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ORIGINAL PAPER

Effect of the algal alkaloid caulerpin on neuropeptide Y (NPY) expression in the central nervous system (CNS) of *Diplodus sargus*

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Abstract
Recent studies have suggested that Mediterranean indigenous fish species are affected by bioactive metabolites coming from marine invasive species via food web interactions. In particular, both physiological and behavioural changes in the white sea bream *Diplodus sargus* were related to caulerpin (CAU), a bisindolic alkaloid particularly abundant in the invasive alga *Caulerpa cylindracea*, on which the fish actively feed. Dietary administration of CAU decreased aggressiveness in *D. sargus*, suggesting an anxiolytic-like effect of CAU possibly mediated by endogenous anxiolytic agents. This hypothesis is supported here by the finding of a significant increase of NPY transcriptional expression in the brain of fish fed with CAU enriched food, shedding more light on the neural mechanisms behind the altered behaviour of *D. sargus*.

Keywords NPY · Caulerpin · Biological invasions · *Caulerpa cylindracea* · *Diplodus sargus*

Introduction

Invasive species are a worldwide leading threat to wildlife conservation (Vitousek et al. 1997; Molnar et al. 2008). Recently, the invasive alga *Caulerpa cylindracea* showed an explosive dispersal and had a deep impact on indigenous communities in the Mediterranean Basin (Piazzi and Cinelli 2001; Piazzi and Balata 2008; Deudero et al. 2011; Vázquez-Luis et al. 2009). This impact includes the secondary effects of the ingestion of metabolites present in the alga tissue (Felline et al. 2017) that become a major diet component of white sea bream, *Diplodus sargus* (Klein and Verlaque 2008; Box et al. 2009; Terlizzi et al. 2011). This alga is known to contain bioactive metabolites (Yang et al. 2015), the most studied of which are the bisindolic alkaloid red pigment caulerpin (CAU), the toxic sesquiterpene caulerpenyne, and the mixture of hydroxy amides caulerpicin, for which a vast panel of biological activities are reported in the literature (Mollo et al. 2015). In particular, it has been established that CAU enters the food chain and accumulates in the fish tissues (Terlizzi et al. 2011; Felline et al. 2012, 2014, 2017; Gorbi et al. 2014). In addition, the level of CAU in *D. sargus* tissues has been related to the appearance of cellular, physiological, and behavioural alterations (Felline et al. 2012, 2017; Gorbi et al. 2014). A reduction of the aggressive behaviour of *D. sargus* has been observed as an effect of dietary administration of CAU (Magliozzi et al. 2017), possibly mediated by endogenous anxiolytic agents.

In vertebrates, neuropeptide Y (NPY) is a highly conserved neuropeptide that, beside to have orexigenic effect (reviewed in Loh et al. 2015), it shows anxiolytic and stress-reducing properties (reviewed in Karl and Herzog 2007; Schmeltzer et al. 2016), also decreasing swimming activity in fish (Matsuda et al. 2011, 2012; Jeong et al. 2018).

Consequently, in this study we investigated the hypothesis that orally administered CAU can affect NPY distribution and gene expression in the brain of *D. sargus*, with the aim to provide evidence for a role of NPY in the altered behaviour of the fish.

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Materials and methods

Extraction of caulerpin in *C. cylindracea*

Caulerpa cylindracea was collected in Italy in the Gulf of Pozzuoli (40°48′N, 14°07′E) and stored at − 20 °C until chemical analyses were performed. The alga was exhaustively extracted with acetone at room temperature following Anjaneyulu et al. (1991). The acetone extract was evaporated at reduced pressure and the residual water was extracted with diethyl ether. The diethyl ether extract was first fractionated on Sephadex column ($CHCl_3$/MeOH 1:1, as eluent) to give a fraction that was further purified by silica-gel column chromatography (gradient of light petroleum ether/Et_2O, as eluent) to give pure caulerpin (CAU), identified by comparison of spectroscopic data with the literature (Maiti et al. 1978). Size-exclusion chromatography was achieved on Sephadex LH-20 column, whereas silica-gel column chromatography was performed using Merck Kieselgel 60 powder. NMR data were recorded on a Bruker Avance-400 spectrometer using an inverse probe fitted with a gradient along the *z* axis. The NMR spectra were acquired both in DMSO-d6, and in $CDCl_3$.

