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CONTENTS

Male mating success in the Omei treefrog (*Rhacophorus omeimontis*): the influence of body size and age

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ABSTRACT. In anuran mate choice, advantaged males are generally known to be larger or older individuals. To test whether male mating success in the foam-nesting treefrog *Rhacophorus omeimontis*, a species distributed in western China, correlated with body size and age, we analysed differences in body size and age among three types of males. Males were classified as mated, joining or unmated at the time of sampling with joining males being additional males joining pairs in amplexus. Our results showed that there were no significant differences in body size among the three types of males. However, age was an important factor, with mated males being significantly older than joining and unmated males, which indicated that older individuals tended to have greater mating success than younger frogs.

KEY WORDS: Sexual selection, age, body size, *Rhacophorus omeimontis*

INTRODUCTION

Sexual selection has interested biologists for more than a century because it works differently to natural selection, leading to the evolution of sexual traits that are usually costly in fitness (DARWIN, 1871; ZAHAVI & ZAHAVI, 1997; ANDERSSON & SIMMONS, 2006). Intra-sexual competition and mate choice have stimulated the majority of recent investigations (WEST-EBERHARD, 1979; HUNT et al., 2009; RAFAEL et al., 2010, LIAO & LU, 2011c). A majority of studies on sexual selection have shown that it usually results from a female choice for a particular sexual display, rarely from male mate choice. Under natural conditions, this sexual display (presence or value) is not only correlated to mating success but also to reproductive success (reviewed by ANDERSSON, 1994; HOSKEN & STOCKLEY, 2004; PLATH et al., 2006; HUNT et al., 2009).

Studies of sexual selection on amphibians have shown that males with relatively high values of traits such as body size, forearm length, thumb-pad width, and advertisement calls will be preferred by females. Individuals of the sex undergoing selection usually intimidate their competitors and thus obtain mating advantages (ANDERSSON, 1994; POSCHADEL et al., 2007; BELL, 2010). Body size represents the ability of an individual to have many resources and is therefore related to competition ability. Individuals with large body size use it in sexual display (HALLIDAY, 1983; ANDERSSON, 1994; LIAO & LU, 2009a; b). In some anurans male mating success is also correlated with age, which results from the indirect consequence that age is positively correlated to body size (TRIVERS, 1972; HÖGLUND & SÄTERBERG, 1989; EGGERT & GUYÉTANT, 2003; OKKO & JENNIONS, 2008; HARRISON et al., 2009; BELL, 2010; SOMASHEKAR & KRISHNA, 2011). In some species (ANDERSSON, 1994; BYRNE & WHITING, 2008), larger and older individuals, which have survived well and have better sexual displays, have more successful egg fertilization than do smaller and younger ones.

The Omei treefrog, *Rhacophorus omeimontis,* an arboreal breeder, is endemic to mountain ranges in the subtropical forests of Sichuan province in western China, where it occurs ranging from 750 to 2,000m a.s.l. (LIU & HU, 1961). During the prolonged breeding season, males gather in a pond and produce irregular advertisement calls to attract females. The males do not compete aggressively for call sites when they aggregate in groups in the pond (LIAO & LU, 2010d). Moreover, this species has a mating system where females will mate with more than one male (LIAO & LU, 2010d). Except for the knowledge presented above, there is no detailed information about the mating behaviour of this species. Here, we tested the hypothesis that body size and age in *R. omeimontis* affected male mating success in a subtropical high-elevation region in western China.

MATERIALS AND METHODS

Study area

The field study was conducted in Fengtongzhai National Nature Reserve in western China (102º48′-103º 00′E, 30º19′-30º 47′N, and total area about 39,039ha). The reserve ranges from 1,000-4,200m above sea level, has an annual average temperature of 5.9-7.2°C and an annual average precipitation of 700-1,300mm (over 60% of which falls during May–August, LIAO, 2009). During the breeding season, from 15 April to 20 May in 2008, we collected treefrogs in a permanent pond at Dengchigou Protection Station at an altitude of 1,700m. The pond was filled to a depth of 25cm with fresh water and was mainly covered by replanted broadleaf forest, which may provide hiding places for *R. omeimontis*.

Sampling

Females are quickly grasped by a male when they enter the breeding pond. The pairs then move to a neighbouring plant and release foam in leaves above the water surface of the pond. Immediately, other males, which we call joining

males here, come to join the pairs. Treefrogs were caught by hand at night whilst they were in amplexus or searching for mates in the pond. Following the protocol of LEE & CRUMP (1981), we conservatively characterized unmated males as unsuccessful although these males may have been successful in securing a mate prior to or after capture. However, unmated males were collected after amplectant males and joining males had been located, thus any differences between mated, joining and unmated males are likely to reflect true differences at that moment. Each adult specimen was sexed by direct observation of the secondary sexual characteristics (the nuptial pads on the first finger for male, the eggs readily visible by the skin of the abdomen for female). We confirmed the three types of males by direct observation at the breeding sites. Body size (from snout to vent, SVL) was measured to the nearest 0.1mm using a calliper. All treefrogs were individually marked by toe clipping and then released at their capture site. Toe clipping can reduce frog survival and affect their behaviour (BYRNE & ROBERTS, 1999), but the technique did not affect or inhibit behaviour of individuals in *R. omeimontis* during two successive years (LIAO & LU, 2010d). The longest toe from the right limb of the hind leg was removed and stored in 10% neutral buffered formalin for skeletochronological analysis. Since there was no substantial regeneration of the toe pads, the individuals could be easily identified throughout the study period. We used the Lincoln-Petersen index to estimate population size (CAUGHLEY, 1977).

Age determination

Skeletochronology was applied to the phalanges of adults (141 males and 28 females). The selected digits were washed in water for 2h and then decalcified in 5% nitric acid for 24h. They were washed in running tap water overnight and then stained with Ehrlich's haematoxylin for 75min. Thin sections (13μm thickness) were selected at the mid-shaft diaphysis of the phalanx with the smallest medullar cavity and mounted on glass slides. The analysis of lines of arrested growth (LAG) was made under a light

microscope by two observers, who had similar experience in the technique and who agreed on the identification criteria and the final age estimation. As recommended by ROZENBLUT & OGIELSKA (2005), we confirmed LAG endosteal resorption based on the occurrence of the Kastschenko Line (KL, the division line between endosteal and periosteal zones). In the samples, endosteal resorption was only found in four individuals, and we added one year to the age of these individuals. Of 169 adult specimens, 166 (138 males and 28 females) exhibited clear LAGs in their bone sections.

Statistical analyses

We also used parametric tests to perform homogeneous analyses for comparing differences in body size and age between the two sexes. We applied ANCOVA with age and sex as factors to test for differences in body size between the sexes. One-way ANOVA was used to test for differences in body size and age among mated, joining and unmated males. We ran an ANCOVA with age as covariate to test whether statistical significance of differences in body size of mated, joining and unmated males still remained after removing the effects of age. The total breeding sex ratio in the population was evaluated using a Chi-square test. All statistical tests were two-tailed and values given are shown as mean ± SD.

RESULTS

During the study period, a total of 169 adult individuals (141 males in amplexus, joining an amplexus or alone, and 28 females) were individually marked by toe-clipping. We recaptured 101 individuals during the subsequent 35-day period. The mark-recapture data produced an estimate of the population size of 284 individuals. Therefore, our sample containing 169 frogs (59.9% of 284) provided a representative part of the adult population. The total breeding sex ratio was significantly male-biased (χ^2=72.62, *df*=1, *P*<0.001). Females were significantly larger than males (Table 1; Students *t*-test: *t*=7.508, *df*=84, *P*<0.001). The average body size of females was 76.7±3.1mm, while males were 64.7±2.4mm. Female frogs were on average 4.3±1.2 yrs in age, and thus significantly older than males with 3.6±1.0 yrs (Students *t*-test: *t*=22.054, *df*=81, *P*<0.001). ANCOVAs
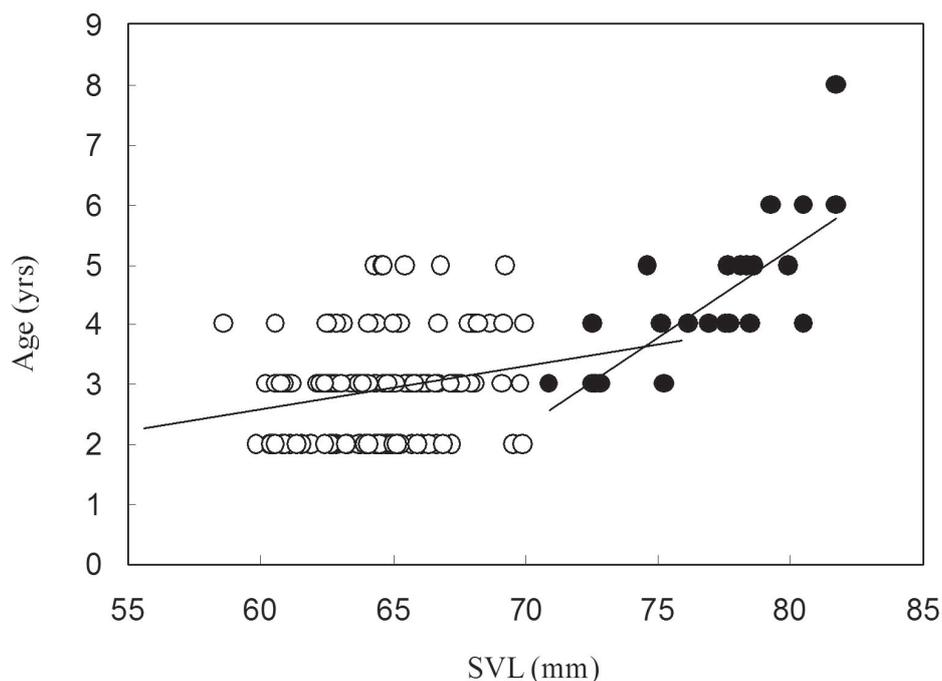
Fig. 1. – The interaction between sex and age of *Rhacophorus omeimontis* (female, black circles; male, white circles) in western China. SVL=Snout-vent length.
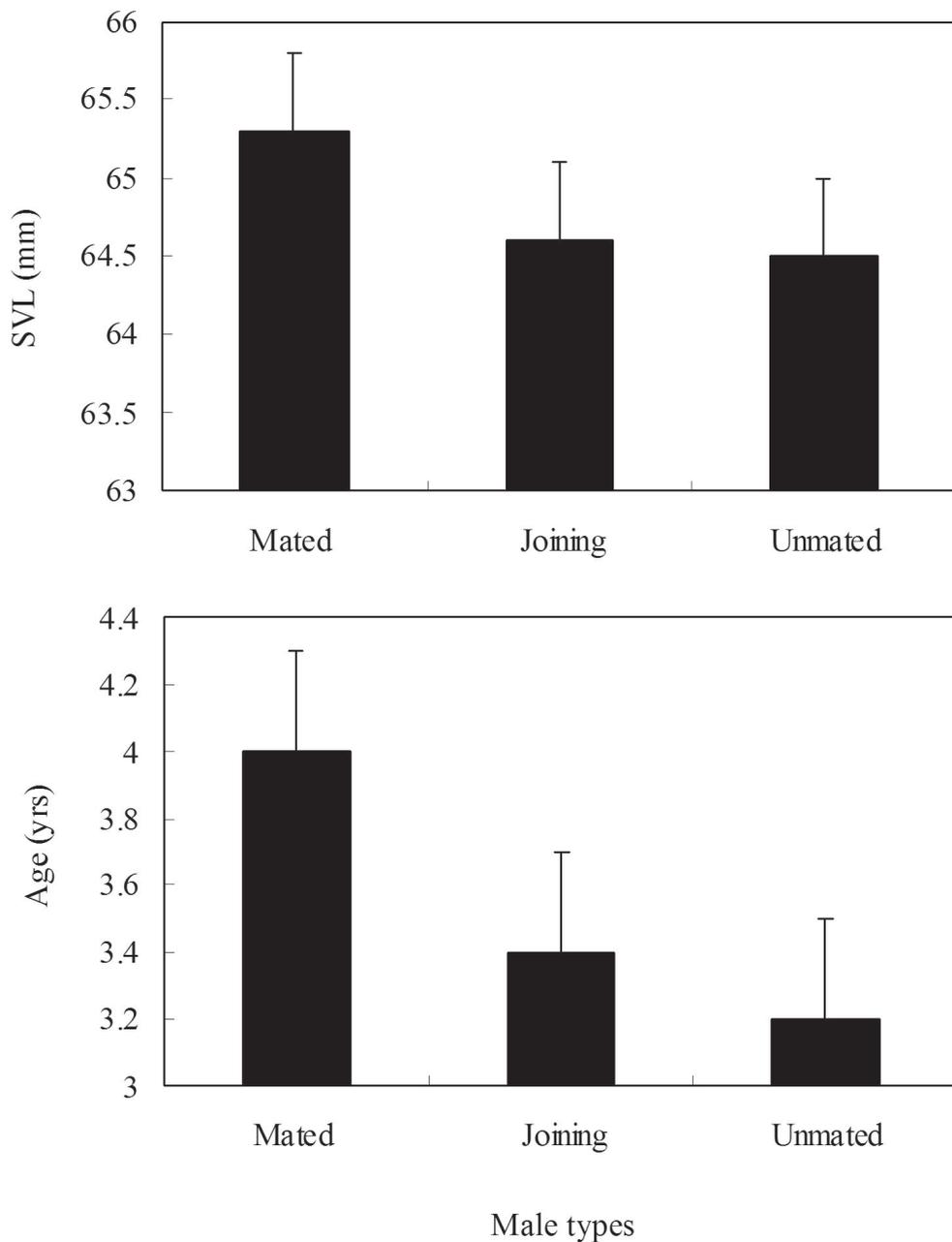
Fig. 2. – Differences in body size and age of *Rhacophorus omeimontis* among mated males, joining males and unmated males in western China. Vertical bars are standard deviations. SVL=Snout-vent length.

with age and sex as factors showed that their interaction was significant (Fig.1; $F_{2, 165}=5.041$, $P=0.008$), and age and sex resulted in significant difference in body size between the sexes (age, $F_{5, 157}=7.182$, $P<0.001$; sex, $F_{1, 165}=304.056$, $P<0.001$). The sexual difference in body size remained significant when the effect of age was controlled ($F_{1, 165}=335.858$, $P<0.001$).

Body sizes did not differ significantly among mated, joining and unmated males (one-way ANOVA: $F_{2,138}=1.12$, $P=0.33$; Fig.2). On the other hand, average age did differ significantly among the three categories of males (one-way ANOVA: $F_{2, 136}=3.86$, $P=0.01$; Table 1), with mated males tending to be older than joining ($P<0.03$ for Tukey HSD post hoc test) or unmated males ($P<0.04$ for test) while there was no significant difference between the average age of joining and unmated males ($P=0.90$ for Tukey HSD post

hoc test). ANCOVAs with age and male types as factors showed that their interaction was not significant ($F_{4, 108}$=0.972, P=0.322). There were no differences in SVL between mated, joining and unmated males even when controlling for the effect of age (Fig.3; ANCOVA: $F_{2, 136}$=0.55, P=0.47).

DISCUSSION

We found a positive correlation between age and body size in both sexes of *Rhacophorus omeimontis*, similar to what has been described from several other anurans (*R. perezi*, ESTEBAN et al., 1996; *Mantidactylus microtympanum*, GUARINO et al., 1998; *R. nigrovittata*, KHONSUE et al., 2000; *R. ridibunda*, KYRIAKOPOULOU-SKLAVOUNOUA et al., 2008; *Amolops mantzorum*, LIAO & LU, 2010a; b; *Hyla annectans chuanxiensis*, LIAO & LU, 2010c; *Rana nigromaculata*, LIAO et al., 2010; *R. omeimontis*, LIAO & LU, 2011a; *Bufo andrewsi*, LIAO & LU, 2011b). However, in our study, it was not possible to predict age from body size because there was considerable overlap of body size among the different age classes (Fig.1).

We found no differences in body size of *R. omeimontis* among mated, joining and

TABLE 1

Body size and age by *Rhacophorus omeimontis* among mated, joining and unmated males in western China.

|		MATED MALES	JOINING MALES	UNMATED MALES
Samplings	n	28	29	81
Snout-vent-length	mm	65.3±2.2	64.6±2.2	64.5±2.6
Age	yrs	4.0±1.1	3.4±0.9	3.5±0.9
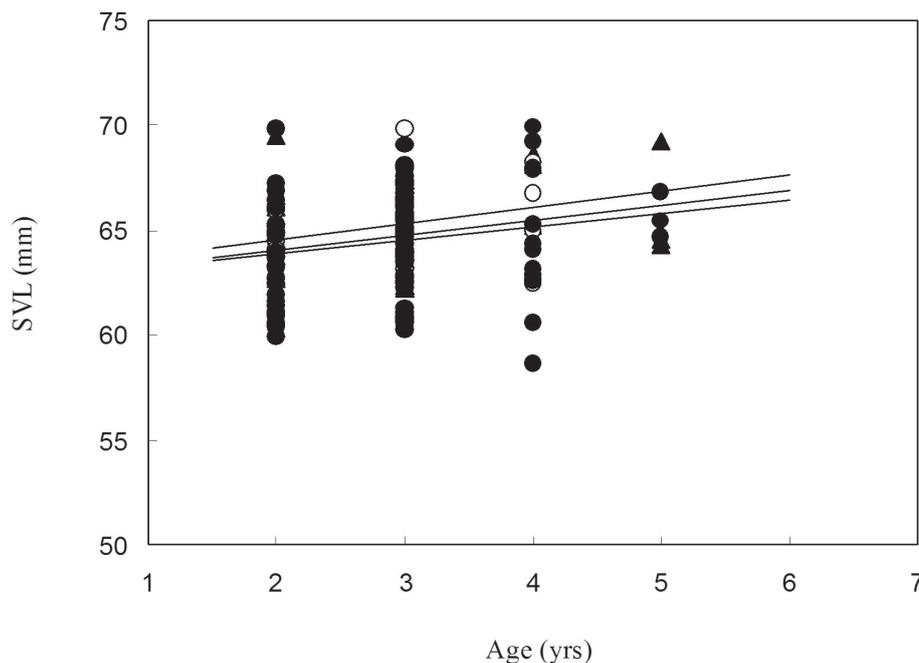
Fig. 3. – The interaction between sex and male type of *Rhacophorus omeimontis* (mated males, black triangles; joining males, white circles; unmated males, black circles) in western China. SVL=Snout-vent length.

unmated males even when controlling for the effect of age, suggesting that larger males did not have higher chances to obtain mates. This is in line with the prediction that sexual selection does not act on body size in the related foam-nest species because female choice depends on male calls (i.e. *R. schlegelii*, FUKUYAMA, 1991; *R. arboreus*, KUSANO et al., 1991; *Chiromantis xerampelina*, JENNIONS et al., 1992). Thus, body size may not be a good indicator of male competing advantages in *R. omeimontis*, suggesting that larger size does not reflect a male's ability to compete for a mate (LIAO, 2009). Similar results have been obtained for other frogs and toads (HALLIDAY & VERRELL, 1988). Moreover, we postulate that the absence of any relationship between body size and mating success could result from our experimental design. Our three defined male categories may not be good indicators for mating success. Future studies should include consideration of calling abilities as an indicator for mating success, as well as age or body size.

Although we did not find any obvious effect of body size on mating success, older males clearly had more success than younger males (see above). Male attributes that females may consider important include resources controlled and genetic quality (HOWARD, 1978). Males with strong competitive abilities are males with high genetic quality. Because these males have "good genes", they have higher mating success and their offspring benefit from these genes (TRIVERS, 1972). In our study on *R. omeimontis,* older males tended to have greater mating success than younger ones. This could be because older males possess genes that have enabled them to survive through a longer succession of different environments in the past (WILBUR et al., 1978; HOWARD, 1984; SOMASHEKAR & KRISHNA, 2011). According to this hypothesis, females that mate with older males would gain indirect genetic benefits in terms of increased offspring fitness.

FELTON et al. (2006) reported that age, independent of body size, was negatively correlated to call dominant frequency and female preference for low dominant frequency equates with choosing older males. In *R. omeimontis*, older males may have more attractive calling because they have larger SVL (WELCH et al., 1998). Previous studies have indicated that female anurans show clear preferences for older males' advertisement calls with a high rate or long duration, because females may gain indirect benefits in terms of increased offspring viability (GERHARDT, 1994; GERHARDT & HUBER, 2002; PROKOP et al., 2007). Indeed, WELCH et al. (1998) found for the grey treefrog, *Hyla versicolor*, that there may be indirect genetic benefits for females choosing males with long calls. Female *R. omeimontis* preferences seem to be (also) based on male advertisement calls (LIAO, 2009). However, data on the relationships between male call rates, female preferences and male age are still missing. Further investigation is warranted.

ACKNOWLEDGEMENTS

REFERENCES

ANDERSSON M (1994). Sexual selection. Princeton University Press, Princeton.

ANDERSSON M & SIMMONS LW (2006). Sexual selection and mate choice. Trends in Ecology & Evolution, 21:296-302.

BELL MBV (2010). Sex and age influence responses to changes in the cost of cooperative care in a social carnivore. Behavioral Ecology, 21:1118-1123.

BYRNE PG & ROBERTS JD (1999). Simultaneous mating with multiple males reduces fertilization success in the myobatrachid frog *Crinia georgiana*. Proceedings of the Royal Society B: Biological Sciences, 266:717-721.

BYRNE PG & WHITING MJ (2008). Simultaneous polyandry increases fertilization success in an African foam-nesting treefrog. Animal Behaviour, 76:1157-1164.

CAUGHLEY G (1977). Analysis of Vertebrate Populations. New York, John Wiley.

DARWIN C (1871). The Descent of Man and Selection in Relation to Sex. John Murray, London, UK.

EGGERT C & GUYÉTANT R (2003). Reproductive behaviour of spadefoot toads (*Pelobates fuscus*): daily sex ratios and males' tactics, ages, and physical condition. Canadian Journal of Zoology, 81:46-51.

ESTEBAN M, PARIS MG & CASTANRT J (1996). Use of bone histology in estimating the age of frogs (*Rana perezi*) from a warm temperate climate area. Canadian Journal of Zoology, 74:1914-1921.

FELTON A, ALFORD RA, FELTON AM & SCHWARZKOPF L (2006). Multiple mate choice criteria and the importance of age for male mating success in the microhylid frog, *Cophixalus ornatus*. Behavioural Ecology and Sociobiology, 59:786-795.

FUKUYAMA K (1991). Spawning behaviour and male mating tactics of a foam-nesting treefrog, *Rhacophorus schlegelii* (Rhacophoridae, Amphibia). Animal Behaviour, 42:193-199.

GERHARDT HC (1994). The evolution of vocalization in frogs and toads. Annual Review in Ecology and Systematics, 25:293-324.

GERHARDT HC & HUBER F (2002). Acoustic Communication in Insects and Anurans. University of Chicago Press, Chicago.

GUARINO FM, ANDERSON F & ANGELINI F (1998). Growth and longevity by skeletochronological analysis in *Mantidactylus microtympanum*, a rainforest anuran from southern Madagascar. Copeia, 1998:194-198.

HALLIDAY TR (1983). The study of mate choice. In: BATESON P (ed) Mate Choice. Cambridge University Press, Cambridge: pp.3-32.

HALLIDAY TR & VERRELL PA (1988). Body size and age in amphibians and reptiles. Journal of Herpetology, 22:253-265.

HARRISON F, BARTA, Z, CUTHILL I & SZÉKELY T (2009). How is sexual conflict over parental care resolved? A meta-analysis. Journal of Evolutionary Biology, 22:1800-1812.

HÖGLUND J & SÄTERBERG L (1989). Sexual selection in common toads: correlates with age and body size. Journal of Evolutionary Biology, 2:267-272.

HOSKEN DJ & STOCKLEY P (2004). Sexual selection and genital evolution. Trends in Ecology & Evolution, 19:87-93.

HOWARD RD (1978). The evolution of mating strategies in bullfrogs, *Rana catesbeiana*. Evolution, 32:850-871.

HOWARD RD (1984). Alternative mating behaviours of young male bullfrogs. American Zoologist, 24:397-406.

HUNT J, BREUKER CJ, SADOWSKI JA & MOORE AJ (2009). Male–male competition, female mate choice and their interaction: determining total sexual selection. Journal of Evolutionary Biology, 22:13-26.

JENNIONS MD, BACKWELL PRY & PASSMORE NI (1992). Breeding behaviour of the African frog, *Chiromantis xerampelina*: multiple spawning and polyandry. Animal Behaviour, 44:1091-1100.

KHONSUE W, MATSUI M & MISAWA Y (2000). Age determination by skeletochronology of *Ratza nigrovittata*, a frog from tropical forest of Thailand. Zoological Science, 17:253-257.

KUSANO T, TODA M & FUKUYAMA K (1991). Testes size and breeding systems in Japanese anurans with special reference to large testes size in the treefrog *Rhacophorus arboreus* (Amphibia, Rhacophoridae). Behavioural Ecology and Sociobiology, 29:27-31.

KYRIAKOPOULOU-SKLAVOUNOUA P, STYLIANOU P & TSIORA A (2008). A skeletochronological study of age, growth and longevity in a population of the frog *Rana ridibunda* from southern Europe. Zoology, 111:30-36.

LEE JC & CRUMP ML (1981). Morphological correlates of male mating success in *Triprion petasatus* and *Hyla marmorata*. Oecologia, 50:153-157.

LIAO WB (2009). Elevational variation in the life-history of anurans in a subtropical montane forest of Sichuan region, southwestern China. Ph.D. thesis. Wuhan University, Wuhan, China.

LIAO WB & LU X (2009a). Male mate choice in the Andrew's toad *Bufo andrewsi*: a preference for larger females. Journal of Ethology, 27:413-417.

LIAO WB & LU X (2009b). Sex recognition by male Andrew's toad *Bufo andrewsi* in a subtropical montane region. Behavioural Processes, 82:100-103.

LIAO WB & LU X (2010a). Age and growth of a subtropical high-altitude torrent frog *Amolops mantzorum* in western China. Journal of Herpetology, 44:72-76.

LIAO WB & LU X (2010b). A skeletochronlogical estimation of age and body size by the Sichuan torrent frog (*Amolops mantzorum*) between two populations at different altitudes. Animal Biology, 60:479-489.

LIAO WB & LU X (2010c). Age structure and body size of the Chuanxi Tree Frog *Hyla annectans chuanxiensis* from two different elevations in Sichuan (China). Zoologischer Anzeiger, 248:255-263.

LIAO WB & LU X (2010d). Breeding behaviour of the Omei tree frog *Rhacophorus omeimontis* (Anura: Rachophoridae) in a subtropical montane region. Journal of Natural History, 44:2929-2940.

LIAO WB & LU X (2011a). Variation in body size, age and growth in the Omei Treefrog (*Rhacophorus omeimontis*) along an altitudinal gradient in western China. Ethology Ecology & Evolution, 23:248-261.

LIAO WB & LU X (2011b). Adult body size = *f* (initial size + growth rate × age): explaining the proximate cause of Bergman's cline in a toad along altitudinal gradients. Evolutionary Ecology, in press, DOI:10.1007/s10682-011-9501-y.

LIAO WB & LU X (2011c). Proximate mechanisms leading to large male-mating advantage in the Andrew's toad, *Bufo andrewsi*. Behaviour, 148:1087-1102

LIAO WB, ZHOU CQ, YANG ZS, HU JC & LU X (2010). Age, size and growth in two populations of the dark-spotted frog *Rana nigromaculata* at different altitudes in southwestern China. Herpetological Journal, 20:77-82.

LIU CZ & HU SQ (1961). The Tailless Amphibians of China. Science Press, Beijing.

OKKO H & JENNIONS MD (2008). Parental investment, sexual selection and sex ratios. Journal of Evolutionary Biology, 21:919-948.

PLATH M, ROHDE M, SCHRÖDER T, TAEBEL-HELLWIG A & SCHLUPP I (2006). Female mating preferences in blind cave tetras *Astyanax fasciatus* (Characidae, Teleostei). Behaviour, 143:15-32.

POSCHADEL JR, RUDOLPH A & PLATH M (2007). Nonvisual mate choice in the Pyrenean mountain newt (*Euproctus asper*): females prefer small males. Acta Ethological, 10:35-40.

PROKOP ZM, STUGLIK M, ZABINSKA I & RADWAN J (2007). Male age, mating probability, and progeny fitness in the bulb mite. Behaviour Ecology, 18:597-601.

RAFAEL M, JAIME B & XAVIER E (2010) Intensity of female preference for call source level in midwife toads *Alytes cisternasii* and *A. obstetricans*. Behaviour, 147:1185-1199.

ROZENBLUT B & OGIELSKA M (2005). Development and growth of long bones in European water frogs (Amphibia: Anura: Ranidae), with remarks on age determination. Journal of Morphology, 265:304-317.

SOMASHEKAR K & KRISHNA MS (2011). Evidence of female preference for older males in *Drosophila bipectinata*. Zoological Studies, 50:1-15.

TRIVERS R (1972). Parental investment and sexual selection. In: CAMPBELL B (ed), Sexual selection and the descent of man 1871-1971. Aldine, Chicago.

WELCH AM, SEMLITSCH RD & GERHARDT HC (1998). Call duration as an indicator of genetic quality in male gray tree frogs. Science, 280:1928-1930.

WEST-EBERHARD MJ (1979). Sexual selection, social competition, and evolution. Proceedings of the American Philosophical Society, 123:222-234.

WILBUR HM, RUBENSTEIN DI & FAIRCHILD L (1978). Sexual selection in toads: the roles of female choice and male body size. Evolution, 32:264-270.

ZAHAVI A & ZAHAVI A (1997). The Handicap Principle: A Missing Piece of Darwin's Puzzle, New York: Oxford University Press.

Analysis of ontogenetic changes in head shape and diet in a catfish with moderately enlarged jaw adductors (*Clariallabes melas*)

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ABSTRACT. Jaw adductor hypertrophy, or the presence of enlarged jaw-closing muscles, has arisen several times independently in African clariid catfish. Previous work has demonstrated that species characterized by enlarged jaw adductors may have unusual ways of foraging such as terrestrial foraging and prey capture, and often include a large proportion of hard and terrestrial prey in their diet. However, relatively little is known about species of the genus *Clariallabes* with an intermediate degree of jaw adductor hypertrophy. In the present study we present data on head shape and diet for a range of sizes of specimens of a poorly known species of *Clariallabes*, *C. melas*. Our data show that growth patterns in this species and the previously studied *C. longicauda* are similar in some ways (e.g. positive allometry of the growth of the jaw muscles) but different in others (negative allometry in hyoid width). However, *C. melas* has a smaller head for its body size in all dimensions. Due to the large number of empty stomachs we encountered, dietary data remain preliminary, but suggest a varied diet including both hard and soft prey. Our data show that a large amount of variation in head shape may exist even among related species and that species with jaw adductor hypertrophy generally show positive allometry in the growth of the jaw adductors and associated structures. Whether jaw muscle hypertrophy is an adaptive trait in clariid catfish awaits further comparative analyses testing for the evolutionary association between jaw adductor hypertrophy and the inclusion of hard prey into the diet.

KEY WORDS: fish, muscle, biting, suction, feeding, ontogeny

INTRODUCTION

In fish, the inclusion of hard prey into the diet (durophagy) is often associated with changes in dentition (e.g. STREELMAN & ALBERTSON, 2006), the presence of enlarged pharyngeal or oral jaw adductor muscles (WAINWRIGHT, 1987; HERNANDEZ & MOTTA, 1997), an overall increase in the robustness of the oral or pharyngeal jaws and the skull (CABUY et al., 1999; GRUBICH, 2003) and in some species durophagy is associated with the presence of a specialized muscle activation pattern (LAUDER, 1983; KORFF & WAINWRIGHT, 2004). Interestingly, hypertrophy of the jaw adductors is often noted in populations showing a higher proportion of molluscs in the diet, suggesting an adaptive evolutionary response of the cranial morphology to diet (TURINGAN et al., 1995). Moreover, a strong plastic response in muscle size to an increase of molluscs in the diet has been demonstrated in some fish (MITTELBACH et al., 1999).

Oral jaw adductor hypertrophy has arisen several times independently in African clariid

catfish (JANSEN et al., 2006; DEVAERE et al., 2007). Indeed, a recent study using combined morphological and molecular data suggests that an increase in jaw adductor size has originated at least four times independently (DEVAERE et al., 2007). Clariid catfish are, however, not known to be mollusc crushers and retain large pointed teeth on the jaws (DEVAERE et al., 2001). Among clariid catfish species, jaw adductor hypertrophy ranges from the normal dorsoventrally-compressed type with the jaw adductors largely covered by the cranial bones (*e.g.* within the genus *Clarias*), to a situation where most of the dorso-lateral cranial bones are strongly reduced to make space for the jaw adductor muscles (*e.g.* within the genus *Channallabes*) (DEVAERE et al., 2001). Although the species with more pronounced degrees of jaw adductor hypertrophy have been relatively well studied, little is known about species with an intermediate degree of jaw adductor hypertrophy, such as those of the genus *Clariallabes* (but see WYCKMANS et al., 2007).

In other vertebrates, an increase in the size of the oral jaw adductor muscles is typically associated with an increase in bite force (HERREL et al., 2007a), and in many vertebrates an increase in bite force is associated with the inclusion of hard or large prey into the diet (HERREL et al., 2002; AGUIRRE et al., 2003; HERREL et al., 2005; HUBER et al., 2009). However, at least for some groups such as lizards, it has been shown that sexual selection for increased fighting capacity may also lead to increased bite force (LAILVAUX et al., 2004; HUYGHE et al., 2005; LAPPIN & HUSAK, 2005) and sexual differences in the size of the jaw adductors (HERREL et al., 2007b). Moreover, in some burrowing mammals, high bite force may be associated with the ability to dig tunnels into hard soils rather than diet (VAN DAELE et al., 2009) suggesting that a multitude of selective pressures may be operating on jaw muscle size and bite force capacity.

Previous work on clariid catfish with enlarged oral jaw adductors has demonstrated that some of these species have unusual ways of foraging including terrestrial prey capture (VAN

WASSENBERGH et al., 2006a), and that they include a large proportion of hard and often terrestrial prey in their diet (HUYSENTRUYT et al., 2004). However, the species with the most extreme degree of jaw adductor hypertrophy appear to swallow their prey whole (HUYSENTRUYT et al., 2004). Previous studies have shown that rapidly-growing and suction-feeding clariid species, such as *Clarias gariepinus* grow isometrically (HERREL et al., 2005). In contrast, the growth of jaw adductors and related structures shows positive allometry in a species with an intermediate degree of jaw hypertrophy, such as *Clariallabes longicauda* (WYKMANS et al., 2007). In order to test the hypothesis that species with intermediate degrees of jaw hypertrophy are characterised by positive allometric growth, we analysed growth patterns in a species with an intermediate degree of jaw adductor hypertrophy (*Clariallabes melas*; BOULENGER 1887). Moreover we compare patterns of growth in *C. melas* to those observed for its congener, *C. longicauda* (BOULENGER, 1902) and present data on diet for a range of sizes of individuals of *C. melas*.

MATERIALS AND METHODS

Material examined

The 51 *Clariallabes melas* used in this study were obtained from the Royal Museum for Central Africa (RMCA - KMMA), Tervuren, Belgium. All specimens (30592-603-1 to 10; 68800-68802-1 to 2; 117776-117780-1 to 5; 119100-101-1 to 2; 29660; 46746-46747-1 to 2; 117770-117775-1 to 2; 178220-230-MEL15, 178220-230-1; 19072-19074-1; 38495-38508-1 to 2; 44916-917-1 to 2; 74381; 79220; 96646-96648-1 to 2; 98931; 119117; 138665; 73-68-P-566-574-1 to 8; 86-06-P-51-53-1 to 2; 88-01-P-1993; 88-01-P-1994-1995-1 to 2) were collected in the Congo River drainage. Fish included in this study range from 116 to 329mm total length and encompass juvenile (i.e. non-reproductive) and adult specimens.

Morphometrics

Body size (both total length and standard length) and head dimensions were measured using digital calipers (Mitutoyo CD-30C and CD-15B; ± 0.01mm). Head length was measured as the distance between the tip of the snout and the caudal edge of the occipital process. Head width and head height were measured just posterior to the jaw adductors.

The surface area taken up by the jaw adductors in dorsal view was determined using digital photographs of each specimen and is used here as a proxy for jaw adductor size (see Fig. 1; Wyckmans et al., 2007). For our analyses, the average of the left and right sides was calculated. Additionally the maximal width of the head and the width of the neurocranium were determined on these pictures. Measurements were carried out using tpsDIG (version 1.40; Rohlf, 2004). A scale bar (1mm grid) was included in each photograph, allowing us to convert pixel measurements to metric units. These measures were taken as previous studies have shown them to be correlated with the inclusion of functionally-different prey into the diet in fish (Wyckmans et al., 2007).

To determine the dimensions of a number of internal skeletal elements, X-ray photographs (dorsoventral view) were made using a Philips Optimus X-ray unit with image intensifier, coupled to a Redlake Imaging MotionPro high resolution digital video camera. On each photograph eight landmarks were digitized using Didge (version 2.2.0.; Cullum A.). Based on the X-Y coordinates, the length and width of the lower jaw and the hyoid, the length of the hyoid bars, the angle between the two hyoid bars and the width of the pectoral girdle were calculated (see Fig. 2; Wyckmans et al., 2007). These measures are thought to reflect the ability of these fish to enlarge the oral cavity and thus their ability to capture elusive prey (Van Wassenberg et al., 2006c;d). As fish having wider pectoral girdles and longer and wider hyoids should be able to increase the volume of the oral cavity to a greater degree, they should be more successful in capturing elusive prey.

Diet

Stomach contents were removed *in situ* through a ventral incision, and preserved in a 70% aqueous ethanol solution. All prey items were sorted and identified using a binocular microscope (Wild M3Z). Because the majority of organisms found in the stomachs were crushed and/or digested, they were identified to the level of order or higher taxonomic level where appropriate.

The number of prey items found in every stomach was counted and for every prey item prey size was estimated. Intact prey items were measured using digital calipers (Mitutoyo CD-15B; ± 0.01mm). Non-intact prey items were grouped in size classes, from 0–45mm, at 5mm intervals. Average prey size, maximal prey size and the proportion of large prey (i.e. > 20mm) consumed were calculated for each individual fish. In addition, every prey group was weighed using an electronic microbalance (Mettler Toledo MT5; ± 0.001mg). For each prey group, a relative importance index (IRI) was calculated as an indicator of the significance of that particular prey group in the diet (Huysentruyt et al., 2004): IRI=(%N+%V)× %Oc where %N and %Oc are, respectively, the numeric abundance and the frequency of occurrence of a particular prey group. %V is the mass that particular prey group contributes to total prey mass. In addition, %IRI was calculated, being the proportion of IRI of each prey group in relation to the total IRI value.

Statistical analysis

To investigate size-related changes in morphology, $\log_{10}$ transformed morphological measures were regressed against the $\log_{10}$ transformed standard length. A two-tailed Student t-test was used to test for differences between the observed slopes obtained from the regression analyses and the slopes expected in the case of isometric growth (Sokal & Rohlf, 1995). Isometry is the expected mode of growth

for ectotherms growing throughout their life and has been demonstrated for non-specialized suction-feeding catfish (*Clarias gariepinus*; see HERREL et al., 2005). Analyses of covariance (ANCOVA) with standard length as covariate were used to test for differences in morphology between data presented here for *Clariallabes melas* and previously published data for *C. longicauda* (WYCKMANS et al., 2007). All analyses were performed using SPSS V15.

of isometric growth. Whereas jaw adductor size (Student t-test, d.f.=50, t=2.02, 0.02<p<0.05) increases faster, lower jaw width (Student t-test, d.f.=50, t=-2.57, 0.01<p<0.02) and hyoid width (Student t-test, d.f.=47, t=-2.44, 0.01<p<0.02) increase slower than predicted by geometric scaling models. Unexpectedly, the angle between the two hyoid bars decreases significantly during growth in *C. melas* (Student t-test, d.f.=47, t=-2.48, 0.01<p<0.02).

RESULTS

Ontogenetic changes in head size and shape in *Clariallabes melas*

All morphological traits examined are correlated with fish body size (all R²>0.120 and p<0.016; Table 1). The observed ontogenetic changes in morphology, however, were not always consistent with those expected for a model

Clariallabes melas versus *C. longicauda*

Significant interspecific differences were detected for all morphological traits examined. Taking into account the observed difference in average body size (SL) (one-way ANOVA; $F_{1,105}$=10.36; p=0.002), it is clear that *C. longicauda,* despite its smaller body size has a broader, more robust head and larger jaw adductor muscles than does *C. melas* (ANCOVA; all p<0.002; see Fig. 1).

TABLE 1

Table summarizing the scaling of head dimensions relative to body size in *Clariallabes melas*.
d.f. = degrees of freedom; S.E. = standard error of the slope.

