2193	86.27	3649	X
Blicca bjoerkna (LINNAEUS, 1758)	29.44	802	28.1	477	X
Carassius carassius (LINNAEUS, 1758)	0.43	1	3.92	8	X
Carassius gibelio (BLOCH, 1782)	12.12	304	35.29	1154	X
Cobitis taenia (LINNAEUS, 1758)	0.00	0	4.58	36	
Cyprinus carpio carpio (LINNAEUS, 1758)	15.15	225	21.57	392	X
Esox lucius (LINNAEUS, 1758)	5.19	16	49.67	563	X
Gasterosteus aculeatus (LINNAEUS, 1758)	0.00	0	6.54	399	X
Gobio gobio (LINNAEUS, 1758)	0.00	0	5.88	39	X
Gymnocephalus cernua (LINNAEUS, 1758)	38.10	2776	35.95	455	X
Lepomis gibbosus (LINNAEUS, 1758)	15.15	874	33.99	2238	
Leucaspius delineatus (HECKEL, 1843)	0.87	3	11.11	195	X
Leuciscus idus (LINNAEUS, 1758)	2.16	5	15.03	45	X
Liza ramada (RISSO, 1827)	0.43	2	0.00	0	
Lota lota (LINNAEUS, 1758)	0.0	0	0.00	0	X
Perca fluviatilis (LINNAEUS, 1758)	60.17	6408	91.50	7577	X
Platichthys flesus (LINNAEUS, 1758)	6.06	21	3.27	9	
Pseudorasbora parva (TEMMINCK & SCHLEGEL, 1846)	6.93	96	9.80	431	
Pungitius pungitius (LINNAEUS, 1758)	0.00	0	2.61	10	X
Rhodeus sericeus (PALLAS, 1776)	6.06	160	18.95	1069	X
Rutilus rutilus (LINNAEUS, 1758)	38.96	2700	79.08	8879	X
Salmo trutta (LINNAEUS, 1758)	0.43	1	0.65	4	
Sander lucioperca (LINNAEUS, 1758)	33.33	1836	15.69	65	X
Scardinius erythrophthalmus (LINNAEUS, 1758)	13.85	303	58.17	1666	X
Silurus glanis (LINNAEUS, 1758)	0.00	0	0.00	0	X
Squalius cephalus (LINNAEUS, 1758)	0.00	0	1.31	2	
Tinca tinca (LINNAEUS, 1758)	15.58	59	43.14	541	X

TABLE C

Reference fish species occurring in reservoirs in Flanders and their guild attribution. * *Perca fluviatilis*: ≥ 13 cm piscivorous; ** not caught in reservoirs yet.

Scientific name	Omnivore	Benthic	Invertivore	Piscivore	Tolerance value	Specialised spawner	Obligatory species
Abramis brama (LINNAEUS, 1758)	x				1		x
Anguilla anguilla (LINNAEUS, 1758)	x	x			2		
Blicca bjoerkna (LINNAEUS, 1758)					2		x
Carassius carassius (LINNAEUS, 1758)					3		
Carassius gibelio (BLOCH, 1782)	x				0		
Cyprinus carpio carpio (LINNAEUS, 1758)	x				2		
Esox lucius (LINNAEUS, 1758)				x	4	x	x
Gasterosteus aculeatus (LINNAEUS, 1758)			x		1	x	
Gobio gobio (LINNAEUS, 1758)			x		3	x	
Gymnocephalus cernua (LINNAEUS, 1758)		x	x		2		
Leucaspius delineatus (HECKEL, 1843)					4		
Leuciscus idus (LINNAEUS, 1758)	x				4	x	
Lota lota (LINNAEUS, 1758)**		x		x	4	x	
Perca fluviatilis (LINNAEUS, 1758)			x	x*	2	x	
Pungitius pungitius (LINNAEUS, 1758)			x		1		x
Rhodeus sericeus (PALLAS, 1776)					3	x	x
Rutilus rutilus (LINNAEUS, 1758)	x				1		x
Sander lucioperca (LINNAEUS, 1758)				x	3		x
Scardinius erythrophthalmus (LINNAEUS, 1758)	x	x			3	x	x
Silurus glanis (LINNAEUS, 1758)**		x		x	2	x	x
Tinca tinca (LINNAEUS, 1758)	x	x			3	x	

Biodiversity and seasonal variations of zooneuston in the northwestern Mediterranean Sea

France Collard[1,2,*], Amandine Collignon[1,3], Jean-Henri Hecq[1,3], Loïc Michel[1] & Anne Goffart[1,3]

[1] Laboratory of Oceanology, MARE Centre, University of Liège, B6C, 4000 Liège, Sart Tilman, Belgium.
[2] Laboratory of Functional and Evolutionary Morphology, AFFISH-RC, University of Liège, B6C, 4000 Liège, Belgium.
[3] Station de Recherches Sous-Marines et Océanographiques (STARESO), BP 33, 20260 Calvi, France.

* Corresponding author: france.collard@ulg.ac.be

ABSTRACT. Neuston includes animals and plants inhabiting the surface layer of the water column. The neustonic area is an accumulation zone for bacteria, organic molecules but also terrestrial debris. The surface layer is also the air/water exchange region. Therefore, neustonic organisms are directly exposed to several constraints such as wind stress and turbulence. The present study aims to characterize the zooneuston in terms of abundance and biodiversity and to evaluate the impacts of wind stress on neustonic abundance. Zooneustonic and zooplanktonic (depth of 5 meters) samples were collected twice a month between 30th August 2011 and 10th July 2012 in Calvi Bay, Corsica. Zooneustonic biodiversity was high and, notably, twenty-eight copepod genera were identified. Among these copepods, several organisms, belonging to the Pontellidae family, were much more frequent in neuston than in underlying plankton and their abundance depended on wind direction. Taxon-specific trends in seasonal abundance variation were present. For example, individuals of the Acantharia *Lithoptera* spp. were found in summer whereas the Pontellidae *Anomalocera patersoni* appeared in winter. Overall, our data provide a first step towards a better knowledge of neuston community structure in the Mediterranean Sea.

KEY WORDS: Neuston, Pontellidae, Mediterranean Sea, Plankton, Wind forcing.

INTRODUCTION

The neuston comprises organisms inhabiting the surface layer of the water column, whereas planktonic organisms inhabit the subsurface layer (NAUMANN, 1917). Neustonic organisms live under a particular, not well-structured surface microlayer (SML), composed of colloids and macromolecules coming from dissolved organic matter and bacteria (SIEBURTH, 1983). The surface layer is a critical zone for marine species, and is notably a feeding area for fish (CARDINALE et al., 2003; PUSINERI et al., 2005). The surface layer is also an accumulation zone of pollutants (GARCIA-FLOR et al., 2008) and debris such as terrestrial debris or (micro)plastics (RYAN et al., 2009) that can threaten marine organisms (LAIST, 1997; MATO et al., 2001; GREGORY,

2009; COLLIGNON et al., 2012 & 2014). Due to its intermediate position between the atmosphere and the water column, the neuston is exposed to many constraints related to atmospheric conditions: high light intensity, wind stress, turbulence and temperature variations. Both physical and chemical parameters of seawater can affect zooneuston, and sea surface temperature and salinity notably influence the distribution of Pontellidae copepods (ZAITSEV, 1971). Copepods are known to migrate vertically according to light (HANEY, 1988), water density (HARADA et al., 1985), oxygen concentration (HERMAN, 1984), or phytoplankton abundance (and therefore nutrient concentration, TISELIUS, 1992). In the Black Sea, neustonic communities resemble the underlying plankton (ZAITSEV, 1971). However, some neustonic species display particular morphological or ecophysiological adaptations.

For example, neustonic bacteria communities are different from planktonic communities (FRANKLIN et al., 2005) and phytoneuston differs from phytoplankton (HARDY & APTS, 1984; LYALYUK & LIPNITSKAYA, 2003). ZAITSEV (1971) also reported that Pontellidae copepods are characteristic of the neuston. Very few studies deal with neuston in the Mediterranean sea (OLIVAR & SABATES, 1997). The diversity and abundance of mediterranean zooneustonic organisms, as well as seasonal and daily variations of these organisms remain unknown to date. In this context, aims of this study were (1) to assess zooneuston diversity in a well-preserved Mediterranean area, (2) to quantify the abundance of dominant organisms at each season, and (3) to examine relations between neuston abundance and environmental parameters. To achieve this goal, neuston community structure in Calvi Bay (NW Corsica, France) was analyzed over a 10-month-long sampling period.

MATERIAL AND METHODS

Sample collection

Zooneuston and zooplankton samples were collected twice a month between 30th August

2011 and 10th July 2012. The sampling site, located near the STARESO oceanographic station (Calvi Bay, NW Corsica; Fig. 1), is characterized by very low anthropogenic influence (GOBERT et al., 2009). Neustonic and planktonic samples were collected with WP2 nets (200 µm mesh). The frame of the neuston net was rectangular (0.60 m x 0.25 m) and the trawl sampled the top 20 cm of the water column. The frame of the plankton net was circular (diameter of 0.60 m) and the trawl sampled at a depth of 5 m. Nets were towed following a trajectory fixed by two points (42°35'7.80''N 8°43'46.37''E and 42°35'5.09''N 8°43'44.39''E; Fig. 1) for 20 minutes between 7.00 AM and 8.00 AM, at a speed of 2 km/h. After collection, samples were concentrated to a volume of 0.2L and fixed in 2.5% formalin.

A short period of ten days (from 30th August to 8th September 2011) was chosen to evaluate the impact of wind stress on zooneustonic abundances. During this period, twelve neuston samples were collected at the same location (Fig. 1), using the methodology described above.

Biovolume measurements and community structure assessment

In order to rapidly and easily estimate the quantity of organisms, their biovolume was measured using a non-destructive method. Organisms were placed in a graduated cylinder, and after a 24 hour sedimentation period, it was possible to visually estimate the biovolume, *i.e.* the volume of the graduated cylinder occupied by all organisms. In two samples, we had to remove exceptional proliferations of Siphonophora (family of Hippopodiidae) and *Velella* that made precise biovolume estimation impossible. Neustonic organisms were examined and counted under a binocular microscope. For each neuston sample, occurring groups were listed. Important taxa (i.e. taxa supposedly characteristic of the neustonic environment and/or proportionally abundant in the samples)
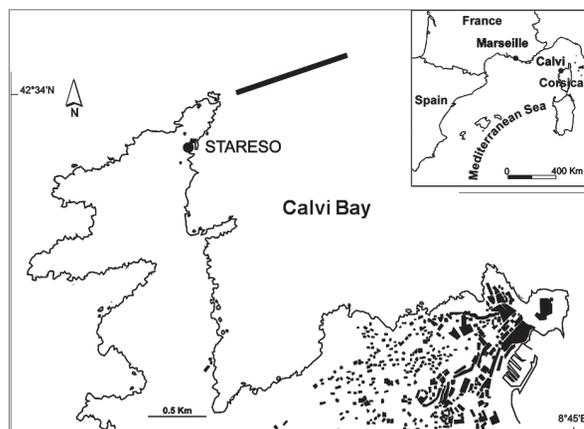

Fig. 1 – Map of Calvi Bay showing the STARESO research station and the sampling transect (solid black line) (modified from Vermeulen et al., 2011).

were counted. Particular attention was given to Pontellidae copepods. The main species identification criteria for this family (Rose, 1933; Tregouboff & Rose, 1957) included the number of ocular lenses, the number of cephalic lateral hooks, the pincer type on males' last right appendages, and the presence and the shape of spikes on the last thoracic segment.

Environmental parameters

Wind speed and wind direction were measured near STARESO (42°34'43.32''N 8°43'8.36''E; altitude 169 m) using an AWS 2700 weather sta-tion (Aanderaa Data Instruments, Bergen, Norway) equipped with a wind speed sensor 2740 and a wind direction sensor 3590. Measurements were done every 20 minutes, and data were averaged over the twelve hours before sampling. Wind speed is one of the most important parameters in the estimation of turbulence. Wind speed to the power of 3 can indeed be used as a turbulence proxy (Stacey & Pond, 1997). Precipitation data were obtained from Météo France. They were taken at Calvi airport (42°31'23.88''N 8°47'30.01''E; altitude 57 m).

Statistical analyses

The relation between zooneuston abundance and environmental parameters was investigated using correlation analysis. Statistical analyses were conducted using Prism 5.03 (Graphpad Software, La Jolla, CA, U.S.A.). Significance threshold was fixed at $\alpha = 0.05$.

RESULTS

Neustonic biodiversity

Twenty-one higher taxa belonging to 9 phyla were identified and counted (Table 1). Arthropoda was the most abundant group in the neuston. It was mainly represented by copepods, particularly the genera *Clausocalanus* and

Paracalanus (regrouped under the "Others" entry, Table 1). Pontellidae copepods were present in 16 of the 22 neuston samples. Contrastingly, they were rarely found in plankton (2 of the 22 samples), and only encountered in autumn. Most of the neustonic Pontellidae were immature individuals that could not be identified to the genus or species level. Besides those, 7 Pontellidae species belonging to 4 genera were observed during the sampling period. *Pontella mediterranea* (Table 1) was the most common adult Pontellidae in neuston samples, and the only one to be encountered in underlying plankton. Small copepods (*Clausocalanus* spp., *Paracalanus* spp., *Oithona* sp., *Acartia* sp.) were more abundant than large ones (*Centropages* sp., *Temora* sp., Pontellidae, *Candacia* sp.). Twenty-eight genera of copepods were found, including four from the Pontellidae family.

Other major zooneustonic groups included Cladocera, Mollusca, Appendicularia, Chaeto-gnatha, ichthyoplankton and Siphonophora, as well as *Collozoum inerme* (Table 1). Members of Foraminifera, Polychaeta, Decapoda (adults), Amphipoda or Ostracoda were more rare.

