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First report of pseudohermaphroditism in cephalopods

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Cephalopods are gonochoristic molluscs that show no hermaphroditism and the animals never change sex.[1] Their main sexual dimorphism is the presence of a hectocotylized arm in males, which transfers the spermatophores to the female. In the incirrate octopods, females have a single ovary with paired oviducts, whereas in males a single duct forms the spermatophoric complex and the terminal organ (or penis). Several malformations have been reported for cephalopods, including internal and external structures. Among others, these refer to malformations of arms,[2–6] of gills and associated vascular organs, of mantle-hyponomal locking cartilages, to dextral displacement of the caecum with respect to the location of the stomach, to duplication of the chitinous lining of the alimentary canal,[7] to the presence of double hectocotylization[6,8,9] (abnormal characteristic in incirrate octopods) and to malformation of the systemic heart complex.[10] However, so far there are no published records of pseudohermaphroditism for the class.

During a study of the reproductive biology of the Patagonian red octopus, *Enteroctopus megalocyathus* (Gould, 1852), a total of 185 females and 143 males were collected during research surveys conducted from June to December 2004 in Nuevo Gulf (42°46′ S 65°02′ W), Atlantic Ocean, Argentina, at 6 to 10 m depth. Each freshly collected animal was weighed, measured and sexed on the basis of the presence of the hectocotylus. Internal organs were removed and immediately fixed in 10% formalin, and the rest of the animal was discarded. Three months after fixation, when preparing internal organs, an abnormality was noticed in one individual.

The abnormal specimen (A) appeared in the sample of August. Because of its size and weight, and the presence of immature oocytes, it was probably immature. For this reason, we compared it with other normal immature females and males (i.e. animals with immature oocytes and without spermatophores respectively) from the same August sample (N). The following measurements and indices were recorded: total body weight, dorsal mantle length, reproductive system weight, spermatophoric complex weight, ovary weight, weight of oviducts and oviducal glands and terminal organ length. For the last four measurements, we calculated a simple 'abnormality index' as $(X_A/\bar{X}_N) * 100$, where X_A is the reproductive tissue of the abnormal individual divided by its total body weight, and $\bar{X}_N$ is the sample mean of the same reproductive tissue divided by mean total body weight for all normal individuals. X_A and $\bar{X}_N$ were used to compare statistically the reproductive tissue of the abnormal individual with that of normal ones, using a special case of the t-test.[11]

To identify the type of gonadal tissue and differences between normal specimens and the abnormal one, histological preparations of several parts of the female and male reproductive system were stained with haematoxylin and eosin. The latter system was defined according to Mann *et al.*[12]

The abnormal specimen was initially sexed as a female, i.e. it did not exhibit a hectocotylus. Internally, it showed male structures with normal genital female characteristics, orientated as in normal octopuses. In its male reproductive system, the testis was absent, the penis was normal and both the Needham sac, and glandular systems I and II displayed an abnormal shape (Fig. 1). In its female reproductive system, the ovary was significantly larger than normal (Table 1). The spermatophoric complex and left female gland and oviduct lay enclosed in a male membranous sac and were joined only between glandular system II and the left distal oviduct. The presence of immature oocytes was also evident through the ovary wall. Histological comparisons did not reveal differences from normal ovaries, and we did not find testicular tissue in the ovary nor sperm stored in oviducal glands. In view of these morphological and histological characteristics, we suggest that this individual shows the first case of pseudohermaphroditism in cephalopods.

The presence of mixed female and male structures may not have caused sterility for the female function since one oviduct was free and showed normal characteristics. Although this