	EXPECTED SLOPE	OBSERVED SLOPE	S.E.	d.f.	t	P
Total length (mm)	1	0.990	0.010	50	-1.000	0.30 < P < 0.50
Cranial length (mm)	1	0.940	0.056	50	-1.071	0.20 < P < 0.30
Cranial width (mm)	1	1.005	0.057	45	0.088	> 0.90
Cranial depth (mm)	1	1.011	0.066	50	0.167	0.80 < P < 0.90
Neurocranial width (mm)	1	0.802	0.099	50	-2.000	0.05 < P < 0.10
Head width (mm)	1	1.005	0.067	47	0.075	> 0.90
Surface area jaw adductors (mm²)	2	2.269	0.133	50	2.023	0.02 < P < 0.05
Width lower jaw (mm)	1	0.879	0.047	50	-2.574	0.01 < P < 0.02
Length lower jaw (mm)	1	0.978	0.063	50	-0.349	0.70 < P < 0.80
Width hyoid (mm)	1	0.883	0.048	47	-2.438	0.01 < P < 0.02
Length hyoid (mm)	1	1.111	0.088	47	1.261	0.20 < P < 0.30
Hyoid angle (°)	0	-0.149	0.060	47	-2.483	0.01 < P < 0.02
Width pectoral girdle (mm)	1	0.959	0.061	48	-0.672	0.50 < P < 0.70

TABLE 2

Table summarizing the diet of *Clariallabes melas*. **Occurrence** reflects the number of stomachs in which a particular prey group was observed; **prey number** reflects the total number of prey found; **mass** reflects the total prey mass of that prey category and **%IRI** indicates the relative importance of a prey group in the diet.

|	OCCURRENCE		PREY NUMBER		MASS		%IRI
	#	%	#	%	mg	%	
Plant/unidentifiable	18	69.23					
Nematoda	2	7.69					
Gastropoda	1	3.85	2	3.23	3.61	1.99	0.85
Isopoda	1	3.85	8	12.90	2.00	1.10	2.26
Chilopoda	1	3.85	3	4.84	1.70	0.94	0.93
Insecta	4	15.38	4	6.45	0.55	0.30	4.35
Coleoptera (adult)	5	19.23	10	16.13	2.34	1.29	14.03
Coleoptera (larval)	1	15.38	12	19.35	46.90	25.81	29.55
Dictyoptera	1	3.85	2	3.23	3.14	1.73	0.81
Diptera (larval)	3	11.54	11	17.74	0.99	0.54	8.82
Ephemeroptera (larval)	1	3.85	1	1.61	0.26	0.14	0.28
Lepidoptera (larval)	2	7.69	2	3.23	66.51	36.60	13.17
Odonata (larval)	2	7.69	3	4.84	7.74	4.26	2.97
Teleostei	4	15.38	4	6.45	45.97	25.30	20.92
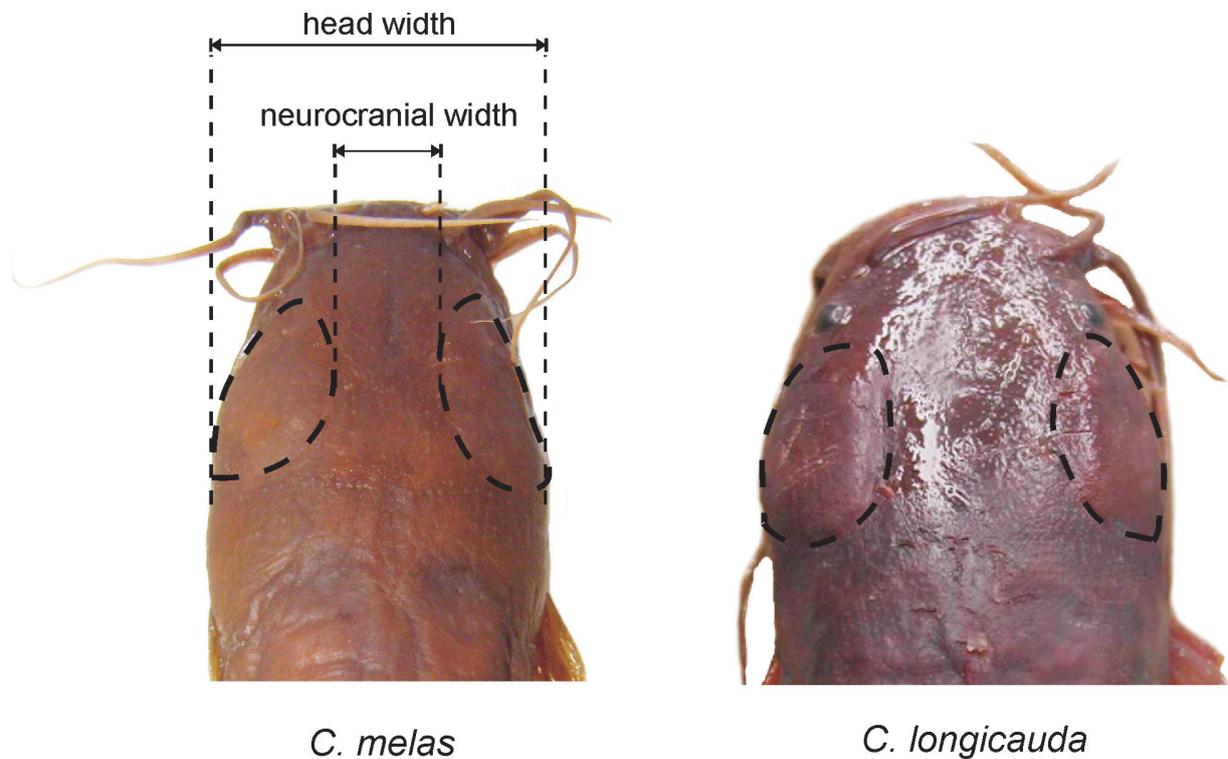
Fig. 1. – Pictures in dorsal view of the heads of *Clariallabes melas* (left) and *C. longicauda* (right). Indicated are the outlines of the jaw adductors, head width and neurocranial width. Note the more robust appearance of the head in *C. longicauda* compared to *C. melas*.

Diet in *Clariallabes melas*

Twenty (43.5%) of the 46 stomachs examined were empty and 13 (28.3%) contained only unidentifiable or plant matter. The content of the remaining 13 stomachs could be identified, although these also regularly contained some unidentifiable or plant matter, which was probably accidentally ingested. Table 2 summarizes the results of the stomach content analysis. The most important prey groups for *C. melas* are larval specimens of Coleoptera (%IRI=29.6), fish (%IRI =20.9), adult specimens of Coleoptera (%IRI=14.0) and larval specimens of Lepidoptera (% IRI=9.1). Over 30% of the preys retrieved from the stomachs were large (over 20mm in length). All hard-shelled prey retrieved from the stomachs had been effectively crushed, as was observed previously for *C. longicauda* (WYCKMANS et al., 2007). Throughout ontogeny no changes in diet, or in the relationships between morphology and diet, could be demonstrated for *C. melas*.

DISCUSSION

Our data on cranial morphometrics for a size range of *Clariallabes melas* indicate that the jaw adductors become disproportionately bigger as fish grow; a phenomenon that was previously also observed for *C. longicauda* but is distinctly different from growth patterns observed for the non-hypertrophied *Clarias gariepinus* (HERREL et al., 2005). Interestingly, and in contrast to the patterns observed for *C. longicauda* (WYCKMANS et al., 2007), jaw width and hyoid width within *C. melas* showed a negative allometry indicating that the heads in the large fish are characterized by large jaw muscles but relatively narrow jaws and hyoids. Why this is the case currently remains unclear, but we suggest that the relatively narrow hyoid may allow for a greater ventral excursion of the mouth floor due to rotation of the hyoid. All else being equal, rotation of a hyoid of a given length characterized by hyoid bars being closer together, as observed here for *C. melas*, will result in a greater ventral excursion of the
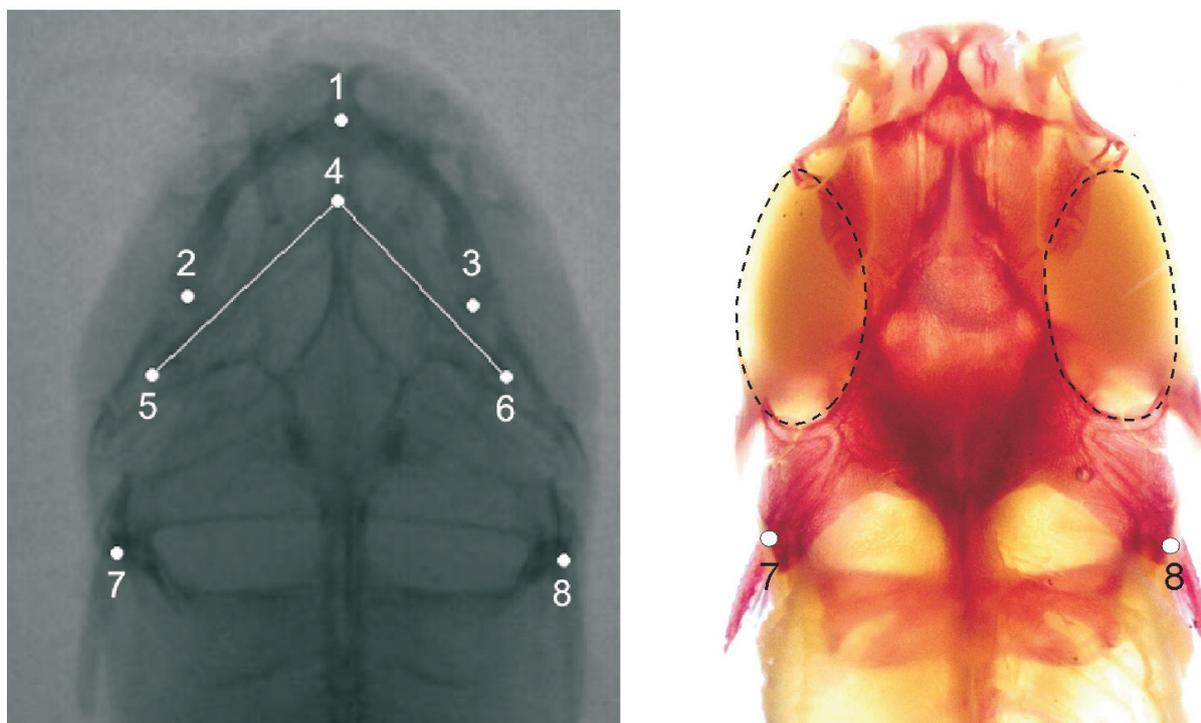
Fig. 2. – To the left a ventral view x-ray of the head of a *Clariallabes melas* is shown. On the X-ray the landmarks used to quantify head and jaw dimensions are indicated. To the right a dorsal view picture of a cleared and stained *C. melas* specimen is shown to illustrate the size of the jaw adductors. Note that the lower jaw and hyoid are not present in this specimen. (1) Lower jaw symphysis; (2, 3) caudal tips of the lower jaw; (4) hyoid symphysis; (5, 6) caudal tips of the hyoid; (7, 8) left and right pectoral fin articulation.
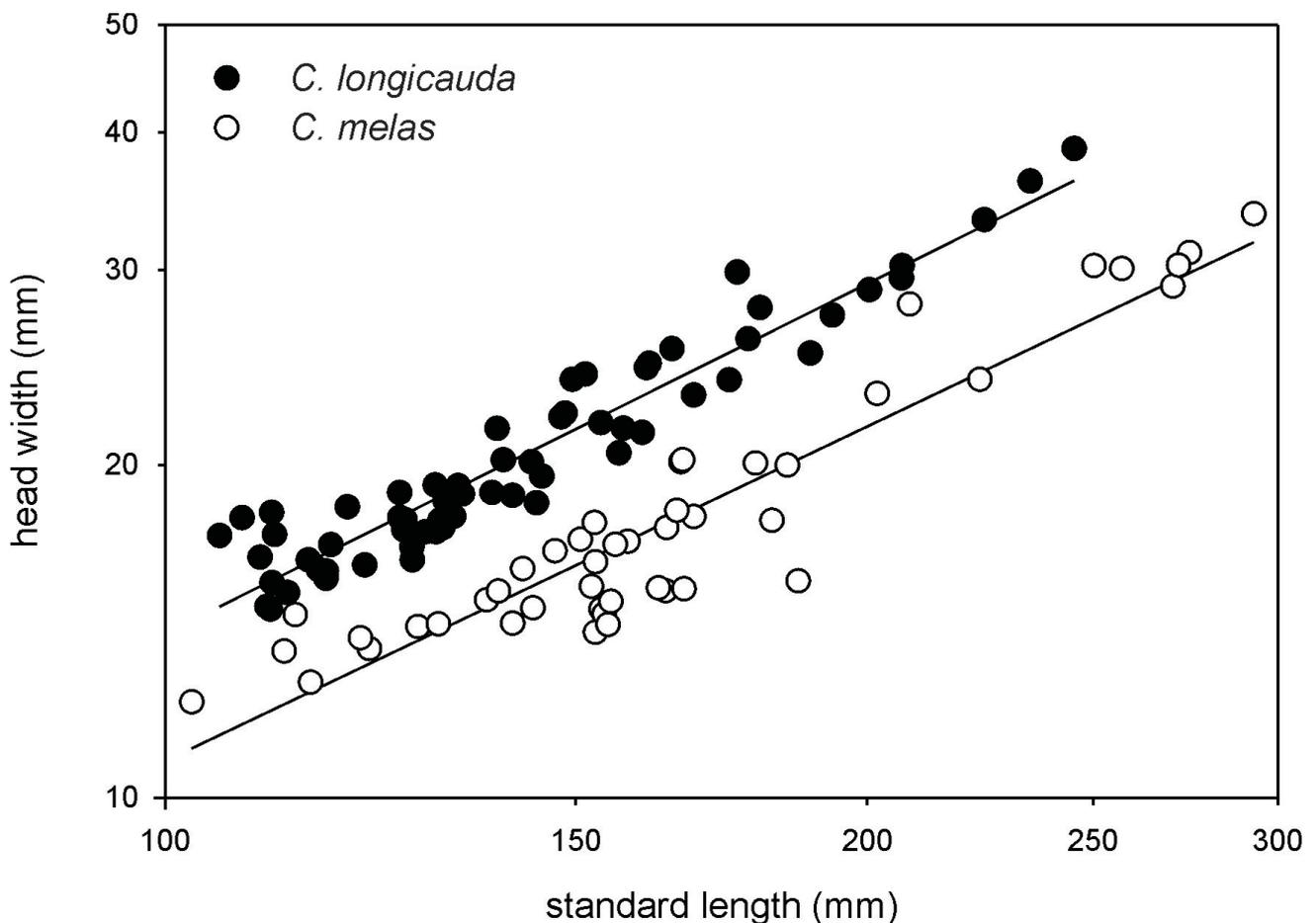
Fig. 3. – Scatterplot illustrating the differences in head width between *Clariallabes melas* and *C. longicauda*. For a given body size (standard length) *C. melas* has a significantly narrower head than *C. longicauda*.

mouth floor. Previously published kinematic data on suction feeding in clariid catfish with enlarged jaw adductors (including *Clariallabes*) do indeed suggest that the expansion of the buccal cavity is largely restricted to a ventrad rotation of the hyoid (VAN WASSENBERGH et al., 2004, 2006b;c) and consequently larger fish are expected to have an improved suction performance. An improvement of suction performance with size has been suggested previously also for *Clarias gariepinus* (VAN WASSENBERGH et al., 2005; 2006d). Although we suggest that larger *C. melas* might prey disproportionately more on relatively large and mobile prey such as teleost fish, our preliminary dietary data cannot confirm this. Additional dietary data are clearly needed to shed further light on the ecological significance of the observed ontogenetic changes in morphology.

Interestingly, our data indicate clear differences in morphology between the two *Clariallabes* species studied here. Even though *C. melas* is on average slightly larger than *C. longicauda* its head is clearly smaller and the jaw adductors are less developed for a given body size (Figs 1; 3). Although it is tempting to speculate about the ecological significance of this difference in morphology, our data for *C. melas* are insufficient to address this in a quantitative manner. Although the diet in *C. longicauda* appears more diverse, this may simply be an artifact of the small sample size in *C. melas*. In both species coleopterans and teleost fish are the most important food items with about 60-80% of the individuals having one or the other prey in their stomachs. Despite having smaller jaw adductors, the *C. melas* examined here crushed all hard prey as evidenced by the crushed

molluscs, and crushed exoskeletons of insects and other arthropods, similar to the situation reported for *C. longicauda* (WYCKMANS et al., 2007). As many of the prey retrieved from the stomachs were large (30% over 20mm in length) this suggests an important role for the hypertrophied jaw adductors.

Our data illustrate how different species of clariid catfish with intermediate jaw adductor hypertrophy may differ dramatically in morphology and growth. Moreover, and in contrast to non-specialized suction-feeding catfish such as *Clarias gariepinus*, the growth of the head and structures functionally relevant to feeding is allometric. Although some tendencies for differences in diet can be discerned, these await further and more robust quantitative analyses. The fact that all hard prey were found crushed in the stomachs of the two *Clariallabes* species is in strong contrast to data for species with more extreme jaw adductor hypertrophy such as *Channalabes* or *Gymnallabes* where hard prey are apparently swallowed whole (HUYSENTRUYT et al., 2004). Thus, whereas the enlarged jaw adductors in these species with intermediate degrees of jaw specialization appear to provide a performance benefit in crushing hard prey, this does not appear to be the case in more specialized forms. Clearly further data on morphology and diet in species with enlarged jaw adductors such as *Platyallabes tihoni* (DEVAERE et al., 2005), *Dolichallabes microphtalmus* (DEVAERE et al., 2004) and their non-specialized sister taxa (DEVAERE et al., 2007) will be crucial to quantitatively test for evolutionary associations between morphology and diet in the group.

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REFERENCES

AGUIRRE LF, HERREL A, VAN DAMME R & MATHYSSEN E (2003). The implications of food hardness for diet in bats. Functional Ecology, 17:201-212.

CABUY E, ADRIAENS D, VERRAES W & TEUGELS GG (1999). Comparative study on the cranial morphology of *Gymnallabes typus* (Siluriformes: Clariidae) and their less anguilliform relatives, *Clariallabes melas* and *Clarias gariepinus*. Journal of Morphology, 240:169-194.

DEVAERE S, ADRIAENS D, TEUGELS GG & VERRAES W (2005). Morphology and spatial constraints in a dorso-ventrally flattened skull, with a revised species description of *Platyallabes tihoni* (Poll, 1944). Journal of Natural History, 39:1653-1673.

DEVAERE S, ADRIAENS D, VERRAES W & TEUGELS GG (2001). Cranial morphology of the anguilliform clariid *Channallabes apus* (Günther, 1873) (Teleostei: Siluriformes): are adaptations related to powerful biting? Journal of Zoology, 255:235-250.

DEVAERE S, JANSEN G, ADRIAENS D & WEEKERS P (2007). Phylogeny of the African representatives of the catfish family Clariidae (Teleostei, Siluriformes) based on a combined analysis: independent evolution towards anguilliformity. Journal of Zoological Systematics and Evolutionary Research, 45:214-229.

DEVAERE S, TEUGELS GG, ADRIAENS D, HUYSENTRUYT F & VERRAES W (2004). Redescription of *Dolichallabes microphthalmus* (Poll, 1942) (Siluriformes, Clariidae). Copeia, 2004:108-115.

GRUBICH J (2003). Morphological convergence of pharyngeal jaw structure in durophagous perciform fish. Biological Journal of the Linnean Society, 80:147-165.

HERNANDEZ LP & MOTTA PJ (1997). Trophic consequences of differential performance: ontogeny of oral jaw crushing performance in the sheepshead, *Archosargus probatocephalus* (Teleostei: Sparidae): Journal of Zoology, 243:737-756.

HERREL A, MCBRAYER LD & LARSON PM (2007b). Functional basis for intersexual differences in bite

force in the lizard *Anolis carolinensis*. Biological Journal of the Linnean Society, 91:111-119.

HERREL A, ADRIAENS D, VERRAES W & AERTS P (2002). Bite performance in clariid fishes with hypertrophied jaw adductors as deduced by bite modeling. Journal of Morphology, 235:196-205.

HERREL A, SCHAERLAEKEN V, MEYERS JJ, METZGER KA & ROSS CA (2007a). The evolution of cranial design and performance in squamates: consequences of skull-bone reduction on feeding behavior. Integrative and Comparative Biology, 47:107-117.

HERREL A, VAN WASSENBERGH S, WOUTERS S, ADRIAENS D & AERTS P (2005). A functional morphological approach to the scaling of the feeding system in the African catfish, *Clarias gariepinus*. Journal of Experimental Biology, 208:2091-2102.

HUBER DR, CLAES JM, MALLEFET J & HERREL A (2009). Is extreme bite performance associated with extreme morphologies in sharks. Physiological and Biochemical Zoology, 82:20-28.

HUYGHE K, VANHOOYDONCK B, SCHEERS H, MOLINA-BORJA M & VAN DAMME R (2005). Morphology, performance and fighting capacity in male lizards, *Gallotia galloti*. Functional Ecology, 19:800-807.

HUYSENTRUYT F, ADRIAENS D, TEUGELS GG, DEVAERE S, HERREL A, VERRAES W & AERTS P (2004). Diet composition in relation to morphology in some African anguilliform clariid catfishes. Belgian Journal of Zoology, 134:41-46.

JANSEN G, DEVAERE S, WEEKERS PHH & ADRIAENS D (2006). Phylogenetic relationships and divergence time estimate of African anguilliform catfish (Siluriformes: Clariidae) inferred from ribosomal gene and spacer sequences. Molecular Phylogenetics and Evolution, 38:65-78.

KORFF WL & WAINWRIGHT PC (2004). Motor pattern control for increasing crushing force in the striped burrfish (*Chilomycterus schoepfi*). Zoology, 107:335-346.

LAILVAUX SP, HERREL A, VANHOOYDONCK B, MEYERS JJ & IRSCHICK DJ (2004). Fighting tactics differ in two distinct male phenotypes in a lizard: Heavyweight and lightweight bouts. Proceedings of the Royal Society London B., 271:2501-2508.

LAPPIN AK & HUSAK JF (2005). Weapon performance, not size, determines mating success and potential reproductive output in the collared lizard (*Crotaphytus collaris*). American Naturalist, 166:426-436.

LAUDER GV (1983). Neuromuscular patterns and the origin of trophic specialization in fishes. Science, 219:1235-1237.

MITTELBACH GG, OSENBERG CW & WAINWRIGHT PC (1999). Variation in feeding morphology between pumpkinseed populations: phenotypic plasticity or evolution? Evolutionary Ecology Research, 1:111-128.

ROHLF FJ (2004). tpsDIG32 version 1.40. Available at http://life.bio.sunysb.edu/morph/index.html

SOKAL RF & ROHLF FJ (1995). Biometry. WH Freeman, New York.

STREELMAN JT & ALBERTSON RC (2006). Evolution of Novelty in the Cichlid Dentition. Journal of Experimental Zoology, 306B:216-26.

TURINGAN RG, WAINWRIGHT PC & HENSLEY DA (1995). Interpopulational variation in prey use and feeding biomechanics in Caribbean triggerfishes. Oecologia, 102:296-304.

VAN DAELE PAAG, HERREL A & ADRIAENS D (2009). Biting performance in teeth-digging African mole-rats (*Fukomys*, Bathyergidae, Rodentia). Physiological and Biochemical Zoology, 82:40-50.

VAN WASSENBERGH S, AERTS P & HERREL A (2005). Scaling of suction-feeding kinematics and dynamics in the African catfish, *Clarias gariepinus*. Journal of Experimental Biology, 208:2103–2114.

VAN WASSENBERGH S, AERTS P & HERREL A (2006d). Scaling of suction feeding performance in the catfish *Clarias gariepinus*. Physiological and Biochemical Zoology, 79:43-56.

VAN WASSENBERGH S, HERREL A, ADRIAENS D & AERTS P (2004). Effects of jaw adductor hypertrophy on buccal expansions during feeding of air breeding catfishes (Teleostei, Clariidae). Zoomorphology, 123:81-93.

VAN WASSENBERGH S, HERREL A, ADRIAENS D & AERTS P (2006b). Modulation and variability of

prey capture kinematics in clariid catfishes Journal of Experimental Zoology, 305A:559-569.

Van Wassenbergh S, Herrel A, Adriaens D & Aerts P (2006c). No trade-off between biting and suction feeding performance in clariid catfish. Journal of Experimental Biology, 210:27-36.

Van Wassenbergh S, Herrel A, Adriaens D, Huysentruyt F, Devaere S & Aerts P (2006a). A catfish that can strike its prey on land. Nature, 440:881.

Wainwright PC (1987). Biomechanical limits to ecological performance: mollusc-crushing by the Caribbean hogfish, *Lachnolaimus maximus* (Labridae). Journal of Zoology, 213:283-297.

Wyckmans M, Van Wassenbergh S, Adriaens D, Van Damme R & Herrel A (2007). Size-related changes in cranial morphology affect diet in the catfish *Clariallabes longicauda*. Biological Journal of the Linnean Society, 92:323-334.

Fish assemblages across a salinity gradient in the Zeeschelde estuary (Belgium)

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ABSTRACT. Between 1991 and 2008 a total of 71 fish species was recorded in the brackish and fresh water zone of the Schelde estuary (Zeeschelde). The results were obtained from fish surveys from the cooling water filter screens of the power plant at Doel (between 1991 and 2008) and fyke net surveys along the length of the estuary between 1995 and 2008. Species abundance in the different salinity zones was analysed using the fyke net data only. The ten most abundant species represent 90.8% of the total number of individuals caught. In decreasing order of abundance: flounder *(Platichthys flesus),* roach (*Rutilus rutilus*), herring (*Clupea harengus),* eel (*Anguilla anguilla*), pike-perch (*Sander lucioperca*), sole (*Solea solea),* common goby (*Pomatoschistus microps*), seabass (*Dicentrarchus labrax*), three-spined stickleback (*Gasterosteus aculeatus*) and white bream (*Blicca bjoerkna*). With fyke nets 33 species were caught in the tidal freshwater zone, 43 species in the oligohaline zone and 59 species in the mesohaline zone. Each salinity zone is characterised by a typical fish assemblage, although some species are shared between all three salinity zones: e.g. three-spined stickleback (*Gasterosteus aculeatus*), Prussian carp (*Carrasius gibelio*), roach (*Rutilus rutilus*) and eel (*Anguilla anguilla*). Diadromous species occur in all zones and make up, on average up 22% of the species richness. Freshwater species comprise about 70% of the species in the tidal freshwater zone. In the oligohaline zone the contribution of the freshwater species to the species richness is less while marine migrants become more abundant. As expected, the contribution of marine migrants and estuarine species is higher in the mesohaline zone. The recent increase in species richness in the freshwater and oligohaline zone coincides with a remarkable increase in dissolved oxygen since 2007.

KEYWORDS: fish assemblages, spatial variation, low salinity zone, functional guilds

INTRODUCTION

Estuaries play a key role in nutrient cycling and transformation, and are an essential habitat in the life cycle of many organisms, in particular fish and waterfowl (COLCLOUGH et al., 2005; MCLUSKY & ELLIOTT, 2004). An estuary is the part of a river that is under tidal influence and is characterised by a continuous salinity gradient (FAIRBRIDGE, 1980). Hence fish assemblages in estuaries are very diverse and composed of marine, estuarine, freshwater and migrating species (HENDERSON, 1988; LOBRY et al., 2003). ELLIOTT & DEWAILLY (1995) assessed the fish assemblage structure in 17 European estuaries. They identified functional guilds according to the habitat use of each fish species encountered. This guild approach facilitates the comparison of fish assemblages across different estuaries (e.g. LOBRY et al., 2003). Recently FRANCO et al. (2008) validated the functional guild approach. Estuaries in Northwest Europe have been the

subject of considerable research focussing on the functioning of the different habitats (e.g. DOLBETH et al., 2007; ELLIOTT et al., 2007). Their role as nursery and feeding areas, refuges and migration routes have been described for specific estuaries such as the Zeeschelde (MAES et al., 2007, 2008), the mudflats in the Westerschelde (CATTRYSSE et al., 1994) and the Forth estuary (ELLIOTT et al., 1990). Other research focused on spatiotemporal patterns in fish composition and assemblage structure indicating that fish communities differ in space and time (POTTER et al., 1997; MARSHALL & ELLIOTT, 1998; ARAÚJO et al., 1999; THIEL & POTTER, 2001; JOVANOVICK et al., 2007; SELLESLAGH & AMARA, 2008; SELLESLAGH et al., 2009). Spatial patterns in estuarine species assemblages are mainly correlated with salinity (HENDERSON, 1989), while temporal variations are mostly the result of migration of young fish (MAES et al., 1998; THIEL & POTTER, 2001).

The fish community in the Zeeschelde, the Belgian part of the Schelde estuary, has been studied since the 1990s. However, studies in the earlier years were generally limited to the mesohaline and oligohaline zone, occasionally including one site in the freshwater zone (e.g. VAN DAMME et al., 1994; MAES et al., 1997; MAES et al., 1998, b; MAES et al., 1999, MAES et al., 2004; STEVENS, 2006; STEVENS et al., 2006; CUVELIERS et al., 2007; BUYSSE et al., 2008 and GUELINCKX et al., 2008). VRIELYNCK et al. (2003) give a historical overview of fish species present in the salt and brackish parts of the Zeeschelde and its tributaries. The Rupel (oligohaline tributary) and Durme (freshwater tributary) have been surveyed annually since 2004 (e.g. VAN THUYNE & BREINE, 2008). Since 2007 volunteers monitor fish all year round at different sites along the salinity gradient of the Zeeschelde, including the tidal freshwater zone.

The main aim of this study is to describe the fish assemblage along the salinity gradient in the Zeeschelde estuary based on sampling results in the mesohaline, oligohaline and tidal freshwater zone and to provide an overview of its temporal

and spatial variation (measured as species richness and abundance).

MATERIAL AND METHODS

Study area

The river Schelde is a tidal lowland river with its origin in the northern part of France (St. Quentin), and its mouth in the North Sea near Vlissingen, The Netherlands. With a total length of 355 km, the fall is approximately 100 m and the mean depth about 10 m (BAEYENS et al., 1998). The main river and tributaries are rain-fed, with a minimal discharge in summer and autumn, causing the salt water to penetrate further upstream in these seasons. At the mouth the average tidal range is 4.2 m (average spring tide in period 2000-2010). The tide penetrates 160 km upstream where the average tidal range is 2.34 m (average spring tide in period 2000-2010).

In the Zeeschelde (the Belgian part of the estuary, Fig. 1) three zones are distinguished based on the Venice system (1959): a mesohaline zone (5-18) between Zandvliet and Antwerpen, an oligohaline zone (0.5-5) between Antwerpen and Temse, including the Rupel tributary, and a tidal freshwater zone till Gent including the Durme tributary. In Gent the effect of the tide is abated by a complex of sluices. The Rupel is an oligohaline tributary. The tidal part of the Durme was interrupted downstream of Lokeren in the 1960's and functions now as a large freshwater tidal creek of the main river. The tidal amplitude in the Durme is quite large (average 5.40 m at Tielrode); therefore habitat conditions change drastically between incoming and outgoing tides. Both Rupel and Durme have important mudflats (26 and 24 ha) and marshes (43 and 100 ha).

The oligohaline zone has been impacted for decades by untreated sewage water from metropolitan Brussels. From 1925 onwards fish was absent in the Rupel river (VRIELYNCK et al., 2003). Also the industrial areas of Lille (France),

Gent and Antwerpen (Belgium) and Vlissingen (The Netherlands) had a major negative impact on the estuarine water quality (VAN ECK et al., 1991). For years the Zeeschelde downstream of Antwerpen remained anoxic, creating an effective barrier for diadromous fish (MAES et al., 2007, 2008). As water treatment efforts increased and diffuse pollution along the river reduced, the water quality improved and a shift in oxygen regime and nutrient cycling was observed (MARIS et al., 2008, VAN DAMME et al., 2005; VAN DEN BERGH et al., 2005). Since March 2007 most sewage water from Brussels is treated and since then the oxygen concentration in the River Rupel increased markedly (VAN THUYNE & BREINE, 2008; STEVENS et al., 2009). However, the Zeeschelde still receives significant discharges of untreated industrial and domestic waste water, as well as diffuse pollution from agricultural runoff, resulting in a poor water quality in a large part of the estuary.

Data collection

Data were collected at 32 different sites in the Zeeschelde and its tributaries (Fig. 1). Samples were taken using fyke nets between 1995-1999 and 2001-2008 in the mesohaline zone and between 1997-1999 and 2001-2008 in the other salinity zones. Collections at the cooling water intakes of the power station at Doel (Fig. 1, #23) were made between 1991-2001 and 2003-2008. The Doel data set was used to complete the species list of the mesohaline zone (presence/absence). All field work was performed by trained fish biologists and trained volunteers using a standardised protocol (see BREINE et al., 2007). All fish were identified to species level, counted and released back into the estuary. Occasional cross examination in the laboratory assured the quality of the fish identification.

Data were collected by assignments from
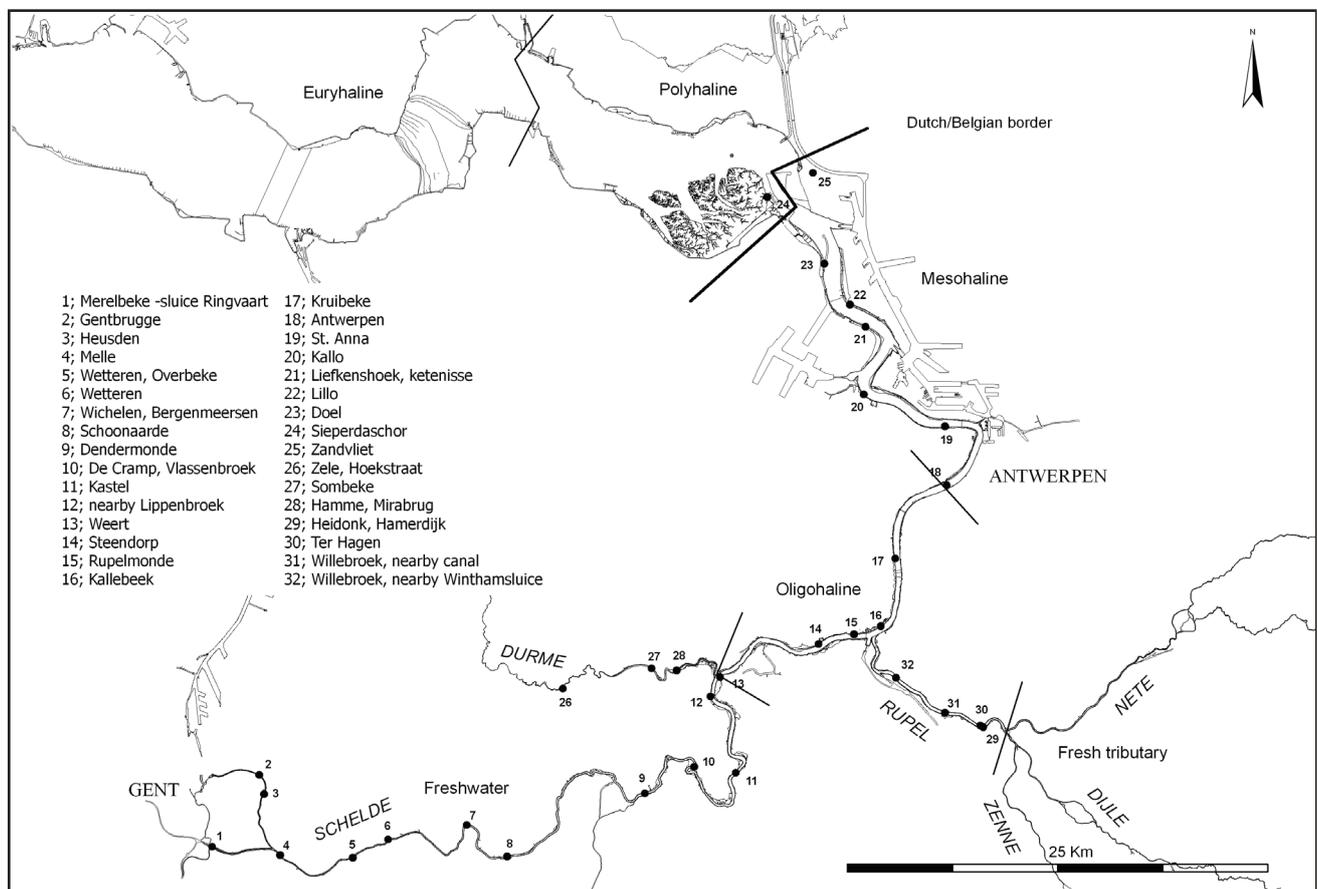
Fig. 1. – Map of the Zeeschelde Estuary with indication of the sampling sites.

TABLE 1

An overview of the sites surveyed between 1991 (including Doel) and 2008. All sites were surveyed with fyke nets except the cooling circuit at Doel.

RIVER	SITE (number in Fig. 1)	PERIOD	NUMBER OF SURVEYS (1 survey = 24 h)	NUMBER OF FYKES
Schelde	Merelbeke (1)	2003	2	1
Schelde	Merelbeke – sluice Ringvaart (1)	2002	12	1
Schelde	Gentbrugge (2)	1997	1	1
Schelde	Heusden (3)	2002	11	1
Schelde	Melle (4)	1997	4	1
Schelde	Melle (4)	2002	12	1
Schelde	Overbeke, Wetteren (5)	2007-2008	4	2
Schelde	Wetteren (6)	2007	4	2
Schelde	Uitbergen, Wichelen (7)	2008	4	2
Schelde	Schoonaarde (8)	1997	2	1
Schelde	Dendermonde (9)	1997	4	1
Schelde	De Cramp, Vlassenbroek (10)	2007-2008	44	1
Schelde	Kastel (11)	2002-2007	17	2
Schelde	Lippenbroek (12)	2006-2008	158	1
Schelde	Weert (13)	2007-2008	43	1
Schelde	Steendorp (Notelaar) (14)	2008	6	2
Schelde	Steendorp (14)	2002-2007	14	2
Schelde	Steendorp (14)	1997	4	1
Schelde	Steendorp (14)	1998	8	1
Schelde	Steendorp (14)	2001	5	1
Schelde	Rupelmonde (15)	2007-2008	62	1
Schelde	Kallebeek (16)	1997	1	1
Schelde	Kruibeke (17)	1997	4	1
Schelde	Antwerpen (18)	1997	4	1
Schelde	Antwerpen (18)	1998	8	1
Schelde	Antwerpen (18)	2001	6	1
Schelde	Antwerpen (18)	2002-2007	12	2
Schelde	Antwerpen (18)	2007-2008	398	1
Schelde	St. Anna (19)	2004-2005	304	1
Schelde	Kallo (20)	1995-1998	11	1
Schelde	Kallo (20)	2008	25	1
Schelde	Liefkenshoek, Ketenisse (21)	2007-2008	185	1
Schelde	Lillo (22)	1995	4	1
Schelde	Doel (23)	1991-2008	170	
Schelde	Sieperdaschor (24)	1997-1999	9	1
Schelde	Zandvliet (25)	1995-2004	197	1
Schelde	Zandvliet (25)	2005-2007	6	2
Durme	Zele (26)	2004-2008	5	2
Durme	Sombeke (27)	2004-2007	4	2
Durme	Hamme, Mirabridge (28)	2004-2008	5	2
Rupel	Heidonk, Hamerdijk (29)	2004-2008	5	2
Rupel	Heidonk, Hamerdijk (29)	2007-2008	56	1
Rupel	Ter Hagen (30)	2007-2008	29	1
Rupel	Willebroek, near canal (31)	2004-2008	5	2
Rupel	Willebroek, Wintham sluice (32)	2004-2008	5	2

the Flemish Environmental Agency (VMM), Association of Industrial Companies of North Antwerpen (VIBNA, Vereniging van de Industriële Bedrijven van Noord-Antwerpen), Department of Mobility and Public Affairs, division Maritime Access (MOW) and the Research Institute for Nature and Forest (INBO, Instituut voor Natuur- en Bosonderzoek). For the period 1997-1999 data from the mesohaline reach near Sieperdaschor (Fig. 1, #24) were obtained from a volunteer fishing with paired fykenets.

Table 1 gives an overview of the survey campaigns at the sites, illustrating differences of sampling effort between the zones.

For each sampling location, monthly oxygen concentrations in the different salinity zones were obtained from the OMES database (http:www.vliz.be/projects/omes/) (i.e. 32). If not available, data from the nearest sites within the zone were used. In the OMES project, tidal-independent oxygen measurements are taken monthly from a boat. Maris et al (2008) compared these point measurements with continuous measurements (2 week periods) and observed no time-related differences in oxygen values. For each year (1997-2008) the annual average values were calculated using this data.

Sampling gear

Fyke nets

At each location one or two 'paired-fyke' nets were deployed near the low-tide mark for two tidal cycles (24 h) and emptied the next day (Table 1). Some sites were surveyed during two successive days. Each paired-fyke consists of two fyke ends of 2.2 m long, linked by an 11 m leader net (0.5 cm mesh size). The largest hoop measures 0.8 m and has an oblate basis of 1.2 m to make sure that the net stays upright. Fish are directed by the leader into the fyke and collected in the last chamber with a mesh size of 8 mm.

Intake screens at the power station Doel

The cooling water is drawn through a multiple intake system ($25 \text{ m}^3\text{s}^{-1}$) at 2 m above the bottom of the estuary and filtered by two vertical travelling screens with a mesh size of 4 mm. The screens prevent larger organisms and debris from obstructing the condensers (Maes et al., 2004).