Seasonal variations

The mean neustonic biovolume was 5.1 ml.(100 m²)$^{-1}$, varying between 0.8 ml.(100 m²)$^{-1}$ and 14.0 ml.(100 m²)$^{-1}$ throughout the studied year (Fig. 2). Neustonic biovolume did not seem to follow a clear seasonal pattern, and high values did not occur consistently: the highest value was found on 16th January 2012 (sample 10), the second highest on 24th April 2012 (sample 16) and the third on 8th May 2012 (sample 17).

No general seasonal pattern was found for total number of species, but some group-specific trends were present (Table 1). The Acantharia *Lithoptera* spp. and the Pontellidae *Pontellopsis regalis* were the only organisms found in a single season. The acantharians were found in summer (beginning of September) and the copepods in autumn. By contrast,

TABLE 1

Abundances and seasonal variations of dominant neustonic taxa. X: present, -: absent.

Taxa	Global mean abundance (ind.100^{-1}m^{-2})	Summer	Autumn	Winter	Spring
Foraminifera					
Globothalamea	-	X	X	-	X
Radiozoa					
Acantharia					
Lithoptera spp.	1.40	X	-	-	-
Amoebozoa					
Lobosa incertae sedis					
Collozoum inerme	30.4	X	X	X	X
Cnidaria					
Hydrozoa					
Siphonophora	24.6	X	X	X	X
Annelida					
Polychaeta	-	-	X	X	X
Mollusca					
Gastropoda					
Creseis sp.	342	X	X	X	-
Chaetognatha					
Sagittoidae	29.2	X	X	X	X
Arthropoda					
Maxillopoda					
Copepoda					
Calanoida					
Pontellidae					
Immature Pontellidae	96	X	X	-	-
Anomalocera patersoni	0.58	-	-	X	X
Labidocera brunescens	30.8	X	X	X	X
Labidocera wollastoni	0.08	X	X	-	-
Pontella lobiancoi	0.07	-	-	-	-
Pontella mediterranea	23.6	X	X	-	-
Pontellopsis regalis	0.02	-	X	-	-
Pontellopsis villosa	0.10	X	X	-	-
Other families					
Acartia sp.	154	X	X	X	X
Candacia sp.	1.70	-	X	X	-
Centropages spp.	163	X	X	X	X
Oithona sp.	314	X	X	X	X
Temora sp.	162	X	X	-	-
Others	9375	X	X	X	X
Cladocera	1024	X	X	-	X
Malacostraca					
Isopoda	1.35	-	X	X	X
Chordata					
Appendicularia					
Salpa spp.	180	X	X	X	X
Actinopterygii					
Eggs	25.6	X	X	X	X
Larvae	2.31	X	X	X	X
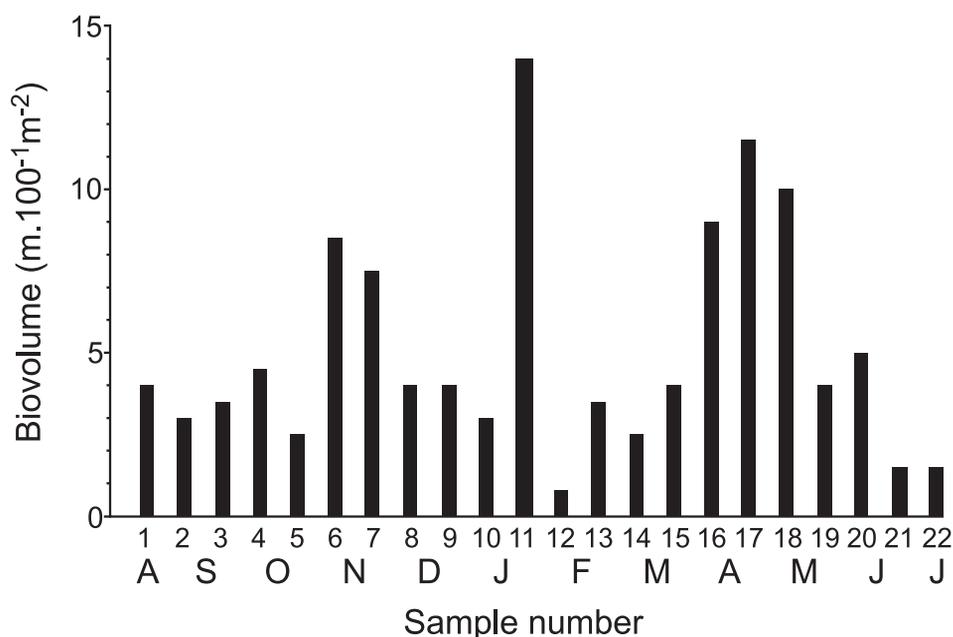
Fig. 2 – Neustonic biovolume variations from August 2011 to July 2012.

several groups were present throughout the whole sampling period: *Collozoum inerme, Paracalanus* spp., *Clausocalanus* spp., *Oithona* sp., *Acartia* sp., *Centropages* spp., Appendicularia, Chaetognatha, Siphonophora, and ichthyoplankton. Most Pontellidae species were found in summer or in autumn, with the exception of *Anomalocera patersoni* which was found in spring and winter. The maximal Pontellidae abundance (1602 ind.$100^{-1}m^{-2}$) occurred on 4th October 2011 (Fig. 3). Molluscan *Creseis* sp. and copepods *Temora* sp. were present in autumn and absent in spring. Cladocerans were abundant in September 2011, June and July 2012. Their numbers strongly decreased in October 2011 and they progressively disappeared from November 2011 to April 2012.

Relationship with wind stress and turbulence

During the ten days of measurement, wind directions associated with neuston sampling events fluctuated between 71° (ENE) and 260° (W) and wind speed ranged between 0.6 m/s and 14.8 m/s.

Although a non-significant negative trend seemed to be present for Pontellidae (data not shown), no correlation was found between turbulence and abundance of any of the neustonic taxa.

Mean wind direction, however, influenced the abundance of Pontellidae (Fig. 4, $r^2 = 0.7848$, p = 0.0001) and Chaetognatha (Fig. 4, $r^2 = 0.4969$, p = 0.0105). Pontellidae were more abundant when wind blew from the north-east than when it blew from the south-west. Inversely, Chaetognatha were less abundant when wind blew from the east (Fig. 4). No correlation between abundance and wind direction was found for any of the other counted taxa.

DISCUSSION

The neustonic environment contains a diverse assemblage of organisms. Crustacea, and mostly copepods, were the most important group in terms of biodiversity and abundance Other major groups included Mollusca, Appendicularia, early life stages of fish, Siphonophora and Amoebozoa. Other species of Cnidaria, Acantharia and
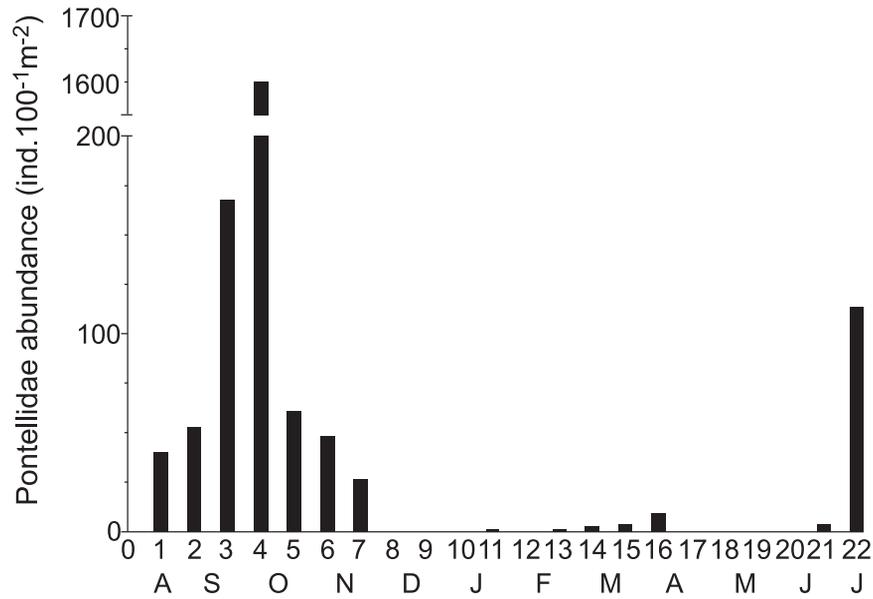
Fig. 3 – Pontellidae abundance variations from August 2011 to July 2012.

Foraminifera were less abundant, but regularly observed. Our results are comparable with another study from the NW Mediterranean (LICANDRO & ICARDI, 2009). Neuston biodiversity highlights the ecological importance of this zone.

Pontellidae copepods are known to be specific to the neuston. In Calvi Bay, they were accordingly much more frequently observed in neuston than in underlying plankton. In the Red Sea, they seem to be absent from samples taken "just beneath the surface" (i.e. a bit deeper than our neuston samples) of the sea (KHALIL et al., 1997), supporting the fact that Pontellidae copepods mostly live in the first centimeters of the water column. Species of Pontellidae have surface attachment structures (SAS), formed by several patches of setae on the dorso-anterior surface of the cephalosome and the second thoracic segment (IANORA et al., 1992).
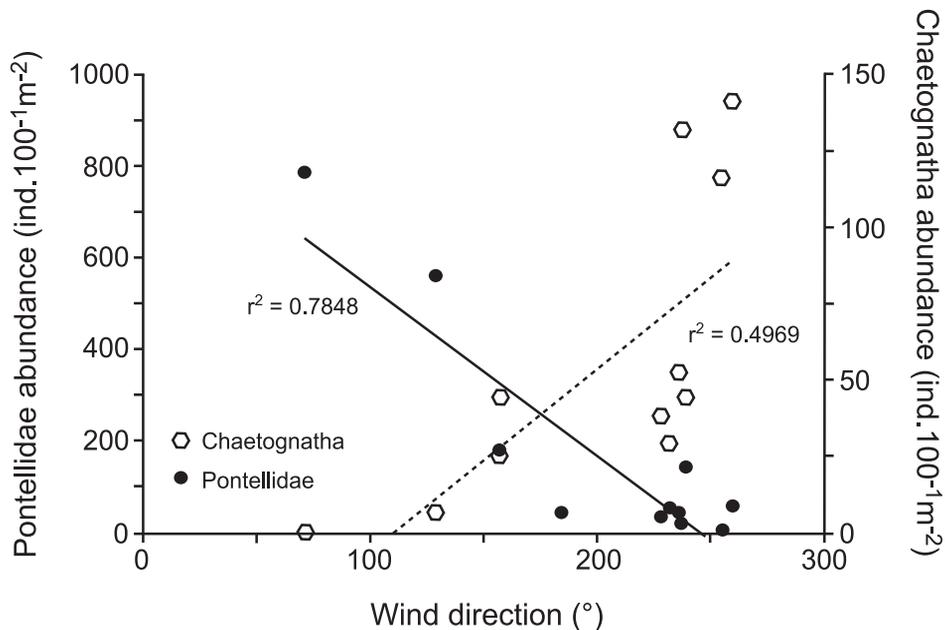
Fig. 4 – Correlations between Pontellidae and Chaetognatha abundances and wind direction.

These SAS are thought to be an energy-saving way to adhere to the surface film, and may therefore represent a morphological adaptation to neustonic lifestyle. SAS are not present in all species of Pontellidae (Pennell, 1973). It appears that only species of Pontellidae with bright colors possess an SAS. This pigmentation is thought to play a protective role against the ultraviolet rays to which Pontellidae with SAS are more exposed.

A negative linear correlation between abundance of Pontellidae and wind direction was highlighted. When wind blows from the north-east, abundance of Pontellidae is high, while abundance is low when wind blows from the south-west. The offshore opening of Calvi Bay is towards the northeast (Fig. 1). A northeasterly wind therefore causes offshore surface water to enter the bay. High Pontellidae abundance in this advected water could therefore explain the observed correlation.

As explained by Holdway & Maddock (1983), abundance and diversity in the neuston vary with the day/night cycle. In their study, some taxa were more abundant during the night (e.g. Amphipoda and Ostracoda) or during the day (e.g. Appendicularia). Several taxa were more numerous at dusk or dawn (e.g. *Lucifer* spp. and Cnidaria except Siphonophora). We are aware of this influence on diversity and abundance. Nevertheless, for practical reasons (boat and staff availability), samples have been collected at the same hour each day. Further studies based on samples taken during multiple times of the day are needed to improve our knowledge of dynamics of Mediterranean communities.

Chaetognatha abundances, on the other hand, were inversely impacted by wind direction. These two correlations should be confirmed by concomitant sampling in the bay and offshore.

Although our study is a first step towards a better knowledge of neuston community structure in the Mediterranean Sea, information about this particular layer of the sea remains scarce. It would be interesting to know what drives dominance of certain taxa in the neuston, and what are their ecological impacts on other compartments of the pelagic ecosystem. The surface layer is indeed a feeding location for fish, as well as an accumulation zone of pollutants. In the context of increasing human population, and consequently of increasing pollution and fishing activities, neuston assemblages could undergo drastic changes. Whether or not these changes could have adverse effects on the underlying parts of the water column is a question that will only be answered through further understanding of neuston ecology.