Subject and holding facilities and acclimation

Juvenile white sea breams were collected from pools in the Atlantic Portuguese coast near Cascais (38°40′N, 9°21′W) and transported in constantly aerated container to the laboratory of ISPA-Instituto Universitário for behavioural experiments. In the laboratory, 12 fish with a mean initial body mass of 1.82 ± 0.1 g and a standard length of 3.99 ± 0.1 cm were randomly housed in sea water aquaria. Experiments were conducted in 12 tanks ($20 \times 40 \times 30$ cm; filled with 20 L of sea water) equipped with recirculating seawater and external filtration system. Water in each aquarium was drained at the bottom to a reservoir tank from which water was then pumped to each aquarium unit after running through a biofilter. Water was exchanged in the aquaria every week. The water physicochemical parameters were maintained as follows: temperature, 20–22 °C; dissolved oxygen, 7 mg L^{-1}, pH 7–8; salinity 33–35 g L^{-1}; NH_4 and NO_2 never exceeded 0.5 mg L^{-1}. Fish were kept under constant conditions of photoperiod (12L:12D) and fed (stick for cichlids JBL, Joachim Bohme in Ludwigshafen) with 0.25 g once a day in the morning. During a period of acclimation, they were accustomed to the artificial food and soon learned to recognize food when it was placed in the tanks.

Behavioural experiments

The experiment lasted 20 days divided into acclimation, and treatment phases (5 and 15 days, respectively); after acclimation, fish were randomly assigned to control (C), low dose (LD) and high dose (HD) feeding groups. During the treatment phase, LD groups were fed with 0.25 g of food enriched with CAU at natural estimated levels in *C. cylindracea* (nominal concentrations of 0.1 mg g^{-1}), while HD subjects were fed with a dose of CAU tenfold higher (nominal concentrations of 1.0 mg g^{-1}). Treated food was prepared by soaking 0.25 g of commercial pellet (stick for cichlids JBL) in 1.5 mL acetone, in which CAU was previously dissolved at the desired concentration and then evaporating the organic solvent under reduced pressure. The same procedure, without adding CAU, was performed for the control food. Leftovers were removed each day from the aquaria. To evaluate CAU possible effect on fish feeding behaviour, tanks had 3 opaque sides to avoid visual interference from other subjects in adjacent tanks. For each fish, behavioural observations of three 5-min sessions were carried out during feeding at 10:00 a.m. daily, by video records.

Video were analysed to determine frequency of feeding behaviour (number of pellets consumed by fish) following fish in each aquarium.

Fish behaviour was analysed with Solomon Coder beta114.05.19 (ELTE TTK, Hungary). A second analysis of 25% of the video recorded was independently performed by another observer to verify the first analysis reliability. Agreement percentage was more than 95%.

One-way analysis of variance (ANOVA) was performed to test if there were statistically significant differences ($P < 0.05$) between experimental and control groups. Following behavioural experiments fish were euthanized with a 400 mg L^{-1} dose of MS222 (tricaine methane sulphonate; Pharmaq, Norway) for subsequent tissue analysis. The brains were immediately dissected and processed (see immunohistochemistry below) for immunohistochemical and molecular analysis.

Immunohistochemistry

Six (two for each group) brains were quickly dissected and fixed in Bouin's fluid fixative for 24 h at room temperature and kept for a week in 75% alcohol, which was changed daily.

