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Effects of dietary caffeine on growth, body composition, somatic indexes, and cerebral distribution of acetyl-cholinesterase and nitric oxide synthase in gilthead sea bream (*Sparus aurata*), reared in winter temperature

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Abstract

This study aims at examining the effect of caffeine administration on growth, feed efficiency, and consumption of sea bream (*Sparus aurata*), reared in winter temperatures. Moreover, it is questioned whether caffeine has a central action in the brain and its effects are partly mediated via central brain mechanisms. For this, we studied the influences of caffeine treatment on the cerebral pattern of the cholinergic neurotransmission and the novel neuromodulator nitric oxide (NO), by means of acetyl-cholinesterase (AchE) and nitric oxide synthase (NOS) histochemistry. Five different diets containing 0.0, 0.1, 1.0, 2.0 and 5.0 g caffeine kg^{-1} of diet were administrated to five groups of fish. Caffeine adversely affected seabream growth at a concentration higher than 1 g kg^{-1} diet and increased feed conversion ratio in the treatments of 2 and 5 g kg^{-1} ($P < 0.05$). The daily consumption of feeds was similar to all groups, indicating that caffeine did not influence diet palatability. AChE- and NADPH-diaphorase histochemistry showed densely labeled cells and fibers mainly in dorsal telencephalon, preoptic, pretectal, hypothalamic areas, optic tectum, reticular formation, cerebellum and motor nuclei. When compared with matched caffeine-treated animals, no differences in the histochemical pattern and cell densities of cerebral AChE and NADPH-diaphorase were found.

KEY WORDS: acetyl-cholinesterase, caffeine, nitric oxide synthase, sea bream

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Introduction

Caffeine (1,3,7-trimethylxanthine) is an alkaloid, part of a group of compounds known as methylxanthines occuring naturally in many plants [e.g. coffee beans (*Coffee Arabica*), tea leaves (*Commelia thea*)]. As an antagonist of adenosine receptors, caffeine is associated with many pharmacological actions on heart, renal, respiratory and central nervous systems, adipose tissue, and behavioral effects (for a review see Garrett & Griffiths 1997 and references therein). In line with animal behavioral studies, there is evidence that exposure to caffeine can alter jewel fish (*Hemichromis bimaculatus*) behavior as judged by an increase in the variability of its spacing behavior (Burgess 1981). In the same species chronic exposure to caffeine during early development can facilitate neuronal growth and complexity as shown by the increased formation of dendritic spines on apical stem dentrites of pyriform tectal neurons (Burgess & Monachello 1983). At a cellular level, caffeine-activated intracellular store of Ca^{2+} can mediate the release of growth hormone in response to gonadotropin releasing hormone in goldfish (*Carassius auratus*) somatotropes (Wong *et al.* 2001). Furthermore, caffeine is used for the induction of triploidy in mussel (*Mytilus galloprovicialis*), Pacific oyster (*Crassostrea gigas*), small abalone (*Haliotis diversicolor*) and Japanese pearl oyster (*Pinctada fucata martensii*) caffeine is used for the induction of triploidy (Durand *et al.* 1990; Yamamoto *et al.* 1990; Chao *et al.* 1993; Scarpa *et al.* 1994).

The interest of caffeine in aquaculture emerged from the attempted use of coffee pulp in aqua feeds (Bayne *et al.* 1976). The inclusion of coffee pulp in diet adversely affected growth and feed conversion efficiency in a number of species such as carp (*Cyprinus carpio* L) and catfish (*Clarias mossambicus*;

Christensen 1981), tilapia (*Oreochromis aureus* Steindachner; Ulloa *et al*. 2002), Nile tilapia (*Oreochromis niloticus*; Moreau *et al*. 2003) and catfish (*Clarias isheriensis*; Fagbenro & Arowosoge 1991) because of antinutritional factors (polyphenols, tannins, and caffeine) and the high level of fiber of coffee pulp (Ulloa *et al*. 2003). The negative effect of coffee pulp inclusion in feeds can be ameliorated if fish are reared in ponds where they have access to natural feed (Ulloa & Verreth 2003).

