Differential Cetacea Circadian Rhythmicity is associated with the molecular erosion of Cortistatin

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Abstract

The ancestors of Cetacea underwent profound morpho-physiological alterations. By displaying an exclusive aquatic existence, cetaceans evolved unique patterns of locomotor activity, vigilant behaviour, thermoregulation and circadian rhythmicity. Deciphering the molecular landscape governing many of these adaptations is key to understand the evolution of phenotypes. Here, we investigate Cortistatin (*CORT*), a neuropeptide displaying an important role mammalian biorhythm regulation. This neuropeptide is a known neuroendocrine factor, stimulating slow-wave sleep, but also involved in the regulation of energy metabolism and hypomotility inducement. We assessed the functional status of *CORT* in 139 mammalian genomes (25 orders), including 30 species of Cetacea. Our findings indicate that cetaceans and other mammals with atypical biorhythms, thermal constraints and/or energy metabolism, have accumulated deleterious mutations in *CORT*. In light of the pleiotropic action of this neuropeptide, we suggest that this inactivation contributed to a plethora of phenotypic adjustments to accommodate adaptive solutions to specific ecological niches.

74 Main text

75 Habitat transitions nourish the emergence of novel phenotypes. In this context, Cetacea (whales
76 and dolphins) are a particularly fascinating group to understand the genomic signatures of
77 adaptation to radical shifts, given their land-to-water evolutionary history and their exclusive
78 reliance on aquatic ecosystems (e.g. McGowen et al. 2014; Huelsmann et al. 2019; McGowen et
79 al. 2020). Specifically, gene loss events, including complete gene absence or sequence gene
80 erosion (Albalat and Cañestro, 2016), seem to stand out as significant triggers of phenotypic
81 adaptations within this group: as reported for disparate processes such as skin remodelling,
82 immunity and inflammation, deep-diving induced hypoxia, blood pressure maintenance, or even
83 circadian rhythmicity and sleep/vigilance behaviours (e.g. Braun et al. 2015; Hecker et al. 2017;
84 Lopes-Marques et al. 2018, 2019a, 2019b, 2019c; Alves et al. 2019; Ehrlich et al., 2019;
85 Huelsmann et al. 2019). Among these, circadian and sleep/vigilance behaviours are particularly
86 challenging since cetaceans require occasional surfacing to breathe. Importantly, mammalian
87 sleep generally prompts several physiological adjustments, which conflict with a fully aquatic
88 lifestyle, including hypomotility, sleep thermoregulation or decreased blood pressure (Giglio et
89 al. 2007; Alves et al. 2019). To offset these constraints, Cetacea exhibit distinctive biological
90 rhythms, allowing the maintenance of vigilant states over long periods of time (Ridgway et al.
91 2006; Branstetter et al. 2012), along with lateralized sleep (Lyamin et al. 2008), or even
92 uninterrupted activity as observed in Delphinidae neonates and mothers (Lyamin et al. 2005;
93 Siegel 2005). In agreement, recent studies have highlighted gene loss signatures related with the
94 maintenance of such an unusual form of mammalian sleep/vigilance behaviours, notably
95 regarding the synthesis and signalling of melatonin, a potent modulator of circadian
96 rhythmicity, affecting multiple physiological and behavioural processes such as sleep
97 entrainment, locomotor activity or thermoregulation (Huelsmann et al. 2019; Lopes-Marques
98 2019c). Here, we investigate Cortistatin (*CORT*), a cyclic neuropeptide which plays an
99 important role in sleep physiology (de Lecea et al. 1996). Belonging to the somatostatin (SST)
100 neuropeptide family, *CORT* was shown to have sleep-promoting properties, stimulating slow-
101 wave sleep, as well as to induce hypomotility (Spier and de Lecea 2000) (Figure 1). Moreover,
102 the diversification of roles attributed to *CORT* also includes regulation of endocrine
103 metabolism, immunomodulation, inflammatory responses, pain perception and cardiovascular
104 protection (Deghengi et al. 2001; Robas et al. 2003; Broglio et al. 2007; Gonzalez-Rey et al.
105 2015; Liang et al. 2019). Despite sharing similarities with SST, such as peptide structure,
106 resulting from proteolytic cleavage, and binding affinity towards SST receptors, *CORT*
107 generally yields antagonizing effects when compared to SST (Spier and de Lecea 2000; Broglio
108 et al. 2007). In this context, we sought to characterize the functional status of *CORT* genes in
109 Cetacea, and other mammalian lineages, to determine whether gene inactivation events have
110 taken place, as previously reported for other genes (e.g. Kim et al. 2014; Shinde et al. 2019). We

