First observations of conjoined-twins in newborn seahorses *Hippocampus guttulatus* Cuvier, 1829

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Running head: First record of seahorse conjoined-twinning

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Occurrence of conjoined-twins or two-headed fish are not uncommon (Hanson 1985), being generally more frequent under artificial conditions than in nature (Owusu-Frimpong & Hargreaves 2000). The origin of conjoined-twins can be natural, occurring when two centers of development arise from one germ ring (Laale 1984), or teratogenic, by environmental pollution or toxicity by mutagenic chemicals in the water (Herrmann 1995; Al-Jufaily, Laith & Al-Az 2005). Since the first recorded case of conjoined-twins among fishes in *Carassius* sp. (Jussieu 1754), twinning has been also reported in fish species such as *Oncorhynchus keta* (Walbaum) (Yamamoto, Kobayashi & Kuramoto 1996), *Oreochromis aureus* (Steindachner) and *O. niloticus* (Linnaeus) (Owusu-Frimpong and Hargreaves 2000), *Arius dussumieri* (Valencienes) (Al-Jufaily *et al.* 2005) and *Brachydanio rerio* (Hamilton) (Herrmann 1995), among other species, both in the wild and under laboratory or farmed conditions (Pavis 1961; Hanson 1985; Al-Jufaily *et al*. 2005). Twinning in fish of the family Syngnathidae has been previously described in the pipefish *Syngnathus floridae* (Jordan & Gilbert) (Cable 1940) but not in seahorses. In the present study, three sets of twins were released in the laboratory from pregnant seahorses *Hippocampus guttulatus* Cuvier 1829. This finding is the first record of conjoined-twins in seahorses.

Three males (N81, N104 and N105) of long-snouted seahorses *Hippocampus guttulatus* Cuvier were hand-caught collected in August and September 2010 in the coast of Galicia (NW Spain) and maintained in captivity under photoperiod and temperature regimes (maximum 20 ºC) simulating natural conditions during the breeding season. Seahorses were fed on enriched adult *Artemia* (EG-brand, Inve Aquaculture, S.A.) (Planas, Chamorro, Quintas & Vilar 2008) supplemented with wild caught shrimps (*Leptomysis* sp. and *Siriella* sp.). Male N81 mated in the laboratory but males N104 and N105 were already pregnant when collected in the wild.

The first release of newborns was recorded in July 2010 (N81). A set of conjoined-twins (CT1) was collected together with 500 well-developed newborns, 8 eggs and 8 pre-mature seahorses. The second and third batches of newborns (N104 and N105, respectively) were released in August and September 2010, respectively. The second batch was composed of 371 well-developed newborns, 128 dead pre-mature seahorses and 3 live pre-mature seahorses. The third batch consisted of 355 well-developed newborns, 50 pre-mature seahorses and 9 embryos. Two conjoined-twins (CT2 and CT3, respectively) were also released with each batch.

Biometrical analyses were performed in conjoined-twins following the standard seahorse measurement protocol (Lourie, Vincent, & Hall 1999). Measurements were made from photos taken under a stereomicroscope (Nikon) and using the software package NIS-Elements (Nikon). Seahorse standard length (SL) was calculated as the sum of body length and head length. Body length was measured from the tip of the tail to the ridge of the operculum and head length from the tip of the snout to the ridge of the operculum. In addition, trunk length (from the ridge of the operculum to immediately below the dorsal fin), tail length (from the tip of the tail to immediately below the dorsal fin), dorsal fin length (from the upper to the lowest tip of the fin), head fusion length (Hf; from the fusion point of the twins to the top of the head) and tail fusion length (Tf; from the fusion point of the twins to the tip of the tail).

Newborn twins were in the post yolk sac-stage and alive after birth. CT1 and CT2 twins were normally developed but ventrally fused and with a shared tail (Fig. 1A and 1B). Conversely, twins CT3 were non-fully developed and unequal-sized (Fig. 1C).

[Figure 1]

59 The results of the measurements performed are summarized in Table 1. The standard
60 length (SL) in all twins was smaller than that of the normally developed *H. guttulatus*
61 newborns (Planas, Quintas, Chamorro & Balcazar 2009). In CT1, each twin had their
62 own pectoral and dorsal fin and a unique well developed tail. On the contrary, a
63 deformation of spinal axis was present in CT2 (Fig. 1B) which showed a separated well
64 developed head, a fused trunk from the jaw and a completely deformed and twisted tail.
65 Differences in size between both twins in CT3 agree with what generally occurs in twin
66 pairs (Hanson 1985), where one of the twins is much smaller than the other. The smaller
67 twin showed an underdeveloped head which appeared to be fused to the abdominal
68 region of the other twin.

69 [Table 1]

70 All twins were processed for histological analysis and submitted to differential staining
71 procedures. Seahorses were fixed overnight at 4ºC in methanol containing 2.5%
72 paraformaldehyde and 5% acetic acid,and embedded in parafin. Sections of 2µm were
73 stained with the Masson's trichrome technique (Masson 1929) and sections of 3µm
74 were stained with hematoxylin-eosin. Only histological sections of CT1 provided a
75 clear fusion point between twins and more accurate histological cuts.

