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# High genetic diversity but limited gene flow in Flemish populations of the crested newt, *Triturus cristatus*

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ABSTRACT. Habitat destruction and fragmentation are among the major causes of amphibian decline. We investigated to what extent geographic distance and barriers affect the genetic composition of the crested newt, *Triturus cristatus* in Flanders (North Belgium), causing inbreeding or loss of genetic diversity. Data from seven microsatellite loci and 170 individuals from seven meta-populations up to 180km apart revealed heterozygosities of 0.53 to 0.67 within populations and moderate levels of genetic divergence between populations ($F_{ST}$ values from 0.074 to 0.141, harmonic means of $D_{est}$ between 0.070 and 0.189). In all Flemish meta-populations, more than 90% of the individuals from a given geographic region were assigned to the same genetic cluster indicating little genetic exchange, even in De Panne where the two populations Oosthoek and Westhoek are only a few kilometres apart. Such sub-structuring on a micro-scale has also been described in other amphibians. Unique alleles in most populations further support the probability that genetic drift has already led to some isolation. With the exception of the Oosthoek population, however, we found no significant evidence for bottlenecks. Connectivity within pool clusters seems essential to the maintenance of genetic diversity in crested newts as is indicated by our findings from Tommelen, the population with the largest number of pools in close proximity, which also shows the highest levels of heterozygosity ($H_e$ and $H_o$) and the second highest number and richness of alleles.

In conclusion, our study indicates that dispersal and migration rates between the Flemish populations of *Triturus cristatus* are limited at the geographic scale studied here but that habitat fragmentation has not yet led to a significant loss of genetic diversity of the studied Flemish populations, possibly because crested newts are relatively long-lived, fragmentation of their habitat is relatively recent in Flanders, and most investigated pools are still connected at the local scale.

KEY WORDS: *Triturus cristatus*, genetic variability, conservation, microsatellite, population genetics, crested newt.

## INTRODUCTION

Worldwide, many populations of amphibians are rapidly declining (HOULAHAN et al., 2000), which means that as a group, they are more threatened than many birds or mammals (STUART et al., 2004, VENCES & KÖHLER, 2008). Although diseases have also been put forward as an explanation (JAMES et al., 2009), in industrial countries the decline is mainly attributed to habitat fragmentation and the loss of suitable breeding habitats (SODHI et al., 2008). Especially in many parts of Western Europe, the number of ponds has declined severely in recent decades (MCLEE & SCAIFE, 1993; THIESMEIER & KUPFER, 2000; NÖLLERT & NÖLLERT, 2001, DECLERCK et al., 2006). Because these small water bodies are essential for many amphibian species, several pond conservation projects have been instigated (e.g. European Pond Conservation Network; http://campus.hesge.ch/epcn/). They have contributed to a growing sense of awareness and understanding of the necessity to conserve ponds in a rapidly changing European landscape in order to protect their fauna and flora (e.g. FROGLIFE, 2001). However, little is known about the genetic constitution of typical pond species, although such knowledge is a prerequisite for effective and efficient conservation practices (JEHLE & ARNTZEN, 2002).

The crested newt, *Triturus cristatus* (Laurenti 1768) has a wide distribution in central and northern Europe (NÖLLERT & NÖLLERT, 2001). Nowadays, it is mostly found inhabiting deep and vegetated pools in extensive agricultural landscapes and is thus readily influenced by human activities (GRIFFITHS, 1996; THIESMEIER & KUPFER, 2000). The crested newt, with a typical amphibian dual lifestyle, spends more of its time in the aquatic phase than other western European newts (THIESMEIER & KUPFER, 2000). Adults often linger in ponds until autumn, while larvae sometimes hibernate during their first year on the bottom of pools. In contrast to many other newts, young, non-reproducing individuals return to the pool in spring to exploit the abundance of food that is found there (GRIFFITHS, 1996; NÖLLERT & NÖLLERT, 2001). *Triturus cristatus* is thus more dependent on suitable ponds than most other newts of the genus *Triturus* in Western Europe.

In Flanders (Northern Belgium), *Triturus cristatus* has a wide but very fragmented distribution (http://www.hylawerkgroep.be/images/website/hi_kamsalamander/kaart.jpg), making the species particularly vulnerable to

habitat disturbance and destruction. For example, in the Flemish region Haspengouw, the species declined by 20% from 1980-89 to 1992 and many pools held only few individuals (BAUWENS & CLAUS, 1996). To develop suitable conservation plans, genetic data are required (BEEBEE, 2005). The crested newt may be even more endangered through inbreeding than most other amphibians, because homomorphism for chromosome 1 leads to 50% mortality in eggs (WALLACE, 1987).

Population genetic theory states that in small, isolated populations, inbreeding may reduce average fitness, and loss of genetic variability from random genetic drift may further reduce future adaptability (LANDE, 1988). Small and isolated populations are therefore expected to show lower levels of genetic variability than large populations that belong to a highly connected meta-population. ALTENTOFT & O' BRIEN (2010) recently showed that genetic variation in 17 amphibian species is linked to fitness, emphasizing the necessity of genetic variability for evolutionary potential (FRANKHAM et al., 1999). A high degree of genetic diversity may be required for populations to respond adequately to changing selective pressures, especially in highly dynamic anthropogenic environments. Due to negative effects of inbreeding and genetic drift, small isolated populations are more likely to disappear over time (e.g., SACCHERI et al., 1998). Assessing the genetic diversity of threatened amphibian species can, therefore, provide clues for the long-term viability of their populations.

Here, we investigated the genetic variation of the crested newt, *Triturus cristatus* in Flanders (Northern Belgium) as an initial indication of the conservation genetic status of this species in Flanders and to facilitate further studies on smaller geographic scales. Genetic variability among populations was estimated using microsatellite markers. The studied populations are distributed over Flanders to give an overall assessment of regional genetic diversity and population genetic structure. We specifically tested predictions from population genetics theory. First, we would expect that areas with fragmentation (large distances between individual pools and/or man-made barriers) show indications for population differentiation, substructuring and low levels of genetic diversity. Areas with well-connected pools, on the other hand, would be expected to harbor higher levels of genetic diversity and facilitate genetic exchange between pools. Second, we would expect the loss of suitable habitats in the last few decades and the fragmented distribution of this species in Flanders to have induced bottlenecks and hence, have led to a loss of genetic diversity in Flemish populations of *T. cristatus*.

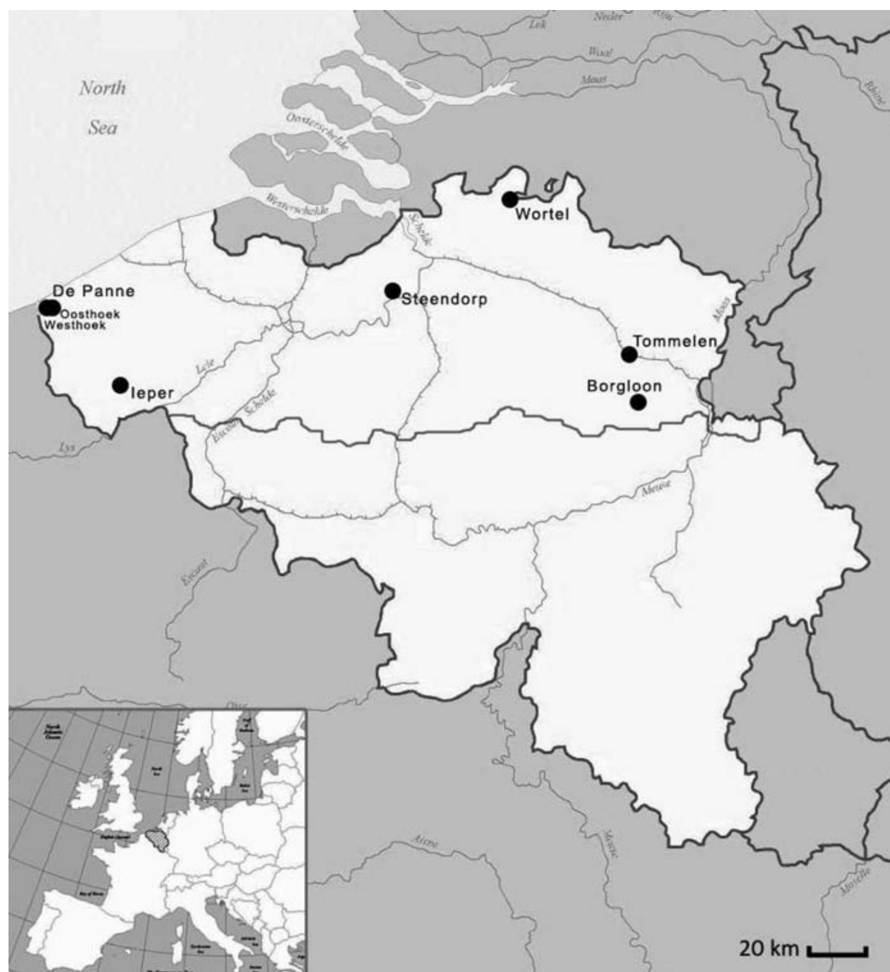


Fig. 1. – Distribution of sample localities in Flanders.

TABLE 1

Overview of sampling localities, numbers and connectivity of sampled ponds, and individuals sampled for two consecutive years.
#=number of ponds; D=maximal geographic distance between individual pools with *Triturus cristatus* in km (in Ieper, pools closer to each other did not contain any *T. cristatus* during our sampling periods); C=connectivity estimated as average number of pools within 1.5km, the dispersal distance of *T. cristatus* (GRIFFITHS, 1996) subtracting 0.1 for every barrier (roads, agricultural areas or human settlements) between pools; N=sampled individuals. The population from De Panne is split into Westhoek and Oosthoek in all subsequent analyses.

| Geographic location | # ponds | Year | D | C | N |
|---|---|---|---|---|---|
| Tommelen | 15 | 2006 | 0.267 | 1.00 | 30 |
| Ieper | 7 | 2006 | 10.500 | 0.30 | 12 |
| | 7 | 2007 | | | 35 |
| Steendorp | 5 | 2006 | 0.931 | 0.70 | 10 |
| | 5 | 2007 | | | 20 |
| De Panne | 8 | 2006 | 3.300 | 0.35 | 16 |
| | 8 | 2007 | | | 40 |
| De Panne/Oosthoek | 6 | 2006 | 0.412 | 1.00 | 12 |
| | 2 | 2007 | | | 10 |
| De Panne/Westhoek | 2 | 2006 | 0.618 | 1.00 | 4 |
| | 6 | 2007 | | | 30 |
| Borgloon | 3 | 2006 | 0.580 | 0.80 | 5 |
| Wortel | 1 | 2006 | 0.098 | 1.00 | 2 |

## MATERIALS AND METHODS

We studied newts from seven localities over Flanders, up to 180km apart (Fig. 1). *Triturus cristatus* is protected by Belgian law; permission to capture specimens and collect tissue samples is administered by the Agency for Nature and Forest of the Flemish Community. In 2006, we were only permitted to sample two larvae per pool. To acquire sufficient individuals for statistically meaningful analyses, we therefore reverted to sampling clusters of breeding ponds, focusing on those pools with the highest numbers of individuals. With the exceptions of Ieper and De Panne, distances between pools with *T. cristatus* within a certain cluster were less than 1km (see Table 1 for the maximal distances between individual pools and Fig. 1S in the supplementary material for maps of the sampled regions). In Ieper, pools in close proximity to each other (less than 1km) did not contain any crested newts during our sampling campaigns. *T. cristatus* is known to migrate up to 1.5km and often wanders among different pools within a cluster during the breeding season (GRIFFITHS, 1996; THIESMEIER & KUPFER, 2000; KUPFER, 1998; but see also GRIFFITHS et al., 2010). We also calculated relative connectivity of pools by dividing the number of pools within dispersal distance of *T. cristatus* by the overall number of sampled pools with *T. cristatus* and by subtracting 0.1 for every barrier between pools (roads, fields, houses etc.; see Table 1 for the sampled regions). In 2007, we obtained permission to sample five larvae per pond. In that year, we re-sampled the populations from the regions De Panne, Ieper and Steendorp. At these localities, we revisited the same ponds as in 2006 and additional ponds in close vicinity. All sampled pools in 2007 were within the same maximal distance to each other as in 2006 and also with the same relative connectivity. In the analyses, we pooled the data obtained in 2006 and 2007. One could wonder whether our sampling regime, which differed in the two years with respect to the number of samples per individual pond, could have biased our results. Preliminary analyses of the 2006 data (RAPSAET, 2007) revealed similar results to those with the combined samples from both years. The fact that we noticed migration between breeding pools in some clusters further supports our sampling design of including several individual pools per cluster rather than sampling high numbers of newts from individual pools.

Tissue from a total of 170 crested newt larvae was collected; Table 1 provides an overview of the number of ponds and specimens. Tissue samples were taken by clipping the tip of the tail of larval *Triturus cristatus*; subsequently, larvae were released at their capture site. This procedure has no long-term effect on the development and survival of newt larvae as clipped tails are readily regrown (ARNTZEN et al., 1999). Tail clips were stored in 95% ethanol at 4°C until further use. DNA was extracted following the standard DNeasy® Blood & Tissue Kit procedure (QIAGEN).

A polymerase chain reaction (PCR) was used to amplify seven microsatellite loci, Tcri13, Tcri27, Tcri29, Tcri32, Tcri35, Tcri36 and Tcri46, following the PCR-protocols of KRUPA et al. (2002). Each forward primer was labeled with a fluorescent dye allowing automatic detection of the amplified microsatellites on an ABI 3130 Genetic Analyzer (Applied Biosystems). Following standard procedures, a 500-Liz size standard (Applied Biosystems) was used to estimate the size of the amplified microsatellites, excluding both the 35bp and 250bp peak.

One μL of PCR-product and 0.5μL of the 500-Liz size standard were diluted in formamide to a total volume of 18μL. Samples were denatured and then loaded onto the automatic genetic analyzer. Data collection and sizing analysis were performed using Genemapper® v4.0 software. Allele sizes were also checked manually. Results of all analyzed microsatellite loci from each individual are given in Table 1S (supplementary material).

TABLE 2

Summary of population genetic parameters for the five investigated populations in Flanders with sufficient numbers of individuals.
N=number of individuals for which microsats were analysed; NAL=number of alleles; AR=average allelic richness; HE=expected heterozygosity; HO:observed heterozygosity; p (HW) average probability for deviations from Hardy Weinberg equilibrium; if significant, this is indicated by *, individual p values and number of microsatellites in brackets with significant p values is provided; FIS=F coefficient; if significant, this is indicated by * and number of microsatellites in brackets with significant values for FIS is provided; AUA=average number of private alleles; AUAR=average unique allelic richness. Averages and standard deviations were calculated from all microsatellites per (meta)population.

| Population | N | NAL | AR | HE | HO | p (HW) | FIS | AUA | AUAR |
|---|---|---|---|---|---|---|---|---|---|
| Tommelen | 29.14± 1.86 | 5.86± 1.57 | 3.17± 0.43 | 0.65± 0.08 | 0.67± 0.12 | 0.56± 0.23 | (0) | 0.10 | 0.42 |
| Ieper | 46.43± 0.79 | 6.57± 2.07 | 3.85± 1.85 | 0.64± 0.18 | 0.61± 0.16 | 0.006* 0.026* 0.0008* (3) | 0.098* 0.190* 0.171* (3) | 0.085 | 0.70 |
| Steendorp | 28.86± 1.86 | 5.14± 2.27 | 3.01± 1.02 | 0.57± 0.25 | 0.59± 0.26 | 0.61± 0.33 | (0) | 0.07 | 0.55 |
| Oosthoek | 21.86± 0.38 | 3.86± 1.07 | 2.61± 0.54 | 0.53± 0.11 | 0.54± 0.10 | 0.67± 0.34 | (0) | 0 | 0 |
| Westhoek | 33.14± 1.86 | 5.57± 1.27 | 3.07± 0.51 | 0.62± 0.13 | 0.66± 0.12 | 0.43± 0.34 | (0) | 0.03 | 0.42 |

### Data analysis

The possible presence of null alleles, stuttering or large allele dropouts was checked with MICROCHECKER (van Oosterhout et al., 2004).

GENEPOP v4.0 (Roussset, 2008) was used to test for linkage disequilibrium and to calculate allelic frequencies of microsatellites across populations. For multiple comparisons with a significant result, the Bonferroni correction was applied (http://www.quantitativeskills.com/sisa/calculations/bonfer.htm). GENEPOP was also used to calculate the observed and expected number of heterozygotes ($H_o$ & $H_e$) for each population. The implemented Markov Chain method was applied to obtain unbiased estimates of Fisher's exact tests for the calculations of $H_o$, $H_e$ and departures from Hardy-Weinberg equilibrium (HWE; following Guo & Thompson, 1992; and others). To distinguish between several possible explanations for deviations from HWE (see below), additional analyses were conducted for those meta-populations with significant deviations from HWE. These meta-populations were subdivided into smaller subunits to determine whether these subunits themselves were in HWE. If so, deviations from HWE were probably due to population sub-structuring. $F_{IS}$ values were calculated for all meta-populations (Table 2). These values can fluctuate between -1 and 1 (Weir & Cockerham, 1984), where a positive value indicates a surplus of homozygotes, a negative value an excess of heterozygotes. Allelic richness and private allelic richness were estimated with HP-Rare (Kalinowski, 2005).

Microsatellite data were analysed with the programme STRUCTURE (Pritchard et al., 2000), which infers population structures by implementing model-based clustering methods. It does so by assigning individual multilocus genotypes to the user-defined number of clusters with certain probabilities. We applied it to estimate the number of genetic clusters (K) in the seven studied populations, assigned individuals to those clusters and estimated the number of individuals with mixed ancestry. With test runs, we checked that all necessary parameters reached equilibrium before the end of the burn-in. For the actual runs, 100,000 burn-in iterations were used, followed by another 900,000 generations and cluster numbers varied from 1 to 10. Independent chains were conducted with and without a model of admixture (mixed ancestry), a constant value for individual admixtures in all clusters and independent allele frequencies between clusters. We conducted eight independent chains of iterations for the five simulations with the highest likelihood values. STRUCTURE was also used to discover hidden genetic structures among individuals and pools in the meta-populations of De Panne and Ieper (see below).

To test whether populations showed signs of a significant recent bottleneck, all data were analysed with the BOTTLENECK software (Cornuet & Luikart, 1996). When populations undergo a size reduction, an excess of heterozygotes develops, which persists for a number of

generations until a new equilibrium is reached between mutation-migration and genetic drift (CORNUET & LUIKART, 1996). BOTTLENECK statistically tests for shifts in heterozygosity, which may indicate past demographic expansions (heterozygote deficiency) or reductions (heterozygote excess). We used the infinite allele mutation model as we explicitly assume in further tests for population differentiation (*F*- and *D*-statistics) that identical alleles among populations are identical by descent, not by chance of having identical mutations. It also relaxes the assumption that no migration among populations takes place, as a new allele in a population may originate through mutation as well as through occasional migration. A one-phased model of mutation (100% IAM (GRIFFITHS, 1979) was used. Mean heterozygosities were calculated across all loci for each population. Levels of significance were determined with a one-tailed Wilcoxon sign-rank test (LUIKART et al., 1998), with $10^4$ iterations.

Genetic differentiation was assessed with two estimates: First, calculating $F_{ST}$ values for pairwise population comparisons according to WEIR & COCKERHAM (1984) using GENETIX v.4.05 (BELKHIR et al., 2004) and estimating levels of significance by using an exact test in GENEPOP for population differentiation to be able to compare our results with published data from other amphibia. Second, estimating $D_{est}$ (JOST, 2008), using the unbiased estimators of NEI & CHESSER (1983) of the overall gene diversity $H_t$ and subpopulation gene diversity $H_s$. Calculations of $D_{est}$ were performed using the online software tool SMOGD (CRAWFORD, 2010). Just like $G_{st}$ or related measures ($F_{ST}$, Theta), $D_{est}$ ranges from zero (no differentiation) to 1 (complete genetic differentiation), but it is not dependent on gene diversity (JOST, 2008), providing a much more accurate measure of genetic differentiation when using highly variable markers such as microsatellites.

A Mantel test (MANTEL, 1967), as implemented in GENEPOP, was used to check for a significant correlation between geographical and genetic distances. $F_{ST}$ and $D_{est}$ statistics were logarithmically converted and fitted with geographic distances in km.

## RESULTS

### Sample size and sampling sites

In two localities, Wortel and Borgloon, only two and five larvae, respectively, could be sampled (Table 1). These sample sizes were too small for reliable statistical analyses with classical population genetic methods, which is why these populations were omitted from the analyses with GENEPOP, BOTTLENECK and the calculations of $F_{st}$ and $D_{est}$. They were, however, included in the analyses with STRUCTURE, which is less sensitive to the effects of sample size.

From the two sampling campaigns in 2006 and 2007, it was obvious that the area of De Panne showed a shifted occupation of pools by larvae. In some pools with ample larvae in 2006, we found no larvae in 2007, whereas other pools from that cluster, where larvae were absent in 2006, contained larvae in 2007. No other sampling area showed comparable shifts.

### Genetic diversity within and between populations

We did not find evidence for null alleles, stuttering or large allele dropouts in any population using MICRO-CHECKER. There was also no evidence for linkage disequilibrium between any pair of loci across all populations except for the locus pair Tcri29 & Tcri46 (Fisher exact test $P$=0.035, df=12), which was no longer significant after the Bonferroni correction. Genotypes from different loci are thus independent from each other. The total number of alleles per locus ranged from 3 to 10 (Table 1S, supplementary material) and the average between 3.86 (Oosthoek) and 6.57 (Ieper) alleles (Table 2) while allelic richness varied between 2.61 (Oosthoek) and 3.85 (Ieper; see Table 2). Locus Tcri36 showed the lowest, locus Tcri27 the highest variability (Table 1S, supplementary material). All loci were in accordance with HWE, with two exceptions for the meta-populations in Ieper and De Panne with significant heterozygosity deficiencies for three and two microsatellites, respectively (Table 1S, supplementary material).

To test whether the Wahlund effect could have caused these deviations, the De Panne meta-population was split into two subpopulations for additional analyses according to geographic location. No more deviation from HWE was detected (Table 2). Henceforth, the population from De Panne was divided into two subpopulations based on their geographical origin, Oosthoek and Westhoek, for all subsequent analyses (see the maps in the supplementary material for their positions). Unraveling sub-structuring of the Ieper meta-population was less straightforward. This meta-population was either divided into two or three subunits comprising several ponds each or into seven subunits with a single pond each. Deviations from HWE disappeared when we split the meta-population into seven subunits according to the individual pools (data not shown but available from the first author on request). Because these subunits only contained five to seven individuals each, it was not possible to conduct subsequent analyses (e.g. estimates of allele number, private alleles, BOTTLENECK etc.) with the subdivided data sets.

When the average number of alleles per population was calculated for all loci (see Table 2), the number was highest in Ieper (6.57), followed by the populations in Tommelen (5.86) and Westhoek (5.57) (see Table 1). Omitting the two populations with limited sample size (Wortel and Borgloon), newts from Oosthoek displayed, with 3.86, the lowest average number of alleles. We found the same patterns for the average allelic richness (Table 2). The frequency of unique alleles in a single population (Table 2) fluctuated between zero for the Oosthoek population to 0.10 for Tommelen and 0.085 for Ieper. Considering the average of unique allelic richness, the population from Oosthoek showed the lowest (0) and the Ieper meta-population the highest value (0.70; Table 2). Tommelen, with 0.42, was less rich in average unique alleles than the population from Steendorp with 0.55.

Values of expected heterozygosity, $H_e$, ranged from 0.53 for the population in Oosthoek to 0.64 for the population in Tommelen (see Table 2). Bottleneck tests showed a significant heterozygote deficiency in the Oosthoek population as well as a mode-shift in the distribution

of alleles (Table 3) but provided no statistically significant evidence for genetic bottlenecks in the other investigated *Triturus* popuations from Flanders.

Simulations with STRUCTURE showed that the data fitted best a model with five genetic clusters (K=5) without admixture (max. Ln P(D)= –3081.7 without admixture at K=5; max. Ln P(D)= –3194.2 with admixture at K=5;), meaning that each individual is derived purely from one of the five clusters. These clusters correspond to the meta-populations of Tommelen, Oosthoek, Westhoek, Ieper and Steendorp (Fig. 2). Average posterior probabilities reached 90% (or more) if the individuals from these populations were assigned to the same cluster according to geographic origin (Fig. 2) except for Wortel for which only two individuals could be analysed. On the whole, standard deviations were low (see Fig. 2) with the exception of Westhoek where two individuals were assigned to other genetic clusters – one to Oosthoek, and the second to Steendorp. If six genetic clusters (K=6) were defined, the maximal Ln P (D) was with -3087.2 only slightly smaller than the Ln with five genetic clusters. Also with six clusters, more than 90% of newts from the meta-populations Ieper, Tommelen, Oosthoek, Westhoek and Steendorp were assigned according to their geographic origin, again with little standard deviation, while the posterior probabilities for assigning individuals from the two populations with limited sample sizes, Wortel and Borgloon, increased (not shown).

TABLE 3

Results of analyses for population bottlenecks.
MHE=Probabilities of mean heterozygosity excess; MHD=Probabilities of mean heterozygosity deficiency. MHDE=Probabilities of mean heterozygosity excess or deficiency. Distribution shape describes the shape of allele frequencies. Bold numbers indicate 5% significance.

| | MHE | MHD | MHDE | distribution shape |
|---|---|---|---|---|
| Tommelen | 0.234 | 0.813 | 0.469 | normal |
| Ieper | 0.188 | 0.852 | 0.375 | normal |
| Steendorp | 0.234 | 0.813 | 0.469 | normal |
| Oosthoek | **0.039** | 0.973 | 0.078 | **shifted** |
| Westhoek | 0.188 | 0.852 | 0.375 | normal |

TABLE 4

Pairwise estimates of $F_{ST}$ and $D_{est}$.
Upper half: pairwise estimates of $F_{ST}$. Lower half: pairwise estimates of the arithmetic mean of $D_{est}$ and the harmonic mean of $D_{est}$ (in italics).

| | Tommelen | Ieper | Westhoek | Oosthoek | Steendorp |
|---|---|---|---|---|---|
| Tommelen | – | 0.079 | 0.074 | 0.110 | 0.079 |
| Ieper | 0.172; *0.070* | – | 0.141 | 0.107 | 0.077 |
| Westhoek | 0.157; *0.104* | 0.274; *0.189* | – | 0.114 | 0.145 |
| Oosthoek | 0.207; *0.103* | 0.207; *0.149* | 0.184; *0.092* | – | 0.107 |
| Steendorp | 0.169; *0.087* | 0.165; *0.070* | 0.270; *0.152* | 0.198; *0.102* | – |

## Genetic divergence between populations

Pairwise genetic differentiations between populations are represented in Table 4 showing that all pairwise comparisons were different from zero. When estimating $F_{ST}$, the allelic distribution of all microsatellite loci was significantly different amongst all investigated populations ($P$<0.0001 for each locus separately; $P$<0.0001, df=14 in the combined test with Fisher's method). $F_{ST}$ values ranged from 0.074 to 0.141 when estimated with GENETIX (see Table 4) and from 0.0647 to 0.2055 when estimated with STRUCTURE. Overall genetic differentiation $D_{est}$ was estimated to be 0.139 (harmonic mean over all loci) and 0.179 (arithmetic mean over all loci; Table 4). Overall differences between populations estimated with $F_{ST}$ were primarily influenced by the allelic variation at loci Tcri35 and Tcri27, and to a lesser extent by locus Tcri46 (see Table 2S, supplementary material) while estimates of $D_{est}$ were primarily influenced by Tcri35 and Tcri 36 (Table 4). The populations from Tommelen and Westhoek had the lowest pairwise $F_{ST}$ values (0.074) (Table 4) although these two populations were geographically the furthest away from each other. Estimates of the arithmetic mean of $D_{est}$ show the same pattern while harmonic means of $D_{est}$ were lowest between Tommelen and Ieper (Table 4). Seven alleles of microsatellites Tcri27, Tcri35 and Tcri46, are exclusively found in either Tommelen or Westhoek, respectively, and are not shared between these two populations (see Table 2S, supplementary material).

No significant correlation between geographical and genetic distances was detected with the Mantel test with either $F_{ST}$ or the arithmetic and harmonic means of $D_{est}$ ($P$=0.82, 0.47, and 0.77, respectively).

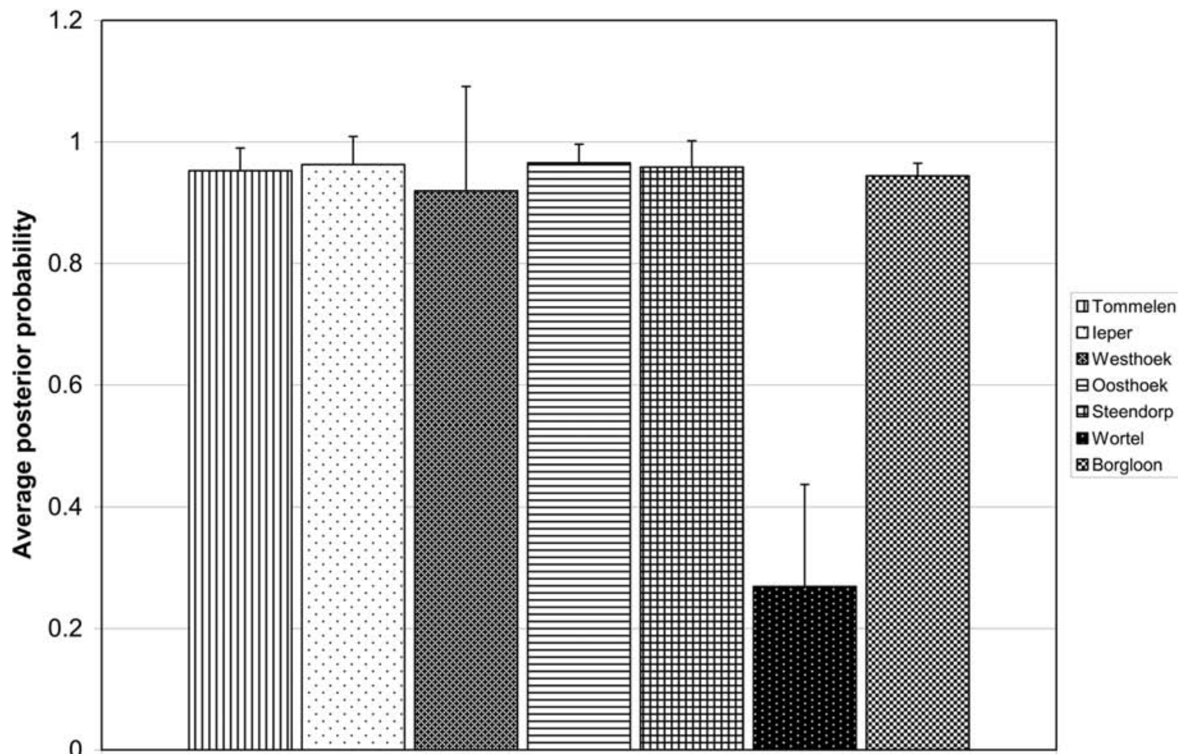


Fig. 2. – Assignment of individual newts to the seven clusters of geographic origin defined by STRUCTURE. The y axis represents the average posterior probability and its standard deviation for all individuals of a certain geographically-defined population to be assigned to this particular genetic cluster. Different black and white patterns indicate the seven genetic clusters. Average and standard deviation were calculated per geographic origin and all eight runs of STRUCTURE.