Brains, after fixation, were dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin (58–60 °C). Serial sections were cut in transversal plane at 7 µm, and mounted on series of albumin-coated slides and processed for localization of NPY immunoreactivity. Sections were deparaffinised, rehydrated in a decreasing ethanol series, washed in 0.1 M phosphate-buffered saline (PBS, pH 7.4) and then treated with 1% normal goat serum (Sigma Chemical) in PBS for 20 min to reduce nonspecific staining and subsequently incubated with primary antiserum (rabbit antiserum raised against NPY; Phoenix) at a dilution of 1:3000 overnight at 4 °C in a dark moist chamber. After two 10-min

rinses in PBS, sections were incubated with biotinylated secondary antibody (goat anti-rabbit IgG, 1:200; Pierce) for 1 h at room temperature. After two 10-min rinses in PBS, purified HRP-streptavidin (1:200; Pierce) was applied to the sections for 1 h. The antigen/antibody complex was visualized by exposure to 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, Milan, Italy) with 0.3% H_2O_2 in TRIS buffer (0.05 M, pH 7.4). Slides were dehydrated and mounted in Bio Mount (Bio Optica). Specificity of the immunostaining was determined by omitting the primary antiserum that led to a complete absence of staining. Immunohistochemistry protocol was tuned on *D. sargus* based on our previous experiences (D'Aniello et al. 2016). Microscopy analysis was performed at Leica DM 6000 R with digital camera Leica DFC340 FX using Leica application suite (LAS) software to observe NPY peptidergic pathways variations in the brain of treated and control sample. The identification of brain structures was based on Munoz-Cueto et al. (2001).

Molecular analysis

Brain tissues were sampled from six individuals (two for each group) in a sterile and RNase-free way, kept on ice immediately, and RNA was extracted and purified using RNeasy Mini Kit (QIAGEN, Valencia, USA). The disruption and homogenization occurred using TissueLyser II (QIAGEN®) and steal beads of 5 mm diameter (QIAGEN®).

The quality and amount of purified RNA was analysed spectrophotometrically with Nanodrop2000 (Thermo Scientific Inc., Waltham, MA USA) and QubitTM RNA Assay Kits on Qubit® 2.0 Fluorometer (Life Technologies). 1000 ng of RNA was reverse transcribed with the QuantiTect® Reverse Transcription Kit (QIAGEN, Valencia, USA), used as described by the manufacturer. Afterwards Real Time PCR was performed using the QuantiTect SYBR Green PCR Kit (QIAGEN, Valencia, USA) in a final volume of 25 µL, with 100 ng of cDNA, 1 µM of each primer, 12.5 µL of QuantiFast SYBR Green PCR Master Mix (2×). PCR cycling profile consisted of a cycle at 95 °C for 5 min, 40 two-step cycles at 95 °C for 15 s, at 60 °C for 60 s. Quantitative RT-PCR analysis was conducted using the $2(-\Delta\Delta C(T))$ method (Livak and Schmittgen 2001). RT-PCR was performed in a Rotor-Gene Q cycler (QIAGEN, Valencia, USA). We analysed a fragment of 84 bp of the coding region of the NPY. The β-actin gene (85 bp) was used as an internal standard (Table 1) (Micale et al. 2012).

At the end of each test, a melting curve analysis was done (plate read every 0.5 °C from 55 to 95 °C) to determine the formation of the specific products. Each sample was tested and run in duplicate.

mRNA levels in the different treatments were compared by one-way analysis of variance (ANOVA), followed by Tukey's test. Significant difference between two means was evaluated using Student's *t* test.

Results

Behavioural experiments

During treatment, within the first 5 min of food administration, feeding frequency showed an increasing trend with results of 55.54 ± 7.91 for C group, 64.79 ± 7.47 for LD group and 77.63 ± 6.18 for HD group (Fig. 1). Statistical analysis reveals no significant differences among groups.