The present research aims at examining the effect of caffeine administration on growth, feed efficiency and feed consumption of sea bream, a species commonly cultured in the Mediterranean Sea. In addition, as caffeine is involved in thermogenesis and has a lipolytic action in humans and homoeothermic animals (Dulloo *et al*. 1989; Dulloo *et al*. 1991; Kobayashi-Hattori *et al*. 2005; Lopez-Garcia *et al*. 2006), its effect on muscle and proximate composition of sea bream during low rearing temperatures in winter was investigated.

Given that caffeine impacts the muscarinic cholinergic system (Sorimachi *et al*. 1992), we questioned whether caffeine treatment has a central action in the brain and whether its effects are partly mediated via cholinergic or nitric oxide producing neurons, as previously suggested for mammals (Corradetti *et al*. 1986; Shi *et al*. 1993; Materi *et al*. 2000; Kayir & Uzbay 2004). In non-mammalian vertebrates, both a constitutive and an inducible nitric oxide synthase (NOS) activity has been described, and NOS-like enzymes have been localized in different tissues by means of NOS immunohistochemistry and NADPH diaphorase (NADPHd) histochemistry (Bruning *et al*. 1995; Villani & Guarnieri 1995; Holmqvist *et al*. 2000; Bordieri *et al*. 2005).

Acetylcholine (ACh) is a neurotransmitter that is widely used in efferent systems as well as in some central circuits (Woolf 1991), synthesized in the cytoplasm of cholinergic neurons by the enzyme choline acetyltransferase (ChAT) and degraded at the synaptic cleft by the enzyme acetylcholinesterase (AChE). In fish, ACh has an integral role in basal cerebral functions and is known to be involved in many functions, such as the alteration of the visual responsiveness of optic circuits (Fite & Wang 1986; King & Schmidt 1991), the processing of gustatory information during feeding (Molist *et al*. 1993), the modulation of the telencephalic circuitry (Pérez *et al*. 2000), or motor information processing (Molist *et al*. 1993). While the distribution of ChAT- containing cells in fish central nervous system has been described extensively in previous studies (Ekström 1987; Wullimann & Roth 1992; Adrio *et al*. 2000; Pérez *et al*. 2000; Clemente *et al*. 2004), reports on the distribution of AChE activity in fish brain are sparse (Contestabile & Zannoni 1975; Villani *et al*. 1987). Nitric oxide (NO) messenger molecule of nervous system is synthesized by nitric oxide synthase (NOS) from L-arginine. The histochemical reaction for nicotinamide adenine dinucleotide phosphate (NADPH-diaphorase) is widely used for the localization of NOS-positive neurons in brain tissue (Dermon & Stamatakis 1994). Nitric oxide is essential for developmental and learning plasticity of the nervous system and although little is known about physiological actions of NO in the teleost brain, NADPH-diaphorase activity was demonstrated in several teleost brain subdivisions (Schober *et al*. 1993; Holmqvist *et al*. 1994; Villani *et al*. 1994; Villani 1999; Jadhao & Malz 2004). To determine the neuronal populations and pathways via which caffeine may act in the sea bream, we studied its influences on cerebral histochemical distribution of cholinergic and cholinoceptive neurons and of the nitric oxide synthase such as enzyme (NO), using acetylcholinesterase (AChE) as a marker for cholinergic and cholinoceptive neurons (Butcher 1983) and NADPH-diaphorase as a NOS-like enzyme (Holmqvist *et al*. 2000; Bordieri *et al*. 2005).

Materials and methods

The experimentation was conducted at the Institute of Aquaculture, Hellenic Centre for Marine Research and at the Biology Department of the University of Crete, using sea bream (*Sparus aurata*) fingerlings. The fish used were from a genetically homogenous stock reared by mesocosm hatchery technology, totally weaned and fully adapted to compound inert diet (INVE, Oerstraat, Belgium). For the experiment, 15 50-L tanks were used, independently supplied with biologically filtered seawater of 40 ppt salinity and oxygenated to saturation by air supply. Temperature and dissolved oxygen were recorded daily and all tanks were cleaned as necessary.