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Effects of Indol-3-Acetic Acid on the biology of *Galleria mellonella* and its endoparasitoid *Pimpla turionellae*

Fevzi Uçkan[1,*], Rabia Özbek[1], Ekrem Ergin[2]

[1] Department of Biology, Faculty of Science and Literature, Kocaeli University, Kocaeli, 41380, TURKEY

[2] Nursing School, Gülhane Military Medical Academy, Ankara, 06018, TURKEY (e-mails: ozbekrabia@gmail.com, ekremxergin@hotmail.com)

* Corresponding author: fevzi.uckan@kocaeli.edu.tr

ABSTRACT. The effects of indol-3-acetic acid (IAA) were investigated on biological parameters of *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) and its endoparasitoid *Pimpla turionellae* (Linnaeus 1758) (Hymenoptera: Ichneumonidae) reared on hosts treated with 50 to 10,000 ppm of IAA in their diet. Percent fecundity of first generation of *G. mellonella* females increased by 12% at 50 ppm whereas the most effective decrease, by >33%, was observed at 5,000 ppm. Percent fertility decreased by >20% at 1,000 ppm. All treatments displayed ±5% variations when compared to controls except for a >20% increase in corrected percent sterility at 1,000 ppm. The results did not reveal any considerable effect of IAA on egg hatching, larval and pupal developmental, or adult emergence times. The most striking effect observed was a decline in second generation pupal developmental time by >47% at 1,000 and 10,000 ppm. No dose-wise alterations were observed in adult longevity, weight, size and female sex ratio of *G. mellonella*. Treatment with IAA caused a slight prolongation in adult emergence and decrease in longevity of *P. turionellae* reared on hosts; longevity of wasps declined by >27% at higher doses. Neither wasp size nor weight displayed significant changes upon IAA treatment.

KEY WORDS: Biology, *Galleria mellonella,* Indole-3-acetic acid, *Pimpla turionellae*.

INTRODUCTION

Synthetic chemicals acting as plant growth regulators (PGRs) have been widely used to obtain high levels of agricultural productivity. However, these chemicals not only affect plant growth and development, but they also negatively affect the development, survival, longevity, reproductive potential, hemocytes, and hemolymph metabolites of insects and other animals (AHMED et al, 2003; PAULSON et al., 2005; GUPTA et al., 2009; UÇKAN et al., 2008, UÇKAN et al., 2011a, b; ALTUNTAŞ et al., 2012). The toxic effects of PGRs, including indole-3-acetic acid (IAA), on the development and reproductive potential of the pest species, *Bactrocera cucurbitae* (COQUILLETT, 1849) were investigated and the authors suggested that these eco-friendly compounds are promising candidates to replace pesticides in Integrated Pest Management (IPM) programs of economically

important pest insect (KAUR & RUP, 2002). GUPTA et al. (2009) also reported that gibberellic acid (GA$_3$) and siapton (an amino acid based plant growth stimulant) caused an increase in the larval period of the hairy caterpillar, *Spilarctia obliqua* (WALKER, 1855) at high doses. However, the overall effects of PGRs on insects still need to be clarified to improve the strategies for pest control and to know what kind of effects these compounds have especially on natural enemies of pest species. For example, UÇKAN et al. (2008; 2011a) demonstrated that egg to adult developmental time of the wasp species, *Apanteles galleriae* (WILKINSON, 1932) reared on *Achoria grisella* (FABRICIUS, 1794) larvae exposed to high doses of GA$_3$ and IAA increased by 40% and 30%, respectively when wasps were reared on larvae exposed to higher doses of the PGRs. Furthermore, the adult longevity of *A. galleriae* decreased by >50% when wasps were reared on IAA- or GA$_3$ -treated hosts (UÇKAN et

al., 2008, 2011a). Recently, Uçkan et al. (2011b) also stated that higher doses of GA$_3$ negatively affected the adult longevity of the wasp species, *Pimpla turionellae* (Linnaeus, 1758) (Hymenoptera: Ichneumonidae). A decrease in length was also apparent at most of the treatment levels. Their results also revealed that hemolymph carbohydrate at most of the dose levels, and lipid at all decreased in host larvae upon exposure to GA$_3$. Kaur & Kaur (2013) reported that the braconid parasitoid, *Bracon hebetor* (Say, 1836) reared on coumarin-treated host, *Spodoptera litura* (Fabricius, 1775) displayed declines in reproductive potential, female life span, and egg hatching with an extended developmental period at higher concentrations. Prado & Frank (2013) also showed that PGRs had adverse effects on parasitoid fitness and caused a decrease in parasitism incidence. Evidence from our and other earlier studies strongly supports the idea that exposure to PGRs via the host diet influences physiological and biochemical mechanisms and plays roles in maintaining survival activities of both pests and their natural enemies.

Many advantages of *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) caterpillars have convinced an increasing number of researchers to favor this species as a powerful, reliable, and proven model system to evaluate the effects of toxic substances on both target species and non-target beneficial ones (Sak et al., 2006; Büyükgüzel et al., 2007; Ergin et al., 2007; Uçkan et al., 2008; 2011a, b; Altuntaş et al., 2012). Larvae of the host species are serious pests in beehives. They damage the combs by boring into the hive and leaving silk-lined tunnels or galleries (Sanford 1987). The solitary idiobiont pupal endoparasitoid *P. turionellae* is utilized for biological control of a number of lepidopteran pest species involving *G. mellonella* (Kansu & Uğur 1984, Fisher, 1987). Upon paralyzation by the wasp species at the time of oviposition (Haeselbarth 1979), the paralyzed host provides food and a living space for larval parasitoids and sometimes food for the adult parasitoid (Slansky, 1986). Some host species of *P. turionellae* also feed on plants

and the adult wasps feed on plant nectar and host pupae in nature. Therefore, the accumulation of environmental pollutants and transmission of these compounds to wasps by both feeding in/on a host directly and by feeding on plant nectar, indirectly, are likely to occur (Sak et al. 2006, Ergin et al. 2007). Thus, the present work was carried out to determine IAA-induced changes in reproductive potential of first generation (F$_1$) of *G. mellonella* and egg hatching time, larval and pupal developmental time, adult emergence time, adult longevity, female sex ratio, adult weight and size of second generation (F$_2$) of *G. mellonella*, as well as immature developmental time, adult longevity, adult weight and size of *P. turionellae*.

MATERIAL AND METHODS

Parasitoid and Host Rearing

Laboratory colonies of the host, *G. mellonella* and the parasitoid, *P. turionellae* were established from adults reared at 25 ± 5°C, 60 ± 5% RH, and with a photoperiod of 12: 12 (L:D) h in our laboratory in Kocaeli University, Turkey. Larvae of *G. mellonella* were maintained by feeding the insects with a diet described by Bronskill (1961) and modified by Sak et al. (2006). A piece of honey comb was also added for egg deposition and feeding of the newly hatched larvae. *P. turionellae* were mass reared on the pupae of the host, *G. mellonella* in cages (25 x 25 x 25 cm). Adults of parasitoids were fed a 30% (wt: vol) honey solution and provided with host pupae (four pupae for every 10 female wasps once every 3 d) (Sak et al., 2006).

Bioassays

An individual mating pair of the host, 1- or 2-day-old *G. mellonella* was placed in 1-L jars containing 2 g honeycomb to provide a mating and oviposition substrate. Adults were removed from the jars on the seventh day. Newly hatched first generation (F$_1$) larvae of *G. mellonella* in

jars were exposed to 10 g of the host diet (SAK et al., 2006) treated with 50, 500, 1,000, 5,000 and 10,000 ppm IAA (Merck 10 g, Darmstadt, Germany) homogenized with doses in separate jars. Larvae reared on IAA-free diet were controls. Both experimental and control group diets were replenished daily.

Last instars of F_1 of *G. mellonella* (0.18 ± 0.02 g) were randomly selected in groups of five, transferred to sterile Petri dishes, and controlled every day until adult emergence. To determine the total number of eggs laid per female, percent fecundity, percent fertility, and corrected percent sterility of F_1 and egg hatching, larval, and pupal developmental time, adult emergence time, adult longevity, sex ratio, adult weight and size of second generation (F_2) of *G. mellonella*, an individual mating pair of F_1 adults of *G. mellonella* (n=30) was placed into 210 ml cups. A piece of paper was placed into each cup as a deposition substrate then cups were covered with gauze to allow air exchange. *G. mellonella* females were allowed to deposit eggs on papers. The papers on which eggs had been oviposited were changed daily and the number of eggs on them was counted every two days for 16 d. The data were tabulated for the total number of eggs deposited by each female, percent fecundity, and percent fertility in experimental and control groups. Corrected percent fertility was calculated according to KAUR & RUP (2002) by applying the following formula:

$$\text{Corrected percent sterility} = \frac{\% \text{ Fertility in control} - \% \text{ Fertility in treatment}}{\% \text{ Fertility in control}} \times 100$$

From each treatment and control group five F_2 eggs were randomly selected and individually transferred to sterile Petri dishes including 2 g of host diet treated with different doses of IAA and observed daily until egg hatching, and the time between egg laying and hatching recorded as egg hatching time. Diet was replenished daily until larvae pupated and the time between egg hatching and pupation recorded as larval developmental time. The pupated individuals were observed until adult emergence, and pupal

developmental times were recorded. Then, all Petri dishes were observed at 24-h intervals until all individuals died, and the time between adult emergence and death were recorded as adult longevity. Female sex ratio and adult weight of the newly emerged adults were also assessed. Adult body size of *G. mellonella* was determined by measuring the length from head to the tip of the abdomen using an Olympus SZ51 (Olympus, center Valley, PA) stereo dissecting microscope equipped with a calibrated eyepiece micrometer.

In a parallel set of experiments, upon molting to the last stage, larvae (0.18 ± 0.02 g) of *G. mellonella* F_1 were randomly selected from the jars and transferred in groups of five to sterile Petri dishes and controlled every day until larvae pupated. Then, parasitization was performed on day 1 or 2 of the host pupae by exposing an individual host pupa to an individual 10- to 20-d-old wasp female. Parasitized pupae were observed until adult emergence. The time required for completion of development from egg deposition to adult eclosion of parasitoids was recorded as immature developmental time. Newly emerged female and male parasitoid weight from each treatment and control group was recorded as adult weight. Then, individual mating pairs of five for each experimental and control group were placed in a 210 ml cup containing a cotton ball saturated in a 30% (wt: vol) honey solution. Cups were covered with a mesh cloth and food was replenished every day. Parasitoids were observed at 24-h intervals until all parasitoids died and adult longevity was recorded. Adult body size of *P. turionellae* was also determined by the method described above for host species.

Statistical Analysis

The experiments were repeated three times with specimens taken from different populations at different times. Means were compared using one-way analysis of variance (ANOVA) of SPSS V.18 for Windows. Means were subjected to Tukey's Honestly Significant Difference (HSD)

Table 1

IAA-related changes in total number of eggs/female, percent fecundity, percent fertility and corrected percent sterility of *G. mellonella*.

IAA (ppm)	Total no. of eggs/female Mean[a]± SE[b]	Percent fecundity Mean[a]± SE[b]	Percent fertility Mean[a]± SE[b]	Corrected percent sterility Mean[a]± SE[b]
0	699.53±77.98a	100.00±0.00ab	88.04±2.00a	—
50	784.67±82.85a	112.17±11.84b	92.75±0.89a	-5.35±1.01a
500	581.67±80.45a	83.15±11.50ab	89.20±1.42a	-1.32±1.61a
1,000	571.53±84.62a	89.47±12.18ab	70.07±8.18b	20.41±9.29b
5,000	465.33±74.05a	66.52±10.59a	83.25±4.01ab	5.45±4.56ab
10,000	546.87±66.14a	78.18±9.46ab	87.57±2.74a	0.53±3.12a

[a]   Means within each column followed by the same letter are not significantly different (P>0.05).
[b]   Average of 15 individuals per treatment.

RESULTS

test when variances were homogenous, but Tamhane T2 tests otherwise were used to assess the significance of the effects of IAA doses (P< 0.05).

The total number of eggs laid by a single host female fed on IAA-free diet was 699.53 ± 77.98. Treatment of IAA did not considerably affect the number of eggs laid by *G. mellonella* regardless of the dose tested (F = 2.164; df = 5, 84; P = 0.066). The number of eggs laid per female did decrease at all dose levels except 50 ppm, however the differences were not significant with respect to controls (Table 1). The percent fecundity of the control group was assumed as 100% in order to determine the relative percent fecundity of assays. The lack of a decrease in total number of eggs at 50 ppm of IAA treatment also led to an increase in percent fecundity and fertility where fecundity was increased to 112.17±11.84% with 92.75±0.89% fertility at the lowest concentration. This trend was not the same for other dose treatments with a fluctuation among doses, reduced significantly at only 5,000 ppm for percent fecundity (F = 2.528; df = 4, 70; P = 0.035) (Table 1). Percent fertility decreased significantly only at 1,000 ppm (F = 3.940; df = 5, 84; P = 0.003) with a considerable increase in corrected percent sterility (F = 4.146; df: 4, 70; P = 0.005) reaching 20.41±9.29% when compared to other doses (Table 1).

Exposure to IAA in host diet resulted in an increase in egg hatching time of F$_2$ females of *G. mellonella* only at 1,000 and 10,000 ppm doses (F = 19.731; df = 5, 264; P = 0.00) (Table 2). Similarly, larval developmental time increased (F = 29.916; df = 5, 264; P = 0.00) at 500 and 5,000 ppm with respect to control (Table 2). However, pupal developmental time of *G. mellonella* decreased (F = 86.128; df = 5, 264; P = 0.00) by >47% at 1,000 and 10,000 ppm (Table 2). On other hand, adult emergence time fluctuated among treatments (F = 48.676; df = 5, 264; P = 0.00) and significantly decreased at 1,000 and 10,000 ppm and increased at 50 and 500 ppm with respect to controls (Table 2).

TABLE 2

IAA-related changes in egg hatching, larval, pupal developmental and adult emergence times (day) of *G. mellonella*.

IAA (ppm)	Egg hatching time Mean[a]± SE[b]	Larval developmental time Mean[a]± SE[b]	Pupal developmental time Mean[a]± SE[b]	Adult emergence time Mean[a]± SE[b]
0	10.16±0.12a	44.13±0.43a	13.18±0.27a	67.47±0.40b
50	9.96±0.07a	45.18±0.20ab	14.78±0.79a	69.91±0.74c
500	10.16±0.05a	46.93±0.20c	13.31±0.26a	70.40±0.30c
1,000	12.84±0.68b	43.00±0.30a	6.80±0.29b	62.78±0.87a
5,000	10.20±0.08a	45.62±0.23b	13.22±0.29a	69.04±0.32bc
10,000	12.27±0.10b	43.27±0.22a	6.36±0.16b	61.89±0.15a

[a] Means within each column followed by the same letter are not significantly different (P>0.05).
[b] Average of 45 individuals per treatment.