111 began by inspecting the open reading frame (ORF) of *CORT* in a selected sub-set of Cetacea
 112 species using PseudoIndex (Alves et al. 2020). This user assistant metric built into the
 113 PseudoChecker pipeline rapidly estimates the erosion condition of the tested genes - discrete
 114 scale from 0 (functional) to 5 (pseudogenized) (Alves et al. 2020). All analysed species revealed
 115 a PseudoIndex equal to 5 within Odontoceti species, except for *Lipotes vexillifer* and
 116 *Delphinapterus leucas*, which displayed a PseudoIndex of 2 and 3 respectively (Supplementary
 117 File 1). This analysis suggested that the ORF of *CORT* includes inactivating mutations. Thus,
 118 we next performed a manual and exhaustive *CORT* sequence analysis in a larger phylogenetic
 119 collection of species and included an additional curated validation step of the mutational
 120 evidence. In detail, genomic *CORT* sequences from 139 terrestrial and aquatic mammalian
 121 species (Supplementary File 2), containing 30 Cetacea (9 Mysticeti and 21 Odontoceti), were
 122 collected from available genomes and manually annotated using *Bos taurus* (phylogenetically
 123 related to Cetacea (McGowen et al. 2019)) or *Homo sapiens* (for other mammals) *CORT* coding
 124 sequences (CDS) as a reference (Lopes-Marques et al. 2017). Predicted ORFs were screened for
 125 disrupting mutations, with these being further validated (when possible) using data from at least
 126 2 independent Sequence Read Archive (SRA) genomic projects or from 2 distinct individuals
 127 (Lopes-Marques et al. 2019a). Gene orthology between Cetacea species and *B. taurus* was
 128 validated through synteny analysis, which essentially revealed *locus* conservation between
 129 species (Supplementary File 3).

130

131 ***CORT* exhibits a conserved premature stop codon in Delphinoidea**

132 In Odontoceti (toothed whales and dolphins), all Delphinoidea, including *Delphinidae*
 133 (dolphins, e.g. *Tursiops truncatus*, *Orcinus orca*), *Phocoenidae* (porpoises, e.g. *Phocoena*
 134 *phocoena*), and *Monodontidae* (narwhal, *Monodon monoceros*, and beluga whale,
 135 *Delphinapterus leucas*), exhibited a conserved stop codon mutation in exon 1 (Figure 1), with
 136 the exception of *O. orca*, which displays an arginine codon in the same position (CGA). Yet,
 137 given the striking conservation of the premature stop codon across analysed Delphinoidea, and
 138 the single nucleotide difference between both codons, the *Orca* exception likely represents a
 139 case of mutational reversion (Stop>Arg, TGA>CGA) (Rosenberg 2001). Nonetheless, in
 140 addition to the premature stop codon, other mutations were identified, confirming the erosion of
 141 *CORT* in *O. orca*, such as frameshifts in exon 1 (31 nucleotide deletion and 2 nucleotide
 142 insertion), conserved in all *Delphinidae* (Figure 1). In *O. orca* and Delphininae species
 143 (*Tursiops* and *Sousa*), exon 2 also presented a frameshift mutation (1 nucleotide deletion), a
 144 pattern not consistent with the species branching tree topology (McGowen et al., 2019).
 145 Additionally, deleterious mutations were also found across all members of *Phocoenidae* and
 146 *Monodontidae* (described in Supplementary File 4). Detected mutations were further validated
 147 by independent SRAs in all species, when available (Supplementary File 5).