Data analysis

The oxygen variation between the salinity zones was assessed with a nested ANOVA (log (x+1) transformed monthly data). The numbers of individuals caught with fyke nets were transformed to catch per unit effort (CPUE); i.e. the total number of individuals is divided by the number of fykes used and the number of days. CPUE data were pooled per month, season and year, log (x+1) transformed and analysed with a non-metric multidimensional scaling (NMDS) ordination to examine the spatial organization of the fish assemblage. Only data from samples that were taken in the same month and year in all three salinity zones were retained for the analysis. This corresponds, for the period 1997-2008, with 28 pooled CPUE data for each zone. Dissimilarity matrices were calculated from log (x+1) transformed fish abundance data, using Bray-Curtis distances. The NMDS ordination was created using random starting configurations and iterated until solutions converged. The vegan package in R 2.6.2 was used for the analysis (Oksanen et al., 2006, R Development Core Team). To reduce the effect of rare species, only the 15 most abundant species in each salinity zone were included for analysis (i.e. 22 species). To test for spatial differences in the fish assemblages a Discriminant Analysis (DA) was applied to the same data. The estimated distinctiveness of fish assemblages was calculated using Wilk's Lambda criterion (λ) (Castillo-Rivera et al., 2002). This value ranges from 1 (similar groups) to 0 (different groups). A PCA with spring (March-May) and autumn (September-November) catches assessed the species contribution within each salinity

TABLE 2

Annual minimum (min), maximum (max) and average dissolved oxygen values (mg l^{-1}) for the different zones in the tidal Zeeschelde between 1997 and 2008 (source OMES).

| YEAR	FRESHWATER			OLIGOHALINE			MESOHALINE		
	min	average	max	min	average	max	min	average	max
1997	1.4	3.3	7.4	1.0	2.2	6.0	5.6	5.6	7.1
1998	1.7	4.6	9.2	1.7	3.4	8.1	4.8	6.8	9.1
1999	0.9	2.8	11.7	0.8	2.1	4.6	3.1	6.0	6.7
2000	1.6	2.3	3.3	2.6	4.3	8.2	3.1	5.6	10.0
2001	1.2	3.6	6.6	1.9	4.2	5.1	3.7	5.3	6.0
2002	4.0	6.1	8.5	1.7	4.0	7.2	4.8	6.4	10.1
2003	4.1	5.1	8.0	3.2	4.4	5.5	5.3	7.7	11.8
2004	6.2	5.5	13.4	2.5	4.0	6.3	2.2	6.5	10.2
2005	5.3	5.8	9.4	1.2	2.5	9.4	1.9	3.9	9.8
2006	1.7	5.7	10.2	0.9	3.0	6.7	2.6	7.9	10.8
2007	4.5	7.0	9.0	1.2	5.2	8.5	1.9	7.0	8.6
2008	4.3	7.8	10.2	2.5	6.1	9.2	5.1	7.1	8.9
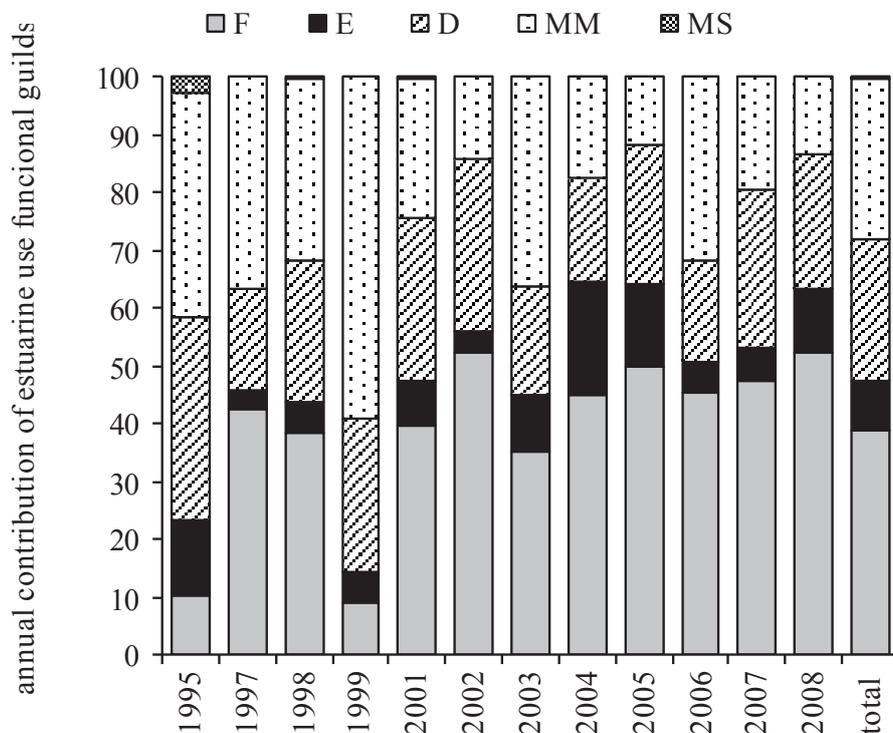
Fig. 2. – The annual contribution (% CPUE) of the estuarine use functional guilds in the Zeeschelde between 1995 and 2008. (F: freshwater species; E: estuarine residents; D: diadromous species; MM: marine migrant species; MS: marine stragglers).

zone. We used the ten most abundant species in each salinity zone for this analysis.

RESULTS

Dissolved oxygen

Minimum, maximum and average annual values, for dissolved oxygen in the different salinity zones for the years 1997-2008 are presented in Table 2. They indicate in general and for all zones an increase in dissolved oxygen over this period. The increase of the average annual dissolved oxygen concentration during the observation period is highest in the freshwater zone. There is a significant difference in monthly oxygen concentration between the different zones (ANOVA: F=10.315; p<0.001). The lowest minimum and average values are recorded in the oligohaline zone.

Fish inventory

Fyke net catches

In total 66 species were caught between 1995 and 2008 (Table A, annex). In the mesohaline zone, 59 species were caught during 741 fishing occasions (day catches) between 1995 and 2008. In the oligohaline 43 species were collected during 632 fishing occasions between 1997 and 2008. In the freshwater zone 33 species were caught during 336 fishing occasions between 1997 and 2008. Figure 2 gives an overview of the total catch in the Zeeschelde (period 1995-2008) with regard to the guild composition.

In the Zeeschelde we distinguished five functional guilds (ELLIOTT et al., 2007; FRANCO

et al., 2008). Their annual contribution changes over the years. In the early 1990's nearly no fish were caught in the freshwater and oligohaline zone. This is reflected by the low contribution of freshwater species. Their contribution became more important from 2001 onwards. The annual guild contribution in each salinity zone is described further in the text.

Zone differences

Between 1997 and 2008, 28 fishing occasions took place in the same month in all the salinity zones (Table 3). During these surveys, 59 species were caught of which 22 were selected for the NMDS ordination.

The NMDS ordination shows a clear distinction between the different zones (Fig. 3). The catches in the different salinity zones form three distinct groups. For the freshwater zone (Dots) summer and autumn catches form two separate groups. The spring catches are scattered alongside these two groups. In the oligohaline zone (Triangles) summer and spring catches form two separate groups. The winter and autumn catches are situated along these groups. In the mesohaline zone (Squares) we observe a large overlap among the seasons with spring and summer catches forming separate groups.

Species such as plaice (*Pleuronectes platessa*), herring (*Clupea harengus*), seabass (*Dicentrarchus labrax*) and smelt (*Osmerus eperlanus*) are typical for the mesohaline zone. In the oligohaline zone the presence of common goby (*Pomatoschistus microps*) and herring is responsible for the differentiation from the freshwater zone, while the presence of freshwater species is responsible for the separation from

TABLE 3

Common fishing occasions in the three salinity zones.

| YEAR	1997	2002	2003	2004	2005	2006	2007	2008
MONTH	3 9 12	3 9	9	3 4 9	3 4	9	3 4 5 6 7 8 10 11 12	1 4 5 6 7 8 9
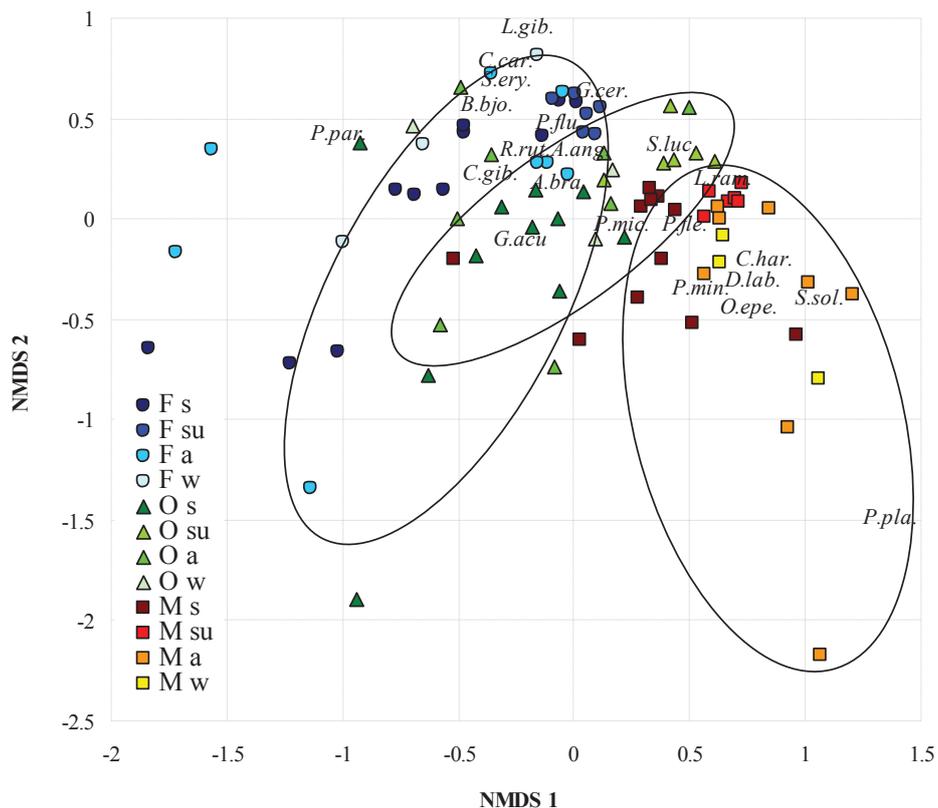
Fig. 3. – Non-metric multidimensial scaling (NMS) ordination of fish abundance data (CPUE) for the different salinity zones of the Zeeschelde estuary between 1997 and 2008 (n=84) (F: freshwater ●; O: oligohaline ▲; M: mesohaline ■; s: spring; su: summer; a: autumn; w: winter). For fish abbreviations see Table A in annex.

the mesohaline zone. Some ordination points of different zones are close together due to species with a comparable abundance in all zones e.g. three-spined stickleback (*Gasterosteus aculeatus*), white bream (*Blicca bjoerkna*), Prussian carp (*Carassius gibelio*), roach (*Rutilus rutilus*) and eel (*Anguilla anguilla*). This explains also the overlap observed for the freshwater and oligohaline zone.

The DA on the log-transformed CPUE of species with zone as grouping variable revealed a significant difference between the zones: λ= 0.029, p<0.0001, with more than 95% correctly classified cases.

Freshwater zone

In the freshwater zone 33 species were collected between 1997 and 2008 (Table A, annex). We grouped fish into guilds or functional groups according to Franco et al. (2008) to

facilitate comparison between the salinity zones. Freshwater species comprised 69.7% of the total species richness and contributed 78.9% to the total number of individuals recorded (Total, Fig. 4). The marine migrants contributed only 0.04% to the total number caught and were only recorded during 2008. Diadromous species make up 18.2% of the species richness and 19.3% of the individuals recorded. In 1997 only a few diadromous specimens were caught but this guild was well represented from 2005 onwards. Two estuarine species (common goby *Pomatoschistus microps* and sand goby *P. minutus*) have been encountered yearly in the freshwater zone since 2006. They had already been recorded occasionally in 1997 and 2004. Estuarine species contributed 1.7% to the total number of individuals caught. The annual guild contribution (relative percentage) is given in figure 4.

A PCA with annual spring and autumn catch
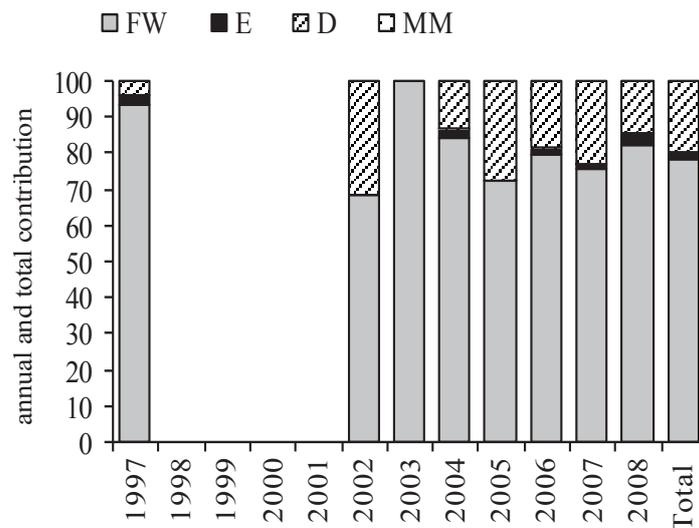
Fig. 4. – The annual and total contribution (% CPUE) of the estuarine use functional guilds in the freshwater zone of the Zeeschelde between 1997 and 2008. (F: freshwater species; E: estuarine residents; D: diadromous species; MM: marine migrant species).

data (CPUE, log (x+1) transformed) groups all the results obtained before 2007 to the right of the first axis (Fig. 5). Factor 1 explains 39.8% and the second factor 17.9% of the variance.

The gradual increase in number of individuals separates the 2007 and 2008 catches from the previous years. The 2002 catches are separated because of the presence of white bream (*Blicca bjoerkna*, factor loadings -0.07;-0.95) and flounder (-0.36;-0.86). The year 2002 was a very wet one (MARIS et al., 2008). The catch results in 2008 are similar to those in 2007 but they are separated in the scatterplot mainly because of pike-perch (-0.94;0.07), rudd (*Scardinius erythrophthalmus*, -0.98;0.04), Prussian carp (*Carrasius gibelio*, -0.95;0.14) and perch (*Perca fluviatilis*, -0.95;-0.01).

The CPUE log (x+1) transformed data of the freshwater zone are represented in figure 6. All survey data are included in order to to show the trend in fish abundance over the years. The figure shows that the abundance and species richness increase from 2004 on and that roach is the most abundant species in the freshwater zone contributing 27.9% of the total number of individuals. An increasing number of flounder (21.5%), pike-perch (8.1%), white bream (7.2%)

and rudd (4.7%) were caught since 2005. At present the most abundantly caught species are flounder, common goby, pike-perch, roach and white bream.

Oligohaline zone

In the oligohaline zone 43 species were caught between 1997 and 2008 (Table A). 53.5% are freshwater species, contributing 62.9% to the total abundance. Nine marine migrant species contribute 5.3% to the total abundance, while they contribute 20.9% to the species richness. Some of the marine migrants, e.g. herring (*Clupea harengus*), were collected yearly but the highest number (CPUE) of marine migrants was caught in 2007 and 2008. Diadromous species make up 19.9% of the species and 14% of the individuals caught. Of this guild only eel and flounder were caught in all years, the other diadromous species were caught regularly since 2007. The two estuarine species, common and sand goby, were recorded in the oligohaline zone since 2003 and 1997 respectively. Since 2007 the greater pipefish (*Syngnathus acus*) was also caught and contributes, together with the two gobies, 11.9% to the total abundance. Occasionally marine stragglers venture in the oligohaline zone, e.g. Lozano's goby (*Pomatoschistus lozanoi*) and the
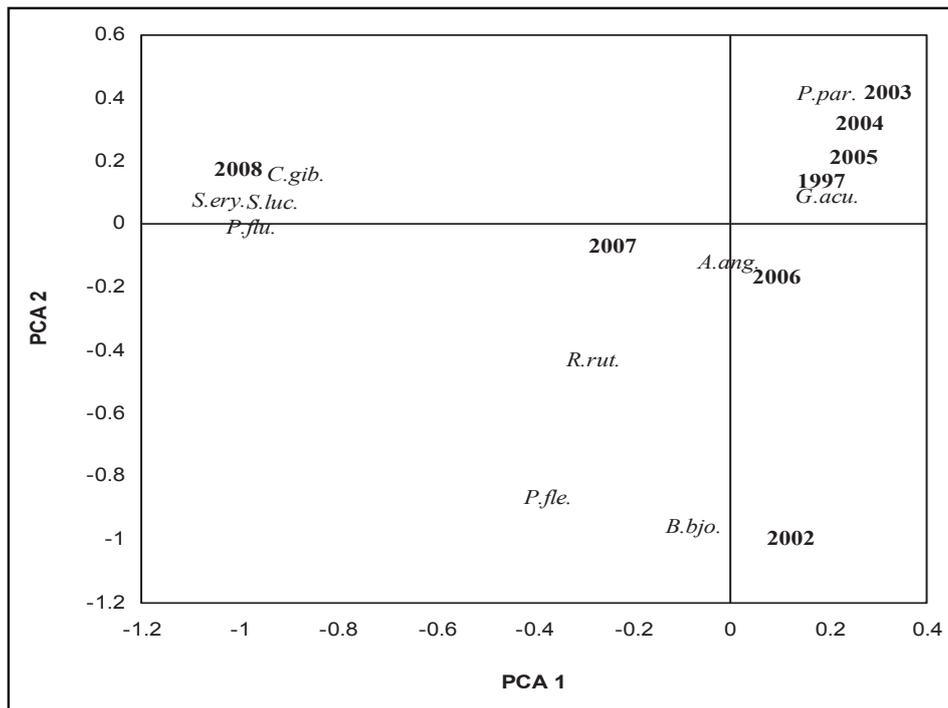
Fig. 5. – Scatterplot of factor loadings categorized by year obtained by PCA with log (x+1) transformed number of individuals caught (CPUE) and factor loadings of the ten most abundant species in the freshwater zone of the Zeeschelde estuary between 1997 and 2008 (spring and autumn catches, n=26). Abbreviations see Table A in annex.
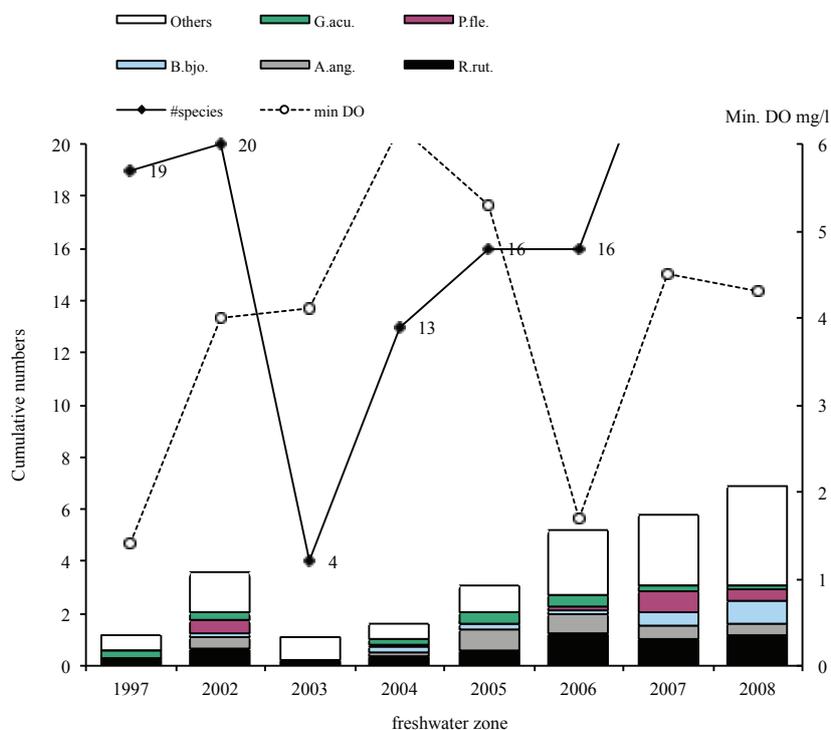
Fig. 6. – Species richness (figures on full line) and the catch per unit effort (cumulative numbers, log (x+1) transformed) for fish species caught in the freshwater zone of the Zeeschelde between 1997 and 2008 (abbreviations see Table A, annex). Only the on average five most abundant species are indicated with a specific pattern. Dotted lines connect the minimum recorded DO for a particular year.
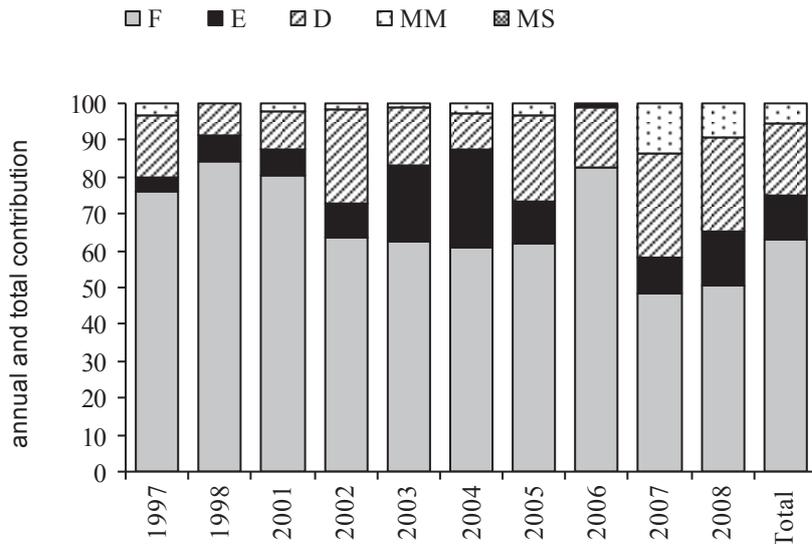
Fig. 7. – The annual and total contribution (% CPUE) of the estuarine use functional guilds in the oligohaline zone of the Zeeschelde between 1997 and 2008. (F: freshwater species; E: estuarine residents; D: diadromous species; MM: marine migrant species; MS: marine stragglers).
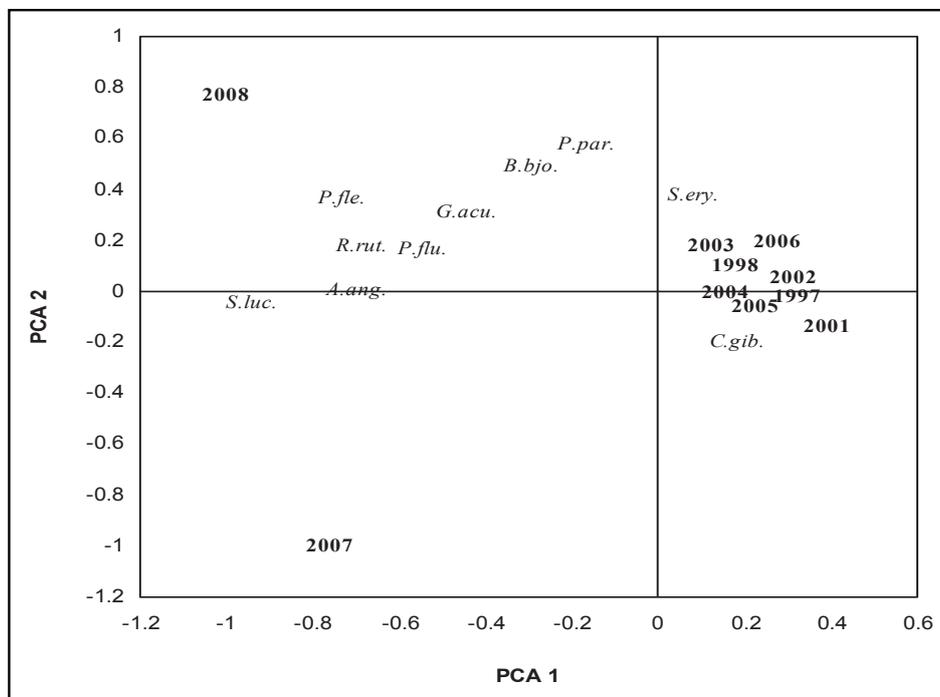

Fig. 8. – Scatterplot of factor loadings categorized by year obtained by PCA with log (x+1) transformed number of individuals caught (CPUE) and factor loadings of the ten most abundant species in the oligohaline zone of the Zeeschelde estuary between 1997 and 2008 (spring and autumn catches, n=26). Abbreviations see Table A in annex.
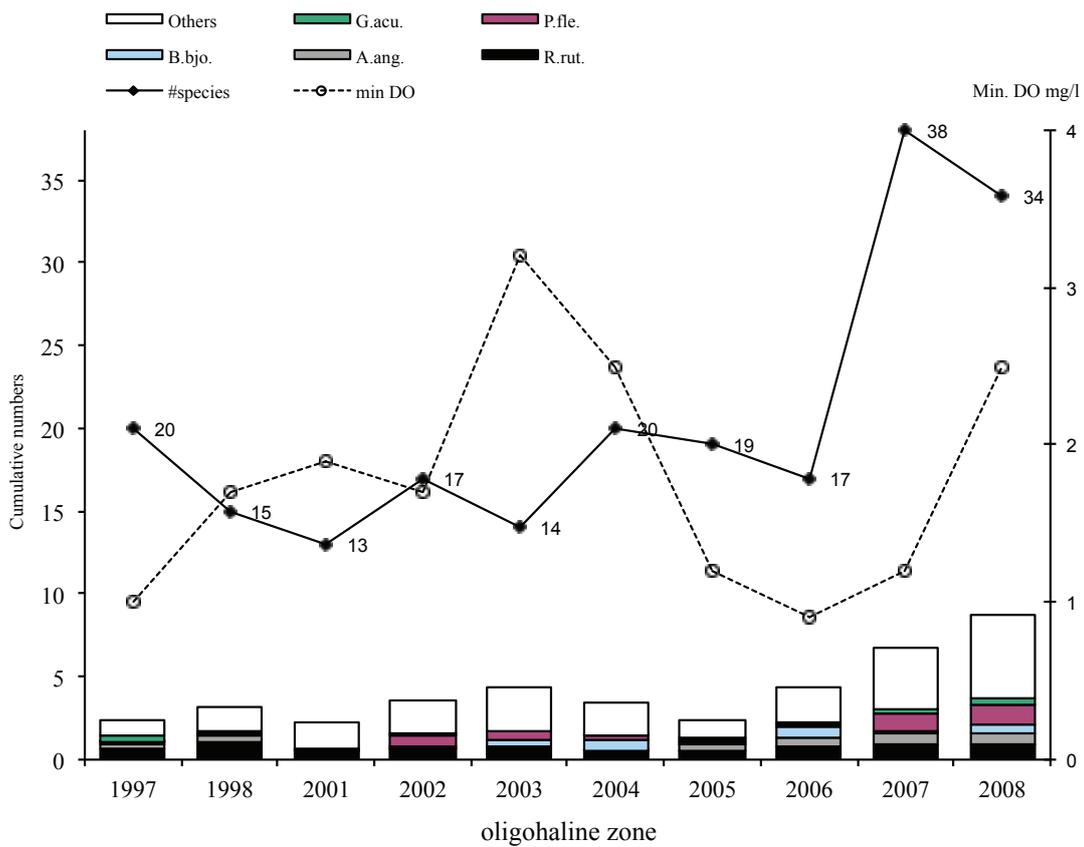
Fig. 9. – Species richness (figures on full line) and the catch per unit effort (cumulative numbers, log (x+1) transformed) for fish species caught in the oligohaline zone of the Zeeschelde between 1997 and 2008 (abbreviations see Table A, annex). Only the on average five most abundant species are indicated with a specific pattern. Dotted lines connect the minimum recorded DO for a particular year.
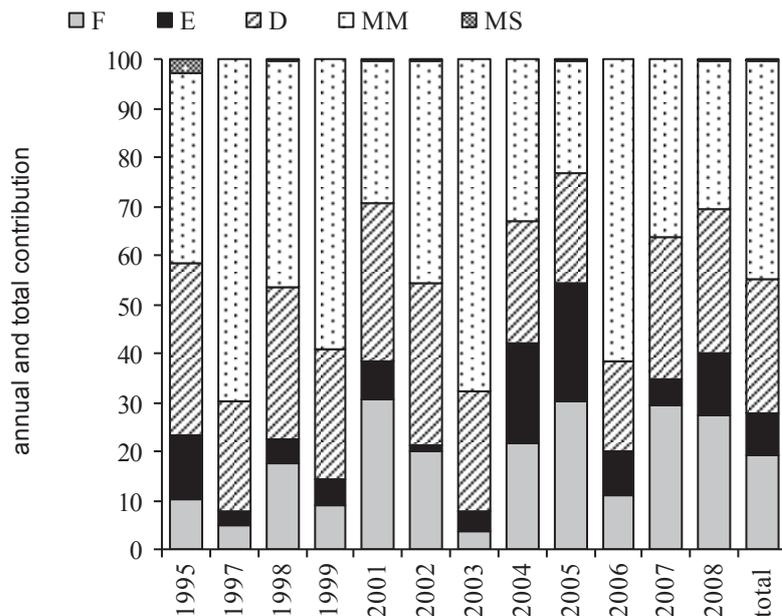
Fig. 10. – The annual and total contribution (% CPUE) of the estuarine use functional guilds in the mesohaline zone of the Zeeschelde between 1995 and 2008. (F: freshwater species; E: estuarine residents; D: diadromous species; MM: marine migrant species; MS: marine stragglers).

lesser weever (*Echiichthys vipera*). The annual and total guild presence (CPUE) is given in figure 7.

Figure 8 shows the scatterplot of a PCA with annual catch data (spring and autumn CPUE, log (x+1) transformed). Most catches cluster together on the right side of the first PCA axis, but the samples of 2007 and 2008 are separated from them and from each other. Factor 1 explains 41.2% and the second factor 17.0% of the variance.

In 2007 and 2008 more species and individuals (CPUE) were caught. They are separated from the other years mainly by the presence of pike-perch (*Sander lucioperca*, -0.93;-0.04). The difference between these two years is the result of differences in numbers caught.

The CPUE log (x+1) transformed data in the oligohaline zone of the Zeeschelde shows a remarkable increase in number of individuals in 2007 and 2008 (Fig. 9). Roach is again the most frequently caught species. The pike-perch and rudd catches increase since 2006. Species richness increased in 2007 but decreased again in 2008.

Mesohaline zone

In the mesohaline zone 59 species were collected between 1995 and 2008 (Table A). Of these, 33.3% are freshwater species, contributing 19.3% to the total abundance. Marine migrant species are well represented, comprising 26.6% of the species and contributing 44.5% to the total number of individuals with herring, flounder and sole as the most abundant species. Marine migrants occurred in all annual catches. About 15% of the species were diadromous species, contributing 27% of the total number of individuals caught. Diadromous species were always present in the annual catches. Ten estuarine species (16.6% of
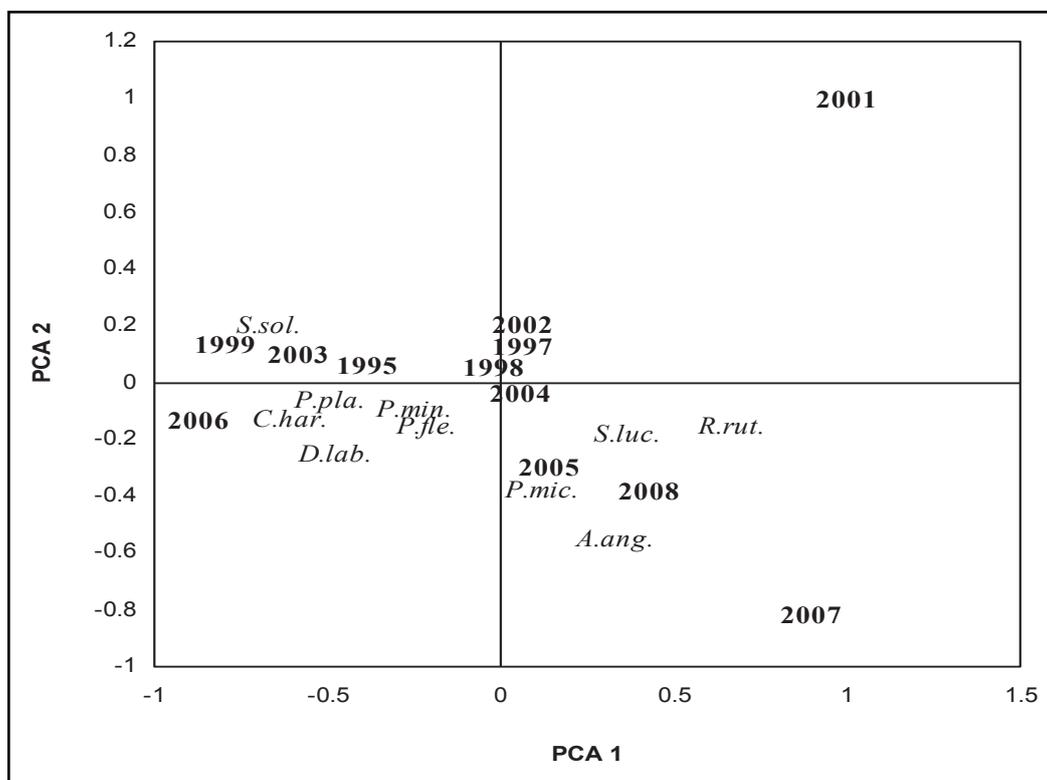
Fig. 11. – Scatterplot of factor loadings categorized by year obtained by PCA with log (x+1) transformed number of individuals caught (CPUE) and factor loadings of the ten most abundant species in the mesohaline zones of the Zeeschelde estuary between 1995 and 2008 (spring and autumn catches n=48). Abbreviations see Table A in annex.

total species number) were caught, contributing 8.8% to the total catch. The marine stragglers contributed 8.3% to the species and 0.2% to the total number of individuals caught. The annual and total guild distribution (relative percentage) is shown in figure 10.

The result of the PCA shows a more dispersed pattern than the ones observed in the freshwater and oligohaline zones (Fig. 11). Factor 1 explains only 18.8% and the second factor 16.0% of the variance. The ordination shows that the data obtained in 2001 and 2007 are separated from the other results. During these years the catches of sole (-0.66;0.20), seabass (-0.47;-0.24), herring (-0.60;-0.12) and plaice (-0.48;-0.05) were low. Catches in 2008 are less distinct than in the other salinity zones due to a decrease in numbers of individuals (Fig. 12) and in species richness.

The more dispersed general pattern reflects the higher annual catch variations in the mesohaline zone compared with the other zones.

The catch per unit effort (log (x+1) transformed) in the mesohaline zone of the Zeeschelde is given in figure 12. The figure shows an increase in CPUE between 1995 and 2001 followed by a decrease until 2005. In 2006 and 2007 the annual CPUE was high, but in 2008 again a decrease was observed.

Doel

At the intake screens of the power station at Doel 66 species were collected between 1991 and 2008 of which snake pipefish (*Entelurus aequoreus* (Linnaeus, 1758)), solenette (*Buglossidium luteum* (Risso, 1810)), painted
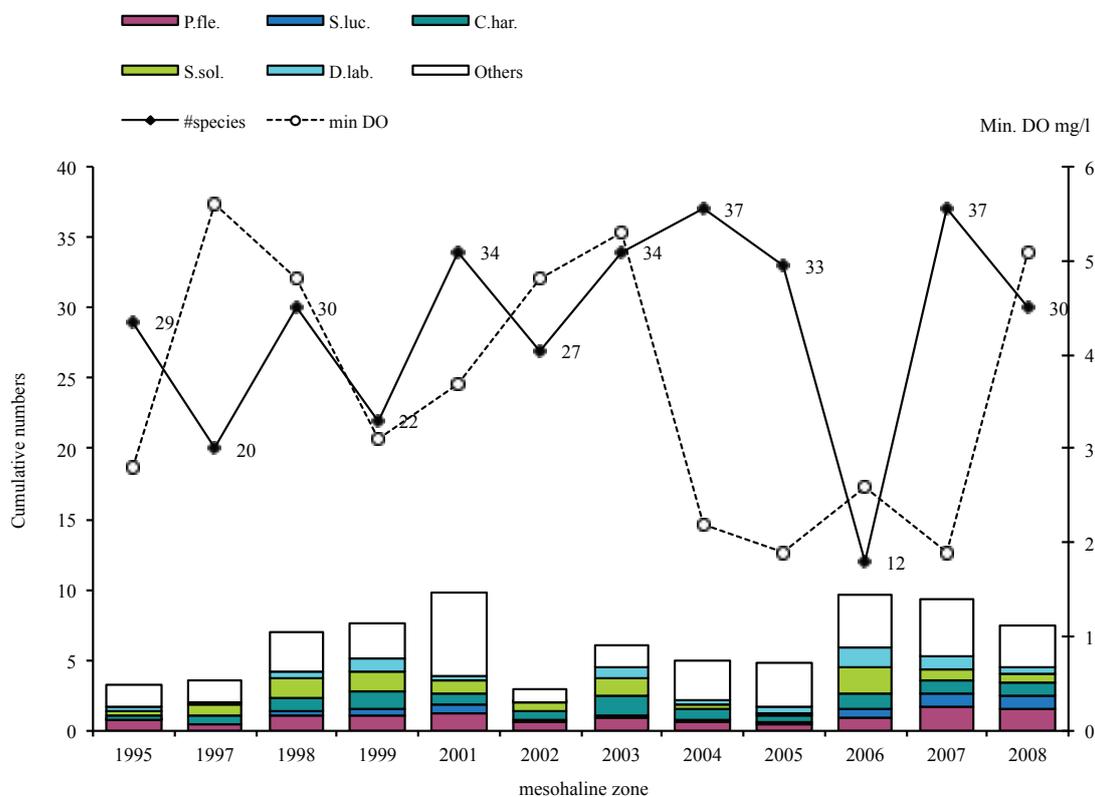
Fig. 12. – Species richness (figures on full line) and the catch per unit effort (cumulative numbers, log (x+1) transformed) for fish species caught in the mesohaline zone of the Zeeschelde between 1995 and 2008 (abbreviations see Table A, annex). Only the on average five most abundant species are indicated with a specific pattern. Dotted lines connect the minimum recorded DO for a particular year.

goby (*Pomatoschistus pictus* (Malm, 1845)), dragonet (*Callionymus lyra* (Linnaeus, 1758)) and great sandeel (*Hyperoplus lanceolatus* (Le Sauvage, 1824)) were not caught with fykes. This brings the total of fish species caught in the Zeeschelde estuary to 71.

DISCUSSION

Dissolved oxygen

One of the main abiotic variables influencing the presence of fish in an estuary is the dissolved oxygen concentration (DO) (MAES et al., 1998; ARAÚJO et al., 2000; TURNPENNY et al., 2006; MAES et al., 2007, 2008). The significant difference in monthly oxygen concentration between the different zones was also observed by SOETAERT et al (2006). DO within the Zeeschelde has increased continuously since 1996 (Table 2, MARIS et al., 2008). The changes observed in the fish assemblages in the freshwater and oligohaline zones become evident from 2007 onwards (Figs. 6 and 9). As a result of the activation of the water purification plant (Brussels North, March 2007) the oxygen concentration in the River Rupel increased strongly and fish started to re-colonise this river (VAN THUYNE & BREINE, 2008). A similar improvement was observed in the Thames estuary where the return of fish species was a striking feature linked with the recovery from pollution (WHEELER, 1969, 1979; ANDREWS & RICKARD, 1980; ATTRILL, 1998). Between 1997 and 2006 the average oxygen concentration in the oligohaline zone remained below 5 mg l^{-1}, i.e. the norm value as stipulated by the MINISTERIE VAN VOLKSGEZONDHEID EN LEEFMILIEU (1987; VLAREM II, 1995). Although in 2007 an improvement was recorded, still 54.6% of the OMES records were below 5 mg l^{-1} (MARIS et al., 2008). The mesohaline zone has a higher oxygen concentration due to oxygen rich water coming in from the Westerschelde. This could explain why, compared to the other zones, in this zone no significant increase in fish catches was observed between 1995 and 2007. In the late 1970s temporal anoxia was common in the

upstream part of the Zeeschelde (SOETAERT et al., 2006). In the freshwater part of the Zeeschelde an improvement of oxygen concentration is observed between 1998 and 2002 which is due to a higher discharge (wet years) and a higher primary production during the summer months (MARIS et al., 2008). Between 2002 and 2007 the biological oxygen demand decreased in the freshwater part (MARIS et al., 2008). Although the freshwater zone has high DO concentrations for the period 2007-2008, even in summer, the norm of 5 mg l^{-1} (VLAREM II, 1995) is not always reached (MARIS et al., 2008).

Zone differences in fish assemblages

The difference in species richness and composition between the different zones is illustrated by the NMDS and the DA. The guild distribution changes gradually with the salinity (Figs. 4, 7 and 10). Although this shift is gradual which is illustrated by the overlap between the freshwater and oligohaline zones, our results indiquate that it is appropriate to distinguish three salinity zones for fish assemblages in the Zeeschelde. The observed shift in distribution is consistent with other estuaries in the North Sea area. THIEL & POTTER (2001) recorded a sequential change in the species composition from the most downstream site (high salinity) to the most upstream one (oligohaline) in the Elbe estuary. SELLESLAGH et al. (2009) reported a variable catch composition between intermediate and upper stations in the Somme estuary.

Freshwater zone

As expected, the fish assemblages in the freshwater zone of the Zeeschelde are dominated by freshwater species (68-100%), corresponding with observations in tidal freshwater along the Atlantic coast of North America (ODUM et al., 1988) and in a freshwater estuary in Estonia (VETEMAA et al., 2006). Freshwater individuals contributed 82.7% to the total catch between 1997 and 2008. The tidal freshwater zone is essentially a habitat for freshwater and diadromous species. An essential fish habitat

consists of both the water column and underlying surface of a particular area. It contains all habitat characteristics essential to the long-term survival and health of particular fishes. Although this zone is characterised by the presence of freshwater species, its fish community is different from non tidal freshwater rivers e.g. thinlip mullet (*Liza ramada*), twaite shad (*Alosa fallax*) and smelt are not caught in non tidal freshwater rivers in Flanders. The difference in fish community is due to morphological characteristics, dynamics and its connection with the oligohaline zone. However, between 1997 and 2008 the number of estuarine species and marine migrants caught were limited to 3.5 and 0.2% respectively. In a highly polluted river like the Zeeschelde oxygen deficiency strongly affects the fish community structure. Over the years a gradual improvement in species richness is observed. A significant and steady increase in species richness and number of individuals is noted since 2004, the worst year observed being 2003 (Fig. 6). In addition concordant with the water quality (DO) improvements, a shift in fish assemblage structure occurred. In 1997 resistant freshwater species such as three-spined stickleback, Prussian carp and roach were dominant in numbers. Another indication of the water quality improvement is the presence of twaite shad, recorded in spring 2007. Other diadromous species observed since 2007 are smelt and thinlip mullet. Since summer 2007 herring and seabass (marine migrants) frequent this zone with abundance peaks in summer. This could be due to an increase in salinity during this season (less freshwater run off) combined with the improved water quality. For some species a seasonal pattern in frequency of occurrence and abundance can be distinguished. Ide (*Leuciscus idus*), Wells catfish (*Siluris glanis*), smelt, thinlip mullet, rudd, eel and pike-perch show a peak in summer. Some species such as lampreys are underestimated because of the low catch efficiency of fykes for this particular group. Concerning trophic level, omnivorous species such as roach, rudd, Prussian carp and eel are dominant in numbers. These are also species tolerant of poor water quality (Breine et al., 2007). Their dominance is an indication that

although the water quality improved the habitat quality is still not optimal (Manolakos et al., 2007).

