1 **Sperm ultrastructure of the hydrothermal vent octopod *Vulcanoctopus***
2 ***hydrothermalis***

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10 Running Title: Sperm ultrastructure of *Vulcanoctopus hydrothermalis*

11
12 **ABSTRACT**

13
14 Sperm ultrastructure of the deep sea hydrothermal vent octopod *Vulcanoctopus*
15 *hydrothermalis* has been carried out by electron microscopy. Spermatozoa of this
16 species have the shortest head observed so far in octopodids. The acrosome possesses a
17 helix with six gyres and the nucleus is short and wide. Noteworthy features along the
18 nucleus are the regularly disposed dense bands of cytoplasm, which haven't been
19 observed before in octopodids. The nuclear fossa is very short and wavy. Mitochondrial
20 sheath has ten elongated mitochondria running parallel to the axoneme-coarse fibers
21 complex (ACF). Sperm morphology of *Vulcanoctopus* is herein discussed considering
22 the new incirrate octopod taxonomy.

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24 **KEY WORDS:** sperm, ultrastructure, *Vulcanoctopus hydrothermalis*, incirrate octopod,
25 taxonomy.

INTRODUCTION

Vulcanoctopus hydrothermalis González & Guerra 1998 is a recently described deep water species inhabiting hydrothermal vents. There are few studies dealing with this species, which are mainly centred on its description (González et al., 1998, 2002, 2008), behaviour (Rocha et al., 2002; Voight, 2008) and ecology (Voight, 2005). Considering anatomical, biogeographical and ecological reasons it was suggested that the most suitable octopod ancestor of *V. hydrothermalis* would be a *Benthoctopus* Grimpe, 1921 or *Bathypolypus* Grimpe, 1921 species (González et al., 2008). This hypothesis was confirmed by a recent study centred in the evolutionary relationships between *Vulcanoctopus* and *Benthoctopus* (Strugnell et al., 2009a). Genetic data provide evidences that *V. hydrothermalis* should be placed within the genus *Benthoctopus*. However this genus has much controversy because, as first noted by Voss and Pearcy (1990) and confirmed by the revision of Muus (2002), the type species of *Benthoctopus* (*Octopus piscatorium* Verrill, 1879) is in fact a junior synonym of *Bathypolypus bairdii* (Verrill 1873). For this reason, *Benthoctopus* is an unavailable name (*nomen nudum*), and in agreement with the rules of the International Code of Zoological Nomenclature, *Vulcanoctopus* should be considered a junior synonym of this genus. Nevertheless, aware of this fact and in connection with the description of five new species of this genus, Voss and Pearcy (1990) plead for the preservation of the name of *Benthoctopus*, which at present includes 20 species worldwide (Sweeney and Roper, 1998). To conserve that generic name, a proposed conservation should be submitted to the International Commission of Zoological Nomenclature. However, until present this has not been done, but this genus was considered valid in the current revision of the state of the octopus taxonomy by Norman and Hochberg (2005).

Depending if sucker seriation was biserial or uniserial, deep-sea incirrate octopods without ink sac were split into the subfamilies Bathypolypodinae and Graneledoninae, respectively (Voss 1988a, b). According to this subdivision and owing to the fact that *Vulcanoctopus* has biserial suckers, it should be placed within the subfamily Bathypolypodinae. Nevertheless, Strugnell *et al.* (2009b) showed that morphological characters used to subdivide the Octopodidae (Voss, 1988a) do not reflect evolutionary history and concluded that the erected subfamilies based on these characters are artificial. Based on molecular analysis, Strugnell *et al.* (2009b) proposed to divide the order Octopoda Leach, 1818 into six families: Octopodidae, Enteroctopodidae, Amphitretidae, Eledonidae, Bathypolypodidae and Graneledonidae. Interestingly, they found that *Enteroctopus*, *Benthoctopus* and *Vulcanoctopus* fall within the same monophyletic group constituting the Enteroctopodidae.