148

149 To further scrutinize the functional condition of *CORT*, we searched for transcriptional evidence
 150 of this gene in *Globicephala melas*, *O. orca* and *D. leucas* by investigating available brain
 151 RNA-Seq projects at the SRA database (Krüger et al. 2020). The collected mRNA reads were
 152 mapped against the corresponding annotated gene and classified as spliced reads (reads partially
 153 covering two exons), exon-intron reads (unspliced reads) and exonic reads (reads containing
 154 only data from a single exon) (Lopes-Marques et al. 2019b). Briefly, we observed a
 155 substantially high proportion of exon-intron reads *versus* spliced reads in stark contrast to the
 156 pattern found in cow (positive control) (Figure 2). In addition, we were able to identify at least
 157 one premature stop codon in the transcripts of all analysed species (Supplementary file 6),
 158 further validating the predicted ORF-abolishing mutations.

159

Figure 1: Distinct physiological roles attributed to *CORT*, effect of *CORT* knockout mice in slow wave sleep time and cetacean idiosyncratic sleeping in the form of lateralized sleep behaviour (A). Mutational landscape of the *CORT* gene in several Odontoceti, *H. amphibius* and *B. taurus* and location of identified mutations (B). Phylogenetic relationships are derived from McGowen et al. (2020). Example of *CORT* open reading frame (ORF) inactivating mutations concerning *Delphinidae*, *Phocoenidae* and *Monodontidae* clades (C). Numbers above characters represent the alignment position index. Silhouettes were recovered from Phylopic (<http://phylopic.org>).

Divergent patterns of functional inactivation are observed in river dolphins

River dolphins represent a polyphyletic group within Odontoceti, including species such as *Lipotes vexillifer*, *Pontoporia blainvillei*, *Inia geoffrensis* or *Platanista gangetica*. Among these, *L. vexillifer* showed a premature stop codon in exon 2, *P. gangetica* presented frameshift

172 mutations in both exons (31 nucleotide deletion in exon 1 and 4 nucleotide insertion in exon 2)
 173 and *I. geoffrensis* displays an indel in exon 2 (Supplementary File 4). Given that no SRAs are
 174 available for these species, SRA validation was not performed. Regarding *P. blainvillei*, on the
 175 other hand, no ORF disrupting mutations were found. Yet, further sequence analysis revealed
 176 amino acid changes that possibly impair canonical peptide function (Supplementary File 7).
 177 *CORT*, similarly to *SST*, contains a carboxyl-terminal FWKT (Phe-Trp-Lys-Thr) tetramer,
 178 essential for binding to *SST* receptors (de Lecea et al. 1996; Spier and de Lecea 2000). Despite
 179 its strict conservation across mammals, amino acid substitutions are observed in *P. blainvillei*,
 180 thus suggesting that, in this species, *SST* receptor binding is possibly impaired (Supplementary
 181 File 7).

182

183 **Variable disruption patterns are observed in the Odontoceti Ziphiidae, Physeteroidea, as** 184 **well as in Mysticeti**