Five different diets containing 0.0, 0.1, 1.0, 2.0 and 5.0 g caffeine (Sigma-Aldrich, Deisenhofer, Germany) kg^{-1} of diet (Table 1) were administered to five groups of fish with three replicates per treatment ($3 \times 5 = 15$ tanks). A higher dose of caffeine at 10 g kg^{-1} was also tested but fish rejected the feed and the treatment was abandoned. The dietary ingredients consisted of fish meal (Triple nine, Esbjerg, Denmark), fish oil (Winterization Europe, Fécamp, France), potato starch, α-cellulose, alginic acid, and choline chloride (Sigma-Aldrich), and vitamins and minerals (Roche, Athens, Greece). To approximate commercial formulations, cellulose was included in diets as a non-nutritive bulk to obtain a final 500 g protein and 160 g fat kg^{-1} diet, on a weight basis. The diets fulfilled the nutrient requirements of sea bream (Oliva-Teles 2000). The dietary ingredients were thoroughly

Table 1 Composition of experimental diets (g kg^{-1} diet)

	Diet				
	1	2	3	4	5
Ingredients					
Fish meal	696.38	696.38	696.38	696.38	696.38
Fish oil	81.75	81.75	81.75	81.75	81.75
α-starch	100.00	100.00	100.00	100.00	100.00
Vitamin–mineral mix[1]	20.00	20.00	20.00	20.00	20.00
Alginic acid	15.00	15.00	15.00	15.00	15.00
Choline chloride	4.00	4.00	4.00	4.00	4.00
Cellulose	82.87	82.77	81.87	80.87	77.87
Caffeine	0.00	0.10	1.00	2.00	5.00
Proximate composition (g kg^{-1})					
Protein	493.63	497.23	495.24	493.81	500.51
Fat	159.00	162.00	161.33	164.67	151.00

[1] Vitamin–Mineral mix/kg premix:VitA 2 500 000 IU, VitD3 1 000 000 IU,VitEacet 100 000 mg, VitB1 15 000 mg VitB2 5000 mg, VitB8(HCL) 15 000 mg, VitB12 12 000 mg, VitK3 50 mg, Nicotinic acid 60 000 mg, Pantothenic acid 25 000 mg, Folic acid 2500 mg, Biotin 500 mg, Co 1000 mg, I 1500 mg, Se 50 mg, Fe 50 000 mg, Mg 40 000 mg, Cu 2000 mg, Zn 50 000 mg.

mixed and moistened by the addition of 75% (w/v) distilled water and then made into pellets by a mincing machine. The pellets were cut into shape manually, dried in an oven at 40 °C for 24 h and stored in a freezer (−16 °C) until used. In each tank, 11 randomly distributed fish (19.9 ± 0.6 g initial weight) were stocked. Before starting the experiment fish were acclimated for 1 week and fed on diet 1 (Table 1). Fish were hand-fed to apparent satiation twice a day for 7 days a week, and were reared under natural water temperature (ranged between 13 and 18 °C) and photoperiod (December–March; Heraklion, 35 ° 20′ latitude, 25 ° 08′ longitude). The experiment lasted for 115 days.

Average weight per tank was assessed every 20 days by weighing all fish individually. Fish were starved for a day before weighing. After each sampling food conversion ratio (FCR = feed consumed/weight increase), daily feed consumption (DFC = feed intake × 100/(((*W*$_{initial}$ + *W*$_{final}$)/2) × days)), specific growth rate (SGR = (ln(*W*$_{final}$)−ln(*W*$_{initial}$)) × 100/days)) were calculated. In addition, at the final sampling, Hepatosomatic Index (HIS = liver weight × 100/body weight), Viscerosomatic Index (VSI = viscera weight × 100/body weight) and Brainsomatic Index (BSI = brain weight × 100/body weight) were also estimated. At the end of the experiment the proximate body composition was determined by measuring protein content (Kjeldahl procedure $N \times 6.25$, Buchi protein measurement apparatuses; Flawil, Switzerland), fat as ether extract (Buchi soxlet fat extraction apparatus; Flawil, Switzerland), moisture (dry at 95 °C until constant weight, Heraeus oven apparatus; Hanau, Germany) and ash (burn at 600 °C, Heraeus laboratory muffle furnace; Hanau, Germany).