IAA-treated *G. mellonella* F$_2$ adults lived for shorter times than controls at all doses tested, however this decline in longevity of adults was not significant (F = 2.196; df = 5, 264; P = 0.055). This trend was also similar in the female sex ratio of adults, showing variations among doses but the ratio did not differ significantly (F = 2.137; df = 5, 12; P = 0.130) upon exposure to different doses of IAA (Table 3). On the other hand, adult weight of *G. mellonella* significantly decreased (F = 4.538; df = 5, 264; P = 0.001) at 500 ppm with respect to controls (Table 3). Adult size of *G. mellonella* did not differ significantly when compared to controls, but it did increase at 10,000 ppm with respect to 500 and 5,000 ppm (F = 3.610; df= 5, 264; P = 0.004) (Table 3).

Immature developmental time of *P. turionellae* females reared on *G. mellonella* pupae exposed to different doses of IAA was not significantly different to that of female parasitoids reared on untreated hosts (F = 1.418; df = 5, 174; P = 0.198) (Table 4). However, males at 1,000 ppm (F = 4.472; df = 5, 174; P = 0.001) completed their immature development later than did those in control and other experimental groups. Wasp

development from egg to adult at 25°C normally required 13-23 d in the control group. Treatment with IAA increased immature developmental time of parasitoids, especially at doses >500 ppm (F = 4.259; df = 5, 354; P = 0.001) and parasitoids reared on hosts exposed to 1,000 ppm IAA emerged 2-4 d later than did controls (Table 4).

The mean longevity of IAA-treated adults decreased significantly at doses of 1,000 and 10,000 ppm (F = 9.292; df = 5, 354; P = 0.000) compared with lower doses tested and wasps reared on untreated hosts. Adults lived 30% and 27% shorter lives than did controls at doses of 1,000 and 10,000 ppm, respectively (Table 4). All IAA-treated females except for those at 500 ppm lived shorter lives than did controls, however there was only a considerable decline in the longevity of females (F = 8.901; df = 5, 174; P = 0.001) at 1,000 and 10,000 ppm. The longevity of females declined by 28 and 33%, respectively. Male longevity fluctuated among doses (F = 4.160; df = 5, 174; P = 0.001) with a significant decrease only at 1,000 ppm such that males lived 33% shorter lives than did controls (Table 4).

Table 3

IAA-related changes in adult longevity, female sex ratio, adult weight and size of *G. mellonella.*

IAA (ppm)	Adult longevity (day) Mean[a]± SE[b]	Female sex ratio (%) Mean[a]± SE[b]	Adult weight (g) Mean[a]± SE[b]	Adult size (mm) Mean[a]± SE[b]
0	22.49±1.29a	86.66±6.67a	0.09±0.00a	12.32±0.19ab
50	18.73±0.96a	93.33±6.67a	0.09±0.00a	12.39±0.17ab
500	19.42±0.89a	88.89±5.88a	0.07±0.00b	11.93±0.18a
1,000	20.38±0.93a	68.89±8.01a	0.08±0.00a	12.61±0.20ab
5,000	22.07±1.13a	84.44±4.44a	0.08±0.00ab	12.10±0.17a
10,000	18.73±1.39a	73.33±6.67a	0.09±0.00a	12.88±0.16b

[a] Means within each column followed by the same letter are not significantly different (P>0.05).
[b] Average of 45 individuals per treatment.

Treatment with IAA did not affect the adult weight of males (F = 1.073; df = 5, 174; P = 0.377) and both sexes combined (F = 1.177; df = 5, 354; P = 0.177) when compared to controls. However, there was only a significant decrease in female weight at 5,000 ppm (F = 2.676; df = 5, 174; P = 0.023) when compared to 500 and 10,000 ppm (Table 5).

Adult wasp females reared on IAA-treated hosts did not differ in length (F = 1.160; df = 5, 174; P = 0.331), while there were only significant

Table 4

IAA-related changes in immature developmental time and adult longevity of *P. turionellae.*

IAA(ppm)	Immature Developmental Time (day)			Adult Longevity (day)		
	Female Mean[a]± SE[b]	Male Mean[a]± SE[b]	Both sexes Mean[c]± SE[b]	Female Mean[a]± SE[b]	Male Mean[a]± SE[b]	Both sexes Mean[c]± SE[b]
0	19.50± 0.31a	17.17 ±0.43a	18.33±0.30a	56.20±3.10ab	42.10±2.24b	49.15±2.11a
50	19.50±0.27a	17.97±0.30ab	18.73±0.22ab	55.60±2.88ab	38.53±2.78ab	47.07±2.27a
500	18.90±0.27a	17.60±0.18a	18.25± 0.18a	63.33±4.72ab	41.20±3.53b	52.27±3.26a
1,000	19.93±0.30a	19.13±0.38b	19.53± 0.25b	40.70±2.59cd	28.03±2.09a	34.37±1.85b
5,000	19.80±0.35a	18.53±0.36ab	19.17± 0.26ab	54.17±3.99bc	33.67±2.88ab	43.92±2.78ab
10,000	19.50± 0.31a	17.17 ±0.43ab	19.05± 0.22ab	37.73±2.87d	33.47±2.06ab	35.60±1.77b

[a] Means within each column followed by the same letter are not significantly different (P>0.05).
[b] Average of 30 individuals per treatment.
[c] Average of 60 individuals per treatment.

Table 5

IAA-related changes in adult weight and adult size of *P. turionellae.*

IAA (ppm)	Adult weight (g)			Adult size (mm)		
	Female	Male	Both sexes	Female	Male	Both sexes
	Mean[a]± SE[b]	Mean[a]±SE[b]	Mean[c]±SE[b]	Mean[a]± SE[b]	Mean[a]± SE[b]	Mean[c]± SE[b]
0	0.03±0.00ab	0.02±0.00a	0.02±0.00a	10.82±0.18a	9.87±0.15ab	10.36±0.13ab
50	0.03±0.00ab	0.02±0.00a	0.02±0.00a	10.52±0.15a	9.85±0.22ab	10.19±0.14ab
500	0.03±0.00b	0.02±0.00a	0.03±0.00a	11.00±0.15a	10.35±0.19b	10.68±0.13b
1,000	0.03±0.00ab	0.02±0.00a	0.02±0.00a	10.602± 0.18a	9.42±0.19a	10.01±0.15a
5,000	0.02±0.00a	0.02±0.00a	0.02±0.00a	10.60±0.19a	10.15±0.20ab	10.38±0.14ab
10,000	0.03±0.00b	0.02±0.00a	0.02±0.00a	10.75±0.15a	9.58±0.20ab	10.17±0.14a

[a] Means within each column followed by the same letter are not significantly different ($P>0.05$).
[b] Average of 30 individuals per treatment.
[c] Average of 60 individuals per treatment.

variations among doses in males (F = 3.183; df = 5, 174; P = 0.000) and both sexes combined (F = 2.724; df = 5, 354; P = 0.020). However, IAA treatment also did not affect the size of males and both sexes combined when compared to that of controls (Table 5).

DISCUSSION

Previous studies have demonstrated that different PGRs influence the well-being of various insects differently, including pests (KAUR & RUP, 2002; 2003a; 2003b; HARIKESH & BHATTACHARYA, 2003; GUPTA et al. 2009) and those species that are beneficial (UÇKAN et al., 2008; UÇKAN et al., 2011a; b), and are considered as ecological life vests (UÇKAN & GÜLEL, 2002). Here, we first attempted to explore the effects of IAA on the reproductive potential of the pest species, *G. mellonella*. Analysis of the data for the total number of eggs laid per female revealed that treatment with different IAA doses did not affect the reproductive potential of the pest species. Percent fecundity increased by 12%

at 50 ppm whereas the most effective decrease by >33% was observed at 5,000 ppm. However, both results were not significantly different from that observed in controls. Percent fertility significantly decreased by >20% at 1,000 ppm. Compatible with percent fertility, all treatments displayed ±5% variations when compared to controls except for a significant increase in corrected percent sterility by >20% at 1,000 ppm.

It is likely that IAA had a low level of adverse activity on the reproductive potential of *G. mellonella*. Similar responses have also been observed in *B. cucurbitae* (KAUR & RUP, 2002) *Dacus dorsalis* (Hendel, 1912) (THAKUR & KUMAR, 1984), *Lipaphis erysimi* (Kaltenbach, 1843) (RUP & DHILLON, 1999) *and Zaprionus paravittiger* (Godbole & Vaidya, 1972) (RUP et al., 1997). Researchers suggested that the impact of IAA may be correlated to its interference with the neurosecretory system, which may affect the reproductive system (THAKUR & KUMAR 1984). Our observations may suggest that IAA does not have a lethal effect toward developing pest progeny across trophic levels.

We previously demonstrated GA$_3$ had consistent negative effects on the pre-adult developmental time of *G. mellonella* with >35% reduction in overall time to adult eclosion largely due to the decrease in egg-larval developmental time at the highest dose of 5,000 ppm tested (UÇKAN et al., 2011b). However, this study with the same pest species did not find a considerable effect of IAA on the egg hatching, larval, pupal developmental and adult emergence times from treatments; rather, only slight increases in egg hatching and larval developmental times, decreases in pupal developmental time, and both in adult emergence time were recorded at some doses tested. The most striking effect observed was a decline in pupal developmental time of *G. mellonella* by >47% at 1,000 and 10,000 ppm. GUPTA et al. (2009) reported that GA$_3$ and siapton caused an increase in the larval period of the hairy caterpillar, *S. oblique* at high doses. Furthermore, KAUR & RUP (1999) observed parallel responses in *B. cucurbitae* by using GA$_3$. However, GUPTA et al. (2009) reported that triacontanal (a saturated long chain alcohol that is known to have a growth promoting activity) did not cause any significant difference in larval or pupal period at any dose tested. Thus, the effects of plant growth regulators on insect pests are variable.

We have previously suggested that GA$_3$ could be used as an insecticide against *G. mellonella* since treatment with GA$_3$ in the diet resulted in a significant influence on the immature developmental time of the pest larva, which is considered the most damaging stage of the pest species (Uçkan et al., 2011b). Further evidence from that study that GA$_3$ did not affect the egg-to-adult developmental time of parasitoids also supports the assumption that GA$_3$ would be a successful chemosterilant against pest species. However, in the present study, IAA had a relatively low level of adverse activity on the reproductive potential and immature developmental times and almost no effect was observed on adult longevity and female sex ratio of the pest species. The differences in adult size and weight were also significant at only some doses tested. On the other hand, we observed that IAA treatment caused a slight prolongation in adult emergence and decrease in adult longevity of parasitoids reared on hosts exposed to different doses; longevity of wasps declined by >27% at higher doses. Our previous work also demonstrated that the egg to adult developmental time of the wasp *A. galleriae* reared on *A. grisella* larvae exposed to high doses (≥ 200 ppm) of GA$_3$ increased by 40% (UÇKAN et al., 2008), and increased by 30% when wasps were reared on larvae exposed to ≥ 500 ppm of IAA (UÇKAN et al., 2011a). Similar responses in the adult longevity of parasitoid wasps have also been observed as a decrease by >50% when wasps were reared on hosts exposed to high doses of IAA and GA$_3$ treatments (UÇKAN et al., 2008, 2011a). KAUR & KAUR (2013) also observed decreased female life span and extended developmental periods of the braconid wasp *B. hebetor* reared on *S. litura* exposed to high doses of coumarin. These results are expected, because previous studies displayed that PGRs reduce the total lipid and carbohydrate levels in insects (RAUP et al., 1998; KAUR & RUP 2003b). We have observed that lipid at all doses, and carbohydrate at most of the doses, decreased in the hemolymph of *G. mellonella* larvae upon exposure to GA$_3$ (UÇKAN et al., 2011b). Recently, we also found that GA$_3$ resulted in different effects on the quantity of free amino acids associated with energy metabolism of *G. mellonella* and *P. turionellae* (ALTUNTAŞ et al., 2014). It is known that stress responses in arthropods are energetically demanding events (KORSLOOT et al., 2004) and RUP et al. (2000, 2002) suggested that PGRs-induced stress may cause decreases in hemolymph components. It is likely that the decrease in energy reserves of the host resulting from IAA-induced stress may cause delay in the immature growth and development and a decline in adult longevity of parasitoid species (UÇKAN & ERGIN 2002; UÇKAN et al., 2007, 2008). Thus, it may be concluded that PGRs influence the life history parameters of various insects differently, depending on a number of factors. In the present case, our data displayed no severe adverse effects on the reproductive potential, development and longevity of pest species, whereas interferences in development

and longevity of wasps were abundant at high doses of IAA treatment. Further effects of IAA should be explored for its overall influence on pest status before proposing this plant growth factor as an environmentally-safe compound for use in the management of lepidopteran pest species. The authors are currently attempting to evaluate the effects of IAA on the hemolytic and phenoloxidase activity, hormones controlling insect development and metamorphosis, and antioxidant enzymes detoxifying free radicals of the pest species *G. mellonella*. In addition, investigation of how the same parameters are affected by IAA via host feeding for *P. turionellae* is with in the scope of new research.

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Early colonization on Artificial Seagrass Units and on *Posidonia oceanica* (L.) Delile leaves

Dorothée C. Pête[1],*, Gilles Lepoint[1], Jean-Marie Bouquegneau[1] & Sylvie Gobert[1]

[1] Laboratory of Oceanology, MARE Center, University of Liège, Institut de Chimie, B6C, Allée du 6 août 13, 4000 Liège, Belgium.