Oligohaline zone

This zone is characterised by a return of fish due to a continuous improvement of the water quality (DO; Maris et al., 2008). Since 2007 species richness is higher than in the freshwater zone and estuarine species and marine migrants have become more important, which corresponds with previous research in oligohaline waters (e.g. Rozas & Hackney, 1983). Between 1997 and 2008 the number of estuarine fish contributed 11.8%, diadromous 19.8% and the marine migrants 5.3% to the total catch. During this period freshwater individuals contributed 62.9% to the total catch. We therefore consider the oligohaline zone as a habitat for freshwater, estuarine, diadromous and marine migrant species. As already discussed, a higher oxygen concentration has been observed in the oligohaline zone since the treatment of Brussels' waste water began, enhancing the presence of fish (Fig. 9). In 1994-1995, the dissolved oxygen concentration was close to zero during most of the year. Only in winter 12 fish species were caught at the cooling water inlets in Schelle, all of which were freshwater species except smelt and eel, which are diadromous species (Maes et al., 1998). Between 1995 and 2007 a gradual increase in species was recorded (Guelinckx et al., 2008). Since 2008 twaite shad is occasionally recorded (Stevens et al., 2009). The anadromous lampreys are easily missed with fyke nets, but they are caught in summer at the lock-weir complex in Gent (Stevens et al., 2009). Upstream spawning grounds for twaite shad and lampreys are absent or inaccessible due to barriers (e.g. sluices, dams,…) (Stevens et al., 2009).

Mesohaline zone

This is the area where estuarine fish complete their life cycle and where fish from the upper and lower estuary seek refuge and food. Especially

mudflats provide food for juveniles (HIDDINK & JAGER, 2002; STEVENS, 2006). As such we find representatives from all estuarine use functional groups. VAN DAMME et al. (1994) presented a checklist of 23 fish species for the mesohaline zone of the Zeeschelde belonging to five ecological guilds: marine migrants (2), diadromous species (3), estuarine species (9), marine stragglers (3) and freshwater species (6). Compared to the checklists of DE SELYS-LONGCHAMPS (38 sp., 1842) and POLL (40 sp., 1945, 1947) more than 15 species had disappeared from the lower Zeeschelde in 1994. The anadromous fishes recorded by DE SELYS-LONGCHAMPS (1842): sea lamprey (*Petromyzon marinus*), allis shad (*Alosa alosa*), twaite shad (*Alosa fallax*), sturgeon (*Acipenser sturio*), shelly (*Coregonus lavaretus*) and the Atlantic salmon (*Salmo salar*) had all, except for twaite shad, already disappeared in 1945 (Poll, 1945). In 1991 the river lamprey was the only anadromous species persisting in the lower Zeeschelde (VAN DAMME et al., 1994). The status of anadromous fish populations remained problematic until recently (MAES et al., 1998, 1999). Overall ten diadromous species have been recorded, some abundantly (eel, smelt, thinlip mullet) but others are rare (river lamprey and sea trout). Species richness shows year by year variations, but a dominance of marine migrants was always observed. From the guild distribution (Fig. 10) we consider the mesohaline zone as an important habitat for most estuarine species, diadromous species and marine migrants. The vast majority of species recorded consisted of juveniles. When combining all our survey results (1995-2008) the guild distribution shows similarities with other European estuaries, e.g. the Elbe (ELLIOTT & DEWAILLY, 1995) and the Gironde (LOBRY et al., 2003). CABRAL et al. (2001) observed a dominance of marine migrants, marine stragglers and estuarine species in the mesohaline zone of the Tagus and SELLESLAGH et al. (2009) observed a dominance of marine migrants and estuarine species in three eastern English Channel macrotidal estuaries (Canche, Authie and Somme). The same authors found also a dominance of marine migrants and estuarine

species in 15 other French estuaries, whereby the freshwater group showed the highest variation ranging from 0 to 37% of species richness.

No overall seasonal effect was observed although for some species a seasonal pattern was present. Sprat and herring are known to be winter migrants (MAES et al., 1998) however; herring is now more abundant in autumn compared to a previous winter. The gradual increase in densities of flounder could be an indication of global warming as described by THIEL et al. (2003). However, the seasonal pattern of this species is complex and not only influenced by temperature. There is an effect of inter-annual variations in recruitment (THIEL & POTTER, 2001) and the availability and abundance of food can also disrupt a seasonal pattern. Observed seasonal patterns can be the result of behavioural responses to changes in predation risk and are probably linked to a size-related behaviour (MAES et al., 1998). The main predators in the mesohaline zone are freshwater species, e.g. pike-perch and perch. Other predators are rarely caught, e.g. juvenile seabass, twaite shad and smelt are occasionally passing through. Large numbers of species enter the estuary to avoid predation (PIHL et al., 2002) and remain there for a short or longer period depending on water quality and food availability. Turbidity may be a driving force for fish migration into the estuary as those fish are attracted by the plume in the sea (MAES et al., 1998). We embrace the hypothesis mentioned by several authors that although some species can be considered as estuarine dependent, a large number of the individuals concerned use the mesohaline zone of an estuary on a facultative or opportunistic basis (POWER & ATRILL, 2003; MAES et al., 2004; GUELINCKX, 2008). Indeed several fish species show variable migration patterns that could be the result of habitat selection (MORRIS, 2003).

We are aware that the fish assemblages in the different salinity zones are also affected by physical habitat characteristics. Supralitoral zones (tidal marshes and flood systems) are most susceptible to human pressure. The loss of mudflats

(dyke reinforcements) combined with dense ship transport and a very dynamic tide, enhance the erosion of tidal marshes (Van Braeckel et al., 2006). These mudflats and marshes are important for fish since they serve as feeding areas and shelter for many species (Cattrijsse & Hampel, 2006; Stevens, 2006). McLusky et al. (1992) commented on the historic loss of inter-tidal habitat and saltmarshes and estimated that the fish population in the Forth estuary was reduced by 66% as a consequence of those losses. The intertidal creek habitat accommodates juvenile fishes during the day, while larger specimens visit the creek by night, resulting in a reduction of space and energy competition (Shenker & Dean, 1979). Colclough et al. (2005) demonstrated that the restored inter-tidal saltmarshes in the Thames and Blackwater estuaries were utilised extensively by juvenile fishes, and species preferences for particular microhabitats were even observed. A decrease of habitat diversity in the freshwater zone is also reflected by impoverished fish diversity (e.g. Jansen et al., 2000; Sindilariu et al., 2006). There are clear differences in juvenile responses to environmental heterogeneity (Grenouillet et al., 2000). This has a direct effect on species richness (Belliard et al., 1999; Schiemer, 2000) and may affect the functional structure of the fish community. Juvenile fish will benefit from structured habitats and avoid substrates lacking any suitable shelter. The creation of shallow intertidal habitats will therefore enhance the restoration of the fish community as these new habitats can be used as nursery and spawning places, shelter and resting areas, as well as feeding grounds (Simoens et al., 2007).

CONCLUSIONS

The fish richness increased over the years 1991 to 2007 in the different salinity zones of the Zeeschelde. A similar observation has been recorded in many of the industrialised countries because of restoration and conservation efforts (Lotze et al., 2006). The gradual increase in oxygen concentration in the different salinity zones of the Zeeschelde estuary seems to have a positive impact on the species richness and confirms the model for diadromous fishes developed by Maes et al. (2007, 2008). A longitudinal shift in fish assemblages, numbers and species richness is mainly explained by the salinity gradient. This allowed us to define estuarine zones for different estuarine fish guilds. However, present fish communities do not reflect the assemblages recorded a century ago. The estuary and its tidal tributaries have been heavily influenced by anthropogenic pressures such as land claim, harbour expansion, dredging activities, embankments and urbanisation (Van Braeckel et al., 2006). The restoration of a natural sustainable fish assemblage will be enhanced by the creation of floodplains as spawning and nursery areas. Protection of the tidal marshes in all zones should be implemented in order to reduce further loss of habitat. Seasonal patterns are complex, which is due in part to a suite of opportunistic behaviour and partly because of external natural variation and human impacts. The most abundant species in the estuary are species that are tolerant of poor water quality. Some species are restricted to one zone while others frequent the whole estuary. Flounder and eel are the only diadromous species found in all zones. Freshwater eurytopic species with a high tolerance to harsh conditions are also present in all surveyed zones. Our results add to the information needed to understand estuarine dependence of fishes.

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REFERENCES

Andrews MJ & Rickard DC (1980). Rehabilitation of the inner Thames estuary. Marine Pollution Bulletin, 11: 327-332.

Araújo FG, Bailey RG & Williams WP (1999). Spatial and temporal variations in fish populations in the upper Thames estuary. Journal of Fish Biology, 55: 836-853.

Araújo FG, Williams WP & Bailey RG (2000). Fish assemblages as indicators of water quality in the middle Thames estuary, England (1980-1989). Estuaries, 23 (3): 305-317.

Attrill MJ (1998). A rehabilitated estuarine ecosystem: The environment and ecology of the Thames estuary. London Kluwer Academic Publishers. 115-139.

Baeyens W, van Eck B, Lambert C, Wollast R & Goeyens L (1998). General description of the Scheldt estuary. Hydrobiologia, 366: 1-14.

Belliard J, Berrebi dit Thomas R & Monnier D (1999). Fish communities and river alteration in the Seine Basin and nearby coastal streams. Hydrobiologia, 400: 155-166.

Breine JJ., Maes J, Quataert P, Van den Bergh E, Simoens I, Van Thuyne G & Belpaire C (2007). A fish-based assessment tool for the ecological quality of the brackish Schelde estuary in Flanders (Belgium). Hydrobiologia, 575: 141-159.

Buysse D, Coeck J & Maes J (2008). Potential re-establishment of diadromous fish species in the River Scheldt (Belgium). Hydrobiologia, 602: 155-159.

Cabral HN, Costa MJ & Salgado JP (2001). Does the Tagus estuary fish community reflect environmental changes? Climate research, 18: 119-126.

Castillo-Rivera M, Zavala-Hurtado JA & Zárate R (2002). Exploration of spatial and temporal patterns of fish diversity and composition in a tropical estuarine system of Mexico. Reviews in Fish Biology and Fisheries, 12: 167-177.

Cattrijsse A & Hampel H (2006). European intertidal marshes: a review of their habitat functioning and value for aquatic organisms. Marine Ecology Progress Series, 324: 293-307.

Cattrijsse A, Makwaia ES, Dankwa HR, Hamerlynck O & Hemminga MA (1994). Nekton communities of an intertidal creek of a European estuarine brackish marsh. Marine Ecology Progress Series, 109: 195-258.

Colclough S, Fonseca L, Astley T, Thomas K & Watts W (2005). Fish utilisation of managed realignments. Fisheries Management and Ecology, 12: 351-360.

Cuveliers E, Stevens M, Guelinckx J, Ollevier F, Breine J & Belpaire C (2007). Opvolging van het visbestand van de Zeeschelde: resultaten voor 2006. Studierapport in opdracht van het Instituut voor Natuur- en Bosonderzoek. INBO.R.2007.48. 42pp. (in Dutch).

de Selys-Longchamps E (1842). Faune belge, 1re partie, Liège. 310 pp.

Dolbeth M, Martinho F, Viegas I, Cabral H & Pardal MA (2007). Estuarine production of resident and nursery fish species: Conditioning by drought events? Estuarine, Coastal and Shelf Science, 78: 51-60.

Elliott M & Dewailly F (1995). The structure and components of European estuarine fish assemblages. Netherlands Journal of Aquatic Ecology, 29 (3-4): 397-417.

Elliott M, O'Reilly MG & Taylor CJL (1990). The Forth estuary: a nursery and overwintering area for the North Sea fishes. Hydrobiologia, 195: 89-103.

Elliott M, Whitfield AK, Potter IC, Blaber SJM, Cyrus DP, Nordlie FG & Harrison TD (2007). The guild approach to categorizing estuarine fish assemblages: a global review. Fish and Fisheries, 8: 241-268.

FAIRBRIDGE R (1980). The estuary, its definition and geodynamic cycle. In: OLAUSSEN E & CATO I (eds). Chemistry and biochemistry of estuaries. Wiley, NY. 1-35.

FRANCO A, ELLIOTT M, FRANZOI P & TORRICELLI P (2008). Life strategies of fishes in European estuaries: the functional guild approach. Marine Ecology Progress Series, 354: 219-228.

GRENOUILLET G, PONT D & OLIVIER JM (2000). Habitat occupancy patterns of juvenile fishes in a large lowland river: interactions with macrophytes. Archives für Hydrobiologie, 149: 307-326.

GUELINCKX J (2008). Estuarine habitat use by a goby species: a geochemical approach. Proefschrift voorgedragen tot het behalen van het doctoraat in de wetenschappen, Katholieke Universiteit Leuven. 163 pp.

GUELINCKX J, CUVELIERS E, STEVENS M, OLLEVIER F, BREINE J & BELPAIRE C (2008). Opvolging van het visbestand van de Zeeschelde: resultaten voor 2007. Studierapport in opdracht van het Instituut voor Natuur- en Bosonderzoek. INBO.R.2008.39. 47 pp. (in Dutch).

HENDERSON PA (1988). The structure of the estuarine fish communities. Journal of Fish Biology, 33: 223-225.

HENDERSON PA (1989). On the structure of the inshore fish community in England and Wales. Journal Marine Biology Association UK, 69: 145-163.

HIDDINK JG & JAGER Z (2002). Abundance and reproduction of Nilsson's pipefish on tidal flats. Journal of Fish Biology, 61: 125-137.

JANSEN W, BÖHMER J, KAPPUS B, BEITER T, BREITINGER B & HOCK C (2000). Benthic invertebrate and fish communities as indicators of morphological integrity in the Enz River (south-west Germany). Hydrobiologia, 422/423: 331-342.

JOVANOVIC B, LONGMORE C, O'LEARY Á & MARIANI S (2007). Fish community structure and distribution in a macro-tidal inshore habitat in the Irish Sea. Estuarine, Coastal and Shelf Science, 75: 135-142.

LOBRY J, MOURAND L, ROCHARD E & ELIE P (2003). Structure of the Gironde estuarine fish assemblages: a comparison of European estuaries perspective. Aquatic Living Resources, 16: 47-58.

LOTZE HK, LENIHAN HS, BOURQUE BJ, BRADBURY RH, COOKE RG, KAY MC, KIDWELL SM, KIRBY MX, PETERSONS CH & JACKSON JBC (2006). Depletion, degradation, and recovery potential of estuaries and coastal seas. Science, 312: 1806-1809.

MAES J, STEVENS M & BREINE J (2007). Modelling the migration opportunities of diadromous fish species along a gradient of dissolved oxygen concentration in a European tidal watershed. Estuarine Coastal and Shelf Science, 75: 151-162.

MAES J, STEVENS M & BREINE J (2008). Poor water quality constrains the distribution and movements of twaite shad *Alosa fallax fallax* (Lacépède, 1803) in the watershed of river Scheldt. Hydrobiologia, 602: 129-143.

MAES J, STEVENS M & OLLEVIER F (2004). The composition and community structure of the ichthyofauna of the upper Scheldt estuary: synthesis of a 10-year data collection (1991-2001). Journal of Applied Ichthyology, 20: 1-8.

MAES J, TAILLIEU A, VAN DAMME PA, COTTENIE K & OLLEVIER F (1998). Seasonal patterns in the fish and crustacean community of a turbid temperate estuary (Zeeschelde estuary, Belgium). Estuarine, Coastal and Shelf Science, 47: 143-151.

MAES J, TAILLIEU A, VAN DAMME PA & OLLEVIER F (1997). The composition of the fish and crustacean community of the Zeeschelde estuary (Belgium). Belgian Journal of Zoology, 127: 47-55.

MAES J, PAS J, TAILLIEU A, VAN DAMME PA & OLLEVIER F (1999). Diel changes in the vertical distribution of juvenile fish in the Zeeschelde estuary. Journal of Fish Biology, 54: 1329-1333.

MANOLOKOS E, VIRANI H & NOVOTNY V (2007). Extracting knowledge on the links between the water body stressors and biotic integrity. Water Research, 41: 4041-4050.

MARIS T, COX T, VAN DAMME S & MEIRE P (2008). Onderzoek naar de gevolgen van het Sigmaplan, baggeractiviteiten en havenuitbreiding in de Zeeschelde op het milieu. Geïntegreerd eindverslag van het onderzoek verricht in 2007-2008. R08-166. Universiteit Antwerpen, 223 pp. (in Dutch).

MARSHALL S & ELLIOTT M (1998). Environmental influences on the fish assemblage of the Humber estuary. Estuarine, Coastal and Shelf Science, 46: 175-184.

MCLUSKY DC, BRYANT DM & ELLIOTT M (1992). The impact of land-claim on macrobenthos, fish and shorebirds on the Forth estuary, eastern Scotland. Aquatic Conservation: Marine and Freshwater Ecosystems, 2:211-222.

McLusky DS & Elliott M (2004). The estuarine ecosystem; ecology threats and Management. Oxford University press NY. 214 pp.

Ministerie van Volksgezondheid en Leefmilieu (1987). "Koninklijk besluit van 4 november 1987 houdende vaststelling van de basiskwaliteitsnormen voor water en van het openbaar hydrografisch net en tot aanpassing van het koninklijk besluit van 3 augustus 1976 houdende algemeen reglement voor het lozen van afvalwater in de gewone oppervlaktewateren, in de openbare riolen en in de kunstmatige afvoerwegen voor regenwater", BS van 21.11.87.

Morris DW (2003). Toward an ecological synthesis: a case for habitat selection. Oecologia, 136: 1-13.

Odum WE, Rosas LP & McIvor CC (1988). A comparison of fish and invertebrate community composition in tidal freshwater and oligohaline marsh systems. In: Hook DD, McKee jr. WH, Smith HK, Gregory J, Burrell jr. VG, DeVoe MR, Sojka RE, Gilbert S, Banks R, Stolzy LH, Brooks C, Matthews TD & Shear TH (eds). The ecology and management of wetlands. Timber Press, Portland Oregon. 561-569.

Oksanen J, Kindt R, Legendre P, O'Hara RB, Henry M & Stevens H (2006). Vegan: community ecology package. R package version 1.8-7. (hhttp://cran.r-project.org).

Pihl L, Cattrijsse A, Codling I, Mathieson S, McLusky DS & Roberts C (2002). Habitat use by fishes in estuaries and other brackish areas. In: Elliott M & Hemingway KL (Editors). Fishes in estuaries, Blackwell Science, London. 636 pp. 10-53.

Poll M (1945). Contribution à la connaissance de la faune ichthyologique du Bas-Escaut. Mededeelingen van het Koninklijk Natuurhistorisch Museum van België. 21: 32 pp. (in French).

Poll M (1947). Faune de Belgique: Poissons marins. Musée Royal d'Histoire Naturelle de Belgique. 452 pp. (in French).

Potter IC, Claridge PN, Hyndes GA & Clarke KR (1997). Seasonal, annual and regional variations in ichthyofaunal composition in the inner Severn Estuary and inner Bristol Channel. Journal of the Marine Biological Association of the United Kingdom, 77: 507-525.

Power M & Attrill MJ (2003). Long-term trends in the estuarine abundance of Nilsson's pipefish (Syngnathus rostellatus Nilsson). Estuarine, Coastal and Shelf Science, 57: 325-333.

Rozas LP & Hackney CT (1983). The importance of oligohaline estuarine wetland habitats to fisheries resources. Wetlands, 3: 77-89.

Schiemer F (2000). Fish as indicator for the assessment of the ecological integrity of large rivers. Hydrobiologia, 422/423: 271-278.

Selleslagh J & Amara R (2008). Environmental factors structuring fish composition and assemblages in a small macrotidal estuary (eastern English Channel). Estuarine, Coastal and Shelf Science, 79: 507-517.

Selleslagh J, Amara R, Laffargue P, Lesourd S & Lepage M (2009). Fish composition and assemblage structure in three Eastern English Channel macrotidal estuaries: A comparison with other French estuaries. Estuarine, Coastal and Shelf Science, 81: 149-159.

Shenker JM & Dean JM (1979). Larval and juvenile fishes in saltmarshes. Estuaries, 2: 154-163.

Simoens I, Breine J, Van Liefferinge C, Stevens M & Belpaire C (2007). Het belang van het Lippenbroek als habitat voor vissen in de Zeeschelde. In Congres Watersysteemkennis 2006-2007. Mogelijkheden voor ecologisch herstel van watersystemen. Water, 30: 68-71 (in Dutch).

Sindilariu P-D, Freyhof J & Wolter C (2006). Habitat use of juvenile fish in the lower Danube and the Danube Delta: implications for ecotone connectivity. Hydrobiologia, 571: 51-61.

Soetaert K, Middelburg JJ, Heip C, Meire P, Van Damme S & Maris T (2006). Long-term change in dissolved inorganic nutrients in the heterotrophic Scheldt estuary (Belgium, The Netherlands). Limnology and Oceanography, 51: 409-423.

Stevens M (2006). Intertidal and basin-wide habitat use of fishes in the Scheldt estuary. Proefschrift voorgedragen tot het behalen van het doctoraat in de wetenschappen, 2006. K.U. Leuven. 150 pp.

Stevens M, Maes J, Guelinckx J, Ollevier F, Breine J & Belpaire C (2006). Opvolging van het visbestand van de Zeeschelde: resultaten voor 2005. Studierapport in opdracht van het Instituut voor Natuur- en Bosonderzoek. 33 pp. (in Dutch).

Stevens M, Van den Neucker T, Mouton A, Buysse D, Martens S, Baeyens R, Jacobs Y, Gelaude E & Coeck J 2009. Onderzoek naar de trekvissoorten in het stroomgebied van de

Schelde. Rapporten van het Instituut voor Natuur- en Bosonderzoek. INBO.R.2009.9. Brussel. 188 pp. (English abstract)

THIEL R, CABRAL H & COSTA MJ (2003). Composition, temporal changes and ecological guild classification of the ichthyofaunas of large European estuaries - a comparison between the Tagus (Portugal) and the Elbe (Germany). Journal of Applied Ichthyology, 19 (5): 330-342.

THIEL R & POTTER IC (2001). The ichthyofaunal composition of the Elbe estuary: An analysis in space and time. Marine Biology, 138 (3): 603-616.

TURNPENNY A, COUGHLAN J & LINEY K (2006). Review of temperature and dissolved oxygen effects on fish in transitional waters. Jacobs Babtie. 81 pp.

VAN BRAECKEL A, PIESSCHAERT F & VAN DEN BERGH E (2006) Historical analysis of the Zeeschelde and tidal tributaries. 19th century till present. INBO.R.2006.29. 178 pp. (in Dutch, with English summary).

VAN DAMME P, HOSTENS C & OLLEVIER F (1994). Fish species of the lower Zeeschelde (Belgium): a comparison with historical checklists. Belgian Journal of Zoology, 124 (2): 93-103.

VAN DAMME S, STRUYF E, MARIS T, YSEBAERT T, DEHAIRS F, TACKX M, HEIP C & MEIRE P (2005). Spatial and temporal patterns of water quality along the estuarine gradient of the Scheldt estuary (Belgium and the Netherlands): results of an integrated monitoring approach. Hydrobiologia, 540: 29-45.

VAN DEN BERGH E, VAN DAMME S, GRAVELAND J, DE JONG D, BATEN I & MEIRE P (2005). Ecological rehabilitation of the Schelde estuary (The Netherlands-Belgium; northwest Europe): linking ecology, safety against floods, and accessibility for port development. Restoration Ecology, 13 (1): 204-214.

VAN ECK GTM, DE PAUW N, VAN LANGENBERGH M & VERREET G (1991). Emissies, gehalten, gedrag en effecten van (micro)verontreinigingen in het stroomgebied van de Schelde en Schelde-estuarium. Water, 60: 164-181 (in Dutch).

VAN THUYNE G & BREINE J (2008). Visbestandopnames in Vlaamse beken en rivieren afgevist in het kader van het 'Meetnet Zoetwatervis' 2007. Rapporten van het Instituut voor Natuur- en Bosonderzoek. INBO.R.2008.21. 154 pp. (English abstract)

VENICE SYSTEM, 1959. The final resolution of the symposium on the classification of brackish waters. Archo Oceanography Limnology, 11 (suppl): 243-248.

VETEMAA M, ESCHBAUM R, VERLIIN A, ALBERT A, EERO M, LILLEMAEGI R, PIHLAK M & SAAT T (2006). Annual and seasonal dynamics of fish in the brackish-water Matsalu Bay, Estonia. Ecology of Freshwater Fish, 15 (2): 211-220.

VLAREM II (1995). Besluit van de Vlaamse regering van 1 juni 1995 houdende algemene en sectorale bepalingen inzake milieuhygiëne 388 pp. (in Dutch).

VRIELYNCK S, BELPAIRE C, STABEL A, BREINE JJ & QUATAERT P (2003). De visbestanden in Vlaanderen anno 1840-1950. Een historische schets van de referentietoestand van onze waterlopen aan de hand van de visstand, ingevoerd in een databank en vergeleken met de actuele toestand. Instituut voor Bosbouw en Wildbeheer en Afdeling Water (AMINAL), Groenendaal. 271 pp. (in Dutch).

WHEELER AC (1969). Fish-life and pollution in the lower Thames: A review and preliminary report. Biological Conservancy, 2: 25-30.

WHEELER AC (1979). The tidal Thames: The history of a river and its fishes. London Routledge & Kegan Paul.

ANNEX: TABLE A

Catch frequency for each fish species, expressed as percentage, for the different salinity zones of the Zeeschelde between 1995 and 2008. M: Mesohaline zone; O: Oligohaline zone and F: freshwater zone with the number of monthly catches for each zone between brackets. The estuarine use guild is given (FRANCO et al., 2008). D: Diadromous species; E: Estuarine species; FW: Freshwater species; MS: Marine stragglers (adventitious visitors); MM: Marine migrants (seasonal or juvenile migrant).

Scientific name	Abbreviation	Guild	Common name	M (90)	O (52)	F (49)
Abramis brama (Linnaeus, 1758)	A.bra.	FW	Bream	46.7	71.2	63.3
Acipenser baeri (Brandt, 1869)	A.bae.	D	Siberian sturgeon	6.7	0.0	0.0
Agonus cataphractus (Linnaeus, 1758)	A.cat.	E	Hook-nose	1.1	0.0	0.0
Alburnus alburnus (Linnaeus, 1758)	A.alb.	FW	Bleak	2.2	5.8	16.3
Alosa fallax (Lacepède, 1803)	A.fal.	D	Twaite shad	44.4	9.6	2.0
Ammodytes tobianus (Linnaeus, 1758)	A.tob.	MS	Sand-eel	3.3	0.0	0.0
Anguilla anguilla (Linnaeus, 1758)	A.ang.	D	Eel	85.6	88.5	85.7
Aphia minuta (Linnaeus, 1758)	A.min.	E	Transparent goby	1.1	0.0	0.0
Atherina presbyter (Risso, 1810)	A.pre.	MM	Sand smelt	21.1	1.9	0.0
Blicca bjoerkna (Linnaeus, 1758)	B.bjo.	FW	White bream	35.6	69.2	79.6
Carassius carassius (Linnaeus, 1758)	C.carr.	FW	Crucian carp	0.0	9.6	2.0
Carrasius gibelio (Bloch, 1782)	C.gib.	FW	Prussian carp	38.9	96.2	81.6
Chelidonichthys lucernus (Linnaeus, 1758)	C.luc.	MM	Tub gurnard	18.9	1.9	0.0
Chelon labrosus (Risso, 1827)	C.lab.	MM	Thick-lipped mullet	0.0	1.9	0.0
Ciliata mustela (Linnaeus, 1758)	C.mus.	MM	Fivebeard rockling	15.6	0.0	0.0
Clupea harengus (Linnaeus, 1758)	C.har.	MM	Herring	88.9	50.0	2.0
Cottus gobio (Linnaeus, 1758)	C.gob.	FW	Bullhead	1.1	3.9	4.1
Cyclopterus lumpus (Linnaeus, 1758)	C.lum.	MM	Lumpsucker	1.1	0.0	0.0
Cyprinus carpio (Linnaeus, 1758)	C.car.	FW	Carp	18.9	61.5	73.5
Dicentrarchus labrax (Linnaeus, 1758)	D.lab.	MM	Seabass	84.4	25.0	2.0
Echiichthys vipera (Cuvier, 1829)	E.vip.	MS	Lesser weever	2.2	3.9	0.0
Engraulis encrasicolus (Linnaeus, 1758)	E.enc.	MM	Anchovy	1.1	0.0	0.0
Esox lucius (Linnaeus, 1758)	E.luc.	FW	Pike	11.1	11.5	14.3
Gadus morhua (Linnaeus, 1758)	G.mor.	MM	Cod	25.6	1.9	0.0
Gasterosteus aculeatus (Linnaeus, 1758)	G.acu.	FW	Three-spined stickleback	46.7	61.5	73.5
Gobio gobio (Linnaeus, 1758)	G.gob.	FW	Gudgeon	0.0	7.7	0.0
Gymnocephalus cernuus (Linnaeus, 1758)	G.cer.	FW	Ruffe	35.6	46.2	59.2
Lampetra fluviatilis (Linnaeus, 1758)	L.flu.	D	River lamprey	5.6	11.5	16.3
Lepomis gibbosus (Linnaeus, 1758)	L.gib.	FW	Pumpkinseed	6.7	26.9	28.6
Leucaspius delineatus (Heckel, 1843)	L.del.	FW	Belica	0.0	7.7	6.1
Leuciscus cephalus (Linnaeus, 1758)	L.cep.	FW	Chub	0.0	0.0	2.0
Leuciscus idus (Linnaeus, 1758)	L.ide.	FW	Ide	7.8	32.7	22.5
Limanda limanda (Linnaeus, 1758)	L.lim.	MM	Dab	6.7	0.0	0.0
Liparis liparis (Linnaeus, 1760)	L.lip.	E	Sea snail	2.2	0.0	0.0
Liza ramada (Risso, 1827)	L.ram.	D	Thinlip mullet	42.2	26.9	8.2
Merlangius merlangus (Linnaeus, 1758)	M.mer.	MM	Whiting	20.0	1.9	0.0
Mullus surmuletus (Linnaeus, 1758)	M.sur.	MS	Red mullet	2.2	0.0	0.0
Myoxocephalus scorpius (Linnaeus, 1758)	M.sco.	E	Bull rout	11.1	0.0	0.0
Oncorhynchus mykiss (Walbaum, 1792)	O.myk.	FW	Rainbow trout	1.1	0.0	0.0

Scientific name	Abbreviation	Guild	Common name	M(90)	O(52)	F(49)
Osmerus eperlanus (Linnaeus, 1758)	O.epe.	D	Smelt	70.0	32.7	8.2
Perca fluviatilis (Linnaeus, 1758)	P.flu.	FW	Perch	61.1	86.5	71.4
Platichthys flesus (Linnaeus, 1758)	P.fle.	D	Flounder	98.9	61.5	61.2
Pleuronectes platessa (Linnaeus, 1758)	P.pla.	MM	Plaice	36.7	0.0	0.0
Pomatoschistus lozanoi (de Buen, 1923)	P.loz.	MS	Lozano's goby	1.1	1.9	0.0
Pomatoschistus microps (Krøyer, 1838)	P.mic.	E	Common goby	30.0	48.1	20.4
Pomatoschistus minutus (Pallas, 1770)	P.min.	E	Sand goby	54.4	40.4	12.2
Pomatoschistus sp.	P.spe.	E	Gobidae sp.	6.7	0.0	0.0
Psetta maxima (Linnaeus, 1758)	P.max.	MM	Turbot	1.1	0.0	0.0
Pseudorasbora parva (Temminck & Schlegel, 1842)	P.par.	FW	Stone morocco	13.3	59.6	77.6
Pungitius pungitius (Linnaeus, 1758)	P.pun.	FW	Nine spine stickleback	12.2	34.6	20.4
Rhodeus sericeus (Bloch, 1782)	R.ser.	FW	Bitterling	13.3	44.2	51.0
Rutilus rutilus (Linnaeus, 1758)	R.rut.	FW	Roach	74.4	100.0	93.9
Salmo salar (Linnaeus, 1758)	S.sal,	D	Salmon	2.2	0.0	0.0
Salmo trutta (Linnaeus, 1758)	S.tru.	D	Sea trout	8.9	0.0	0.0
Sander lucioperca (Linnaeus, 1758)	S.luc.	FW	Pike-perch	77.8	57.7	61.2
Scardinius erythrophthalmus (Linnaeus, 1758)	S.ery.	FW	Rudd	36.7	63.5	81.6
Scophthalmus rhombus (Linnaeus, 1758)	S.rho.	MM	Brill	7.8	0.0	0.0
Silurus glanis (Linnaeus, 1758)	S.gla.	FW	Wels catfish	0.0	13.5	12.2
Solea solea (Linnaeus, 1758)	S.sol.	MM	Sole	84.4	13.5	0.0
Sprattus sprattus (Linnaeus, 1758)	S.spr.	MM	Sprat	6.7	0.0	0.0
Syngnathus acus (Linnaeus, 1758)	S.acu.	E	Greater pipefish	17.8	9.6	0.0
Syngnathus rostellatus (Nilsson, 1855)	S.ros.	E	Nilsson's pipefish	1.1	0.0	0.0
Tinca tinca (Linnaeus, 1758)	T.tin.	FW	Tench	6.7	5.8	8.2
Trachurus trachurus (Linnaeus, 1758)	T.tra.	MS	Scad	6.7	0.0	0.0
Trisopterus luscus (Linnaeus, 1758)	T.lus.	MM	Pouting	34.4	7.7	0.0
Zoarces viviparus (Linnaeus, 1758)	Z.viv.	E	Viviparous blenny	6.7	0.0	0.0

Effects of foraging mode and group pattern on vigilance behavior in water birds: a case study of mallard and black-winged stilt

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ABSTRACT. Vigilance behavior is affected by many factors including foraging mode and group pattern which we attempted to understand through a case study of mallard *Anas platyrhynchos* and black-winged stilt *Himantopus himantopus* in the Hengshui Lake National Nature Reserve, China. Mallard, a swimming bird, forages in irregular and loose circular groups in open water and its foraging mode (with its head underwater looking for food) does not allow for simultaneous monitoring of potential threats above the water surface, which is why its foraging is called "blind period of foraging". In contrast, black-winged stilt is a wading bird and forages along riversides in long linear foraging groups. Its long beak, neck and legs enable it to scan around while foraging without any obviously blind period. We used the focal sampling method and measured vigilance behavior by the time spent vigilant, vigilance frequency, average duration of vigilance, alert distance and flee distance for the two species. We predicted higher levels of vigilance and longer alert and flee distance in mallard with long blind periods of foraging. Our results indicated significant differences ($p<0.05$) in vigilance behavior between the two species. The effects of group size and gender on vigilance were also checked but showed no significant differences. Higher-level vigilance in mallard was mainly due to its foraging mode with long 'blind periods' and circular foraging group pattern. We suggested that buffer zones be established in ecotourism regions in order to protect birds from human interference.

KEYWORDS: alert distance, *Anas platyrhynchos*, blind period of foraging, flee distance, *Himantopus himantopus*

INTRODUCTION

Vigilance behavior plays an important role in ensuring the fitness of animals and their offspring (TREVES, 2000; BEAUCHAMP, 2001). If animals monitor their surroundings and detect threats earlier, they may have a better chance to survive. However, high-level vigilance is often at the expense of other activities crucial for their maintenance (INGER et al., 2006). Therefore, animals should balance the tradeoff between vigilance and other activities, especially feeding and resting (BACHMAN, 1993; ILLIUS & FITZGIBBON, 1994; GAUTHIER-CLERC et al., 2000; INGER et al., 2006; BENHAIEM et al., 2008).

Many factors affect vigilance pattern and how animals balance the tradeoff (GAUTHIER-CLERC et al., 2000; BOYSEN et al., 2001; ROLANDO et al., 2001; CHILDRESS & LUNG, 2003; LAZARUS, 2003; LI & JIANG, 2008). Investigation of the effects of foraging posture (or body posture) has received the attention of a number of researchers (KRAUSE & GODIN, 1996; KABY & LIND, 2003; MAKOWSKA & KRAMER, 2007). Animals with different foraging postures differ in their efficiency of detecting predation risk during feeding known as low-quality vigilance and high-quality vigilance suggested by LIMA & BEDNEKOFF (1999). During foraging, the visual field is, to some extent, obstructed (METCALFE,

1984) resulting in lower-quality vigilance. Compared with foraging posture, more emphasis should be put on foraging mode, which has more obvious influence on vigilance pattern. Group geometry is another important factor influencing vigilance behavior. Individual position and distance-to-neighbor have been found to affect vigilance behavior (LAZARUS, 2003; DIAS, 2006; PROCTOR et al., 2006; FERNÁNDEZ-JURICIC et al., 2007; ÖST et al., 2007). In addition, group pattern (or group shape) is also a potential factor of concern. Birds foraging in linear foraging group are more vigilant and change their heads and body positions more often than do those foraging in a circular group (BEKOFF, 1995; BAHR & BEKOFF, 1999).

Along with percentage time, frequency and duration of vigilance, alert and flee distance are also indices for vigilance level in animals (WALTHER, 1969; LI et al., 2007) and reflect tolerance to intrusions (ERWIN, 1989; FERNÁNDEZ-JURICIC et al., 2002). Alert distance was defined as the minimum distance between an animal and the approaching threat when the animal started to be alert toward the threat (COOPER, 2008). Flee distance is referred to as the minimum distance when the animal can not tolerate the approaching threat and flees away (FERNÁNDEZ-JURICIC et al., 2002).

Mallard *Anas platyrhynchos* Linnaeus, 1758 and black-winged stilt *Himantopus himantopus* Linnaeus, 1758 differ in both foraging mode and group pattern, which makes them ideal models for investigating the effects of these two factors on vigilance behavior. Mallard is a swimming bird, that forages in irregular and loose circular groups in open water. During foraging, it keeps its head underwater looking for food. This kind of foraging mode blindfolds its eyes from monitoring potential threats above the water surface, which is why this foraging behavior is called "blind period of foraging". In contrast, black-winged stilt has a long beak, neck and legs, which are all typical characteristics of a wading bird. This species forages in linear foraging groups at the waterfront along the riverside.

The morphological characteristics and foraging behavior enable black-winged stilts to scan the surroundings while foraging without any evident blind period.

In the present study, we hypothesized that foraging mode and group pattern influence vigilance behavior in mallard and black-winged stilt. We predicted that mallard with long "blind period of foraging" would have a higher vigilance level, a longer alert distance and a larger fleeing distance.

MATERIALS AND METHODS

Study site and animals

We conducted our field work in the Hengshui Lake National Nature Reserve (37°31′40″-37°41′56″N, 115°27′50″-115°42′51″E), Hebei Province, China. The climate in the reserve is characterized as a temperate continental monsoon climate. The nature reserve is composed of a mixed landscape with lakes, rivers, swamps, mudflats, meadows, field crops and woodlands. Hengshui Lake is a crucial foraging and resting site for migratory birds. For observation of black-winged stilt, we selected a foraging site along a riverside in the northern part of the reserve. An open lake about 4 000m² was selected to observe the mallard's behavior. The lake is surrounded by wetlands and several ponds, and separated from the selected foraging site of black-winged stilt by a dam. Mallard and black-winged stilt, focal species in our study, were the dominant species at the two sites. Other sympatric birds included spotbill duck *Anas poecilorhyncha* Forster, 1781, little grebe *Tachybaptus ruficollis* Pallas, 1764, white-eyed pochard *Aythya nyroca* Güldenstädt, 1770, common moorhen *Gallinula chloropus* Linnaeus, 1758, common snipe *Gallinago gallinago* Linnaeus, 1758 and long-toed stint *Calidris subminuta* Middendorff, 1853. Predators of mallard or black-winged stilt were rarely seen at the study site. Occasionally, there were human intrusions but the activities of the birds were rarely disturbed.

Behavior observation

We used the focal sampling method (ALTMANN, 1974) to observe and record behaviors of mallard and black-winged stilt from sunrise to sunset during sunny days between April 12 and May 13, 2007. Observations were conducted over all daylight hours equally to avoid the probable effect of time period on vigilance during daytime (ELGAR, 1989; LI & JIANG, 2008). In order to avoid disturbing the normal behavior of the focal individual, we hid behind bushes or reeds about 100m away on the bank and conducted observations of birds with a pair of binoculars (8-16×40) a few minutes after a group of birds entered into the observation area.

We defined a focal group as a flock of individuals of a single species with a visually estimated distance between intra-group members being shorter than ten meters. Due to the difficulties of individual identification, we numbered group members from left to right, randomly selected focal individuals according to their sequence number and kept eyesight focused on the focal individuals through each observation session. This random selection process resulted in focal individuals with random spatial locations in groups, which avoided the effects of distance-to-neighbour and position in group on vigilance behavior (ROLANDO et al., 2001; LAZARUS, 2003; PROCTOR et al., 2006; FERNÁNDEZ-JURICIC et al., 2007). We randomly selected one but never more than two individuals in a single focal group as focal observation to lower the probability of repeated observation. Each focal individual was observed for ten minutes unless it ran out of sight or the focal group composition changed. Observations shorter than ten minutes were discarded.

For each observation, we recorded date, time, location, gender, and group size. When one behavior state occurred, we immediately recorded the behavior and the time. The observation and recording tasks were carried out by the same person. For both mallard and black-winged stilt, we recorded 13 different types of behavior states, which were then categorized into four types: feeding, resting, vigilance and others (see details in Table 1). No juveniles of either species was observed during our field work, thus all the data were of adult birds. During the field work, we adhered to the "Guidelines for the use of animals in research" published in *Animal Behaviour* (1991), and also adhered to the Wildlife Protection Law of People's Republic of China.

TABLE 1

Behavior definition for mallard (*Anas platyrhynchos*) and black-winged stilt (*Himantopus himantopus*).

Behavior Category	Behavior Definition
Feeding	Mallard: drilling its head into deep water or exploring with its beak in shallow water
	Black-winged stilt: searching and foraging along the riverside wetland during a foraging session with its head down
Resting	Standing still on one leg, lying down and preening feathers
Vigilance	Scanning around with head up, alarm calling and flushing
Others	Twitter, flying, bathing, swimming, fighting, and chasing

Alert and flee distance measurement

To measure the alert and flee distance, one observer approached the focal group slowly (always in the same clothes and at approximately the same speed, c. one kilometer per hour) and recorded the focal group size and the distance data measured by a laser range finder (WCJ-2, Leiyuan Electronic Industry Co., Ltd, China, guaranteed range: 30-4,000m, accuracy: ±1m). We defined alert distance as the minimum distance when approximately 50% individuals in the focal group became alert toward the observer, and flee distance as the minimum distance when approximately 50% individuals in the focal group fled away (ERWIN, 1989). When the distance was shorter than the detection range of the range finder, we measured distance with tapelines after focal individuals fled away. We measured the alert and flee distances in mallard and black-winged stilt on two successive days at the same time of day: early morning and late afternoon when birds were most active.