185 In *Ziphiidae* (*Mesoplodon bidens* and *Ziphius cavirostris*) or *Kogia breviceps* (pygmy sperm
 186 whale), no ORF disrupting mutations, nor radical amino acid replacements, were detected
 187 (Supplementary File 4). On the other hand, *Physeter macrocephalus* presented a single
 188 frameshift mutation in exon 2, validated by SRA (Supplementary file 4). In Mysticeti,
 189 *Balaenoptera acutorostrata* presented a premature stop codon in exon 2 (Supplementary File 4).
 190 Also, a 22-nucleotide deletion was detected in exon 1 of *Eschrichtius robustus*, *Megaptera*
 191 *novaeangliae* and *Balaenoptera musculus*, while for *Balaenoptera physalus* we identified three
 192 indel mutations also in exon 1. These mutations were validated by SRA searches in *B.*
 193 *acutorostrata* and *E. robustus* (Supplementary File 5). Besides these ORF-disrupting mutations,
 194 a conserved loss of start codon (ACG - Thr) was also detected for the full set of examined
 195 Mysticeti species, except for *B. acutorostrata*. However, an ATG (methionine) codon could be
 196 found downstream from the threonine in the same translational reading frame (Supplementary
 197 File 4). Thus, in species presenting no more disruptive mutations - namely in *Eubalaena*
 198 *glacialis*, *Eubalaena japonica*, *Balaenoptera bonaerensis* and *Balaenoptera edeni*, we cannot
 199 rule out the possibility of a functional *CORT* gene. In *Balaena mysticetus*, the fragmentation of
 200 the genomic region (Ns) corresponding to exon 2 from *CORT* impeded us to infer the coding
 201 status in this species. Additionally, for *B. acutorostrata*, mapping of transcriptional reads from a
 202 brain RNA-Seq project yielded a high proportion of exon-intron reads versus spliced reads
 203 (Figure 2) and further corroborated the presence of the premature stop codon in exon 2. For the
 204 remaining analysed species, no deleterious mutations were unequivocally found. Nonetheless,
 205 besides ORF disruptive mutations, other processes can lead to the non-functionalization of a
 206 gene: i.e. lack of transcription, RNA decay, or even protein degradation (e.g. Sadier et al. 2018).
 207 Yet, we were unable to verify the transcriptomic profile of *CORT* in these species given the
 208 absence of adequate transcriptomic data.

209

210

211 **Figure 2:** Gene expression of *CORT* across represented Cetacea species: mapping of the NCBI Sequence Read
212 Archive (SRA) brain RNA-Seq reads (black) for each of the 5 represented species against the corresponding *CORT*
213 annotated gene (in blue). Overall count of RNA-Seq mapped reads for each species is also represented. Reads are
214 classified into spliced reads (SR), exon-intron reads (UR), and exonic reads (ER).

215

216 Selection analysis

217 To find evidence of some relaxation of purifying selection typically associated with events of
218 gene pseudogenization, RELAX analysis using the HyPhy package was performed (Pond et al.
219 2005; Wertheim et al. 2014). We used the predicted *CORT* sequences from 43 species,
220 comprising all Cetartiodactyla species analysed in this study (Supplementary File 2). As
221 RELAX compares a background set of species with a foreground set of species over a
222 hypothesis-testing framework, we targeted the ancestral branch of the cetacean lineages, using
223 the branches from all non-cetacean species as a reference. Although no significant
224 intensification or relaxation has been found (Supplementary File 8 – Table S1), it can be seen
225 through the general descriptive model (Supplementary File 8 – Figure S1) that the ancestor of
226 all cetaceans experienced relaxed selection ($k < 1$), coinciding with substantial changes in
227 *CORT* amino acidic composition. Moreover, we detected signs of intermittent relaxed selection
228 ($k < 1$) within various cetacean lineages, which could result from differences in gene
229 length/composition across branches. This could be due to an intensification of
230 positive/diversifying selection prior to the observed pseudogenization in the terminal cetacean
231 branches.