* Corresponding author: Dorothee.Pete@ulg.ac.be

ABSTRACT. Many epiphytes grow on *Posidonia oceanica* (L.) Delile leaves but early stages of that colonization are not well known. To study this early colonization without destroying the plant, Artificial Seagrass Units (ASUs) were utilised. The first nine days of colonization by macroscopic eukaryotic organisms on natural *P. oceanica* leaves and on ASUs were studied. The capability of those ASUs to mimic *P. oceanica* in the long term was also evaluated. Indeed, early colonists of a substrate can influence the settling of later ones by "priority effects". Thus if the pioneer community is the same on both substrates, they will more likely be the same after a longer exposure time. On both substrates, colonization began by the settling of crustose-calcareous algae and foraminiferans. The number of organisms increased more quickly on ASUs than on natural leaves but Shannon-Wiener diversity index was higher for *P. oceanica* leaves. The low colonization rate on natural leaves may have been due to different microclimatic conditions on the two substrates and to a less developed biofilm than on ASUs. The high diversity observed on natural leaves was mainly related to the presence of bryozoan ancestrulae, which were absent on ASUs. Different microhabitats on each substrate (different algae morphotypes) can explain this difference. Thus, at such an early colonization stage, pioneer communities were different on the two substrates, suggesting that later communities would be different too. However, ASUs could be used in environmental perturbation studies instead of natural leaves, thanks to their high colonization rate.

KEY WORDS: seagrass, artificial substrata, epiphytes, colonization, substrate preferences.

INTRODUCTION

In the Mediterranean coastal zone, the seagrass *Posidonia oceanica* (L.) Delile forms dense meadows. The leaves of this seagrass are long (up to 130 cm) and have the longest life span among seagrasses (up to 56 weeks) (THELIN & BOUDOURESQUE, 1983; GOBERT et al., 2005). Thus, they can support an important epiphytic community that is composed of many organisms such as bacteria, diatoms, dinoflagellates, red and brown macroalgae, protists, sponges, bryozoans, hydrozoans and annelids (KERNEIS, 1960; MAZZELLA et al., 1981; CASOLA et al., 1987; MAZZELLA et al., 1989; JACQUEMART & DEMOULIN, 2006; MABROUK et al., 2011; LEPOINT et al., 2014). In total they can represent up to 40 % of the leaf biomass (GOBERT et al., 1995) and their density increases with leaf age (NOVAK, 1984). *P. oceanica* leaves grow from a basal meristem. Youngest parts of the plant are situated at the basis of the leaves and in the centre of the shoots. They grow from the centre to the external part of the shoot, tightly packed with older leaves. Epiphytes are thus more abundant on the outer leaves of the shoots than on the inner ones and on the leaf tips than on the bases (VAN DER BEN, 1971; CASOLA et al., 1987). On the young parts of the plant, the epiphyte cover is mainly constituted of bacteria and diatoms that form a biofilm (NOVAK, 1984). Biofilms are important in marine environments because they condition the settling of organisms on immerged substrates (CRISP & RYLAND, 1960; KEOUGH & RAIMONDI, 1995; DE TROCH et al., 2005). On older parts of *P. oceanica*, macroepiphytes become dominant, even if they can be secondarily colonized by bacteria or other micro-organisms (NOVAK, 1984).

Epiphytes also contribute to the food webs associated with the *P. oceanica* ecosystems (MAZZELLA et al., 1992; LEPOINT et al., 2000; MICHEL, 2011). Their biomass, biodiversity, abundance and/or coverage are used in pollution studies because they react more quickly to environmental perturbations than does the plant itself, thanks to their rapid turnover and their high nutrient uptake rates (MORRI, 1991; PERGENT-MARTINI et al., 2005; LEPOINT et al., 2007; GOBERT et al., 2009).

The daily evolution of early epiphyte settling on *P. oceanica* leaves is not known, although it can influence subsequent epiphytic assemblages. Indeed, according to the theory of CONNELL & SLATYER (1977), the first colonists of a substrate influence later colonists by facilitating or inhibiting their settling and persistence. First colonizers are able to influence the recruitment of other organisms by creating some specific conditions in the environment or by limiting the possible interspecific interactions with newly-arriving species (BOLOGNA & HECK, 1999; IRVING et al., 2007). These phenomena are called "priority effects" (BELYEA & LANCASTER, 1999). The identification of early colonizers is thus essential to understanding the dynamics of the epiphytic community living on *P. oceanica* leaves.

Artificial Seagrass Units (ASUs) are interesting in the study of the epiphyte colonization of seagrass leaves because they can be set up anywhere, regardless of conditions (BARBER et al., 1979; LEE et al., 2001) and they allow the control of parameters such as leaf number, shoot density, leaf length and width, as well as the surface available for colonization (BOLOGNA & HECK, 1999; TRAUTMAN & BOROWITZKA, 1999). Moreover, they represent a non-destructive method, which is particularly important when working with protected species such as *P. oceanica* (DONNARUMMA et al., 2014).

The aims of this study were to identify the first sessile macroscopic eukaryotic organisms settling on the basal parts of *P. oceanica* leaves and ASUs, day by day during nine days, and then to determine if those ASUs are able to mimic natural leaves, in order to use them instead of the natural plant to study the colonization of *P. oceanica* by epiphytes or to detect environmental perturbations. Indeed, if the pioneer community composition and abundances are the same on ASUs as on *P. oceanica*, subsequent colonists will more likely be the same on both substrates too (CONNELL & SLATYER, 1977; IRVING et al., 2007; MAGGI et al., 2011).

MATERIALS AND METHODS

This study was realised from March 5th to March 14th 2005, at a depth of 10 m, in front
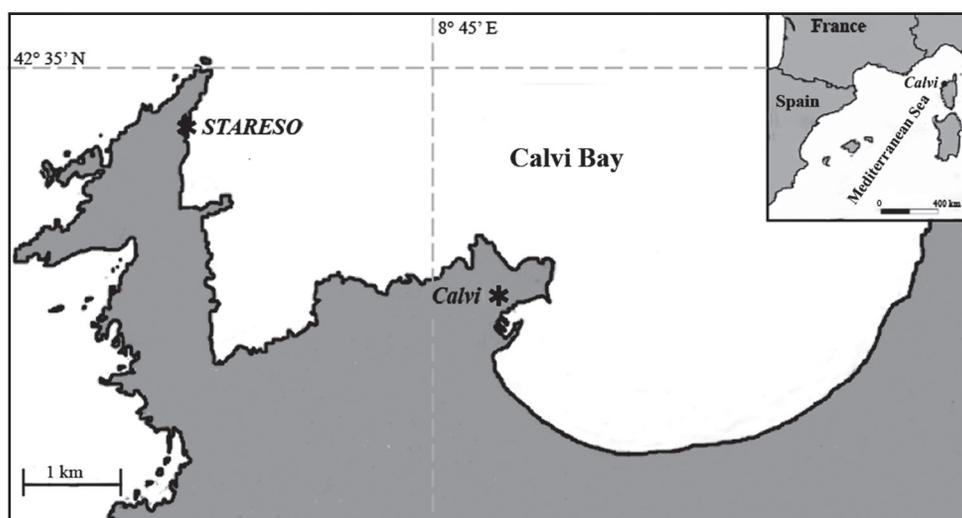

Fig. 1 – Sampling site. The sampling site is situated in front of the research station STARESO, Corsica, France, in the *P. oceanica* meadow, at a depth of 10 m.

of the STARESO research station (Fig.1, Calvi Bay, Corsica, France, 42°35' N, 8°43' E). This site is characterized by a continuous *P. oceanica* meadow where the shoot density varies from 400 to 700 shoots.m^{-2} at a depth of 10 m (SOULLARD et al., 1994).

Every Artificial Seagrass Unit (ASU, Fig. 2) was made of a black PVC ribbon (width = 1 cm, length = 50 cm, thickness = 0.5 mm) with a float (Eppendorf tube) at the free end to maintain those ASUs in the same position as natural leaves. They were fixed on a ballasted PVC tube and the whole structure was put inside the meadow. At the same time (day 0 of the experiment), ten *P. oceanica* shoots were marked with the punching hole method (ZIEMAN & WETZEL, 1980) to assure that both substrates had been exposed to the same pool of colonists during the same period. Indeed, as *P. oceanica* leaves grow from a meristem situated in the centre of the shoot, it was assumed that the zone of the youngest marked leaf (GIRAUD, 1979) situated under the hole experienced the same exposure time as the ASUs. As the youngest parts of *P. oceanica* leaves are wrapped in the sheaths of old leaves, it was impossible to define more precisely this newly exposed zone.

Every day during nine days (after day 0), one ASU and one marked natural shoot were picked up by scuba diving. The youngest punched leaf of each natural shoot was selected (GIRAUD, 1979) and the inner and outer surfaces of the freshly exposed zone were observed (0 to 4.2 cm^2) with a magnification of fifty times, to determine the density of sessile macroscopic eukaryotic organisms. To compare with natural leaves, only the basal parts (0-5 cm) of ASUs were observed.

Organisms were determined at phylum level. To compare the contribution of each phylum on both substrates, relative abundances (percentage of the total number of organisms in each sample) were calculated. Sample diversity was also estimated using Shannon-Wiener diversity index, calculated with a DIVERSE analysis in PRIMER 6 (CLARKE & WARWICK, 2001).

A preliminary study was carried out on the samples collected after four days of exposure using scanning electron microscopy (SEM), in order to illustrate some epiphytes and the development of the biofilm on every substrate. A portion of 0.5 cm^2 was selected at the bases of natural and artificial leaves and fixed in 4% seawater glutaraldehyde for a few days, at 4°C.
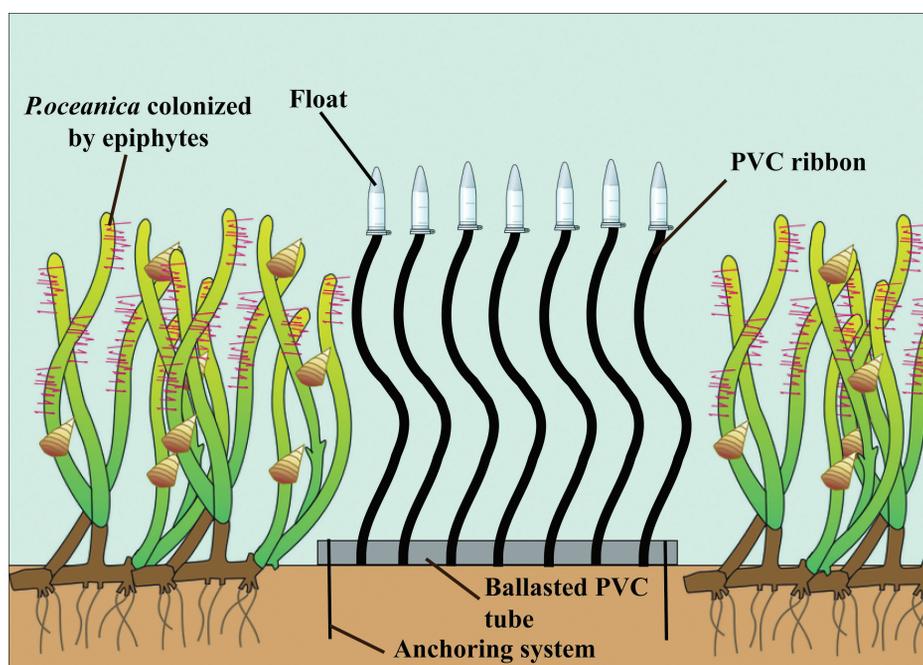

Fig. 2 – Experimental device for the Artificial Seagrass Units (symbols used with the courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/symbols/)).

TABLE 1

Shannon-Wiener diversity index (H') calculated for each sampling day from phylum abundances on ASUs and *P. oceanica* natural leaves (N.D.: no data).

Duration of the colonization (days)	1	2	3	4	5	6	7	8	9
ASUs	/	0.26	0.17	0.29	0.12	0.56	0.60	N.D.	0.68
Natural leaves	/	0.00	0.00	0.64	0.64	N.D.	0.67	N.D.	0.92

They were then rinsed in seawater filtered on a 0.22 μm filter, post-fixed in 1% seawater OsO_4, rinsed again in seawater and critical point dried. They were mounted on aluminium stubs and coated with a thin layer of gold-palladium for the SEM observation. It is worth mentioning that the use of distilled water in the preparation of SEM samples of both studied substrates seemed to wash out most of the organisms. It should therefore be avoided.

RESULTS

On both substrates (Fig. 3), the first sessile macroscopic eukaryotic organisms settled after 2 days. The number of colonists increased with time but this increase was more important for

Fig. 3 – Evolution of epiphyte density (organisms.cm⁻²) over time on the bases of natural leaves (triangles) and ASUs (squares).

ASUs. Indeed, the colonist density was only 29 organisms per square centimetre (org.cm⁻²) on *P. oceanica* leaves after nine days while it reached 355 org.cm⁻² on ASUs. Except for the third sampling day, the total abundance was always 3 to 12 times higher on ASUs than on natural leaves.

Shannon-Wiener diversity index (H', Table 1) increased throughout the experiment for natural leaves while this increase was only clear after 5 days of exposure for ASUs. From the fourth sampling day, H' was always higher for the basal parts of natural leaves than for those of ASUs.

After only 4 days (Table 1, Fig 4), sessile communities colonizing natural leaves were more diverse than those settling on ASUs. This fact is also clear when looking at phyla relative abundances. On both substrates, the observed phyla were macroalgae, ciliates, foraminiferans and bryozoans, but their relative abundances (Fig. 4) were different.

On natural leaves (Fig. 4A), the community showed a dominance of algae (20 % after 5 days to 100 % after 3 days) and foraminiferans (15 % after 7 days to 100 % after 2 days). After 7 days, bryozoans became important too (5-6 %).