Immunohistochemistry

The distribution of NPY immunoreactivity (-ir) in the brain of *D. sargus* reveals no appreciable differences among different treated groups and it is summarized in Fig. 2. In agreement with the existing literature (Mathieu et al. 2002; Pirone et al. 2008) NPY-ir cells were regionally located in discrete nuclei while a wide presence of positive fibers was seen throughout the brain, except the cerebellum. Proceeding from the anterior to posterior part of the brain, NPY-ir cells were detected in the telencephalic ventrolateral nucleus (Ntv) (Figs. 2d, 3), some scattered cells in the dorsal telencephalon (medial pallium) and in the anterior commissure. Some NPY-ir cells are found in the posterior part of the preoptic area, in the hypothalamus, in the nucleus posterioris periventricularis (Npp) (Figs. 2h, 4). NPY-ir fibres were found in the pretectum, surrounding the nucleus pretectalis superficialis. Fibres are also detected in the optic chiasma and tracts until the retina where several amacrine cells result NPY-ir. Within the mesencephalon a conspicuous number of positive cells are detected in the nucleus tegmentalis

Table 1 Primer sequences of the genes used for RT-PCR

	Description	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon length (bp)
β-Actin	Housekeeping genes, Actin	GCACCCTGTCCTGCTCACA	GTTGAAGGTCTCGAACATGATCTG	85
NPY	Neuropeptide Y	GGAGGAGCTGGCCAAGTACTACTCA	TCTGGACCTCTTTCCATACCT	84

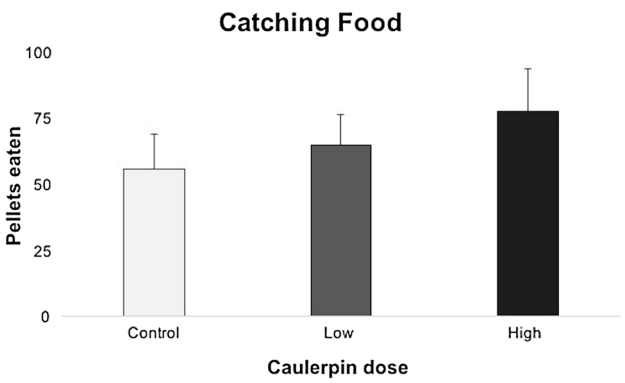

Fig. 1 Diagram showing catching-food frequency in control (white), low dose (gray), high dose (black) during treatment period

dorsalis (Ntd) (Figs. 2j, 5). No positive cells neither fibres were detected in the cerebellum while in rhombencephalon NPY-ir cells appear in the nucleus gustatorius secundarius (Ngs) together with a network of ir-fibres (Figs. 2k, 6).

Numerous cells and fibres NPY-ir were observed in the neurohypophysis at the pars distalis and pars intermedia level (Figs. 2g, 7).

Molecular analysis

The expression of the NPY gene was analyzed by real time PCR on mRNAs from different samples using the control samples to normalize the amount of mRNA. Compared to control samples, the relative expression of the NPY gene was higher in both LD and HD groups, about 1.5-folds for LD and twofolds for HD ones ($p < 0.05$) (Fig. 8).