## DISCUSSION

Contrary to what we expected, we did not find evidence for the significant loss of genetic diversity in the screened Flemish populations. There is one exception, however, the population from Oosthoek that is genetically very diverse from the nearby Westhoek population and shows some indications for a genetic bottleneck. It is also the population with the lowest number of alleles, the lowest estimates for allele richness, observed and expected heterozygosity and has no private alleles (Table 2).

The lack of evidence for bottlenecks in the other Flemish populations could have several explanations. We analysed seven microsatellite loci, whereas more loci are generally advised (CORNUET & LUIKART, 1996). Moreover, the statistical power of a bottleneck test from a single sample is lower than for a test that includes historical samples pre-dating the bottleneck event. This implies that only really severe bottlenecks will be detected (LUIKART et al., 1998). In the case of the tree frog, *Hyla arborea*, from Denmark (ANDERSEN et al., 2004), however, bottlenecks could be demonstrated; these populations had not only lost genetic diversity but also showed signs of low fitness. Recent bottlenecks can furthermore be masked by an increasing number of rare alleles (BURNS et al., 2004). It is also possible that demographic changes are of too recent origin in the *T. cristatus* populations in Flanders to allow their statistical detection. Given that habitat loss and fragmentation of crested newt habitats have mostly taken place during the past decades (BAUWENS, pers. comm.), that the life span of *T. cristatus* is 13 to 18 years (BLITTERSWIJK et al., 2005), and that the severity of a bottleneck depends not only on the minimum population size but also on the duration of the reduction in generations (FRANKHAM et al., 2002), the loss of genetic diversity through drift may still be moderate. Overall, our results are encouraging because they indicate that the loss of neutral genetic diversity, local and regional, is not yet worrisome in most of the studied populations and that restoration measures leading to population expansion may be sufficient for the long-term conservation and viability of crested newt populations in Flanders.

### Genetic variability within and between Flemish populations

We proposed that higher connectivity and shorter distances between individual pools within a pool cluster would facilitate genetic exchange and hence, prevent the loss of genetic diversity. Contrary to this hypothesis, newts from the region with the largest distances between individual pools and the least relative connectivity, Ieper, also displayed the highest numbers of alleles and allelic richness in all analysed populations (Table 2). However, at the same time, this meta-population showed significant deviations from HWE and significant heterozygote deficiency (positive $F_{IS;}$ Table 2). Such deviations may be owing to a variety of causes such as selection, the presence of null alleles (due to failure of PCR) or inbreeding.

In the present study, we can probably eliminate selection as a possible explanation for deviations from HWE, because microsatellites are supposed to be neutral markers (GOLDSTEIN & SCHLÖTTERER, 1999). Also, no null alleles were identified in the dataset by MICRO-CHECKER. These deviations can also be caused by the Wahlund-effect, the mixing of genetically distinct (sub) populations (WAHLUND, 1928), which mostly results from sampling artefacts. This is in our opinion the most likely explanation for our results from Ieper because as described above, we had a second region, namely De Panne (see above), with significant deviations from HWE and significant heterozygote deficiency, which were no longer observed when we split the newts from De Panne into Oosthoek and Westhoek.

A similar subdivision to that in De Panne was unfortunately not possible for the Ieper meta-population – splitting it into two or three subunits did not remove the significant heterozygote deficiencies. Distributing newts according to the seven different pools would not have left sufficient sample numbers per pool for meaningful statistical analyses. STRUCTURE did not provide any indication for more than one genetic cluster in the Ieper area because no individuals were assigned to other clusters and also the standard deviation was not higher than in the other meta-populations (Fig. 2). It seems that additional sampling will be required to conclusively test for population substructuring in the Ieper area.

If we exclude the results from Ieper for testing our hypothesis mentioned above, the second most diverse crested newt population in Flanders is Tommelen with the largest $H_e$ and $H_o$, the highest number of alleles and unique alleles and allelic richness. In Tommelen, distances between pools in the cluster are shortest (see Table 1), individual pools are surrounded by the highest number of other pools (Table 1) and barriers are absent (see Fig. 1S, supplementary material). The population size there was estimated between 575 and 1037 individuals (males=369-521; females=206–516; DESCAMPS & BAERT, 2003) although the effective (genetic) population size is not known. For the other regions, data on population size are not yet available. The data from Tommelen thus support our hypothesis on the importance of connectivity for maintaining genetic diversity in crested newts.

This is further corroborated by the clustering analyses with STRUCTURE, which showed that newts from Westhoek and Oosthoek formed two distinct genetic clusters with only one shared individual, despite their geographic proximity of 3.3km. However, the two areas are separated by roads, agricultural areas and human settlements (see Fig. 1S in the supplementary material). We believe that most of the roads were built at the latest in the 1950's together with the adventure park of De Panne. This would mean that within 50 years, habitat fragmentation might effectively have blocked genetic exchange between crested newt populations close to each other. Road infrastructure, even when there is little or no traffic seems to negatively affect the migration abilities of newts between ponds (THIESMEIER & KUPFER, 2000). *Triturus cristatus* has been known to occur for decades in the Oost- and Westhoek region but we cannot exclude the possibility that the introduction of newts from elsewhere into this region by tourists, for example for gardens of holiday houses (VERSCHOORE, pers. comm.) might have further contributed to the genetic differentiation between the Oost- and Westhoek populations. Additional research on population histories will be required to unravel this strong genetic differentiation.

Such differentiation at the micro-geographical scale (less than 10km) has also been observed in other amphibians such as frogs (*Geocrinia* – DRISCOLL, 1998; MONSEN & BLOUIN, 2003), the tiger salamander, *Ambystoma tigrinum* (ROUTMAN, 1993) and toads (*Bufo canorus* – SHAFFER et al., 2000; *Bufo calmita* – ROWE et al., 2000). In the spotted salamander, *Ambystoma maculatum*, (ZAMUDIO & WIECZOREK, 2007), significant correlations between genetic and geographic distances were only found for populations that were separated by 4.8km or less. This could explain why we failed to detect a significant correlation between geographic and genetic distances in Flemish populations of *Triturus cristatus*. However, other amphibian species seem to depart from this pattern of strong population sub-structuring (*Bufo bufo* – SEPPA & LAURILA, 1999; *Ambystoma macrodactylum* – TALLMON et al., 2000; *Rana sylvatica* – NEWMAN & SQUIRE, 2001; *Litoria aurea* – BURNS et al., 2004).

Genetic differentiation between Flemish populations was considerable, irrespective of the measure used (see Table 4). There was no significant correlation between geographic distance and genetic differentiation from the Mantel test, probably because comparisons between the close populations Oost- and Westhoek showed similar $F_{st}$ and $D_{est}$ estimates as did comparisons with the distant meta-populations Tommelen, Ieper and Steendorp, which are separated by major rivers (IJzer, Scheldt).

The marked pairwise genetic differentiation between all study areas (except those with very few sampled individuals) is supported by the results from STRUCTURE (see above) showing that all populations formed effective genetic clusters with little (Westhoek/Oosthoek and Westhoek/Steendorp) or no exchange of genotypes among populations. Individual newts were always clearly assigned (>90%) to one of the five genetic clusters with asymmetrically assigned proportions and small standard deviations (Fig. 2), strongly indicating that the observed population structure with five clusters is real. The STRUCTURE results also support our sampling scheme of considering clusters of pools as one meta-population, with the exceptions of De Panne and Ieper as discussed above. STRUCTURE assignments were less obvious for the two populations Borgloon and Wortel but the limited sample numbers (2 and 5, respectively) from these areas probably provide sufficient explanation for this pattern.

Four out of seven microsatellite loci shared common alleles in all analyzed Flemish *Triturus cristatus* populations (Table 2S, supplementary material). This genetic conformity indicates that the Flemish populations once shared common ancestors and this is further supported by our relatively low $F_{st}$ and $D_{est}$ values (Table 4) when compared to the study by MALETZKY et al. (2010) on *T. cristatus* from Germany and Austria from a much smaller geographic area.

From our sampling campaign, we cannot conclude if the Flemish *Triturus cristatus* populations are in migra-

tion-drift equilibrium. Therefore, we can also not infer whether the observed genetic differentiation is a remnant of persistent founder effects after postglacial recolonizations or whether it reflects the balance between gene flow/mutation and genetic drift in the investigated populations. The observed population genetic differentiation nevertheless indicates that either sufficient numbers of individuals colonized the pools during postglacial expansion such that founder effects are not too prevalent and/or that gene flow in the past has been sufficiently high to maintain these relatively low levels of genetic differentiation among populations.

### Genetic variability of *Triturus cristatus* at a larger scale

One major aim of this study was to assess the genetic variability of *Triturus cristatus* within and among Flemish populations. If estimates of genetic diversity are expressed as expected heterozygosity, $H_e$, to allow comparisons with other studies, we would expect lower values for the Flemish populations, because most of them are small, fragmented and isolated owing to anthropogenic influences (BAUWENS, pers. commun.; http://www.inbo.be/content/page.asp?pid=FAU_AMF_VER_kamsalamander). Values of $H_e$ in our study varied from 0.53 to 0.64 (Table 2) and were thus close to estimates from another investigation on *T. cristatus* from France (0.49 and 0.65- JEHLE et al., 2005). However, our estimated $H_e$ were clearly larger than those of MALETZKY et al. (2010) from Austria and Germany (0.34-0.59) but lower than the data by MEYER (2005) on German and by MIKULICEK (2005) on Czech and Slovakian crested newts, who estimated $H_e$ of 0.53-0.81 and 0.50-0.84, respectively. Also allelic richness of Flemish *T. cristatus*, with 2.61 to 3.86, was higher than that observed by MALETZKY et al. (2010; 2.0 to 3.75). The number of alleles for each analyzed microsatellite locus in Flemish populations varied from 3 to 10 (Table 1S, supplementary material) while French populations exhibited 4 (Tcri13 and Tcri36) to 9 (Tcri43) alleles per locus with the same microsats (JEHLE et al., 2001). German populations showed a slightly elevated allelic variation, ranging from 7 (Tcri29) to 13 (Tcri43) alleles per locus and up to 29 alleles in the study by MALETZKY et al. (2010). Thus, we can conclude that genetic diversity of *T. cristatus* populations from Flanders is similar to that of French populations, slightly lower than that of German, Czech and Slovakian and higher than that of Austrian/German populations despite the fact that Belgium has a much more fragmented landscape with extensive urbanized areas intersected by agriculture (MAES & VAN DYCK, 2001).

In comparison to other European amphibians, Flemish *Triturus cristatus* showed higher $H_e$ values (0.5 to 0.81). *Triturus marmoratus*, a close relative to *T. cristatus*, exhibited $H_e$ values ranging from 0.20 to 0.55 (JEHLE et al., 2005) whereas $H_e$ values of *Bufo calamita* varied from 0.24 to 0.39 (ROWE et al., 2000). ANDERSEN et al. (2004) found $H_e$ between 0.35 and 0.53 for *Hyla arborea* populations in Denmark and VOS et al. (2001) reported $H_e$ of 0.2 to 0.6 for *Rana arvalis*. *Alytes muletensis* showed lower minimal values than *T. cristatus* ranging between 0.38 and 0.71 (KRAAIJVELD-SMIT et al., 2005).

While MALETZKY et al. (2010) have detected decreasing genetic diversity of crested newts in their study area including a river valley and hills, none of the studies of *T. cristatus* in Flanders, France, Germany, Czech Republic and Slovakia showed any strong evidence for loss of genetic diversity. However, we studied only those Flemish populations with the highest densities. Our data illustrate that in two sites where large numbers were expected, namely Wortel and Borgloon, only few larvae could be found. Thus, pools with lower numbers of *T. cristatutus* might already have lost genetic diversity but this will be difficult to demonstrate because limited sample numbers will hamper meaningful, statistical analyses.

## CONCLUSIONS AND PROSPECTS

We have found evidence that connectivity (proximity of suitable pools and the absence of barriers at a local scale) is crucial for preventing loss of genetic diversity in crested newts. However, at the larger scale, Flemish populations of *Triturus cristatus* are already genetically isolated to a large extent as is illustrated by our results from the genetic clustering and the finding of unique alleles in most populations. The expectation in the long term is that genetic differentiation between populations will increase, mainly because of drift and a continued lack of genetic exchange at small geographic scales. Most of all, however, we expect that genetic signs of population bottlenecks will manifest themselves, also in the other populations if no suitable conservation measures are taken. Because the persistence of populations depends on their evolutionary abilities to adapt to changing environments, genetic variation is required (FRANKEL & SOULÉ, 1981). If small subpopulations become endangered because of genetic isolation, then this will also have negative effects on the persistence of meta-populations. Such effects are well-documented (FAHRIG, 2002; FAHRIG & MERRIAM, 1985; WILCOX & MURPHY, 1985). Further monitoring of the genetic diversity will be necessary, especially at local geographic scales, to provide reliable data on the genetic exchange and migration rates among sub-populations of *T. cristatus*.

It furthermore seems advisable to take additional action to protect *Triturus cristatus* in Flanders. Firstly, appropriate geographical units for conservation management should be identified, which will in the case of *T. cristatus* sometimes consist of (sub) populations at a very local scale (as in De Panne and Ieper) or include closely connected clusters of pools as in Tommelen. Habitat fragmentation has a negative effect on genetic diversity of amphibian populations (HITCHINGS & BEEBEE, 1998; VOS & CHARDON, 1998; ANDERSEN et al., 2004; CUSHMAN, 2006), mainly because of the limited dispersal and gene flow (VOS et al., 2001; BURNS et al., 2004). Also our results illustrate that gene flow and dispersal can be significantly reduced, even between neighboring sites. Although the crested newt is rapidly diminishing in number, for the time being, it still contains relatively large genetic variability at the Flemish scale, at least in the studied populations. This genetic variability might have persisted up to now, because population fragmentation has only taken place recently. However, genetic variabil-

ity could vanish fast if isolation is further increased and no measures to promote migration and genetic exchange are put into place, for example by creating and maintaining suitable pools. The connectivity between habitats seems to be one of the key factors to enable the survival of *T. cristatus* in Flanders and elsewhere in Europe.

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# Establishment of Big Bone chicken fibroblast cell bank and study of its biological characteristics

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ABSTRACT. A fibroblast bank of Big Bone chicken was established using tissue adherent culture method. This cell bank included 29 embryo samples, had stocks of 147 cryogenically-preserved vials each containing 2~3×10$^6$ cells, and met all the cell line quality control standards established by the American Type Culture Collection (ATCC). The cells cultured *in vitro* showed the typical morphology of fibroblasts. The growth curve assumed the “S” shape, and the cell population doubling time (PDT) was about 30h. Cell viability was 98.1% before cryopreservation and 96.6% after recovery. All tests for microbial contamination were negative. The isoenzyme pattern was of species specificity. The frequency of diploid cells was 91%. The transfection efficiency of three fluorescent protein genes fluctuated between 10.6% and 26.5%. These results showed that the cells cultured *in vitro* grew well and had stable genetic properties. This research thus does not only preserve the poultry genetic resources of Big Bone chicken at the cell level, but also opens new ways for preserving important genetic resources of endangered animals in the form of somatic cells.

KEY WORDS: Big Bone chicken, fibroblast cell bank, biological characteristics

## INTRODUCTION

China has a total of 596 livestock and poultry species, among which 17 species have gone extinct, 336 species are subject to threats of different degrees and only 243 species are considered to be relatively safe (Ma & Feng, 2002). If we do not take effective protection measures immediately, these endangered domestic animals will become extinct in the next 20 years (Scherf, 2000). Therefore, it is imperative to launch the protection work of genetic resources of endangered livestock and poultry in China and elsewhere in the world.

At present, preservation of individual animals, semen, embryos, genomic libraries and cDNA libraries are all practical methods for preserving animal genetic resources. However, due to certain restrictions these methods still have some drawbacks. With the development and maturity of somatic cell cloning, animal somatic cells receive more attention as a supplement to the preservation of animal genetic resources (Liu et al., 2008; Wu et al., 2008; Li et al., 2009a). Establishing somatic cell banks of special livestock and poultry species could not only preserve the genetic resources at the cell level effectively and permanently, but also provide valuable experimental material for research in the life sciences of these species, such as cell biology, genomics, post-genomics and embryo engineering.

The Big Bone chicken, which is also named Zhuanghe chicken, is mainly distributed throughout the Zhuanghe County in the Liaoning Province (Xu & Chen, 2003). This breed was listed among the 138 nationally-protected domestic animals by the Chinese government in 2006. It has a burly physique, broad chest, a wide, long back, sturdy legs and a plump abdomen. Its outstanding advantage is its big eggs, which have thick solid shells and low breakage rates. Moreover, it has good meat with well-distributed subcutaneous fat and fresh flavour. In a word, the Big Bone chicken is an excellent native breed raised for meat and egg.

The purpose of this study is to preserve the genetic resources of Big Bone chicken breed in the long term in the form of fibroblasts and to provide technical and theoretical references for the cellular preservation of other poultry breeds. In this study, we established a fibroblast bank of this chicken breed and checked the quality of cells during cultivation and cryo-preservation.

## MATERIALS AND METHODS

### Materials

The embryos of Big Bone chicken were provided by the Poultry Institute of Jiangsu Province.

### Primary culture

Eight days old embryos of Big Bone chicken were rinsed 3 times with phosphate buffered saline (PBS) and the brain, eyes, limbs and viscera were removed. Then, the tissue samples were rinsed again three times with PBS and cropped into pieces of about 1mm$^3$ in size. These small pieces were uniformly seeded on the bottom wall of culture flasks. The flasks were inverted and incubated at 37°C, 5% $CO_2$ and at saturated humidity for about 4 ~ 5h to let the tissue pieces adhere to the flasks. When tissue samples adhered firmly, the flasks were turned over and complete minimum essential medium (MEM) containing

10% foetal bovine serum (FBS) was added to produce the primary culture.

### Subculture

When cell confluence (measurement for cell density in the culture) reached 80% ~ 90%, flasks were sub-cultured. The cells were rinsed three times with PBS after the culture medium had been removed, then 0.10% trypsin (m/v) was added and cells were incubated at 37°C for 5min with the flask turned over. When we observed under phase contrast microscopy that a large number of cells were retracted into a round shape and intercellular space had enlarged, the flask was shaken gently to detach the cells from the bottom wall. Complete medium was added to stop the trypsinization when most cells detached. The cells were split into new flasks at a ratio of 1:2 or 1:3 and continually incubated.

### Cryogenic preservation and recovery

The culture medium was changed 24h prior to freezing. Cells were collected conventionally when the cell confluence reached 80% ~ 90%, and then centrifuged at 1000rpm for 8min. Afterwards, the supernatant was discarded and the appropriate amount of freezing medium (10% dimethyl sulfoxide (DMSO) + 50% FBS +40% MEM) was added, adjusting the cell density to $4 \times 10^6$ cells/mL. Then the single cell suspension was dispensed into 1mL aliquots in 2mL cryovials. The cryovials were placed sequentially at 4°C for 1h, at -20°C for 1h, at -70°C for 12h, and then transferred to liquid nitrogen for long-term preservation.

The vials were taken out from the liquid nitrogen and quickly thawed in a water bath of 42°C. When the ice clump had thawed to bean size, the cells were transferred into a flask with complete medium and gently pipetted into uniform suspension and cultured at 37°C and 5% $CO_2$. The medium was refreshed 24h later (FRESHNEY, 2000).

### Microorganism detection

Cells were cultured in medium without antibiotics and tested for the presence of bacteria and fungus three days after subculturing. Following the method of DOYLE et al. (1990), cells were seeded on tryptone and wort medium, respectively, to detect possible bacteria or fungus contamination of the cells with cells deliberately infected with bacteria and fungus as positive controls.

In accordance with the DNA fluorescent staining method of ATCC, cells were stained with Hoechst 33258 and observed under the fluorescence microscope (FRESHNEY, 2006). For confirming the accuracy of the results, the mycoplasma detection kit (Roche) was used, which can identify the four most common *mycoplasma* species: *M. arginini, M. hyorhinis, A. laidlawii,* and *M. orale*.

Routine examinations for virus injuries such as plaque or barren spot were performed under the phase contrast microscope in the daily culture. In addition, some cells were randomly selected for hemadsorption tests (checking *in-vitro* for hemagglutinating viruses).

### Cell viability

The trypan blue dye exclusion test was used to determine cell viability before cryo-preservation and after recovery (XUE, 2001). One thousand cells were counted to calculate cell viability.

### Growth curve

Cells were seeded in 24-well plates at a density of approximately $1.5 \times 10^5$ cells/mL and cultured for 10 days. The cell density was counted every 24h, each time for three wells. The average cell density at each observation point was plotted against time and the cell population doubling time (PDT) was determined from this curve.

### Chromosome analysis

According to the method of SUN et al. (2006b), chromosomal preparations were carried out using cell monolayers that reached 80% ~ 90% confluence and were in the exponential growth phase. After Giemsa staining, 100 well-spread metaphases were observed under the oil immersion lens. The relative length, arm ratio index, and centromere index were measured and calculated according to CONFERENCE (1960) and the standard of LEVAN et al. (1964) to determine the centromere type.

### Isoenzyme analysis

In the process of cell culture, several breeds are cultured at the same time using the same apparatus and reagent, making cross-contamination a possibility (NIMS et al., 1998). Because isoenzymes vary within species and even within individuals or tissues of the same species, NIMS et al. (1998) suggested that even if only 10% of cells are contaminated, this could still be detected by isoenzyme analysis. Currently, analysis of isoenzyme polymorphisms has been adopted as a standard method for detection of interspecies cross-contamination by many world-famous culture collection centres such as ATCC, ECACC and DSMZ (DREXLER et al., 1999). In animals, lactic dehydrogenase (LDH) and malic dehydrogenase (MDH) are important enzymes of the metabolic pathways glycolysis and tricarboxylic acid cycle. The electrophoretic mobilities of these two isoenzymes, LDH and MDH, were determined using polyacrylamide gel electrophoresis (FRESHNEY, 2000) with some modifications. Isoenzyme patterns of Big Bone chicken were obtained and compared with other breeds simultaneously cultured in the laboratory. The technical details of the procedure were as follows:

### Sample preparation

Cells were harvested and rinsed three times with PBS, then the protein extraction solution was added, adjusting the cell density to $5 \times 10^7$ cells/mL. After centrifugation at 4°C and 1000rpm for 2min, the supernatant was aliquoted and stored at –70°C.

### Electrophoresis

Every sample well was loaded with 20 ~ 50μL loading solution, which was prepared by mixing the sample with 40% sucrose solution (1:1) and 2.5μL bromophenol blue

solution. Then the gel electrophoresis took place at 4°C and 120V. When the bromophenol blue migrated into the gel below the spacer, the voltage was changed to 220V. When the bromophenol blue had migrated to the bottom (0.5 ~ 1cm), the electrophoresis was finished.

### Staining

The spacer gel was cut off and the gel rinsed twice with distilled water. After that, the gel was placed at 37°C for 2h to stain in the dark inside an incubator. Afterwards photographs were taken (HE & ZHANG, 1999).

### Transfection of fluorescent protein genes

Using the lipofectamine-mediated method with some modifications (TSUCHIYA et al., 2002), three fluorescent protein genes including DsRed1, EGFP and EYFP were transfected into the in-vitro fibroblasts of Big Bone chicken with 2μg plasmid DNA (pDsRed1-N1, pEGFP-C1, pEYFP-N1, respectively) and 6μL lipofectamine (Lipofectamine 2000). Transfection was conducted when cell confluence reached 60% ~ 70%.

Six to eight hours after transfection, the transfection solution was discarded and complete MEM containing 10% serum was added. The transfection results were observed at 24h, 48h, 72h, 1 week, and 2 weeks after transfection, under a laser scanning confocal microscope. The transfection efficiency was calculated by choosing 10 visual fields in every well and counting the total cells and the positive cells in every visual field. The distribution of fluorescent proteins in positive cells was determined by detecting the fluorescent intensity under the high power lens of the laser scanning confocal microscope.

## RESULTS

### Cell morphology

In primary culture, the cells grew out of the edge of tissue blocks 1 day after seeding, and soon after the cells rapidly multiplied outwards around tissue blocks in a radiated manner. After 2 to 3 days, cells spread along the flask wall and the confluence reached 80% ~ 90%. In primary culture, fibroblasts mingled with epithelial cells. With sequential passages, epithelial cells were excluded from the culture, and we obtained pure fibroblasts. Primary cells had typical fibroblast morphology, showing the shape of a shuttle or irregular triangle with plump cytoplasm and a clear nucleolus (Fig. 1A). After subculture, cells grew well and showed the flame-like or whirlpool-like shapes (Fig. 1B).

### Microorganism detection

#### Detection of bacteria and fungus

In the actual test group and negative control, the medium was clear, showing no turbidity or other abnormal changes. In contrast, apparent turbidity and precipitation was observed in the positive control group.

#### Detection of mycoplasmas

After being stained with Hoechst 33258, the cells showed smooth surfaces, round or oval nuclei with blue fluorescence, and no filamentous blue fluorescence around nuclei when observed under fluorescence microscope (Fig. 2). This result indicates that the *in vitro* cultured fibroblasts were free of *mycoplasma*. To reconfirm this result, the mycoplasma detection kit (Roche) was used, also with a negative outcome.

#### Detection of viruses

Virus injuries were not found in cells under the phase contrast microscope, and also the result of hemadsorption test was negative.

### Cell viability

The viability detected by trypan blue staining was 98.1% before cryo-preservation and 96.6% after recovery, with a non-significant difference (P>0.05). Cells after recovery grew well and adhered to the flask wall in 30 ~ 60min, and had spread along the bottom wall by 48h later. Growth velocity and morphology of cells before cryo-preservation and after recovery showed no significant difference.

### Growth curve

The growth curve of Big Bone chicken assumed an "S" shape. Thus, cells experienced the latent phase, the exponential growth phase, the stationary phase and the decline phase, and the PDT was about 30h (Fig. 3). In addition, the growth curve reflected the dynamic growth state of cells. Cells were at the latent phase within 0 ~ 2d and reached the exponential growth phase afterwards. Cell density was at its maximum at day 4. From the 5$^{th}$ day onwards, cells entered the stationary phase and began to degenerate and die after day 7.

### Karyotype analysis

This study analyzed 100 well-spread metaphases of the P1 ~ P3 fibroblasts of Big Bone chicken. From the karyotype and chromosome parameters, no abnormality in chromosome structures was detected (Fig. 4, Table1). The frequency of diploid cells was 91%. Generally, if cells with a certain chromosome number account for more than 75% of the total, this chromosome number can therefore be regarded as the number of diploids. Therefore, it could be confirmed that the cultured cells were stable diploid and the chromosome number of diploid was 78, in agreement with studies on other chicken breeds.

### Isoenzyme analysis

In the LDH isoenzymogram, four bands representing LDH1, LDH2, LDH3 and LDH4 were distinct from the anode to the cathode. The LDH activity of the four breeds was similar, LDH2, LDH3 and LDH4 displaying heavy shading and LDH1 light shading (Fig. 5). In the MDH isoenzymogram, there was a cellular solute form (s-MDH) near the anode and a mitochondrial form (m-MDH) near the cathode (Fig. 6). Relative enzyme mobility of the four breeds was similar although small differ-

ences were visible. These results indicate that each breed had its characteristic bands and that there was no cross-contamination between different breeds.

### Transfection analysis of three fluorescent protein genes

The three fluorescent genes pDsRed1-N1, pEGFP-C1 and pEYFP-N1 are widely used in living cells and organisms as marker genes to dynamically observe the expression, distribution and function of target proteins (HEIM et al., 1995; CHENG et al., 2003). Here, the expression of these genes in the Big Bone chicken fibroblasts was observed at 24h, 48h, 72h, 1 week, and 2 weeks after transfection under the laser scanning confocal microscope. The results showed that the highest number of cells with fluorescent signals, the strongest fluorescence intensity and the highest transfection efficiency appeared 48h after transfection (Fig. 7, Table 2).

Twenty four hours after transfection, the exogenous genes had begun to become expressed in some cells and all three experimental groups showed obvious fluorescent signal. Besides, a few positive cells became shrunken, deciduous and disintegrated. Forty eight hours after transfection, the number of positive cells increased in all three groups, many bright dots could be seen in the visual field, and the transfection efficiency reached its maximum at this time. Seventy two hours after transfection, there was no further increase in positive cells, and in most of the positive cells fluorescent proteins were not expressed in the vacuoles. From the 7$^{th}$ day onwards, the number of positive cells decreased and fluorescence intensity weakened gradually. However, two weeks later, there were still a small number of cells expressing fluorescence.

The red fluorescence was distributed in dots around the nucleus (Fig. 7D, arrow a; b) and uniformly in other parts (Fig. 7D, arrow c) and showed the weakest fluorescence intensity. Intensity of the green fluorescence was the strongest, with a stronger expression in the nucleus (Fig. 7E, arrow d) and some vacuoles, but without expression in the cytoplasm (Fig. 7E, arrow e). The yellow fluorescence had a slightly stronger expression in the nucleus (Fig. 7F, arrow f) than in the cytoplasm, where a ribbon-like texture with a large number of non-expressed vacuoles and empty strip zones (Fig. 7F, arrow g) appeared.

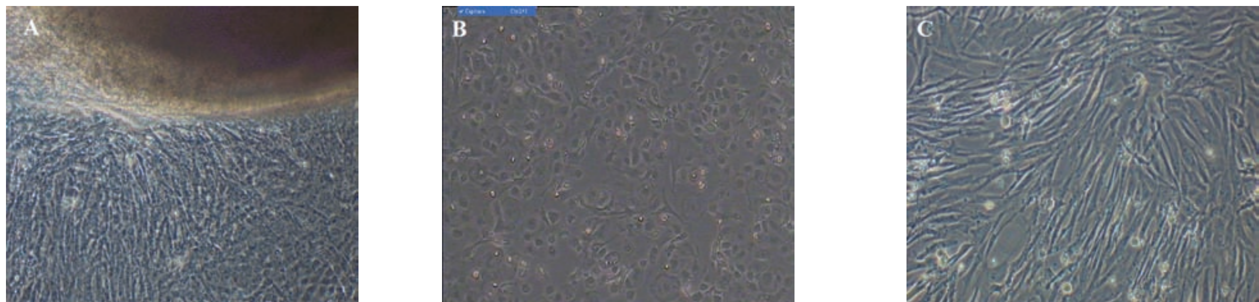


Fig. 1. – Primary and subcultured cells of Big Bone chicken (100×). A: Primary Cells grew out from the edge of tissue blocks at 1d after seeding. B: Primary cells: fibroblasts mingled with epithelial cells. C: Pure subcultured fibroblasts.

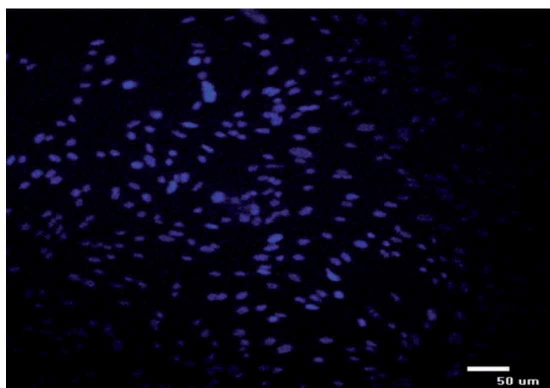


Fig. 2. – Detection of mycoplasmas by Hoechst33258 staining. This figure shows *mycoplasma* negative fibroblasts of Big Bone chicken.