232

233

234

235 **Episodes of *CORT* ORF-disruption are found in other mammal species**

236 We next interrogated the genomes of other mammalian species. The implemented assessment
 237 revealed a number of ORF-disrupting mutations in 14 species (Supplementary File 9). For
 238 example, the sequence of *CORT* in *Ursus maritimus* (polar bear) revealed a single nucleotide
 239 deletion in exon 2 and in *Procavia capensis* (rock hyrax) a premature stop codon in exon 2
 240 (confirmed by SRA search in *U. maritimus*; Supplementary File 10); in the Pholidota *Manis*
 241 *javanica* and *Manis pentadactyla* (pangolins), a shared frameshift mutation in exon 1 and a
 242 premature stop codon was identified (validated by SRA in *M. javanica*; Supplementary File 10).
 243 In the Carnivora *Canis lupus* (wolf) and *Lycaon pictus* (African wild dog), conserved single
 244 nucleotide insertions were found in exon 2, whereas *Felis catus* (cat) and *Acynonix jubatus*
 245 (cheetah), exhibit a conserved 2 nucleotide insertion in exon 1, all validated by independent
 246 sequencing reads. Other examples include *Dasypus novemcinctus* (nine banded armadillo) with
 247 an indel and a premature stop codon in exon 2, *Choleopus hoffmanni* (Hoffmann's two-toed
 248 sloth) with single nucleotide insertions and deletions in both exons, and *Condylura cristata*
 249 (star-nosed mole) for which exon 2 was not found.

250

251 **Metabolic Homeostasis and Circadian rhythmicity**

252 Our study provides clear evidence for the dismantling of *CORT* gene sequence within most
 253 Cetacea and in several other mammalian lineages. Curiously, various of these species display
 254 particular biological rhythms and/or labile body temperatures: the hibernating polar bear; the
 255 subterranean star-nosed mole; nocturnal species with variable and relatively low body
 256 temperatures such as the two-toed sloth, the nine banded armadillo, pangolins, or the rock hyrax
 257 that undergoes nocturnal hypothermia (Ralph 1975; Brown and Downs 2007; Ware et al. 2013).
 258 Thus, it is plausible to hypothesize that, in these species, *CORT* inactivation is related with
 259 changes in circadian rhythmicity, affecting sleep/vigilance behaviours, body temperature
 260 maintenance and/or activity patterns. Interestingly, these species also display some degree of
 261 reduction or even complete atrophy of the pineal gland, the dominant organ for melatonin
 262 synthesis (Pévet 2002). In Cetacea, constituting a group with a remarkably altered sleep
 263 physiology, *CORT* pseudogenization possibly paralleled additional losses, notably regarding the
 264 melatonin synthesis and signalling genes (Lopes-Marques et al. 2019c; Huelsmann et al. 2019).
 265 Unexpectedly, we also found robust evidence supporting *CORT* pseudogenization in some
 266 Carnivora species, particularly felids and canids. However, and in addition to SST receptors,
 267 *CORT* was also suggested to bind other G-protein-coupled receptors such as the ghrelin receptor
 268 growth hormone secretagogue receptor type 1a (GHSR-1a), which participates in energy
 269 homeostasis regulation: namely in the control of food intake and energy metabolism
 270 (Deghenghi et al. 2001; Broglio et al. 2007). Mice lacking *CORT* gene showed alterations in

whole-body metabolism, in a gender-dependent manner, notably resulting in the increase of acylated ghrelin levels in female, or higher glucose levels and insulin resistance in males (Córdoba-Chacón et al. 2011; Luque et al. 2016). In addition, *CORT* action was also proposed to be conditioned by the underlying metabolic status (i.e. fasting or obesity) (Luque et al. 2016). These observations are in agreement with the metabolic profile observed in felids, associated with a low-carbohydrate consumption and deficit in hepatic glucokinase activity, yielding fasting hyperglycaemia and insulin resistance (Schermerhorn 2013). Both hyperglycaemia and insulin resistance were also reported in dolphins, also displaying reduced carbohydrate intake (Schermerhorn 2013). Overall, our results put forward that the evolutionary loss of *CORT*, along with additional genomic and phenotypic signatures, paralleled the adjustment of circadian rhythmicity and energy homeostasis to accommodate adaptations to specific ecological niches and life-history traits.

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