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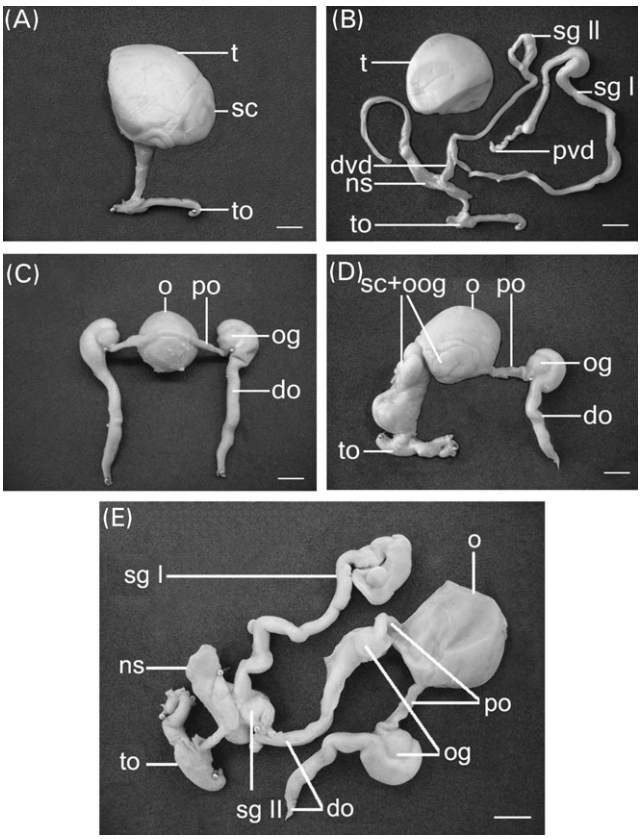

Figure 1. Reproductive system of *Enteroctopus megalocyathus*. **A.** Normal male. **B.** Unwrapped normal male. **C.** Normal female. **D.** Abnormal specimen. **E.** Unwrapped abnormal specimen. Abbreviations: do, distal oviduct; dvd, distal vas deferens; ns, needham sac; o, ovary; og, oviducal gland; oog, oviduct and oviducal gland; po, proximal oviduct; pvd, proximal vas deferens; sc, spermatophoric complex; sg I, glandular system I; sg II, glandular system II; t, testis; to, terminal organ. Note that a membranous sac encloses the genital organs in the normal male and the abnormal specimen. Scale bars **A**, **B**, **C**, **D**, **E** = 10 mm.

normal morphology suggests that the animal was able to mate, the lack of sperm in the oviducal glands might indicate that mating was impaired for other reasons. Thus, we are unable to tell whether the animal was able to reproduce successfully.

Pseudohermaphroditism or imposex is an abnormality of the gastropod reproductive system that can be caused by the effects of an environmental pollutant, tributyltin (TBT), in which parts of the male reproductive tract develop in females.[13] The Nuevo Gulf is home to a busy industrial harbour in which traffic has been increasing over the last two decades. Big vessels and harbours are painted with antifouling paints, producing the main source of TBT contamination, and a TBT concentration of 4 ng/g has been recorded in sediments.[14,15] This TBT contamination likely has acted as an endocrine disruptor, because imposex has been recorded in the Gulf in two species of gastropod, with the percentage of females with imposex at 100% between the years 2002–2004.[14] Bioaccumulation of TBT in molluscs such as gastropods and bivalves occurs by both dietary uptake and direct uptake from water.[16,17] Although octopuses mainly feed on molluscs and crabs,[18] we have no direct evidence to support the routes of TBT uptake in the abnormal animal. In fact, this kind of malformation in molluscs might also be caused by other factors including copper, paint matrix and environmental stress.[19]

As far as we know, the only record of an effect of TBT on cephalopods refers to a malformation of the systemic heart complex of *Sepia officinalis* (Linnaeus) in the Bay of Arcachon (Atlantic coast of France).[10] However, in spite of the low frequency of pseudohermaphroditism in the total sample (0.3% of all the animals and 0.5% of the females), given the morphological characteristics and the common presence of imposex in gastropods in Nuevo Gulf, we suggest that TBT should not be discarded as an explanation for the observed malformation.

According to Wells & Wells[20] for *Octopus vulgaris* (Cuvier) and Olivares *et al*.[21] for immature specimens of *O. mimus* (Gould), the development of the spermatophoric complex and a terminal organ are not under the control of male gonadal hormones in immature incirrate octopuses. Since we observed these structures in the abnormal individual in the absence of a testis, our abnormal individual supports their results.

Table 1. Measurements, weights, indices and significance of differences for normal and one abnormal *Enteroctopus megalocyathus* of the sample selected for comparisons.