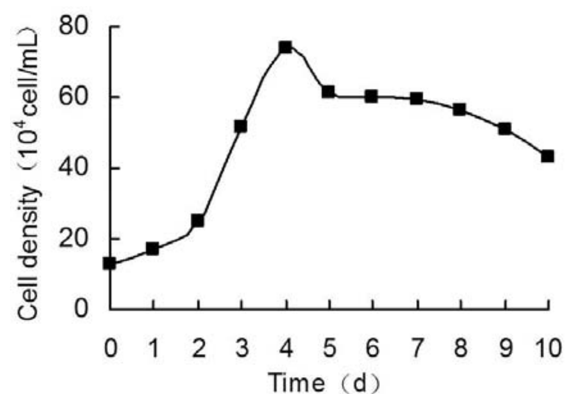


Fig. 3. – Growth curve of Big Bone chicken fibroblasts.

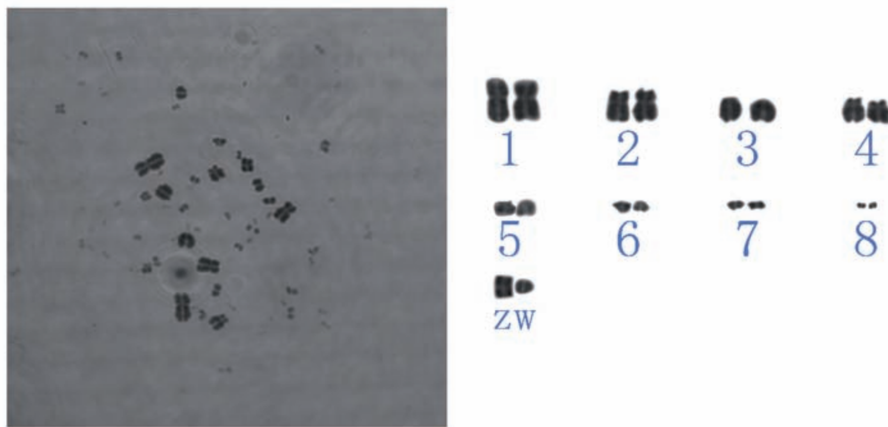


Fig. 4. – Chromosome karyotype of Big Bone chicken ZW (♀)

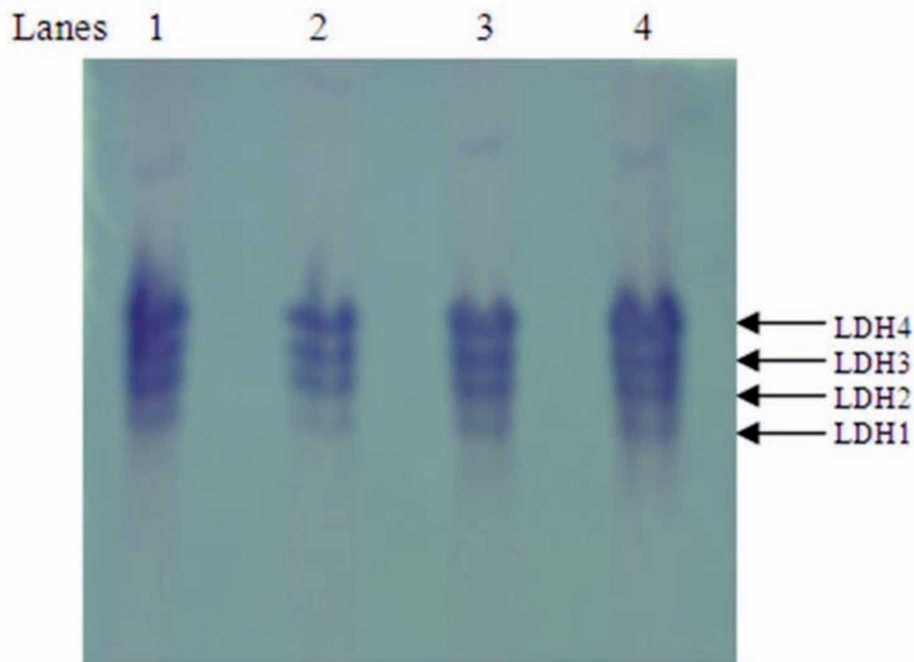


Fig. 5. – LDH isoenzyme typing of four chicken breeds. Lane 1: Youxima chicken. Lane 2: Chinese Game chicken. Lane 3: Qingkedan chicken. Lane 4: Big Bone chicken.

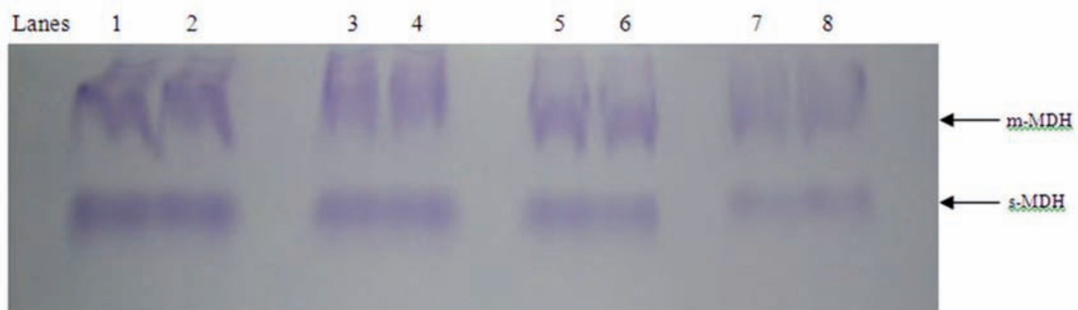


Fig. 6. – MDH isoenzyme typing of four chicken breeds. Lanes 1 & 2: Youxima chicken. Lanes 3 & 4: Chinese Game chicken. Lanes 5 & 6: Qingkedan chicken. Lanes 7 & 8: Big Bone chicken.

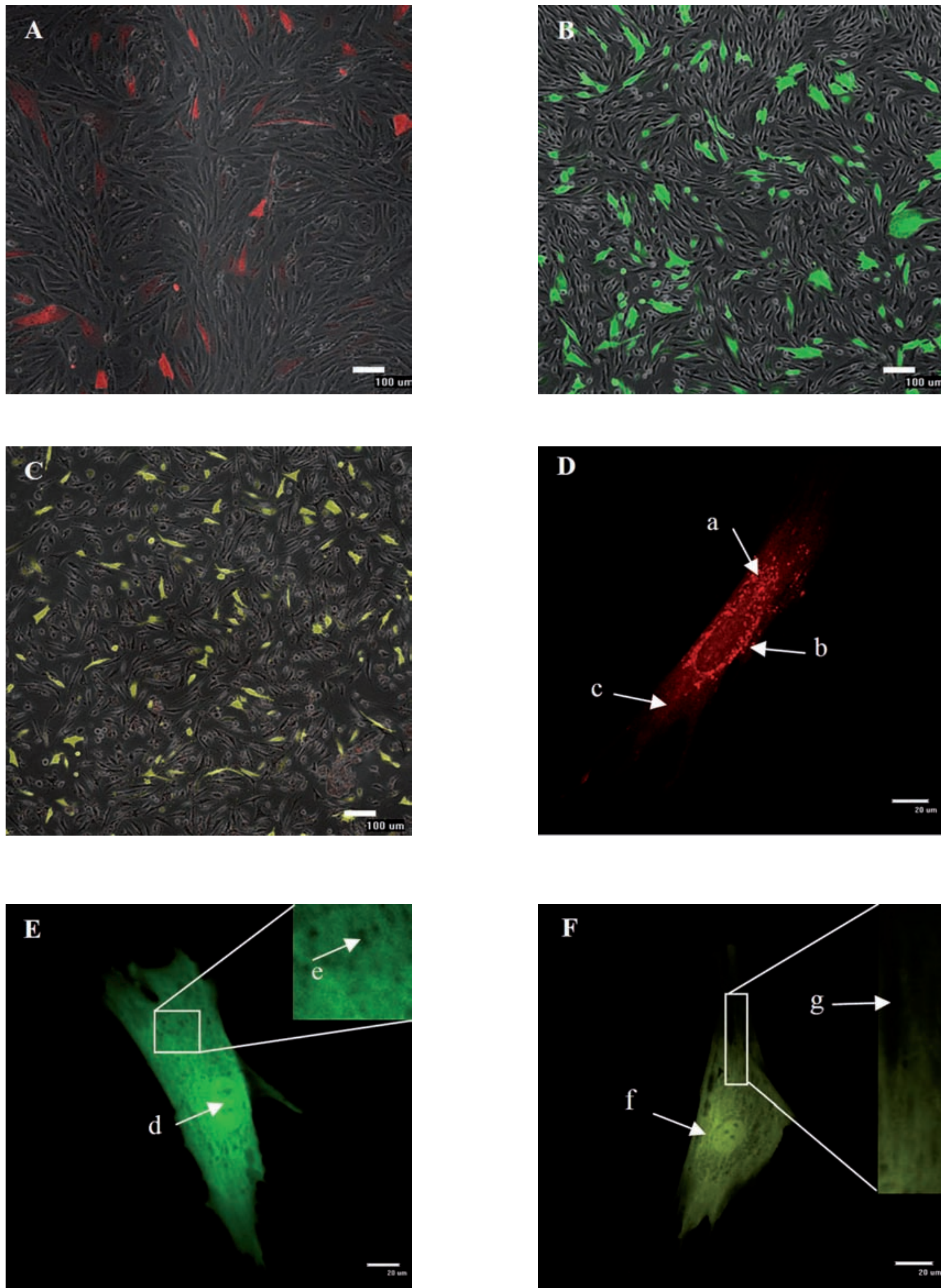


Fig. 7. – The expression of DsRed1, EGFP and EYFP in fibroblasts of Big Bone chicken. A & D: The transfection results of DsRed at 48h (100×, 400×). B & E: The transfection results of EGFP at 48h (100×, 400×). C & F: The transfection results of EYFP at 48h (100×, 400×). DsRed1, EGFP and EYFP are the red, green, and yellow fluorescent protein genes being located in plasmid DNA of pDsRed1-N1, pEGFP-C1, pEYFP-N1, respectively. D, E, F: The distribution of the three fluorescent proteins in cells. Arrows a and b show dot-like, red fluorescence while arrow c indicates the uniform fluorescent signal. Arrow d points towards the stronger green fluorescence in the nucleus and arrow e shows the vacuoles without fluorescence. Arrow f indicates the stronger yellow fluorescence in the nucleus and arrow g the empty strip zones without fluorescence.

TABLE 1

Chromosome parameters of Big Bone chicken (♀)

| Number | Relative length (%) | Arm ratio | Centromere index (%) | Centromere type |
|---|---|---|---|---|
| 1 | 21.73 | 1.27 | 44.05 | M |
| 2 | 16.52 | 1.71 | 36.90 | SM |
| 3 | 12.52 | ∞ | 0 | T |
| 4 | 10.95 | 1.86 | 34.97 | SM |
| 5 | 8.70 | ∞ | 0 | T |
| 6 | 7.00 | ∞ | 0 | T |
| 7 | 6.61 | ∞ | 0 | T |
| 8 | 5.39 | ∞ | 0 | T |
| Z | 10.61 | 1.03 | 49.26 | M |
| W | 7.04 | 1.19 | 45.66 | M |

* relative length = $\frac{\text{a single chromosome length}}{\text{all haploid chromosome length} + \text{chromosome X length}} \times 100\%$

arm ratio index = $\frac{\text{the long arm length}}{\text{the short arm length}}$

centromere index = $\frac{\text{the short arm length}}{\text{the chromosome length}} \times 100\%$

According to the Levan criteria for classification, the ones with arm ratio index in the range of 1.0 ~ 1.7 and centromere index in the range of 50.0% ~ 37.5% are metacentric chromosomes (M), the ones with arm ratio index in the range of 1.7 ~ 3.0 and centromere index in the range of 37.5% ~ 25.0% are submetacentric chromosomes (SM), the ones with arm ratio index >7.0 and centromere index in the range of 12.5% ~ 0.0% are telocentric chromosomes (T).

TABLE 2

Transfection efficiencies of three fluorescent protein genes in Big Bone chicken fibroblasts

| Transfection time (h) | pDsRed1-N1 (%) | pEGFP-C1 (%) | pEYFP-N1 (%) |
|---|---|---|---|
| 24 | 10.6 | 22.9 | 19.6 |
| 48 | 17.8 | 26.5 | 23.4 |
| 72 | 16.2 | 25.1 | 22.6 |

# DISCUSSION

## Cell culture

Tissue adherent culturing method and enzyme digestion are two frequently used methods for primary cell culture (FRESHNEY, 2006). The concentration and action time changes, to some extent, in enzyme-injured cells and thus affects normal development of cells severely (LI et al., 2009b; YANG et al., 2009). In comparison, the tissue adherent culturing method is simple and feasible, avoiding the injury of enzyme digestion. Furthermore, cells that are cultured using this method have good homogenicity and strong vitality. We used the tissue adherent culturing method to primary culture fibroblasts of Big Bone chicken, and managed to grow cells well with typical fibroblast morphology. Passage changes the growing environment of cells, and trypsinization causes some injuries (RYAN & MAXWELL, 1986; PARK et al., 2006; CHO et al., 2008). Consequently after high numbers of passages, cells usually vary in their biological characteristics, especially in genetic parameters (SUN et al., 2006a). For preservation, times of passage should be decreased considerably, keeping the number to a maximum of five passages. In addition, for ensuring the cell viability after recovery, the freezing density should be more than $1\times10^6$ cells/mL (FRESHNEY, 2006).

Because the tolerance of epithelial cells and fibroblasts to trypsin is different when trypsinized, fibroblasts can be detached from the flask wall more readily and adhere more quickly after passage, whereas most epithelial cells need the support of growing matrices like collagen or other extracellular matrix components. Otherwise, they fail to adhere in a short time and are easily shed off when using gentle mechanical agitation. Because of these differences, fibroblasts will quickly outgrow their epithelial counterparts. Consistent with earlier studies, pure fibroblasts of Big Bone Chicken were obtained here after two to three passages, and in order to minimize the injuries caused by trypsinization, passage numbers were limited to five to guarantee the best vitality (GUAN et al., 2005; ZHOU et al., 2005).

## Micro-organism detection

Micro-organism contamination is a common problem in cell culture and may originate from air, equipment, operation, serum and tissue samples. Bacteria and fungus grow rapidly, consume nutrients, produce toxins that inhibit cell growth, and kill cells within a short time (FRESHNEY, 2000). After normal culturing for five days, we did not observe, by naked eye and phase contrast

microscopy, any turbidity or other evidence of contamination from bacteria and fungi. Using indirect DNA fluorescence staining, a convenient and reliable protocol, the existence of *mycoplasma* was also ruled out (MASOVER & BECKER, 1998; FRESHNEY, 2000). These results suggest that the Big Bone Chicken fibroblasts are free of infections from microorganisms and suitable for genetic resource preservation.

### Cell viability before freezing and after recovery

Generative cells, somatic cells, stem cells, zygotes and embryos can all be cryopreserved in cell banks (GUAN et al., 2007). In the freezing process when the temperature goes down to 0°C, cells suffer from physical and chemical injuries (HAN & BISCHOL, 2004). Adding cryoprotectant during freezing can prevent ice crystals and injuries caused by them. In this study, we prepared the freezing solution as 10% DMSO, 50% FBS, 40% MEM. After conventional harvest of cells, an appropriate amount of freezing solution was added to prepare the cell suspension. Then cells were placed at 4°C for 1h, at -20°C for 1h, at -70°C for 12h in order, and transferred to liquid nitrogen for long-term preservation. For recovery, the freezing tube was taken out from liquid nitrogen and thawed quickly in a 42°C water bath. The key point for cryogenic preservation and recovery of cells was programmed freezing and quick thawing. We found a difference between cell viabilities before cryo-preservation and after recovery, which could probably be related to injuries from freezing and recovery. Nevertheless, the average viability after recovery was 96.6% which indicated that freezing and recovery had little influence on the overall cell viability. Thus, it seems feasible to preserve fibroblast cell banks of Big Bone chicken using such long-term cryo-preservation techniques.

### Growth curve

After seeding, there was a latent phase of about 48h, which was probably the adaptive phase during which cells recovered from injuries caused by trypsinization. Afterwards, cells entered the exponential growth phase. Finally, from the $4^{th}$ day on, after reaching the maximum cell density, cell growth became slow and stopped while some cells were floating. This was probably the stationary phase. The available growing space was gradually occupied, which generated contact and density inhibition, eventually causing the culture to enter the stationary phase and ultimately, the decline phase.

### Karyotype analysis

Chromosome number and karyotype are the basis of cytogenetics. They are reliable indices for identifying the taxonomic and sexual origin of a cell line, and also allow assessing whether the cell line is stable or variable. Thus, they represent the characteristics of a certain cell line and are not only used to analyse genetic stability but also to test for cross-contamination. According to the international karyotype standard, poultry contain eight pairs of macrochromosomes, the sex chromosomes Z and W, and 30 pairs of microchromosomes (LADJALI-MOHAMMADI et al., 1999). Because the purpose of our experiment is to conserve the genetic resources of Big Bone chicken, the fibroblasts must maintain their diplont character similar to *in vivo* cells. We improved the freezing technique and decreased the passages necessary to obtain a stable diploid cell line. Karyotypic analysis was conducted on 100 cells, and our results indicate that the Big Bone chicken cells are diploid with nine pairs of macrochromosomes and 30 pairs of microchromosomes; 91% of the cells examined were diploid. A small percentage of cells displayed abnormal chromosomes, presumably as a result of chromosome loss or overlap during preparation or chromosomal damage during culture and passage *in-vitro*. Because most chromosomes are microchromosomes, which are easily missed in the process of slide making, chromosome number and morphology of chicken karyotypes are difficult to determine.

### Isoenzyme analysis

We analysed the isoenzyme patterns of LDH and MDH in our study and obtained clear, distinct bands.

ZENG & CHEN (1997) retrieved five to eight clear bands in the electrophoretic analysis of eight kinds of chicken tissues; in the pectoral muscle, five bands were found, among which LDH4 and LDH5 predominated over others. This pattern might coincide with characteristics of these breeds, which are not good at flying. LIU & YU (1997) analysed LDH isoenzymes in the cardiac muscle, in the liver and from blood samples of Chinese *Gallus gallus* using thin layer isoelectric focusing electrophoresis, and found five bands in all three types of tissues. In contrast, we obtained four bands solely from fibroblasts of Big Bone chicken embryos. This could be either explained by breed difference or the heterogenous origins of the fibroblasts. However, the LDH bands of the four breeds were clear and distinct, thus suggesting that the Big Bone Chicken fibroblast cell line we established is not contaminated by cells from other breeds in the same lab.

Similarly to mammals, MDH of birds includes a cellular solute form (s-MDH) and a mitochondrial form (m-MDH), and the migration rate of the former is higher than that of the latter (JOHN & CHARLES, 1987). In our experiment, there were two MDH bands, which is in accordance with the MDH activity of chicken embryos during the first 16 days of development (MA & QIU, 1995), indicating that cells cultured *in-vitro* have similar MDH activity to biological tissues.

### Expression of exogenous genes

The three enhanced fluorescent protein genes screened in our experiment have stable structures, high-level expression and are not dependent on the germ-line (HEIM et al., 1995; BAIRD et al., 2000; CARRIE et al., 2003). Concentrations of DNA and lipofectin, the DNA incubation time, lipofectin-plasmid DNA complexes, and the presence of serum all can affect the efficiency of transfection, as shown by research on Vero cells, HeLa cells and various other cell lines (TSENG et al., 1999; RUI et al., 2006; SHU et al., 2007). In our experiment, the transfer efficiency varied from 10.6% to 26.5% with the optimized ratio of plasmid and lipofactamine. After 2 weeks a few dispersed positive cells remained showing fluorescent

signals. This means some that cells gain the ability to express DsRed, EGFP and EYFP stably. After transfection, cells at different dividing phases could be seen, and the growth of transfected cells was not different to the control group, while some transfected cells changed their morphology. This result showed that the transfected cells were to some extent not affected by fluorescent protein. It is very possible that when cells accumulate enough exogenous fluorescent protein, morphologically-changed cells will greatly increase, which leads to the growth changes of cells finally. Furthermore, fluorescent proteins were not homogeneously distributed; for example, EGFP was mainly found in the nucleus and DsRed expressed surrounding the nuclear membrane and forming a red ring profile (see Fig. 7). These differences of fluorescent proteins are typical for molecular tags (labelling) for investigating the functional role of the genes of interest. From these results, it can be concluded that Big Bone chicken fibroblasts possess the potential to be used in transgenic studies.

## CONCLUSIONS

In conclusion, our results strongly indicate that the established fibroblast cell line of Big Bone chicken embryos has stable genetic properties and normal biological characteristics. Moreover, we have been able to preserve the unique genetic resource of the Big Bone chicken at the cellular level, and thus provide effective technical and theoretical references and suitable biological material for related studies.

## ACKNOWLEDGEMENTS

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# Comparison of waterbird communities in a Mediterranean salina – saltmarsh complex

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ABSTRACT. Coastal wetlands provide habitat for large numbers and many species of waterbirds. Man-made salinas are a particular habitat type often found in such wetlands. This study is an initiative to understand the differences in bird communities between a salina (including evaporation ponds and prebasin) and a saltmarsh. Bird counts and nest surveys took place in the wetlands of Angelochori, Thessaloniki, Greece, in 1991, when the salina was inactive, and in 1997, 2000-01 when it was active. Counts in evaporation ponds were richer in species, abundance and nests compared to the prebasin and the saltmarsh. These three wetland types supported different bird communities. Similarities among bird communities depended on the inundation of the salina with seawater. Evaporation ponds in their inactive period presented low similarity with the communities of the prebasin and the saltmarsh; in the active period this was observed only for the saltmarsh. Species showing clear selection for the evaporation ponds were *Charadrius alexandrinus*, *Calidris alpina*, *Calidris minuta, Recurvirostra avosetta*, *Sterna hirundo*, *Sternula albifrons*, *Sterna sandvicensis* and *Haematopus ostralegus*; the prebasin was preferred by *Phoenicopterus roseus* and *Anas platyrhynchos,* and the saltmarsh by *Anas querquedula*, *Anas clypeata*, *Plegadis falcinellus, Tringa totanus*, *Tringa glareola*, *Tringa stagnatilis* and *Himantopus himantopus*.

KEY WORDS: conservation, shorebirds, waders, waterfowl, estuary

## INTRODUCTION

Ecologists' knowledge of the functioning of ecosystems is needed to broaden the scientific basis of decisions on land use and management (Dale et al., 2000). In Europe, it is estimated that half to 2/3 of wetland areas have been destroyed, while degradation of the remaining wetlands continues (Commission of European Communities, 1995). The need to maintain wetlands becomes imperative particularly for coastal wetlands due to increasing intensification of agriculture and pressures originating from housing needs and tourism (Lovvorn & Baldwin, 1996; European Environment Agency, 2000).

Among other uses of coastal wetlands, salt collection in salinas is often a major activity. Coastal salinas (also known as solar ponds or saltworks) are anthropogenic habitats where salt is extracted from marine water through evaporation caused by solar radiation. They occur in large expanses in several geographical regions, extending, for example, over 1000 km² in the Mediterranean basin (Sadoul et al., 1998).

In the Mediterranean, over half of the approximately 500,000 migratory and wintering shorebirds that occur in the region use salinas (Sadoul et al., 1998). The latter provide important nesting, foraging and roosting habitats to waterbirds that prefer saline or hypersaline habitats (Rufino et al., 1984; Goutner & Papakostas, 1992; Masero & Perez-Hurtado, 2001). Findings suggest that salinas may be of particular value for some species, including waders and the greater flamingo (*Phoenicopterus roseus*) (Masero et al., 2000; Masero & Pérez-Hurtado, 2001; Paracuellos et al., 2002; Bechet et al., 2009; Dias, 2009), but not for some others such as grebes and ducks (Paracuellos et al., 2002).

In some cases, salinas are constructed in saltmarsh areas (Sadoul et al., 1998), a major, widely distributed, coastal natural habitat. Saltmarshes with high structural and plant diversity, especially on sites where freshwater seepages provide a transition from fresh to brackish conditions, are particularly important for avifauna (Milsom et al., 2000; Sokos, 2006). For centuries saltmarshes have been subject to modification or destruction because of human activities (e.g. Goutner, 1997); further impacts on saltmarshes are expected by global sea level rise and warming (Adam, 2002).

Although various studies have focused on birds either in salinas or saltmarshes (e.g. Milsom et al., 2000; Paracuellos et al., 2002), the simultaneous comparison of waterbird communities in both land uses rarely takes place (e.g. Bollman & Thelin, 1970; Catry et al., 2004) and that is the aim of the present study. Additionally, the observed cessation and reactivation of the salina in our study area provided an excellent opportunity to detect the effect of that short-term habitat change in waterbird populations.

## MATERIAL AND METHODS

### The study area

The wider area of Central Macedonia is characterized by the presence of important wetlands for birds. The wetland of Angelochori (40° 29' N – 22° 49' E) is located on the east coast of the Thermaikos Gulf, near the city of Thessaloniki, Macedonia, Hellas (Greece). The climate is Mediterranean with a drought period during the summer. In 1991 (first year of our study), the mean air temperature during spring was 13.1ºC and precipitation 185.2mm (March-April-May). In the 1997, 2000-01 period, the mean air temperature during spring was 14.4ºC and precipitation 120.8mm (mean values for the three years according to the Meteorological Station of Thessaloniki Airport). The wetland is surrounded by a non-tidal coast in the west and cultivated fields in the east (Fig. 1). The study area included both the salina with its evaporation ponds and prebasin (Angelochori salina) and the saltmarsh.

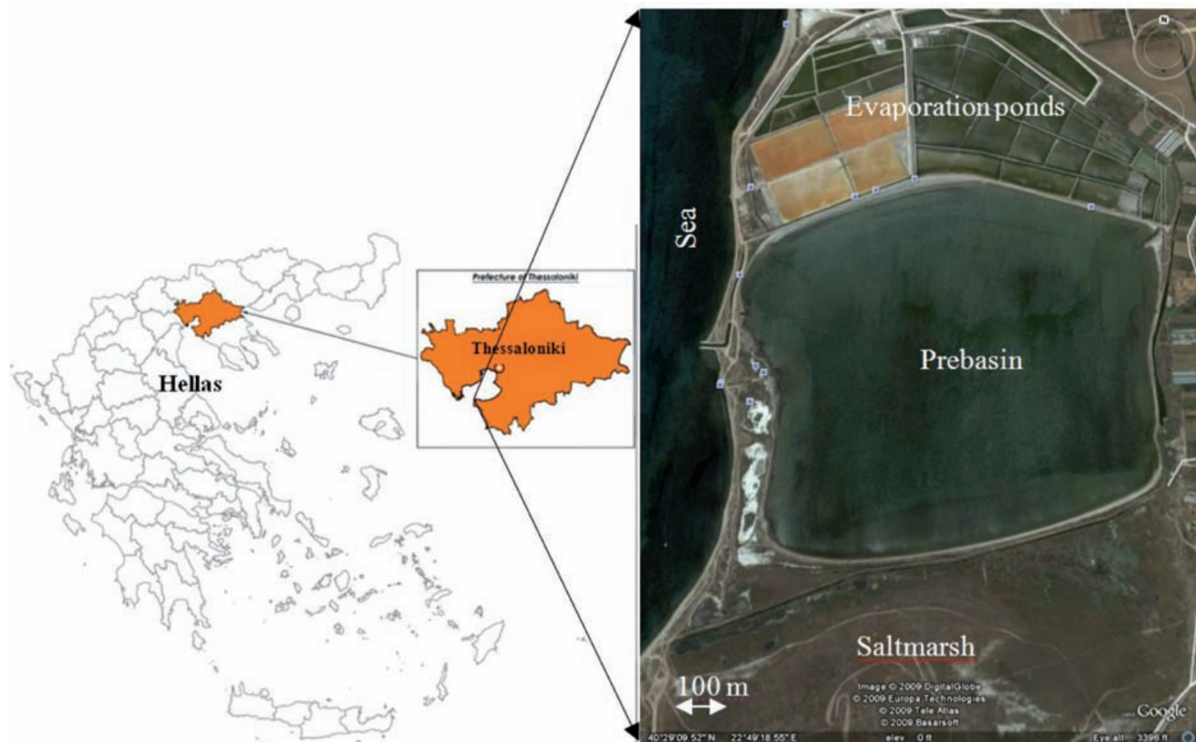


Fig. 1. – Satellite image of the study area (with map showing its location in Greece) with the localities of the three wetland types: evaporation ponds, prebasin and saltmarsh (source: Google Earth).

The evaporation ponds are delineated by small dykes without vegetation, about 30cm high, and the water depth varies from 0 to 15-20cm. Works aiming to improve salt harvesting in the salina prevented salt collection from 1989 to 1992. Thus, in the spring period of 1991, the evaporation ponds were not supplied with seawater and their flooded part extended over approx. 11ha, depending on the rainfalls. When the salina was active, in the spring periods of 1997, 2000-01, most of the ponds were flooded with seawater through the prebasin in late March – early April, and occupied an area of 20ha. Salt is usually collected during October; therefore in spring there is generally no or little human disturbance to birds using the wetlands.

The prebasin is a man-made lagoon with a total area 50ha, of which one third includes a muddy shore. According to Hellenic Saltworks Office in Angelochori, water depths vary from zero to a few centimetres in muddy areas, and up to 1.2m in the centre of the prebasin. In spring 1991, surface seawater did not enter the prebasin; the contrary happened in the spring study periods of 1997, 2000-01.

To the south of the prebasin there is a saltmarsh, with an area of approx. 30ha, covered with halophytic vegetation (mainly *Salicornia fruticosa, Tamarix* spp. and *Arthrocnemum glaucum*), which is flooded during the rainy period and dries out in early summer. The extent of flooding varied from 17ha in the wet spring of 1991 to approx. 5ha in the dry periods of 1997 and 2000-01. The saltmarsh relief varies, thus creating ponds and small islands. The saltmarsh is grazed by cattle, which in some cases trample bird nests and chicks.

According Dias (2009) water depth, pond area and water salinity were the most important factors that explain the presence of the bird species on the salinas. In our study area the extent of flooding, water depth and salinity in the three wetland types depends on rainfall, evaporation and seawater entry. Salinity is highest in the ponds, with an average of 170ppt, medium in the prebasin (60-70ppt) and lowest in the saltmarsh (3-5ppt). During

the inactive period of 1991, salinity decreased in the ponds and the prebasin due to lack of entry of seawater in spring, although seawater did enter on one occasion during the summer (Hellenic Saltworks Office in Angelochori).

### Waterbird surveys

Waterbird counts were carried out during spring in the study area when many wintering species, passage migrants and breeders coincide (GOUTNER et al., 2005). Waterbird surveys were conducted every 10 to 30 days during spring periods (5/3-29/5). In total, nine censuses were carried out in 1991, when the salina was inactive, and 16 in the 1997, 2000-01 period (five in 1997, six in 2000 and five in 2001) when the salina was active. These numbers of counts are adequate for the aims of such study (TUCKER, 1990; POMEROY & DRANZOA, 1997; MURIAS et al., 2002).