76 Histological cuts show a clear fusion point of the spinal cord in CT1 (Fig. 2A and 2B).
77 Figure 2B shows a deformation in the backbone of the twins with two additional
78 vertebrae at the fusion point. Figure 2 (A and B) also shows the presence of two kidneys
79 (K_1 and K_2) with a shared anus (AN).

80 [Figure 2]

81 Conjoining-twins is a phenomenon that has been subject of discussion for a long time
82 (Laale 1984) and it is known that several causes are responsible of conjoining (Arbuatti,

Salda & Romanucci 2011). As for most abnormalities in early developmental stages of fish, the occurrence of twinning can be due to different causes, such as egg over-ripening (Witschi 1952), pollutants, low dissolved oxygen levels or thermal shock induction (Owusu-Frimpong and Hargreaves 2000). The formation of multiple development centers in eggs, probably due to any disturbance during the early development axis formation, results in polyembryony (Laale 1984; Arbuatti *et al.* 2011). In this regard, it has been suggested that the main cause of polyembryony is environmental stress (Kaufmann 2004; Arbuatti *et al.* 2011) during development, both in oviparous (Herrmann 1995; Owusu-Frimpong and Hargreaves 2000; Al-Jufaily *et al.* 2005) and ovoviviparous fish (Cable 1940; Arbuatti *et al.* 2011). It has been demonstrated in the laboratory that wild fish are sensitive to possible teratogenic effects causing mitotic abnormalities on embryos or specific locus mutations during oogenesis and spermatogenesis (Longwell, Chang, Hebert, Hughes & Perry 1992). However, polyembryony in fish also occurs in nature (Laale 1984).

Twins described in the present paper corresponded to monozygotic conjoined-twins, morphologically described as *anadidymus* type (Laale 1984), since they were two headed but shared a unique tail.Besides this,, twins CT1 and CT2 were also normally shaped seahorses (*autosita* type*)* whereas twins CT3 were *parasita* type, in which one of the twins is a parasite´like of the other normally shaped twin (Laale 1984). The occurrence of twin conjoining in fish of the Syngnathidae family has been already reported in an embryo of the pipefish *Syngnathus floridae* (Cable 1940) but our observations are the first record of this feature in seahorses.

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157 mechanism. *Canadian Journal of Zoology*. **74**, 485-491.
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Figure captions

Figure 1 Conjoined-twins CT1, CT2 and CT3.

Figure 2 Histological sections in CT1 showing the fusion point oftwins ($CT1_1$ and $CT1_2$). A: Hematoxylin-eosin staining. B: and Masson's trichromic staining. VC: Vertebral Column. K: Kidney. AN: Anus.

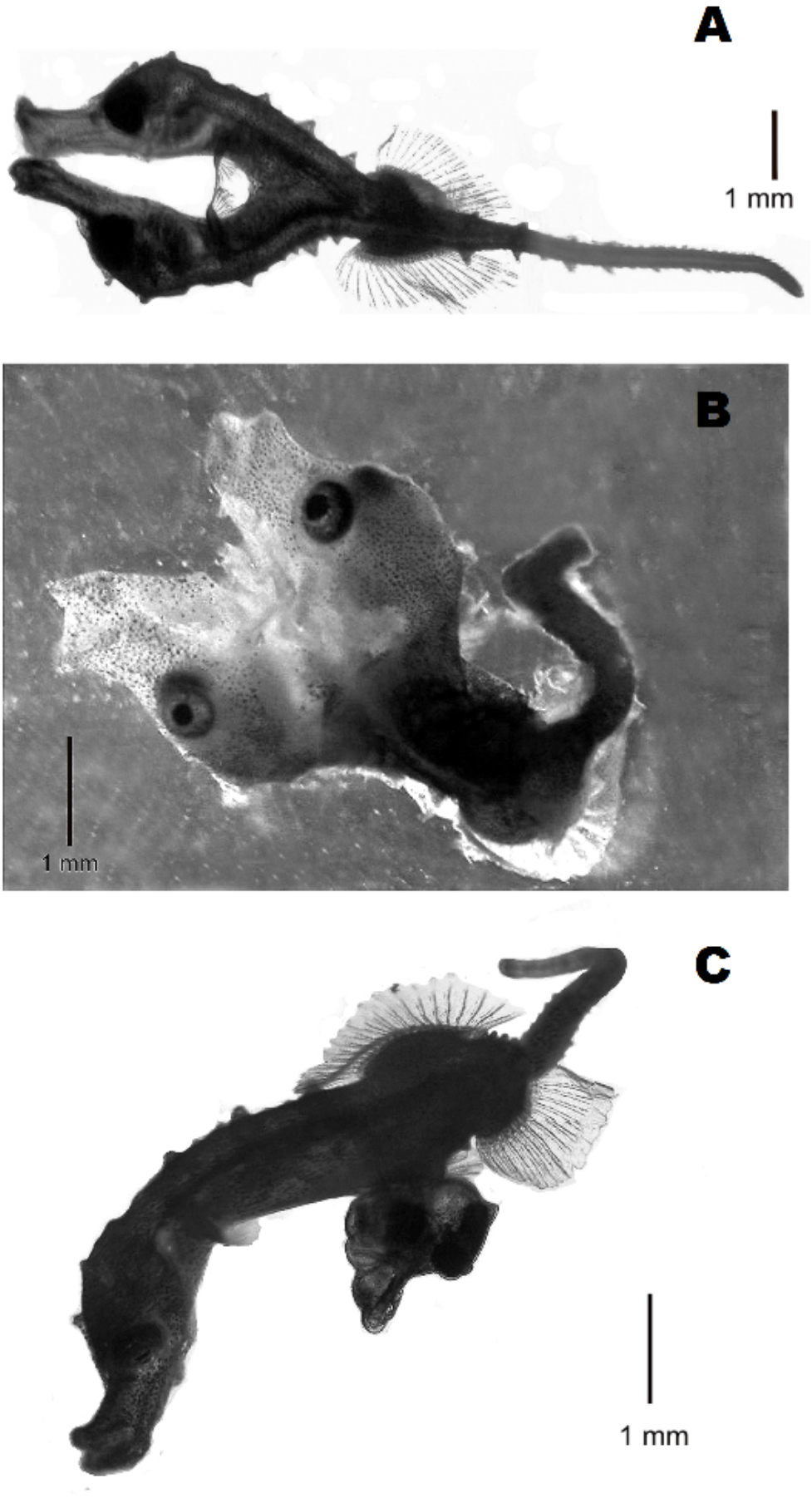
165 **Table 1**. Biometrics (lenght in mm) in Conjoined -twins CT1, CT2 and CT3. Hf and Tf:
166 Distance from the twins fusion point to head and tail, respectively. nd = not determined
167 (impossible to measure); SL: Standard Length.