	Sex	*n*	Mean ± SD	Range	Index (%)	*P*
Total weight (g)	F	31	836 ± 203	439–1165	–	
	M	20	868 ± 161	596–1157	–	–
	A	1	674	–	–	
Mantle length (mm)	F	30	113 ± 13.0	90.0–143.0	–	
	M	20	122 ± 10.4	99.5–138.0	–	–
	A	1	116	–	–	
Reproductive system weight (g)	F	31	6.17 ± 3.48	1.40–19.60	–	
	M	19	25.7 ± 8.16	8.30–40.60	–	–
	A	1	(F) 9.67 + (M) 8.25	–	–	
Spermatophoric complex weight (g)	M	20	5.48 ± 1.72	2.01–8.33		ns
	A	1	3.95	–	92.7	
Terminal organ length (mm)	M	20	40.0 ± 6.85	15.2–40.8		ns
	A	1	43.0	–	132.4	
Oviducts and oviducal glands weight (g)	F	31	2.23 ± 1.04	0.46–5.17		ns
	A	1	2.47	–	142.1	
Ovary weight (g)	F	31	3.32 ± 2.02	0.75–10.75		<0.01
	A	1	6.41	–	252.6	

Abbreviations: F, female; M, male; A, abnormal specimen.

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Accurate identification of cryptic slug taxa of the *Arion subfuscus/fuscus* complex by PCR-RFLP (Pulmonata: Arionidae)

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Shell characters (e.g. colour, banding pattern, size, shape) are commonly used to discriminate among closely related gastropod species. However, in slugs the shell is internal, often reduced and taxonomically uninformative.[1] Therefore, body colour pattern and the size and shape of the (proximal) genitalia are the most important taxonomic markers in slugs. However, body colour and the development of stylommatophoran reproductive organs is subject to considerable intraspecific, seasonal and physiological variation.[2,3,4,5] The identification of immature slugs is therefore often especially problematic. Two biological species of land slugs of the arionid subgenus *Mesarion*, viz. *Arion subfuscus* (Draparnaud, 1805) and *A. fuscus* (Müller, 1774), are very similar morphologically and have overlapping distributional ranges in northwestern Europe.[6] Using allozyme data and gonad morphology, both species can be consistently delineated, even in sympatry.[6] *Arion fuscus* is widespread throughout Central, North and East Europe,[7] whereas *A. subfuscus* is restricted to West Europe.[8] DNA sequence variation in nuclear (ITS-1) and mitochondrial (16S rDNA) genes revealed two major evolutionary lineages in *A. fuscus*,[7] one in the Balkan region and another in the Alps and the rest of Europe, and five evolutionary lineages (S1-S5) with largely allopatric distributions in *A. subfuscus*.[8] Two of the *A. subfuscus* lineages (S1 and S2) have overlapping distributions and may hybridize.[8]

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Both allozyme electrophoresis and DNA sequencing have a number of disadvantages. Allozyme data can only be used when fresh or deep-frozen tissue is available. Although electrophoretic zymograms afford excellent markers for distinguishing *A. subfuscus* from *A. fuscus*, they do not allow accurate discrimination of the evolutionary lineages. The sequencing of DNA requires PCR-amplification using specific primers, followed by purification of the PCR product and sequencing of the amplified region. This procedure is often expensive and time-consuming. Hence, when large numbers of (immature) individuals have to be analysed, a rapid technique allowing reliable identifications of the cryptic species and lineages within the *A. subfuscus/fuscus* complex is needed. Here we present an easy and convenient molecular marker based on a restriction-fragment length digestion of a PCR-amplified 16S rDNA stretch (PCR-RFLP), which allows practical and reliable identification of the northwestern European evolutionary lineages within the *A. subfuscus/fuscus* complex.

In the period February–May 2004, a total of 175 *A. subfuscus/fuscus* individuals of different life-stages were collected in 25 Belgian and one German locality. A list of the localities and the number of individuals sampled per locality are available upon request. Individuals were frozen at −80°C. All specimens were identified by sequencing of the mitochondrial 16S rDNA[7,8] and, whenever enough digestive gland tissue was available, using allozyme analysis.[6] DNA sequencing was also used to assign all individuals to one of the evolutionary lineages. Then, a portion of foot tissue of each individual was digested