Wetlands were surveyed in their total area by observers walking along specific transects and visiting specific positions around and inside the study area. Surveys were conducted from 9 a.m. to midday (12:00). All waterbird species were identified and counted with the use of either a 16-60 × 70 telescope or 10 × 50 binoculars. Given the open space of the study area, these counts were expected to lead to accurate assessments of abundance for most species (DAVIS & SMITH, 1998). Species unlikely to be detected with this census method, due to their dispersal or concealment in vegetation, are not considered here (e.g. *Gallinago gallinago*) or were not included in statistical analyses (*Charadrius alexadrinus*).

### Nest surveys

Seven nest surveys were conducted only in 1991, from April 24$^{th}$ to August 11$^{th}$ by systematic rapid ground searching aimed at locating the nests without risking significant disturbance to nesting birds or chicks. Nest positions were marked on a map. Due to the difficulty in finding Redshank (*Tringa totanus*) nests, these were estimated indirectly from the number of birds (couples found) and their breeding behaviour (characteristic alarming calls near the nests).

### Statistical analysis for the number of species and abundances

The aim of the present study was to compare the three wetland types as land uses, independently of the microhabitats provided by each one of them to different bird species. For instance, evaporation ponds were expected to have higher availability of preferred microhabitat for wader species than the prebasin due to their shallow waters, but the opposite was expected for *Phoenicopterus roseus*, which prefers deeper waters. For that reason and as the three wetland types had similar sizes, absolute numbers of species and abundances were used in statistical comparisons.

The one factor within subjects analysis of variance (ANOVA) (MEYERS et al., 2006) was used to compare the number of species and abundances per census, measured at the same date simultaneously for the three wetland types, thus compensating for the influence of weather conditions and other factors on bird presence. The number of waterbird species was chosen as the response variable, the period as the within subject factor and the wetland type as the between subject factor. Normality and equality of variances were checked with the Kolmogorov-Smirnov and the Levene's test respectively (ZAR, 1996). The homogeneity of covariance was tested with the Mauchly Sphericity test. Whenever this test was significant, the F value was estimated with the Greenhouse – Geisser correction. In two cases, when some of the variables were not normally distributed, the non-parametric Friedman test was used. Post hoc tests were performed using the Bonferroni test, when there were multiple comparisons.

### Statistical analysis for bird community similarity between wetland types

Similarity between waterbird communities of the three wetland types was investigated with the Bray-Curtis index. This widely-used technique reliably compares similarity in species composition and abundance between sample pairs (KREBS, 1999). The similarity was tested in each pair of wetland types for each count according to whether the salina was inactive (1991) or active (1997, 2000-01). Average similarities were compared via the one factor within subjects analysis of variance (ANOVA) (MEYERS et al., 2006).

Correspondence analysis (HAIR et al., 2006) was also used to explore relationships between the wetland types with regard to bird species and numbers. The variable "bird species" was weighted using the variable "number of species". The former was combined with the variable "wetland type" and then correspondence analysis was performed. In order to find the strength of the relationship between the categories of each variable, their distances were measured using the chi square method. The normalization for the distances was performed with the symmetrical method. The solution for the optimum number of dimensions (axes) was found by using the criterion of the variable with the fewer categories. This criterion defines that one should use "categories minus one" dimensions to represent the relationships (GARSON, 2008).

### Statistical analysis for habitat selection

The Jacobs preference index (JACOBS, 1974) was used to make an assessment of the preference for the three wetland types by bird species taking into account the status of the function of the salina (inactive in 1991, active in 1997, 2000-01). The index (D) ranges from -1 (complete avoidance) to +1 (total selection); it is calculated by the form $D = (r - p) / (r + p - 2rp)$, where r is the proportion of individuals (of a specific bird species) in wetland type A, and p the proportion of the habitat area covered by wetland type A. The Jacobs index was calculated only for the species that were found in more than one wetland type. For species that were found in three or more censuses and with more than ten individuals in each period (inactive period in 1991, active period in 1997, 2000-01), index values between the two periods were compared via an independent samples T test. When there were violations of the assumptions of normality/equality of the variances, the alternative Mann-Whitney Test was used (ZAR, 1996).

## RESULTS

### Numbers and abundance of bird species – Bird community similarities between wetland types

In the 25 censuses carried out during the four spring seasons, 9528 birds belonging to 27 species were recorded in the three wetland types (Table 1); 21 species were observed in the evaporation ponds, 20 in the prebasin and 20 in the saltmarsh. In 1991, we observed 24 species and in the 1997, 2000-01 periods we found 21 species. The most numerous species were *Recurvirostra avosetta*, *Himantopus himantopus*, *Charadrius alexadrinus, Phoenicopterus roseus*, *Tringa totanus, Philomachus pugnax*, *Tadorna tadorna* and *Anas querquedula*.

TABLE 1

List of bird species and total numbers of individuals in each wetland type (all visits included).

| Species[a] | Species codes[b] | Evap. ponds | Prebasin | Saltmarsh |
|---|---|---|---|---|
| *Ardea cinerea* Linnaeus, 1758 | Ard cin | 10 | 13 | 11 |
| *Egretta garzetta* Linnaeus, 1766 | Egr garz | 7 | 7 | 4 |
| *Plegadis falcinellus* Linnaeus, 1766 | Pleg falc | 0 | 0 | 34 |
| *Phoenicopterus roseus* Pallas, 1811 | Phoen ros | 40 | 925 | 8 |
| *Anas platyrhynchos* Linnaeus, 1758 | Anas plat | 1 | 54 | 20 |
| *Anas penelope* Linnaeus, 1758 | Anas pen | 0 | 4 | 0 |
| *Anas acuta* Linnaeus, 1758 | Anas ac | 0 | 4 | 0 |
| *Anas querquedula* Linnaeus, 1758 | Anas querq | 2 | 256 | 278 |
| *Anas clypeata* Linnaeus, 1758 | Anas clyp | 0 | 4 | 48 |
| *Tadorna tadorna* Linnaeus, 1758 | Tad tad | 308 | 226 | 105 |
| *Fulica atra* Linnaeus, 1758 | Ful at | 0 | 13 | 0 |
| *Haematopus ostralegus* Linnaeus, 1758 | Haem ostr | 19 | 1 | 2 |
| *Charadrius alexandrinus* Linnaeus, 1758 | - | 744 | >302 | >177 |
| *Calidris minuta* Leisler, 1812 | Cal min | 66 | 0 | 50 |
| *Calidris alpina* Linnaeus, 1758 | Cal alp | 44 | 0 | 0 |
| *Calidris ferruginea* Pontoppidan, 1763 | Cal fer | 35 | 0 | 29 |
| *Philomachus pugnax* Linnaeus, 1758 | Phil pugn | 236 | 370 | 165 |
| *Tringa totanus* Linnaeus, 1758 | Trin tot | 197 | 316 | 440 |
| *Tringa nebularia* Gunnerus, 1767 | Trin nebul | 4 | 0 | 0 |
| *Tringa glareola* Linnaeus, 1758 | Trin glareo | 10 | 6 | 66 |
| *Tringa stagnatilis* Bechstein, 1803 | Trin stagn | 0 | 0 | 34 |
| *Recurvirostra avosetta* Linnaeus, 1758 | Recur avos | 2034 | 78 | 56 |
| *Himantopus himantopus* Linnaeus, 1758 | Him him | 206 | 403 | 642 |
| *Burhinus oedicnemus* Linnaeus, 1758 | Burh ohed | 4 | 4 | 7 |
| *Sterna hirundo* Linnaeus, 1758 | St hir | 97 | 8 | 2 |
| *Sterna sandvicensis* Latham, 1787 | St sandv | 118 | 0 | 0 |
| *Sternula albifrons* Pallas, 1764 | St albifr | 164 | 10 | 0 |
| **Total** | | **4346** | **3004** | **2178** |

[a]Species names according to Legakis and Maragou (2009)
[b]Species codes as used in Figs 3-4 are given in this column.

The mean number of waterbird species per census between wetland types was slightly higher in the ponds (Table 2), but the application of the one factor within subjects ANOVA did not reveal significant differences, either in the 1991 or in the 1997, 2000-01 period [1991: Greenhouse-Geisser F (1.209, 9.672)=1.258, p>0.05; 1997, 2000-01: Sphericity assumed F (2, 20)=2.482, p>0.05)].

There was no significant difference in mean waterbird abundance per census between the wetlands in 1991 (Friedman $X^2(2)=3.556$, exact p>0.05). For the 1997, 2000-01 period, however, there were differences between the wetlands (Friedman $X^2(2)=10.364$, exact p=0.004<0.01). The Bonferroni test revealed that the difference in abundance was significant only between ponds and saltmarsh (p=0.005<0.01). For the same wetland types, no significant differences were found in the number of waterbird species and abundance within the study periods (p>>0.05).

Bray-Curtis percent similarities are showed in Fig. 2. Mean similarity values of wetland pairs were different in both periods (1991: Sphericity assumed F (2, 16)=41.5, p=0.000, 1997, 2000-01: Sphericity assumed F (2, 30)=8.3, p=0.001). During 1991, the Prebasin-to-Saltmarsh mean similarity was higher than Ponds-to-Prebasin (p=0.001) and Ponds-to-Saltmarsh (p=0.000). Between Ponds-to-Prebasin and Ponds-to-Saltmarsh we did not find significant difference (p=1). During 1997, 2000-01 Ponds-to-Saltmarsh mean similarity was lower than

Ponds-to-Prebasin (p=0.003) and Prebasin-to-Saltmarsh (p=0.037). Ponds-to-Prebasin similarity was higher than the Prebasin-to-Saltmarsh, which, however, was not statistically significant (ANOVA, p = 0.41).

TABLE 2

Mean number (± SE) of waterbird species (sp.) and abundance (ab.) per count in each wetland type of the Angelochori wetland in the spring of 1991 (inactive salina) and in the springs of 1997, 2000-01 (active salina) (Spring 1991: n=9 counts; Springs 1997, 2000-01: n=16 counts).

| | **Ponds** | | **Prebasin** | | **Saltmarsh** | |
|---|---|---|---|---|---|---|
| | **´91** | **´97, ´00 -´01** | **´91** | **´97, ´00 -´01** | **´91** | **´97, ´00 -´01** |
| sp. | 6.56±0.44 | 6±0.81 | 5±0.6 | 4.64±0.87 | 5.67±0.99 | 3.91±1.06 |
| ab. | 125.8±21.5 | 209.9±40.5[a] | 84.1±12.4 | 157.91±63.5 | 91.56±22.4 | 105.82±41[b] |

The two letters ([a, b]) indicate the only significant different counts at the 0.05 level.

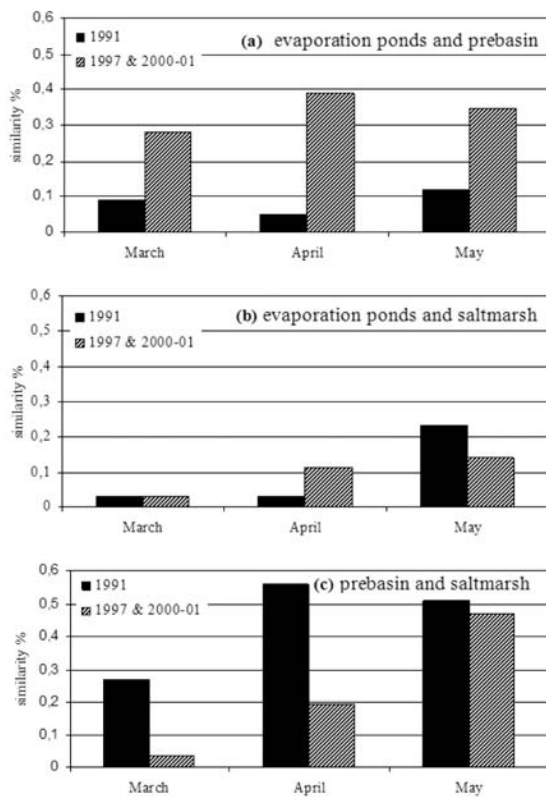


Fig. 2. – Bray-Curtis percent similarity between a) evaporation ponds and prebasin, b) evaporation ponds and saltmarsh, c) prebasin and saltmarsh, for each month.

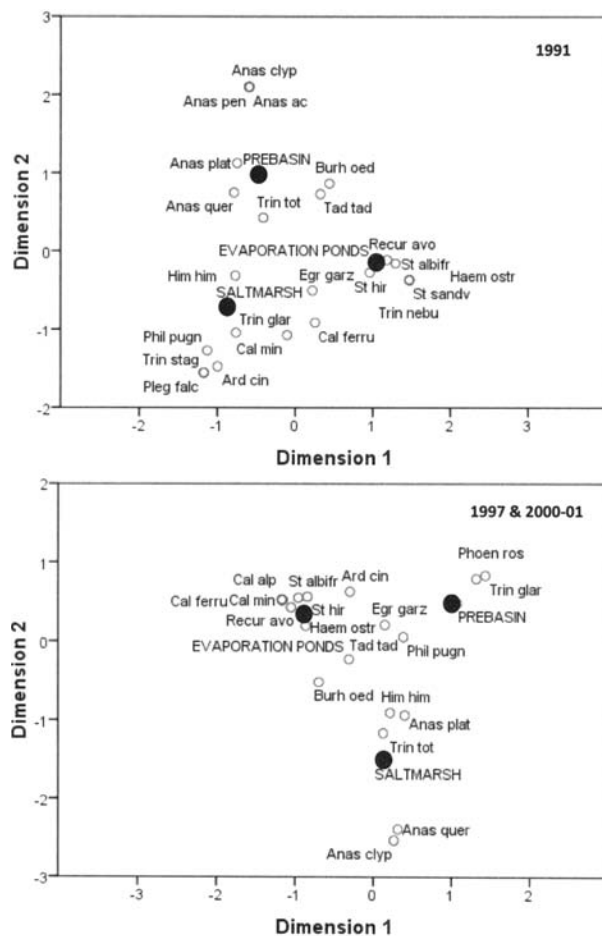


Fig. 3. – Correspondence analysis' bi-plot of wetland type with regard to bird species during the spring of 1991 (salina inactive) and springs 1997, 2000-01 (salina active).

The correspondence analysis was applied twice (Fig. 3). Since the variable with the fewer categories had three categories, a two dimension solution was chosen. For the 1991 period, the two-dimension solution fitted well to the data (Type I error < 0.1%) ($X^2(44)=1406.75$, p=0.000<0.001). The first dimension accounted for 54% of the total variance, while the second for 21.3%. For the 1997, 2000-01 period, the two-dimension solution also fitted well to the data (Type I error < 0.1%) ($X^2(38)=4428.02$, p=0.000<0.001). The first dimension accounted for 56.2% of the total variance, while the second for 33.4%. The correspondence analysis identified different bird communities in each wetland type and that some species, such as *Ardea cinerea*, selected other wetland type between the two periods.

## Habitat selection

The selection of habitat by 18 waterbird species that used more than one wetland type, are examined in Fig. 4. Jacobs preference indices are not significantly different between the 1991 and 1997, 2000-01 periods for most of the species (T test or Mann-Whitney U: -0.122–3,7, df: 3–17, p=0.059–0.844), except for *P. pugnax* (Mann-Whitney U: 1.1, df: 9, p=0.018) and *Ardea cinerea* (Mann-Whitney U: 0, df: 5, p=0.042). *P. pugnax* selected the salt-

marsh in 1991, but in 1997, 2000-01 it did not show any particular preference for any wetland type. *A. cinerea* selected the saltmarsh in 1991, but in 1997, 2000-01 it selected the ponds.

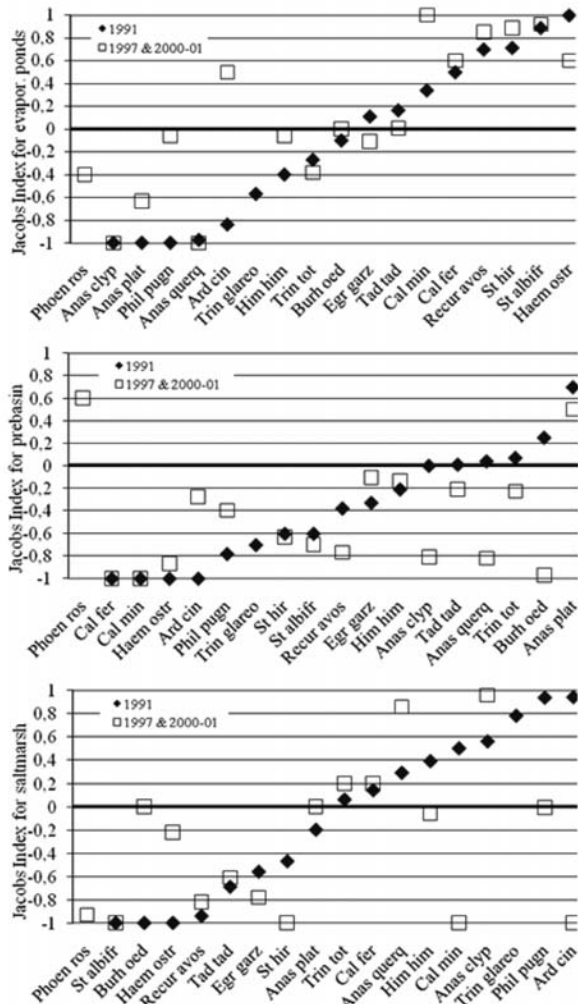


Fig. 4. – Wetland type selection of 18 bird species (codes names given in Table 1) as shown by the Jacobs preference indices. Points show mean index values from each count in which the species was found during the spring of 1991 (salina inactive) and the springs of 1997, 2000-01 (salina active).

### Nest survey

In total, 335 nests belonging to seven waterbird species were found (Table 3). Most of the nests were found in the evaporation ponds, on the dykes and also at the bottom of dry ponds. At the saltmarsh, the nests of *H. himantopus* were in halophytic vegetation. The same was concluded for *T. totanus* based on the observed breeding individuals located in the saltmarsh and their breeding behaviour.

Thus, species with a mean Jacobs index of more than 0.6 are:

- seven for the evaporation ponds: *Calidris minuta, C. alpina*, *R. avosetta*, *Sterna hirundo*, *Sternula albifrons*, *S. sandvicensis* and *Haematopus ostralegus*,
- two for the prebasin: *Anas platyrhynchos, P. roseus*, and
- five for the saltmarsh: *Anas querquedula*, *A. clypeata*, *Plegadis falcinellus*, *Tringa glareola*, *T. stagnatilis*.

TABLE 3

Minimum number of nests in each wetland type during the spring and summer of 1991.

| | Ponds | Prebasin | Saltmarsh |
|---|---|---|---|
| *Recurvirostra avosetta* | 118 | | |
| *Charadrius alexadrinus* | 69 | 16 | |
| *Sternula albifrons* | 41 | | |
| *Sterna hirundo* | 30 | | |
| *Burhinus oedicnemus* | 2 | | |
| *Himantopus himantopus* | | | 46 |
| *Tringa totanus** | | | 13 |
| **Total** | **260** | **16** | **59** |

*Nests were estimated indirectly from the number of couples found and their characteristic alarming calls near the nests.

## DISCUSSION

The correspondence analysis identified different bird communities in each wetland type. However, significantly higher mean abundance was found in the 1997, 2000-01 period in active evaporation ponds in comparison with the saltmarsh (Table 2). This difference was caused by the selection of ponds shown by *R. avosetta*, *Calidris* spp. and *Sterna* spp.. It is also noteworthy that *P. pugnax* and *A. cinerea* selected the evaporation ponds more when these were active, that is in the 1997, 2000-01 period. This can be attributed mainly to the flooding of the salina with seawater and the increase of its flooded area (Dias et al., 2009). In contrast, the flooded area of the saltmarsh decreased due to lower precipitation in comparison with the 1991 period. Studies have shown that shorebirds respond to variation in prey density, with a positive correlation between prey density and bird density (Velasquez, 1992; Masero, 2003; Sanchez et al., 2006). Bird counts in San Francisco Bay from 1964 to 1966 showed the highest densities of birds in salt ponds, followed by tidal flats, open water, and tidal marshes (Bollman & Thelin, 1970). The same pattern appears to occur in our study area, where the higher salinity and water levels, in the 1997, 2000-01 period, seem to have improved food availability for many waterbird species in ponds.

Nevertheless, different waterbird species or groups respond to salinity in various ways (Stralberg et al., 2003). Thus, in our study area, avoidance of the prebasin increased for *A. clypeata* and *A. querquedula* after its inundation with seawater. The same has also been found in other studies (Murias et al., 2002, Paracuellos et al., 2002, Stralberg et al., 2003).

The bird community of the prebasin is of intermediate similarity to the bird communities of the ponds and the saltmarsh (Figs 2-3). This could be attributed to the position of the prebasin between the other two wetlands, although the distance between them is only a few hundred

meters and thus a waterbird requires minimal effort to fly from one to the other. Therefore, this explanation does not seem very likely. Another reason may be water salinity; when the salina was inactive and the prebasin water came from rainfalls, its bird community resembled more the saltmarsh bird community. When the ponds were irrigated with seawater through the prebasin (1997, 2000-01), their bird community similarity increased, but not significantly (Fig. 2). Similarly, PARACUELLOS et al. (2002) and MURIAS et al. (2002) have found that the abandonment of a salina causes changes in the waterbird community, such as the decrease of some waders and greater flamingo and the increase of ducks and diving bird species.

The observed use of available breeding habitat was different between waterbird species, with most species nesting in the ponds. Four species made their nests in the evaporation ponds, one both in ponds and the prebasin, and two species in the saltmarsh (1991). Most of the nests found in the ponds and the saltmarsh belonged to *R. avosetta* and to *H. himantopus,* respectively. RINTOUL et al. (2003) found that *Recurvirostra americana* made greater use of ponds than saltmarsh, but for *Himantopus mexicanus* no preference was found. *Himantopus* spp. prefer vegetated areas for their nests (RINTOUL et al., 2003), a feature lacking from the dykes of the evaporation ponds in our study area.

## CONCLUSIONS AND WETLAND COMPLEX CONSERVATION

From a bird conservation point of view, the objective is to determine how management of the wetland complex could improve for birds, especially for threatened species. Firstly, all three different wetland types have their own value for bird species. However, based on wetland type and breeding habitat selection, the evaporation ponds, followed by the saltmarsh, have higher value than the prebasin. Consequently, habitat improvement and partial expansion of, firstly, the evaporation ponds and, secondly, the saltmarsh area at the expense of the presently larger prebasin area, are actions that could potentially increase waterbird diversity. Nevertheless, it is essential to gain a better understanding of bird requirements and carrying capacities in different microhabitats, for at least the threatened species, as proposed in other studies (e.g. GOSS-CUSTARD et al., 1996). For example, the establishment of herbaceous vegetation on the dykes of the evaporation ponds is expected to improve the breeding habitat for *H. himantopus* (RINTOUL et al., 2003). However, this may degrade the habitat for some other species that prefer to nest on bare dykes.

The abandonment of the salina operation by the salt industry for two years did not significantly change bird diversity and abundance; however, changes in habitat selection by certain species were recorded. Therefore, if management of the salina – apart from its salt production target –aims not only at general avian conservation, but focuses on the conservation of specific species (such as threatened or flag species), the manager should take special measures.

A better understanding of the ecological requirements of waterbirds at the microhabitat level will contribute to the effective management of such a wetland complex. The current trend to cease or consolidate salt production in many parts of the Mediterranean has created many inactive and intermittently exploited salinas with hydrological infrastructures falling into ruins (PETANIDOU & DALAKA, 2009). Thus, in order to conserve salina waterbird species the inundation of salinas with seawater before the egg laying period (to protect nests from flooding) would be advisable. With regard to the saltmarsh birds, the implementation of habitat improvement techniques for the increase and retention of rainfall waters would ameliorate conditions for wintering waterfowl and nesting waterbirds (*H. himantopus, T. totanus*). Such activities can also be favourable to the development of sustainable human activities, as in the case of wetlands in the Camargue, France (SKINNER & ZALEWSKI, 1995; CUFF & RAYMENT, 1997).

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# Statistical relationships of cnidocyst sizes in the sea anemone *Oulactis muscosa* (Actiniaria: Actiniidae)

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ABSTRACT. This study analysis the multivariate statistical relationships among cnidocyst types and tissues in terms of cnidocyst capsule size in the sea anemone *O. muscosa*. In a total of 12 individuals we measured the length of 30 unfired capsules of each cnida type, taken randomly from each of the following tissues: tentacles, acrorhagi, column, actinopharynx and mesenterial filaments. In these tissues, we found spirocysts, basitrichs, microbasic b-mastigophores, microbasic p-mastigophores and holotrichs. Thus, a total of 5400 measurements were made. Looking for a common pattern of variation, a principal components analysis (PCA) was performed on the data matrix, among cnidocyst types for each tissue and between tissues for each cnidocyst type. The results showed that the lengths of most cnidocysts, whether from the same or different tissues, are not statistically related to each other, suggesting that different forces (cnidogenesis, replacement dynamics, etc.), could affect in various forms the development and therefore the length of each cnida, depending on the type or tissue of origin.

KEY WORDS: cnidae, PCA, *Oulactis muscosa,* intertidal, Argentina.

## INTRODUCTION

Cnidocysts (nematocysts, spirocysts and ptychocysts) are formed from the secretions of specialized cells that reside mainly in the tentacles of cnidarians, such as sea anemones, cerianthids, jellyfish, corals and hydroids. These stinging organelles, for which Cnidaria is named, are the chief instruments by which the animals in this taxon obtain food, defend against predation, adhere to solid substrata during locomotion or settlement, or build protective tubes (Kass-Simon & Scappaticci, 2002). These authors proposed that the persistence of cnidarians in marine and freshwater niches since the Cambrian is due not least to the effectiveness with which their nematocysts-bearing tentacles act as lethal weapons of defense and predation.

Each cnidocyst is secreted by a cnidoblast, which matures into an epithelial cell, the cnidocyte. A mature cnidocyst consists of a three-layered wall that encloses a dense lumen containing an inverted tubule which, depending on the cyst type, features specialized structures such as shaft, barbs, spines, stylets, etc. In response to appropriate stimuli, a cnidocyst explosively evaginates its tubule. This enables the everting tubule to penetrate through the integument or skin of invertebrates, vertebrates and even humans. By this mechanism toxins and enzymes contained in the cysts can be introduced into the body of either the aggressor, or prey organism (Weber, 1989).

The cnidome refers to the assemblage of cnidae present in any cnidarian. A diagrammatic way of presenting information covering both the types of cnidae present and their sizes was developed by Hand (1955a; 1955b; 1956) and has become a standard procedure in the taxonomic description of sea anemones (Ryland et al., 2004). However, in this group of cnidarians their value has been debated (Fautin, 1988; Zamponi & Acuña, 1991; Acuña et al., 2003; 2004), mainly due to their high statistical variation. Several studies reported on this variation and other statistical parameters (Zamponi & Acuña, 1994; Acuna & Zamponi, 1997; Chintiroglou et al., 1997; Chintiroglou & Karalis, 2000; Williams, 2000;), while a few authors (Francis, 2004; Acuña et al., 2007) have related aspects of the cnidae to the body size of sea anemones. A complete review of the structural diversity, systematics and evolution of cnidae can be found in Fautin (2009).

The biometry of the sea anemone *Oulactis muscosa* (Drayton in Dana, 1846) was studied by Acuña et al. (2007). They showed that cnida length varies with specimen, tissue and cnida, and their results do not support a functional relationship between cnida length and anemone body weight. However, possible correlations between the lengths of different cnidocysts from all tissues of the sea anemone were not examined. Therefore, the aim of this study was to perform a principal components analysis (PCA) to explore the multivariate statistical relationships of cnidocyst capsule sizes among different types of cnidocysts and tissues in *O. muscosa*. To our knowledge, similar studies for other sea anemone species do not exist.

## MATERIALS AND METHODS

### Sampling

Individuals of *O. muscosa* were collected randomly from the rocky intertidal zone of Punta Cantera (38º 05' S, 57º 32' W), Mar del Plata, Argentina. Specimens were kept in aquaria with aerated sea water for up to two days, then anesthetized by the addition of crystals of magnesium chloride, and preserved in 5% formalin. A total of 12 individuals were used. For each specimen, we measured the length of 30 unfired capsules of each cnida type (we usually take N=30 since this is a statistically significant number), taken randomly, from each of the following tissues: tentacles, acrorhagi, column, actinopharynx and mesenterial filaments. In these tissues, we found spirocysts, basitrichs, microbasic b-mastigophores, microbasic p-mastigophores and holotrichs (see ACUÑA et al., 2007); terminology based on that of ENGLAND (1991). Thus, a total of 5400 measurements were made using a Zeiss Axiolab Microscope with micrometric eyepiece at a magnification of 1000X (oil immersion). Where two or more discrete nematocyst sizes were present within a tissue, they were counted separately and suffixed I, II, etc., according to size. For tentacle tissue squashes, we used the tips, since the base is a cnidogenesis zone with cnidocysts in different stages of development and consequently with high variation in sizes. For more details see ACUÑA et al. (2007).

### Statistical analysis

A PCA was performed on the data matrix, among cnidocyst types for each tissue and among tissues for each cnidocyst type. Calculations were based on the correlation matrices, and the goal of this analysis was to express variance-covariance structures of the original variables (lengths of cnidocyst from different types and locations) through a few linear combinations or principal components. These components are determined by the eigenvectors of the correlation matrix and indicate the maximum variability directions that define the new factorial planes. Coordinates in the new system are called loadings and they are given by the correlations between each original variable and each principal component. Much of the total information contained in the data can be accounted for in the first principal components (see JOHNSON & WICHERN, 1992 for a detailed description of PCA). The proportion of the total variance due to each component was also calculated. All calculations were done with the R statistical package (R, 2008).

## RESULTS

### Variation among cnidocysts within each type of tissue

In the tentacles there were only two types of cnidocysts (spirocysts and basitrichs), so for this location the analysis was just bivariate. Spirocyst and basitrich sizes were significantly correlated ($\rho$=0.78, p-value=0.003). In acrorhagi, the following types of cnida were present: holotichs (I and II), basitrichs and spirocysts. The bivariate association between each pair of cnidocysts, with their corresponding p-values is shown in the correlation matrix (Table 1); the highest correlation is between pairs holotrichs I – II. The associations between holotrichs I – basitrichs, holotrichs II – spirocysts and holotrichs II – basitrichs were weak, significant only at 10%. Fig. 1 illustrates the results of the multivariate PCA on this matrix. The first component (horizontal axis) is associated with holotrichs I and II, representing 54% of total variability or information. The second component (vertical axis) comprises the opposition between the latter two cnidocysts, while the third component (not shown in the figure) is associated to spirocysts with near 15% of total variability. Holotrichs, and basitrichs (I and II) are the cnida present in the column. Significant association at 5% was not observed (see Table 2), but the correlation between basitrich I and II was significant at 10%. As expected, given the correlation matrix, each component was strongly associated with one tissue, so the three-dimensional problem could not be reduced. The first component, represented by the horizontal axis in Fig. 2, comprised 56% of total variability and it represented the holotrichs. The second component (vertical axis) had 30% of variability, strongly comprising basitrichs II. Finally the third component explained 14% of the remaining variability and it was highly associated with basitrichs I and weakly with basitrichs II. In the actinopharynx, microbasic b-mastogophores, microbasic p-mastigophores and basitrichs were present. Significant correlations were not observed, similarly to the situation observed in the column (Table 3). Due to the low correlations, each component represented a moderate proportion of the variability. The first component represented microbasic b-mastigophores and explained near 50% of variability, the second was associated with microbasic p-mastigophores and the third with basitrichs (Fig. 3). The same types of cnida present in the actinopharynx were present in mesenterial filament, but they showed a different correlation matrix (compare Tables 3 and 4). Negative correlations between microbasic b-mastigophores and basitrichs, and microbasic p-mastigophores and basitrichs, were present, both significant at 5%. This means that large microbasic b-mastigophores and microbasic p-mastigophores were associated with small basitrichs. This means that large values of microbasic b-mastigophores are associated with large values of microbasic p-mastigophores. The three principal components represent each cnidocyst type, microbasic b-mastigophores, basitrichs and microbasic p-mastigophores, respectively. Given these correlations, 70% of the information of the three types of cnida can be represented by a single principal component (Fig. 4).