Sperm morphology has provided important clues defining taxonomic position and phylogenetic relationships between many groups of molluscs including cephalopods (Franzén, 1955; Hou and Maxell, 1992; Healy, 1988, 1989, 1990a, b, 1993). Sperm morphology from the Octopodidea studied to date has been centred in the families Octopodidae and Eledonidae (Galangau and Tuzet, 1968a, b; Longo and Anderson, 1970; Maxwell, 1974; Martin *et al.*, 1970; Healy, 1989; Selmi, 1996; Ribes *et al.*, 2002; Zhu *et al.*, 2005). In order to recognize the different sperm morphologies within the Octopodidea, Roura *et al.* (2009a, b) have described the sperm morphology of specimens from the families Bathypolypodidae and Graneledonidae.

The aim of this work is to describe the mature sperm morphology of *V. hydrothermalis*, and collect data for a complete comparison within the Octopoda. Furthermore, its taxonomic position within the incirrate octopods is discussed.

MATERIAL AND METHODS

The animals were collected during the French cruise HOPE 99 from 1-21 May 1999, at 2631 m depth, in a hydrothermal vent site named Genesis, located at the East Pacific Rise (12°48.7'N, 103°56.4W). The specimens were fixed in formaldehyde (4% buffered in sea water) for 24 hours and then preserved in 70% ethanol. Spermatophores were extracted from two mature males (44.3 and 45.8 mm mantle length with 40 and 24 spermatophores, respectively).

For transmission electron microscopy (TEM), sections of spermatophores were fixed in 3.0% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2 for 12 h at 4°C, washed in the same buffer for 4 h at 4°C and then post-fixed in buffered 2.0% osmium tetroxide for 4 h at the same temperature. After dehydration in a graded ethanol series, the fragments were embedded in Epon, sectioned with diamond knife, double-stained with uranyl acetate and lead citrate, and observed in a JEOL 100CXII TEM operated at 80 kV.

Spermatozoa measurements were taken using the imaging data processor NIS-Elements D 2.30. In order to describe the helicoidal structure of the acrosome we measured the distance between spires in 9 acrosomes of both specimens. The distance between spires was compared with the number of spires using simple linear regression.

RESULTS

Measurements made to both animals are summarized in Table 1. The acrosome equation that related the separation between gyres (y) with the number of gyres (x) is:

$$y = 1.0361 - 0.0359 x; r^2=0.9035, p<0.0001, n=45.$$

102 This equation shows that the separation between gyres decreases in a constant
103 rate towards the anterior tip (Fig.1). Longitudinal sections of the acrosome show a
104 single helix arranged in 5 gyres around an inner cone, which exhibit dense striations
105 perpendicularly arranged to the long axis of the spermatozoon (Fig.2a). The basal part
106 of the acrosome has almost the same diameter as the nucleus, decreasing in diameter
107 anteriorly. Between the plasma membrane and the acrosome membrane there is a little
108 space with electron-dense cytoplasm or periacrosomal material (Fig. 2a, b).

109 The nucleus shows in longitudinal sections regularly disposed dense bands
110 throughout its length (Fig. 2c, arrow heads). As seen in detailed, these bands are
111 composed by electro dense cytoplasm placed between the plasma membrane and the
112 nuclear membrane (no se ve en la plancha Fig. 2e, arrow heads). The neck corresponds
113 to the posterior part of the nucleus, where there exists an indentation called centriolar
114 fossa that accommodates the centriole (Fig. 2d, e). The nuclear fossa emerges anteriorly
115 from the centriole, while posteriorly emerges the axoneme (Fig. 2e). The nuclear fossa
116 is very short and wavy, constituting slightly more than the tenth part of the nucleus. It is
117 coated by the nuclear membrane (Fig. 2e). The axoneme shows the typical arrangement
118 of nine microtubular doublets surrounding a central doublet (Fig. 2d, arrow head). The
119 anterior part of the axoneme is surrounded by undefined material (Fig. 2d), which
120 become ordered distally into nine well defined coarse fibers (Fig. 2f). The nine coarse
121 fibers together with the axoneme constitute the axoneme-coarse fibers complex (ACF)
122 that is the axis of the flagellum (Fig. 2e).

