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First report of the scavenging isopod *Natatolana neglecta* (Crustacea: Isopoda: Cirolanidae) feeding on a sea turtle

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Abstract

A juvenile male loggerhead sea turtle (*Caretta caretta*) was found dead in April 2015, entangled in a trammel net on the Mediterranean coast of Spain. Post-mortem examination revealed the presence of ninety-five isopods dispersed in the coelomic cavity, and inside the oesophagus and skull. All individuals found scavenging on the sea turtle were identified as *Natatolana neglecta* (Hansen, 1890) (Isopoda: Cirolanidae). Genetic analysis of the isopod gut contents showed that they were feeding on turtle tissue, confirming that *N. neglecta* can also attack dead sea turtles. This study shows the value of cirolanids as potential indicators of the cause of death in stranded sea turtles.

Keywords: Bycatch; *Caretta caretta*; Isopod; Mediterranean; *Natatolana neglecta*; Scavenger.

Introduction

Isopods of the family Cirolanidae are distributed worldwide, including marine, estuarine and some freshwater environments (Bruce *et al*., 2002). Some cirolanids are micro-predators on the surface of fishes or invertebrates (Bruce, 1986; Brusca *et al*., 1995), other species are scavengers on marine fauna with no apparent preference for particular carrion types (Stepien & Brusca, 1985; Bruce, 1986; Berrow, 1994; Keable, 1995; Wong & Moore, 1995, Marsden, 1999). For some species it is not clear if they are only scavengers or also predators (Bunkley-Williams & Williams, 1998). Observations on cirolanids associated with sea turtles are apparently frequent, but the species are seldom identified or reported (Williams *et al*., 1996). There are records of cirolanids from loggerhead (*Caretta caretta*), hawksbill (*Eretmochelys imbricata*) and green (*Chelonia mydas*) sea turtles (Bustard, 1976; Williams *et al*., 1996), all of them are cases of specimens located on the exterior of the turtles. Association of cirolanids with sea turtles has been considered to result from opportunistic behavior, particularly to turtles injured in nets or resting on the sea floor (Williams *et al*., 1996). However, to the best of our knowledge, none of these hypotheses have been confirmed.

In this study we report a swarming attack by isopods on a dead loggerhead sea turtle and identification of the isopods based on morphological and molecular methods. The nature of interaction of the isopods with the turtle, and the value of cirolanids as potential indicators of the cause of death in stranded turtles, is also discussed.

Material and Methods

In April 2015, a juvenile male loggerhead sea turtle (curved carapace length notch to tip = 34.8 cm) was found dead, entangled in a trammel net in Vinaros (40.466667° N, 0.466667° W), Valencian Community, Mediterranean coast of Spain (Fig. 1A). During necropsy, the oesophagus, stomach, and intestinal tract were removed from the body cavity and all organs were examined for lesions and parasites. Isopods were found inside the oesophagus and dispersed in the coelomic cavity (Fig. 1B-C).

The isopods were identified morphologically based on the descriptions, diagnoses and keys of Bruce (1981), Bruce (1986), Keable & Bruce (1997) and Keable (2006). An investigation using DNA barcoding was undertaken to support this identification. For this analysis 10 individuals were used to extract the DNA using a Qiagen DNeasy® Blood & Tissue kit following the manufacturer's instructions (Qiagen, Germany). To compare with other sequenced isopods, cytochrome c oxidase subunit 1 (CO1) was amplified using primers LCO1490 and HCO2198 from Folmer *et al*. (1994).