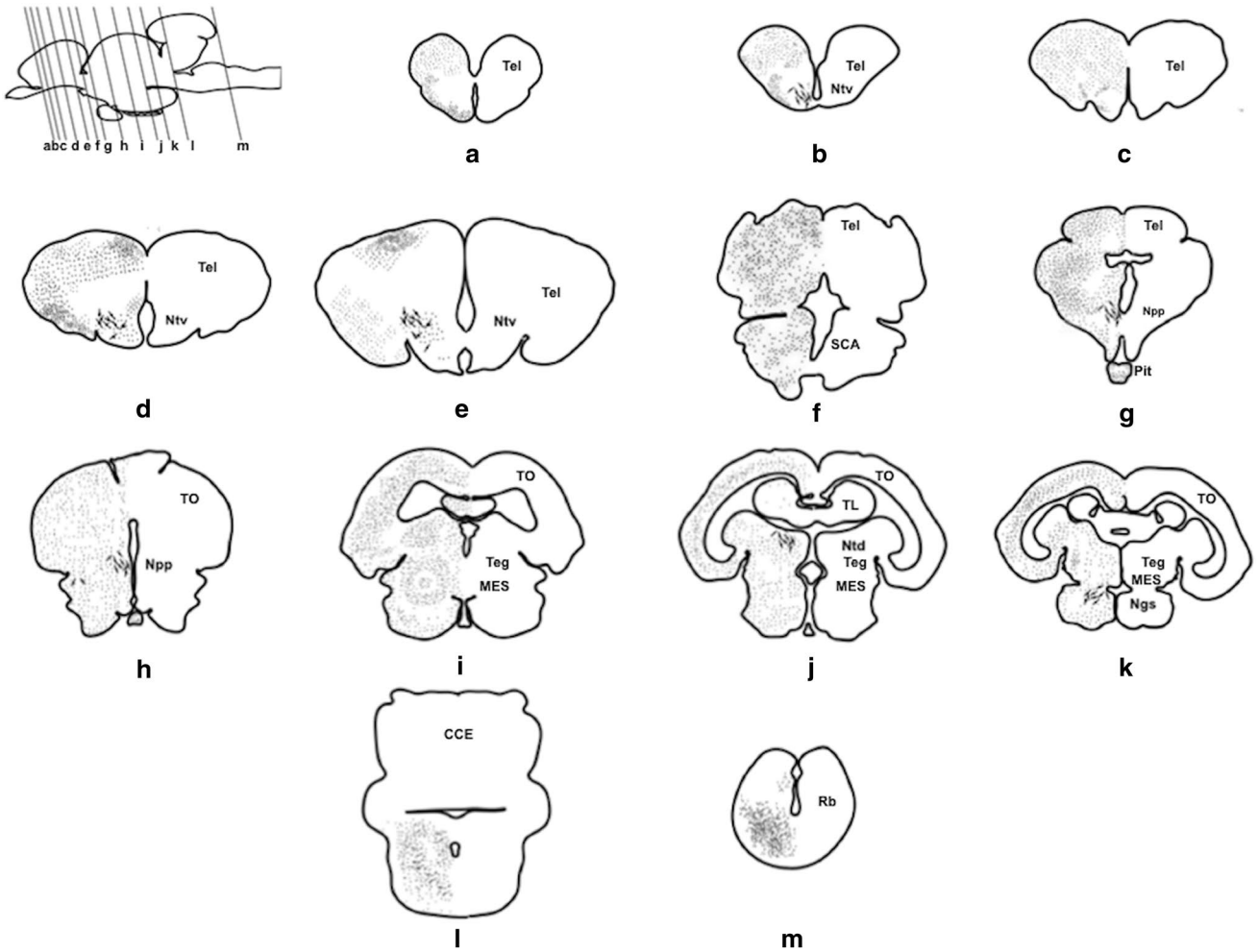

Fig. 2 Schematic representation of *Diplodus sargus* brain transversal sections showing NPY-immunoreactive (ir) fibers and cells. *Cce* cerebellum, *MES* mesencephalon, *Ngs* nucleus gustatorius secundarius, *Npp* nucleus posterioris periventricularis, *Ntd* nucleus tegmentalis dorsalis, *Ntv* telencephalic ventrolateral nucleus, *Pit* pituitaria, *Rb* rhombencephalon, *SCA* area suprachiasmatica, *Teg* tegmentum, *Tel* telencephalon, *TL* torus longitudinalis, *TO* tectum opticum