Standard histochemical methods were used for the localization of the AchE activity and NADPH-diaphorase (that is a NOS-like enzyme) in the brain of the control group and the group to which caffeine caused growth retardation at its lowest concentration and was not aversive (2.0 g caffeine kg^{-1} of diet Table 2; $n = 4$). Briefly, the fish were anesthetized with 0.04% tricaine methanesulfonate (MS222; Sigma-Aldrich) and intracardially perfused with saline followed by 0.1 M phosphate-buffered paraformaldehyde (4 °C, pH 8). After extraction and postfixation for about 4 h, the brains were placed in a cryoprotective solution (0.1 M phosphate-buffered 20% sucrose solution) for about 14 h before being frozen and stored at −80 °C. Using a cryostat (−20 °C;

Table 2 Growth data and somatic indexes of sea-bream fed diets containing different amounts of caffeine for 115 days

	Diet (g caffeine kg^{-1} food)				
	0.0	0.1	1.0	2.0	5.0
Initial weight (g)	19.7 ± 1.1 a	20.1 ± 0.3 a	20.1 ± 0.4 a	19.6 ± 0.5 a	20.0 ± 0.6 a
Final weight (g)	43.2 ± 2.9 a	46.0 ± 3.1 a	45.0 ± 2.9 a	37.5 ± 1.7 b	29.2 ± 1.9 c
FCR[1]	2.1 ± 0.1 a	2.0 ± 0.2 a	1.8 ± 0.1 a	2.6 ± 0.6 a	4.4 ± 1.1 c
DFC (%)[2]	1.2 ± 0.2 a	1.3 ± 0.0 a	1.2 ± 0.1 a	1.3 ± 0.1 a	1.2 ± 0.1 a
SGR(%/day)[2]	0.7 ± 0.0 a	0.7 ± 0.1 a	0.7 ± 0.1 a	0.6 ± 0.0 b	0.3 ± 0.0 c
HSI (%)[4]	1.3 ± 0.2 ab	1.2 ± 0.1 a	1.5 ± 0.2 b	1.3 ± 0.2 ab	1.0 ± 0.2 a
VSI (%)[5]	5.3 ± 0.9 a	5.5 ± 0.8 a	6.4 ± 2.3 a	5.5 ± 0.3 a	4.7 ± 0.8 a
BSI (%)[6]	0.5 ± 0.1 a	0.5 ± 0.1 a	0.5 ± 0.1 a	0.5 ± 0.1 a	0.7 ± 0.1 a

Values are means ± standard deviation. Within the same line values with different letters differ significantly ($P < 0.05$).

[1] Food conversion ratio (FCR = feed consumed/weight increase).

[2] Daily feed consumption (DFC = feed intake × 100/(((*W*$_{initial}$ + *W*$_{final}$)/2) × days)).

[3] Specific growth rate (SGR = (ln(*W*$_{final}$)−ln(*W*$_{initial}$)) × 100/days)).

[4] Hepatosomatic (HSI = liver weight × 100/body weight).

[5] Viscerosomatic Index (VSI = viscera weight × 100/body weight).

[6] Brainsomatic Index (BSI = brain weight × 100/body weight).

Leica CM1500, Wetzlar, Germany), the brains were cut into 40 μm thick coronal sections which were collected on PBS solution (0.01 M, pH 7.4; phosphate buffer saline).