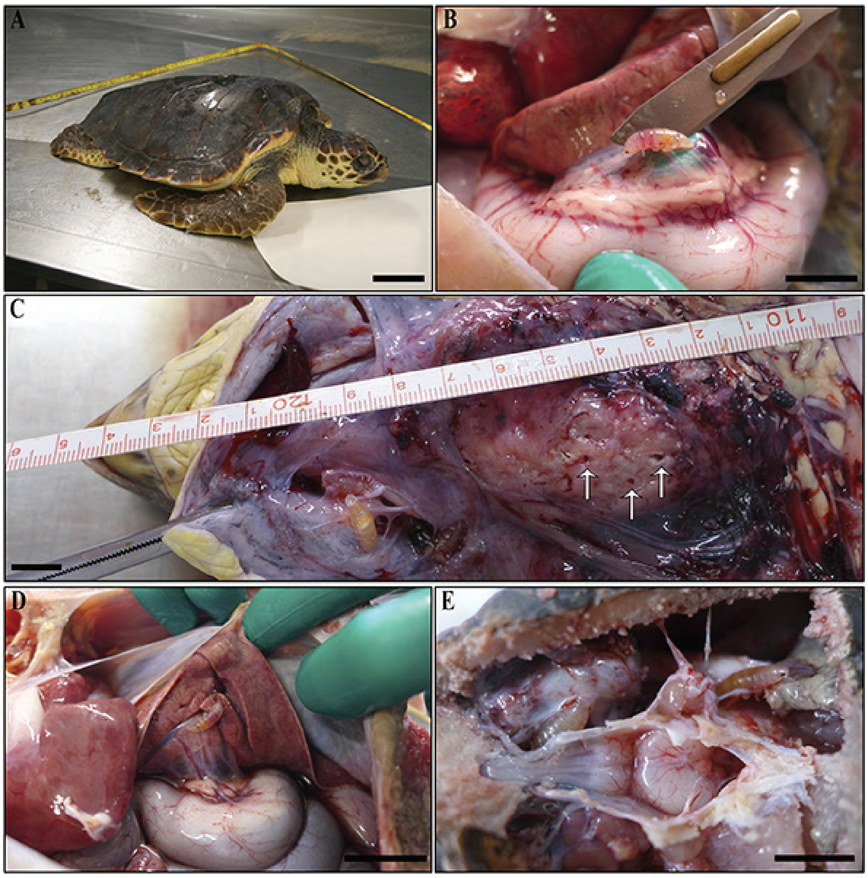

Fig. 1: Isopods of the species *Natatolana neglecta* scavenging on a loggerhead sea turtle *Caretta caretta*. A: juvenile loggerhead stranded in Spain (western Mediterranean). B: *N. neglecta* individual found in the coelomic cavity of the turtle. C: Detail of the holes (arrows) observed in the oesophagus wall. D: specimen found feeding on the turtle's liver. E: specimen found inside the skull, feeding on the turtle's salt gland. Scale bars 10 cm (A) and 1 cm (B, C and E) and 2 cm (D).

A further analysis utilising DNA barcoding was performed to confirm that the gut contents found in two of the isopods was blood and tissue of the turtle. These contents were removed from the isopods, and their DNA extracted with the same technique described above. To compare with sequences available at GenBank the R35 intron was sequenced using the primers R35Ex1 and R35Ex2 from Fujita *et al*. (2004). PCR amplifications were performed with the ready-to-use MyFi Mix (Bioline Ltd., United Kingdom).

The thermocycling profiles were applied following Raupach *et al*. (2015) for CO1, and Fujita *et al*. (2004) for the R35 intron. PCR amplicons were purified using a Macherey–Nagel NucleoSpin® Gel and PCR Clean-Up kit (Macherey-Nagel, Germany), and PCR primers were used for sequencing. Sequencing was performed by the commercial sequence provider Macrogen (Netherlands). Contigs were assembled using BioEdit v.7.2.5 (Hall, 1999). Consensus sequences of CO1 regions were submitted to GenBank (accession numbers MH509756, MH509757, MH509758). A BLAST (Basic Local Alignment Search Tool) was used in order to try to determine the species of isopod, and/or the more similar ones.

The new generated sequences of CO1 were aligned with those of other isopods, including *Natatolana* spp., recorded by Raupach *et al*. (2015) using MAFFT ver. 7 software (Katoh & Standley, 2013), and revised in MEGA 6.0 (Tamura *et al*., 2013). The brachyuran crustacean *Cancer pagurus* was included as an outgroup (Raupach *et al*., 2015). The total alignment comprised 25 sequences and 645 nucleotide positions. The nucleotide substitution model was estimated using jModelTest ver. 2.1.6 (Darriba *et al*., 2012). Based on the Akaike information criterion (AIC), the model GTR+Γ (general-time-reversible model with gamma distributed among-site rate variation) was found to be the best fitting the dataset.