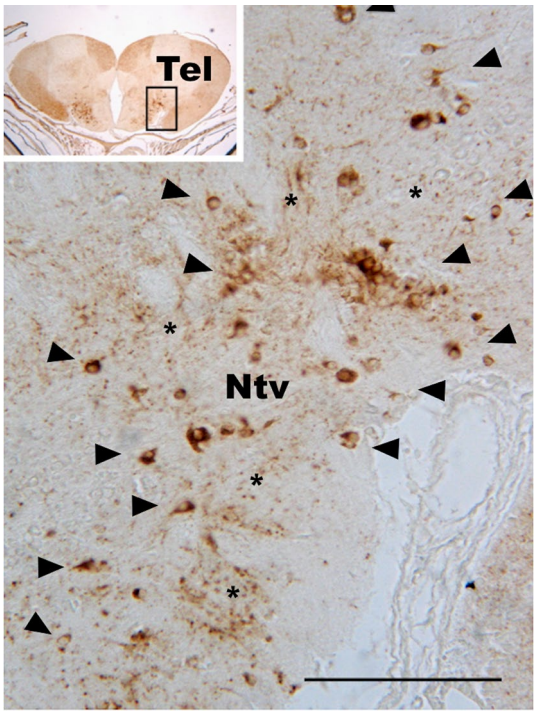

Fig. 3 NPY-ir neurons (arrowheads) and fibers (asterisk) in telencephalic ventrolateral nucleus. Scale bar: 100 µm. *Ntv* telencephalic ventrolateral nucleus, *Tel* telencephalon

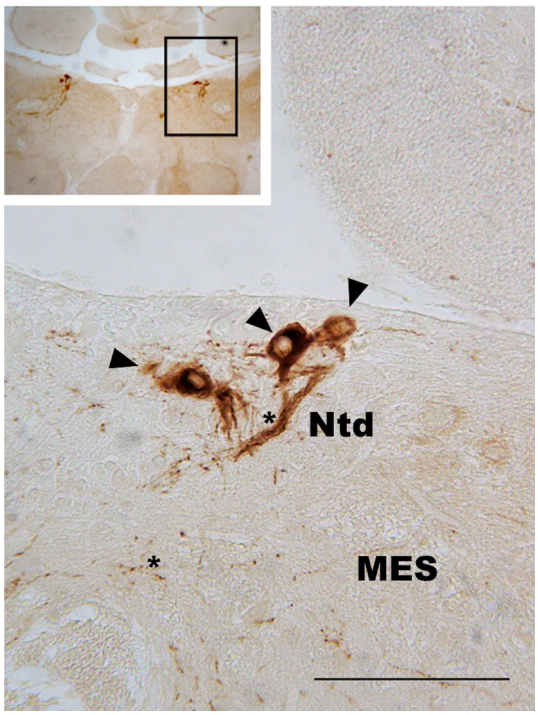

Fig. 5 NPY-ir neurons in the dorsal nucleus tegmentalis. Scale bar: 100 µm. *MES* mesencephalon, *Ntd* nucleus tegmentalis dorsalis

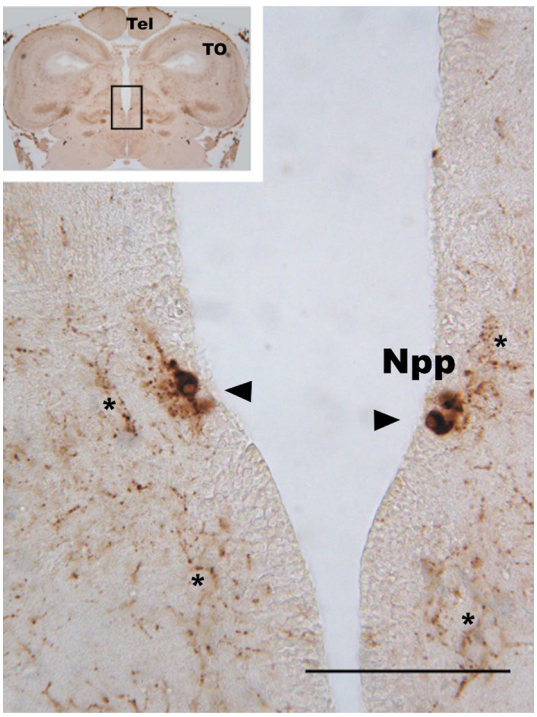

Fig. 4 NPY-ir neurons (arrowheads) in the nucleus posterioris periventricularis of the hypothalamus. Scale bar: 100 µm. *Npp* nucleus posterioris periventricularis, *Tel* telencephalon, *TO* tectum opticum