On ASUs, the dominant phylum, before 6 days of exposure, was algae (Fig. 4B, between 85 % after 4 days and 97 % after 5 days). From the 6th sampling day to the end of the experiment, there was a switch between algae (39 % after 9 days) and foraminiferans (61 % after 9 days).
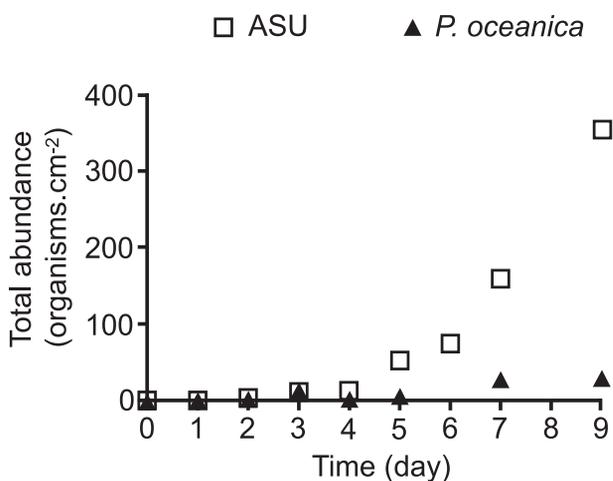
Whenever other phyla were observed, they represented less than 2 % of the total abundance on both substrates.

DISCUSSION

Epiphyte biomass on *P. oceanica* leaves increases with leaf age and follows a sigmoid distribution (CEBRIÁN et al., 1999). The stabilization of the curve is reached when there is a balance between epiphyte growth and losses, due, for example, to grazing pressure (VAN MONTFRANS et al., 1984; BORUM, 1987; ALCOVERRO et al., 1997). In this study, the obtained curves (Fig. 3) are in the exponential part of the sigmoid curve because of the shortness of the experiment (BORUM 1987). Indeed, the exposure time here is 9 days while CEBRIÁN et al. (1999) have shown that epiphyte biomass on *P. oceanica* leaves reaches the upper part of the sigmoid distribution after 270 days.

The curve for ASUs showed a highly increasing density of organisms towards the end of the experiment, suggesting that colonization was in an active phase. This rapid increase in colonist density may be due to a "snowball effect". Indeed, it was proven that colonization modifies the leaf surface and favours the recruitment of new colonizers (NOVAK, 1984; BOLOGNA & HECK, 1999; IRVING et al., 2007). So, in the early phase of colonization, if more organisms settle on a substrate, the colonization by new organisms is favoured.

In comparison with ASUs, the increase in epiphyte density on *P. oceanica* leaves was low, although the Shannon-Wiener diversity index was higher. These observations are in accordance with those of other authors comparing ASUs with aquatic macrophytes (CATTANEO & KALFF, 1978; NOVAK, 1984; EDGAR, 1991). The difference between both types of substrates can be explained by the existence of a longer lag phase before active epiphyte colonization for *P. oceanica* than for ASUs. This lag phase is the period needed by a substrate to be ready to be colonized. It depends on bacterial communities living on the leaves and on the maturity of the biofilm, which grows importantly during the early stages of colonization (KEVERN et al., 1966; NOVAK, 1984; KEOUGH & RAIMONDI, 1995, 1996; DAHMS et al., 2004). SEM observations of samples of each substrate, after 4 days of exposure, confirmed that a more complex biofilm was present on ASUs than on natural leaves (Fig. 5A–B).

Another reason for a lower colonization rate of natural leaves in comparison with ASUs is the production of secondary metabolites, such as phenolic compounds, by the plant. Those chemicals can influence the biofilm and the epiphytic cover that settle on seagrasses by acting as an antifouling agent or by selecting species adapted to resist those compounds (HARRISON & CHAN, 1980; NOVAK, 1984; JENSEN et al., 1998). For *P. oceanica*, the production of phenolic compounds presents a seasonal pattern, depends on environmental stress (competition, sewage…) and is higher in intermediate leaves than in adult
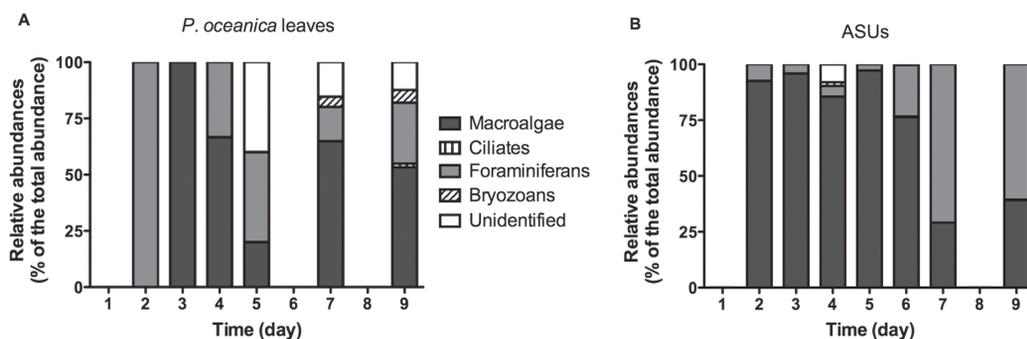

Fig. 4 – Evolution of relative abundances of the observed phyla (% of the total abundance) over time for natural leaves (**A**) and ASUs (**B**). Unidentified: phyla unidentified at the used magnification level.

ones (CUNY et al., 1995; AGOSTINI et al., 1998; DUMAY et al., 2004). As this study focused on the bases of intermediate *P. oceanica* leaves, the high production of phenolic compounds in those leaves could have influenced the epiphytic cover, either directly or by modifying the biofilm composition.

The difference in microclimatic conditions on the surface of the two substrates could be responsible for the observed differences too. Natural leaves are slightly curved, so the water circulation and the light conditions on their surfaces are probably different to those existing on the completely flat ASUs. The dominant bryozoan on *P. oceanica* leaves, an obligate epiphyte of this plant, *Electra posidoniae*, is

almost entirely restricted to the plant internal curved face (MATRICARDI et al., 1991). So, the scarcity of bryozoans on ASUs and particularly of *E. posidoniae*, also observed by GAMBI et al. (2011) and DONNARUMMA et al. (2014), is probably due to the flatness of ASUs as well as to the absence of the chemicals cited above.

Different microclimatic conditions can also be created on a substrate by the existence of different microhabitat structures that can influence the colonization by micro- or macro-organisms (NOVAK, 1984; GARTNER et al., 2013). According to CASOLA et al. (1987), after the biofilm development, the epiphytic community succession on *P. oceanica* leaves is characterized by, at first crustose-calcareous
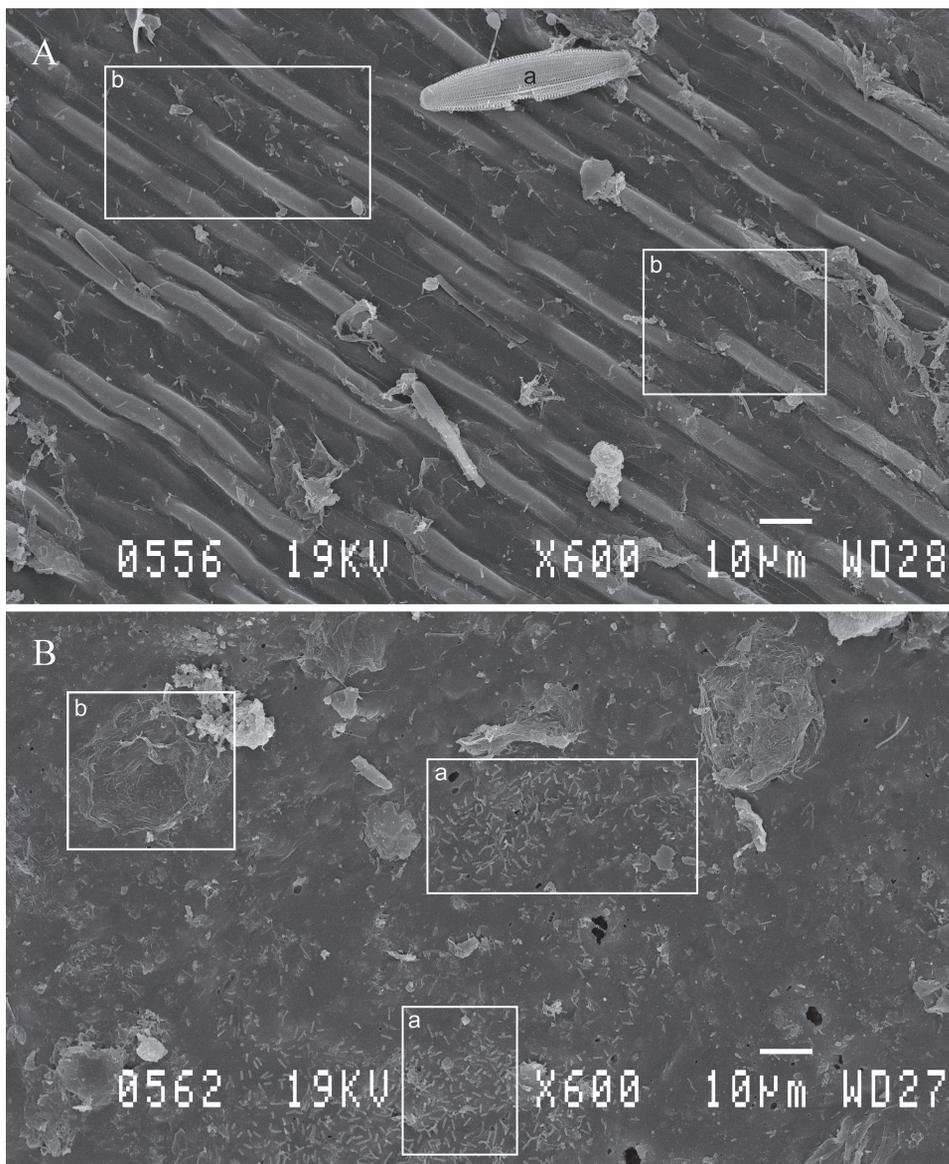
Fig. 5 – SEM pictures (magnification: 600x) of the basal portions of the *P. oceanica* leaf (**A**) and the ASU (**B**), showing the difference in microhabitat structure between the two substrates after 4 days of exposure. **A**. a = diatom, b = bacterial biofilm in formation. **B**. a = developed bacterial biofilm, b = biofilm agglutinated by mucus.

forms (foraminiferans, bryozoans, crustose-calcareous algae), then crustose-noncalcareous forms and finally erect forms. So the community goes from a simple habitat structure (i. e. mono-layered biofilm) to a more complex one (i. e. multi-layered structure). Here, the morphology of the algal community was different between the two substrates (Fig. 6). On natural leaves, the classical succession was observed. Crustose-noncalcareous algae became important after 7 days of exposure and some filamentous and erect algae were observed too. On ASUs, crustose-calcareous algae were dominant throughout the exposure period. Thus, as the microhabitat structures and the microclimatic conditions were different on both substrates, the observed communities were different too.

The dominance of crustose-calcareous algae and the scarcity of sessile fauna on artificial *Posidonia* leaves have also been observed by MICHEL (2011) and DONNARUMMA et al. (2014), after respectively 76 days and three months of *in situ* exposure. This suggests that the community structure that is observed here on ASUs is already representative of the later communities. That also underlines the role of microhabitat structure and microclimatic conditions in the establishment of colonists on immersed substrates.

Leaf movements can also influence algal epiphyte biomass (LAVERY et al., 2007). Here, every ASU was formed of a single PVC blade ending with a float. Thus, each ASU probably moved more in the water column than would a natural leaf, especially an intermediate one,

which is tightly packed with the other leaves of the seagrass shoot. However, the high density of epiphytes observed on ASUs is in contradiction with results of LAVERY et al. (2007), suggesting that movement was not a limiting factor in our experimental design.

However, the experimental protocol can explain the low colonization rate observed on natural leaves in comparison with ASUs. The very young parts of *P. oceanica* studied here were situated in the centre of the tuft formed by the leaves. In that zone, leaves are tightly arranged side by side so the leaf surface is less accessible for epiphyte propagules or larvae than on ASUs that are not organised in tufts. This low epiphytic cover of the youngest and basal parts of *P. oceanica* shoots has already been observed by CASOLA et al. (1987). It explains only the difference in colonization rate between the two substrates but not the difference in community structures. Indeed, the classical succession on *P. oceanica* leaves was identified on natural leaves after only nine days of exposure while the community structure on ASUs was different (see above).

CONCLUSIONS

At the end of this study, we can hypothesize that the community present on ASUs after a few months inside the meadow would be different to the one on natural leaves, because the first colonization stages are not the same. Despite the absence of replication and the shortness of this study (nine days of exposure), first steps of
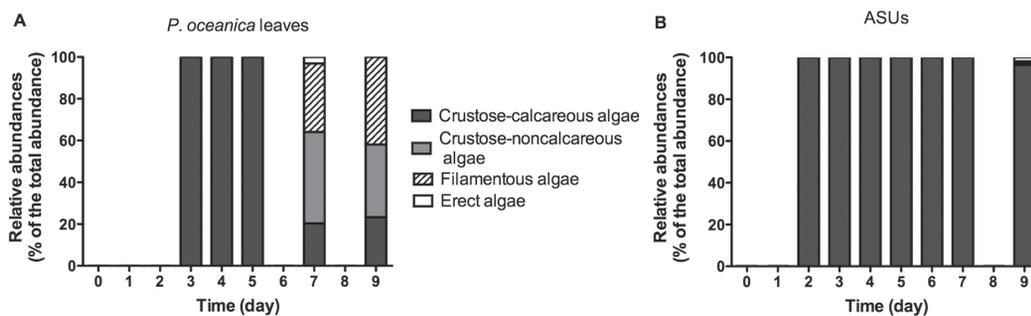

Fig. 6 – Evolution of relative abundances (% of the total abundance of algae) of the different morphological groups of algae observed on the bases of natural leaves (A) and ASUs (B) over time.

the classical epiphyte succession on *P. oceanica* leaves (Casola et al., 1987) were observed, while the community on ASUs was different. Moreover, dominant (crustose calcareous algae) and absent (bryozoans) groups observed on ASUs in this study were the same as those observed by other authors after a longer exposure time: Michel (2011), 76 days, same ASUs as here; Donnarumma et al. (2014), 3 months, different ASUs), suggesting that our results and conclusions are reliable. However, the same study should be performed with more replicates in order to strengthen these conclusions. It would also be interesting to follow the settling of colonists on a longer time scale and to use SEM to study quantitatively the composition and evolution of the biofilm.