TABLE 1

Correlation matrix between cnidocysts from acrorhagi and p-values corresponding to the null hypotheses of no correlation.

| | Basitrich | Holotrich I | Holotrich II | Spirocyst |
|---|---|---|---|---|
| Basitrich | 1.000(0.000) | | | |
| Holotrich I | 0.497(0.100) | 1.000(0.000) | | |
| Holotrich II | 0.522(0.082) | 0.612(0.034) | 1.000(0.000) | |
| Spirocyst | 0.160(0.620) | 0.450(0.143) | 0.524(0.080) | 1.000(0.000) |

TABLE 2

Correlation matrix between cnidocysts from column and p-values corresponding to the null hypotheses of no correlation.

| | Basitrich I | Basitrich II | Holotrich |
|---|---|---|---|
| Basitrich I | 1.000(0.000) | | |
| Basitrich II | 0.516(0.086) | 1.000(0.000) | |
| Holotrich | 0.287(0.365) | 0.131(0.684) | 1.000(0.000) |

TABLE 3

Correlation matrix between cnidocysts from actinopharynx and p-values corresponding to the null hypotheses of no correlation.

| | Microb. b-mastigophore | Basitrich | Microb. p-mastigophore |
|---|---|---|---|
| Microb. p-mastigophore | 1.000(0.000) | | |
| Basitrich | 0.488(0.107) | 1.000(0.000) | |
| Microb. p-mastigophore | 0.440(0.152) | 0.003(0.993) | 1.000(0.000) |

TABLE 4

Correlation matrix between cnidocysts from mesenterial filament and p-values corresponding to the null hypotheses of no correlation.

| | Microb. b-mastigophore | Basitrich | Microb. p-mastigophore |
|---|---|---|---|
| Microb. b-mastigophore | 1.000(0.000) | | |
| Basitrich | -0.700(0.011) | 1.000(0.000) | |
| Microb. p-mastigophore | 0.676(0.016) | -0.586(0.045) | 1.000(0.000) |

TABLE 5

Correlation matrix between basitrichs from different tissues and p-values corresponding to the null hypotheses of no correlation.

| | Tentacle | Acrorhagi | Column | Actinopharynx | Mes. filament |
|---|---|---|---|---|---|
| Tentacle | 1.000(0.000) | | | | |
| Acrorhagi | 0.798(0.002) | 1.000(0.000) | | | |
| Column | 0.636(0.026) | 0.278(0.381) | 1.000(0.000) | | |
| Actinopharynx | 0.409(0.187) | 0.387(0.214) | -0.161(0.616) | 1.000(0.000) | |
| Mes. filament | 0.715(0.009) | 0.398(0.199) | 0.538(0.071) | 0.442(0.151) | 1.000(0.000) |

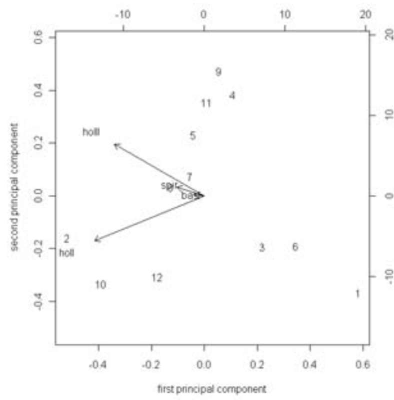


Fig. 1. – Scatterplot of the two first principal components for acrorhagi. Arrows represent the mean values for each cnidocyst. holI: holotrich I, holII: holotrich II, basI: basitrich I, spiro: spirocyst.

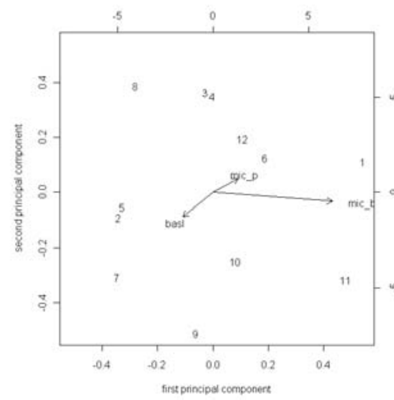


Fig. 4. – Scatterplot of the two first principal components for mesenterial filament. Arrows represent the mean values for each cnidocyst. mic_p: microbasic p-mastigophore, mic_b: microbasic b-mastigophore. basI: basitrich.

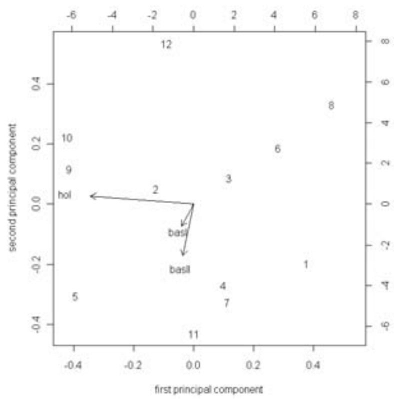


Fig. 2. – Scatterplot of the two first principal components for column. Arrows represent the mean values for each cnidocyst. hol: holotrich, basI: basitrich I, basII: basitrich II.

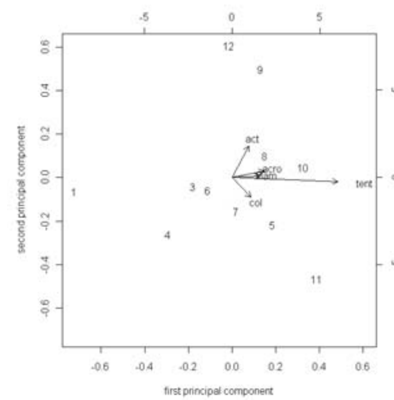


Fig. 5. – Scatterplot of the two first principal components for basitrichs. Arrows represent the mean values for each tissue. acro: acrorhagi, tent: tentacle, col: column, filam: mesenterial filament, act: actinopahrynx.

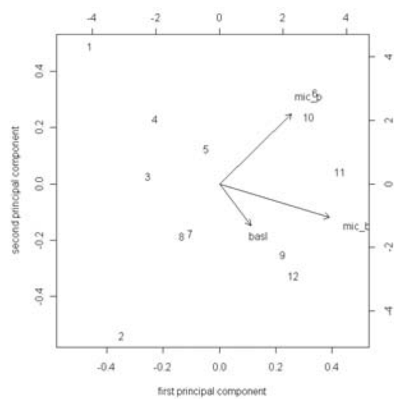


Fig. 3. – Scatterplot of the two first principal components for actionpharynx. Arrows represent the mean values for each cnidocyst. mic_p: microbasic p-mastigophore, basI: basitrich, mic_b: microbasic b-mastigophore.

## Variation within each type of cnidocyst from different tissues

Basitrichs were present in all sampled tissues. The capsule lengths measured in tentacles were significantly associated with acrorhagi, column and mesenterial filament and this last tissue was also associated with column (Table 5). Actinopharynx was not significantly correlated with any other cnida. The PCA showed a first component explaining 82.5% of total variability, strongly associated with tentacles (Fig. 5, horizontal axis). This was due to the fact that tentacle lengths have a very high variance (Acuña et al., 2007) and so it determines the first component. Spirocysts were present in tentacles and acrorhagi; the correlation between the two localities was $\rho=0.622$ and its p-value=0.031, meaning that big spirocysts appeared simultaneously in tentacles and acrorhagi and the same for small spirocysts. The cnidocyst holotrich

was present in acrorhagi and column. The calculated correlation between these tissues was moderately significant: ρ=0.571, p-value=0.052. Microbasic b-mastigophores were recorded in actinopharynx and mesenterial filament; their corresponding lengths were not correlated (ρ=0.142, p-value=0.659). Microbasic p-mastigophores were also in actinopharynx and mesenterial filament; their correlation was a little higher, but still not significant (ρ=0.486, p-value=0.109).

## DISCUSSION

Sea anemones are equipped with two major types of cnidocysts (nematocysts and spirocysts), with functions in food capture, defence, aggression and larval settlement. The description of the size and distribution of cnidocysts is commonplace in taxonomic studies of these marine invertebrates, but its value is discussed here. In a previous paper we examined all 15 types and sizes of cnida distributed in all different tissue from specimens of different sizes of the sea anemone *O. muscosa* (Acuña et al., 2007). Complementary to this, the goal of this paper was to study the multivariate statistical relationships of the sizes, between types in each tissue and between tissues for each type of cnidocyst.

An important correlation was observed between spirocysts and basitrichs from tentacles, despite being different types, while the holotrichs I and II from acrorhagi showed a moderate correlation. This could be explained by a similar cnidogenesis and replacement dynamics. In the other tissues no significant correlations were observed between the different cnidocysts. The strong correlations observed between lengths of basitrichs measured from tentacles and acrorhagi are possibly due to ontogeny of the last structure, since Bigger (1982) concluded that they are homologs of tentacles. However, Daly (2003) mentioned that an acrorhagus must not be considered a highly modified tentacle, as it is topologically and anatomically distinct. Also the basitrichs from tentacles are significantly associated with those from column and mesenterial filament, but not with those from actinopharynx. It is important to mention that the actinopharynx is a tissue with a complex pattern of nematocyst size-distribution (Ardelean & Fautin, 2004).

These results showed that the lengths of most cnidocysts, both from the same or different tissues, are not statistically related; suggesting that different forces (cnidogenesis, replacement dynamics, etc.); could be affecting in various forms the development and therefore the length of each cnida, depending on the type or tissue of origin. Doumenc et al. (1989) concluded that for the genus *Telmatactis*, nematocyst biometry is associated to a significant degree with the “relative age” of the anemone. According with Chintiroglou (1996) in his study on *Edwardsia claparedii,* it appears that certain nematocyst dimensions of sea anemones can be affected by various physiological conditions. Also Karalis & Chintiroglou (1997) suggested that each sea anemone species uses a different mode of cnidome construction. More studies involving other species and types of cnida could shed light on this issue. The cnidome and the distribution and dimensions of the cnidocysts are now considered essential to any taxonomic description or redescription of a cnidarian species of most taxa (Fautin, 1988; Östman, 2000). However the extremely complex biometry of these subcellular organelles reduces their value as taxonomical tools at least in sea anemones. Usefulness of cnidome size investigations in Medusozoa (hydra, hydroids, jellyfishes, etc.) and Anthozoa (including sea anemones, cerianthids, zoanthids, corals, etc.) vary considerably. In Anthozoa, given the continuous growth of cnidocytes (perhaps explaining the large size variability), the taxonomical value of this character is greatly reduced and it requires time-consuming investigation, whereas in Medusozoa cnidocyst size may be a key to discrimination between sister species or geographically isolated populations (Östman et al, 1987). Further understanding of which morphological aspects are informative is needed for their systematic and phylogenetic value to be understood and their potential as reflections of evolution to be realized (Fautin, 2009).

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# The effect of nesting habitat on reproductive output of the Barn Swallow (*Hirundo rustica*). A comparative study of populations from atypical and typical nesting habitats in western Poland

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ABSTRACT. The aim of this study was to discover if atypical nesting places such as abandoned second world war bomb shelters and conditions occurring within, can constitute suitable and good quality alternative habitat for the Barn Swallow. To answer this question, the time of breeding, clutch size and the mean survival probability of nest contents were compared between swallows nesting in shelters and in farm outbuildings – typical nesting habitat. The study showed that bunkers do constitute a suitable and relatively good quality alternative habitat for the Barn Swallow but they are poorer nesting places than pigsties or cowsheds. Mean survival rate of nest contents (eggs/nestlings) was higher in farm outbuildings than in bunkers, but only differences in the first broods were recorded. The results are most probably the effect of different conditions occurring in the two kinds of nesting habitat, especially at the beginning of the breeding season, when the unfavourable weather conditions can negatively influence breeding swallows to a higher degree in bunkers than in outbuildings.

KEY WORDS: Barn Swallow, *Hirundo rustica*, reproduction, shelters, survival analysis

## INTRODUCTION

The Barn Swallow *Hirundo rustica* is a farmland, long distance migratory, insectivorous passerine bird that has declined in some parts of its European breeding range during recent decades (Møller, 1989; Marchant et al., 1990; Tucker & Heath, 1994; Møller & Vansteenwegen, 1997; Ambrosini et al., 2002a; Papazoglou et al., 2004; Wretenberg et al., 2006). The main causes for decline in abundance in its breeding range are most probably intensive agricultural practices, disappearance of individual livestock farms, especially those raising cattle, and the removal and refurbishment of old farm buildings leading to a loss of nesting opportunities (Marchant et al., 1990; Weggler & Widmer, 2000; Møller, 2001; Ambrosini et al., 2002b; Evans et al., 2003; Robinson et al., 2003). By virtue of its large range and population size, the Barn Swallow is not a species of special concern (Papazoglou et al., 2004), but extremely high breeding philopatry (Saino et al., 2002) and social facilitation of breeding habitat choice may delay response of populations to rapidly changing ecological conditions in anthropogenic habitats (Ambrosini et al., 2002b). In the case of habitats with superabundant insects, where availability is not exactly related to grazing animals (eg. flooded river valleys), the removal and refurbishment of old farm buildings – typical nesting places, seems to be the most important factor responsible for distribution and abundance of the Barn Swallow. In such circumstances, the alternative nesting places create breeding opportunities and to some degree can compensate for changes in habitat and simultaneously increase the chances for the local populations to survive.

To date, studies on breeding biology and ecology of the Barn Swallow have been carried out in the most typical nest sites used by this species, such as inside derelict buildings, cowsheds, pigsties, stables and other similar outbuildings where animals are raised, as well as in different kinds of domestic buildings (Adams, 1957; Kuźniak, 1967; Löhrl & Gutscher, 1973; Møller, 1982; Turner, 1982; Bańbura & Zielński, 1998; Giacchini & Piangerelli, 2001; Møller, 2001). Furthermore, it is known that Barn Swallows nest in less specific, atypical places such as under roofs of different kinds of buildings, in rock crevices, under bridges, in mine shafts, wells, jetties and in culverts (Vietinghoff-Riesch, 1955; Weiner 1967; Davies & Tucker 1984; Tryjanowski & Lorek, 1992; Turner, 2006). However, in most cases there is a complete lack of data about the breeding biology or ecology from such nesting habitats. Other atypical breeding habitats of the Barn Swallow are abandoned second world war bomb shelters (Czechowski & Zduniak, 2005). Such places differ from the typical nesting habitats with regard to light and water conditions, temperature, predation pressure and direct access to food such as flying insects. They also, to some degree, resemble primeval nesting places of the Barn Swallow such as caves, grottos and rocky crevices (Turner, 2006).

The aim of this study was to investigate whether atypical places such as abandoned second world war bomb shelters and conditions occurring within, can constitute suitable and good quality alternative habitat for the Barn

Swallow. This is of interest especially in the context of areas with decreasing numbers of the Barn Swallow, where alternative, atypical nesting places can increase the chance for the local populations to survive. To answer the question, the main breeding parameters of swallows nesting in shelters were compared to populations occurring in typical nesting habitats.

## MATERIAL AND METHODS

The breeding biology of the Barn Swallow has been studied by many researches in many places and at different times. These differences make it difficult to directly compare the results obtained from other studies. For example, there are differences in habitat or atmospheric conditions influencing food resources, which in turn affect many breeding parameters (e.g., KUŹNIAK, 1967; MØLLER, 1984; TURNER, 2006). Hence, the only solution to this kind of difficulty is to compare the breeding parameters of populations studied at the same time, located close to each other and in similar environmental conditions such as the same river valley. For this reason during the study of the Barn Swallows in bunkers, another nearby but typical nesting population of this species was studied using the same methods.

The fieldwork was carried out during four breeding seasons (from the end of April to the end of August) in 2004-2007 simultaneously in two areas in the Odra river valley, W. Poland, located 52km from each other. The first of the areas was situated near Czerwińsk (52°01'N, 15°26'E), where Barn Swallows nested in 13, abandoned second world war shelters built in 1939, being a part of the Odra war embankment (detailed description of bunkers in CZECHOWSKI & ZDUNIAK, 2005). The second area was Kłopot (52°7'N, 14°43'E) – a small village located 1km from the channel of the Odra river, where there are 50 small farms with a population of 200 inhabitants. In this area, swallows nest in the farm outbuildings. The number of farms and outbuildings visited varied during the study period from three to 10 and from nine to 22, respectively. On average in 89.1% (SE=1.21, range: 86.4-92.3; n=4) of the outbuildings animals were raised (mainly pigs, cows, rabbits, chickens) and 10.9 % (SE=1.21, range: 7.7-13.6; n=4) of the outbuildings were derelict.

In comparison to the outbuildings, the shelters were characterised by relatively low and stable air temperature and water occurring inside especially in the first stage of the breeding season. The shelters were on average 5ºC cooler than outbuildings (Wilcoxon matched-pairs test, Z=2.20, n=6, p=0.028; mean temperature values in ºC for the same 6 days and the same time during the breeding season; shelters: $\overline{x}$=13.5±0.34; outbuildings: $\overline{x}$=17.7±0.84).

The study sites were visited at intervals depending on the stage of broods and were shorter (4-5 days) during the most important events such as egg laying and nestling rearing periods and longer (8-9 days) during the incubation period and between the first and second broods. The mean number of visits per season in the four years of study was 14.0±1.87 (range: 10-19) in bunkers and 13.0±0.82 (11-15) in outbuildings. During each visit, all nests in individual shelters and outbuildings were checked and in every active nest the number of eggs, and later the number of nestlings was recorded. The mean number of first and second broods observed each year was 38.2±4.97 (range: 30-50) and 22.5±3.92 (17-34) in bunkers and 33.3±4.21 (23-43) and 21.3±1.43 (17-23) in outbuildings, respectively.

The reproductive parameters such as clutch size and the mean survival probability of nest contents expressing the reproductive output were compared between populations breeding in bunkers and typical nesting population of this species.

### Data processing and analysis

The ages of eggs and nestlings were estimated on the basis of the date of laying of the first egg, established by direct observation or calculated assuming that one egg was laid per day (i.e. KUŹNIAK, 1967; TURNER, 2006). The age estimations of eggs and nestlings were supported by the use of the field instruction "Euring Swallow Project" from 2001 prepared by the Ornithological Station of the Museum and Institute of Polish Academy of Sciences.

Survival was calculated on the basis of changes in the number of eggs and nestlings in the nests observed and the life tables method was applied (KLEINBAUM, 1996). This method assumes that survival is a function of time, which enables the identification of the critical moments occurring during the reproduction period in the populations studied. The best element of the life tables showing the critical periods is hazard rate defined as the probability per time unit that an individual that has survived to the beginning of the respective interval will die in that interval. In general, the higher the hazard rate the higher the risk of failure and the same the lower survival, whereas the lower the values of hazard rate the higher the survival.

The survival time of each individual (egg/nestling) was estimated from the day when the egg was laid in the nest to the day of the last visit of each nest. The day when eggs or nestlings failed was calculated as half way between two subsequent nest visits (ZDUNIAK, 2010).

To compare survival between many groups, the multiple sample test was used, which is an extension of Gehan's generalized Wilcoxon test (GEHAN, 1965), Peto and Peto's generalized Wilcoxon test, and the log-rank test (PETO & PETO, 1972; LEE, 1980). Multiple comparisons between two groups were made using the Cox-Mantel test (COX, 1959; 1972; MANTEL, 1966) and the Bonferroni correction was applied.

Overall, the material was analysed using life tables including two cohorts of a total number of 1041 eggs from 222 clutches (376 eggs in 2004, 251 in 2005, 199 in 2006 and 215 in 2007) observed in bunkers and 953 eggs from 204 nests (289 eggs in 2004, 260 in 2005, 220 in 2006 and 184 in 2007) recorded in farm outbuildings. To check if the data from the four years of study can be pooled, the possible effect of a year on the survival was tested with the use of the Cox's proportional hazard model (COX, 1972). This model assumed that hazard rate is a function of independent variables. Use of this model allows the estimation of regression coefficients for independent variables. In general, variables with positive

coefficients are associated with a higher risk of failure and decreased survival, whereas variables with negative coefficients are connected with a lower risk of death and increased survival (ZDUNIAK, 2010).

Besides the survival, differences at the time of breeding were also analysed (expressed as Julian days of the first egg laying in each nest) and clutch size between study areas using factorial ANOVA, where the year effect was also controlled. It was not possible to acquire the complete set of information for each nest, and thus sample sizes varied in analyses. The standard statistical methods used in this paper were described by SOKAL & ROHLF (1995). Throughout the text, all mean values are presented with standard errors (±SE). All calculations were performed using STATISTICA for Windows (STATSOFT INC, 2008).

## RESULTS

### Time of breeding and clutch size

The time of the first broods initiation did not differ between study areas (factorial ANOVA, $F_{1,245}$=2.85, p=0.09; bunkers: $\overline{x}$=51.5±1.3, n=134, outbuildings: $\overline{x}$=48.4±1.3, n=119). The same was found for the second broods ($F_{1,160}$=1.99, p=0.16, bunkers: $\overline{x}$=100.1±1.6, n=83, outbuildings: $\overline{x}$=97±1.5, n=85).

Mean clutch size in the first broods was higher than in second broods (factorial ANOVA, $F_{1.418}$=88.41, p<0.001) and was 4.98±0.05 (n=264) and 4.31±0.06 (n=170), respectively. Moreover, mean clutch size in both broods did not differ between study areas ($F_{1.418}$=0.94, p=0.33).

### Survival of eggs and nestlings

The initial analysis with the use of the Cox's proportional hazard model, where year and study area were the factors, showed significant differences in survival between study areas and no effect of the year (whole model: chi-square =12.65, df=2, p<0.002; study area: ß = -0.16±0.04, Wald statistic =12.21, p<0.001; year: ß = -0.01±0.02, Wald statistic =0.20, p=0.65). For this reason the data from four years' study were pooled.

Mean survival rate of nest contents (eggs/nestlings) for the whole nesting period (40 days – from the egg laid to fledgling) for both study areas was 0.754±0.010 (n=1994), and was higher in outbuildings than in bunkers (Cox-Mantel test = -7.30, p<0.001). However, the recorded differences concerned first broods only (Cox-Mantel test = -8.12, n=1271, p<0.001; Fig. 1) and second broods did not differ between study sites (Cox-Mantel test = -1.16, n=723, p>0.25). Simultaneously, in outbuildings survival rate in first broods was higher than in second broods (Cox-Mantel test =2.62, n=953, p<0.009). The inverse result was obtained in bunkers, where survival rate in second broods was higher than in first broods (Cox-Mantel test = -3.39, n=1041, p<0.001; Fig. 1).

The differences in survival rate of nest contents (eggs/nestlings) between study areas and between broods were mostly determined at the hatching stage of nestlings (Fig. 2).

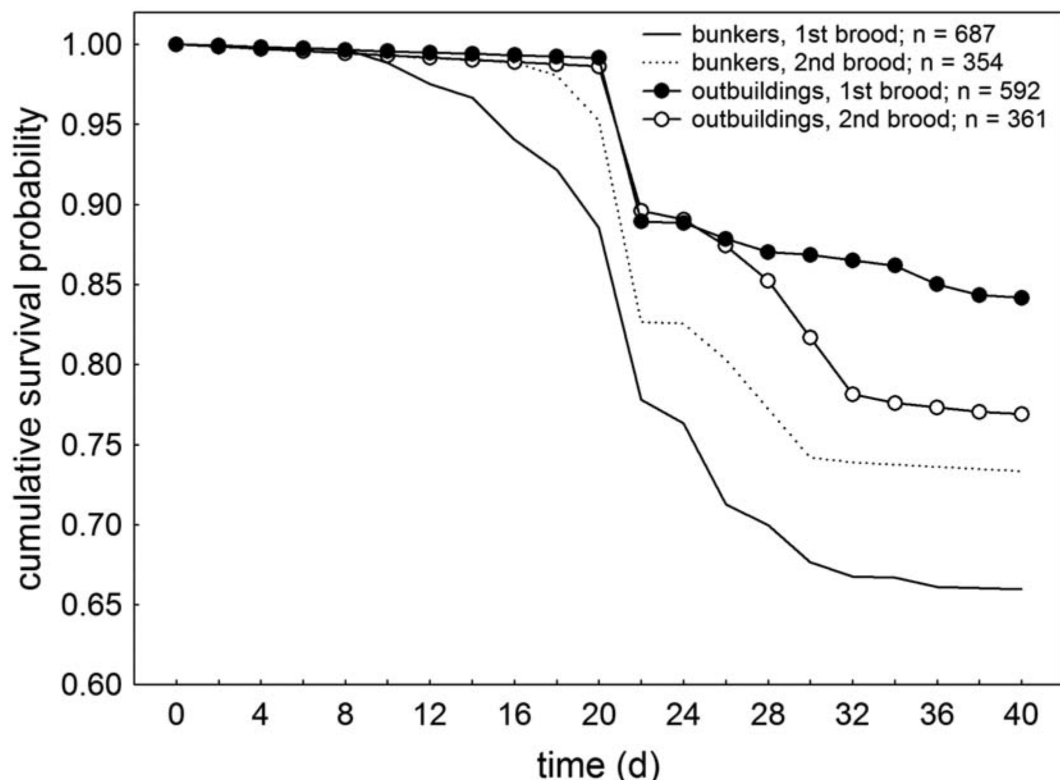


Fig. 1. – Differences in survival probability (survival curves) of Barn Swallow eggs and nestlings from first and second broods in bunkers and farm outbuildings.

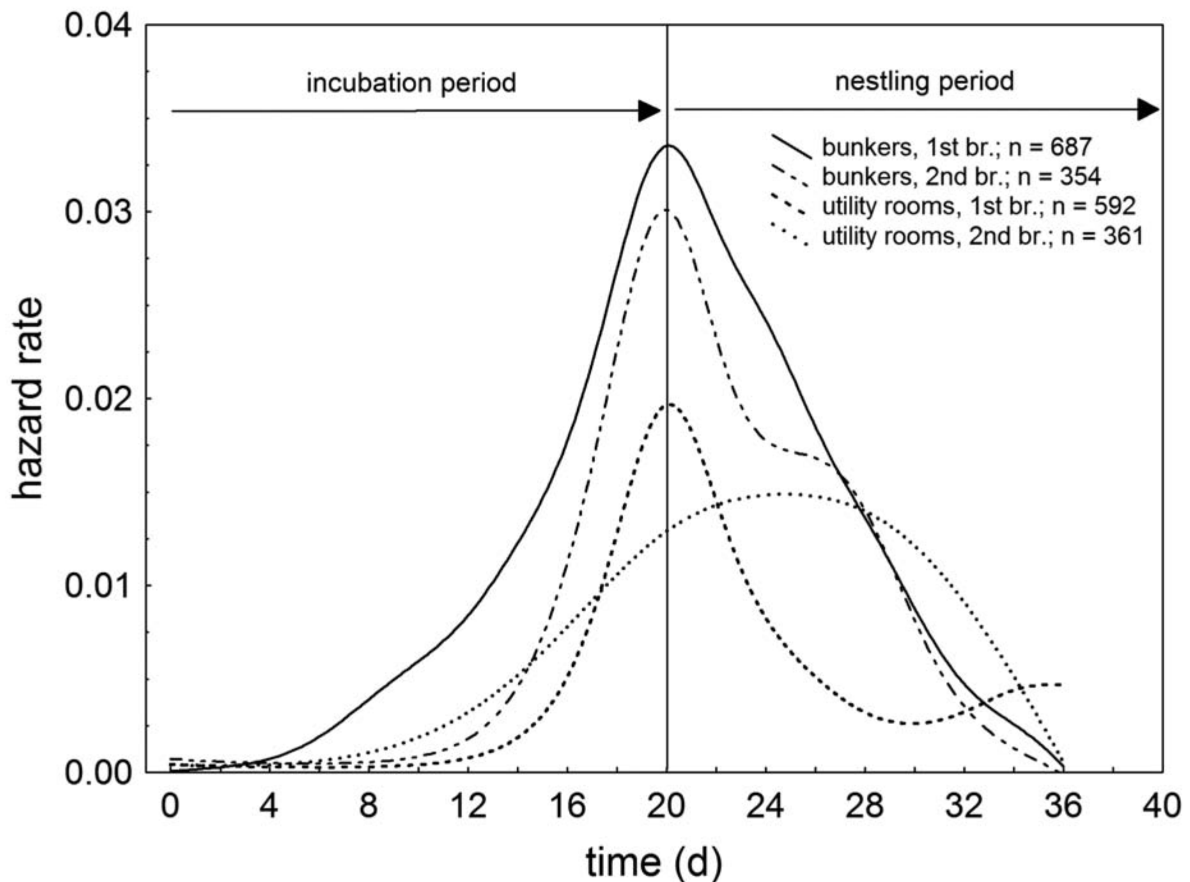


Fig. 2. – Hazard rate in the following days of egg and nestling life from first and second broods in bunkers and farm outbuildings; the curves are fitted using the distance weighted least squares smoothing technique; time in days since laying of the first eggs in clutches.

## DISCUSSION

This study showed that bunkers constitute suitable and relatively good quality alternative habitats for the Barn Swallow but they are poorer nesting places than farm outbuildings. Time of breeding and mean clutch size in both broods did not differ between study areas. However, mean survival rate of nest contents (eggs/nestlings) was higher in farm outbuildings than in bunkers but the differences concerned first broods only. The results are most probably the effect of different conditions occurring in these two kinds of nesting habitat, perceptible especially at the beginning of the breeding season. At this time, weather conditions are often unfavourable (low temperatures connected with high precipitation) and are much more changeable than in later stages of the breeding season, when the temperatures are higher and much more stable. In the incubation period, during rainy and cold days, females more often have breaks from foraging and as a result of the thermal conditions prevailing inside the bunkers there is an increased risk of eggs cooling than in the farm outbuildings. This may reduce hatching success (e.g. Williams & Ricklefs, 1984; Reid et al., 1999) or can negatively influence post-hatch growth (Sockman & Schwabl, 1998) and thereby nestling survival. Unfavourable weather conditions also have an influence on insects (Turner, 2006) and force adults to go further from the nest to localised feeding sites (Bryant & Turner, 1982). Adults spending more time on foraging can have a negative effect on small nestlings, especially in bunkers. Pigsties or cowsheds protect incubating females, their eggs and small nestlings better and have much more stable thermal conditions than bunkers at times of low outside temperatures. Firstly, these results arise from differences in the construction between these types of buildings. Secondly, during unfavourable weather the presence of farm animals increases the temperature inside the outbuildings and provides access to flying insects. Furthermore, relatively low temperatures in the bunkers, mostly at the first stage of the breeding season, are also heightened by the presence of water. Therefore, survival of first broods reared in pigsties or cowsheds was higher than in bunkers. During the later stages of the breeding season, temperatures are higher and weather conditions are much more stable than at the beginning of the breeding season and broods are less exposed to the effects of unfavourable weather conditions. Therefore, there are no differences in survival rates between second broods reared in the two study sites. Differences in thermal conditions can also explain the results that in farm outbuildings survival rate

in first broods was higher than in second broods and inversely in bunkers. The importance of nesting places' microclimate was also found in Tree Swallows (*Tachycineta bicolor*) population, where birds preferred warmer nesting places but only during the first half of the breeding season, characterized by lower ambient temperatures than the second half of the breeding season, when swallows selected nesting sites based on their availability (Ardia et al., 2006). Authors suggest that warmer nest temperatures may provide fitness benefits, especially at the beginning of the breeding season, which also was found in this study.