Phylogenetic analyses of CO1 sequences were performed using Bayesian Inference (BI) and Maximum Likelihood (ML) criteria. ML analyses were performed using PhyML ver. 3.0 software (Guindon *et al*., 2010). Branch support was estimated by bootstrap analysis with 1000 replicates. BI analyses were performed using MrBayes ver. 3.2 (Ronquist *et al*., 2012) with four Markov Chain Monte Carlo (MCMC) chains run for one million generations with a sampling frequency of 500 and a "burn-in" value of 25% of the stored trees. A majority rule consensus tree was built after discarding the first 25% of the trees. The nodal support was estimated as posterior probabilities (PP) (Huelsenbeck *et al*., 2001).

Voucher specimens of the isopods are deposited in the Australian Museum, Sydney, Australia, registration number P.101627.

Results and Discussion

Ninety-five individual isopods were found in the turtle carcass during the necropsy. All of the specimens were found inside the turtle, dispersed in the coelomic cavity, on the external surface of organs, glands and tissues. The examination of the turtle's organs showed several holes in the oesophagus wall, which were probably made by mechanical penetration of the isopods to reach the coelomic cavity (Fig. 1C). Feeding activity was also observed on the turtle's liver (Fig. 1D). Individuals were also found inside the skull, where they had presumably eaten the salt gland of the right side (Fig. 1E). A hole found in the right eye of the turtle could be the entry route followed by the isopods, at least for the ones found in the skull. Other possible points of access could be the mouth and nose.

Specimens agreed with the morphological diagnostic and descriptive generic characters of *Natatolana* provided by Bruce (1981), Bruce (1986), Brusca *et al*. (1995) and Keable (2006), including the setation of pereopod 7, the short antennule and details of the frontal lamina that Keable (2006) highlighted as particularly distinctive. Out of the six species of the genus *Natatolana* reported in the North Atlantic and Mediterranean, the specimens were identified as *Natatolana neglecta* (Hansen, 1890) based on the keys, descriptions and diagnosis provided by Keable & Bruce (1997) and Keable (2006). The pleotelson shape (anterolateral margins almost straight and angling posteriorly toward the midline; posterolateral margins straight, markedly angled to anterolateral margins and meeting at an obtuse angle), with a high number (10 to 14) of robust setae on the margins, is especially characteristic. This coupled with the well-developed eyes, lack of robust setae on the propodal palm of pereopods two and three (although single setae were found on these pereopods in a few individuals) and relatively narrow basis of pereopod 7 (distance between the anterior margin and medial carina less than between posterior margin and medial carina – following the orientation indicated in Keable (2006) fig. 3B) are sufficient to separate the material examined from other species of *Natatolana* that are known from the area and also from similar species occurring elsewhere.

The BLAST tool showed a close similarity of our species to the other three species of the genus *Natatolana* (*N. borealis*, *N. gallica* and *N. rossi*) available at GenBank, and the phylogenetic tree obtained (Fig. 2) shows a distinct clade formed by the four species of *Natatolana*. This supports the identification of our specimens as a species of *Natatolana*. The results of the analysis of the R35 intron from the blood and tissue remains found in the gut of some of the specimens, confirm that they belonged to a loggerhead sea turtle.

Whether the turtle was scavenged alive or already dead in the net is unknown, but *Natatolana* species are opportunistic and voracious scavengers (references summarized in Keable (2006)), and they usually swarm in vast numbers and attack damaged fish, particularly at dusk or at night (Wong & Moore, 1995; Stepien & Brusca, 1985). They can cause damage to the commercial fishing operations (Bird, 1981; Stepien & Brusca, 1985; Berrow, 1994; Johansen & Brattegard, 1998; Poore & Bruce, 2012) and have been described feeding on dying fishes trapped in trammel nets and long-line fisheries, including a case reported in Italy and involving *N. neglecta* (Mizzan, 1995). Local fishermen in the study area have also reported the presence of scavenging isopods on fishes trapped in trammel nets (authors, pers. obs.). Hence, the association of