123 The flagellum is divided into three parts, middle, principal and end pieces. The
124 anterior part or middle piece (Fig. 2c, e), is composed of enlarged mitochondria running
125 parallel to the ACF. Cross section at this level shows that there are ten mitochondria
126 constituting the mitochondrial sheath (Fig. 2f). Middle piece end is marked by the

annulus, which encircles the ACF (Fig. 2g). Principal piece follows middle piece and shows no mitochondrial sheath. There are not highlighting features at principal and end pieces that differ from other cephalopods.

DISCUSSION

This work describes for the first time the sperm morphology of a hydrothermal vent cephalopod. Among the spermatozoa of the Octopoda studied so far, *V. hydrothermalis* acrosome arrangement resembles those present in the families Graneledonidae (Roura et al., in press) and Octopodidae (Galangau and Tuzet, 1968; Longo and Anderson, 1970; Healy, 1989; Zhu et al., 2005), in contrast with the double helix arrangement showed in Bathypolypodidae (Roura et al., in press), and the totally torsioned acrosome of Eledonidae (Maxwell, 1974; Selmi, 1996; Ribes et al., 2002). However, within the Octopodidae, there exists a notable plasticity related with the acrosome arrangement. While the number of gyres in *Octopus vulgaris* acrosome ranges between three and four (Galangau and Tuzet, 1969; Healy, 1989; Ribes et al., 2002), it increases in *O. tankahkei* up to nine gyres (Zhu et al., 2005; Li et al., in press) and reaches up to thirteen in *O. bimaculatus* (Longo and Anderson, 1970). The presence of the equidistant striations perpendicularly disposed along the acrosome of *V. hydrothermalis* reinforces the hypothesis of Selmi (1996), who indicated that these striations are a peculiarity of octopod spermatozoa.

One of the most remarkable features of *V. hydrothermalis* mature sperm is the presence of dense periodic accumulations along its nucleus, a character that has not been described before in cephalopod sperm morphology. *Vulcanoctopus* has the smallest nucleus among the Octopoda. Nuclear length gets this species closer to those found in

Octopodidae (Galangau and Tuzet, 1968; Longo and Anderson, 1970; Martin et al., 1970; Healy, 1989; Zhu et al., 2005). On the contrary, large nucleus presents in Bathypolypodidae (Roura et al, in press), Graneledonidae (Roura et al., in press) and Eledonidae (Maxwell, 1974; Selmi, 1996, Ribes et al., 2002) distance *Vulcanoctopus* sperm from these subfamilies.

Vulcanoctopus nuclear fossa becomes a useful morphological character in order to place this species in their correct family. Although features of the acrosome and nucleus seems to place *Vulcanoctopus* among the Octopodidae, nuclear fossa of *Octopus* species is wide and large (Galangau and Tuzet, 1968; Longo and Anderson, 1970; Healy, 1989; Zhu et al., 2005), while in *Vulcanoctopus* is thin and short. The family that shares this peculiar nuclear fossa is Eledonidae, where the nuclear fossa is composed of some microtubules immersed in a plug of very short dense material (Selmi, 1996). Nevertheless, it is quite unlikely that *Vulcanoctopus* belongs to Eledoninae, due to the unmistakable differences in the acrosome and nucleus arrangement.