ASUs could, however, be useful for the study of environmental perturbations without the use of *P. oceanica*, thanks to the rapidity of their colonization. In that case, further experiments should first characterize the communities present on ASUs in pristine and perturbed locations.

ACKNOWLEDGEMENTS

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Strategies of food detection in a captive cathemeral lemur, *Eulemur rubriventer*

Mats Perrenoud[1], Anthony Herrel[1], Antony Borel[2,3] & Emmanuelle Pouydebat[1,*]

[1] UMR 7179 C.N.R.S/M.N.H.N., Département d'Ecologie et de Gestion de la Biodiversité, 57 rue Cuvier, Case postale 55, 75231, Paris Cedex 5, France.
[2] Institute of Archaeological Sciences, Eötvös Loránd University, Budapest, Hungary.
[3] Department of Prehistory, UMR 7194-CNRS/ National Museum of Natural History, Paris, France.

[*] Corresponding author: epouydebat@mnhn.fr

ABSTRACT. The senses involved in food detection in primates in general, and lemurs in particular, remain poorly investigated. However, as lemurs include diurnal, nocturnal, and cathemeral species they represent a good model to test whether prey detection is dependent on activity pattern. As both diurnal and nocturnal species have been investigated previously we here aim to quantify the relative importance of different sensory modalities during prey detection in a cathemeral species, the red-bellied lemur (*Eulemeur rubriventer*). A series of experiments was performed using a group of four *Eulemur rubriventer* (Zoo de La Londe les Maures, France) to test the role of visual, olfactory and acoustic cues in prey detection. Both unimodal and multimodal cues were tested. The responses obtained in the different experiments show that visual cues are essential for prey detection in this species, at least in captivity. However, the use of multiple sensory modalities improves the success of detection suggesting that cathemeral species may benefit from the use of multiple sensory modalities.

KEY words: food detection, visual cues, cathemeral species, *Eulemur rubriventer*.

INTRODUCTION

The sensory modalities used during food detection in primates, and in lemurs in particular, have been poorly investigated (but see ERICKSON 1991, 1994; ERICKSON et al. 1998; BICCA-MARQUES & GARBER 2004; GOERLITZ & SIEMERS 2007; SIEMERS et al. 2007, PIEP et al. 2008). As lemurs include diurnal, nocturnal, and cathemeral species, they represent a good model to test the effect of activity pattern on prey detection. Whereas diurnal and nocturnal species have been studied previously (e.g. PIEP et al. 2008), little is known about the senses that guide food detection in cathemeral species. Here, we aim to quantify the involvement of the different sensory systems during prey detection in a cathemeral lemur, the red-bellied lemur (*Eulemeur rubriventer*), a species that forages at dusk to find fruits and insects. As this species forages at dusk we hypothesize that they preferentially use vision and potentially olfaction for food detection (SIEMERS et al. 2007). The large eye size and visual acuity

of lemurs suggests that the contribution of the visual system in food detection and prey capture success may indeed depend on daily activity patterns (ROSS & KIRK 2006). However, the pronounced use of olfactory markings and communication by growls (MITTERMEIER et al. 2006) in this species suggests high sensitivity to sounds and odours, and leads us to predict that olfactory and acoustic cues may also be used in food detection. Although the structure and the function of acoustic signals in primates have been previously investigated, only a handful of studies have explored the way that primates hear and use these stimuli (RAMSIER & DOMINY 2010). Interestingly, it appears that the amplitude of sounds emitted by insects can inform nocturnal lemurs, such as the mouse lemur, on the size and mass of their prey (GOERLITZ & SIEMERS 2007).

Surprisingly, no sensory detection studies have been performed with members of the *Lemuridae* rendering our understanding about the evolution of sensory modalities in response to activity patterns in primates limited. Here we experimentally test,

for one captive species of cathemeral lemur, which sensory modalities affect prey capture success, and discuss the use of sensory cues in relation to activity patterns in primates.

MATERIALS AND METHODS

Animals and Housing

The red-bellied lemur (*Eulemur rubriventer*) is an exclusively arboreal species that lives in the rainforests of eastern Madagascar. In this study, a group of six captive individuals, five males (M) and one female (F) of the tropical zoo of La Londe les Maures (France) were observed; four were used in the experiments. Of these three were males and one was a female. Given that only a single female was used, we could not test for differences between sexes. This will have to be investigated using more individuals in future research.

The placement of the boxes was random and placement was decided using a random number generator implemented in R.

Behavioural Experiments

The prey used during our trials consisted of crickets as they allowed us to differentiate between the three types of sensory modalities. Red-bellied lemurs were isolated and placed in front of a pair of boxes with a prey placed randomly in one of them. The individual's response was noted as positive if the box containing the prey was opened and as negative if the empty box was chosen. For every test, two habituation trials were performed before the real experiment. Four boxes (17 x 20 x 15 cm) were made and covered by a thick plastic cover slightly longer than the box, which facilitated its opening by the lemurs (Fig. 1). Two boxes were opaque and two were transparent. The boxes were cleaned every day with bleach to avoid any olfactory bias. Three parameters were tested: visual, olfactory and acoustic cues. Their

effect was tested in unimodal and multimodal conditions: vision, olfaction, audition, vision-olfaction, vision-audition, olfaction-audition. A control experiment with all cues present was also conducted. The seven experiments are summarized in Table 1.
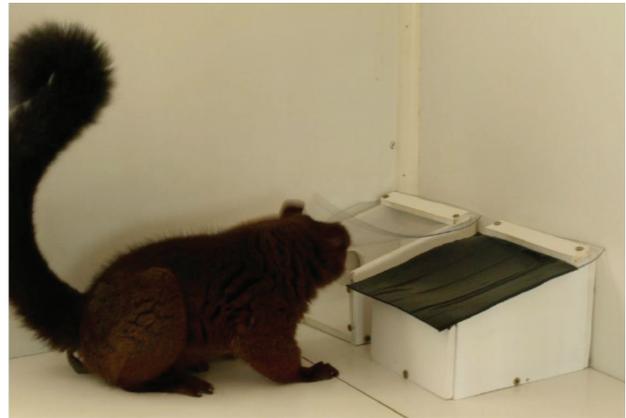

Fig. 1 – Opening of a box by a test subject. The use of an opaque versus transparent lid allowed us to test for the use of visual cues.

To test the use of visual stimuli (V), the pair of boxes was opaque or transparent and crickets were dead and contained in sealed plastic bags to avoid olfactory and auditory stimuli. To test the use of olfaction (O), dead crickets were placed directly in the opaque box or in a transparent hermetically sealed plastic bag that was then put in the box. To test the effect of auditory stimuli (A), live crickets that were moving (and thus making sounds) were placed in one of the opaque boxes in sealed bags, whereas the other box contained dead crickets, also in sealed bags to avoid olfactory stimuli. Seven different experiments were performed with each individual, with four replicates for each experiment, one replicate representing a session of 10 crickets (10 individual responses). Altogether, 1120 responses were analysed (280 responses per individual).

Statistical analyses

All analyses were performed using R (R Development Core Team, 2011). Mac Nemar

TABLE 1

Testing protocol for the different cues. VOA = vision-olfaction-audition, AO = audition-olfaction, VO = vision-olfaction, VA = vision-audition, O = olfaction, A = audition, V = vision.

Sensory modalities	Box type	Prey status	Prey conditioning
VOA	Transparent	Alive	Free
AO	Opaque	Alive	Free
VO	Transparent	Dead	Free
VA	Transparent	Alive	Locked (bag)
A	Opaque	Alive	Locked (bag)
O	Opaque	Dead	Free
V	Transparent	Dead	Locked (bag)

tests (comparison of proportions for dependent series) were used to test if there was learning during the detection experiments. We compared the proportions of successful detections during each half of each experiment for each individual and each parameter. This test is used to treat two qualitative variables in two classes (positive/negative response and $1^{st}/2^{nd}$ part of the experiment). The chance factor was tested to verify if the response was random or not. The observed proportions were compared with theoretical proportions (50 % of success) by means of a binomial test (e.g. MILLOT 2009). Tests were performed for each situation at the individual and group level. Finally, to test the effect of the availability of one or several sensory modalities on the proportion of successful detections a G-test with multiple comparisons was performed. A Holm correction was applied to the p-values to take in account the multiple pairwise comparisons (e.g. MILLOT 2009).

RESULTS

The Mac Nemar tests showed that no learning was apparent for any of the individuals during the experiment (Table 2).

Moreover, the binomial tests showed that the responses were not random at the group level when visual cues were available (Table 3). The situations involving audition, olfaction and audition-olfaction did not give responses diverging from chance. The same results were obtained at the individual level but the responses of one individual (Ernest) were also significantly different from chance when olfaction was available as a unimodal cue.

TABLE 2

Comparison of the proportions of successful detections for each subject according to the available senses. *P*-values for Mac Nemar tests (with df=1). VOA = vision-olfaction-audition, AO = audition-olfaction, VO = vision-olfaction, VA = vision-audition, O = olfaction, A = audition, V = vision.

Individual	VOA	AO	VO	VA	O	A	V
Ernest	1	1	0.13	1	0.75	0.72	1
Bart	1	0.75	1	0.505	1	0.55	1
Jumpy	1	1	0.07	0.72	0.1	1	0.37
Pimousse	0.37	0.72	1	1	1	0.29	0.22

TABLE 3

Comparison of the proportions of successful detections with the theoretical proportions corresponding to a performance level expected by chance. *P*-values for Binomial tests (with df = 1). *= significant values, VOA = vision-olfaction-audition, AO = audition-olfaction, VO = vision-olfaction, VA = vision-audition, O = olfaction, A = audition, V = vision.

Individual	A	O	V	AO	VA	VO	VOA
Bart	0.87	0.87	3.21e-03*	0.13	1.92e-02*	9.28e-08*	6.91e-07*
Ernest	0.99	0.01*	3.21e-03*	0.44	6.91e-07*	9.28e-08*	3.39e-04*
Jumpy	0.92	0.68	3.39e-04*	0.13	9.11e-05*	2.11e-05*	6.91e-07*
Pimousse	0.56	0.68	4.18e-06*	0.98	6.91e-07*	3.73e-11*	6.91e-07*
Group level	0.99	0.35	2.73e-12*	0.35	1.03e-15*	3.12e-27*	1.72e-20*

In order to visualize the role of each sensory modality, the averages of the proportions of successful detection for each unimodal and multimodal task were calculated, and are presented in Figure 2.

The worst performance of the lemurs was recorded when only audition was available (40 % of successful responses). The situations where the olfaction (O) and audition-olfaction (AO) parameters were involved resulted in success rates slightly higher than 50 %. In the four other situations (V, VA, VO, VAO), those in which visual cues were involved, more than 75 % of the responses were correct. Vision thus seems to be the dominant sense used in prey detection for this species (77 % of correct responses for unimodal experiments involving vision). The results also show that the association of vision with other senses improves the detection performance and thus the access to food. The best performance was recorded when both vision and olfaction were available (90 % of correct responses). The addition of audition to these two modalities reduced the performance of the lemurs (85 % of correct responses). Moreover, if vision was associated with audition only, the performance decreased to 80 %.
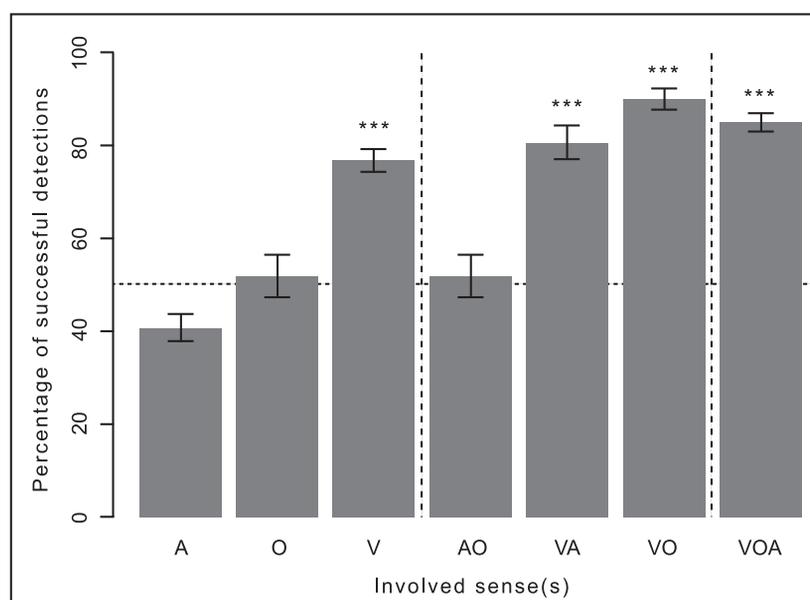

Fig. 2 – Averages of the success proportions of all the subjects (N=4) for each sensory modality tested with standard errors. *** = significantly different from chance, VOA = vision-olfaction-audition, AO = audition-olfaction, VO = vision-olfaction, VA = vision-audition, O = olfaction, A = audition, V = vision.

TABLE 4

Significance of the difference of the proportion of successful detections according to the available modalities associated with vision. *P*-values of G-tests with multiple comparisons and Holm corrections. * = significant results (*p* < 0.05), VOA = vision-olfaction-audition, VO = vision-olfaction, VA = vision-audition, V = vision.