Statistical treatment

We assembled the following data for both mallard and black-winged stilt: time spent vigilant, vigilance frequency, average duration of vigilance, alert distance and flee distance. Time spent vigilant in each observation session was calculated and expressed as percentage of the total time being vigilant to the entire duration of the observation session. Vigilance frequency was defined as the number of vigilance behaviors per minute. Average duration of one vigilant bout was calculated by dividing the time spent vigilant by the corresponding number of vigilance states.

Data were first tested for normality with a one-sample Shapiro-Wilk test. Linear regression analysis was used to test the correlation between
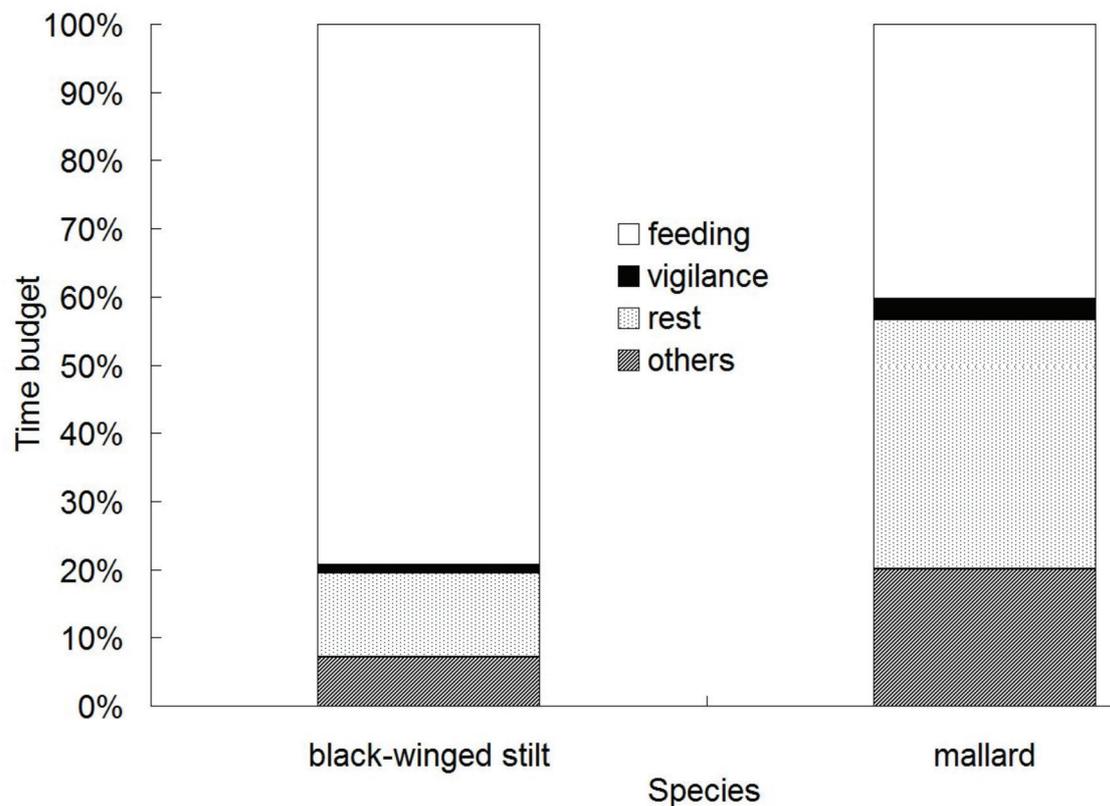

Fig. 1. – Percentage time spent being vigilant in mallard (*Anas platyrhynchos*) and black-winged stilt (*Himantopus himantopus*).
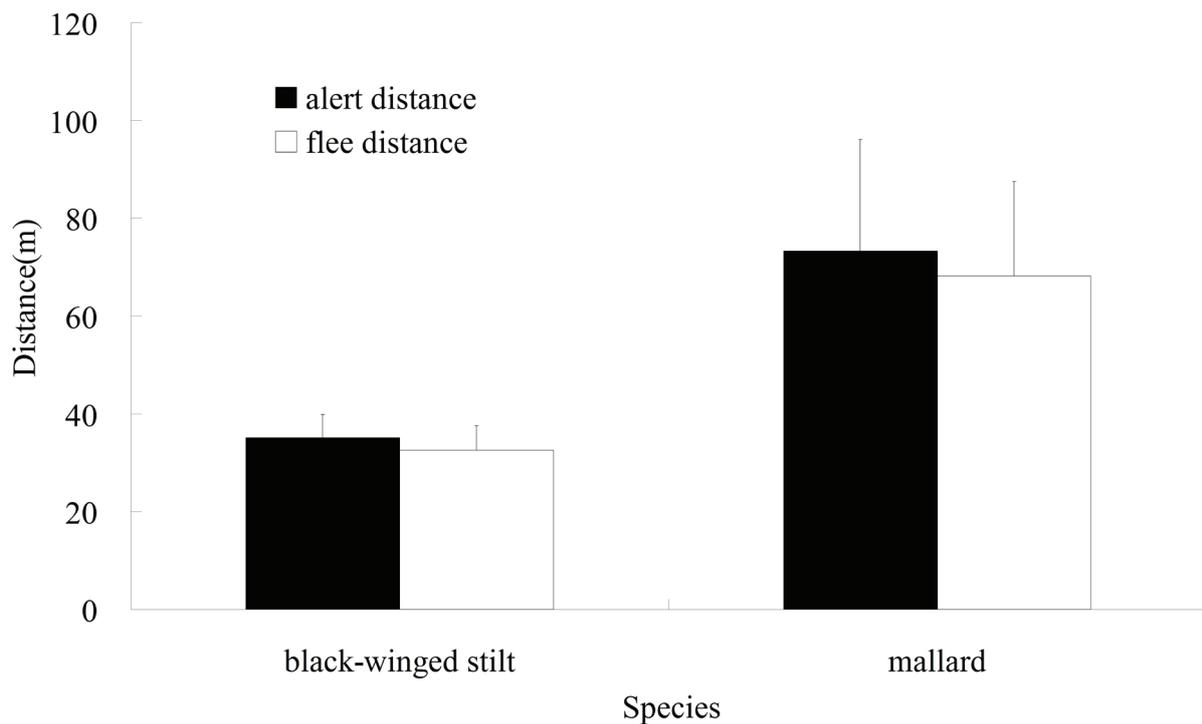
Fig. 2. – Alert and flee distances of mallard (*Anas platyrhynchos*) and black-winged stilt (*Himantopus himantopus*).

alert and flee distance, $\log_{10}$ transformed average duration of vigilance and group size (continuous factor) or gender (discrete factor). Because these tests gave no significant results ($p>0.05$ for all), we removed the effects of gender and group size in the subsequent analyses. The interaction between alert distance and flee distance was also tested using the linear regression analysis. Then we performed the General Linear Model (GLM) to test the effect of the blind period (two level, 1 for black-winged stilt and 2 for mallard) on average duration of vigilance. GLM was also used to test the effect of the blind period on alert and flee distances. Time spent vigilant and vigilance frequency were tested with nonparametric tests because they failed to fit normal distribution even after transformations. Statistical analyses were carried out with SAS 8.1 (Cary, NC, USA). All significant differences were set at $p=0.05$.

RESULTS

In total, we conducted 163 (1,630min) and 72 (720min) focal observations (each observation session lasted ten minutes) after discarding those less than ten minutes for black-winged stilt and mallard, respectively. Twenty-nine sets of alert distance and flee distances were measured for each of the two species. The focal group size of black-winged stilt and mallard ranged from 13 to 53 (37 ± 8, mean ± SE) and from 8 to 33 (17 ± 7), respectively. Alert distance, flee distance and $\log_{10}$ transformed average duration of vigilance were normally distributed ($p>0.05$).

Duration of each vigilant bout averaged 17.9 ± 15.6s ranging from 0 to 65s for black-winged stilt and 23.4 ± 16.0s ranging from 0 to 72s for mallard. The GLM process gave a significant result indicating that average vigilance duration of black-winged stilt was significantly shorter than that of mallard ($F_{1,85}=3.04, p=0.0349<0.05$). Vigilance frequency in each observation session of mallard ranged from 0 to 0.4min^{-1} and averaged 0.06min^{-1}. Percentage of time spent vigilant in the entire duration time of each observation session for mallard ranged from 0 to 48% and

was 2.95% on average, with two-thirds of observations less than the average. Vigilance of black-winged stilt was less frequent, i.e. 0.04min^{-1} on average and ranging from 0 to 0.3min^{-1}. Black-winged stilt spent 1.22% on average of total observation time on vigilance ranging from 0 to 22% with three-quarter observations less than the average (Fig. 1). Nonparametric tests showed that differences in both time spent vigilant and vigilance frequency between the two species were significant ($p=0.0098<0.05$ and $p=0.0276<0.05$, respectively). Black-winged stilt spent 79.3% of the total time feeding while mallard spent 40.2% on this activity ($p<0.0001$). In contrast, mallard spent nearly three times as much time resting as black-winged stilt: 36.6% and 12.3%, respectively ($p<0.0001$).

Alert distance and flee distance of black-winged stilt ranged from 24m to 44m with an average of 35.1 ± 4.8m and from 23m to 43m with an average of 32.6 ± 5.0m, respectively. Mallard had longer alert and flee distances: 73.3 ± 22.8m of alert distance ranging from 27m to 128m and 68.3 ± 19.2m of flee distance ranging from 25m to 103m (Fig. 2). The GLM analysis for the alert and flee distances between the two species also indicated both distances were significantly longer for mallard ($F_{1,\ 57}=78.38$, $p<0.001$ for alert distances and $F_{1,\ 57}=93.51$, $p<0.001$ for flee distances). Differences between alert distance and flee distance ranged from 1m to 13m with an average of 2.52 ± 2.46m for black-winged stilt and from 1m to 33m with an average of 5.21 ± 6.52m for mallard. Flee distance was linearly correlated with alert distance (β=-0.83 ± 0.09, $p<0.001$ and β=-1.14 ± 0.06, $p<0.001$, respectively).

DISCUSSION

Cost of vigilance behavior and blind period of foraging

Vigilance behavior plays a crucial role in maintenance and offspring protection in animals, but it is often costly and conflicts with energy intake and preservation (BACHMAN, 1993; GAUTHIER-CLERC et al., 2000; LAZARUS, 2003; INGER et al., 2006). Almost no prey animals can successfully detect approaching threats in its surroundings while concentrating on foraging or resting (BEAUCHAMP & LIVOREIL, 1997; JONES, 1998). However, vigilance behavior is not completely incompatible with other activities for many animals (LIMA & BEDNEKOFF, 1999). For instance, ungulates are able to chew cud with their heads up scanning around (FRID, 1997; TREVES, 2000). In practice, however, it is often difficult to determine whether the focal animal is vigilant or not while foraging. In a previous study, the view field of dairy cattle was manually restricted so that they could not feed and scan simultaneously (WELP et al., 2004). Obviously this is not feasible when studying wild animals in the field.

Mallard is a dabbling duck that usually feeds by pulling and tearing underwater plants (GUILLEMAIN et al., 2000). When a mallard has a blind period of foraging, it must spend time after each feeding dive to carefully scan its surroundings above water surface. In contrast, black-winged stilt is a typical wading bird with long beak, long neck and long legs. It mainly forages in waterfronts along riversides (ZHANG et al, 2003). These morphological and ecological characteristics enable black-winged stilt to scan its surroundings more conveniently while it forages. Black-winged stilt neglect mild disturbances that are often misjudged as serious risks and given undue attention by mallard. This advantage results from foraging mode. Compared with mallard, black-winged stilt have a reduced cost of vigilance and are able to spend more time engaged in other crucial behaviors such as foraging and resting.

We found that mallard were significantly more vigilant than black-winged stilt. Mallard spent more time ($p=0.0098<0.05$) on vigilance behavior (2.95% of the total time) than did black-winged stilt (1.22%). In addition, mallard scanned more often ($p=0.0276<0.05$) (0.06min^{-1} versus 0.04min^{-1}). The frequent interruption

to foraging sessions could cost extra energy because of the stop and start nature. The process of switching between the two activities was much longer for mallard than black-winged stilt, which easily switched from scanning to feeding with simple changes to their head position.

Furthermore, mallard were forced to stay alert longer after they perceived any approaching threat. This was apparent by the fact that the mean vigilance duration of mallard ($23.4 \pm 16.0s$) was significantly ($F_{1, 85}=3.04$, $p=0.0349<0.05$) longer than that of black-winged stilt ($17.9 \pm 15.6s$). These results revealed that cost of vigilance was not equal among different species. Animals with long blind periods of foraging may allocate more energy in vigilance and the cost of vigilance behavior may be higher than in the case of animals without blind periods. Thus, blind periods of foraging contribute substantially to the higher level of vigilance of mallard.

Group pattern and vigilance behavior

Group geometry is another promising area to be investigated (ARENZ, 2003). Many studies have shown that position in the group (LAZARUS, 2003; PROCTOR et al., 2006) and the distance-to-neighbor (ROLANDO et al., 2001; FERNÁNDEZ-JURICIC et al., 2007) affect vigilance pattern. These findings may be more applicable to species that feed in a circular group such as mallard. Individuals arranged in a circular pattern are closer to each other. This pattern allows individuals to receive signals given by alert neighbors and thus guarantees the rapid transmission of social information (TREVES, 2000; BEKOFF, 2003; FERNÁNDEZ-JURICIC et al., 2007). As for the species foraging in linear arrays such as black-winged stilt, individuals in a group do not have more than two neighbors (BAHR & BEKOFF, 1999). In this case, signal transfer will be frustrated and individuals foraging in this kind of group pattern may rely more on their own detection rather than on associates (TREVES, 2000). BEKOFF (1995) found that, compared with those foraging in a circle, evening grosbeaks *Coccothraustes vespertinus* Cooper 1825 in

a linear array reacted to changes in group size more slowly, showed less coordination in head movements and more variability in all measures. Social information transfer inefficiency in linear group pattern hinders the synchronization of behavior among group members, resulting in unreliability of collective detection (SIROT, 2006).

The two species we studied represent two distinct foraging group patterns (GUILLEMAIN et al., 2000; ZHANG et al, 2003). Comparison between these two species provided some valuable insights that, in fact, group pattern and foraging mode can independently as well as jointly affect vigilance behavior. We found in this study that longer blind periods of foraging increased the vigilance level of mallard, but the group pattern of a circle shape palliated the effect of blind period. However, the mixed effects of foraging mode and group pattern cannot be easily differentiated without robust evidence from laboratory experiments.

Alert distance and flee distance

Trade-off between vigilance behavior and other activities is also reflected by alert and flee distance (WALTHER, 1969; LI et al., 2007). Animals will not immediately flee upon detection of an approaching predator or other threat. Rather they will stare at or listen to the approaching threat for a short time, which is called the detectability period (GUTZWILLER et al., 1998). If they flee at once, they will waste time and energy and may miss a feeding chance. Continuing to forage will save them some energy, but at the risk of being captured by a predator. When the potential prey can no longer tolerate the approaching predator, they will flee. Difference between alert and flee distance reflects the tolerance of prey animals to predation pressure (WANG et al., 2004; HOLMES et al., 2005).

The tolerance to approaching threats differs among species and reflects biological, ecological and even historical characteristics of the species. Like the other aspects of vigilance behavior, alert

and flee distances are affected by many factors, such as seasonal reproductive status (WHITE & BERGER, 2001; WOLFF & VAN HORN, 2003) and nutritional status, habitat type, experience of the specific animal to threats (TREVES, 2003), body size, and time of the day (TAYLOR & KNIGHT, 2003). In the present study, we found that in mallards the longer alert and flee distances coincided with higher vigilance levels. The results were due to differences in foraging mode and group pattern of the two species but not to group size effects (ERWIN, 1989).

Management implications

Animals express higher levels of vigilance where predation risk or intrusion is frequent (CREEL et al., 2008). However, higher vigilance level is at the expense of feeding or resting time (INGER et al., 2006). Efficient and convenient vigilance behavior saves time for other activities resulting in increased fitness. Management measures should be taken to protect animals from intense intrusions and especially from human interference. Analysis of vigilance behavior for animals is one of the necessary steps when formulating efficient management measures. The results of our present study on mallard and black-winged stilt could be generalized for other taxa. Based on our findings, we suggest the establishment of a buffer zone around feeding sites of wild animals. As for the protection of Anatidae and shorebirds in ecotourism regions, we suggest buffer zones being 100m and 50m wide, respectively.

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REFERENCES

ALTMANN J (1974). Observational study of behavior: sampling methods. Behaviour, 49: 227-267.

ARENZ CL (2003). The group size effect on vigilance: many unanswered questions. Behavioural Processes, 63: 123-124.

BACHMAN GC (1993). The effect of body condition on the trade-off between vigilance and foraging in Belding's ground squirrels. Animal Behaviour, 46: 233-244.

BAHR DB & BEKOFF M (1999). Predicting flock vigilance from simple passerine interactions: modelling with cellular automata. Animal Behaviour, 58: 831-839.

BEAUCHAMP G (2001). Should vigilance always decrease with group size? Behavioral Ecology and Sociobiology, 51: 47-52.

BEAUCHAMP G & LIVOREIL B (1997). The effect of group size on vigilance and feeding rate in spice finches (*Lonchura punctulata*). Canadian Journal of Zoology, 75: 1526-1531.

BEKOFF M (1995). Vigilance, flock size, and flock geometry: Information gathering by western evening grosbeaks (Aves, Fringillidae). Ethology, 13: 150-161.

BEKOFF M (2003). Where's the brain? Geometric and cognitive underpinnings of group-size effects on vigilance. Behavioural Processes, 63: 139-140.

BENHAIEM S, DELON M, LOURTET B, CARGNELUTTI B, AULAGNIER S, HEWISON A, MORELLET N & VERHEYDEN H (2008). Hunting increases vigilance levels in roe deer and modifies feeding site selection. Animal Behaviour, 76: 611-618.

BOYSEN AF, LIMA SL & BAKKEN GS (2001). Does the thermal environment influence vigilance behavior in dark-eyed juncos (*Junco hyemalis*)? An approach using standard operative temperature. Jouranl of Thermal Biology, 26: 605-612.

CHILDRESS MJ & LUNG MA (2003). Predation risk, gender and the group size effect: does elk vigilance

depend upon the behaviour of conspecifics? Animal Behaviour, 66: 389-398.

COOPER WE (2008). Visual monitoring of predators: occurrence, cost and benefit for escape. Animal Behaviour, 76: 1365-1372.

CREEL S, WINNIE JR JA, CHRISTIANSON D & LILEY S (2008). Time and space in general models of antipredator response: tests with wolves and elk. Animal Behaviour, 76: 1139-1146.

DIAS RI (2006). Effects of position and flock size on vigilance and foraging behaviour of the scaled dove *Columbina squammata*. Behavioural Processes, 73: 248-252.

ELGAR MA (1989). Predator vigilance and group size in mammals and birds: A critical review of the empirical evidence. Biological Review, 64: 13-33.

ERWIN R (1989). Responses to human intruders by birds nesting in colonies: experimental results and management guidelines. Colonial Waterbirds, 12: 104-108.

FERNÁNDEZ-JURICIC E, BEAUCHAMP G & BASTAIN B (2007). Group-size and distance-to-neighbour effects on feeding and vigilance in brown-headed cowbirds. Animal Behaviour, 73: 771-778.

FERNÁNDEZ-JURICIC E, JIMENEZ M & LUCAS E (2002). Alert distance as an alternative measure of bird tolerance to human disturbance: implications for park design. Environmental Conservation, 28: 263-269.

FRID A (1997). Vigilance by female Dall's sheep: interactions between predation risk factors. Animal Behaviour, 53: 799-808.

GAUTHIER-CLERC M, TAMISIER A & CÉZILLY F (2000). Sleep-vigilance trade-off in gadwall during the winter period. Condor, 102: 307-313.

GUILLEMAIN M, FRITZ H & BLAIS S (2000). Foraging methods can affect patch choice: an experimental study in Mallar (*Anas platyrhynchos*). Behavioural Processes, 50: 123-129.

GUTZWILLER KJ, MARCUM HA, HARVEY HB, ROTH JD & ANDERSON SH (1998). Bird tolerance to human intrusion in Wyoming Montane forests. Condor, 100: 519-527.

HOLMES N, GIESE M & KRIWOKEN LK (2005). Testing the minimum approach distance guidelines for incubating Royal penguins *Eudyptes schlegeli*. Biology Conservation, 126: 339-350.

ILLIUS AW & FITZGIBBON C (1994). Costs of vigilance in foraging ungulates. Animal Behaviour, 47: 481-484.

INGER R, BEARHOP S, ROBINSON JA & RUXTON G (2006). Prey choice affects the trade-off balance between predation and starvation in an avian herbivore. Animal Behaviour, 71: 1335-1341.

JONES ME (1998). The function of vigilance in sympatric marsupial carnivores: the eastern quoll and the Tasmanian devil. Animal Behaviour, 56: 1279-1284.

KABY U & LIND J (2003). What limits predator detection in blue tits (*Parus caeruleus*): posture, task or orientation? Behavioral Ecology and Sociobiology, 54: 534-538.

KRAUSE J & GODIN JGJ (1996). Influence of prey foraging posture on flight behavior and predation risk: predators take advantage of unwary prey. Behavioral Ecology, 7: 264-271.

LAZARUS J (2003). Vigilance and group size: early studies, the edge effect, secondary defences, the double advantage trade-off and the future. Behavioural Processes, 63: 129-131.

LI C, JIANG Z, TANG S & ZENG Y (2007). Evidence of effects of human disturbance on alert response in Pere David's deer (*Elaphurus davidianus*). Zoo Biology, 26: 461-470.

LI Z & JIANG Z (2008). Group size effect on vigilance: Evidence from Tibetan gazelle in Upper Buha River, Qinghai-Tibet Plateau. Behavioural Processes, 78: 25-28.

LIMA SL & BEDNEKOFF PA (1999). Back to the basics of antipredatory vigilance: can nonvigilant animals detect attack? Animal Behaviour, 58: 537-543.

MAKOWSKA I & KRAMER D (2007). Vigilance during food handling in grey squirrels, *Sciurus carolinensis*. Animal Behaviour, 74: 153-158.

METCALFE NB (1984). The effects of habitat on the vigilance of shorebirds: is visibility important? Animal Behaviour, 32: 981-985.

ÖST M, JAATINEN K & STEELE B (2007). Aggressive females seize central positions and show increased vigilance in brood-rearing coalitions of eiders. Animal Behaviour, 73: 239-247.

PROCTOR CJ, BROOM M & RUXTON GD (2006). Antipredator vigilance in birds: Modelling the "edge effect". Mathematical Biosciences, 199: 79-96.

ROLANDO A, CALDONI R, DE SANCTIS A & LAIOLO P (2001). Vigilance and neighbour distance in foraging flocks of red-billed choughs, *Pyrrhocorax pyrrhocorax*. Journal of Zoology, 253: 225-232.

SIROT E (2006). Social information, antipredatory vigilance and flight in bird flocks. Animal Behaviour, 72: 373-382.

TAYLOR AR & KNIGHT RL (2003). Wildlife responses to recreation and associated visitor perceptions. Ecological Application, 13: 951-963.

TREVES A (2000). Theory and method in studies of vigilance and aggregation. Animal Behaviour, 60: 711-722.

TREVES A (2003). Modeling vigilance remains unrealistic. Behavioural Processes, 63: 137-138.

WALTHER FR (1969). Flight behaviour and avoidance of predators in thomson's gazelle (*Gazella Thomsoni* Guenther 1884). Behaviour, 34: 184-220.

WANG Y, CHEN S & DING P (2004). Flush distance: bird tolerance to human intrusion in Hangzhou. Zoological Research, 25: 214-220.

WELP T, RUSHEN J, KRAMER DL, FESTA-BIANCHET M & DE PASSILL AMB (2004). Vigilance as a measure of fear in dairy cattle. Applied Animal Behaviour Science, 87: 1-13.

WHITE KS & BERGER J (2001). Antipredator strategies of Alaskan moose: are maternal trade-offs influenced by offspring activity? Canadian Journal of Zoology, 79: 2055-2062.

WOLFF JO & VAN HORN T (2003). Vigilance and foraging patterns of American elk during the rut in habitats with and without predators. Canadian Journal of Zoology, 81: 266-271.

ZHANG Y, WU Y, WU M, LI D, GAO Q, JIA F, LV S & LIU Z (2003). Observation on breeding habits of *Himantopus himatopus* in Nandagang of Hebei province. Chinese Journal of Zoology. 38: 103-105.

Description of a new genus and two new species of Darwinulidae (Crustacea, Ostracoda), from Christmas Island (Indian Ocean) with some considerations on the morphological evolution of ancient asexuals

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ABSTRACT. Darwinulidae is believed to be one of the few metazoan taxa in which fully asexual reproduction might have persisted for millions of years. Although rare males in a single darwinulid species have recently been found, they may be non-functional atavisms. The representatives of this family are characterized by a slow evolutionary rate, resulting in a conservative morphology in the different lineages over long time frames and across wide geographic ranges. Differences between species and genera, although often based on small details of valve morphology and chaetotaxy, are nevertheless well-recognizable. Five recent genera (*Darwinula*, *Alicenula*, *Vestalenula*, *Penthesilenula* and *Microdarwinula*) and about 35 living species, including also those left in open nomenclature, are included in this family. Previous phylogenetic analyses using both morphological characters and molecular data confirmed that the five genera are good phyletic units.

Here, we report on the results of a study on darwinulid ostracods from Christmas Island (Indian Ocean). The taxonomic investigation led to the description of a new genus and two new species, namely *Isabenula humphreysi* gen. nov. sp. nov. and *Vestalenula* sp. E, this latter being left in open nomenclature, because only one individual was available. A new phylogeny of the family, using 30 morphological characters of 23 described species, is here presented. A key to the genera of Recent Darwinulidae, based on the morphology of valves and soft parts in adult females, is also provided. The discovery of *Isabenula* gen. nov., which shares intermediate morphological features with the genera *Vestalenula* and *Penthesilenula*, opens new opportunities to critically reconsider previous hypotheses on tempo and mode of evolution in the family Darwinulidae and on the phylogenetic relationships between its representatives.

KEY WORDS: Ostracods, ancient asexuals, morphology, taxonomy, evolution, *Isabenula humphreysi* gen. nov. sp. nov., *Vestalenula* sp. E.

INTRODUCTION

A few metazoan lineages, namely bdelloid rotifers, some groups in oribatid mites, some lineages in the stick insects, strains of the brine shrimp *Artemia salina* and darwinulid ostracods, are considered to be putative "ancient asexuals", i.e. animal groups that have been reproducing without males (obligate parthenogenesis) over long geological time scales (see reviews in SCHÖN et al., 2009 and SCHWANDER et al., 2011). They represent evolutionary scandals (JUDSON & NORMARK, 1996; SCHÖN et al., 2009), because they violate ruling evolutionary hypotheses which predict that fully asexual lineages are doomed to early extinction (MAYNARD SMITH, 1978). Nevertheless, in the Darwinulidae, the presence of long-lived asexual genera (e.g., >145 Myr for *Alicenula*) and species (20-25 Myr for the type species *Darwinula stevensoni*) is widely supported by fossil evidence (MARTENS et al., 2003; SCHÖN & MARTENS, 2003) although the asexual status of the family as a whole is still debated (MARTENS & SCHÖN, 2008)

Darwinulidae occur in freshwater (including lentic, lotic, and interstitial environments), but also in (semi-)terrestrial habitats and occasionally in brackish waters. Both ecological specialists and generalists, as well as intermediate forms, exist in this family, and taxa with the broadest ecological tolerance also have the widest geographical distribution (VAN DONINCK et al., 2002, 2003).

Darwinulidae are thought to reproduce apomictically (BUTLIN et al., 1998; SCHÖN et al., 1998, but see also GORELICK, 2003 for an alternative view). At least some species show extremely low rates of molecular and morphological evolution (SCHÖN et al., 2003) and a possible explanation of that could be the presence of highly efficient DNA repair mechanisms in this family (SCHÖN & MARTENS, 1998; 2003).

The existence of a continuous fossil record in the Darwinulidae is of the utmost importance for research on tempo and mode of biological evolution in organisms lacking sexual reproduction (SCHÖN et al., 2009). According to MARTENS et al. (2003), the presence of putative males in fossil Darwinulidae must be rejected for all post-Triassic (c. 208 Myr) records.

Rare males in a single darwinulid living species, *Vestalenula cornelia*, have recently been described by SMITH et al. (2006), but they may be non-functional, as they seem to have rudimentary reproductive organs, namely ovate structures that possibly represent vestigial or remnants of the Zenker organs, and hemipenes lacking a labyrinth and tubular structures differently from other Recent podocopid ostracods (Figs 3h and 3i in SMITH et al., 2006); in addition, no spermatozoa have been observed in either males or sympatric females (SMITH et al., *loc. cit.*).

The presence of rare males does not necessarily affect the status of "ancient asexuals" of the Darwinulidae. Long before the finding of those phenotypic males in living Darwinulidae, ROSSETTI & MARTENS (1996) stated that even if rare males would occur in this family, it is probable that they are atavistic and non-functional, as is known in other asexual taxa such as *Artemia salina* (e.g., BROWNE, 1992; HALKETT et al., 2005). The occurrence of rare males has been reported in other parthenogenetic species of non-marine ostracods (GEIGER et al., 1998; YIN et al., 1999; NAMIOTKO et al., 2005), as well as in other groups of ancient asexuals, such as the oribatid mites (SCHÖN et al., 2008; HEETHOFF et al., 2009). These males may be dysfunctional, especially in older asexual lineages, since mutations are likely to accumulate in pathways necessary for exclusively male functions (BUTLIN et al., 1998). In some arthropod taxa, ancestral genes for male production can be maintained unexpressed over evolutionary time, and the loss of the complex process of sexuality may not be irreversible: for example, there is evidence that the Crotoniidae, a family of oribatid mites, have re-evolved sexuality from parthenogenetic ancestors (DOMES et al., 2007; but see GOLDBERG & ISIC, 2008). The discovery of males in *V. cornelia* does allow the decisive rejection of the presence of male specimens of *Darwinula stevensoni*, figured by BRADY & ROBERTSON (1870) and TURNER (1895), because the morphology of the copulatory appendages in *Vestalenula* clearly indicates that the presumed hemipenes of the *D. stevensoni* male constituted different parts of its anatomy (MARTENS & SCHÖN, 2008).

A worldwide taxonomic revision of the Recent Darwinulidae, based on both valve and soft part characters, has been performed by ROSSETTI & MARTENS (1998). Before that, all Recent darwinulid species were grouped in two genera, i.e. *Darwinula* and *Microdarwinula*. New synonymies were proposed and all the retained species were allocated to five genera, three of which were erected as new (*Alicenula*, *Penthesilenula* and *Vestalenula*, the latter two comprising two species-groups each).

In the subsequent years, new species of Recent Darwinulidae have been described, mainly from interstitial or semi-terrestrial habitats (ROSSETTI & MARTENS, 1999; MARTENS & ROSSETTI, 2002; PINTO et al,. 2003, 2004, 2005; ARTHEAU, 2007; SMITH et al., 2006); these studies brought the total number of species of the family to

c. 35, representing roughly less than 2% of the global specific diversity of freshwater ostracods (MARTENS et al., 2008). Using the set of diagnostic morphological characters suggested by ROSSETTI & MARTENS (1998), all of these new species were easily accommodated into existing species-groups and genera, the latter being confirmed to be good phyletic units through cladistic analysis of both morphological characters and molecular data (MARTENS et al., 2005).

Here, we present new data on darwinulids collected in groundwater habitats of Christmas Island, a small, elevated coral-capped island of the Indian Ocean. The analysis of samples led to the description of a new genus and two new species; other material, consisting either of immature or incomplete specimens, was identified at the generic level. In particular, the new genus shows intermediate morphological features in the limb chaetotaxy between two extant darwinulid genera (*Vestalenula* and *Penthesilenula*), and its valve morphology is not sufficient to separate it from a species-group of the genus *Vestalenula*. This unexpected finding opens intriguing questions on the patterns of morphological evolution and speciation in the Darwinulidae and on the origin and dispersal abilities of these ancient asexuals.

MATERIALS AND METHODS

Study site and sampling locations

Christmas Island (135 km^2) is located in the Indian Ocean, about 350 km south-west of Java and 1200 km west of the Australian continent (Fig. 1), on an oceanic plate fixed to the northern flank of the Australian plate, with which it has drifted north at the same speed (in the Eocene, the northern edge of the Australian plate was about 2000 to 2800 km south of its current location) (LUNT, 2003). The island is the summit of a submarine mountain, formed by volcanic activity that began about 80 million years ago. It is an example of an isolated intra-plate island, resulting from a single volcano breaking off the ridge with part of the sub-ridge magma chamber beneath it (WHITTAKER, 1998). Its surface is mostly characterised by Tertiary carbonate

Fig. 1. – Map of Christmas Island. Its location in the Indian Ocean is shown by a star.

sediments deposited over the core of basaltic rocks. A detailed description of the island geology is provided by Namiotko et al. (2004). Water drainage is mostly underground and karstic (Grimes, 2001). The subterranean system of the islands includes a number of freshwater, marine, anchialine and terrestrial habitats. The island hosts a diversified fauna, with rare and endemic species (Humphreys & Eberhard, 2001; Namiotko et al., 2004).

Ostracods used for this work were provided by W.F. Humphreys (Western Australian Museum) at Christmas Island in 1998 and preserved in alcohol. Four samples out of a total of ten were considered in the present study, i.e. only those containing darwinulids (as complete specimens, empty carapaces or isolated valves). The material was generally scarce and occasionally also damaged. In total, only three complete adult females were recovered. Sampling sites and collection methods are listed below.

- Grants Well, cave # CI-11, approx., cave # CI-64, approx. coordinates 10°28’08”S, 105°39’01”E. Sample BES 5740. Collected on March 28, 1998 by W.F. Humphreys, 24 hour filter on stream flow.
- Henderson’s Spring, cave # CI-64, approx. coordinates 10°29’13”S, 105°40’40”E. Sample BES 5837. Collected on April 6, 1998 by W.F. Humphreys and R. Webb, net over outlet for 24 hours.
- Pump house at Ross Hill Gardens, cave # CI-64,, approx. coordinates 10°29’13”S, 105°40’40”E. Sample BES 5758. Collected on March 3, 1998 by W.F. Humphreys, outflow.
- Hugh’s Dale (Dale No 2), Cave # CI-77, approx. coordinates 10°28’07”S, 105°33’40”E. Sample BES 5864. Collected on April 7, 1998 by S.M. Eberhard, hand net.

Taxonomic analysis

Dissections were done under a stereo-microscope. Valves were used for Scanning Electron Microscopy (SEM) and then stored dry in micropalaeontological slides; soft parts were dissected in glycerine and sealed in permanent glass slides. Descriptions of chaetotaxy used the

nomenclature proposed by Danielopol (1968, 1970) and adapted by Rossetti & Martens (1998), Pinto et al. (2004) and Smith & Kamyia (2008). Only morphological features that allow discrimination between darwinulid genera and species were considered here. All the analysed material is lodged in the Ostracod Collection (OC) of the Royal Belgian Institute of Natural Sciences (RBINSc), Brussels.

Phylogenetic analysis using morphological data

Methods used for the construction of phylogenetic trees mostly conform to the criteria adopted by Martens et al. (2005); a new matrix was constructed from literature data to include additional species that have recently been described. Twenty-three species were retained, i.e. those with detailed description of both valve (characters 1-12) and soft part (characters 13-30) morphology (see Tables 1 and 2). Polarizations of character states are given in Table 1, but these are not necessarily the polarizations used by the programs during the tree-building. Phylogenetic trees were constructed for all morphological data combined. Input order of taxa was as in the matrix in Table 2. *Darwinula stevensoni* was used as outgroup, based on the results presented in Martens et al. (2005). A non-darwinulid would have been preferable as an outgroup, but the morphology of the Darwinulidae is too specialised to allow inclusion of non-darwinulid ostracod species and at the same time test for relationships within the Darwinulidae (Horne et al., 2005). It must thus be understood that the topology of obtained trees is relative to the outgroup position of *D. stevensoni*.

Three different analyses were performed: (1) maximum parsimony analysis, without character weighting and without Dollo-up option; (2) neighbour joining analysis, without character weighting and without Dollo-up option; (3) neighbour joining analysis, with character weighing and with Dollo-up option. In the latter (3rd) analysis, all characters were designated ‘Dollo-up’, meaning that we assume that character states can change from plesiomorphic

TABLE 1

List of morphological characters and polarization of character states used for the phylogenetic analyses.

A. Valves
1   Lateral view: sloping (0), sub-squarish (1) or rounded (2)
2   L/R (0) or R/L (1) overlap
3   Postero-ventral keel on RV absent (0), short (1) or long (2)
4   Ventro-frontal internal tooth (mostly in LV) long (2), short (1) or absent (1)
5   Posterior internal tooth (mostly in LV) in ventral position present (0) or absent (1)
6   Posterior internal tooth (mostly in LV) in caudal position present (0) or absent (1)
7   Hinge adont (0), with simple large cardinal teeth (1) or subdivided large cardinal teeth (2)
8   Size: >0.6 mm (0) or =<0.6 mm (1)
9   Le/H ratio >2.2 (0), between 1.8 and 2.2 (1) or <1.8 (2)
10   Brooding space externally visible (0) or not (1)
11   Position of Cms: towards the front (0) or central (1)
12   Muscle scars: average number of scars >8 (0) or =<8 (1)

B. Soft parts
13   A1, first segment with 2 (0) or 1 (1) dorsal seta(e)
14   A1, "exopodite" with 3 (0) or 2 (1) setae
15   A1, second segment with (0) or without (1) dorso-apical seta
16   A1, third segment with (0) or without (1) ventro-apical seta
17   A1, fourth segment with (0) or without (1) ventro-apical seta
18   A1, fourth segment with 2 (0) or 1 (1) large dorsal seta(e)
19   A2, exopodite with 2 (0) or 1 (1) seta(e) (+spine)
20   A2, first segment of endopodite with 2 (0) or 1 (1) ventro-apical seta(e)
21   Md-palp, penultimate segment: seta *z* long (0) or short (1)
22   Md-palp, penultimate segment: seta *y* long (0), short (1) or absent (2)
23   Md-palp, last segment: «poil stevensoni» present (0) or absent (1)
24   Md-palp, last segment: number of apical claws: 5 (0), 4 (1) or 3 (2)
25   Md-palp, last segment: seta *a* claw-like (0), spine-like (1), absent (2)
26   Md-palp, last segment: seta *c* present (0) or absent (1)
27   T1, penultimate palp segment: number of seta(e): 2 (0) or 1 (1)
28   CR: number of seta(e): 2 (0), 1 (1) or absent (2)
29   P-abd: with projections (0), smooth (1) or absent (2)
30   Caudal seta present (0) or absent (1)

to apomorphic conditions, but that such changes are irreversible. Soft part characters were weighted five times more than valve characters, because the limb morphology appears to be more conservative and therefore more informative regarding deep-rooted relationships in Darwinulidae (MARTENS et al., 2005). The phylogenetic trees were validated using bootstrapping (10,000 replicates). Analyses 1 and 2 were done using PaupUp for Windows (CALENDI & MARTIN, 2005), the third analyses used PAUP* 4.0b10 (SWOFFORD, 2002).

Abbreviations used in the text and in the figures

Valves
Cp   carapace
LV   left valve
RV   right valve
Cms   central muscle scar(s)
iv   internal view
lv   lateral view
H   height of valves
Le   length of valves

TABLE 2

Data matrix used in the phylogenetic analyses. Character numbers as in Table 1.

Taxa	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
D. stevensoni	(0 1)	1	2	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	2	1	0
A. serricaudata	0	0	2	1	1	0	0	(0 1)	0	0	0	1	0	0	0	0	0	0	0	0	0	2	1	2	2	0	1	1	0	1
A. furcabdominis	0	0	2	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	2	1	1	2	1	1	1	0	1
A. inversa	0	1	2	1	1	0	0	1	0	0	0	1	0	0	0	0	0	1	1	0	1	2	1	2	2	0	1	1	1	1
V. boteai	1	0	1	1	1	1	0	1	0	0	0	1	1	1	1	1	1	1	1	0	0	1	1	0	2	0	1	1	1	1
V. botocuda	1	0	1	1	1	1	0	1	1	0	0	1	1	1	1	0	1	1	1	0	0	1	1	0	2	0	1	0	0	0
V. irajai	1	0	1	1	1	1	0	1	0	0	0	1	1	1	1	0	1	1	1	0	0	1	1	0	2	0	1	1	0	1
V. marmonieri	0	0	1	1	1	1	0	1	0	0	0	0	1	1	1	1	1	1	1	0	0	1	1	0	2	0	1	1	1	1
V. molopoensis	0	0	1	1	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	0	0	1	1	0	2	0	1	1	1	1
V. pagliolii	(0 1)	0	1	1	1	1	0	1	1	0	0	(0 1)	1	1	1	0	1	1	1	0	0	1	1	0	2	0	1	1	0	0
V. sp. D	1	0	1	1	1	1	0	1	0	0	0	?	1	1	1	1	1	1	1	0	0	1	1	0	2	0	1	1	1	1
V. matildae	1	0	0	0	1	1	0	1	1	0	0	(0 1)	1	1	1	0	1	1	1	0	0	1	1	0	2	0	1	1	1	0
V. sp. E	1	0	0	0	1	1	0	1	0	0	0	1	1	1	1	1	1	1	1	0	0	1	1	0	2	0	1	1	2	1
V. cornelia	1	0	0	0	1	1	0	1	1	0	0	(0 1)	1	1	1	1	1	1	1	0	0	1	1	0	2	0	1	1	1	1
V. carveli	1	0	0	0	1	1	0	1	0	0	0	0	1	1	1	1	1	1	1	0	0	1	1	0	2	0	1	1	1	1
I. humphreysi	1	0	1	1	1	1	0	0	1	0	0	1	0	1	1	1	1	1	1	0	0	0	1	0	2	0	1	1	0	1
P. incae	1	0	2	2	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	2	1
P. aotearoa	1	0	2	1	1	0	0	(0 1)	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	2	1
P. kohanga	(0 1)	0	2	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	2	1
P. brasiliensis	1	0	2	(1 2)	0	1	0	1	(0 1)	0	0	(0 1)	1	0	0	0	0	0	0	0	0	0	1	0	1	0	1	(0 1)	2	1
P. reidae	(0 1)	0	2	1	0	1	0	1	(0 1)	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	1	1	2	1
M. zimmeri	2	0	2	1	0	1	1	1	(1 2)	1	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	2	2	1
M. inexpectata	(1 2)	0	2	1	0	1	2	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	2	2	1

Limbs and soft parts
A1   Antennula
A2   Antenna
Md   Mandibula
CR   caudal ramus
P-abd   post-abdomen
b, c, w, x, y, z   specific setae on Md-palp

RESULTS

Taxonomic account

Class Ostracoda LATREILLE, 1802
Subclass Podocopa G.W. MÜLLER, 1894
Order Podocopida SARS, 1866
Suborder Darwinulocopina SOHN, 1988
Superfamily Darwinuloidea
BRADY & NORMAN, 1889
Family Darwinulidae BRADY & NORMAN, 1889

Genus *Vestalenula* ROSSETTI & MARTENS, 1998

Diagnosis (after ROSSETTI & MARTENS, 1998)
Small darwinulids, with valves elongate or short and subquadrate. RV with a posteroventral, external keel, the latter short (*boteai*-group) or elongate (*danielopoli*-group). LV with internal anteroventral tooth, no caudal or caudoventral internal teeth. Hinge adont. Mostly c. 0.6 mm or less in length. Valves with L/R overlap along anterior, ventral and posterior margins. Dorsal margin straight over part of length. Cms always situated towards the front in adult females. Large caudal brooding cavity externally visible. First segment of A1 with one dorsal seta, second segment with two ventral setae; fourth segment without ventro-apical seta. A2 with one long seta and a spine on exopodite. Seta *y* on penultimate Md-palp segment short; last segment with five apical claws. Adults mostly with CR, P-abd smooth or absent.