Adjacent brain sections were subjected to AChE and NADPH-diaphorase histochemistry. In the AChE histochemistry, the brain slices were immersed in acetylcholinesterase solution (pH 5.0) containing 0.3 mM ethopropazine (E-2880; Sigma-Aldrich), 2 nM acetylthiocholine iodide (substrate; A-5751; Sigma-Aldrich), 10 mM glycine, 2 mM cupric sulfate (S-1297; Sigma-Aldrich), and sodium acetate (S-7545; Sigma-Aldrich), for 1 h at room temperature. After incubation, the slides were rinsed six times by distilled water (1 min each time). Then, slides were transferred in a 1.25% sodium sulfide solution for 1 min and rinsed six times by distilled water (1 min each time). Slides were transferred to a 1% silver nitrate solution in distilled water for 1 min and rinsed again six times by distilled water (1 min each time). In the NADPH-diaphorase histochemistry, the brain sections rinsed in 0.1 M Tris–HCl buffer (pH = 7.7) for three times (10 min each time) at 37 °C. Then, the sections were incubated in diaphorase solution consisting of 0.8 mM β-nicotinamide adenine dinucleotide phosphate [β-NADPH (substrate); N-1630; Sigma-Aldrich], 0.9 mM nitro blue tetrazolium (NBT dyes as a chromogen; Sigma-Aldrich N-6876), 0.08% Triton X-100 (Sigma-Aldrich) and 10 mM malic acid (L-hydroxybutanedioic acid; M-6413; Sigma-Aldrich) in Tris–HCl buffer, at 45 °C for 1.5–4.0 h. The sections were then rinsed three times in cold (4 °C) Tris–HCl buffer for 10 min. Following the histochemistry, the sections were mounted on gelatin-coated slides, dried and coverslipped. Analysis of the positive-cell distribution was performed blindly to the experimental protocol. The brain sections were examined under a light microscope (NIKON Optiphot 2, Tokyo, Japan) equipped with a camera lucida and drawings of the morphological features of the labelled neurons and their dendritic arbors were made at 40× magnification. All stained cell structures (cells and fibers) in the whole area of each brain region of interest were recorded and initial mapping of the histochemical distribution of the enzymes under study did not show any significant differences in the pattern or intensity of labeling between matched sections of control and caffeine-treated animals. For further validation, quantification of the AChE and NADPH-diaphorase positive cells was performed in selected brain areas, with the aid of a camera lucida attached to a microscope (Nikon, Eclipse E800; Tokyo, Japan) at 600× magnification. Labeled cells were determined using a counting frame (0.04×0.04 mm, 0.0016 mm^2 area) with exclusion of the edges, in six representative serial coronal sections of the selected arousal and motor related areas (Dc, Vc, Purkinje cell layer, NIII and Rm) in matched control and caffeine-treated animals. Neuroanatomical regions were identified based on nomenclature used in a previous study on the teleost brain by Zikopoulos & Dermon (2005).

All images were captured using a microscope (Nikon, Eclipse E800, Tokyo, Japan) via a color 3CCD camera (Sony DXC-950P; Tokyo, Japan) to a PC (Microsoft, Windows XP, Redmond, WA, USA). Stacks of optically sliced images were generated using Scion image software (Scion Image B4.0.2; Scion Corp., Frederick, MD, USA). Size and luminosity of the figures were modified with Macintosh version of Adobe Photoshop CS2 (Adobe System Inc., San Jose, CA, USA).

Data are presented as means of three replicates and were analyzed by analysis of variance, ANOVA. When tests for normality and equal variance were rejected, a logarithmic transformation was performed and if this was not sufficient a Kruskal–Willis ANOVA on ranks test was used. Differences between treatment means were identified by Holm–Sidak or Student–Neuman Keuls' multiple range tests at the level of $P < 0.05$. The results are presented as means ± standard deviation. All statistical analysis was performed with SigmaStat statistical package (Systat Software, Inc., Point Richmond, CA, USA).

Differences in cell densities of stained cells of the selected studied areas were statistically tested by *t*-test (two-tailed hypothesis; STATGRAPHIC plus v5.0 (Statistical Graphics Corp., Herndon, VA, USA), $P < 0.05$). All experimental procedures were in accordance with the European Communities council directive (86/609/EEC) for the care and use of laboratory animals.