Available modalities 1	Available modalities 2	p-values with Holm correction
V	VA	0.60
V	VO	0.01*
V	VOA	0.25
VA	VO	0.08
VA	VOA	0.60
VO	VOA	0.52

To test the significance of the effect of the sensory modalities available (in unimodal and multimodal tasks) on the rate of positive response of the subjects, a G-test on the results concerning only the situations with responses different from those expected by chance at the group level was performed. It gave significant results (G = 11.33; df: 3; P = 0.01). To understand which modality or association of modalities had an effect on performance, differences were tested using a G-test with multiple comparisons and Holm correction, the results of which are presented in Table 4.

Results from the experiment with all cues available (VOA) were not significantly different (p > 0.05) from the other situations (vision-audition, vision-olfaction and vision) in terms of the proportion of correct responses. The same results were obtained with the bi-modal tasks involving vision and audition (p > 0.05). Nevertheless, a significant difference of performance existed between experiments with visual and olfactory cues and those with only visual cues (p < 0.05). Vision combined with olfaction improved the detection performance compared to situations where only visual cues were available. Therefore, both vision and olfaction are important in prey detection for *Eulemur rubriventer*.

DISCUSSION

The objective of this study was to explore the sensory modalities employed by a cathemeral captive lemur to detect insect prey. The captive environment of the species, the relatively simple testing conditions, and the low variability in the food offered (crickets only) could have led to habituation and learning by the lemurs (DUKAS & KAMIL 2001). However, our results showed that no bias or learning effect could be detected. The statistical analyses also revealed a better detection performance when vision was involved and that these responses were different from chance. In the absence of visual cues, the responses were not different from what could be expected by chance, however. The use of vision in prey detection appears essential but detection performance is improved with the availability of other sensory cues. The data obtained for wild (SIEMERS et al. 2007) and captive (PIEP et al. 2008) mouse lemurs offer similar results concerning the dominance of vision in the detection of prey by lemurs. Yet, it should be noted that our sample size was limited (four individuals) and may thus potentially be biased at the individual level. Thus further experiments using additional individuals would be needed to confirm our results.

Visual parameters

Visual stimuli appear to dominate prey detection in this lemur species in captivity. A protocol allowing the assessment of sensory

stimuli involved in prey detection in wild conditions would be interesting to validate our results for captive animals. Previous studies predicted the dominance of visual cues in prey detection in lemurs and highlighted the importance of movement in the detection of prey (CARTMILL 1972, 1974, 1992). In the present study, movement was not tested, similar to the conditions in PIEP et al. (2008). Indeed, in the condition without auditory cues prey were dead to avoid rustling. Therefore, it would be interesting to test detection performance in the presence of moving prey.

Audition

Crickets were chosen as these insects are very mobile thus favouring the emission of rustling sounds. Whereas olfactory and visual cues remained stable during the tests, acoustic cues could have been variable due to the fact that insects stopped moving once placed in the experimental boxes. Thus, we could have underestimated the detection performance based on auditory cues due to behavioural variability of the prey. Indeed, the study conducted in the wild on *Microcebus murinus* (GOERLITZ & SIEMERS 2007) suggests a higher acoustic detection performance than observed here. This discrepancy can potentially be explained by the presence of leaf litter in natural conditions producing more noise upon movement of insect prey, thus aiding acoustic detection. Similarly, many bats depend on the vegetation to detect their prey by echolocation, even if some of them, such as *M. bechsteinii* and *M. myotis,* use passive listening to detect their prey via the noise the insects themselves produce (e.g. fluttering sound in moths) and the noise that is made by the insects when touching vegetation (e.g. rustling sound produced when beetles walk over substrate or moths touching leaves with their wing) (SIEMERS & SWIFT 2006). In addition, for bat species using only echolocation to detect prey (e.g. *M. nattereri*) the vegetation is rather hindering because it produces lots of echoes in which the echo of the prey can be embedded (backward masking effect). These bat species adapt their echolocation calls in a way to better deal with the problem of backward masking (e.g. calls of broad bandwidth, short duration). In primates, studies performed on Tarsiers and Galagos also demonstrated the importance of audition in these nocturnal insectivorous predators (DOYLE 1974; NIEMITZ 1979). *Eulemur rubriventer* is a species for which social communication involving growls is important (MITTERMEIER et al. 2006). The use of these sounds during communication could potentially also interfere with the acoustic cues emitted by potential prey. Finally, nocturnal species may be more dependent on auditory cues than diurnal or cathemeral species, yet this remains to be tested explicitly.

Olfactory cues

Diet likely has a significant effect on the cues used in food detection in lemuriforms. Indeed, a similar experiment performed with mouse lemurs and using pieces of fruit instead of live prey demonstrated higher detection performance using olfaction (SIEMERS et al. 2007) than we observed in red-bellied lemur. Frugivorous lemurs are likely more sensitive to smells derived from fruits than from insects, and thus it would be important to perform a similar experiment using fruits. Field observations showed that slender loris (*Loris tardigradus*) use olfaction to detect insects emitting strongly smelling repulsive substances (NEKARIS 2005). Thus, olfaction in addition to vision may be used by the red-bellied lemur in order to detect strongly smelling toxic prey such as millipeds, which they appear to like (MITTERMEIER et al. 2006).

CONCLUSIONS

Although our results suggest a dominant role of vision in prey detection in a cathemeral lemur species, a comparison with other primates differing in their daily activity cycle and utilising similar dietary resources is needed to test the generality of these results. Moreover, more

comparative data are needed to gain insights into the evolution of prey detection in primates.

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SHORT NOTE

A first report of introduced non-native damselfly species (Zygoptera, Coenagrionidae) for Belgium

Tim Adriaens[1],* & Geert De Knijf[1]

[1] Research Institute for Nature and Forest (INBO), Kliniekstraat 25, B-1070 Brussels, Belgium.
* Corresponding author: im.adriaens@inbo.be (tel.: +32 2 525 02 04, fax: +32 2 525 03 00)

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The introduction and spread of invasive alien species (IAS) constitutes one of the most important drivers affecting global biodiversity and ecosystem services (1). The rate of biological invasions is accelerated by the world-wide movement of people and goods (2). It is widely recognized that an important first step in developing a strategy for addressing the IAS problem is to document alien species already present as well as those likely to arrive in a particular region. Therefore, reporting on the occurrence of the non-invasive segment of non-native species remains important, since non-natives can become established and behave invasive in time. Moreover, data on pathways and commodities of introductions are crucial to inform preventive strategies to reduce the arrival of new and potentially damaging alien species (3). Information on the routes and mechanisms of invasions is also used in alien species risk assessments, management, monitoring and surveillance (4). Pathway analysis represents a first and important step of curtailing the accidental spread of non-native species and is becoming increasingly important with the newly adopted European Union regulation No 1143/2014 on the prevention and management of the introduction and spread of IAS (5, 6). This regulation includes provisions for the drafting of

action plans on priority pathways of unintentional species introductions. Here, we report on the introduction of two species of damselfly alien to Belgium: *Ischnura senegalensis* and *Pseudagrion microcephalum*.

One *Ischnura senegalensis* (RAMBUR, 1842) individual emerged from a small home aquarium (28 litre, 28 °C) in Buggenhout (East Flanders) on 19 March 2013. This animal died five days later. The aquarium was used for rearing *Betta* fish and contained some waterplants. The native range of *I. senegalensis* includes the (sub)tropical belt from Africa to Japan. The species is widespread, often abundant and is resistant to anthropogenic pressures such as disturbance and pollution. *Ischnura senegalensis* occupies a broad range of stagnant and slow-flowing habitats and also occurs in saline waters (7, 8). In its Asian range, it is quick to colonise and is often found in ponds in urban areas (9). Reports of natural occurrences closest to Belgium are from Mauritania, Egypt and Israel (10). Also, presence of the species on the Canary Islands has been confirmed (11). The species was reported as incidental for Finland (12), Germany (13, 14) and Great Britain (15). The combination of bright green thorax underside and antehumeral stripes, markings on segment 2, and the black abdomen bearing a bright blue ring on segment 8 provide good field characteristics for the adult male *I. senegalensis* (16, 17). Females are polymorphic and identification needs to be confirmed by examination of the hind margin of the pronotum (18).

TABLE 1

Records of two non-native damselflies in Belgium. Pathway terminology based on Hulme *et al*. (2008) (25).

Species	Locality	Date	Pathway	Commodity
Ischnura senegalensis	Buggenhout (East Flanders)	19-24 March 2013	Contaminant	Live plant material
Pseudagrion microcephalum	Opitter (Limburg)	24 Feb 2012; 15 March 2012	Contaminant	Live plant material

Two *Pseudagrion microcephalum* (Rambur) larvae were observed in an aquarium (240 litre, 25 °C) of a private house in Opitter (Limburg, Flanders) from November 2011. The aquarium was designed to represent an Asian freshwater habitat. The materials for aquascaping had been ordered online (Aquarium-Planten.com) and comprised the following plants, ferns and mosses: *Vallisneria spiralis*, *Microsorium pteropus*, *Blyxa japonica*, *Vesicularia montagnei*, *Vesicularia dubyana*, *Limnophila aromatica*, *Vesicularia* sp., *Riccardia chamedryfolia*. The plant material was delivered early November 2011. Two individuals, at least one female, emerged on 24 February 2012. Both of them died, one during emergence, the other several days later. A third larva was observed on March 14th 2012, but did not succeed to emerge. The native range of *P. microcephalum* includes India, South East Asia, China, Japan, Indonesia, New Guinea and Australia (19, 20). The species is widespread in its native range and occurs in freshwater ponds, lakes and streams (21). *Pseudagrion microcephalum* was one of the most frequently encountered exotic damselfly taxa in glasshouses of a plant trader near Wels, Austria (22) and in aquaria in western Germany (23). The genus *Pseudagrion* comprises over 140 species worldwide (24). There is much variation between the species and they are prone to specialisation resulting in many endemics.

Both species are widespread and common generalists in their native ranges and often occur in anthropogenically disturbed habitats there. Larvae of both species were accidentally imported as contaminants of live plant materials for use in aquariums and ponds (Table 1). These cases represent the first documented introductions of non-native Odonata for Belgium. The vast majority of non-native invertebrates in Europe are introduced unintentionally as contaminants, hitchhikers or stowaways, and roughly three quarters of the invasive alien species that came into the EU unintentionally entered via these pathways (25).

Valtonen (1985) (12) already mentioned the emergence of both species at an aquarium shop in Tampere (Finland) (*P. microcephalum* in 1982 and 1983; *I. senegalensis* in 1983 and 1984). These represent the first documented records of both species for Europe (22). The specimens arrived as larvae (or eggs) in water plants from Singapore. Successful larval development was also reported in London greenhouses (Great Britain) (15).

Wasscher & Bos (2000) (26) presented the first synthesis of Odonata introductions in Europe and mentioned 20 species. This number further increased to 41 taxa by 2013 (22). In this 13-year study in glasshouses *P. microcephalum* and *I. senegalensis* were among the most commonly encountered damselflies. Furthermore, Laister *et al*. (2014) showed that a large proportion of exotic odonates originate from eggs or larvae associated with aquarist trade of live plant materials. Other forms of introduction, such as accidental import of live imagines as hitchhikers in banana boxes, on airplanes (e.g. 7) or on ships (27), or deliberate releases for biocontrol (28), are comparatively rare. The majority of species were

of Southeast Asian origin, with many species emerging from home aquaria and glasshouses. Thus, the occurrences of both species reported here are well in line with general introduction histories of other damselflies in Europe.

Despite available reports, the DAISIE and NOBANIS species registries, which represent some of the most comprehensive and up-to-date registers of non-native species in Europe, currently do not comprise any non-native Odonata. Odonata are also lacking from the European Alien Species Information Network (EASIN), which compiles data from various European IAS databases (29). One reason for this might be that, so far, none of the reported accidental introductions of damselflies and dragonflies in Europe has resulted in established populations of non-natives and the phenomenon is therefore regarded as not ecologically relevant (22). However, introductions of non-native dragonflies into similar climate zones outside their native range may lead to the establishment and expansion of odonates (30, 31, 32). Secondly, whilst impacts of other non-native species such as zebra mussel *Dreissena polymorpha* (Pallas, 1771) and exotic crayfish on several species of dragonflies have been documented (e.g. 33, 34, 35), we could not find reports of introductions of dragonflies with negative impact on native biota (cf. 36). Introductions of non-native Odonata are therefore usually regarded as incidental and benign. Nonetheless, in Japan, *Ceriagrion auranticum ryukyuanum (*Asahina, 1967) established and spread outside its main range following accidental introductions with aquatic plants (32). Here, despite lack of evidence on impact (pers. comm. N. Ishizawa), eradication is ongoing based on the argument of potential impact on the threatened *C. nipponicum* (Asahina, 1967) (pers. comm. R. Futahashi).

Several authors have shown Odonata to expand their distribution ranges as a result of increasing temperatures (37, 38). Therefore, with global warming, successful establishment of accidentally introduced damselflies with live plant materials or as aquarium escapes remains a possibility. Subsequently, as both larval and adult Odonata can be important keystone predators (39, 40, 41), there would be the potential for impact on native biota. As many introductions of non-native Odonata occur indoors, accidental import is likely to be underestimated. Adult dragonflies and damselflies are highly mobile organisms. Long distance dispersal events, autonomously or aided by air currents, are not uncommon in this group (42). Therefore, in Odonata, there is also the probability of confusing human-mediated spread with autonomous species dispersal. Currently, tropical plants for aquarists are mainly imported to Europe from Singapore, Indonesia and Thailand (22, 43). However, the introduction of plant material from temperate regions (e.g. North America, China) could pose a risk for temperate zones in Europe. With regards to the species described here, we hypothesize that these subtropical species have the potential to establish at least in the mediterranean bioregion in Europe. We recommend that non-native Odonata should be incorporated in the registers of non-native species introductions in Europe.

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