Vestalenula sp. E
(Figs 2, 4F-H)

Material investigated
One ovigerous female, with valves stored dry in a micropalaeontological slide (used for SEM) and soft parts dissected in glycerine and kept in a sealed slide (OC3269), collected at Grants Well (see above for details).

Remark
The genus *Vestalenula* includes four as-yet-unnamed species: sp. A and sp. B (DANIELOPOL, 1980) belonging to the *danielopoli*-group, and sp. C and sp. D (ROSSETTI & MARTENS, 1999) belonging to the *boteai*-group. Therefore, in the present paper we name our species in open nomenclature as *Vestalenula* sp. E. This species belongs to the *danielopoli*-group within this genus.

Diagnosis
Small darwinulid (<0.4 mm). Valves sub-quadrate in lv, Le/H c 2.12. LV overlapping RV. Cp posteriorly enlarged to form a brooding pouch. Hinge adont. RV with an elongate ventral keel (*danielopoli*-group) relatively distant from the postero-ventral corner. LV with a long antero-ventral tooth. Cms with 6-7 spots arranged in a circular rosette. Third segment of A1 without ventro-apical seta. First endopodal segment of A2 with one ventro-apical seta. Penultimate segment of Md-palp with seta *y* short and seta *z* extending beyond last segment; this latter with five apical claws, setae *b* and *c* present. CR a hirsute base carrying a seta. P-abd absent. Males unknown.

Measurements
Le(LV)=373 μm, H(LV)=176 μm, Le(RV)=358 μm, H(RV)=169 μm (n=1).

Differential diagnosis
Vestalenula sp. E can be easily differentiated from most of its congeners by its tiny size. In fact, all the other species in this genus are 0.40-0.60 mm, apart from *V. matildae* (0.38 mm) (ROSSETTI & MARTENS, 1998, 1999; MARTENS & ROSSETTI, 2002; PINTO et al., 2003). This latter species is indeed the closest congener of *V.* sp. E, but it has a P-abd (not present in *V.* sp. E), slightly more elongated valves in lv (Le/H >2.2), and a higher number of spots (c. 9) in the Cms.

Distribution
The species is known from its type locality only.

Genus *Isabenula* gen. nov.

Derivation of name
We are pleased to name the genus after Dr Isabelle Schön (RBINSc, Brussels), the leading world authority in the application of DNA-based molecular techniques to non-marine ostracods, for her outstanding contribution to the study of genetics and ecology of the Darwinulidae. The name is a combination of 'Isabe' (from Isabelle) and 'nula' for assonance with other darwinulid generic names. The etymology of the name Isabelle can be traced to Jezebel, which is transliterated from both the Hebrew Iyzebel and the Greek Iezabel, and means, among other things, "chaste". This name seems most appropriate for a genus of supposedly ancient asexuals.
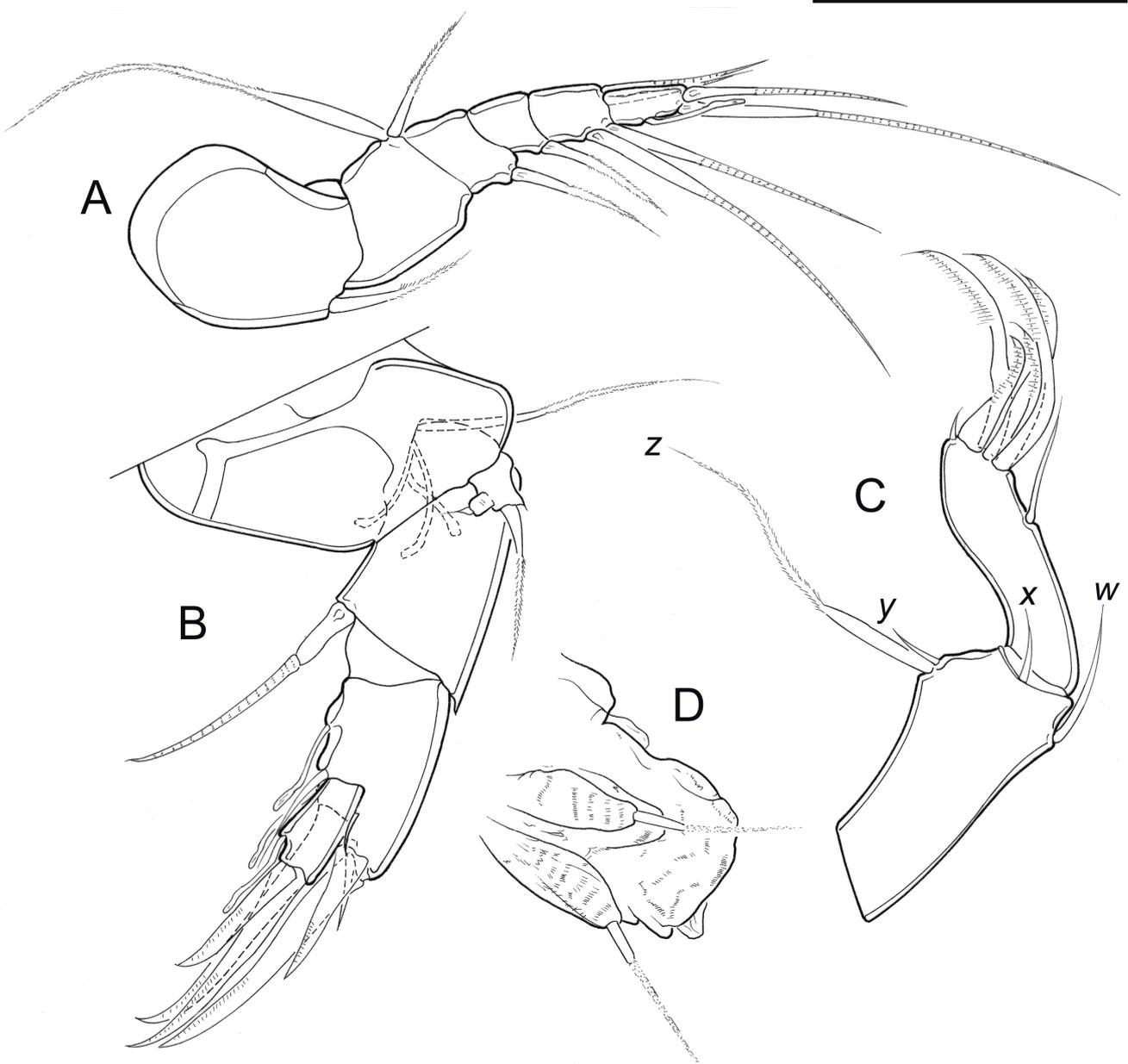
Fig. 2. – *Vestalenula* sp. E. A: A1 (OC3269). B: A2 (idem). C: Md-palp (idem). D: CR (idem). Scale bar: 50 μm.

TABLE 3

Differences in the soft part morphology of A1, A2 and Md-palp between the genera *Penthesilenula*, *Isabenula* n.gen., and *Vestalenula*. Shaded areas indicate more plesiomorphic states for the selected characters (see Table 1 and 2).

		Penthesilenula	*Isabenula* n.gen.	*Vestalenula*
A1, first segment	dorsal seta(e)	2	2	1
A1, second segment	ventral setae	3	2	2
	dorsal seta	present	present	absent
A1, third segment	ventral seta	present	absent	absent
A1, fourth segment	ventral seta	present	absent	absent
	dorsal seta(e)	2	1	1
A2, "exopodite"	long seta(e)	2	1	1
A2, third segment	ventral seta(e)	2	2	1 or 2
Md-palp, penultimate segment	setae z and y, relative length	$z≈y$	$z≈y$	$z»y$
Md-palp, last segment	seta a	present	absent	absent

Diagnosis
Medium-sized darwinulids, Cp sub-quadrate in lv, with externally visible brood pouch. RV with short postero-ventral keel, LV with antero-ventral internal tooth. A1 with two dorsal setae on first segment; second segment with dorso-apical seta. A2 exopodite with one seta and a lateral spine. Penultimate segment of Md-palp with setae z and y both long, last segment lacking seta a.

Type species (here designated):
Isabenula humphreysi Rossetti, Pinto & Martens sp. nov.

Differential diagnosis
Isabenula gen. nov. can be distinguished from the genera *Darwinula*, *Alicenula*, *Penthesile -nula* and *Microdarwinula* by the presence of a short keel on RV. Since this feature is also found in the *boteai*-group of the genus *Vestalenula*, the valve morphology is not sufficient to separate *Isabenula* gen. nov. from *Vestalenula*. Nevertheless, given the apparent morphological stasis of the soft parts in the whole family Darwinulidae, *Isabenula* gen. nov. and *Vestalenula* can clearly be distinguished by the chaetotaxy of A1 (two dorsal setae on first segment and one dorsal seta on second segment in *Isabenula* gen. nov., one dorsal seta on first segment and dorsal seta absent on second segment in *Vestalenula*) and Md-palp (penultimate segment with setae z and y both long in *Isabenula* gen. nov., seta z considerably longer than seta y in *Vestalenula*) (see Table 3).

Isabenula humphreysi sp. nov.
(Figs 3, 4A-E)

Type locality
Hugh's Dale (Dale No 2), Cave # CI-77 (see above for details).

Holotype
An ovigerous female, with valves stored dry in a micropaleontological slide (used for SEM) and soft parts dissected in glycerine and kept in a sealed slide. Material deposited in the Ostracod Collection of the Royal Belgian Institute of Natural Sciences, Brussels (OC3270).

Paratype
An adult female, with valves stored dry in a micropal slide (used for SEM) and soft parts dissected in glycerine and kept in a sealed slide (OC3271).

Derivation of name
This species is named after Dr William F. Humphreys (Western Australian Museum, Perth) for his important studies on the fauna of subterranean water systems and for providing us with the material described in the present paper.
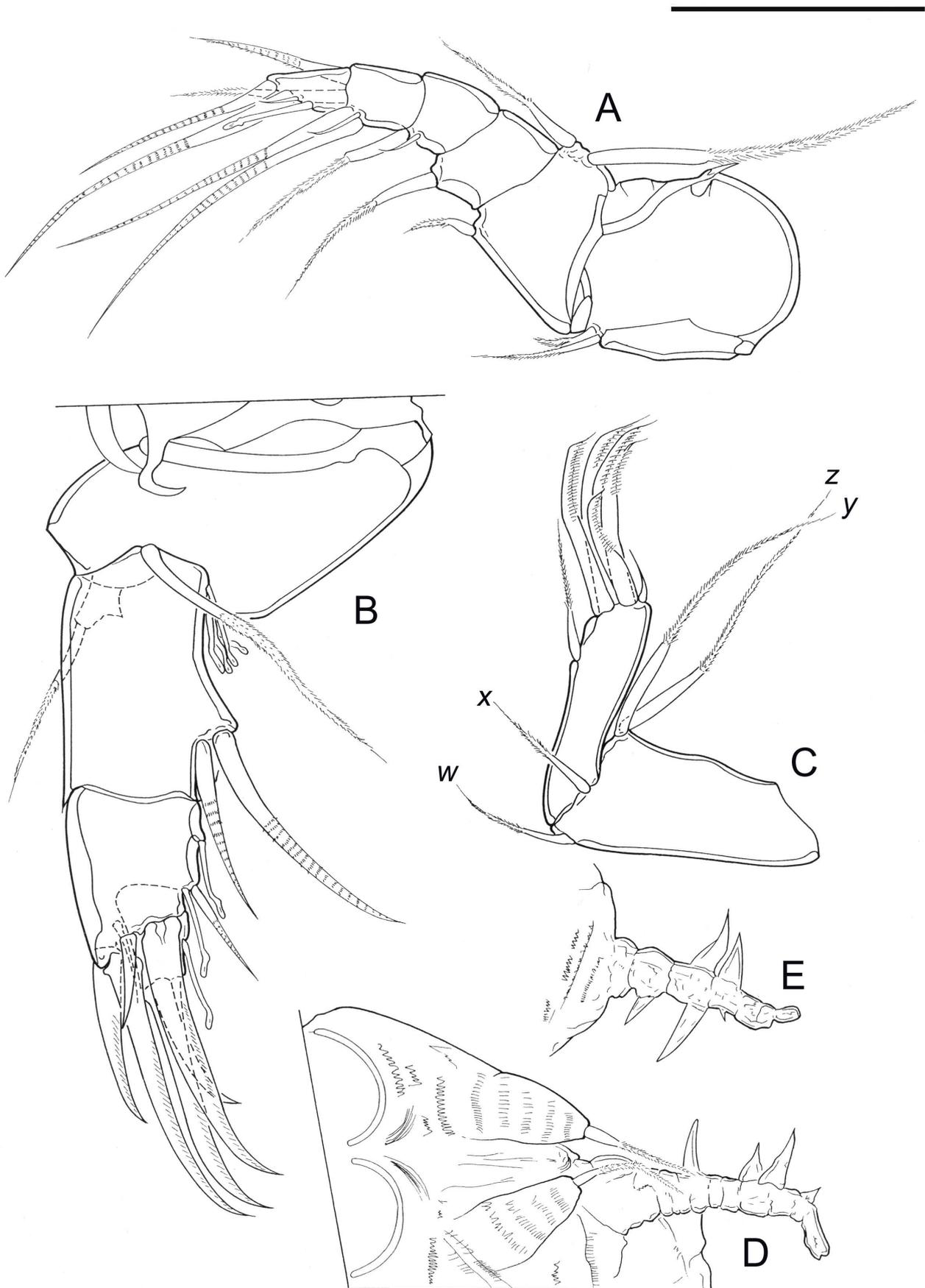
Fig. 3. – *Isabenula humphreysi* gen. nov.sp. nov. A: A1 (OC3270). B: A2 (idem). C: Md-palp (idem). D: CR and P-abd (idem). E: P-abd (OC3271). Scale bar: 50 μm.
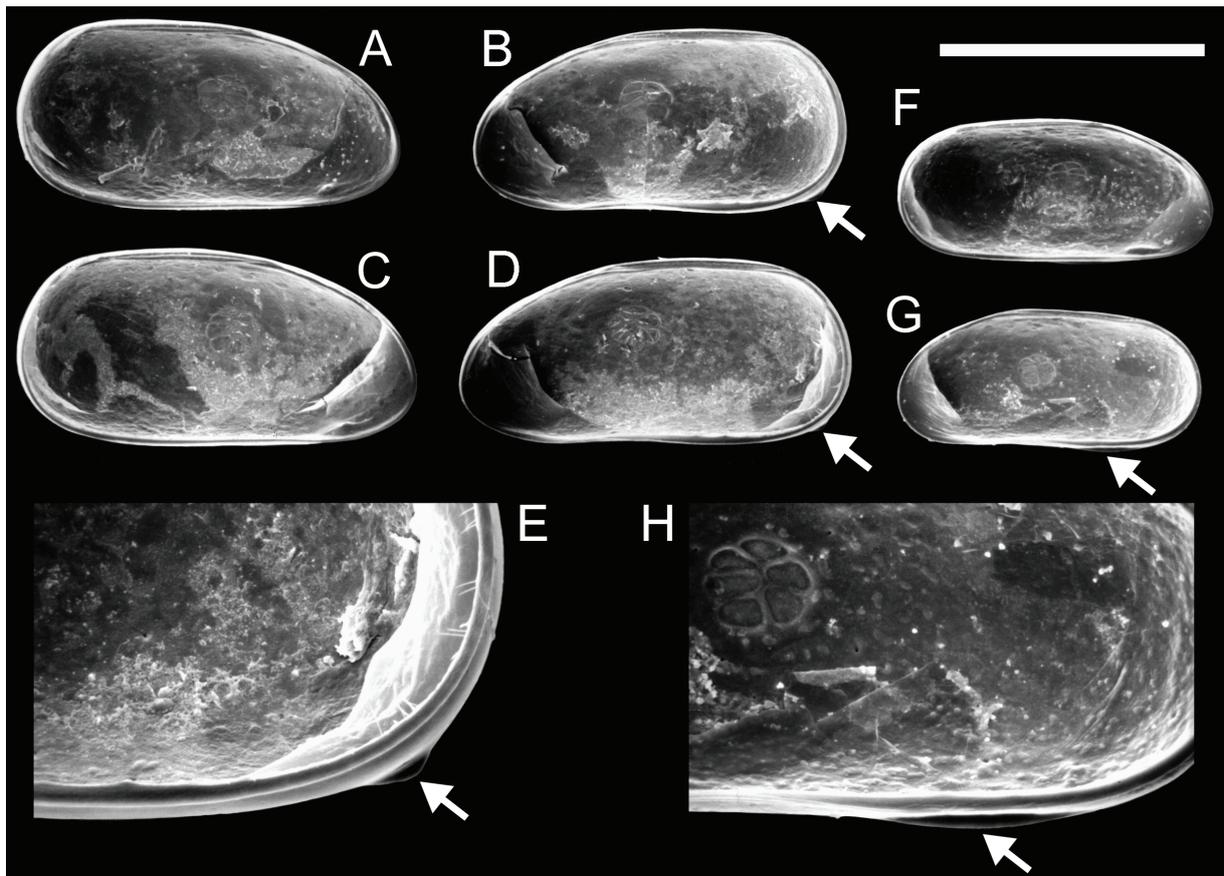
Fig. 4. – A-E: *Isabenula humphreysi* gen. nov.sp. nov. F-H: *Vestalenula* sp. E. A: LV, iv (OC3271). B: RV, iv (idem). C: LV, iv (OC3270). D: RV, iv (idem). E: RV, iv, detail postero-ventral corner (idem). F: LV, iv (OC3269). G: RV, iv (idem). H: RV, iv detail posterior part of ventral margin (idem). Arrows indicate the position of the keel on RV. Scale bar: 300 μm for A-D, F,G; 100 μm for E, H.

Diagnosis
Medium-sized species. Cp sub-quadrate in lv. LV overlapping RV. Hinge adont. Caudal brooding chamber externally visible. RV with short keel inserted at the postero-ventral corner, LV with antero-ventral internal tooth. Cms with c 7 spots arranged in a rosette. A1 with two dorsal setae on first segment, second segment with one dorso-apical seta and two ventral setae, one c half as long as the other; third and fourth segment with one long dorsal seta each. A2 exopodite with one seta and a lateral spine, first endopodal segment with ventral-apical setae. Penultimate segment of Md-palp with setae *z* and *y* both long, subequal; last segment with five terminal claws, setae *b* and *c* present. CR a broad conical base carrying a seta. P-abd long and cylindrical, with clear lateral projections. Males unknown.

Measurements
Le(LV)= 458-479 μm, H(LV)=232-246 μm.

Le(RV)=443-465 μm, H(RV)= 224-229 μm (n=2).

Distribution
The species is known from its type locality only.

Differential diagnosis
As for the genus.

Remarks
1. KLIE (1932, Figs 74 and 76) illustrated the Md-palp, CR and P-abd of a darwinulid species from Sunda Islands that he identified as *Darwinula malayica* MENZEL, 1923; this species was then transferred to the genus *Penthesilenula* by ROSSETTI & MARTENS (1998). In particular, KLIE (*loc. cit.*) figured the presence of two long *z* and *y* setae on the penultimate Md-palp segment, associated with the absence of seta *a*, i.e. a unique combination of characters now only known for *Isabenula humphreysi* sp. nov.

(Table 1). Actually, also the seta *c* on penultimate Md-palp segment appeared to be missing. Differences with *Isabenula humphreysi* sp. nov. can be found in the length of the CR setae and the shape of the P-Abd, which is respectively shorter and with lateral projections in this latter species. Due to the brief (and possibly erroneous) description by KLIE (1932) and the absence of reference material, the correct determination of this darwinulid species remains doubtful, but its synonymy with *Isabenula humphreysi* sp. nov. can almost certainly be excluded.

2. *Vestalenula flexuosa* was placed in the *boteai*-group by ROSSETTI & MARTENS (1999) because of the presence of a short keel on RV. However, after the description of *Isabenula* gen. nov., this species could belong either to *Vestalenula* or *Isabenula* gen. nov., as only the valves of this species are known. Only the discovery of animals with preserved soft parts will allow its precise allocation.

Other material investigated (not illustrated)
Two immature specimens (one with damaged LV) and an empty Cp of a juvenile from Henderson's Spring; one immature specimen from Pump house at Ross Hill Gardens; an isolated LV from Hugh's Dale (Dale No 2) (see above for details on sampling stations). All these specimens are provisionally assigned to the *africana*-group of the genus *Penthesilenula* ROSSETTI & MARTENS, 1998, due to the sub-quadrate shape of valves in lv and the presence of two rounded internal teeth (antero-ventral and postero-ventral) on LV. These teeth, however, can be also observed in larval specimens of the genus *Vestalenula*. Differences in valve morphology suggest the possible presence of two species. Due to the scarcity of the material, no further description is given here.

Key to genera of Recent Darwinulidae based on the morphology of valves and soft parts in adult females

1a. Cp rounded or slightly elongated in lv, no externally visible brood pouch, Cms centrally positioned .... ***Microdarwinula***

b. Cp elongate or sub-quadrate in lv, with externally visible brood pouch, Cms situated towards the front 2

2a. RV without postero-ventral keel, LV with or without internal teeth. A1 with two dorsal setae on first segment; second segment with one dorso-apical seta and three ventral setae. A2 expodite with two setae and a spine 3

b. RV with postero-ventral keel, LV with antero-ventral internal tooth. A2 expodite with one seta and a spine. Md-palp, seta *a* absent 4

3a. Last segment of Md-palp with less than five claws (three or four), penultimate segment with seta *y* short or absent 5

b. Last segment of Md-palp with five claws, penultimate segment with seta *y* long .... ***Penthesilenula***

4a. A1 with one dorsal seta on first segment; second segment with dorso-apical seta absent. Md-palp, penultimate segment with seta *z* long, seta *y* short .... ***Vestalenula***

b. A1 with two dorsal setae on first segment; second segment with dorso-apical seta. Md-palp, penultimate segment with setae *z* and *y* long ....***Isabenula* gen. nov.**

5a. RV overlapping LV, Le>0.65 mm. LV without internal teeth. Second segment of A1 endopodite with two large dorsal setae. Md-palp, penultimate segment with seta *z* long, seta *y* short; last segment with 'poil stevensoni'; setae *a* and *b* present .... ***Darwinula***

b. RV overlapping LV or LV overlapping RV, Le≤0.65 mm. Md-palp, penultimate segment with seta *z* short, seta *y* absent; last segment without 'poil stevensoni', seta *a* absent .... ***Alicenula***

Cladistic analysis of darwinulid species based on morphological features

Figure 5 shows the cladogram constructed with the parsimony criterion from all morphological data combined (valve and soft part characters), and using *Darwinula stevensoni* as outgroup. The tree shows a separation into two main groups: *Alicenula – Vestalenula – Isabenula* gen. nov. on one hand, and *Penthesilenula – Microdarwinula* on the other, although bootstrap support for each of these two clusters is low. *Darwinula* remains outside of either group, even when it is not designated as outgroup (results not shown). In
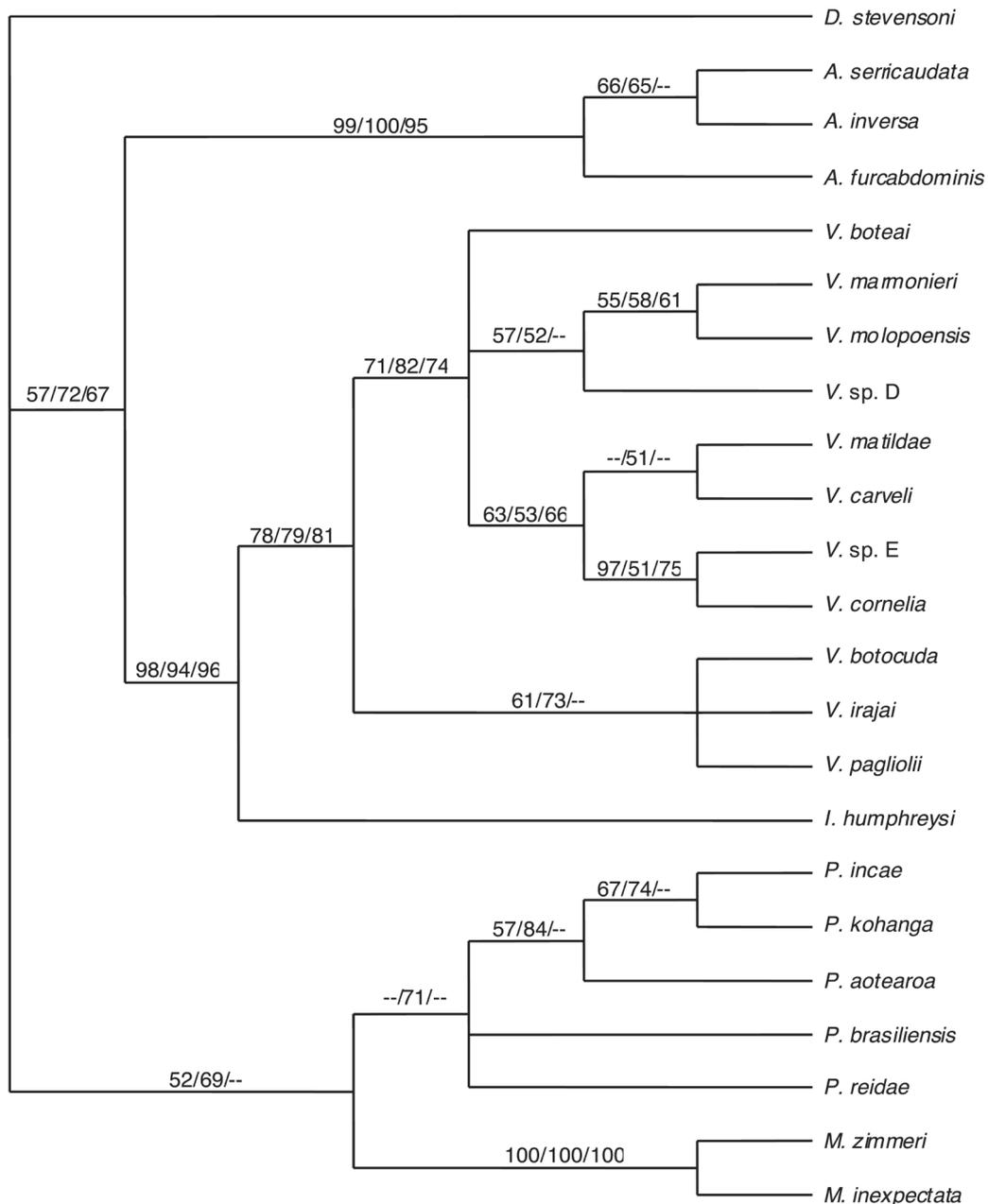
Fig. 5. – Cladogram constructed with parsimony criterion in PaupUp, using the morphological dataset in Table 3. *Darwinula stevensoni* is selected as outgroup. Numbers above branching events are % bootstrap values of 10,000 replicas as follows (1/2/3): (1) bootstrap (BS) values of neighbour joining (NJ) without special settings/ (2) BS values of NJ with character weighting and Dollo-up option/ (3) BS values of maximum parsimony analysis without special settings. ” –“ means the value was below 50%.

particular, *Isabenula* gen. nov. is a sister group to *Vestalenula*; the bootstrap values of the branch point for these two genera are 98/94/96 (see Figure 5 for explanation).

DISCUSSION

Distribution and colonisation

Samples from interstitial habitats of Christmas Island possibly yielded four different species of Darwinulidae. Such a high incidence of darwinulid species in a small area is quite uncommon, although coexistence of different species and genera has been previously reported, even from single locations (MARTENS et al., 1997; HIGUTI et al., 2007).

Darwinulids have a reduced mobility, and no dispersal mechanisms in the form of resting stages are known to date. Nevertheless, clonal reproduction theoretically allows the establishment of populations from a single individual; in addition, antiquity of the group and movement of land masses through geologic time could account for the intercontinental distribution of some species of darwinulids. Paleontological evidence confirms that the Darwinulidae, supposedly consisting entirely of non-marine genera (with the possible unique exception of the monospecific and highly enigmatic genus *Semidarwinula* (CHOE, 1988)), is the only surviving family of Darwinuloidea since the Permian-Triassic mass extinctions (MARTENS et al., 2003). On the other hand, in Christmas Island the main limestone deposits were laid down from the Late Oligocene onwards and a karst and underground drainage system developed only in the Miocene (GRIMES, 2001; NAMIOTKO et al., 2004). The origin of the island's darwinulid fauna is most likely a consequence of repeated events of colonisation, rather than of local speciation events, as we know that evolution in general in this group is slow (SCHÖN et al., 1998, 2003), while the availability of suitable habitats on this island is relatively recent in geological terms. Although accidental introduction through human

activity cannot in principle be discarded, long-distance transport by birds of living ostracod surviving the gut passage (e.g., PROCTOR et al., 1967; FRISCH et al., 2007) is the most probable way of dispersal. HORNE & SMITH (2004) documented that disjunctive distributions of freshwater ostracods may be associated with bird migration routes over broad geographic ranges. Distances of Christmas Island from both Indonesian islands and mainland Australia easily fall within the range of non-stop flights for many land- and seabirds (BERTHOLD, 2001). If we accept such a view, darwinulid ostracods found in subterranean habitats of Christmas Island could also inhabit (or have inhabited) epigeic waters of surrounding lands, where they can have been ingested by birds. Some darwinulid species are known to colonize both surface and subterranean aquatic habitats. For example, *Penthesilenula brasiliensis* was found in lakes, rivers and streamlets, interstitial and (semi-) terrestrial habitats (ROSSETTI & MARTENS, 1998; ROSSETTI et al., 1998; MARTENS & ROSSETTI, 2002; VAN DONINCK et al., 2003; PINTO et al., 2004).

Most of the known and described darwinulid species are assumed to be of Gondwanian origin and are more common in the Southern hemisphere. In particular, *Penthesilenula* and *Vestalenula* are by far the most speciose genera of the Darwinulidae (representing c. 80% of the known species of Recent darwinulids), and have numerous representatives in southeast Asia, Oceania and the West Pacific (ROSSETTI & MARTENS, 1998; MARTENS & ROSSETTI, 2002; MEISCH et al., 2007; REEVES et al., 2007). The discovery of *Isabenula* gen. nov., of which the phylogenetic relationships with *Penthesilenula* and *Vestalenula* are determined here, further reinforces the role of that geographic area as a biodiversity hotspot for the darwinulid fauna.

Characters used

The revision of Recent Darwinulidae by ROSSETTI & MARTENS (1998) took into account both valve and soft characteristics, since it was conceived as a tool for neontological and

paleontological classifications of this family. However, it is clear that the identification at the genus/species level of fossils, exclusively based on valve morphology, may in some cases lead to incorrect conclusions. In fact, soft part morphology in the Darwinulidae is more conservative and thus more informative regarding phylogenetic and evolutionary relationships, while valve morphology is more plastic and more readily adaptable to changing environmental conditions. Therefore, chaetotaxy of limbs is generally used to characterise genera, while valve shapes are better suited as specific characteristics (MARTENS et al., 2005). For example, the presence of a keel on the RV, sub-quadrate valves and small Cp size are features found in *Isabenula* gen. nov. and in several species of *Vestalenula*. Extreme caution is therefore needed when synonymising fossil darwinulids with Recent taxa, as was for example done for. *V. cylindrica* with *V. pagliolii* by GROSS (2008).

Phylogenetic analysis

MARTENS et al. (2005) performed a phylogenetic analysis of Recent Darwinulidae using both molecular and morphological data. The resulting trees showed a high degree of congruence, indicating that *Alicenula* and *Vestalenula* form one group, while *Penthesilenula* and *Microdarwinula* constitute another; the monospecific genus *Darwinula* is more closely related to the former cluster; exclusive morphological characters, mainly in the soft parts, seem to indicate that *Microdarwinula* has probably a monophyletic origin and is a relatively recent spin-off from a *Penthesilenula*-like ancestor, meaning that the latter genus has to be considered paraphyletic (MARTENS et al., 2005; PINTO et al., 2005).

Our phylogenetic analysis confirms the taxonomic separation of *Isabenula* gen. nov. from the other existing genera of Darwinulidae. Trees obtained combining valve and soft part characters indicate the presence of two main clusters (*Penthesilenula* + *Microdarwinula*, and *Alicenula* + *Vestalenula* + *Isabenula* gen.

nov.) and the isolated position of *Darwinula*, showing topologies that confirm those generated by MARTENS et al. (2005, Fig. 1) using morphological characters.

The diagnostic characters of the A1, A2 and Md-palp used to separate the genera *Vestalenula*, *Penthesilenula* and *Isabenula* gen. nov. (Table 3) show a clear pattern: they are plesiomorphic in *Penthesilenula* and apomorphic in *Vestalenula*, while in *Isabenula* gen. nov. there is a combination of ancestral and derived states (Table 1). Also the presence of an external keel on the RV in both *Vestalenula* and *Isabenula* gen. nov. (not present in *Penthesilenula*) as remnant of the outer list has to be considered a plesiomorphism (MARTENS et al. 2005). These morphological features and the results of the phylogenetic analysis thus indicate that *Vestalenula* could be a spin-off from an *Isabenula*-like ancestor, which in turn shares a common ancestor with *Penthesilenula*.

The new cladistic analysis does not allow us to identify species-groups (*boteai*- and *danielopoli*-groups in the genus *Vestalenula* and *incae*- and *africana*-group in the genus *Penthesilenula*), based on morphological features of the valves (relative length of the external keel in the RV and number and position of internal teeth in the LV) (ROSSETTI & MARTENS 1998). These species-groups have been created for practical convenience, and no taxonomic rank should be allocated to them.

Phenotypic evolution in darwinulids

Some changes in the phenotypic evolution of darwinulids can be seen as a result of natural selection. For example, miniaturization of the carapace may reflect an adaptation to reduce the risk of detection by visually-hunting predators and to permit the colonization of interstitial habitats. On the other hand, it is not immediately apparent how changes in chaetotaxy, often minimal (e.g., variation in the relative length or loss of setae), can be related to functional aspects, for example feeding and locomotion activities. In addition, in clonal organisms such morphological differences

cannot be the results of selective pressure on mate recognition systems or barriers to avoid cross breeding. Nevertheless, it is evident that the main causes of speciation invoked for sexual organisms, for example divergent selection due to niche-specific adaptations, also operate in ancient asexual lineages, although their rate and magnitude might differ (FONTANETO et al., 2007). As reported above, our present knowledge on darwinulids indicates that the soft part morphology is very conservative, also in long-lived taxa with intercontinental distribution. An alternative view states that natural selection may weed out variants that do not conform to a limited number of "models", i.e. those arrangements of characters that are found and that can be used to unambiguously identify distinct taxonomic units. This is even more relevant in an animal group that reproduces fully asexually and for which any neutral or non-deleterious mutation might theoretically be clonally transmitted. Individuals with abnormalities in valve or limb morphology have been observed in some darwinulid populations. Such deviant morphologies are thought to be mostly epigenetic and due to developmental errors during the moulting process rather than be genetically transmitted (ROSSETTI & MARTENS, 1998, PINTO et al., 2007), but a genetic basis cannot be excluded *a priori* (see below).

A more detailed examination of data used for the present cladistic analysis (see Tables 2 and 3) reveals that in the Recent representatives of the Darwinulidae:

1) the body structure is very conservative, especially in the limb chaetotaxy;
2) few possible character states are known for each character;
3) morphological "gaps" are easily recognizable and allow the definition of genera, species-groups and species (see MARTENS et al., 1998; ROSSETTI & MARTENS, 1998 and FONTANETO et al., 2007 for a discussion on the applicability of the species concept and other taxonomic categories to clonal lineages);
4) in several clades, plesiomorphies and apomorphies coexist (as do intermediate states), and characters seem to evolve independently and probably at different rates. In other terms, clades result from a mosaic of "ancestral" and more derived characters.

According to ROSSETTI & MARTENS (1998), two possible causes can be singled out to account for clear morphological gaps between species and genera in the Holocene and Recent Darwinulidae. Firstly, slower molecular evolution means that fewer intermediate forms originate. Secondly, it also means that natural selection has longer time spans to weed out the (potentially less fit) intermediate forms. The morphology of *Isabenula* gen. nov., although unexpected on the basis of existing knowledge, and intermediate between those of *Vestalenula* and *Penthesilenula*, can be simply viewed as a new combination of already described characters. It can be also hypothesized that the limited number of "attainable" morphological states reflects a condition of an evolutionary dead end, or at least of a low evolutionary potential, probably linked to obligate asexuality. An alternative view is that there are molecular mechanisms to slow down or even stop genetic (and probably also morphological) evolution in the Darwinulidae to preserve combinations of ecologically relevant genes which confer broad tolerance to diverse environmental conditions (VAN DONINCK et al., 2004). For example, highly efficient DNA repair has been invoked to explain the slow molecular evolution observed in some darwinulid species (SCHÖN & MARTENS, 1998, 2003). The wide geographical and ecological distribution of *Darwinula stevensoni*, the only representative of the genus that has persisted through geological time, seems to strengthen the hypothesis that natural selection actually acts to preserve the same successful, invariant model of organism (VAN DONINCK et al., 2002). The same argument can be extended at least to those darwinulid genera that are monospecific (*Isabenula* gen. nov.) or consist of few species (*Microdarwinula* and *Alicenula*).

It should also be evaluated, if for the

Darwinulidae, a pure phylogenetic approach is applicable to morphological data and under which conditions. Our cladistic analysis, which used the Dollo-up option, was based on the assumption that characters can change from plesiomorphic to apomorphic conditions, but are irreversible. DOMES et al. (2007) argue that Dollo's law is not applicable to putative ancient asexuals, because some oribatid mites might have regained the ability to reproduce sexually after an asexual period, but GOLDBERG & IGIC (2008) argued that both models, with and without reversal to sex, are equally likely.

There is some evidence that morphological evolution in Darwinulidae may not be completely unidirectional, or at least that some lineages could be an exception to general rules. Indeed, the absence of an externally-visible brooding pouch in the genus *Microdarwinula* can be seen as a paedomorphic feature (PINTO et al., 2005), and the occasional re-activation of genes regulating the expression of atavistic characters (e.g., the occurrence of rare males in *V. cornelia*) seems to be possible. If such silent genes are occasionally switched on, they can introduce strong bias into any phylogenetic scheme (ROSSETTI & MARTENS, 1998). However, the observed morphological abnormalities in the Darwinulidae seem to occur at the intraspecific level only, and are relatively easily recognisable amongst the various other features that do comply with the general diagnosis of species clusters (either genera or species-groups within genera), thus allowing the reconstruction of phylogenetically-valid taxonomies (ROSSETTI & MARTENS *loc. cit.*). The present phylogenetic reconstructions using two different criteria (maximum parsimony and neighbour joining) and with or without character weighting and Dollo-up options, all produce very similar topologies and bootstrap supports. Apart from demonstrating the robustness of the retained nodes, it may also be that the discussions on irreversibility of loss of character states (including the ability to reproduce sexually) are largely academic.

CONCLUSIONS

In the last fifteen years, research on taxonomy, systematics, genetics and ecology of the Recent Darwinulidae has greatly expanded. Nevertheless, our knowledge is still far from sufficient in many fields. Further phylogenetic reconstructions using molecular data (nuclear and mitochondrial sequences) from a large number of darwinulid species are needed to give new insight into the still-puzzling evolutionary scenario of the Darwinulidae. We need to better calibrate the time of splitting of major lineages, and to assess the level of congruence between phylogenies constructed using morphological and molecular characters. A phylogeographic approach is severely hampered by the scarce information on the distribution of the Darwinulidae, most of them being known from their type locality only. A greater sampling effort in aquatic ecosystems that are generally scarcely studied, such as groundwater habitats, may lead to a more precise estimate of the taxonomic diversity of darwinulids. Finally, only a deeper integration of paleontological, morphological and molecular approaches will allow us to test hypotheses of the evolutionary processes in the Darwinulidae that remain partly speculative at this stage.

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REFERENCES

Artheau M (2007). Geographical review of the ostracod genus *Vestalenula* (Darwinulidae) and a new subterranean species from southern France. Invertebrate Systematics, 21: 471-486.

Berthold P (2001). Bird Migration: A General Survey. 2nd ed., Oxford University Press, Oxford.