168

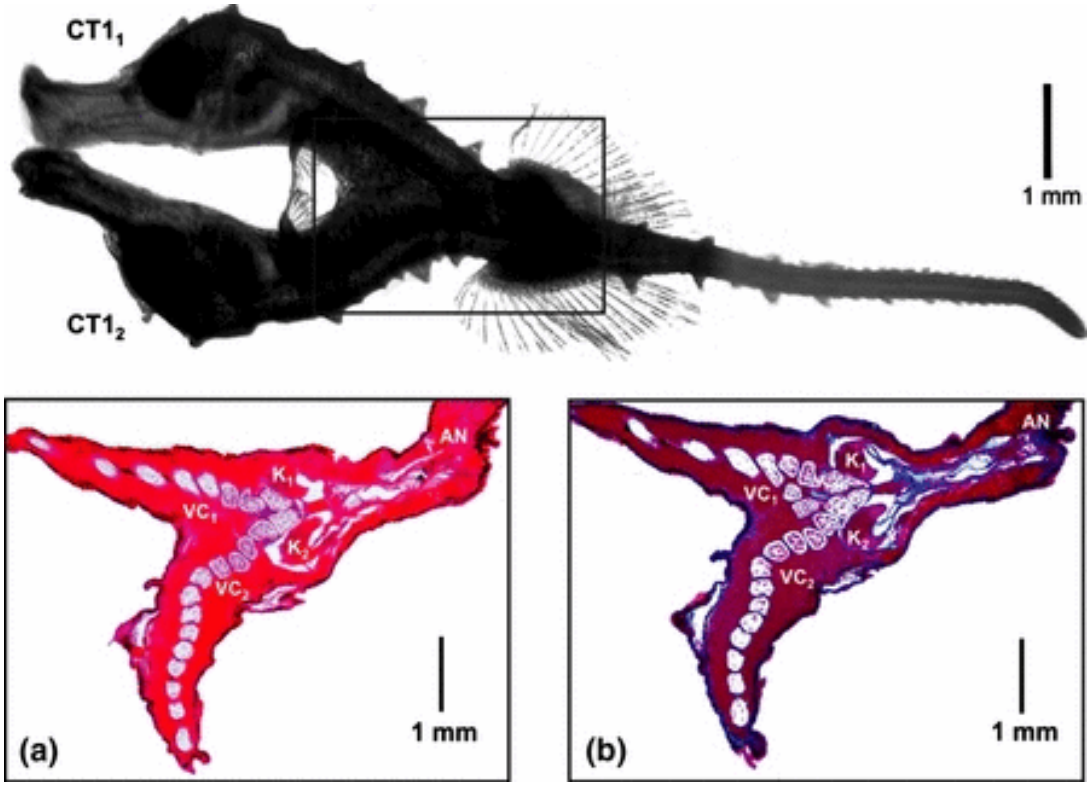
	SL	Head	Trunk	Tail	Dorsal fin	Hf	Tf
CT1	11.42	2.80 - 2.99	3.65 - 3.27	5.18	2.05 - 2.63	3.61	7.83
CT2	7.76	2.59 - 2.78	2.33	2.69	2.13 - nd	1.44	6.32
CT3	6.24	2.02 - 1.71	3.74 - nd	2.50	2.34 - 1.35	3.74 - 0	4.12

169

170 **Figure 1**



223 **Figure 2**



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