Results and discussion

The use of caffeine-containing ingredients in fish farming has been studied in the past without been made possible to elucidate the effects of caffeine on fish growth and feed utilization as they were masked by other dietary factors such as tannins, polyphenols or fiber. The present study showed that caffeine adversely affected sea-bream growth at a concentration higher than 1 g kg^{-1} diet (Table 2) judging by the reduced specific growth rate of fish. Sea bream exhibited normal growth for the winter rearing temperatures (13–18 °C) and the growth retarding effect of caffeine was evident during the winter period of low growth. Apparently any possible induction of thermogenesis in sea bream – similar to the one observed in homoeothermic animals – was not substantial to elevate body temperature and thereby influence its growth. Caffeine also caused an increase in feed conversion ratio in the treatments of

2 and 5 g kg^{-1} diet. On the other hand, the daily consumption of feeds was similar to all groups, an indication that caffeine – at doses tasted – did not influence diet palatability, implying that the reduction in growth at 2 and 5 g kg^{-1} doses of caffeine was because of reduced diet utilization. Throughout the feeding period the fish in all experimental groups were in good health- and dose-related mortalities were not observed, indicating that sea bream can tolerate high caffeine doses (up to 5 g kg^{-1} diet) albeit with reduced growth rate and increased feed conversion ratio.

The adverse effect of caffeine on fish growth was implied in tilapia (*Oreochromis aureaus*) when coffee pulp was offered to fish at doses of 130, 260 and 390 g kg^{-1} diet, which correspond to 2.34, 4.68, and 7.02 g caffeine kg^{-1} of diet considering that coffee pulp contains 18 g kg^{-1} caffeine (Ulloa & Verreth 2003). The growth reduction caused by caffeine at 2 g kg^{-1} in sea bream is in agreement with a previous study of Ulloa & Verreth (2003) where it was observed that the 130 g kg^{-1} (2.34 g kg^{-1} caffeine) inclusion level of coffee pulp reduced the growth of tilapia. However, the growth retardation effect of caffeine may be confined to fish species as chronic caffeine treatment in rats did not change animal growth patterns (Corradetti *et al*. 1986).

It has been inferred that caffeine in coffee pulp, together with polyphenols and tannins can deter feed consumption in fish (Ulloa & Verreth 2003). possibly because of its bitter taste usually perceived by animals (Mazzafera 2002; Frank *et al*. 2004). Indeed caffeine inhibited the feeding behavior of turbot (Mackie 1982; cited by Kasumyan & Døving 2003) and in goldfish (*Carassius auratus*) caffeine at concentration higher than $10^{-2.5}$ M in gelatine-pellets was an aversive tastant (Lamb & Finger 1995). In our study, sea bream did not accept the caffeine flavored feed at a 10 g kg^{-1} dose but at doses at or lower to 5 g kg^{-1} caffeine appeared not to have a deterrent effect.

Therefore, the negative effect of caffeine on sea bream growth can be traced in its increased feed conversion ratio. In various past studies with fish, chicken, or cattle the inclusion of coffee pulp in feeds lead to reduced conversion efficiency (Cabezas *et al*. 1976; Donkoh *et al*. 1988; Fagbenro & Arowosoge 1991; Moreau *et al*. 2003). Unfortunately, cases with direct experimentation with caffeine in fish are lacking but it could be hypothesized that caffeine could interfere with nutrient bioavailability and utilization of fish metabolism. In rainbow trout (*Oncorhynchus mykiss*), long-term exposure to caffeine could lead to lipid peroxidation (Gagne *et al*. 2006) while caffeine is an inhibitor of glycogen phosphorylase in the mantle tissue of mussel (*Mytilus galloprovincialis*; Serrano *et al*. 1995) and also of lactate dehydrogenase in the muscle of rabbit (Gardiner & Whiteley 1985). Another hypothesis for the increase of FCR value in caffeine-treated fish could be the increase in fish activity. To test this hypothesis, the employment of behavioral techniques (analyses of video images) is required. However this was not at the scope of the present investigation. A visual observation of fish showed no apparent differences in fish activity between groups.