The overall survival and the same breeding success can be influenced by the phenotypic quality of breeders where high-quality individuals are often more fecund than the poor-quality ones. This is also true for Barn Swallow females whose fecundity is directly related to the body condition (Møller, 1994). Moreover, the breeding performance can be also affected by the age and therefore the experience of breeding Barn Swallow females (Balbontín et al., 2007). However, it seems not to be the case in this study. Unfortunately, the quality of breeders was not evaluated and their age is unknown, but there were no differences in breeding time and clutch size between the two kinds of nesting places. It would seem that parental quality and quality of birds in general does not differ between bunkers and outbuildings. Therefore, the thermal conditions occurring inside the nesting places are most probably the main factor influencing the differences in survival between study areas. The significance of thermal conditions on the survival in both study sites also is supported by the estimated hazard functions. In the case of both study areas, the hazard rate was the highest in the period of nestlings' hatching. However, higher values for this period were recorded in bunkers than in outbuildings. Such results suggest that the incubation and hatching periods are the most critical moments in reproduction and determine the higher overall reproductive output of barn swallows in bunkers than in outbuildings.

The effect of different kinds of nesting place on breeding success was found also for other bird species, where natural cavities were compared with nest boxes (e.g. Purcell et al., 1997, Robertson & Rendell, 1990, Evans et al., 2002, Czeszczewik, 2004). Nest boxes are often cleaner, dryer and warmer than natural holes. On the other hand, they are more visible and broods are more exposed to predators. The study has shown that differences in microclimate can exist also between the unnatural nesting places such as bunkers and outbuildings used by Barn Swallows as nesting places, and these determine the differences in the breeding output.

Studying the differences in breeding output of swallows between two kinds of nesting habitats, local adaptations and possible differences in the trade-off between fecundity (expressed here as number of eggs laid) and survival need to be taken into account. One of the possible adaptations to poor nesting conditions could be, for example, an investment in a smaller but better surviving brood. Because the clutch size did not differ between nest-site habitats but differed only in survival rate, no clear trade-off between the fecundity and survival in this study was found.

In conclusion, bunkers offering worse conditions for breeding than farm outbuildings, nevertheless provide a suitable and good quality alternative nesting habitat for Barn Swallows, where they achieve a relatively high breeding output, which is important for local populations of this species to survive. In the light of the mentioned declining numbers of the Barn Swallow during recent decades, all the atypical nesting places used by this species with success, such as the studied bunkers, should be protected. This is especially important in areas where the access to typical nesting places has strongly decreased over recent years. In the case of Poland, this is a result of access to European Union and restrictive regulations about animal husbandry.

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# A reference list of fish species for a heavily modified transitional water: The Zeeschelde (Belgium)

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ABSTRACT. A crucial step in the development of a fish-based index for the ecological assessment of water bodies as provided by the European Water Framework Directive is to define a reference list of fish species occurring in pristine rivers. The aim of this study was to elaborate such a list. The reference corresponds to an ecological status that is referred to as Good or Maximal Ecological Potential (GEP/MEP). Based on historically-reported fish survey data of the Zeeschelde estuary (Belgian part of the Schelde estuary) and its tributaries, i.e. an affluent system, under tidal influence, presence/absence reference lists were compiled for different salinity zones and adjusted using information from recent catches. In addition, an MEP list of fishes occurring in the Westerschelde (Dutch part of the Schelde estuary), developed by Jager & Kranenbarg (2004), is provided to present a complete overview of the Schelde estuary. Inclusion of fish species in the reference lists depended on their natural geographical distribution and ecological demands. These reference lists contain guild-specific information for the different zones within the estuary and its tidal tributaries.

KEY WORDS: ecological potential, fish reference list, Schelde estuary and tidal tributaries, Water Framework Directive

## INTRODUCTION

All transitional waters in Flanders have been identified as heavily modified water bodies (HMWB) because their nature has changed fundamentally as a result of physical anthropogenic alterations. According to Article 4(3) of the European Water Framework Directive (WFD) the principal environmental objective for HMWB and artificial water bodies is to obtain a “good ecological potential” (GEP) and “good surface water chemical status” 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; Borja & Elliott, 2007). 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 hydromorphological and associated physico-chemical conditions. Borja & Elliott (2007) considered the MEP as the reference conditions for HMWB. For an HMWB to be classified as attaining GEP status no more than slight changes in the values of the relevant biological quality elements must be observed as compared to their values at MEP. The biological potential can be defined once the hydromorphological and physical chemical potentials are described. The different paths of the decision procedure are illustrated in Fig. 1

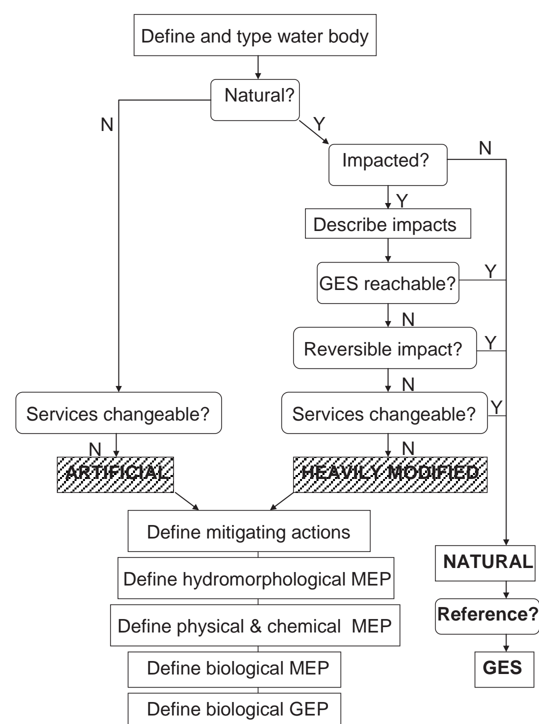


Fig. 1. – Flow diagram: guidelines to describe MEP/GEP adapted from a report of the Dutch Ministry of Transport, Public Works and Water Management (RIZA, 2006). MEP: Maximum Ecological Potential, GEP: Good Ecological Potential and GES: Good Ecological Status. Y stands for ‘yes’ to follow the indicated path; N stands for no to abort the next step.

During an international workshop on the WFD and hydromorphology held in Prague 2005 it was concluded that these biological MEP/GEP conditions can also be defined from the current status (KAMPA & KRANZ, 2005). A key difference in this approach is that the GEP is derived directly from the effect of mitigation measures, i.e. measures that reduce or remedy effects of human activities, and not indirectly from the specification and prediction of biological quality elements at MEP (KAMPA & LAASER, 2009). For the benthos in the Westerschelde, the part of the Schelde estuary that is situated in The Netherlands, ESCARAVAGE et al. (2004) suggested that when a reference based on historically pristine conditions is absent, the MEP has to be based on the knowledge of the ecosystem functioning. This concept was further elaborated by VAN DEN BERGH et al. (2005) using a scale-dependent approach. In particular ESCARAVAGE et al. (2004) defined MEP/GEP at an ecosystem scale, an ecotope scale and a macrobenthic community scale. For the Zeeschelde, the Belgian part of the Schelde estuary, BRYS et al. (2005) applied a similar hierarchical approach to define MEP/GEP for macrobenthic invertebrates and macrophytes on tidal marshes. In addition and according to the Common Implementation Strategy, they established the hydromorphological conditions required for these MEP/GEP conditions, but not for fish. We take the habitat requirements described in BREINE et al. (2008) at the guild level as the MEP/GEP conditions in estuaries for fish. Here we compile a species list for fish that should occur in the Zeeschelde estuary and its tributaries when it reaches GEP or MEP condition.

## MATERIALS AND METHODS

The study area comprises the Zeeschelde estuary and its tributaries under tidal influence. JAGER & KRANENBARG (2004) defined the reference for the Westerschelde to which we add the reference list for the Belgian part of the estuary.

We defined five different zones based on the Venice system (1959, Fig. 2): the polyhaline and mesohaline part of the Zeeschelde, the oligohaline part of the Zeeschelde including the River Rupel, the freshwater part of the Zeeschelde and Durme and the freshwater tributaries under tidal influence (Rivers Dijle, Zenne, Nete, Grote Nete, Kleine Nete). Like the estuary, all tidal tributaries are heavily modified.

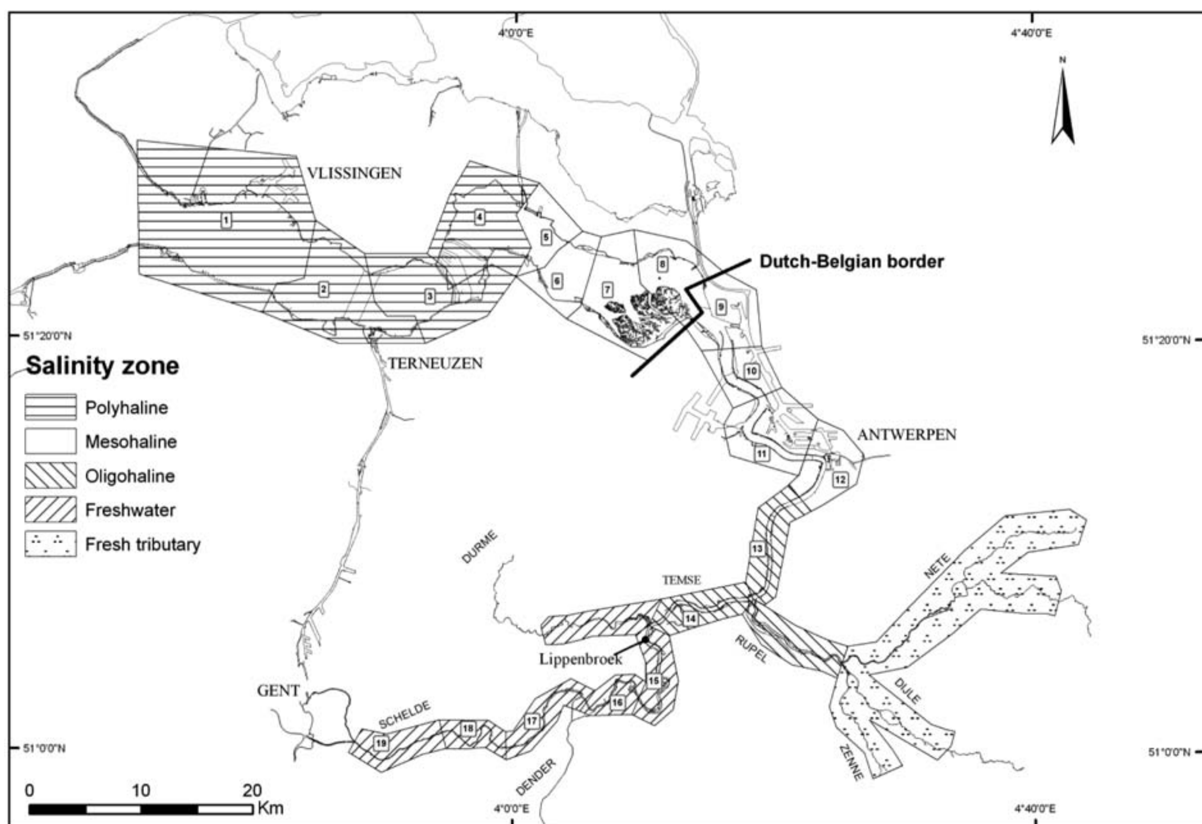


Fig. 2. – Salinity zones and Omes segments (numbers, HOFFMANN & MEIRE, 1979) in the Schelde. Omes segments are different units of the Zeeschelde that were defined in modelling studies. The Dutch-Belgian border separates the Westerschelde (downstream) from the Zeeschelde (upstream).

Next, we compiled historical records of fish that occurred in each zone of the Zeeschelde between 1842 and 1947. This list was then adjusted to an MEP/GEP reference list based on data from recent sampling programmes using fyke nets (1995-2007) and the cooling-water intake screens at the Doel power plant, situated in the mesohaline part of the Zeeschelde estuary (1991-2007). As an additional resource, we used information from peer-reviewed and grey literature reporting on non-regular sampling campaigns (Table 1). All fish species were assigned to functional groups or guilds following ELLIOTT et al. (2007) and FRANCO et al. (2008) according to their particular niche within their particular salinity zone.

TABLE 1

References, in chronological order of appearance, used to assess the presence of fish species in the Zeeschelde and tidal tributaries, classified by salinity zone.

| Salinity zone | Literature |
|---|---|
| Mesohaline | DE SELYS-LONGCHAMPS (1842); POLL (1945; 1947); VAN DAMME et al. (1994); MAES et al. (1997); BREINE et al. (2001); MAES et al. (2001); ADRIAENSSENS et al. (2002); BREINE et al. (2007; 2010a) |
| Oligohaline | MAES et al. (1997); VRIELYNCK et al. (2003); BREINE & VAN THUYNE (2004); MAES et al. (2005); BREINE & VAN THUYNE (2005); BREINE et al. (2006); SIMOENS et al. (2006); BREINE et al. (2007; 2010a) |
| Freshwater | VAN DEN BOGAERDE (1825); BREINE et al. (2001); VRIELYNCK et al. (2003); BREINE & VAN THUYNE (2005); MAES et al. (2005); BREINE et al. (2006); SIMOENS et al. (2006); BREINE et al. (2007; 2010a) |
| Nete | YSEBOODT & MEIRE (1999); VAN LIEFFERINGHE et al. (2000); BREINE et al. (2001); VAN THUYNE & BREINE (2003a); VRIELYNCK et al. (2003); VAN LIEFFERINGHE et al. (2005); BUYSSE et al. (2007); VAN THUYNE & BREINE (2008) |
| Dijle and Zenne | BREINE et al. (2001); VAN THUYNE & BREINE (2003b); VRIELYNCK et al. (2003); BUYSSE et al. (2007); VAN THUYNE & BREINE 2008) |

A species was included in the MEP/GEP lists if historical data indicate its presence in a particular zone or if its habitat needs correspond to the habitat potentials of that particular zone (BREINE et al., 2001; 2008). In addition, the catch frequency was considered and species that are no longer, or rarely, caught (<5% catch frequency defined by expert judgment) are retained only in the MEP list (Fig. 3). Eurytopic species, i.e. fishes that are able to tolerate a wide range of conditions, and species being tolerant to extreme conditions (e.g. low oxygen concentration) are placed in both lists. The GEP list differs from the MEP list since it should reflect a small anthropogenic impact. These historical MEP/GEP fish record lists were then adjusted following the criteria stipulated by RAMM (1990). We applied three conditions to omit some species from both the MEP and the GEP list even if they previously occurred in a particular zone: 1) fish are locally or regionally extirpated, 2) the presence in a particular zone is not an indication of good status (potential), 3) the zone is not their preferred habitat.

Stragglers or occasional visitors were not listed either since they do not depend on the estuary to complete their life cycle (ELLIOTT et al., 2007). Nevertheless, some interesting observations are reported here: e.g. the snake pipefish (*Entelurus aequoreus*) was quite rare in the Zeeschelde but is now captured more frequently at Doel. DE SELYS-LONGCHAMPS (1842) and POLL (1947) stated that the greater weaver (*Trachinus draco*) was common, in contrast with POLL (1945) where it was considered as an irregular guest. This species was never caught in recent surveys in the estuary.

All exotic species were omitted since they are indicators of disturbance (KARR, 1981), with the exception of pike-perch (*Sander lucioperca*) because this species can be considered as naturalised and has a high demand concerning oxygen concentrations (FAO, 1984). Exotic species were defined according to VERREYCKEN et al. (2007). Marine species that occur in the North Sea but were never reported in the river were also omitted.

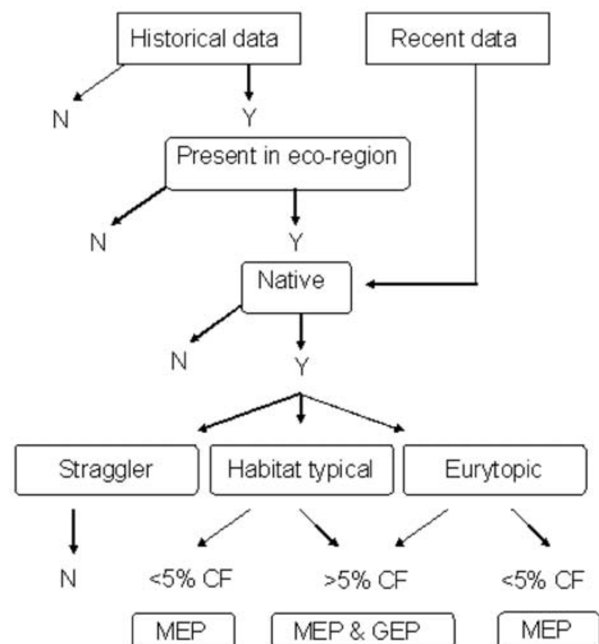


Fig. 3. – Decision tree used to allocate fish species to the Maximum Ecological Potential (MEP) and the Good Ecological Potential (GEP) list. At each level, the answer 'yes' or 'no' indicates the path along the tree. Finally, the attribution to the MEP or GEP depends on the catch frequency (CF).

TABLE 2

Historical and recent presence (1) - absence (0) fish data for the Zeeschelde estuary and GEP and MEP lists for the polyhaline zone (Jager & Kranenbarg, 2004), mesohaline, oligohaline, freshwater zones and tidal estuaries. Fish guilds are based on Franco et al. (2008). For each data source it is indicated whether the study deals with the mesohaline (M), oligohaline (O), freshwater (F) zone or (T) tributary of the Schelde. Empty cells mean that no data are available; figures in italics indicate few catches or records (<5sp.); * no longer in the Zeeschelde; ** exotic species; PC: personal communication. DS: Diadromous species; ER: Estuarine species; FW: Freshwater species; MS: Marine stragglers (adventitious visitors); MM: Marine migrants (seasonal or juvenile migrant).

| Scientific name | **MEP Westerschelde** Jager & Kranenbarg, 2004 | (M) fyke nets surveys, 1995-2006 | (M) surveys cooling-water Doel 1991-2007 | **MEP Mesohaline zone** | **GEP Mesohaline zone** | (M–O) de Selys-Longchamps, 1842 | (M–O) Poll, 1945 | (M–O) Poll, 1947 | (M-0) Simoens et al., 2006 | (M-O) Breine et al., 2007 | (M–O–F) Breine et al., 2001 | (O) fyke nets surveys, 2005-2007 | (O) River Rupel Breine et al., 2005-2007 | (O) River Rupel Simoens et al., 2006 | **MEP Oligohaline zone** | **GEP Oligohaline zone** | (F) Maes et al, 2005 | (F) Simoens et al., 2006 | (F) Durme Simoens et al., 2006 | (F) River Durme Breine et al., 2005-2007 | (F) fyke nets surveys, 2005-2007 | **MEP Freshwater zone** | **GEP Freshwater zone** | (T) Breine et al., 2001 | (T) Vrielynck et al. (2003) | (T) Nete Simoens et al., 2006 | (T) Dijle & Zenne Simoens et al., 2006 | (T) River Nete Buyse et al., 2007 & PC | (T) River Dijle Buyse et al., 2007 & PC | **MEP Tributaries** | **GEP Tributaries** | Guild |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Abramis brama* (Linnaeus, 1758) | **0** | 1 | 1 | **0** | **0** | 1 | 1 | | 1 | 1 | 1 | 1 | 1 | 1 | **1** | **1** | 1 | 1 | 0 | 1 | 1 | **1** | **1** | 1 | 1 | 0 | 0 | 1 | 1 | **1** | **1** | FW |
| *Acipenser baerii* (Brandt, 1869) | **1** | | | **1** | **0** | 1 | *1* | *1* | 0 | | 1 | | | 0 | **1** | **0** | 0 | 0 | 0 | | | **1** | **0** | 1 | 1 | 0 | 0 | | | **1** | **0** | DS* |
| *Agonus cataphractus* (Linnaeus, 1758) | **1** | 1 | *1* | **1** | **0** | | 1 | 1 | 0 | 1 | 1 | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | ER |
| *Alburnus alburnus* (Linnaeus, 1758) | **0** | | | **0** | **0** | 1 | 0 | | 0 | 1 | | *1* | 0 | 0 | **0** | **0** | | 0 | 1 | 1 | | **1** | **0** | | 1 | 1 | 1 | *1* | 0 | **1** | **0** | FW |
| *Alosa alosa* (Linnaeus, 1758) | **1** | | | **1** | **0** | 1 | *1* | *1* | 0 | *1* | 1 | | | 0 | **1** | **0** | 0 | 0 | 0 | | | **1** | **0** | 1 | 1 | 0 | 0 | | | **1** | **0** | DS* |
| *Alosa fallax* (Lacepède, 1803) | **1** | 1 | 1 | **1** | **1** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | 1 | **1** | **1** | 0 | 1 | 1 | | 1 | **1** | **1** | 1 | 1 | 1 | 0 | | | **1** | **1** | DS |
| *Ammodytes tobianus* (Linnaeus, 1758) | **1** | | 1 | **1** | **0** | 1 | 1 | 1 | 0 | 1 | 1 | *1* | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MM |
| *Anguilla anguilla* (Linnaeus, 1758) | **1** | 1 | 1 | **1** | **1** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | **1** | **1** | 1 | 1 | 1 | 1 | 1 | **1** | **1** | 1 | 1 | 1 | 1 | 1 | 1 | **1** | **1** | DS |
| *Aphia minuta* (Risso, 1810) | **0** | | 1 | **1** | **1** | | 1 | 1 | 1 | 1 | 1 | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | ER/MM |
| *Arnoglossus laterna* (Walbaum, 1792) | **0** | | | **0** | **0** | | 1 | *1* | 0 | 1 | 1 | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MS |
| *Atherina presbyter* (Risso, 1810) | **0** | 1 | 1 | **1** | **0** | | 0 | | 0 | 1 | | 1 | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MM |
| *Balistes capriscus* (Gmelin, 1789) | **0** | | | **0** | **0** | | 0 | *1* | 0 | | | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MS |
| *Barbatula barbatula* (Linnaeus, 1758) | **0** | | | **0** | **0** | 1 | 0 | | 0 | | | | | 1 | **0** | **0** | | 1 | 0 | | 1 | **0** | **0** | | | 0 | 0 | 1 | | **1** | **0** | FW |
| *Barbus barbus* (Linnaeus, 1758) | **0** | | | **0** | **0** | 1 | 0 | | 0 | | | | | 1 | **0** | **0** | | 1 | 0 | | | **0** | **0** | | *1* | 1 | 1 | | | **0** | **0** | FW |
| *Belone belone* (Linnaeus, 1761) | **1** | | | **1** | **0** | 1 | *1* | *1* | 1 | 1 | 1 | | | | **0** | **0** | | 1 | | | | **0** | **0** | | | | | | | **0** | **0** | MM |
| *Blicca bjoerkna* (Linnaeus, 1758) | **0** | 1 | 1 | **0** | **0** | | 1 | | 1 | 1 | | 1 | 1 | 1 | **1** | **1** | | 1 | 1 | 1 | 1 | **1** | **1** | | 1 | 1 | 1 | 1 | 1 | **1** | **1** | FW |
| *Callionymus lyra* (Linnaeus, 1758) | **0** | | *1* | **0** | **0** | 1 | *1* | *1* | 0 | 1 | 1 | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MS |
| *Carassius carassius* (Linnaeus, 1758) | **0** | | *1* | **0** | **0** | | 0 | | 0 | 1 | | | 0 | 1 | **0** | **0** | | 1 | 0 | *1* | 1 | **1** | **1** | | | 0 | 0 | | | **1** | **1** | FW |
| *Carrasius gibelio* (Bloch, 1782) | **0** | 1 | *1* | **0** | **0** | 1 | | | 0 | 1 | | 1 | 1 | 1 | **0** | **0** | | 0 | 0 | 1 | 1 | **0** | **0** | | | | | | | **0** | **0** | FW** |
| *Chelidonichthys lucernus* (Linnaeus, 1758) | **1** | 1 | 1 | **1** | **1** | 1 | 1 | 1 | 0 | 1 | 1 | *1* | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MM |
| *Chelon labrosus* (Risso, 1827) | **1** | | | **1** | **0** | | 0 | *1* | 0 | | | *1* | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MM |
| *Ciliata mustela* (Linnaeus, 1758) | **1** | 1 | 1 | **1** | **0** | | *1* | *1* | 1 | 1 | | *1* | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MM |
| *Clupea harengus* (Linnaeus, 1758) | **1** | 1 | 1 | **1** | **1** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | **1** | **1** | | 1 | | | | **0** | **0** | | | | | | | **0** | **0** | MM |
| *Cobitis taenia* (Linnaeus, 1758) | **0** | | | **0** | **0** | 1 | 0 | | 0 | | | | | 1 | **0** | **0** | | 1 | 1 | | | **0** | **0** | | | 1 | 1 | 1 | | **1** | **1** | FW |
| *Conger conger* (Linnaeus, 1758) | **0** | | | **0** | **0** | 1 | *1* | *1* | 0 | 1 | 1 | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MS |
| *Coregonus oxyrinchus* (Linnaeus, 1758) | **1** | | | **0** | **0** | 1 | *1* | *1* | 0 | | | | | | **0** | **0** | 0 | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | ER* |
| *Cottus gobio* (Linnaeus, 1758) | **0** | 1 | *1* | **0** | **0** | 1 | *1* | | 0 | 1 | | | | 0 | **0** | **0** | | 0 | 1 | | 1 | **1** | **0** | | | 1 | 1 | 1 | | **1** | **0** | FW |
| *Crystallogobius linearis* (Düben, 1845) | **0** | | | **0** | **0** | | | *1* | 0 | | | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MS |
| *Ctenolabrus rupestris* (Linnaeus, 1758 | **0** | | | **0** | **0** | | | *1* | 0 | | | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MS |
| *Cyclopterus lumpus* (Linnaeus, 1758) | **1** | | 1 | **1** | **0** | | *1* | *1* | 0 | 1 | 1 | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MM |

| Scientific name | **MEP Westerschelde** JAGER & KRANENBARG, 2004 | (M) fyke nets surveys, 1995-2006 | (M) surveys cooling-water Doel 1991-2007 | **MEP Mesohaline zone** | **GEP Mesohaline zone** | (M–O) DE SELYS-LONGCHAMPS, 1842 | (M–O) POLL, 1945 | (M–O) POLL, 1947 | (M-0) SIMOENS et al., 2006 | (M-O) BREINE et al., 2007 | (M–O–F) BREINE et al., 2001 | (O) fyke nets surveys, 2005-2007 | (O) River Rupel BREINE et al., 2005-2007 | (O) River Rupel SIMOENS et al., 2006 | **MEP Oligohaline zone** | **GEP Oligohaline zone** | (F) MAES et al, 2005 | (F) SIMOENS et al., 2006 | (F) Durme SIMOENS et al., 2006 | (F) River Durme BREINE et al., 2005-2007 | (F) fyke nets surveys, 2005-2007 | **MEP Freshwater zone** | **GEP Freshwater zone** | (T) BREINE et al., 2001 | (T) VRIELYNCK et al. (2003) | (T) Nete SIMOENS et al., 2006 | (T) Dijle & Zenne SIMOENS et al., 2006 | (T) River Nete BUYSE et al., 2007 & PC | (T) River Dijle BUYSE et al., 2007 & PC | **MEP Tributaries** | **GEP Tributaries** | Guild |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Dasyatis pastinaca* (Linnaeus, 1758) | 1 | | | 0 | 0 | | | *1* | 0 | | | | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | MM |
| *Dicentrarchus labrax* (Linnaeus, 1758) | 1 | 1 | 1 | 1 | 1 | | 1 | 1 | 0 | 1 | 1 | 1 | | | 1 | 1 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | MM |
| *Echiichthys vipera* (Cuvier, 1829) | 0 | | 1 | 0 | 0 | | 1 | 1 | 0 | 1 | 1 | *1* | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | MS |
| *Enchelyopus cimbrius* (Linnaeus, 1766) | 0 | | | 0 | 0 | | 0 | *1* | 0 | | | | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | MS |
| *Engraulis encrasicolus* (Linnaeus, 1758) | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | 0 | 0 | | 1 | | | | 0 | 0 | | | | | | | 0 | 0 | MM |
| *Entelurus aequoreus* (Linnaeus, 1758) | 0 | | 1 | 0 | 0 | | | *1* | 0 | | | | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | MS |
| *Esox lucius* (Linnaeus, 1758) | 0 | 1 | | 0 | 0 | 1 | 1 | | 1 | 1 | 1 | *1* | | 1 | 1 | 1 | 0 | 1 | 1 | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | FW |
| *Eutrigla gurnardus* (Linnaeus, 1758) | 0 | | *1* | 0 | 0 | | *1* | | 0 | | | | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | MM |
| *Gadus morhua* (Linnaeus, 1758) | 1 | 1 | 1 | 1 | 0 | 1 | *1* | *1* | 0 | 1 | 1 | | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | MM |
| *Gasterosteus aculeatus* (Linnaeus, 1758) | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | 1 | 1 | 1 | 1 | | | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | FW/DS |
| *Glyptocephalus cynoglossus* (Linnaeus, 1758) | | | | 0 | 0 | | | *1* | 0 | | | | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | MS |
| *Gobio gobio* (Linnaeus, 1758) | 0 | | | 0 | 0 | 1 | 0 | | 0 | | | | | 1 | 0 | 0 | | 1 | 1 | | 1 | 0 | 0 | | 1 | 1 | 1 | 1 | *1* | 1 | 1 | FW |
| *Gymnocephalus cernua* (Linnaeus, 1758) | 1 | 1 | 1 | 1 | 1 | 1 | 0 | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | | | 1 | 1 | FW |
| *Hippocampus guttulatus* (Cuvier, 1829) | 0 | | | 0 | 0 | | | *1* | 0 | 1 | 1 | | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | ER |
| *Hippocampus hippocampus* (Linnaeus, 1758) | 0 | | 1 | 0 | 0 | 1 | *1* | | 0 | | | | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | MS |
| *Hippoglossus hippoglossus* (Linnaeus, 1758) | | | | 0 | 0 | | | *1* | 0 | | | | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | MS |
| *Hyperoplus lanceolatus* (Le Sauvage, 1824) | 0 | | | 0 | 0 | 1 | *1* | | 0 | 1 | 1 | | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | MS |
| *Lampetra fluviatilis* (Linnaeus, 1758) | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | 1 | 1 | 1 | 1 | 1 | 0 | | | 1 | 1 | 1 | *1* | 0 | 0 | *1* | 0 | 1 | 0 | DS |
| *Lampetra planeri* (Bloch, 1748) | 0 | | | 0 | 0 | 1 | | | 0 | | | | | 0 | 0 | 0 | | 0 | 1 | | 1 | 0 | 0 | | 1 | 0 | 1 | | | 1 | 0 | FW |
| *Leucaspius delineatus* (Heckel, 1843) | 0 | | | 0 | 0 | | | | 0 | 1 | | *1* | 0 | 0 | 0 | 0 | | 0 | 1 | 1 | 1 | 0 | 0 | | | 0 | 1 | | | 1 | 0 | FW |
| *Leuciscus idus* (Linnaeus, 1758) | 0 | 1 | 1 | 0 | 0 | | 0 | | 0 | 1 | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | | 1 | 1 | | 1 | 0 | 0 | *1* | 0 | 1 | 1 | FW |
| *Leuciscus leuciscus* (Linnaeus, 1758) | 0 | | *1* | 0 | 0 | 1 | 0 | | 0 | | | | | 0 | 0 | 0 | | 0 | 1 | | 1 | 0 | 0 | | | 1 | 1 | 1 | | 1 | 0 | FW |
| *Limanda limanda* (Linnaeus, 1758) | 1 | 1 | 1 | 1 | 0 | | *1* | 0 | 0 | 1 | 1 | | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | MM |
| *Liparis liparis* (Linnaeus, 1766) | 1 | 1 | 1 | 1 | 1 | | 1 | 1 | 0 | 1 | 1 | | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | ER |
| *Liza ramada* (Risso, 1827) | 1 | 1 | 1 | 1 | 1 | | 1 | 1 | 1 | 1 | 1 | 1 | | 1 | 1 | 1 | 1 | 1 | 0 | | 1 | 1 | 1 | 1 | | 0 | 0 | | | 1 | 1 | DS |
| *Lota lota* (Linnaeus, 1758) | | | | 1 | 0 | 1 | 0 | | 0 | | | | | 0 | 1 | 0 | | 0 | 0 | | | 1 | 0 | | *1* | 0 | 0 | | | 1 | 0 | FW |
| *Melanogrammus aeglefinus* (Linnaeus, 1758) | 0 | | | 0 | 0 | 1 | *1* | *1* | 0 | | | | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | MS |
| *Merlangius merlangus* (Linnaeus, 1758) | 1 | 1 | 1 | 1 | 1 | 1 | *1* | 1 | 0 | 1 | 1 | *1* | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | MM |
| *Misgurnus fossilis* (Linnaeus, 1758) | 0 | | | 0 | 0 | 1 | *1* | | 1 | 1 | 1 | | | 1 | 1 | 1 | | 1 | 1 | | | 1 | 1 | 1 | 1 | 1 | 1 | *1* | | 1 | 1 | FW |
| *Mullus surmuletus* (Linnaeus, 1758) | 0 | 1 | 1 | 0 | 0 | | | 0 | 0 | 1 | | | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | MS |
| *Myoxocephalus scorpius* (Linnaeus, 1758) | 1 | 1 | 1 | 1 | 1 | | 1 | 1 | 0 | 1 | 1 | | | | 1 | 1 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | ER |
| *Nerophis ophidion* (Linnaeus, 1758) | 0 | | | 0 | 0 | | | *1* | 0 | | | | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | ER |
| *Osmerus eperlanus* (Linnaeus, 1758) | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | DS |
| *Perca fluviatilis* (Linnaeus, 1758) | 0 | 1 | 1 | 1 | 1 | 1 | 1 | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | FW |
| *Petromyzon marinus* (Linnaeus, 1758) | 1 | | | 1 | 1 | 1 | *1* | *1* | 1 | 1 | 1 | | | 1 | 1 | 1 | 0 | 1 | 0 | | | 1 | 1 | 1 | | 0 | 0 | | | 1 | 0 | DS |
| *Pholis gunnellus* (Linnaeus, 1758) | 1 | | | 1 | 0 | | 1 | 1 | 0 | | | | | | 0 | 0 | | 0 | | | | 0 | 0 | | | | | | | 0 | 0 | ER |
| *Phoxinus phoxinus* (Linnaeus, 1758) | 0 | | | 0 | 0 | | 0 | | 0 | | | | | 0 | 0 | 0 | | 0 | 1 | | | 0 | 0 | | 1 | 1 | 1 | | | 1 | 0 | FW |
| *Platichthys flesus* (Linnaeus, 1758) | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | 1 | 1 | DS |