Because *Vulcanoctopus* has biserial sucker seriation it should be assigned to the subfamily Bathypolypodinae (Voss, 1988a, b). However, as explained before, recent molecular studies proved that the sucker seriation is a taxonomic invalid character (Strugnell et al., 2009b). Our study clearly shows that the sperm morphology of *Bathypolypus* species differs from that of *Vulcanoctopus*. Therefore, according to the sperm morphology *Vulcanoctopus* does not belong to the Bathypolypodinae, which agrees with the results found by Strugnell *et al.* (2009a, b)

Despite it was made using light microscopy, it is important to underline that the work of Martin et al. (1970) with *Octopus dofleini* (Wulker, 1910) shows spermatozoa that resemble those found in *Vulcanoctopus*, with small nucleus and an acrosome with few gyres. After the revisions of Hochberg (1998), the former species is now placed in

the genus *Enteroctopus* Rochebrune and Mabile, 1889. For this reason, *O. dofleini* does not far belong to the family Octopodidae, but to the recently established family Enteroctopodidae (Strugnell et al., 2009b). Thus, *V. hydrothermalis* sperm is similar to that of the Enteroctopodidae. This reinforces the new arising taxonomy suggested by Strugnell et al. (2009b), and even more, gives worth to the sperm morphology as a taxonomical character, very useful in resolving taxonomic position in species inquirendae and nomina dubida, or where genetic studies cannot be applied (i.e. formalin fixed collection specimens). However this should be considered cautiously, until electron microscopy of other specimens from the Enteroctopodidae (such as *Enteroctopus* or *Benthoctopus*) will be carried out.

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LITERATURE CITED

- Franzén Ä. 1955. Comparative morphological investigations into the spermiogenesis among Mollusca. Zool Bidr Uppsala 30: 399-456.
- Galangau MV, Tuzet O. 1968a. L'Acrosome d'*Octopus vulgaris* L. Observations au microscope électronique. CR Acad Sc Paris sér D 267: 1462-1467.

200 Galangau MV, Tuzet O. 1968b. Les mitochondries pendant la spermatogenèse
 201 d'*Octopus vulgaris* L. : recherches au microscope électronique. CR Acad Sc Paris
 202 sér D 267: 1735-1737.

203 Giménez-Bonafé P, Martínez-Soler F, Buesa C, Sautière PE, Ausió J, Kouach M,
 204 Kasinsky HE, Chiva M. 2004. Chromatin organization during spermiogenesis in
 205 *Octopus vulgaris* II: DNA-Interacting proteins. Mol Reprod Dev 68: 232-239.

206 González AF, Guerra A, Pascual S, Briand P. 1998. *Vulcanoctopus hydrothermalis* gen.
 207 et sp. nov. (Mollusca, Cephalopoda): An octopus from deep-sea hydrothermal vent
 208 site. Cah Biol Mar 39:169–184.

209 González AF, Guerra A, Rocha F, Briand P. 2002. Morphological variation in males of
 210 *Vulcanoctopus hydrothermalis* (Mollusca, Cephalopoda). Bull Mar Sci 71: 289–
 211 298.

212 González AF, Guerra A, Pascual S, Segonzac M. 2008. Female description of the
 213 hydrothermal vent cephalopod *Vulcanoctopus hydrothermalis*. J Mar Biol Assoc
 214 UK 88: 375-379.

215 Guerra A, González AF, Cherel Y. 2000. *Graneledone gonzalezi* sp.nov. (Mollusca:
 216 Cephalopoda): a new octopod from the Îles Kerguelen. Antarc Sci 12: 33-40.

217 Healy JM. 1988. Sperm morphology and its systematic importance in Gastropoda.
 218 Malacol Rev 4: 251-266.

219 Healy JM. 1989. Spermatozoa of the deep-sea cephalopod *Vampyroteuthis infernalis*
 220 Chun: Ultrastructure and possible phylogenetic significance. Philos Trans R Soc B
 221 323: 589-600.

222 Healy JM. 1990a. Ultrastructure of spermatozoa and spermiogenesis in *Spirula spirula*
 223 (L.): systematic importance and comparison with other cephalopods. Hel Meer 44:
 224 109-123.