Brady GS & Robertson D (1870). The Ostracoda and Foraminifera of Tidal Rivers. Annals and Magazine of Natural History, ser. 4, 6: 1-33.

Browne RA (1992). Population genetics and ecology of *Artemia*: insights into parthenogenetic reproduction. Trends in Ecology & Evolution, 7: 232-237.

Butlin RK, Schön I & Martens K (1998). Asexual reproduction in nonmarine ostracods. Heredity, 81: 473-480.

Calendini F & Martin J-F (2005). PaupUP v1.0.3.1 A free graphical frontend for Paup* Dos software.

Choe K (1988). On ostracod biofacies and five new genera in Korean Seas. In: Hanai T, Ikeya N & Ishizaki K (eds), Evolutionary biology of Ostracoda: Its fundamentals and applications, Elsevier, Amsterdam and Kodansha, Tokyo: 121-133.

Danielopol DL (1968). *Microdarwinula* n.g. et quelques remarques sur la répartition de la famille Darwinulidae Br. et Norm. (Crustacea, Ostracoda). Annales de Limnologie - International Journal of Limnology, 4: 153-174.

Danielopol DL (1970). Une nouvelle espèce du genre *Darwinula* des eaux souterraines de Roumanie et quelques remarques sur la morphologie des Darwinulidae (Ostracoda-Podocopida). Travaux de l'Institut de Spéléologie Emile Racovitza, 9 : 135-149.

Danielopol DL (1980). An essay to assess the age of the freshwater interstitial ostracods of Europe. Bijdragen tot de Dierkunde, 50: 243-291.

Domes K, Norton RA, Maraun M. & Scheu S (2007). Reevolution of sexuality breaks Dollo's law. Proceedings of the National Academy of Sciences of the United States of America, 104: 7139-7144.

Fontaneto D, Herniou E, Boschetti C, Caprioli M, Melone G, Ricci C & Barraclough TG 2007). Independently evolving species in asexual bdelloid rotifers. PLoS Biology, 5: e87.

Frisch D, Green AJ & Figuerola J (2007). High dispersal capacity of a broad spectrum of aquatic invertebrates via waterbirds. Aquatic Sciences, 69: 568-574.

Goldberg EE & Igic B (2008). On Phylogenetic Tests of Irreversible Evolution. Evolution 62: 2727-2741.

Geiger W, Otero M & Rossi V (1998). Clonal ecological diversity. In: Martens K (ed.), Sex and Parthenogenesis - Evolutionary ecology of reproductive modes in non-marine ostracods, Backhuys, Leiden: 243-256.

Gorelick R (2003). Transposable elements suppress recombination in all meiotic eukaryotes, including automictic ancient asexuals: a reply to Schön and Martens. Journal of Natural History, 37: 903-909.

Grimes KG (2001). Karst features of Christmas Island (Indian Ocean). Helictite, 37: 41-58.

Gross M (2008). A limnic ostracod fauna from the surroundings of the Central Paratethys (Late Middle Miocene/Early Late Miocene; Styrian Basin; Austria). Palaeogeography, Palaeoclimatology, Palaeoecology, 264: 263-276.

Halkett F, Simon JC & Balloux F (2005). Tackling the population genetics of clonal and partially clonal organisms. Trends in Ecology & Evolution, 20: 194-201.

Heethoff M, Norton RA, Scheu S & Mauraun M (2009). Parthenogenesis in oribatid mites (Acari, Oribatida): evolution without sex. In Schön I, Martens K, Van Dijk P. (Eds), Lost Sex, The evolutionary biology of parthenogenesis. Springer, 241-258.

Higuti J, Machado Velho LF, Lansac-Tôha FA & Martens K (2007). Pleuston communities are buffered from regional flood pulses: the example of ostracods in the Paraná River floodplain, Brazil. Freshwater Biology, 52: 1930-1943.

Horne DJ & Smith RJ (2004). First British record of *Potamocypris humilis* (Sars, 1924), a freshwater ostracod with a disjunct distribution in Europe and southern Africa. Bollettino della Società Paleontologica Italiana, 43: 297-306.

Horne DJ, Schön I, Smith RJ & Martens K (2005). What are ostracoda? A cladistic analysis of the extant superfamilies of the subclasses Myodocopa and Podocopa (Crustacea: Ostracoda) In: Koenemann S & Jenner RA (Eds), Crustacea and arthropod relationships. Crustacean issiues. Vol. 16. Taylor & Francis.

Humphreys WF & Eberhard S (2001). Subterranean fauna of Christmas Island, Indian Ocean. Helictite, 37: 59-74.

Judson OP & Normark B (1996). Ancient asexual scandals. Trends in Ecology & Evolution, 11: 41-45.

Klie W (1932). Die Ostracoden der Deutschen Limnologischen Sunda-Expedition. Archiv für Hydrobiologie, Supplement, 11: 447-502.

Lunt P (2003). Biogeography of some Eocene larger Foraminifera, and their application in distinguishing geological plates. Palaeontologia Electronica, 6: 22. (Internet address: http://palaeo-electronica.org/2003_2/geo/geo.pdf/).

Martens K & Rossetti G (2002). On the Darwinulidae (Crustacea: Ostracoda) from Oceania. Invertebrate Systematics, 16: 195-208.

Martens K & Schön I (2008). Ancient asexuals: darwinulids not exposed! Nature, 453: 587.

Martens K, Rossetti G & Fuhrmann R (1997). Pleistocene and Recent species of the family Darwinulidae Brady & Norman, 1889 (Crustacea, Ostracoda) in Europe. Hydrobiologia, 357: 99-116.

Martens K, Rossetti G & Baltanas A (1998). Reproductive modes and taxonomy. In: Martens K (ed.), Sex and Parthenogenesis - Evolutionary ecology of reproductive modes in non-marine ostracods, Backhuys, Leiden: 197-214.

Martens K., Rossetti G & Horne DJ (2003). How ancient are ancient asexuals? Proceedings of the Royal Society of London B: Biological Sciences, 270: 723-729.

Martens K, Rossetti G, Butlin RK & Schön I (2005). Molecular and morphological phylogeny of the ancient asexual Darwinulidae (Crustacea, Ostracoda). Hydrobiologia, 538: 153-165.

Martens K, Schön I, Meisch C & Horne DJ (2008). Global diversity of ostracods (Ostracoda, Crustacea) in freshwater. Hydrobiologia, 595: 185-193.

Martens K & Schön I (2008). Ancient asexuals: darwinulids not exposed! Nature, 453 : 587.

Maynard Smith J (1978). The evolution of sex. Cambridge University Press, Cambridge.

Meisch C, Mary-Sasal N, Colin J-P & Wouters K (2007). Freshwater Ostracoda (Crustacea) collected from the islands of Futuna and Wallis, Pacific Ocean, with a checklist of the non-marine Ostracoda of the Pacific Islands. Bulletin de la Société des naturalistes luxembourgeois, 108: 89-103.

Namiotko T, Wouters K, Danielopol DL & Humphreys WF (2004). On the origin and the evolution of a new anchialine stygobitic Microceratina species (Crustacea, Ostracoda) from Christmas Island (Indian Ocean). Journal of Micropalaeontology, 23: 49-59.

Namiotko T, Marmonier P, Danielopol DL, Horne DJ & Artheau M (2005). New records of rare males of Cryptocandona vavrai Kaufmann, 1900 (Crustacea, Ostracoda), with further additions to the description of the species. Annales de Limnologie - International Journal of Limnology, 41: 203-219.

Pinto RL, Rocha CEF & Martens K (2003). On two new species of the genus Vestalenula Rossetti & Martens, 1998 (Crustacea, Ostracoda, Darwinulidae) from semiterrestrial habitats in São Paulo State (Brazil). Zoological Journal of the Linnean Society, 139: 305-313.

Pinto RL, Rocha CEF & Martens K (2004). On the genus Penthesilenula Rossetti & Martens, 1998 (Crustacea, Ostracoda, Darwinulidae) from (semi-) terrestrial habitats in São Paulo State (Brazil), with a description of a new species. Journal of Natural History, 38: 2657-2589.

Pinto RL, Rocha CEF & Martens K (2005). On the evolution of the genus Microdarwinula Danielopol, 1968 (Ostracoda, Darwinulidae) with a description of a new species from semi-terrestrial habitats in São Paulo State (Brazil). Crustaceana, 78: 975-986.

Pinto RL, Rocha CEF & Martens K (2007). Early release of eggs and embryos in a brooding ancient asexual ostracod: brood selection or a gambling strategy to increase fecundity? Hydrobiologia, 585: 249-253.

Proctor VW, Malone CR & DeVlaming VL (1967). Dispersal of aquatic organisms: viability of disseminules recovered from the intestinal tract of captive killdeer. Ecology, 48: 672-676.

Reeves JM, De Deckker P & Halse SA (2007). Groundwater Ostracods from the arid Pilbara region of northwestern Australia: distribution and water chemistry. Hydrobiologia, 585: 99-118.

Rossetti G & Martens K (1996). Redescription and morphological variability of Darwinula stevensoni (Brady & Robertson, 1870) (Crustacea, Ostracoda). Bulletin van het Koninklijk Belgisch Instituut voor Natuurwetenschappen, Biologie, 66: 73-92.

Rossetti G & Martens K (1998). Taxonomic revision of the Recent and Holocene representatives of the family Darwinulidae (Crustacea, Ostracoda), with a description of three new genera. Bulletin van het Koninklijk Belgisch Instituut voor Natuurwetenschappen, Biologie, 68: 55-110.

Rossetti G & Martens K (1999). Contribution to taxonomy and distribution of the genus *Vestalenula* Rossetti & Martens, 1998 (Crustacea, Ostracoda, Darwinulidae), with the description of two new species. Bulletin van het Koninklijk Belgisch Instituut voor Natuurwetenschappen, Biologie, 68: 57-66.

Rossetti G, Eagar S & Martens K (1998). On two new species of the genus *Darwinula* (Crustacea, Ostracoda) from New Zealand. Italian Journal of Zoology, 65: 325-332.

Schön I & Martens K (1998). Opinion: DNA-repair in ancient asexuals: a new solution to an old problem? Journal of Natural History, 32: 943-948.

Schön I & Martens K (2003). No slave to sex. Proceedings of the Royal Society of London B: Biological Sciences, 270: 827-833.

Schön I, Butlin RK, Griffiths HI & Martens K (1998). Slow molecular evolution in an ancient asexual ostracod. Proceedings of the Royal Society of London B: Biological Sciences, 265: 235-242.

Schön I, Martens K, Van Doninck K & Butlin RK (2003). Evolution in the slow lane: molecular rates of evolution in sexual and asexual ostracods (Crustacea: Ostracoda). Biological Journal of the Linnean Society, 79: 93-100.

Schön I, Lamatsch D & Martens K (2008). Lessons to learn from Ancient Asexuals. In: Egel R, Lankenau DH (Eds) Recombination and meiosis. Models, means and evolution. Springer Berlin Heidelberg, 341-376.

Schön I, Rossetti G & Martens K (2009). Darwinulid ostracods: ancient asexual scandal or scandalous gossip? In: Schön I, Martens K & Van Dijk P (eds), Lost Sex. The Evolutionary Biology of Parthenogenesis, Springer, Dordrecht: 217-240.

Schwander T, Henry L & Crespy BJ (2011). Molecular Evidence for Ancient Asexuality in *Timema* Stick Insects. Current Biology 21, 1129-1134.

Smith RJ & Kamiya T (2008). The ontogeny of two species of Darwinuloidea (Ostracoda, Crustacea). Zoologischer Anzeiger, 247: 275-302.

Smith RJ, Kamiya T & Horne DJ (2006). Living males of the 'ancient asexual' Darwinulidae (Ostracoda: Crustacea). Proceedings of the Royal Society of London B: Biological Sciences, 273: 1569-1578.

Swofford D L (2002). 'PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods) Version 4.0b10. Sinauer Associates, Sunderland.

Turner CH (1895). Fresh-water Ostracoda of the United States. Minnesota Geological and Natural History Survey, Zoological Series, 2: 277-337.

Van Doninck K, De Bruyn L, Schön I & Martens K (2002). A general purpose genotype in an ancient asexual. Oecologia, 132: 205-212.

Van Doninck K, Schön I, Maes F, De Bruyn L & Martens K (2003). Ecological strategies in the ancient asexual animal group Darwinulidae (Crustacea, Ostracoda). Freshwater Biology, 48: 1285-1294.

Van Doninck K, Schön I, Martens K & Backeljau T (2004). Clonal diversity in the ancient asexual ostracod *Darwinula stevensoni* assessed by RAPD-PCR. Heredity, 93: 154-160.

Whittaker RJ (1998). Island Biogeography: Ecology, Evolution, and Conservation. Oxford University Press, Oxford.

Yin Y, Geiger W & Martens K (1999). Effects of genotype and environment on phenotypic variability in *Limnocythere inopinata* (Crustacea: Ostracoda). Hydrobiologia, 400: 85-114.

The harbour porpoise *Phocoena phocoena* in the Belgian part of the North Sea: trends in abundance and distribution

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ABSTRACT. The harbour porpoise *Phocoena phocoena* is currently common in the Belgian part of the North Sea (BPNS), after decades of virtual absence. This article describes the results of aerial surveys to assess its distribution and density in Belgian waters, in concert with the first results of tests with passive acoustic monitoring devices (Porpoise Detectors - PoDs), and a basic analysis of strandings. The strandings data over four decades clearly demonstrate an increase of harbour porpoises in the BPNS, with only few stranded animals between 1970 and 1997 (0 to 6 per year), increasing numbers between 1998 and 2004 (8 to 40 per year), and even higher numbers between 2005 and 2009 (62 to 94 per year). The combined results of aerial surveys, strandings monitoring and tests with passive acoustic monitoring reveal a seasonal pattern, with harbour porpoises being abundant from February to April, and more scarce from May to January. Average densities in 2008 and 2009, as estimated by aerial monitoring covering most of the BPNS (with the exclusion of the nearshore 5 km strip), ranged from 0.05 animals per km², or in total less than 200 animals in an area equivalent to the BPNS in August 2009, to 1.01 animals per km², or in total almost 3,700 animals in an area equivalent to the BPNS during April 2008. In the first quarter of the year porpoises occur throughout the BPNS, including territorial waters (12 mile zone), whereas they are restricted to more offshore and northerly waters later in the year. Erratic invasions in the BPNS however blur general seasonal spatio-temporal patterns, which complicates our understanding of spatial distribution and migration.

KEY WORDS: Harbour porpoise, *Phocoena phocoena*, Belgian part of the North Sea, abundance, seasonal distribution

INTRODUCTION

The harbour porpoise *Phocoena phocoena* (Linnaeus, 1758) is by far the most common marine mammal in Belgian waters (CAMPHUYSEN & PEET, 2006; HAELTERS, 2009). Like most marine mammals, it is wide-ranging and highly mobile. A wide-scale southward shift in the summer distribution of harbour porpoises within the North Sea has been observed between 1994 and 2005 (SCANS II, 2008). Up to this day, the movements of harbour porpoises in the North Sea throughout the year have remained unclear, as have the driving forces behind them and behind the shift in distribution (SCANS II, 2008;

HAELTERS & CAMPHUYSEN, 2009). Yet, a good knowledge of the occurrence and distribution of harbour porpoises, and reliable predictive models are a prerequisite for the assessment of the conservation status of the species, as required for instance in the framework of the European Habitats Directive (92/43/EEC) and the assessment of the impact of human activities such as fisheries and the construction of offshore windfarms. This paper examines patterns in population size, spatial distribution and temporal variability of the harbour porpoise in Belgian waters, using quantitative observations and semi-quantitative detection methods.

MATERIALS AND METHODS

The current baseline situation (population size, spatial and temporal distribution) of the harbour porpoise in the Belgian part of the North Sea (BPNS) was studied through a combination of methods: (1) aerial line transect sampling to assess population size and spatial distribution; (2) passive acoustic monitoring (PAM) to investigate local short- to medium-term variability (weeks to months) in (relative) abundance, and (3) strandings data analysis to assess medium- to long-term trends in general occurrence.

Such a combined approach is needed given the difficulties in elucidating the population dynamics and behaviour of harbour porpoises, even if they are the most common marine mammal in one of the best studied marine areas in the world.

Aerial line transect sampling

The strategy used in aerial surveys is line transect sampling (BUCKLAND et al., 2001), in which a number of predefined tracks are flown and observations are recorded together with their perpendicular distance to the observation platform. The aircraft used was a high-winged two-engine Norman Britten Islander, owned by the Royal Belgian Institute of Natural Sciences (RBINS). This aircraft was equipped with one bubble window up to spring 2009, and from then onwards with two bubble windows, accommodating two observers. The survey altitude was 600 feet (183 m), and the groundspeed was kept at 100 knots (185 km/h). Flights were only performed during good to moderate observation conditions (sea states of 0 - 3). The surveys covered parallel track lines, 5 km apart and perpendicular to the coastline, to follow a presumed onshore–offshore gradient of porpoise density. For practical and flight-technical reasons (frequent other air traffic along the coast, vicinity of the Ostend and Koksijde airport and presence of a military shooting range), and given a sometimes high turbidity of coastal waters, survey tracks only started 5 km from the shore.

We measured the perpendicular angle from the trackline to the animals observed with a hand-held SUUNTO PM-5/360PC clinometer. From this angle, the shortest distance at which the aircraft passed the animal(s) was calculated. The flight track and the position of the observations were recorded by GPS. The programme DISTANCE (Version 6.0, Release 2) (THOMAS et al., 2009) was used to determine the most suitable detection model for the data collected, and to estimate average density and number of animals in the survey area. A similar methodology was used in SCANS II (2008) and during aerial surveys in neighbouring countries (eg. SIEBERT et al., 2006; SCHEIDAT et al., 2008; SCHEIDAT & VERDAAT, 2009).

During 2008 and 2009 five surveys were performed: 8-9 April 2008, 5 May 2008, 18-19 February 2009, 14-20 May 2009 and 4-5 August 2009. The surveys covered most of the BPNS (with a total surface of approximately 3,600 km²), only excluding a 5 km wide strip along the coast. The survey of 5 May 2008 covered a smaller area. The distances covered on track ranged from 448 km to 661 km (10 to 13 tracks), while the 5 May 2008 survey only covered 265 km (6 tracks). The individual tracks varied in length between 37 km and 63 km.

Given the relatively low number of detections, it was necessary to pool data across surveys, to make some assumptions, and to apply some parameters taken from literature:
(1) The probability of detecting animals is independent from group size.
(2) The detection probability remains constant over the area surveyed, season, time of day, density of animals, and between observers.
(3) All data can safely be pooled in order to establish a detection model. A hazard rate cosine adjusted distribution was selected as the detection function, on the basis of the Akaike Information Criterion (AIC) (BUCKLAND et al., 2001).
(4) As not all animals are observed on the track (perception bias), and some are not visible because they are too deep to be observed

(availability bias), a correction factor for the probability of seeing an animal or a group of animals at distance 0 needs to be applied. The correction factor, g(0), was given the value 0.45, as estimated by Hiby (2008) for similar surveys during good observation conditions; it was not possible to calculate this correction factor for the surveys undertaken, and no confidence values were applied to g(0), which needs to be taken account of when interpreting the CI presented for density and numbers.

(5) Given that a survey cannot be performed in a single flight, two single flights performed within a week and covering complementary tracks were treated as one survey, assuming that abundance and distribution remained similar during that week.

Passive Acoustic Monitoring

For PAM we used C-PoDs (porpoise detectors), which consist of a hydrophone, a processor, batteries and a digital timing and logging system, and have an autonomy of up to four months (www.chelonia.co.uk). A C-PoD does not record sound itself, but generates raw files with sound event characteristics, i.e. time, duration, dominant frequency and sound pressure level. In 2009 PoDs were anchored under water at two locations, on moorings of opportunity. The raw files generated were analyzed with the software CPOD.exe (Version 2.009), which applied a filter to only retain sounds identified as being porpoise clicks (within a certain probability). The data obtained provided an indication of the (relative) abundance of harbour porpoises in the vicinity of the device, up to a distance of approximately 300 m.

The PoDs provided continuous information over a short- to medium-term period, independent of weather conditions, for two locations. For the mooring of both PoDs, a tripod (VAN DEN EYNDE et al., 2010) was used: the PoD was attached to the central column of the tripod, at 1.5 m above the seafloor. A first PoD was moored from 19 October 2009 to 9 December 2009 at the Gootebank (51°26.9'N, 002°52.6'E;

21.4 km offshore; depth of 22 m below Mean Low Low Water Spring - MLLWS). The second PoD was moored, with short interruptions for servicing, from 6 November 2009 to 22 July 2010 at a location closely inshore (51°21.4'N, 003°07.0'E; 4.5 km offshore; depth of 6.5 m below MLLWS).

For the data analysis, only data of high and moderate train quality (high and moderate detection probability) were used, and the species filter was set to harbour porpoises (long narrow-band clicks in the 110-160 kHz range). All detections were visually inspected to remove any false positives, although it cannot be excluded that a small number remains, especially in noisy locations. The data obtained during the day of the mooring and the day of the retrieval of the PoD were excluded, given that the mooring platform (RV BELGICA) often remained in the vicinity for many hours after the placement of the mooring, or prior to the retrieval of the mooring, and thus possibly kept porpoises at a distance.

As a measure for harbour porpoise presence we used the number of detection positive 10 minutes per day (dp10m/d), or the number of 10 minute blocks per day in which porpoises were detected by the C-PoD. This measure was chosen over the number of detection positive minutes per day (dpm/d) as the number of detections was very low in many cases, and as this measure minimises the effects of variation between C-PoDs (C-PoD manual, www.chelonia.co.uk).

Strandings data

A last source of information included in this paper is strandings data. A trend in strandings can reflect a trend in the number of harbour porpoises at sea and can reveal seasonal patterns. Being legally protected, stranded and accidentally caught marine mammals must be reported to the authorities, represented by the Royal Belgian Institute of Natural Sciences (RBINS). To all possible extent, the carcasses are collected and made available for scientific research purposes. As a consequence of the legal requirements,

in combination with the easy public access to the Belgian shoreline and the fact that coastal authorities and members of the public are well informed, the marine mammal strandings database managed by the RBINS can be considered as fairly complete from 1990 onwards.

For this paper, general trends in monthly and yearly (i.e. medium- to long-term variability) strandings are presented. The data include a very small number of animals found dead at sea, and animals accidentally caught, and brought to port by fishermen. They also include accidentally caught animals that were discarded and subsequently washed ashore.

RESULTS

Aerial surveys

The detection model was based on 223 observations of a total of 264 porpoises. The resulting estimate of the Effective half strip width (ESW) was 144 m (95% CI: 129 m - 162 m). Between three and 43 harbour porpoises were detected per survey, which renders a population size estimate for an area equivalent to the BPNS, and covering it for the largest part, ranging from 186 to 3,697 individuals (Table 1). The average group size varied between 1.00 and 1.35 individuals. The May 2008, May 2009 and August 2009 surveys (i.e. late spring and summer) indicated the lowest density of harbour porpoises (max. 0.29 ind./km²), whereas the aerial surveys of April 2008 and February 2009 (i.e. late winter and early spring) yielded a much higher density (min. 0.63 ind./km²).

In April 2008 and February 2009, harbour porpoises were present both in territorial waters (12 nautical miles) and in waters further offshore, whereas observations during the May 2008, May 2009 and August 2009 surveys were virtually restricted to the northern half of the BPNS, further offshore (Figs 1-2).

TABLE 1

Results of the aerial surveys for marine mammals - overview of the observations of harbour porpoises: number of groups (number of animals) and estimates of the average group size (individuals, 95% Confidence Interval (CI)), density (ind./km², 95% CI) and abundance (number of animals, 95% CI) within a surface area equivalent to the Belgian part of the North Sea, i.e. 3,600 km². No estimate of the number of animals was made for the survey of 5 May 2008, given the incomplete coverage of the study area.

| Survey	Observations	Group size	Density	Abundance
8-9 April 2008	40 (43)	1.08 (1.00-1.16)	1.03 (0.65-1.63)	3,697 (2,330-5,867)
5 May 2008	5 (5)	1 (-)	0.29 (0.05-1.80)	-
18-19 February 2009	20 (27)	1.35 (1.04-1.75)	0.63 (0.40-1.00)	2,265 (1,429-3,592)
14-20 May 2009	12 (13)	1.08 (1.00-1.29)	0.15 (0.07-0.33)	556 (261-1,184)
4-5 August 2009	3 (3)	1 (-)	0.05 (0.02-0.15)	186 (62-554)
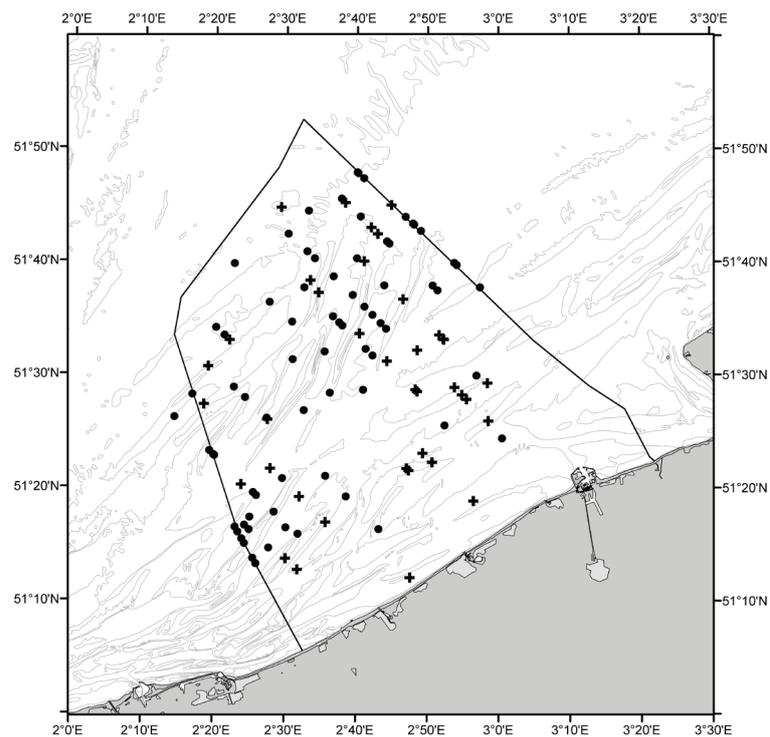
Fig. 1. – Detections of harbour porpoises during the survey of 8-9 April 2008 (circles) and 18-19 February 2009 (crosses). Observations made off track, as well as those made by the observer sitting at the side of the aircraft without bubble window, are included in the figure, but were not used in the analysis.
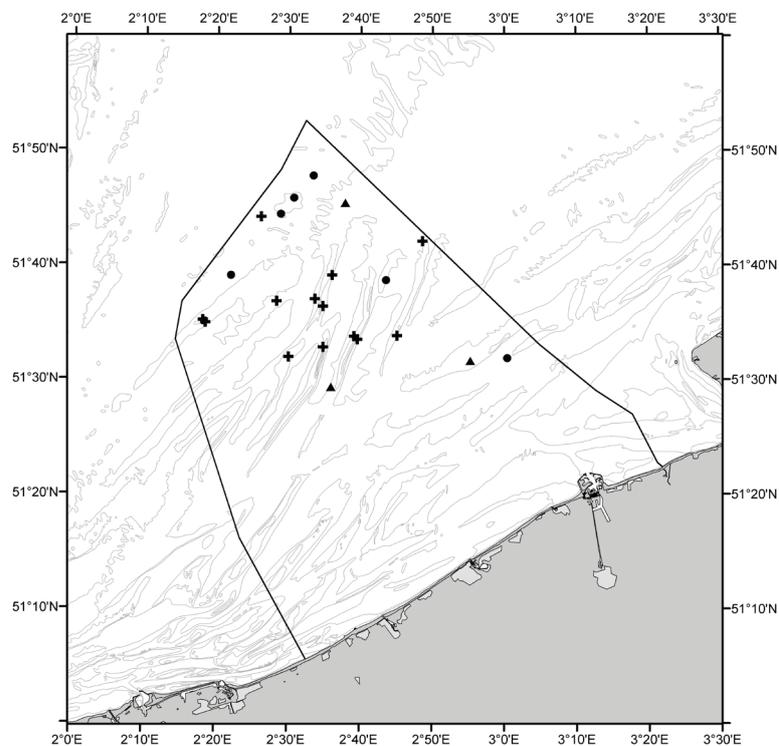

Fig. 2. – Detections of harbour porpoises during the survey of 5 May 2008 (circles), 14-20 May 2009 (crosses) and 4-5 August 2009 (triangles). Observations made off track, as well as those made by the observer sitting at the side of the aircraft without bubble window, are included in the figure, but were not used in the analysis.

Passive acoustic monitoring

Although the PoD moorings should be considered as a trial, given the low number used and the short period of time of one of the moorings, the analysis of the dp10m/d indicated a higher frequency of occurrence of harbour porpoises in the location further offshore than in the inshore location in November to the beginning of December 2009 (Wilcoxon signed rank test: p < 0.001) (Fig. 3). Furthermore, an increase in the number of dp10m/d was observed from October to December in the offshore location (on average 13 dp10m/d between mid-October to mid-November 2009, and 26 dp10m/d between mid-November and early December 2009). In the nearshore location, the number of detections was generally low (on average 4 dp10m/d between early November 2009 and the end of July 2010). Periods with slightly higher detection rates at MOW1 were the last fortnight of December 2009, short periods between the end of January and the beginning of February, and the end of March to mid-April 2010. The number of detections from May 2010 to the end of the mooring in July 2010 was very low, indicating very low numbers of harbour porpoises.

Strandings data

The total number of recorded stranded harbour porpoises between 1970 and 2009 was 597. The yearly number clearly increased from the late 1990s onwards: from only few animals between 1970 and 1997, to a peak in numbers in the period 2005-2007, with respectively 89, 94 and 86 animals (Fig. 4). In 2008 and 2009, the increase was interrupted, with respectively 62 and 66 ind./y.

Figure 4 also indicates a seasonality in strandings. The monthly number of stranded and bycaught animals peaked from March to May (monthly average: 14% of all animals) and in August (13% of all animals stranded). Only few animals were collected in June (8%) and July (6%) and between September and February (monthly average: 5% of all animals).
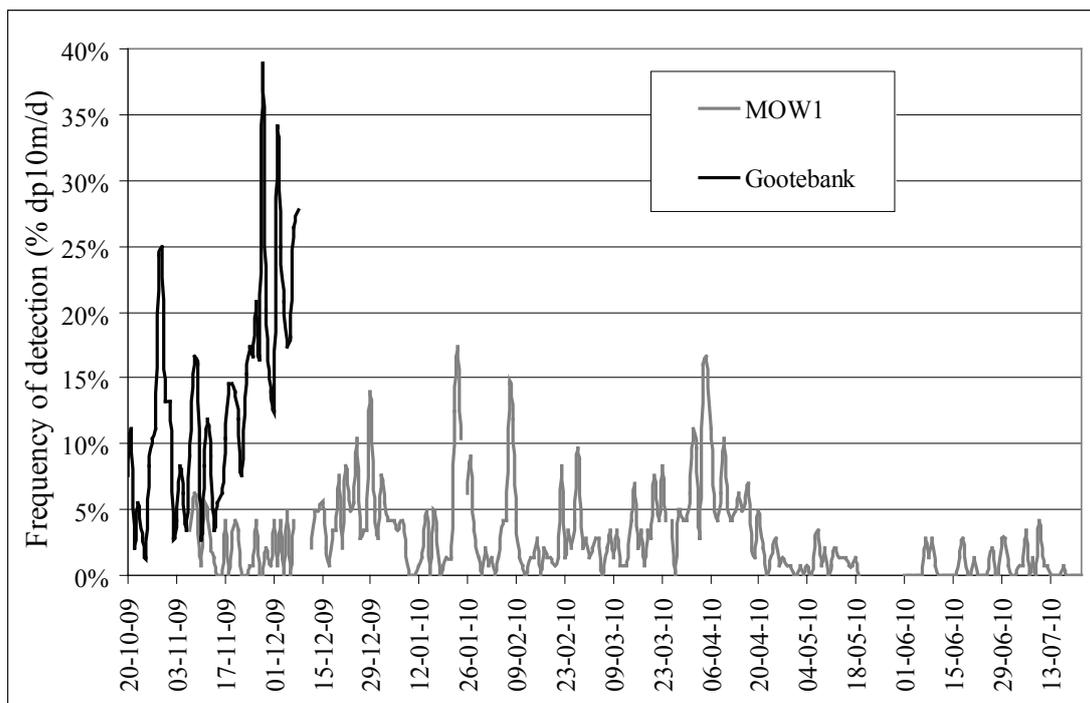

Fig. 3. − Results of the PoD moorings: frequency of detection (dp10m/d), expressed in % of the 144 blocks of 10 minutes per day, at the offshore Gootebank site (20 October to 8 December 2009: black line) and at the nearshore MOW1 site (7 November to 22 July 2010, with short interruptions for servicing the PoD: grey line).

	1970 to 1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	Total
Jan	0	1	1							1		2		1	4	1	3	2	1	1	6	24
Feb	3	1									3		4	1		1	6	2	9	3	3	36
Mar	3							1	1		3	1	3	1	4	8	4	20	9	11	6	75
Apr	3		1		2		1		1		3	1	4		7	8	21	18	13	9	5	97
May	4							1		2	1		5	2	4	6	18	17	14	8	5	87
Jun	0		1	2				1		1	1	1	2	2	1	2	7	9	4	7	5	46
Jul	0	1					1	2	1	1	1	2		1	4	7	3	1	4	5	3	37
Aug	3		1			1				1		1	1	2	8	5	13	13	13	4	10	76
Sep	1							2					1	4	2		6	5	12	3	13	49
Oct	3			1						1					1		4	1	4	8	8	31
Nov	2		1		1						4		1		2	1	2	3	3	3	1	24
Dec	2	1		1		1					2				1	1	2	3			1	15
Total	24	4	4	4	3	2	3	6	3	8	18	8	21	14	38	40	89	94	86	62	66	597

Fig. 4. – Number of harbour porpoises stranded on Belgian beaches per month from 1970 to 2009 (including bycaught animals), and totals per month and per year.

DISCUSSION

Monitoring strategy

Aerial surveys were chosen over ship-based surveys because predefined track lines can be covered easily, without having to take account of shipping lanes, anchorage areas and shallows. Also, a large area could be covered in a short period of time. For short- to medium-term (i.e. weeks to months) monitoring of cetaceans, PAM was chosen, a method which at present can only yield relative estimates of density. Although general trends can be discerned in the few PAM data that are available up to now, large day to day variations occurred in the harbour porpoise encounter rate. This illustrates that the distribution and density data obtained through aerial surveys should be considered with caution: accurate though only snapshot data.

The importance of Belgian waters for harbour porpoises

The harbour porpoise density estimates obtained from five aerial surveys in an area equivalent to, and covering almost the entire BPNS, range from 186 to 3,697 animals/km². These are the first absolute estimates of the number of harbour porpoises present in Belgian waters. This means that at least during part of the year, this species commonly occurred in the BPNS, whereas during other periods it was much scarcer. The estimates indicate that seasonally a number equivalent to 1.5% of the North Sea (summer) population of harbour porpoises, estimated at a quarter of a million individuals (SCANS II, 2008), can be found in the BPNS. Given its significant presence and its protection status, at the Belgian level (Royal Decree of 21 December 2001; Decision of the Flemish Government of 15 May 2009) as well as at the European level (Habitats Directive 92/43/EEC), it is clear that the harbour porpoise legitimately takes an important position in the assessment of the effects of human activities, such as fisheries and offshore construction.

The harbour porpoise showed a tenfold increase in the yearly number of stranded animals from 1970 to the first decade of the 21st century, an increase that started during the last years of the 20th century. This suggests a recent increase in harbour porpoise numbers in Belgian and surrounding waters. One should however take into account that strandings data as a relative estimate of density at sea is biased by meteorological conditions, incidental catches and a high mortality rate of juveniles. Between 2003 and 2006, for example, the proportion of

bycaught animals among the stranded animals for which a cause of death could be identified, rose from 19% to 63% (JAUNIAUX et al., 2008, HAELTERS & CAMPHUYSEN, 2009). Despite this, a similar increase in strandings has been observed in Dutch waters (WITTE et al., 1998; CAMPHUYSEN & PEET, 2006), and it should be interpreted as an increase of harbour porpoises in the Southern North Sea. The reappearance of this species seems to be due to a shift in the distribution of the population, rather than an increase in the population size (CAMPHUYSEN, 2004; SCANS II, 2008), and may have been caused by local reductions in prey availability, especially in the northern part of the North Sea. These reductions are probably the consequence of changes in environmental conditions (GREENE & PERSHING, 2000; BEAUGRAND et al., 2002; MACLEOD et al., 2007; SIMMONDS & ELLIOTT, 2009).

Spatial and temporal patterns

The spatial and temporal distribution of the harbour porpoises in Belgian waters indicates that harbour porpoises are relatively abundant in the BPNS from February to April, including in territorial waters. From May to August their numbers are lower, and they tend to stay in more offshore and more northerly waters. The few PoD measurements also indicated (1) a higher density offshore than inshore between October and December, (2) an increasing density from October to December, (3) a regular occurrence closely inshore between November and April, and (4) a low density inshore from May to July.

The strandings data showed a peak from March to May, which could be linked to higher nearshore densities in that period. The peak of strandings in May can partly be explained by the washing ashore of many decomposed animals, many of which probably died earlier in April (HAELTERS et al., 2006). To a large extent, the peak of strandings in August also concerned decomposed carcasses, mostly juveniles, probably drifted in from more offshore waters (HAELTERS & CAMPHUYSEN, 2009), although account should

be taken of a more rapid decomposition during the months with higher water temperatures.

The seasonal cycle in density and distribution, as described above, seems to be blurred by more erratic events, complicating our understanding of harbour porpoise's movements. Two examples of such erratic events were detected during the present study. Firstly, a dip in the number of strandings (a decrease of approximately 30%) was observed in 2008 and 2009. This dip could be due to a more offshore distribution of harbour porpoises during the first months of 2008 and 2009 in comparison to the previous years (HAELTERS, 2009; HAELTERS & CAMPHUYSEN, 2009). It should hence not necessarily be interpreted as a decrease in population size. Secondly, the relatively high proportion of stranded animals between August and October 2009 suggests a short intrusion into Belgian waters.

CONCLUSIONS

The assessment of strandings data, in combination with the results of the first aerial and PAM efforts in the BPNS have allowed us to draw some initial conclusions about the current spatio-temporal pattern of harbour porpoises in the BPNS. To draw firmer conclusions, more efforts are needed, especially in the field of PAM and aerial monitoring.

Harbour porpoises do not show a random spatio-temporal distribution in the BPNS: in general they are currently found abundantly throughout the whole BPNS from February to April, in numbers amounting up to 1.5% of the North Sea population (the North Sea population as estimated during summer surveys), whereas lower numbers tend to occur in more offshore waters during the rest of the year. The pattern observed might be described as migration, random movement, dispersal or avoidance of areas with temporarily poor feeding conditions. This pattern does not seem to be stable, possibly as a consequence of the small surface of Belgian

waters compared to the distribution range of the harbour porpoise, the fact that it is a highly mobile species, and that Belgian waters are at the edge of their distribution range in the North Sea. Therefore studies covering a larger spatial (e.g. the whole North Sea) and temporal scale are necessary, in combination with studies on harbour porpoise population dynamics and on the distribution and abundance of their dominant prey species.

ACKNOWLEDGEMENTS

REFERENCES

Beaugrand G, Reid PC, Ibañez F, Lindley JA & Edwards M (2002). Reorganization of North Atlantic Marine Copepod Biodiversity and Climate. Science, 296(31): 1692-1694.

Buckland ST, Anderson DR, Burnham KP, Laake JL, Borchers DL & Thomas L (2001). Introduction to Distance Sampling: estimating abundance of biological populations. Oxford University Press.

Camphuysen CJ (2004). The return of the harbour porpoise (*Phocoena phocoena*) in Dutch coastal waters. Lutra, 47(2): 113-122.

Camphuysen CJ & Peet G (2006). Whales and dolphins of the North Sea. Fontaine Uitgevers, Kortenhoef.

Greene CH & Pershing AJ (2000). The response of *Calanus finmarchicus* populations to climate variability in the Northwest Atlantic: basin-scale forcing associated with the North Atlantic Oscillation. ICES Journal of Marine Science, 57: 1536-1544.

Haelters J (2009). Monitoring of marine mammals in the framework of the construction and exploitation of offshore wind farms in Belgian marine waters. In: Degraer S & Brabant R (Eds.). Offshore wind farms in the Belgian part of the North Sea: State of the art after two years of environmental monitoring. Royal Belgian Institute of Natural Sciences, Department MUMM, 237-266.

Haelters J & Camphuysen K (2009). The harbour porpoise in the Southern North Sea: abundance, threats and research- & management proposals. Royal Belgian Institute of Natural Sciences (RBINS/MUMM) and the Royal Netherlands Institute for Sea Research (NIOZ); report commissioned by the International Fund for Animal Welfare (IFAW).

Haelters J, Jauniaux T, Kerckhof F, Ozer J & Scory S (2006). Using models to investigate a harbour porpoise bycatch problem in the Southern North Sea–eastern Channel in spring 2005. ICES CM 2006/L:03.

Hiby L (2008). Effective strip half-width estimates from aerial survey data. In: SCANS II, 2008. Small Cetaceans in the European Atlantic and North Sea (SCANS II). Final Report to the European Commission, Appendix D3.1.

Jauniaux T, Berguerie H, Camphuysen K, Daoust P-Y, Drouguet O, Ghisbain T, Garcia-Hartmann M, Grondin A, Haelters J, Jacques T, Kiszka J, Leopold M, Pezeril S, Schnitzler J & Coignoul F (2008). Causes of death of harbor porpoises (*Phocoena phocoena*) stranded on the continental coastline of the southern North Sea (Belgium, France, and Dutch

coasts) between 1990 and 2007. ICES Annual Science Conference, Halifax, Canada, ICES CM 2008/D:09.

MacLeod C, Santos M, Reid RJ, Scott B & Pierce GJ (2007). Linking sandeel consumption and the likelihood of starvation in harbour porpoises in the Scottish North Sea: could climate change mean more starving porpoises? Biology Letters, 3: 185–188.