Treatment means of hepatosomatic index varied and a clear effect of caffeine on this index was not observed while viscerosomatic and brainsomatic indexes were not affected by caffeine dose (Table 2). In addition, in all doses tested caffeine did not influence the proximate composition of white muscle and liver (Table 3). Although caffeine is reported to induce lipolysis and thereby reduce the body fat mass and body fat percentage in Sprague–Dawley rats fed on a high fat diet (Kobayashi-Hattori *et al*. 2005) and KK mice caffeine upregulates uncoupling protein family expression contributing to thermogenesis (Dulloo *et al*. 1991; Kogure *et al*. 2002), our data do not indicate that caffeine can reduce the lipid content of white muscle and

Table 3 Proximate composition of muscle and liver in g kg^{-1} wet weight (mean ± standard deviation) of sea-bream fed diets containing different amounts of caffeine for 115 days

	Diet (g caffeine kg^{-1} food)				
	0.0	0.1	1.0	2.0	5.0
Muscle					
Moisture	752.64 ± 8.87	775.56 ± 34.89	746.96 ± 12.56	758.36 ± 8.23	776.07 ± 25.85
Protein	188.42 ± 1.42	196.35 ± 19.78	183.98 ± 0.68	192.37 ± 3.31	186.00 ± 3.61
Fat	32.98 ± 10.43	15.24 ± 7.53	31.56 ± 4.80	27.24 ± 2.25	24.67 ± 2.52
Ash	14.92 ± 0.07	14.95 ± 0.23	14.67 ± 0.69	15.25 ± 0.68	17.20 ± 2.81
Liver					
Moisture	588.67 ± 7.48	643.10 ± 5.83	630.51 ± 36.31	646.55 ± 27.99	628.12 ± 34.99
Protein	126.00 ± 4.58	123.00 ± 4.00	119.33 ± 10.21	121.33 ± 7.51	124.00 ± 4.58
Fat	140.33 ± 13.58	139.33 ± 11.50	140.72 ± 5.58	142.00 ± 9.54	139.33 ± 11.50
Ash	15.13 ± 4.66	13.42 ± 1.88	14.15 ± 1.03	17.84 ± 2.56	17.09 ± 6.56

Within the same line values do not differ significantly ($P > 0.05$).

liver in heterotherm sea bream when reared in low winter temperatures.

Microscopic observation of AChE and NADPH-diaphorase showed neuronal (both cell somata and fibers) localization of the enzymes, while no glial cells were labeled in adult sea-bream brain. The localization of NADPH-diaphorase positive neurons, compared with the distribution of AchE, indicated that the production of nitric oxide was mostly found in the brain areas where cholinergic circuits (cholinegic and cholinoceptive neurons) were located, as previously suggested (Villani & Guarnieri 1995), with the exception of the cells of the ganglionic layer (Purkinje, euroderdoid cell bodies) and the parallel fibers of cerebellum. Specifically, in the telencephalon, AChE-positive small round