| Scientific name | **MEP Westerschelde** Jager & Kranenbarg, 2004 | (M) fyke nets surveys, 1995-2006 | (M) surveys cooling-water Doel 1991-2007 | **MEP Mesohaline zone** | **GEP Mesohaline zone** | (M–O) de Selys-Longchamps, 1842 | (M–O) Poll, 1945 | (M–O) Poll, 1947 | (M-0) Simoens et al., 2006 | (M-O) Breine et al., 2007 | (M–O–F) Breine et al., 2001 | (O) fyke nets surveys, 2005-2007 | (O) River Rupel Breine et al., 2005-2007 | (O) River Rupel Simoens et al., 2006 | **MEP Oligohaline zone** | **GEP Oligohaline zone** | (F) Maes et al, 2005 | (F) Simoens et al., 2006 | (F) Durme Simoens et al., 2006 | (F) River Durme Breine et al., 2005-2007 | (F) fyke nets surveys, 2005-2007 | **MEP Freshwater zone** | **GEP Freshwater zone** | (T) Breine et al., 2001 | (T) Vrielynck et al. (2003) | (T) Nete Simoens et al., 2006 | (T) Dijle & Zenne Simoens et al., 2006 | (T) River Nete Buyse et al., 2007 & PC | (T) River Dijle Buyse et al., 2007 & PC | **MEP Tributaries** | **GEP Tributaries** | Guild |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Pleuronectes platessa* (Linnaeus, 1758) | **1** | 1 | 1 | **1** | **1** | 1 | 1 | 1 | 1 | 1 | 1 | | | | **0** | **0** | | 1 | | | | **0** | **0** | | | | | | | **0** | **0** | MM |
| *Pollachius pollachius* (Linnaeus, 1758) | **0** | | *1* | **0** | **0** | | | *1* | 0 | | | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MM |
| *Pomatoschistus lozanoi* (de Buen, 1923) | **0** | 1 | 1 | **1** | **0** | | | | 0 | 1 | 1 | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MM |
| *Pomatoschistus microps* (Krøyer, 1838) | **1** | 1 | 1 | **1** | **1** | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | **1** | **1** | | 1 | 1 | 1 | 1 | **1** | **0** | | | | | | | **0** | **0** | ER |
| *Pomatoschistus minutus* (Pallas, 1770) | **1** | 1 | 1 | **1** | **1** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | **1** | **1** | | 1 | | | *1* | **0** | **0** | | | | | | | **0** | **0** | ER |
| *Psetta maxima* (Linnaeus, 1758) | **1** | | | **1** | **0** | 1 | *1* | 1 | 1 | 1 | 1 | | | | **0** | **0** | | 1 | | | | **0** | **0** | | | | | | | **0** | **0** | MM |
| *Pungitius pungitius* (Linnaeus, 1758) | **0** | 1 | 1 | **0** | **0** | 1 | *1* | | 1 | 1 | 1 | 1 | 1 | 1 | **1** | **1** | 1 | 1 | 0 | 0 | 1 | **1** | **1** | 1 | 1 | 1 | 0 | *1* | | **1** | **1** | FW |
| *Raja clavata* (Linnaeus, 1758) | **0** | | | **0** | **0** | 1 | *1* | 1 | 0 | 1 | 1 | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MS |
| *Raniceps raninus* (Linnaeus, 1758) | **0** | | | **0** | **0** | 1 | | | 0 | | | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | ER |
| *Rhodeus sericeus* (Pallas, 1776) | **0** | 1 | 1 | **0** | **0** | | 0 | | 0 | 1 | | 1 | 1 | 1 | **1** | **1** | | 1 | 0 | 1 | 1 | **1** | **1** | | | 0 | 0 | 1 | *1* | **1** | **1** | FW |
| *Rutilus rutilus* (Linnaeus, 1758) | **0** | 1 | 1 | **0** | **1** | 1 | 1 | | 1 | 1 | 1 | 1 | 1 | 1 | **1** | **1** | 1 | 1 | 1 | 1 | 1 | **1** | **1** | 1 | 1 | 1 | 1 | 1 | 1 | **1** | **1** | FW |
| *Salmo salar* (Linnaeus, 1758) | **1** | 1 | | **1** | **0** | 1 | *1* | *1* | 0 | 1 | 1 | | | 0 | **1** | **0** | 0 | 0 | 0 | | | **1** | **0** | 1 | 1 | 0 | 0 | | | **1** | **0** | DS* |
| *Salmo trutta* (Linnaeus, 1758) | **1** | 1 | | **1** | **1** | | *1* | *1* | 0 | 1 | 1 | 1 | | 0 | **1** | **1** | 0 | 0 | 0 | | | **1** | **1** | 1 | 1 | 0 | 0 | | | **1** | **0** | DS |
| *Sander lucioperca* (Linnaeus, 1758) | **0** | 1 | 1 | **0** | **1** | | *1* | | 0 | 1 | 1 | 1 | | | **0** | **1** | 1 | 0 | | | 1 | **0** | **1** | | | | | | | **0** | **0** | FW** |
| *Sardina pilchardus* (Walbaum, 1792) | **0** | | | **0** | **0** | | 0 | *1* | 0 | | | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MM |
| *Scardinius erythrophthalmus* (Linnaeus, 1758) | **0** | | 1 | **0** | **0** | 1 | 1 | | 1 | 1 | 1 | 1 | 1 | 1 | **1** | **1** | | 1 | 1 | 1 | 1 | **1** | **1** | 1 | 1 | 1 | 1 | *1* | *1* | **1** | **1** | FW |
| *Scomberesox saurus* (Walbaum, 1792) | **0** | | | **0** | **0** | | *1* | *1* | 0 | 1 | | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MS |
| *Scophthalmus rhombus* (Linnaeus, 1758) | **1** | 1 | | **1** | **0** | 1 | *1* | *1* | 1 | 1 | 1 | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MS |
| *Scyliorhinus canicula* (Linnaeus, 1758) | **0** | | | **0** | **0** | 1 | *1* | *1* | 0 | | | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MS |
| *Scyliorhinus stellaris* (Linnaeus, 1758) | | | | **0** | **0** | | *1* | *1* | 0 | | | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MS |
| *Silurus glanis* (Linnaeus, 1758) | **0** | | | **0** | **0** | | | | 0 | | | | 1 | 0 | **1** | **1** | 0 | 0 | 1 | 0 | 1 | **1** | **1** | | | 1 | 0 | 1 | *1* | **1** | **1** | FW |
| *Solea solea* (Linnaeus, 1758) | **1** | 1 | 1 | **1** | **1** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | **1** | **1** | | 1 | | | | **0** | **0** | | | | | | | **0** | **0** | MM |
| *Spinachia spinachia* (Linnaeus, 1758) | **0** | | *1* | **0** | **0** | | 0 | 0 | 0 | | | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | ER |
| *Sprattus sprattus* (Linnaeus, 1758) | **1** | 1 | 1 | **1** | **1** | 1 | 1 | 1 | 1 | 1 | 1 | | | | **1** | **1** | | 1 | | | | **0** | **0** | | | | | | | **0** | **0** | MM |
| *Squalius cephalus* (Linnaeus, 1758) | **0** | | | **0** | **0** | 1 | 0 | | 0 | | | | 0 | 1 | **0** | **0** | | 1 | 0 | *1* | 1 | **0** | **0** | | | 0 | 0 | 1 | 0 | **1** | **0** | FW |
| *Symphodus melops* (Linnaeus, 1758) | **0** | | | **0** | **0** | | 0 | *1* | 0 | | | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MS |
| *Syngnathus acus* (Linnaeus, 1758) | **1** | 1 | 1 | **1** | **1** | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | **1** | **1** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | ER |
| *Syngnathus rostellatus* (Nilsson, 1855) | **1** | | 1 | **1** | **1** | 1 | 1 | 1 | 0 | 1 | 1 | | | | **1** | **1** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | ER |
| *Tinca tinca* (Linnaeus, 1758) | **0** | | *1* | **0** | **0** | 1 | 0 | | 0 | | | *1* | | 1 | **0** | **0** | | 1 | 1 | | | **0** | **0** | | 1 | 1 | 1 | *1* | 1 | **1** | **1** | FW |
| *Trachinus draco* (Linnaeus, 1758) | **0** | | | **0** | **0** | 1 | *1* | 1 | 0 | | | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MS |
| *Trachurus trachurus* (Linnaeus, 1758) | **0** | 1 | 1 | **0** | **0** | | *1* | 1 | 0 | | 1 | 1 | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MS |
| *Trigloporus lastoviza* (Bonnaterre, 1788) | | | | **0** | **0** | | | *1* | 0 | | | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MS |
| *Trisopterus luscus* (Linnaeus, 1758) | **1** | 1 | 1 | **1** | **1** | 1 | 1 | 1 | 0 | 1 | 1 | 1 | | | **1** | **1** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MM |
| *Trisopterus minutus* (Linnaeus, 1758) | **0** | | | **0** | **0** | | | *1* | 0 | | | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MS |
| *Xiphias gladius* (Linnaeus, 1758) | | | | **0** | **0** | | *1* | | 0 | | | | | | **0** | **0** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | MS |
| *Zoarces viviparus* (Linnaeus, 1758) | **1** | 1 | 1 | **1** | **1** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | **1** | **1** | | 0 | | | | **0** | **0** | | | | | | | **0** | **0** | ER |

## RESULTS

Table 2 presents the reference lists for the different zones of the Zeeschelde. Species are ordered in alphabetical order. An MEP list of fishes occurring in the Westerschelde (Jager & Kranenbarg, 2004) is also given to provide a complete overview of the Schelde estuary. The MEP and GEP lists for the Zeeschelde are grouped by salinity zone and in the last column the guild attribution is given.

## DISCUSSION

We structured the discussion of these lists using the ecological guild of estuarine usage (Elliott et al., 2007; Franco et al., 2008). We did not include information from archaeological studies (e.g. Van Neer & Ervynck, 1993; 1994) as anthropogenic impact in the Schelde estuary has been almost continuous since the ninth century; therefore it is scientifically impossible to trace how an unimpaired Schelde estuary would have developed. Shifts in fish assemblages can occur due to climate effects of oceans and estuaries (Tulp et al., 2008). The increase in temperature and decrease in salinity in the Baltic Sea, for example, caused a decrease in abundance of marine species and increase of freshwater species (MacKenzie et al., 2007). Daufresne & Boët (2007) described new evidence that climate change impacted fish communities in large rivers in France. At present, no data on fish community changes due to climate change in the Zeeschelde are available. However, the decision tree (see Fig. 3) used to allocate fish species indirectly considers possible changes.

### 3.1 Estuarine species

Estuarine species can complete their life cycle in the estuary. Estuarine resident species are tolerant to widely varying environmental conditions that typically characterize these transitional waters (Elliott et al., 2007). However, they are sensitive to the disappearance of specific estuarine habitats such as intertidal mudflats, creeks and marshes and to the accumulation of toxic substances. Therefore an estuary in MEP or in GEP status should accommodate these species. The habitat preferences for estuarine species are not fulfilled in the tributaries. According to Poll (1945; 1947), the common goby (*Pomatoschistus microps*) was quite rare in the Schelde but this species and also the sand goby (*Pomatoschistus minutus*) are at present very common (Guelinckx et al., 2008). The common goby is regularly found far upstream, but freshwater is not its preferred habitat. The sand goby is less common in the freshwater part and is therefore not kept in the freshwater lists. Transparent goby (*Aphia minuta*) is an estuarine/marine migrant species that should normally occur in the Schelde and is regularly caught in the mesohaline zone. This species prefers a polyhaline and mesohaline habitat (van Emmerik, 2003) and is therefore only included in the mesohaline GEP and MEP list, contrary to the list proposed by Jager & Kranenbarg (2004). Straight-nosed pipefish (*Nerophis ophidion*) was only occasionally caught in the Schelde (Poll, 1947) and has never been caught in recent surveys. This species is not retained in the Westerschelde reference list (Jager & Kranenbarg, 2004) and hence it is not considered as a GEP or MEP species here either. The greater pipefish (*Syngnathus acus*), Nilsson's pipefish (*Syngnathus rostellatus*) and the viviparous blenny (*Zoarces viviparus*) are estuarine resident species that occurred in the Schelde in the past (de Selys-Longchamps, 1842; Poll, 1945; 1947). At present, they are caught as far upstream as Antwerpen. These species avoid freshwater (van Emmerik, 2003) and therefore are only included in the mesohaline and oligohaline MEP and GEP lists. The hooknose (*Agonus cataphractus*) is an estuarine resident species that is reported to be rare in the Schelde (Poll, 1945), which also corresponds with our catch results. Hooknose is therefore retained only in the mesohaline MEP and the polyhaline lists. Bull rout (*Myoxocephalus scorpius*) was quite common in the Schelde estuary (Poll, 1945) and is still caught from time to time. This species is included in both meso- and oligohaline GEP and MEP lists. Butterfish (*Pholis gunnellus*) is included in the reference list for the Westerschelde (Jager & Kranenbarg, 2004). Poll (1945) stated that the species was present, but it was never caught in recent samples, which is why we excluded this species from the GEP list but included it in the mesohaline MEP list. Striped seasnail (*Liparis liparis*) used to be common in the Schelde (Poll, 1947) preferring poly- and mesohaline water. Seasnail was occasionally caught in recent campaigns and is therefore considered to be a mesohaline GEP and MEP species. Both seahorse (*Hippocampus guttulatus*) and tadpole fish (*Raniceps raninus*) are absent from the lists. In the past, seahorse was caught nearby the sea (Poll, 1945) and was considered as rare. This species prefers polyhaline water and at present is rarely caught in the Zeeschelde. Tadpole fish has been recorded for the first time in the Schelde in 1943 (Poll, 1945) Now this species is believed to be very rare in the estuary but more common in nearby Dutch coastal waters. Fifteen-spined stickleback (*Spinachia spinachia*) was not reported by de Selys-Longchamps (1842) or by Poll (1945) and it was only once caught in Doel. Thus it is not considered as being a GEP or MEP species.

### 3.2 Diadromous species

Diadromous fishes are migrating species that use both marine and freshwater habitats during their life cycles. Estuaries have a crucial role as migration routes (Able, 2005). According to the season different diadromous species occur in different estuarine zones. Absence of diadromous species is caused by human impacts, disrupting the connectivity and resulting in an estuary not being considered to reach the MEP or GEP status. Thus, diadromous species are included in both lists and all zones when not extirpated in the estuary or nearby estuaries. If all physical and chemical barriers were to disappear, these species should be able to swim all along the tributaries (see Table 2). The decline of sturgeon (*Acipenser sturio*), Atlantic salmon (*Salmo salar*) and allis shad (*Alosa alosa*) was already described by Poll (1945). Now, they are extirpated in the Schelde basin and are not considered as GEP species. However, it is not impossible to restore their required habitat in the Schelde basin, especially since

these species are present in some North East estuaries of the Atlantic. Their possible return would indicate MEP condition. Houting (*Coregonus oxyrinchus*) was considered as very rare or in danger of extinction by POLL (1945; 1947). At present, this species is considered to have disappeared (red list) or to be extinct (International Union for Conservation of Nature and Nature Resources: IUCN); hence, it is not in our lists. The habitat area of this species is also situated more to the north (MAITLAND, 2000). All the other diadromous species (see below) are present in the lists because they can be expected to frequent the estuary and tributaries once the habitat conditions improve (MAES et al., 2007). The brown trout (*Salmo trutta*) population was already declining in 1945 (POLL, 1945) and now individuals are rarely caught. However, their presence would indicate an MEP status as they are pollution-intolerant species. Eel (*Anguilla anguilla*) and flounder (*Platichthys flesus*) were common in the River Schelde (DE SELYS-LONGCHAMPS, 1842; POLL, 1945). Three-spined stickleback (*Gasterosteus aculeatus*) is known to be a species that is common in all types of waters in Flanders. In the mesohaline zone of the Zeeschelde, three types occur (RAEYMAEKERS et al., 2007) including the diadromous type. The Western three-spine stickleback (*Gasterosteus gymnurus*) is probably extremely rare or extinct in our study area. This species has never been observed during sampling campaigns by the Research Institute for Nature and Forest (INBO), or by other scientists intensively studying sticklebacks from Belgium and the Netherlands (RAEYMAEKERS, pers. comm.; RAEYMAEKERS et al., 2005; 2007; 2008a; 2008b; 2009; 2011). Thinlip mullet (*Liza ramada*) was previously often confounded with thicklip grey mullet (*Chelon labrosus*), a marine seasonal migrant. POLL (1945) stated that the species was abundant nearby the Belgian coast. At present specimens are recorded far upstream of Antwerpen. River lamprey (*Lampetra fluviatilis*), twaite shad (*Alosa fallax*) and smelt (*Osmerus eperlanus*) are indicators of good water quality and connectivity as well as good ecological functioning of the estuary (e.g. suitable spawning locations). They are again regularly caught in different parts of the Schelde (BREINE et al., 2010a). Sea lamprey (*Petromyzon marinus*), which was abundant according to DE SELYS-LONGCHAMPS (1842), is at present scarce (<5% catch frequency) and is kept in the MEP lists.

### 3.3 Freshwater species

Freshwater resident species can complete their life cycle in the tidal freshwater part of the estuary. They reproduce, grow up and feed in freshwater, but can also exploit the oligohaline zone. This is why they are also included in the oligohaline MEP/GEP list. The Zeeschelde has an important freshwater tidal zone and therefore freshwater species occupy various zones but the spatial distribution is species-dependent. Some freshwater species make regular use of different zones within the estuaries, such as seasonal migrations, nursery or feeding migrations, reproductive migrations through the estuary or the use of the estuary as a refuge (ELLIOTT et al., 2007). Freshwater stragglers are species that occupy the mesohaline zone irregularly and only for a short time. ELLIOTT et al. (2007) considered them analogous to marine stragglers but entering the estuary from the opposite end. For the tributaries, 25 freshwater species are recorded in the MEP list and 16 in the GEP list. The freshwater species ruffe (*Gymnocephalus cernua*) is mentioned by DE SELYS-LONGCHAMPS (1842) but not by POLL (1945). Presently, this species is caught in the Zeeschelde along its entire salinity gradient. POLL (1945) considered perch (*Perca fluviatilis*) to be very common in the freshwater and brackish reaches of the Zeeschelde up to Zandvliet. Recently, perch was caught all over the Zeeschelde. Roach (*Rutilus rutilus*) is captured in all zones but is not typical for the mesohaline zone, though specimens were captured in Doel and Zandvliet. Therefore its presence is justified in all GEP lists but not in the mesohaline MEP list. Bream (*Abramis brama*) and nine-spined stickleback (*Pungitius pungitius*) are typical lowland freshwater species with a tolerance for brackish water. They are opportunistic species that were caught all over the river Schelde. These species are not typical for mesohaline water and were therefore omitted from the mesohaline GEP and MEP lists. Though nine-spined stickleback is less common than the three-spined stickleback, it is to be found in all tributaries. As already mentioned above; three-spined stickleback is common in all zones. Bitterling (*Rhodeus sericeus*) is a freshwater species preferring stagnant or slow moving water with plants. Though POLL (1945) did not mention its presence in the Schelde, it has been collected in different places in the Zeeschelde (BREINE et al., 2010a). SIMOENS et al. (2006) placed this species in the reference list for fresh tidal water but not for the brackish part of the Schelde. We included it only in the oligohaline and freshwater MEP and GEP lists. Wels catfish (*Silurus glanis*) is now frequently caught all along the tidal freshwater part of the Schelde. Though this species can stand brackish water, it is kept in the freshwater and oligohaline GEP and MEP lists only, since the mesohaline is not its preferred habitat (FRIMODT, 1995). The weatherfish (*Misgurnus fossilis*) is now only caught in the tributaries. DE SELYS-LONGCHAMPS (1842) mentioned its presence in the Schelde and POLL (1945) stated that three specimens were collected in the Schelde. This species should not be present in the mesohaline zone but its presence could be indicative in the other zones. Carp (*Cyprinus carpio*) was reported by DE SELYS-LONGCHAMPS (1842) and POLL (1945) and is still caught in the freshwater and oligohaline zones. The species does not occur in our lists since it has an exotic origin and is tolerant to extreme conditions. Species such as white bream (*Blicca bjoerkna*), pike (*Esox lucius*) and rudd (*Scardinius erythrophthalmus*) were mentioned by POLL (1945) to be present in the Schelde. They are still caught in the Zeeschelde and even occasionally in Zandvliet (GUELINCKX et al., 2008). These freshwater species are not part of the mesohaline fish population but can occur in the oligohaline zone. Therefore, all three of them are kept in the oligohaline and freshwater GEP and MEP lists. Ide (*Leuciscus idus*) is a rheophilic B species i.e. some stages of its life history are confined to connected backwaters (VAN EMMERIK, 2003) with a relatively high tolerance value (BREINE et al., 2007). This species is frequently encountered in the oligohaline zone. Ide is found all along the River Schelde and in most of its tributaries. However, its abundance might be underestimated because of a possible confusion with roach. Ide is considered as being repre-

sentative for oligohaline, freshwater and tributaries GEP and MEP lists. We keep Crucian carp (*Carassius carassius*) in the freshwater list since it is occasionally captured (>5% catch frequency) in the Zeeschelde (SIMOENS et al., 2006). Pike-perch (*Sander lucioperca*) is an exotic freshwater species, which is considered as a recent native species in the Netherlands (VAN EMMERIK, 2003). This species can tolerate brackish water and is quite common along the salinity gradient. Pike-perch is sensitive to temperature changes and intolerant to oxygen deficiency and can be used as an indicator for eutrophication (VAN EMMERIK, 2003). The species prefers deeper water than provided by the tributaries and is therefore kept in the GEP lists of the main channel only. Bullhead (*Cottus gobio*) has been reported to be present over the entire salinity gradient (DE SELYS-LONGCHAMPS, 1842; POLL, 1945; 1947) and was also recently caught in Zandvliet while BUYSSE et al. (2007) caught it in the Nete. This non-obligate rheophilic species (i.e. it also tolerates slow running water) lives in freshwater but can stand brackish water. SIMOENS et al. (2006) did not consider bullhead a reference species for the Schelde and its tributaries. This intolerant species has a low range of acceptable habitats (GRANDMOTTET, 1983) and prefers a hard substrate with gravel and stones. At present only the River Nete has a water quality that meets the demands of this species, but the morphological characteristics and substrate of the tributaries are not really optimal. We keep it as an indicator for the MEP status in the freshwater zone and tributaries. Burbot (*Lota lota*) has recently been reintroduced into the upper Nete. It is possible that this species will be caught in the Zeeschelde in the future, because POLL (1945) mentioned that it can tolerate mesohaline conditions. Burbot is retained in the MEP lists since it is an intolerant species. Dace (*Leuciscus leuciscus*) was not mentioned by DE SELYS-LONGCHAMPS (1842) or POLL (1945; 1947) and is only caught in the freshwater tributaries. Because of its rarity and ecological demands, this species is included in the MEP lists for tributaries only (TURNPENNY et al., 2004). The same reasoning applies for spined loach (*Cobitis taenia*) frequently caught in the River Nete but not found in the main channel. Bleak (*Alburnus alburnus*) is a freshwater species that is occasionally fished in the freshwater part of the main river and in the River Nete. DE SELYS-LONGCHAMPS (1842) mentioned its presence in the Schelde while POLL (1945; 1947) did not. According to BREINE et al. (2007), bleak has a low pollution tolerance, which is why it is only included in the freshwater and tributaries MEP lists. Stone loach (*Barbatula barbatula*) is presently caught in the freshwater tributaries only, where it indicates an MEP status (<5% CF). DE SELYS-LONGCHAMPS (1842) reported on barbel (*Barbus barbus*) and brook lamprey (*Lampetra planeri*) while POLL (1945) did not. The Zeeschelde is not their habitat, and MAES et al. (2005) and BREINE et al. (2007) did not include these two species in their reference lists. Barbel is a rheophilic A species preferring fast running water, which is not typical for the Schelde tributaries. This species has not been caught recently and it was decided not to retain barbel in the lists since the tributaries do not offer the required habitat demands. Brook lamprey was caught in the tributaries and is therefore kept in its MEP list. Eurasian minnow (*Phoxinus phoxinus*) is an intolerant species typical for upstream water (BREINE et al., 2004; 2007), preferring well-oxygenated water and gravel substrate (VOSTRADOVSKY, 1973). Minnow has never been caught in the Zeeschelde. European chub (*Squalius cephalus*) and gudgeon (*Gobio gobio*) are species mentioned by DE SELYS-LONGCHAMPS (1842) but not by POLL (1945; 1947). They were caught in the freshwater tributaries (BUYSSE et al., 2007; BREINE et al., 2007). European chub is a rheophilic A species typically occurring in creeks and fast flowing rivers (BILLARD, 1997) and its presence indicates MEP status. Belica (*Leucaspius delineatus*) is caught occasionally in the freshwater part of the Schelde but was not reported by DE SELYS-LONGCHAMPS (1842) or POLL (1945; 1947). Belica is a stagnophilic species that needs the presence of plants, which are not present in the Schelde. Therefore, this species is included in the tributaries list only. Tench (*Tinca tinca*) has been caught around Antwerpen but is considered as a species being typical for standing waters and upstream in the tributaries (ALLEN et al., 2002). Therefore, it is only included in the tributary MEP/GEP lists.