SCANS II (2008). Small Cetaceans in the European Atlantic and North Sea (SCANS II). Final Report to the European Commission under project LIFE04NAT/GB/000245. Available from SMRU, Gatty Marine Laboratory, University of St Andrews, St Andrews, Fife, KY16 8LB, UK.

Scheidat M, Gilles A, Kock KH & Siebert U (2008). Harbour porpoise *Phocoena phocoena* abundance in the southwestern Baltic Sea. Endangered Species Research, 5: 215–223.

Scheidat M & Verdaat H (2009). Distribution and density of harbour porpoises in Dutch North Sea waters. IMARES Report N° C125/09, Wageningen.

Siebert U, Gilles A, Lucke K, Ludwig M, Benke H, Kock KH & Scheidat M (2006). A decade of harbour porpoise occurrence in German waters - Analyses of aerial surveys, incidental sightings and strandings. Journal of Sea Research 56(1): 65-80.

Simmonds MP & Elliot W (2009). Climate change and cetaceans: concerns and recent developments. Journal of the Marine Biological Association of the United Kingdom, 89(1): 203-210.

Thomas L, Laake JL, Rexstad E, Strindberg S, Marques FFC, Buckland ST, Borchers DL, Anderson DR, Burnham KP, Burt ML, Hedley SL, Pollard JH, Bishop JRB & Marques TA (2009). Distance 6.0. Release 2. Research Unit for Wildlife Population Assessment, University of St. Andrews, UK. http://www.ruwpa.st-and.ac.uk/distance/

Van den Eynde D, Brabant R, Fettweis M, Francken F, Van Lancker V, Sas M & Melotte J (2010). Monitoring of hydrodynamic and morphological changes at the C-Power and Belwind offshore windmill sites – A synthesis. In: Degraer S, Brabant R & Rumes B (Eds.). Offshore wind farms in the Belgian part of the North Sea: Early environmental impact assessment and spatio-temporal variability. Royal Belgian Institute of Natural Sciences, Department MUMM, 19-36.

Witte RH, Baptist HJM & Bot PVM (1998). Increase of the harbour porpoise *Phocoena phocoena* in the Dutch sector of the North Sea. Lutra, 40: 33-40.

SHORT NOTES

Facultative nesting in *Rhinella spinulosa* (Anura: Bufonidae): strategy to avoid dehydration of offspring

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KEY WORDS: *Rhinella spinulosa*; facultative nesting; San Juan; reproductive mode; dehydration; hot climate.

The diversity of reproductive modes in amphibians is greater than that observed in other groups of vertebrates. Reproductive strategies of anurans are likewise varied and related to the characteristics of the environment as well as to the presence of vegetation and variation in the water depth, among others (DUELLMAN & TRUEB, 1986). LAVILLA & ROUGES (1992) described eighteen reproductive modes for Argentinean amphibians, but nest building was not included. The construction of nests (or breeding-stalls) has been described for different species of Hylidae such as *Hypsiboas pardalis*, *Hypsiboas faber*, *Hypsiboas boans*, *Hypsiboas wavrini* and *Hypsiboas rosenbergi* (LUTZ, 1960; DUELLMAN, 1970; CRUMP, 1974; KLUGE, 1981; MARTINS & MOREIRA, 1991; HADDAD & HOLD, 1997).

The toad *Rhinella spinulosa* (WIEGMANN, 1834) inhabits the cordilleran and pre-cordilleran regions of Argentina, Chile, Bolivia and Peru (FROST, 2011). In Argentina, *R. spinulosa* is reported from Catamarca, Jujuy, La Rioja, Mendoza, Salta, San Juan and Tucuman, in the 900-4,000 masl altitudinal range (CEI, 1980).

This species usually deposits egg chains at the bottom of water bodies (SINSCH, 1988; 1990), where the embryonic development and hatching occurs (LAVILLA & ROUGES, 1992). Here, we report and describe for the first time the construction of breeding-stalls for a population of *R. spinulosa* inhabiting desert environments of San Juan Province, Argentina.

The study area is located 70km west of San Juan city, in "Sierra de la Dehesa" (31.3497 W; 68.8333 S; Datum: WGS84, elevation: 1,560m). The environment is characterized by numerous shallow streams (water depth: 2- 12cm). Stream vegetation is dominated by *Cortadera rudiscula* and *Baccharis salicifolia*. Marginal vegetation is typical of the xeric environments with low cover. The region belongs to the Monte Phytogeographic Province, characterised by an arid climate with a mean annual temperature of 17.3ºC, a maximum mean annual temperature of 25.7ºC, a minimum mean annual temperature of 10.4ºC and a mean annual rainfall of 89mm, concentrated mainly in summer (CABRERA, 1976). According to the Köppen Climate Classification System, Monte desert belongs to the BWw climate zone, which represents a desert with summer precipitation (POBLETE & MINETTI, 1999).

We visited the study area for three consecutive days in August 2006 in order to characterize nests of *R. spinulosa*. The current selection was

based on accessibility and the number of nests that could be easily found in this place. Also, the distance between streams was at least five kilometres, which made daily measurements of water levels of the streams possible. We measured water depth of the stream flow every three hours (eight times a day) using a digital calliper (Essex; ±0.1mm). A total of fifteen nests were studied, and we measured the following variables using the digital calliper: wall height, water depth in the centre of the nest, water depth outside the nest. We recorded water temperature inside the nest and under the egg mass, and outside the nest with two digital thermometers (Barnant model 600-1040, USA; ±0.1ºC). Nest surface was calculated as $A=\pi*r^2$ (where: A=area; π=3.14; r=radius). Furthermore, nest position (under shade or sun-exposed) and position of the egg chain within the nest (centre or side) were also registered. All measurements of the nest were taken between 1400 to 1800 hours. All variables are expressed as mean ± standard error. We conducted Sign tests to compare measurements of water temperature and water depth between inside and outside of nests; the generalized linear model (GLM) was used to test differences in the water depth of a stream during the day. We used the R statistical software (R Development Core Team, 2008) for data analysis.

Stream water depth showed significant changes during the day [GLM: water deep ~ hours, family = Gamma (log)], a minimum depth record at 1 400 (p<0.001), 1 700 (p<0.001) and 2 000 (p<0.001) hours (Fig. 1; Table 1). Water depth variation within mountain streams in the xeric environment is insufficiently studied. We had
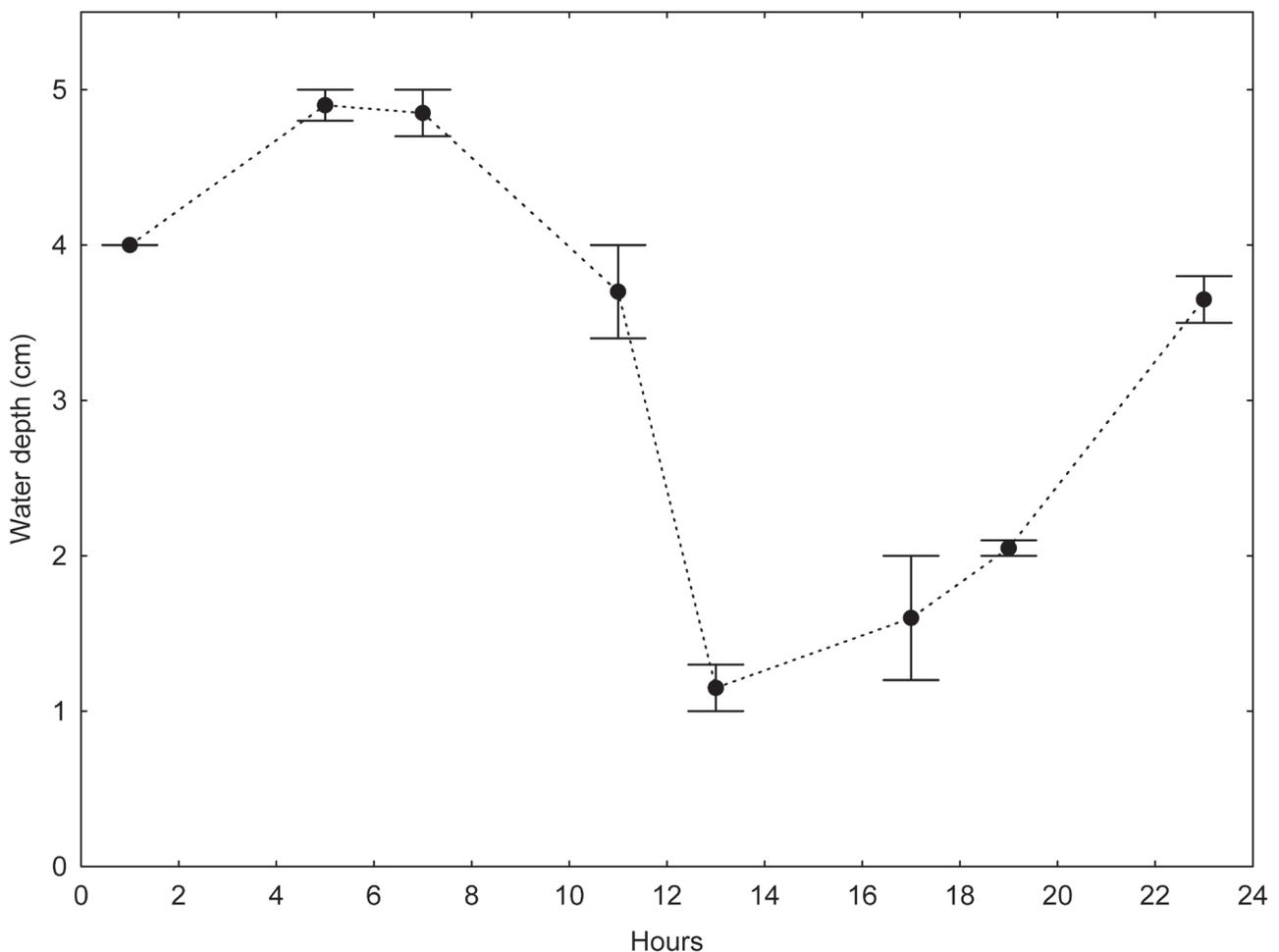

Fig. 1. – Variation of water depth of the studied streams. The level of the water was measured every three hours during two days.

TABLE 1

Results from generalized linear models testing effects of hours and stream water depth in Sierra de la Dehesa, San Juan, Argentina.

	ESTIMATE	STD. ERROR	T-VALUE	p	WATER LEVEL (CM)
Intercept	1.58924	0.09232	17.215	0.001	
1100 h	0.27193	0.11918	-2.282	0.05	3.73±0.18
1400 h	-1.52470	0.11918	-12.793	0.001	1.07±0.12
1700 h	-1.14028	0.11918	-9.568	0.001	1.57±0.23
2000 h	-0.81605	0.11918	-6.847	0.001	2.17±0.12
2300 h	-0.26304	0.11918	-2.207	0.05	3.77±0.15
0200 h	-0.19464	0.11918	-1.633	>0.05	4.03±0.03
0500 h	0.01351	0.11918	0.113	>0.05	4.97±0.09

observed this circadian variation of water depth in other streams of Monte desert, where marked reductions of the water level coincided with the hours of maximum insolation.

The nests of *R. spinulosa* were circular, pot-shaped, built on sandy soils, and had a mean surface area of 762.36±168cm² and a mean diameter of 30.1±8.5cm. The wall that surrounded the clutch was 3.5±1.2cm high. Mean water temperature in the centre of the nest was 20±1.2ºC, while the external water temperature was 18.3±1.5ºC. We did not detect significant differences between water temperature inside and outside of the nests (Sign test: Z=1.22, P=0.22). Mean depth in the centre of nests was 3.5±0.63cm and 1±0.5cm outside of them. Depth differences were statistically significant (Sign test: Z=2.04, P<0.004). All the nests were found sun-exposed with egg chains laid in the centre of the nests, where the water is deeper (Fig. 2).

There are two hypotheses on the benefits of nest-building. The first one is based on the isolation of eggs and embryos to protect them from potential predators, including cannibalism from conspecific tadpoles (CRUMP, 1974; KLUGE, 1981). However, *R. spinulosa* is the only anuran species inhabiting these streams and predatory fish are absent. *R. arenarum,* like *R. spinulosa,* begins reproduction at the end of winter, by middle August (SANABRIA et al., 2005) when temperatures are low (about 12.7°C). It is likely that it minimizes the predation of eggs and tadpoles this way, because invertebrate predators are not active at the end of the winter (HEYER et al., 1975). The second hypothesis suggests that environmental temperature within the breeding-stall is higher than in the surroundings, which implies accelerated embryonic growth (LAMOTTE & LESCURE, 1997). All the clutches of *R. spinulosa* in this study were located at sun-exposed sites. In desert environments, the high radiation during daylight (WARNER, 2004) provides enough energy for the fast development of embryos and tadpoles. Female *R. arenarum* selectively lay eggs on open sites (SANABRIA et al., 2007), behaviour that promotes the early development of embryos, which behave in this sense as black bodies, enhancing radiation absorption (SAVAGE, 1975; SEALE, 1982). Although clutches of *R. spinulosa* are in the sun, the differences in the temperature between inside and outside the nest were not significant in our study. We therefore propose the hypothesis that adults of *R. spinulosa* build nests to avoid stream
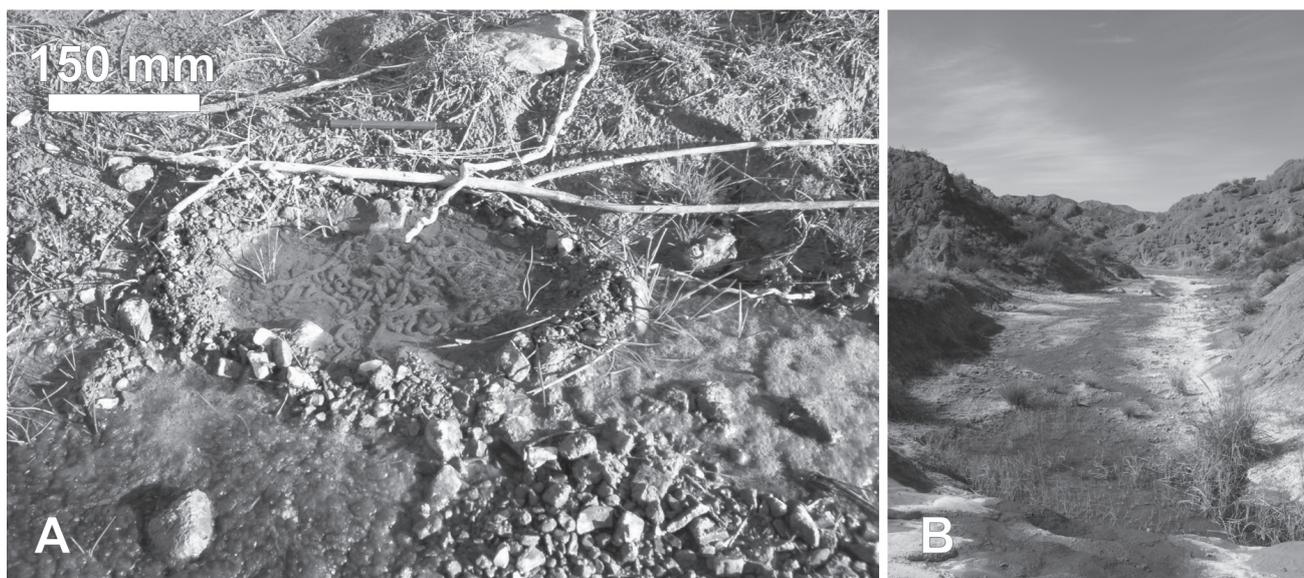
Fig. 2. – (A): The nest of *Rhinella spinulosa*. Eggs are deposited in the centre of the nest. Water level outside the nest water is lower than inside. (B): Typical environment where adults of *R. spinulosa* build the facultative-nest.

water fluctuations throughout the day, assuring in that way the survival of their offspring by avoiding dehydration of eggs and tadpoles. During hours of maximum insolation (1400-2000) streams show a minimum water depth. This effect is seen in a narrowing of the stream and the consequent decrease of water around the nest. Sanabria and collaborators (2005) observed in *R. arenarum* that the repeated decrease of water depth in natural environments caused the death of eggs and tadpoles. The facultative nest-building may be an adaptation of *R. spinulosa* for ensuring the successful raising of offspring in different habitats and in different seasons. This facultative behaviour would allow this species to conserve energy by not constructing nests in environments and periods where the water level is more stable.

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REFERENCES

1. CABRERA AL (1976). Enciclopedia Argentina de Agricultura y Jardinería, Tomo II, Regiones Fitogeograficas Argentinas, Editorial ACME S.A.C.I, Argentina.

2. CEI JM (1980). Amphibians of Argentina. Monographs 2. Monitore Zoologico Italiano (NS), Italy.

3. CRUMP ML (1974). Reproductive strategies in a tropical anuran community. University of Kansas Museum Natural History Miscellaneous Publication, 61:1-68.

4. DUELLMAN W & TRUEB L (1986). Biology of Amphibians. MacGraw-Hill Book Company, London.

5. DUELLMAN WE (1970). The hylid frogs of Middle America. Monographs, 1, University of Kansas Museum Natural History, USA.

6. FROST DR (2011). Amphibian Species of the World: an Online Reference. (Internet address: http://research.amnh.org/vz/herpetology/amphibia/)

7. HADDAD CFB & HOLD W (1997). New reproductive mode in Anurans: Bubble nest in *Chiasmocleis leucosticta* (Microhylidae). Copeia, 1997:585-588.

8. HEYER WR, MCDIARMID RW & WEIGMANN DL (1975). Tadpoles predation and pond habitats in the tropics. Biotropica, 7:100-111.

9. KLUGE AG (1981). The life history, social organization, and parental behavior of *Hyla rosenbergi Boulenger*, a nest-building gladiator frog. Miscellaneous Publication of Museum of Zoology University of Michigan, USA.

10. LAMOTTE M & LESCURE J (1977). Tendances adaptatives a L´affranchissement du milieu aquatique chez las amphibiens anoures. Terre Vie, 31:225-331.

11. LAVILLA EO & ROUGES M (1992). Reproducción y desarrollo de anfibios argentinos. Editorial: Asociación Herpetológica Argentina, Argentina.

12. LUTZ B (1960). The clay nests of *Hyla paradalis* Spix. Copeia, 1960:61-63.

13. MARTINS M & MOREIRA G (1991). The nest and the tadpole of *Hyla wavrini*, Parker (Amphibia: Anura). Memórias do Instituto Butantan, 53:197-204.

14. POBLETE GA & MINETTI JL (1999). Configuración Espacial del Clima de San Juan. CD Síntesis del Cuaternario de la Provincia de San Juan. INGEO Universidad Nacional de San Juan.

15. R DEVELOPMENT CORE TEAM (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

16. SANABRIA E, QUIROGA L & ACOSTA JC (2005). Patrones de actividad temporal estacional y uso de microhábitat de una población de adultos de *Bufo arenarum*, en los humedales de Zonda, San Juan, Argentina. Boletín Sociedad Herpetología Mexicana, 13:61-65.

17. SANABRIA E, QUIROGA L & ACOSTA JC (2007). Sitios de Oviposición y Estimación del Esfuerzo Reproductivo en *Chaunus arenarum*, en el Desierto del Monte, Argentina. Revista Española de Herpetolgía, 21:49-53.

18. SAVAGE RM (1975). A thermal function of the envelope of the egg of common frog, *Rana temporaria temporaria* (Limm) with observations on the structure of the eggs clusters. Brazilian Journal of Herpetology, 1:57-66.

19. SEALE DB (1982). Physical factors influencing oviposition by the woodfrog, *Rana sylvatica*, in Pennsylvania. Copeia, 1982:627-635.

20. SINSCH U (1988). El sapo andino *Bufo spinulosus*: análisis preliminar de su orientación hacia sus lugares de reproducción. Boletín de Lima 57:83-91.

21. SINSCH U (1990). Froschlurche (Anura) der zentralperuanischen Anden: Artdiagnose, Taxonomie, Habitate, Verhaltensökologie. Salamandra, 26:177-214.

22. WARNER TT (2004). Desert Meteorology. Cambridge University Press. New York.

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One more alien freshwater fish species in New Caledonia: the three-spot gourami *Trichogaster trichopterus* (Teleostei: Osphronemidae)

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Reporting the occurrences of non-native species established in a new territory is an important first step to thereafter evaluating both the spread of potential invaders and their consequences for native biodiversity. This is of particular relevance when the newly colonized areas are hotspots of biodiversity. Located in the southern Pacific, the hydrological drainages of the Grande-Terre of New Caledonia (i.e. the main and largest island of the New Caledonia Archipelago, Fig. 1) are recognized as hosting a vast diversity of freshwater fish and crustacean species. Among the 104 recovered species (reviewed by MARQUET et al., 2003 (1)), around one quarter are endemic, with some of them having been very recently described (e.g., *Stiphodon mele* KEITH et al., 2009 (2)). These species sometimes co-exist with introduced taxa that pose a threat for their survival (3; 4). Here, we report the presence of an alien osphronemid fish species identified as
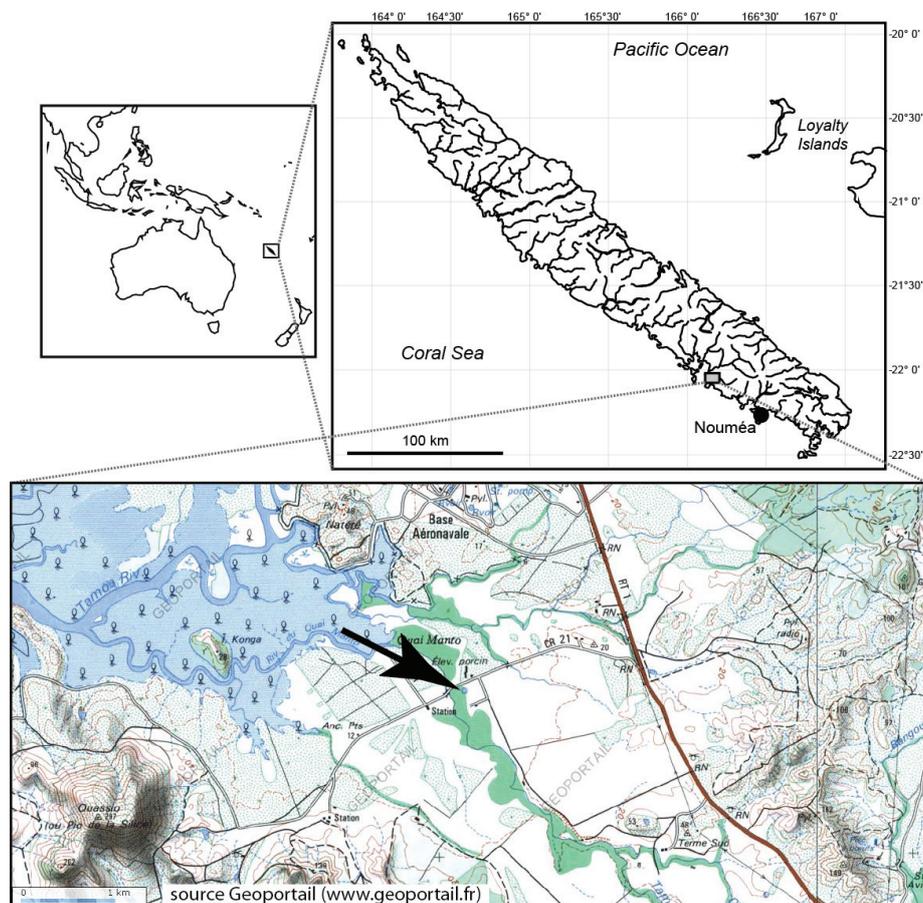

Fig. 1. – Geographic localization of New Caledonia and the pond hosting a population of *Trichogaster trichopterus*. The arrow points the exact location of the pond.

Trichogaster trichopterus (Pallas, 1770) for the first time in New Caledonia.

In two hours fishing effort by one of us (C.F.), 22 individuals were caught using a small-mesh cast-net in a freshwater pond located within the lower reach of the Tamoa drainage (Province Sud, coordinates: 166° 13' 1.77" E, 22° 2' 27.15" S; Fig. 1). These specimens were captured along with a second alien species, the Mozambique tilapia *Oreochromis mossambicus* (Peter, 1852) (*n*=35).

One representative specimen is depicted in Fig. 2. The taxonomic identification is based on descriptions of RAINBOTH (5). The captured specimens exhibit a dorsal fin anteriorly starting far behind the anal fin and a long filamentous pelvic fin. Together, these are characteristic features of the genus *Trichogaster*. The presence of two large dark spots on the side (one at the centre of the body and the other at the basis of the caudal peduncle) and of vertical bars on the anterior part allow us to unambiguously identify this species as the three-spot gourami *Trichogaster trichopterus*. This is further supported by comparisons with *T. trichopterus*

museum specimens hosted in the Bavarian State Collections of Zoology, Munich (ZSM) from Malaysia (*n*=3) and Borneo (*n*=5) which clearly exhibit similar gross morphologies.

Two other species of Osphronemidae have already been introduced in New Caledonia (1). While the giant gourami *Osphronemus gouramy* (Lacépède, 1801) is probably not established, populations of the snakeskin gourami *Trichogaster pectoralis* (Regan, 1910) were recently recovered in one drainage in the northern part of the island (1). Until now, there had been no report of the occurrence of *T. trichopterus* in New Caledonia (1; 4). *T. trichopterus* originates from the Mekong basin where it is found in sluggish or standing-water habitats. It is reported as 'established' (here synonym of 'naturalized', i.e. a species sustaining self-reproducing populations, see e.g. ref. (6)) out of its native range in Asia (Taiwan, Sri Lanka, Philippines), Oceania (Papua New Guinea), Africa (Namibia) and the New World (Columbia, Dominican Republic) (7). To our knowledge, the presence of *T. trichopterus* in New Caledonia is the second occurrence of this species in Oceania (after New Guinea), and the first clue for its spread south-eastward in the Pacific.
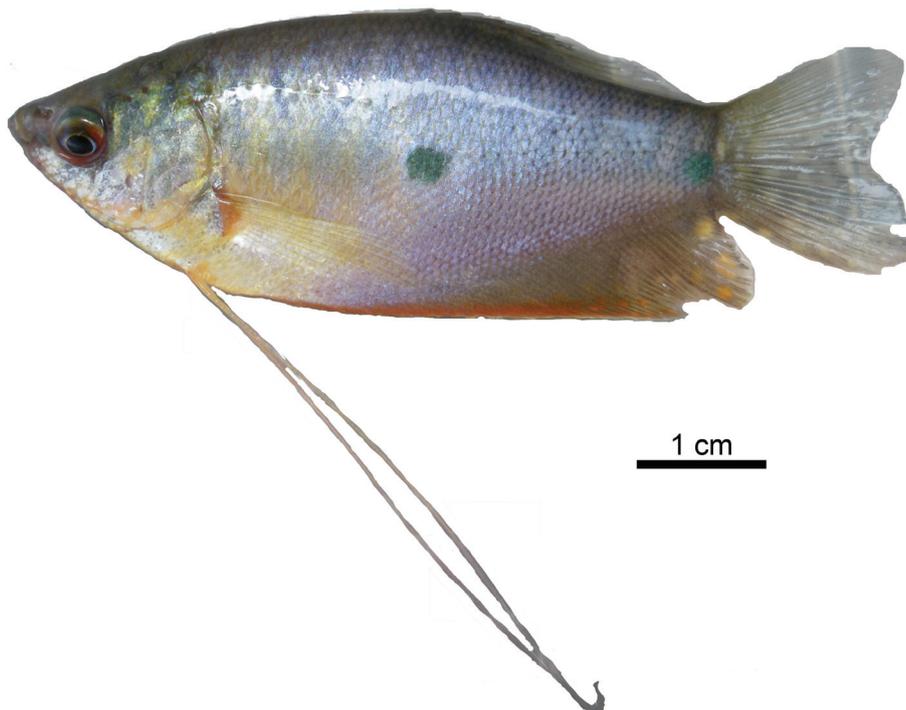

Fig. 2. – An adult specimen of *Trichogaster trichopterus* collected in New Caledonia.

T. trichopterus is widespread in aquarium fish trade and was probably transported to New Caledonia as an ornamental species and then released in the wild. The pond that provided specimens is connected with a tributary of the Tamoa River during the period of floods, suggesting that this species has already spread locally in the drainage. The relatively high number of captured specimens and the already-reported presence of naturalized populations out of the native range (7) suggest that *T. trichopterus* is probably also established in New Caledonia. If other specimens are found in neighbouring drainages, the species could thereafter be considered as established. At least two features of this species would favour its dispersal: (i) Its aesthetic qualities that could stimulate its capture and human-induced transfers as ornamental fish across New Caledonia (or on a larger scale) and (ii) its air-breathing capacity (8), which could favour its survival in eutrophic ponds or swamps before its natural dispersal during flood periods.

T. trichopterus individuals mainly feed on crustaceans, zooplankton and insect larvae (5). At the present time, potential impacts on aquatic invertebrate communities are difficult to evaluate but a potential negative effect can be hypothesized and could be investigated (e.g. through analyses of stomach contents). According to the recent survey of Keith (4), *T. trichopterus* is the 14th freshwater fish species introduced in New Caledonia and could be the 7th one to become established. Estimating its actual repartition, alerting the public to its presence and its potential negative impacts and encouraging people to remove and kill encountered specimens could represent first steps to management of this new alien species.

REFERENCES

1. Marquet G, Keith P & Vigneux E (2003). Atlas des poissons et des crustacés d'eau douce de Nouvelle-Calédonie. Patrimoines Naturels, Paris: 282 pp.

2. Keith P, Marquet G & Pouilly M (2009). *Stiphodon mele* n. sp., a new species of freshwater goby from Vanuatu and New Caledonia (Teleostei, Gobiidae, Sicydiinae), and comments about amphidromy and regional dispersion. Zoosystema, 31:471-483.

3. Keith P (2002). Threatened fishes of the world: *Galaxias neocaledonicus* Weber & de Beaufort, 1913 (Galaxiidae). Environmental Biology of Fishes, 63:26-26.

4. Keith P (2005). Introduction of freshwater fishes and decapod crustaceans in New Caledonia, a review. Revue d'Ecologie - la Terre et la Vie, 60: 45-55.

5. Rainboth WJ (1996). Fishes of the Cambodian Mekong, Rome, Italy: 265 pp.

6. Richardson DM, Pysek P, Rejmanek M, Barbour M, Panneta F & West C (2000). Naturalization and invasion of alien plants: concepts and definitions. Diversity and Distributions, 6:93-107.

7. Fishbase (2011). Fishbase. See http://www.fishbase.org/home.htm. Accessed: 01 April 2011.

8. Herbert NA & Wells RMG (2001). The aerobic physiology of the air-breathing blue gourami, *Trichogaster trichopterus*, necessitates behavioural regulation of breath-hold limits during hypoxic stress and predatory challenge. Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology, 171:603-612.

Salivary amino acid concentrations in zebus (*Bos indicus)* and zebu hybrids (*Bos indicus × Bos taurus*) fed a tannin-rich diet

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Many animals show adaptation to tannins in the form of tannin-binding salivary proteins (1). Among ruminants, such proteins have been demonstrated in saliva of several species (usually browsers and intermediate feeders) (2, 3, 4, 13). There is some circumstantial evidence to suggest that zebu cattle (*Bos indicus*) are different from temperate cattle breeds with respect to their salivary and digestive physiology. Apart from differences in susceptibility to heat and tropical disease (5), a difference in salivary anti-tannin defenses (and a resulting difference in rumen physiology) could be another reason zebu cattle are particularly suited for agricultural systems in the tropics, where available forages often contain high levels of tannins (6, 7). Although non-proline-rich proteins exist that also have affinity for tannins (1, 8), it is interesting to compare the proline content of different cattle breeds. Here, we report such a screening for a comparison of zebu cattle and zebu-Holstein-Friesian in the Jimma area located at 7°40′N and 36°50′E at 1760 masl in southwest Ethiopia. For the study eight heifers were used: four were zebu (100% *Bos indicus*) and four were zebu × Holstein Friesian (HF) crosses. The blood level of crossbreed heifers (*Bos indicus × Bos taurus*) were composed of 70% HF+30% zebu, heifer 1; 66% HF+ 34% zebu, heifer 2; 68% HF+ 32% zebu, heifer 3 and 70% HF+ 30% zebu, heifer 4. The animals were 2.5 years old with comparable body weight and similar body condition scores.

The body condition score was evaluated based on 1-9 point score scale (9). They were fed on a diet that included the tannin-rich plant *Albizia gummifera* for 28 days. The animals were fed on a local hay mixture as a basal diet and experimental diet of leaves of *A. gummifera*. The diets were composed weekly to ensure that cattle would consume *A.gummifera* at a rate of 10% of total dry matter (DM) requirement, estimated as 2.5% of live body weight. To minimize selectivity by the animals, the *A. gummifera* forage was provided in the morning (8:00) whereas hay mixture was offered only later at 10:30. After 21 days, saliva samples were collected from the animals' mouths using a sponge. When the sponge was saturated with saliva, it was squeezed manually (with the investigator wearing fresh latex gloves), allowing the collection of a minimum of 10 ml saliva into a plastic cup with screw top. The saliva was then passed through a tea sieve to remove feed particles, and stored at -43°C. When the samples were thawed for analysis, they were passed through a 0.3µm syringe filter to remove bacteria. Amino acids were determined according to Hendriks et al. (2002). From these data, the proportion of proline in the total amount of measured amino acids was calculated. Differences between genotypes were evaluated by means of a Student's t-test. Significant differences were considered at $P < 0.05$.

The chemical composition of a test diet (*A. gummifera)* and the hay mixture included in the study (g/kg DM) are presented in Table 1. Saliva from pure zebu always had either similar or numerically higher concentrations of

TABLE 1

Chemical composition of *Albizia gummifera* and the hay mixture applied in the study (g/kg DM).
DM: dry matter; OM: organic matter; CP: crude protein; EE: ether extract; CF: crude fibre: NFE: nitrogen free extract (16); NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; HC: Hemicellulose (17); CT: condensed tannins as measured by the butanol-HCl-iron method (18).

FEED STUFF	DM	OM	Ash	CP	EE	CF	NFE	NDF	ADF	ADL	HC	CT
A. gummifera	904	955	45	294	13.3	380	268	740	575	116	165	72
Hay mixture	944	886	114	227	4.9	249	405	892	687	201	205	-

TABLE 2

Salivary amino acid concentrations (mg/l) in pure zebu cows and zebu x Holstein crossbreeds fed a tannin-rich diet.

	ZEBU (N=4)		CROSSBREED (N=4)		*P*	%DIFFERENCE
	MEAN	SD	MEAN	SD		
proline	1.8	0.4	1.0	0.2	0.014	80
threonine	2.8	0.8	1.6	0.2	0.030	75
serine	2.2	0.6	1.4	0.1	0.039	57
glycine	1.9	0.6	1.2	0.2	0.112	58
arginine	1.6	0.4	1.2	0.1	0.149	33
alanine	2.2	0.7	1.7	0.3	0.210	29
histidine	1.2	0.3	0.9	0.1	0.231	33
valine	2.4	0.7	1.9	0.3	0.280	26
isoleucine	1.5	0.5	1.2	0.2	0.364	25
leucine	2.8	0.8	2.3	0.3	0.356	22
glutamate	4.5	1.3	3.8	0.5	0.372	18
tyrosine	1.1	0.4	1.0	0.1	0.746	10
aspartate	3.1	0.9	3.0	0.4	0.862	3
lysine	2.0	0.6	2.0	0.3	0.996	0
phenylalanine	1.3	0.4	1.3	0.2	0.929	0
Sum	32.4	9.4	25.5	3.5	0.24	27

individual or total amino acids than did saliva from crossbreeds (Table 2). The difference was significant for proline, threonine and serine ($P<0.05$). Correspondingly, when the concentration of proline was expressed as a proportion of all amino acids, pure zebu had a significantly higher proportion of proline in saliva than did crossbreeds (P<0.05; Fig. 1).

These findings suggest that differences exist between zebu and Holstein-Friesian breeds with respect to salivary amino acid composition; the differences are strongly suggestive of a difference in salivary anti-tannin defense, although this needs to be further substantiated by studies on tannin-binding capacities (2, 3). Natural browsers such as goats or deer are better adapted to tannin-rich diets than are domestic cattle, which are grazers, because of a lack of tannin-binding proteins in the cattle (2, 6, 12, 13). Threonine (14) and serine (15) are two of the amino acids least affected by tannin; however, in our finding zebu cattle heifers which had a long exposure to a tannin-rich diet had a higher concentration of these amino acids in their saliva as compared to crossbreeds.

In this study, the experimental animals consistently ingested a diet that contained a certain amount of condensed tannins. In view of previous reports on ruminants, this precaution may not have been necessary – in all cases where the presence of tannin-binding salivary proteins has been investigated in ruminants so far, their occurrence did not vary with previous exposure to dietary tannins (2, 4, 7). In this respect, ruminants seem to differ from other herbivores in which tannin-binding salivary proteins can be induced by the diet (1). Whether the diet used in this study had an effect on the amino acid composition of the saliva would have to be tested in experiments with different diets.

The results of this study must be considered preliminary. However, they could stimulate a series of experiments with zebu cattle, in which their readiness to consume tannin-containing forages in cafeteria trials was compared to other domestic cattle breeds, and in which their digestive efficiency and food conversion rate on such forages were determined. Although *Bos taurus* and *Bos indicus* diverged 0.3-0.8 million years ago and are the two most closely related bovine species (11), we still may detect relevant physiological differences that could allow us to make differentiated use of these species in various environments.

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REFERENCES

1. SHIMADA T (2006) Salivary proteins as a defense against dietary tannins. Journal of Chemical Ecology 32:1149-1163.

2. AUSTIN PJ, SUCHAR LA, ROBBINS CT, & HAGERMANN AE (1989) Tannin-binding proteins in saliva of deer and their absence in saliva of sheep and cattle. Journal of Chemical Ecology 15:1335-1347.
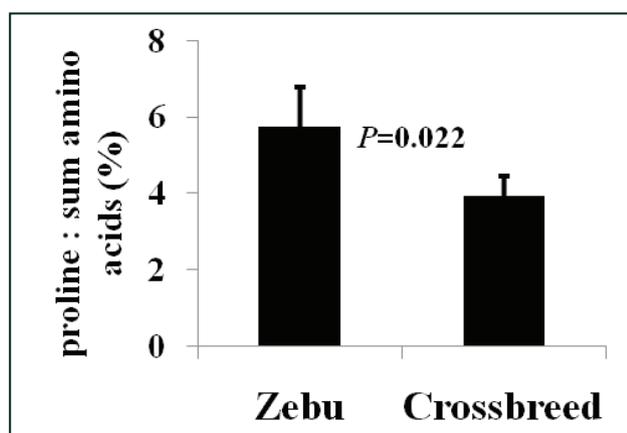

Fig. 1. – The percentage of proline in the total of measured amino acids (weight:weight) in saliva of pure zebu cattle and zebu x Holstein crossbreeds fed a tannin-rich diet.

3. FICKEL J, GORITZ F, JOEST BA, HILDEBRANDT T, HOFMANN RR, & BREVES G (1998) Analysis of parotid and mixed saliva in roe deer (*Capreolus capreolus*). Journal of Comparative Physiology B 168:257-264.

4. CLAUSS M, LASON K, GEHRKE J, LECHNER-DOLL M, FICKEL J, GRUNE T, & STREICH WJ (2003) Captive roe deer (*Capreolus capreolus*) select for low amounts of tannic acid but not quebracho: fluctuation of preferences and potential benefits. Comparative Biochemistry and Physiology B 136:369-382.

5. TURNER JW (1980) Genetic and biological aspects of zebu adaptability. Journal of Animal Science 50:1201-1205.

6. KUMAR R, & SINGH M (1984) Tannins: their adverse role in ruminant nutrition. Journal of Agricultural Food Chemistry 32:447-453.

7. MAKKAR HPS (2003) Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Ruminant Research 49:241-256.

8. MAKKAR HPS, & BECKER K (1998) Adaptation of cattle to tannins: Role of proline-rich proteins in oak-fed cattle. Animal Science 67:277-281.

9. NICHOLSON, M J, BUTTERWORTH, M H (1986) A guide to body condition score of Zebu cattle, ILCA Addis Ababa.

10. HENDRIKS WH, BUTTS CA, THOMAS DV, JAMES KAC, MOREL PCA, & VERSTEGEN MWA (2002). Nutritional quality and variation of meat and bone meal. Asian-Australian Journal of Animal Science 15:1507-1516.

11. RITZ LR, GLOWATZKI-MULLIS ML, MACHUGH DE, & GAILLARD C (2000) Phylogenetic analysis of the tribe Bovini using microsatellites. Animal Genetics 31:178-185

12. MEHANSHO H (1992) Tannin mediated induction of proline-rich protein synthesis. Journal of Agricultural and Food Chemistry 40:93-97.

13. LAMY E, RAWEL H, SCHWEIGERT FJ, CAPELA E SILVA F, FERREIRA A, RODRIGUES COSTA, ANTUNES C, MARTINHO ALMEIDA A, VARELA COELHO A & SALES-BAPTISTA E (2011) The Effect of Tannins on Mediterranean Ruminant Ingestive Behavior: The Role of the Oral Cavity. Molecules. 16:2766-2784. www.mdpi.com/journal/molecules

14. MANSOORI B & ACAMOVIC T (2009) Influence of tannic acid and polyethylene glycol on the excretion and digestibility of amino acids in gelatin-fed broilers. British Poultry Science 50(2):199-2006.

15. MANSOORI B & ACAMOVIC T (2007) The effect of tannic acid on the excretion of endogenous methionine, histidine and lysine with broilers. Animal Feed Science and Technology 134(3-4): 198-210.

16. AOAC (1997) Official methods of analysis. Association of Official Analytical Chemists, Arlington, VA, USA.

17. VAN SOEST PJ, ROBERTSON JB, & LEWIS BA (1991) Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74:3583-3597.

18. MAKKAR HPS (2001) Chemical protein precipitation and bioassays for tannins, effects and fate of tannins and strategies to overcome detrimental effects of feeding tannins-rich feeds. Proceedings of the FAO-CIHEAM Meeting on Sheep and Goat Nutrition, Hammamet, Tunisia 9:40-41.