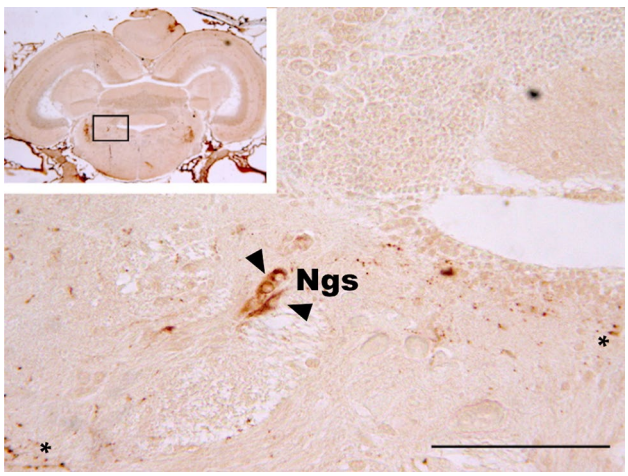

Fig. 6 NPY-ir neurons cells in the nucleus gustatorius secundarius. Scale bar: 100 µm. *Ngs* nucleus gustatorius secundarius

Discussion

Different organisms face a range of challenging biotic and abiotic stressors leading them to adjust behaviour or physiology accordingly (Killen et al. 2013). As a direct consequence of environment and climate change, *C. cylindracea* is one of the main invasive seaweeds introduced in the

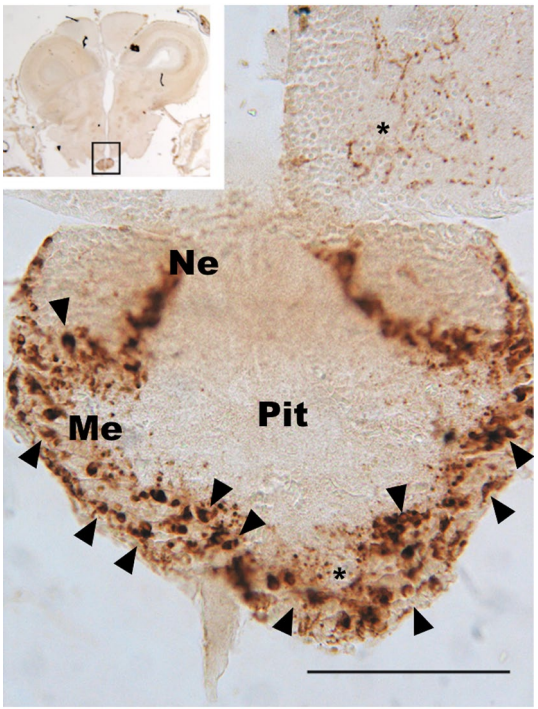

Fig. 7 NPY-ir fibers and cells in pars distalis and pars intermedia of neurohypophysis. Scale bar: 100 µm. *Me* metaadenohypophysis, *Ne* neurohypophysis, *Pit* pituitaria

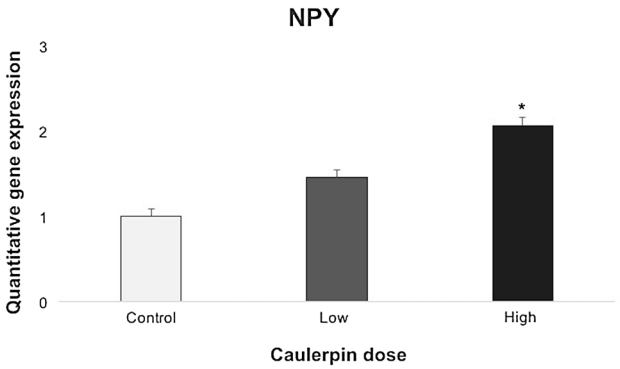

Fig. 8 Diagram showing the quantitative gene expression, by real time PCR, for neuropeptide Y (NPY). Actin B (ActB) was used as a normalizer. Asse Y (relative fold changes in mRNA expression)