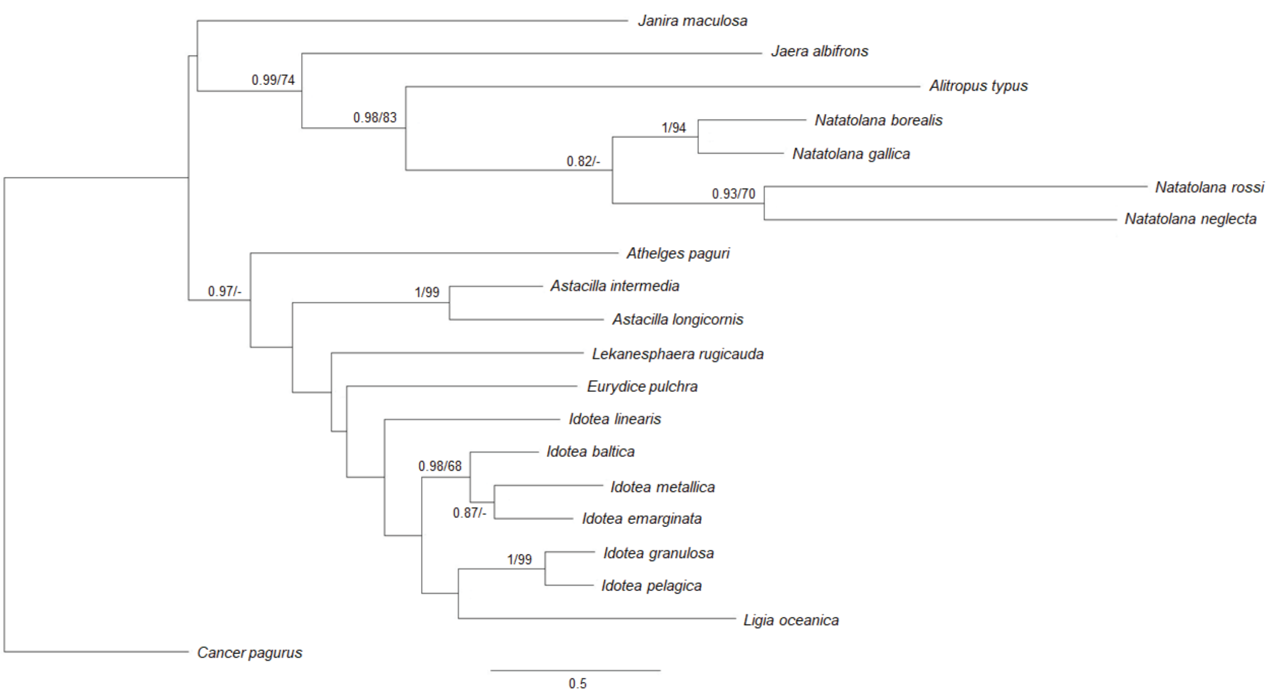

Fig. 2: Phylogenetic tree of Isopoda spp. derived from Bayesian Inference and Maximum Likelihood analysis using CO1 region. Posterior probability values are indicated above the branches, followed by maximum likelihood bootstrap values (in %). Posterior probabilities <0.80 and bootstrap values <60% are not reported.

Natatolana specimens with this entangled turtle is consistent with the ecology and feeding pattern of these cirolanids (Johansen & Brattegard, 1998). Soak time of the net in which the necropsied turtle was found was eight hours between 20:00 hr to 06:00 hr; the turtle may have been entangled for several hours and isopods started to scavenge when the turtle was dead or near dead, without movement.

Sea turtle strandings have been used to identify mortality causes, allowing inferences on patterns of bycatch (Panagopoulos *et al*., 2003; Witt *et al*., 2007). However, determining a specific cause of death is not possible for most of the stranded turtles, due to the absence of external evidence or the degree of decomposition. A common procedure to diagnose the death as due to bycatch uses several criteria [e.g. presence of lines of nylon wrapped around their necks and flippers (Blasi *et al*., 2016); imbedded fishing hook (Parga, 2012); gas bubbles within tissues (Fahlman *et al*., 2017)]. Because presence of cirolanid isopods has been used in forensic assessment of cadavers retrieved from the sea (Nagano, 1963; Colombage & Telisinghe, 2010; Tiemensma *et al*., 2017), occurrences of scavenging isopods in sea turtle carcasses, or the signals of their activity described here, could be added as further important evidence in examination protocols to diagnose instances of bycatch in trammel nets involving sea turtles in future necropsies.

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