225 Healy JM. 1990b. Ultrastructure of spermiogenesis in *Vampyroteuthis infernalis* Chun,
 226 a relic cephalopod mollusc. *Helv. Zool. J.* 44: 95-107

227 Healy JM. 1993. Sperm and spermiogenesis in *Opisthoteuthis persephone* (Octopoda:
 228 Cirrata): Ultrastructure, comparison with other cephalopods and evolutionary
 229 significance. *J. Moll. Stud.* 59: 105-115.

230 Hochberg FG. 1998. Class Cephalopoda. pp 175-236. In: Scott, P.V. and J.A. Blake.
 231 Taxonomic atlas of the benthic fauna of the Santa Maria Basin and the western
 232 Santa Barbara Channel, volume 8. Santa Barbara Museum of Natural History. 250
 233 pp.

234 Hou ST, Maxwell WL. 1992. Evidence for an intermediate type of spermatozoon:
 235 Ultrastructural studies of spermiogenesis in the cuttlefish *Rossia macrosoma*
 236 (Cephalopoda, Decabrachia). *Zoomorphology* 112: 207-215.

237 Li Z, Zhu JQ, Yang WX. 2009. Acrosome reaction in *Octopus tankahkei* induced by
 238 calcium ionophore A23187 and a possible role of the acrosomal screw. *Micron*,
 239 doi:10.1016/j.micron.2009.08.001

240 Longo FJ, Anderson A. 1970. Structural and cytochemical features of the sperm of the
 241 cephalopod *Octopus bimaculatus*. *J. Ultra. Mol. Struct.* 32: 94-106.

242 Martin AW Jr, Thiersch JB, Dott HM, Harrison RAP, Mann T. 1970. Spermatozoa of
 243 the giant octopus of the North Pacific *Octopus dofleini* Martini. *Proc. Roy. Soc. Lond.*
 244 *B Biol.* 175: 63-68.

245 Maxwell WL. 1974. Spermiogenesis of *Eledone cirrhosa* Lamarck (Cephalopoda,
 246 Octopoda). *Proc. Roy. Soc. Lond. B Biol.* 186: 181-190.

247 Norman MD, Hochberg FG. 2005. The current state of the octopus taxonomy. *Phuket*
 248 *Mar. Biol. Center Res. Bull.* 66: 127-154.

249 Ribes E, Giménez-Bonafé P, Zamora MJ, González A, Kasinsky H, Chiva M. 2002.
 250 Evolution of Octopod sperm II: Comparison of Acrosomal Morphogenesis in
 251 *Eledone* and *Octopus*. Mol Reprod Dev 62: 363-367.

252 Roura A, Guerra A, González AF, Pascual S. In press. Sperm ultrastructure in
 253 *Bathypolypus bairdii* and *B. sponsalis* (Cephalopoda: Octopoda). J Morph. DOI:
 254 10.1002/jmor.10787

255 Rocha F, González AF, Segonzac M, Guerra A. 2002. Behavioural observations of the
 256 cephalopod *Vulcanoctopus hydrothermalis*. Cah Biol Mar 43: 299–302.

257 Selmi MG. 1996. Spermatozoa of two *Eledone* species (Cephalopoda, Octopoda).
 258 Tissue Cell, 28: 613-620.

259 Strugnell J, Voight JR, Collins PR, Allcock L. 2009a. Molecular phylogenetic analysis
 260 of a known and a new hydrothermal vent octopod: their relationships with the genus
 261 *Benthooctopus* (Cephalopoda: Octopodidae). Zootaxa 2096: 442-459.

262 Strugnell J, Norman M, Vecchione M, Guzik M, Allcock L. 2009b. The octopod ink sac
 263 clouds its evolutionary history. Cephalopod International Advisory Council
 264 Symposium (CIAC'09). Abstract book: 42.

265 Sweeny MJ, Roper CFE. 1998. Classification, type localities, and type repositories of
 266 Recent Cephalopoda. Smithsonian Contrib Zool 586: 561-599.