### 3.4 Marine migrants

ELLIOTT et al. (2007) no longer distinguished between marine seasonal migrants and marine juvenile migrants since larval and 0+ juvenile migrations into estuaries tend to be seasonal for many marine species. Either way, estuaries in MEP or GEP status are used by these migrants as feeding areas and refugia. Tributaries (i.e. the river affluent) do not offer suitable habitats for marine migrants. Herring (*Clupea harengus*) is a marine species abundant in the juvenile stage (POLL, 1945; 1947; MAES et al., 1997; 2001) and swims upstream as far as the oligohaline zone. Plaice (*Pleuronectes platessa*) was described by POLL (1945) as being very abundant in the Schelde, although adults were rarely caught. The species is now collected in small numbers at Doel and is retained in the mesohaline GEP and MEP lists. Sole (*Solea solea*) penetrated as juveniles quite far into the estuary and numerous adults were caught (POLL, 1945). Sole is now found in the mesohaline and oligohaline zones and is retained in both the GEP and MEP list. Juveniles of the marine species tub gurnard (*Chelidonichthys lucernus*) and whiting (*Merlangius merlangus*) have been reported in the Schelde by DE SELYS-LONGCHAMPS (1842) and POLL (1945; 1947). Also currently, mostly juveniles are caught. The oligohaline zone is not their habitat and they are therefore retained only for the mesohaline GEP and MEP lists. At present, seabass (*Dicentrarchus labrax*) is one of the most common species caught in the Schelde, which is in line with POLL (1945) who reported high numbers of juveniles. This species figures in the GEP and MEP lists of meso- and oligohaline waters. Pouting (*Trisopterus luscus*) is a marine species, the juveniles of which were frequently observed in the Schelde (POLL, 1945, 1947), and they are still captured up to Antwerpen. The species is included in the meso- and oligohaline GEP and MEP lists. Only juveniles of brill (*Scophthalmus rhombus*) are now found in the Zeeschelde. This species was not common in the past (POLL, 1945). Consequently, it is only included in the mesohaline MEP list. Sand smelt (*Atherina presbyter* or *A. boyeri* Risso, 1826) was reported to be quite abundant in Belgian coastal waters (POLL, 1947) and is now regu-

larly caught in the Zeeschelde. Therefore, sand smelt stays on the mesohaline MEP list. Cod (*Gadus morhua*) is an uncommon seasonal migrant, of which only juveniles wander into the estuary. Cod is included in the mesohaline MEP list only. POLL (1947) reported the occasional presence of the marine juvenile migrant dab (*Limanda limanda*). In recent surveys, this species is rarely caught and is therefore retained only for the mesohaline MEP list. Turbot (*Psetta maxima*) is rarely caught and if so, only as juveniles. Turbot is included in the Dutch list (JAGER & KRANENBARG, 2004) but kept in our mesohaline MEP list only. Pollack (*Pollachius pollachius*) was described as being rare in Belgian coastal waters (POLL, 1947); there are also no records from DE SELYS-LONGCHAMPS (1842) and POLL (1945). Pollack was not collected during recent fish campaigns in the Zeeschelde and is therefore omitted from our lists. In the past, sprat (*Sprattus sprattus*) entered the estuary between January and July in large numbers (DE SELYS-LONGCHAMPS, 1842; POLL, 1945; 1947). This species is still caught often and is a reference species for the Westerschelde (JAGER & KRANENBARG, 2004). We included it in our meso- and oligohaline GEP and MEP lists. Following POLL (1947), anchovy (*Engraulis encrasicolus*) was a seasonal guest from April to August, visiting the estuary in large numbers to spawn. At present, it is rarely caught upstream of Doel, which is why they are retained in the mesohaline MEP and GEP lists. POLL (1947) considered thicklip grey mullet (*Chelon labrosus*) as rare in the Schelde but it was occasionally caught (<5% CF) in recent surveys and is therefore included in the mesohaline MEP list. Garpike (*Belone belone*) was uncommon in the estuary (POLL, 1945). Though it was not caught recently, it has a place in the mesohaline MEP list as an indicator of good water quality and as part of the reference list for the Westerschelde (JAGER & KRANENBARG, 2004). The lumpsucker (*Cyclopterus lumpus*) was rarely caught in the 1940's (POLL, 1945; 1947) and this is still the case. Nevertheless, we kept this species in the mesohaline MEP list as it is an indicator of an undisturbed habitat. They are sensitive to dredging activities as they are nest spawners and guarders. The five-beard rockling (*Ciliata mustela*) was rarely caught in the past (POLL, 1945; 1947) but is now regularly caught in Doel and is included in the Mesohaline MEP list. Grey gurnard (*Eutrigla gurnardus*), sting ray (*Dasyatis pastinaca*) and pilchard (*Sardina pilchardus*) were only encountered occasionally in the estuary (POLL, 1945; 1947). Of them, only grey gurnard was caught erratically in Doel and consequently, none of the three species is kept in the lists. Small sandeel (*Ammodytes tobianus* or *A. lancea*) was common in the Schelde estuary (POLL, 1945). This species is occasionally caught today and therefore remains on the mesohaline MEP list. Lozano's goby (*Pomatoschistus lozanoi*) is not mentioned in historical reports but was recently caught regularly in the mesohaline zone (BREINE et al., 2001). Therefore, it is kept in the mesohaline MEP list.

## CONCLUSIONS

To assess the ecological status of heavily modified transitional waters, the European Water Framework Directive requires definitions of Maximal and Good Ecological Potential (MEP/GEP) and the design of classification tools for specified biological quality elements. The hydromorphological, physical and chemical MEP/GEP are described by BRYS et al. (2005). Their approach was also used to define the guild-specific habitat needs (qualitatively) for fish in the Zeeschelde (BREINE et al., 2008). If these habitat needs are fulfilled because of restoration and mitigating actions, then we consider the estuary to be in MEP condition for fish. Near fulfilment brings it into the GEP condition. Based on a literature review in combination with recent fish catch data, we have made guild-specific qualitative MEP/GEP lists for the different salinity zones within the Zeeschelde estuary and its tidal tributaries. For each fish species, the relevance of its presence in each salinity zone was examined. The geographical spreading and ecological demands were assessed and used for the acceptance of a specific species for a certain list. The ecological knowledge of the assessed species is available and sufficient to reduce the risk of mistakes in attribution. The proposed lists should be considered as a starting point to developing quantitative guild lists i.e. including numbers instead of only presence/absence information. BREINE et al. (2010b) attributed threshold values to these quantitative lists. Thresholds for the good ecological potential (GEP) were defined from these references allowing expression of the ecological status as an ecological quality ratio (EQR) between 0 and 1. The guild approach facilitates the development of such an assessment tool. We are aware that by grouping fish into guilds particular information can be lost. On the other hand the guild approach is widely used and accepted in the development of robust assessment tools for the ecological status of surface waters. Such an evaluation system normally assesses the deviation between a reference condition and the actual condition. Therefore, the approach of defining the lists presented here can be used for other estuaries and be helpful in the development of fish-based indices.

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# SHORT NOTES

## Interspecific behaviour in temperate ungulates: an alien adult male associates with a group of non-conspecifics

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Group associations between two or more species have been described across a wide range of taxa in the wild, especially in birds and fishes. However, they have also been documented in many species of mammals, mainly primates and cetaceans, but rarely in ungulates – see review in (1) –. Interspecific mammal groups are usually formed by several individuals of different species (e.g., 2; 3; 4). Nevertheless, on rare occasions a single animal of a given species has been reported to associate with a group of non-conspecifics, in cetaceans (5) and in ungulates (6; 7).

We report here for the first time, an instance of group association between the aoudad *Ammotragus lervia* (Pallas, 1777) and the European mouflon *Ovis orientalis musimon* (Gmelin, 1774), where a mixed group (adult males and females, and on some occasions subadults) of mouflons associated with a solitary adult aoudad male. Both species are exotic in the study area.

The study site is a private rangeland (7.24km$^2$) located in central Spain (38º55'N 0º36'E; 660-820m above sea level), where three species of large herbivores live in sympatry: red deer *Cervus elaphus* (Linnaeus, 1758), aoudads and European mouflons. We followed a routine of observing their behaviour, distinguishing different groups according to sex and age classes: mixed groups (adult males/females and subadults), single male groups and female groups (including subadults). Sampling routine involved regular visits to the study site: four days a week, two months per season, during 1.5 years. We focused our efforts at dawn and sunset hours, when peaks of animal activity take place. During our visits, we witnessed for several days an association of an adult aoudad male and a variable group of mouflons. Sex-age structure and size of the mouflon group varied, although in all cases it was mixed (see Table 1). The aoudad registered was always the same individual, an adult male over six years old (sensu 8). There was only one small aoudad group of around 20 individuals in the study area, so that it was easy to recognize conspicuous animals, such as this large male. We registered this association seven times on five sampling days in October 2006 (Table 1).

TABLE 1

Sightings of the mouflon group when in the company of the adult aoudad male.

| Date | Adults | | Subadults | Total | Main group behaviour | Individual behaviours |
|---|---|---|---|---|---|---|
| | males | females | | | | |
| 16 Oct 2006 | 5 | 3 | 0 | 8 | feeding | – |
| 16 Oct 2006 | ? | ? | ? | 14 | moving | Aoudad: chasing and flehmen towards mouflon female. 2 mouflon males: chasing the latter mouflon female. |
| 17 Oct 2006 | 4 | 8 | 0 | 12 | moving | – |
| 17 Oct 2006 | ? | ? | 0 | 25 | escaping | – |
| 18 Oct 2006 | 5 | 2 | 1 | 8 | feeding | – |
| 25 Oct 2006 | 7 | 3 | 1 | 11 | moving | – |
| 26 Oct 2006 | 4 | 2 | 2 | 8 | guarding | Aoudad: guiding mouflon group. Chasing a mouflon female (see Video 2) |

The aoudad and mouflons were usually seen feeding, moving or guarding together. However, in some instances the aoudad male was observed guiding the whole group (see Video 1). We also recorded this male following a mouflon female on two occasions: on 16 October 2006, the aoudad chased and displayed flehmen behaviour towards an adult mouflon female, which was also pursued by two mouflon males at the same time; on 26 October 2006, the aoudad chased another mouflon female (Video 2). Finally, on 26 October 2006, after the solitary

aoudad had been observed in company with the mouflon group for one and a half hours, a nursery aoudad group joined them to form a new multispecific group devoted to resting and foraging together (Video 3).

Before discussing possible causes for this association, we should point out the relatively small size of the private rangeland where the study was undertaken, which may promote a high frequency of encounters of individuals of different species. In this context, we do not consider a single observed association of an aoudad group, made up of adult females and youngsters, with the aoudad male-mouflon herd on October 26 to be a mixed group, because it appeared to be a random association without a functional purpose (9).

Several factors have been described as possible causes for non-random mixed-species associations to occur. **Antipredator advantage** appears to be the most common explanation in different kinds of mammals and especially in ungulates (2; 7; 10; 11; 12). Thus, ungulate species can reduce predation risk by remaining together for mutual protection and forming multispecific herds. This protection against predation can be attained by a given ungulate species through different mechanisms, such as joining species that are preferred prey for carnivores (2), or improving predator detection by associating with species having different antipredator abilities (7; 11). The association may result in improved foraging behaviour since shared vigilance behaviour increases available feeding time (7).

Old aoudad males, such as the one observed, tend to abandon their conspecific herd and show solitary behaviour (13). Solitude could carry an increased predation risk because of the lack of group help in vigilance behaviour, which might explain this association with mouflons. However, the “oddity effect” (14; 15; 16) predicts a higher risk of attack and capture of individuals that differ in size or colour from other group members, through reduced ‘confusion effect’ (17). Hence, the aoudad’s visibility to predators and therefore its vulnerability to predation would increase because of lack of conspecifics in the group.

In the study site no natural predators are present. Human hunting activity is mainly focused on a specific season (autumn and early winter), so that, as “predator” pressure is relatively low, it does not appear to be a plausible factor shaping ungulate grouping (1; 18).

Some studies conducted in other mammal orders have shown two other potential advantages of non-random interspecific associations that may explain the aoudad-mouflon association we observed: social advantage and increased foraging efficiency.

Associating with a species of higher resource detection ability or one that facilitates resource access may increase **foraging efficiency** of the herdmates(12). Sinclair (2) and Stensland et al. (1) considered that since grazers and browsers eat widely dispersed food they would not obtain any food detection benefit by associating with herds of other species. However, in accord with studies on herbivore diet, a hierarchical selection of resources has been reported for herbivores (19; 20; 21). Foraging behaviour is a hierarchical process where plant selection is optimized at the landscape level, patches at the community level and individual and plant parts at the bite scale (19; 22). Such hierarchical resource selection takes place not only at the herbivore individual level but also at the group level (19; 23; 24). Hence, vegetal resources are actually distributed in differential palatability patches and different animals may have different abilities in their detection. Association between aoudads and mouflons may well respond to the greater ability of either species in food searching. Nutritional analyses made on comparative diet composition between aoudads, mouflons and red deer showed a seasonal-dependent foraging efficiency (25). In autumn, when the aoudad-mouflon association was observed, mouflon were significantly more efficient than the other two species in obtaining plants of high nutritional value (25). This may advantage aoudads associating with mouflons (as seen on Video 3), although we did observe the male aoudad apparently in a leading role (Video 1).

The **social advantage** hypothesis could also apply to our study case. The behaviour we filmed took place in autumn, the rutting season for both mouflons (26) and aoudads (27). Sexual behaviour of the aoudad male towards a mouflon female was observed on two occasions (see Table 1). Interspecific sexual behaviour and resulting hybridization have often been documented in domestic ungulates (e.g., 28) and sporadically between domestic and free-ranging wild species (e.g., 29) or between free-living ungulates (30; 31). Two different situations have been reported as possible catalysts for this unusual behaviour: 1) when access to conspecifics is denied and 2) where breeding groups of allopatric species are artificially in contact with one another. The latter explanation appears to be more plausible in the case of our study since the two species are naturally allopatric but have been brought into artificial contact. Moreover, the genera *Ammotragus* and *Ovis* are phylogenetically close, with similar behavioural patterns (32) although according to Geist (33), hybridization would not be feasible because of reproductive barriers between the two species.

Finally, it is worth pointing out that in the study site the aoudad and mouflon populations share space and resources with a much larger population of red deer. It is postulated that between these three species in sympatry, the mouflon is subordinate in access to resources, due to their smaller body size and lower competitive abilities (Sicilia et al., unpublished data). Thus, there may be an advantage in associating with aoudad individuals. However, this hypothesis needs further study.

From our limited number of observations, we cannot identify the dominant factor causing mouflon and aoudad to associate. However, we suggest that under the particular conditions of resource availability, hunting regime and density of the different ungulate species in the study area, the association may have conferred social advantages and feeding improvements.

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# First Record of Albinism in *Rhinella fernandezae* (Gallardo, 1957)

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Albinism is a rare congenital and inherited condition characterized by an alteration in the amount and distribution of dermal pigment via recessive allele expression that causes an enzyme deficiency involving the metabolism of melanin during prenatal development. Phenotypically, this condition is expressed as: (a) total albinism: absence of melanin involving the entire body; or (b) leucistism or dilute or partial albinism: absence of melanin in part of the body or reduction of melanin in the entire body or a part of it (1; 2; 3).

The different kinds of albinism have been widely reported among the major vertebrate lineages, including cartilaginous fishes, snakes and lizards (*e.g.* 4; 5; 6). There are also reports in amphibians, principally salamanders (*e.g.* 7; 8; 9), and a lower number on anurans (10; 11; 12). For Argentinean wild batrachian fauna there is only one previous report of albinism (13).

In this paper we report the first known case of albinism in the yellow belly toad *Rhinella fernandezae* (Anura, Bufonidae).

On 01 September 2009 in the course of a faunal survey in the Lower Paraná River flood plain valley, ten adults of *R. fernandezae* were sampled at El Rico Island (Santa Fe Province, Argentina) (32º16´36.34´´S, 60º41´8.16´´W; 19m of altitude). The habitat is a flood plain with an open lake surrounded by a forest of predominantly *Salix sp.*. The climate is warm temperate and damp (14).

In the normal colour pattern, live adults of *R. fernandezae* are ventrally blackish yellow and dorsally dark to clear green or chestnut with darker blotches and a yellowish vertebral line (15). The decoloured specimen collected at El Rico Island differed in having a whitish body with darker but transparent blotches in the flanks and chest (Fig. 1). However, its eyes were normally pigmented, indicating a case of leucistism (1; 2; 3). The leucistic specimen (gravid female, 52.7mm SVL) (deposited in the Amphibian Reference Collection of INALI: INALI-777) was captured together with nine normally-pigmented toads (seven males: 57.4±2.7mm SVL; and two females: 47.5±3.54mm SVL). Although we have surveyed amphibians in Paraná River flood plain localities intensively for several years, this is the first leucistic specimen found, indicating that albinism is not common in this environment.

Albinism occurs as a result of inherited gene alterations occurring in several forms. It can be inherited when the alleles are autosomal and recessive, autosomal and dominant, or sex-linked. If a population is very isolated and albinism is more prevalent than normal in that population, the expression of this recessive gene may be explained by this isolation and potentially low genetic flow. However, the El Rico Island population of *R. fernandezae* is widely connected with other populations of the extensive flood plain valley of the Paraná River, forming part of a metapopulation matrix. Likely, this specimen represents a single rare case in an otherwise normal pigmented population.

The behaviour, in both field and captivity, of our leucistic toad appeared typical in every respect. It showed normal feeding during the two months of captivity and none of the diminished activity described in albino specimens of other amphibian species (16). However, these deviant phenotypes are assumed more likely to be selected against in nature (17). In amphibians, albinism seems to be more frequent in larvae (*e.g.* 3; 12; 13; 18) than in adults (19; 20; 21), perhaps indicative of a difference in survival rate between normal and albino larvae. Ultimately, for those albino larvae that achieve metamorphosis, the nocturnal activity patterns of adults probably reduce any disadvantage of the white colour (21).

Although albinism has been reported in different species of anurans, the phenomenon remains quite rare considering the total number of species in the order. It is notable that most of the reports come from North American species (3) and, therefore, it is crucial that herpetologists be encouraged to report albinism in this order to increase our understanding of the spread, frequency and ecological implications of this condition.

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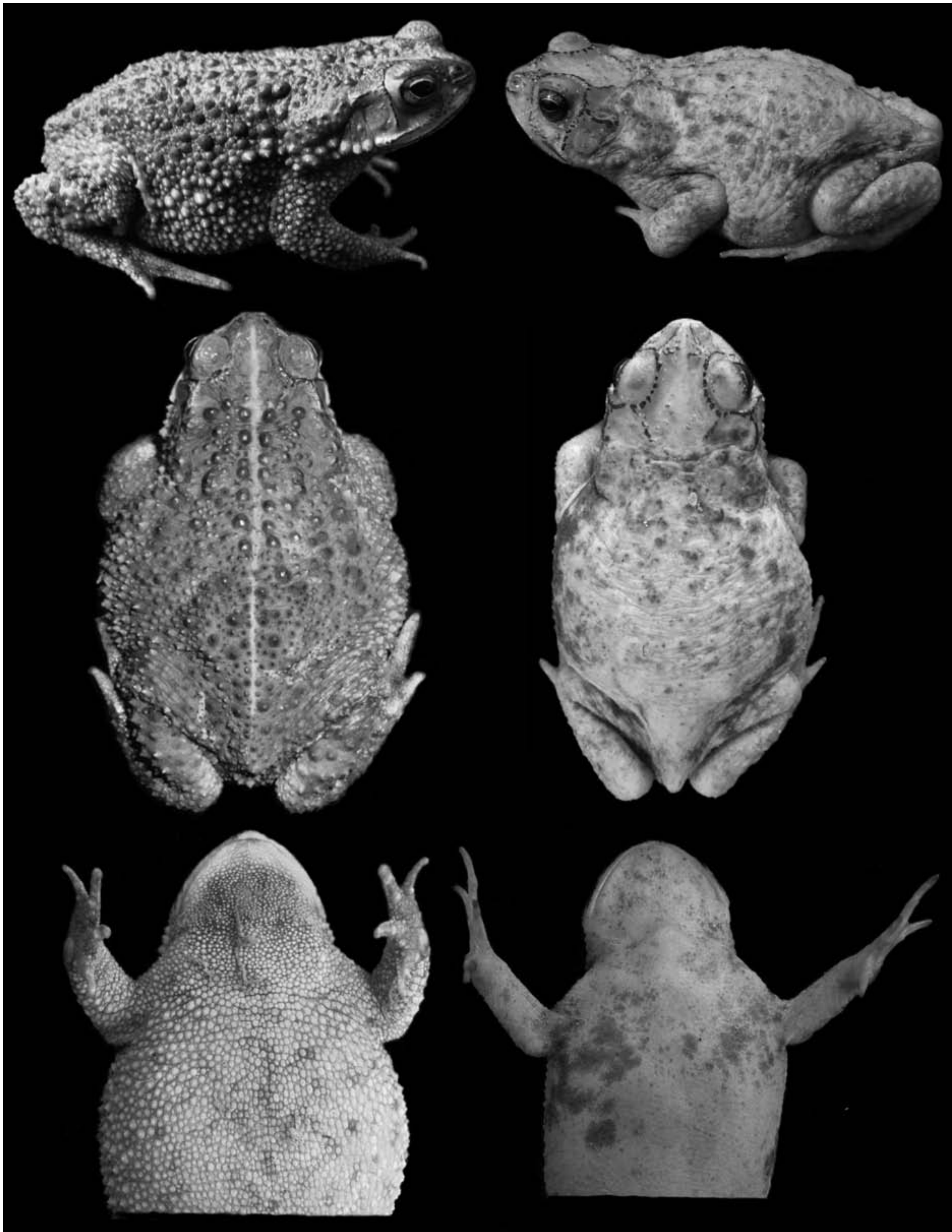

Fig. 1. – Dorsal, lateral and ventral view of a normal (A) and the leucistic specimen (B) of *Rhinella fernandezae*. Note in leucistic specimen the absence of yellowish vertebral line and the presence of darker blotches in the flanks and chest but normally-pigmented eyes. Body granulation is also less evident in leucistic specimen.

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# First record of Nemertodermatida from Belgian marine waters

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Nemertodermatida is a small taxon of marine worms, comprising only eight described species. They are characterised by a nemertine-like epidermis (hence the name) and a statocyst containing (mostly) two statoliths (1). The phylogenetic relationships of the taxon have been (and still are) the subject of debate. Initially, they were placed within the acoel Platyhelminthes (2), but Karling (1, 3) considered them to be substantially different from the acoels in morphology of the gut and the statocyst, and placed the taxon Nemertodermatida outside the Acoela (3). In the first phylogenetic analysis of the Platyhelminthes by Ehlers (4), Nemertodermatida formed a sister group relationship with Acoela, together forming the taxon Acoelomorpha, at that time considered to be the sister group of Rhabditophora (4). This view was followed in most subsequent morphological studies (for a review: see 5). Smith et al. (6) suggested the possible polyphyly of the taxon Platyhelminthes, based on the absence of clear synapomorphies, and viewed the Acoelomorpha as one of the three well-defined monophyletic groups within the Platyhelminthes, the two others being Catenulida and Rhabditophora. Recent molecular phylogenetic studies (7; 8) corroborate the polyphyly of the Platyhelminthes and show the Nemertodermatida and the Acoela in a basal position within Bilateria. The presence of the small Hox cluster in both Acoela (9) and Nemertodermatida (10) is concordant with this phylogenetic position. Based on analyses of 18S rRNA and mitochondrial genes (7) and 18S and 28S rRNA sequences (11), the taxon Acoelomorpha was dismissed because it appeared to be paraphyletic; Nemertodermatida and Acoela are separate branches at the base of the Bilateria. A recent phylogeny based on a large number of taxa and multiple nuclear genes, however, provides support for a monophyletic Acoelomorpha, albeit also positioned at the base of the Bilateria (12). Because of this basal phylogenetic position, the study of Nemertodermatida is important for the understanding of the characteristics of the bilaterian ancestor (11) and it makes them important study-objects for morphologists and molecular biologists alike. Note that the latest study on the phylogenetic position of Acoelomorpha (13) challenges their basal position and places them within the Deuterostomia, together with *Xenoturbella*. In the most recent comprehensive taxonomic overview of Nemertodermatida (14), Sterrer revealed considerable intraspecific variation for characters such as size, colour, presence/absence of glands, etc. Hence, species delimitation is often problematic and also cryptic diversity cannot be excluded, which makes nemertodermatids also interesting from a (molecular) taxonomical point of view. Regrettably, Nemertodermatida are known from a few distinct sampling spots only: the Swedish west coast, the east coast of North America, the Adriatic and the Mediterranean, which suggests that representatives of the taxon are difficult to collect.

The Belgian Continental Shelf, situated in the southern bight of the North Sea and characterised by a mainly sandy substrate, has not previously been sampled for turbellarians specifically, in contrast to the sandy beaches of the Belgian coast, the turbellarian fauna of which is very well known (15; 16). Recently, during a two-year period (2007 – 2009), researchers from the UHasselt and UGent sampled the Belgian continental shelf, in order to investigate the turbellarian fauna of sublittoral sandy sediments. The samples were taken with the research vessel "Zeeleeuw" of the Flanders Marine Institute (VLIZ) in different seasons: autumn (October 2007), winter (December 2007 and January 2008), summer (July 2008 and September 2008) and spring (March 2009). Sublittoral sand samples were collected with a Van Veen grab, after which the samples were left to rest for a few days. Afterwards, subsamples were scooped and animals were extracted from these subsamples following the $MgCl_2$ method (17). Animals were collected under a stereomicroscope and identified using an Olympus BX 51 microscope.

In these samples, a total of 60 specimens of Nemertodermatida were discovered, occurring in 10 sampling stations (Fig. 1), and three different species representing both families of Nemertodermatida could be discerned (already mentioned in 16): *Sterreria psammicola* (Sterrer, 1970) Lundin, 2000 (fam. Nemertodermatidae), *Nemertinoides elongatus* Riser 1987 (fam. Nemertodermatidae) and *Flagellophora apelti* Faubel & Dörjes 1978 (fam. Ascopariidae). Table 1 gives an overview of the sampling sites showing the number of specimens of each species at each of the sampling sites, depth and date of the sampling. In total, 11 specimens of *N. elongatus*, 2 specimens of *S. psammicola* and 20 specimens of *F. apelti* were found. In addition, 27 specimens were found that could not be identified, either because they were fragmented or not yet (fully) mature. Sterrer (14) also mentioned these same problems concerning species identification.

Despite the fact that the Belgian Continental Shelf is the subject of many scientific studies, this is the first record of Nemertodermatida in this area, thus expanding their known geographical distribution. An overview of the worldwide distribution of each of the three species can be found following these links:

*Sterreria psammicola*:

http://maps.google.nl/maps/ms?ie=UTF8&hl=nl&msa=0&msid=100800064963713004739.00046b093b8f2cfa9dfa0&ll=10.983886,-87.553265&spn=173.668929,360&z=1

*Nemertinoides elongatus*:

http://maps.google.nl/maps/ms?ie=UTF8&hl=nl&msa=0&msid=100800064963713004739.00046af8c2fae451945c7&z=3

*Flagellophora apelti*:

http://maps.google.nl/maps/ms?ie=UTF8&hl=nl&msa=0&msid=100800064963713004739.0004694f3788119941f72&ll=10.983886,-87.553265&spn=173.668929,360&z=1

The obvious explanation for the fact that specimens of Nemertodermatida have not been discovered earlier in the Belgian part of the North Sea is the lack of study on these animals in this area (and many other areas worldwide). Their occurrence in this area is, however, not unexpected, since the investigated sediments are of the same type as of the records elsewhere in the world. All three species have been found earlier in sandy bottoms, ranging from fine sand to coarse sand containing shell gravel (18), and this sediment is the main sediment type on the Belgian Continental Shelf. As to depth (see Table 1), the records for *F. apelti* in Belgium fall in the depth range of 2m to 400m mentioned in earlier literature (14), while we found specimens of *S. psammicola* and *N. elongatus* deeper than was known from earlier records (14). The presence of the genus *Ascoparia* Sterrer, 1998, a genus also occurring in sandy substrates, could not be proven in this study, but given the large fraction of unidentifiable specimens (27 out of 60), we cannot rule out their occurrence in this part of the North Sea. Further research is needed to determine whether the species typical of soft, muddy bottoms (*Nemertoderma bathycola* Steinböck, 1930 and *N. westbladi* Steinböck, 1938) are present on the Belgian Continental Shelf. This present study indicates that nemertodermatids could be present in many more localities than their known distribution suggests.

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TABLE 1

Overview of the sampling dates, the sampling sites with their depth and the number of each species found at every site. Species A: *Nemertinoides elongatus*, species B: *Sterreria psammicola*, species C: *Flagellophora apelti*, species D: unknown / unidentifiable.

| Date | Sampling site | Latitude | Longitude | Depth | Species | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | A | B | C | D |
| 17/10/2007 | 330 | 51°26.111 | 2°48.565 | 23m | 2 | | | |
| 17/10/2007 | Bleigh Bank | 51°35.475 | 2°45.120 | sublittoral | | | | 2 |
| 17/10/2007 | 435 | 51°34.662 | 2°47.424 | 31-33m | | | | 1 |
| 30/10/2007 | UH09 | 51°26.772 | 2°49.500 | 17-19m | 4 | | 5 | 4 |
| 30/10/2007 | UH07 | 51°20.272 | 2°45.641 | 16m | | | | 1 |
| 30/10/2007 | UH10 | 51°23.471 | 2°51.677 | 15m | | | 3 | 4 |
| 17/12/2008 | 330 | 51°26.111 | 2°48.565 | 22-24m | 2 | 1 | | 4 |
| 21/01/2008 | Kwintebank | 51°19.635 | 2°40.737 | sublittoral | | | | 2 |
| 21/01/2008 | Middelkerkebank | 51°19.411 | 2°45.509 | 12m | | | 2 | |
| 21/01/2008 | Stroombank | 51°12.174 | 2°43.799 | 10m | | 1 | | 2 |
| 03/07/2008 | UH07 | 51°20.272 | 2°45.641 | 16m | | | 3 | 2 |
| 01/09/2008 | Middelkerkebank | 51°19.411 | 2°45.509 | sublittoral | | | | 1 |
| 01/09/2008 | UH09 | 51°26.772 | 2°49.500 | 20m | | | 2 | 1 |
| 17/03/2009 | UH09 | 51°26.772 | 2°49.500 | 21m | 2 | | | |
| 17/03/2009 | UH07 | 51°20.272 | 2°45.641 | 20-21m | 1 | | 3 | 2 |
| 17/03/2009 | UH03 | 51°18.638 | 2°36.126 | 18-23m | | | 2 | 1 |
| | | | | Subtotal | 11 | 2 | 20 | 27 |
| | | | | Total number: 60 | | | | |

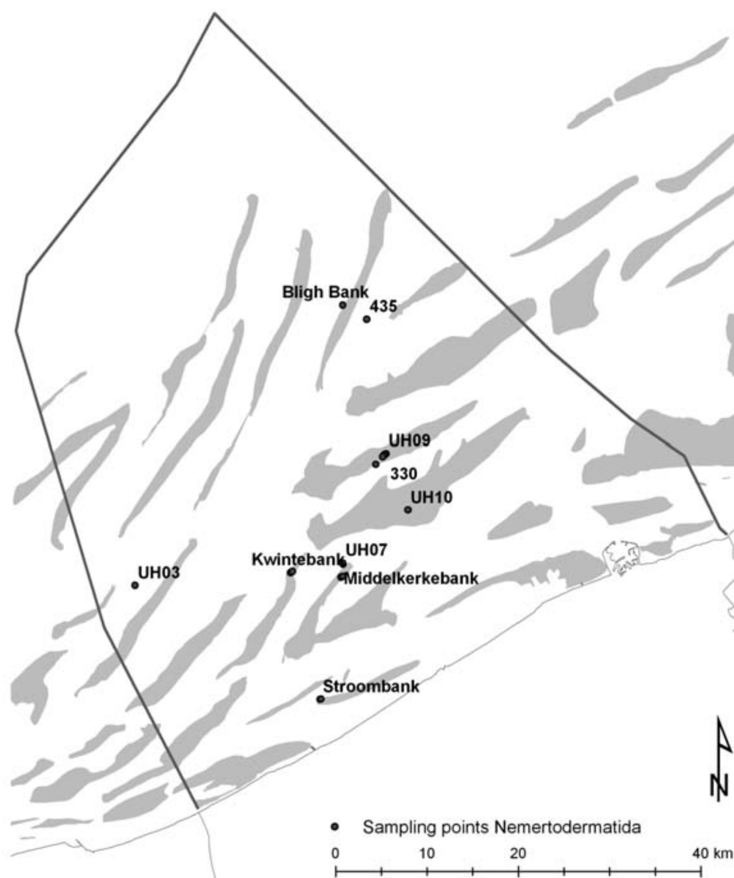


Fig. 1. – Map of the Belgian Continental Shelf and its sandbanks (dark grey) with the locations of the Nemertodermatida records (black dots).


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