Mediterranean Sea (Verlaque et al. 2003; Klein and Verlaque 2008; Ivesa et al. 2015) and caulerpin (CAU) is one of its most abundant metabolites. This metabolite is known to influence physiological and cellular processes (Terlizzi et al. 2011; Felline et al. 2012, 2014; Gorbi et al. 2014; Vitale et al. 2018) and it is noteworthy that disruption of metabolic processes influences normal fish behaviour (Killen et al. 2013). Highlighting the influence of CAU on this omnivorous fish species social behaviour, a recent study showed that the aggressiveness of juvenile *D. sargus* decreased with increasing consumption of CAU (Magliozzi et al. 2017). Furthermore, the natural diet of *D. sargus* includes a percentage of algae ranging from 12% (Sala and Ballesteros 1997) to 33% (Terlizzi et al. 2011). This later study was performed in a region of the Mediterranean Sea extensively impacted by this invasive alga. Changes in behavioural patterns may have consequences on fish survival since variation in aggression among individuals can determine their relative position within social hierarchies, affecting growth, reproductive success, and survival (McCormick and Meekan 2010; McCormick 2012; Huntingford et al. 2012). Indeed, social interactions in fish are an important component of their social structure (Winberg et al. 1996) with several direct impacts on individuals that compete for food and space (Holm and Refstie 1990; Jorgensen et al. 1993; Gonçalves et al. 2015).

Changes in behavioural patterns in *D. sargus* due to a bioactive natural product from an invasive species are likely to be important in crucial phases of feeding, reproduction and controlling shelters where they hide to escape predators (Gonçalves et al. 2015). Disruption of behaviours associated with predator avoidance and social interactions may pose serious risks to fish populations (e.g., Papoutsoglou et al. 2006) and, through a mechanism of trophic cascade, to the functioning of the subtidal communities.

Neuropeptide Y is known to have several functions such as modulate circadian rhythms, gastrointestinal motility, memory, nociception, blood pressure, reproduction and feeding behaviour (Volkoff 2016). Interestingly in goldfish, intracerebroventricular administration of NPY affects locomotion, and induces a decrease of swimming activity (Matsuda 2009), confirming that NPY not only acts as an appetite enhancer but also possesses anxiolytic functions (Matsuda et al. 2011). In our previous article (Magliozzi et al. 2017) performed on the same animals used in this work, mirror experiments revealed that fish with higher doses of CAU reduced the frequency of displays and spent less time acting aggressively towards their image. Here we found that NPY transcriptional level expression has higher values (1.5- to twofold) in *D. sargus* groups fed with CAU (LD and HD, respectively). Among other functions already mentioned it has been found that NPY also alter the expression and secretion of other neurotransmitters such as serotonin [5-hydroxytryptamine (5-HT)] (Karl et al. 2004). Interestingly it has been also shown that CAU share structural similarities with endogenous amines such as 5-HT (Cavalcante et al. 2013) suggesting that it could affect social behaviour acting on NPY transcriptional levels or directly on 5-HT receptors.

Immunohistochemical analysis of NPY distribution in Central Nervous System (CNS) of *D. sargus* revealed no differences showing a complete overlapping pathway in all experimental groups: NPY immunoreactivity is widely detected throughout the brain except for the cerebellum. In

agreement with the behavioural results from other works in which extracts from *C. cylindracea* and its metabolite CAU did not affect feeding behaviour in fish (Paul et al. 1987, 1990; Wylie and Paul 1988; Meyer and Paul 1992) our experiment shows a positive, but non-significant, trend of catching-food frequency in fish fed with increasing dose of CAU.

In this study, we provide additional evidence to support the hypothesis that an invasive algae induces changes in the expression level of NPY on the CNS underlining one of the pathways through which the consumption of substances such as CAU could have multiple effects on physiology, nutritional value and behavioural responses of *D. sargus*. This represents one additional step towards the understanding of the impacts of cryptic invasive molecules on native fish populations.

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