267 Voight JR. 2005. Hydrothermal vent octopuses of *Vulcanoctopus hydrothermalis*, feed
 268 on bathypelagic amphipods of *Halice hesmonectes*. J Mar Biol Assoc UK 85: 985-
 269 988.

270 Voight JR. 2008. Observations of deep-sea octopodid behavior from undersea vehicles.
 271 Am Malacol Bull 24: 43-50.

272 Voss GL. 1988a. Evolution and phylogenetic relationships of deep-sea Octopods
 273 (Cirrata and Incirrata), 253-276. In: Clarke, MR and Trueman, ER eds.

274 Paleontology and Neontology of Cephalopods. Wilbur, K.M (ed.). The Mollusca.
275 Vol 12, Academic Press, London: 355 pp.

276 Voss GL. 1988 b. The Biogeography of the Deep-Sea Octopoda. Malacologia 29: 295-
277 307.

278 Voss GL, Pearcy WG. 1990. Deep-water Octopods (Mollusca: Cephalopoda) of the
279 Northeastern Pacific. Proc California Acad Sci 47: 47-90.

280 Zhu JQ, Yang WX, You ZJ, Jiao HF. 2005. The ultrastructure of the spermatozoon of
281 *Octopus tankahkeei*. J Shellfish Res 24: 1203-1207.

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Figure legends

Fig. 1. – Plot of the distance between gyres (µm) against number of gyres measured from 9 acrosomes.

Fig. 2. a. Acrosome longitudinal section of *Vulcanoctopus hydrothermalis*, showing the periodic striations along its length. b. Cross section of the acrosome showing the periacrosomal material placed between the plasma membrane and the acrosome membrane. c. Longitudinal section of the spermatozoon showing the acrosome, nucleus and middle piece. Arrow heads: electro-dense bands of cytoplasm along the nucleus. d. Nucleus cross section at the neck level, showing the undefined coarse fibers and the central microtubular doublet (arrow head). e. Longitudinal section through the neck and middle piece, showing the small nuclear fossa and the insertion of the tail. Arrow heads: electro-dense bands of cytoplasm. f. Middle piece cross section. g. Annulus cross section. Abbreviations: Ac, acrosome; AM, acrosome membrane; ACF, axoneme-coarse fibres complex; An, annulus; Ax, axoneme; Ce, centriole; CF, centriolar fossa; Cr,

316 chromatin; CFi, coarse fibers; MP, middle piece; M, mitochondria; NF, nuclear fossa;
 317 NM, nuclear membrane; N, nucleus; PA, periacrosomal material; PM, plasma
 318 membrane. Scale bars: a = 1 μ m; b = 100 nm; c = 1 μ m; d = 200 nm; e = 400 nm; f =
 319 100 nm; g = 100 nm.

Table 1. Sperm cell measures (µm) of *Vulcanoctopus hydrothermalis*. Mean, standard deviation, number of specimens.

Measures	<i>V. hydrothermalis</i>
Head length ¹	15.62
Acrosome (Acr.) length	5.68 ± 0.08 (n=4)
Acr. Pitch ²	0.88
Acr. Width	0.91 ± 0.02 (n=8)
Acr. striation separation	54.66 ± 1.81 nm (n=18)
Nucleus length	9.94 ± 0.02 (n=5)
Nucleus width	1.00 ± 0.10 (n=19)
Nuclear fossa length	1.21 ± 0.04 (n=6)
Neck length	0.83 ± 0.01 (n=19)
Neck width	0.85 ± 0.02 (n=7)
Middle piece length	5.80 ± 0.20 (n=4)
Middle piece width	0.46 ± 0.02 (n=8)
Annulus width	0.65 ± 0.02 (n=6)
Principal piece diameter	0.36 ± 0.01 (n=10)

¹ Acrosome length + Nucleus length

² Number of spires/acrosome length