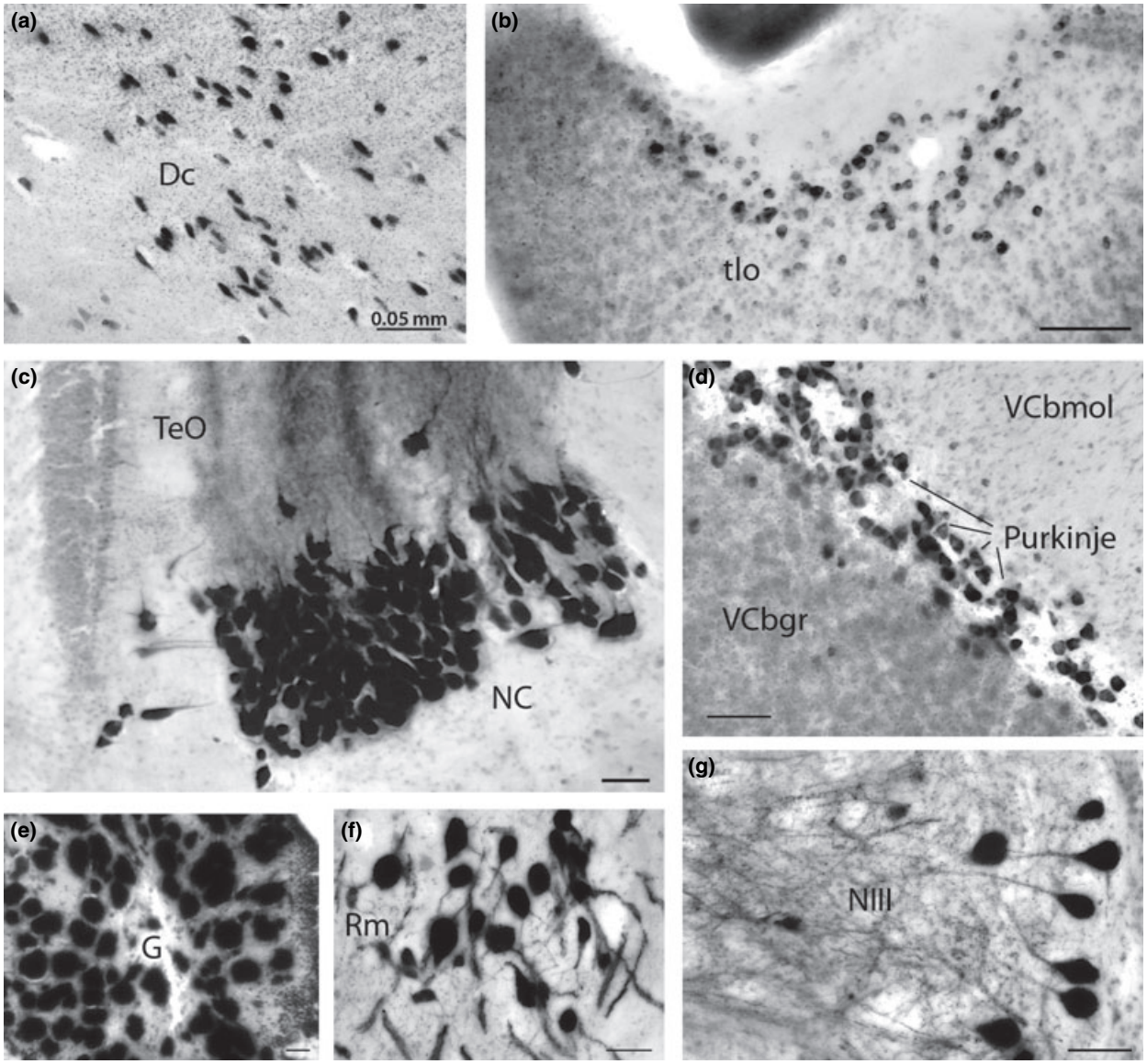

Figure 1 Photomicrographs of selected sections showing the distribution of AChE-positive cell structures in the adult gilthead sea-bream brain. (a) AChE-positive cells in the central zone of the dorsal telencephalic area (Dc). (b) AChE-positive small round-shaped cells in the torus longitudinalis (tlo). (c) Detail of AChE histostained cells and fibers in the cortical nucleus (NC) and in the optic tectum (TeO). (d) Showing details of AChE-positive nuclei of the Purkinje cells in the ganglionic cell layer between the molecular (VCbmol) and ranular layer (VCbgr) of the valvula cerebellum (VCb). (e) High magnification image showing the AChE stained cells in the glumerular nucleus (G). (f) Cells of the medial raphe nucleus (Rm). (g) Section showing the AChE cells with their axons in the oculomotor nucleus (NIII), 170 × 157 mm (300 × 300 DPI)

shaped neurons were observed in the olfactory bulb and in the dorsal (pallium) and ventral (subpallium) telencephalic hemispheres (Fig. 1a). In the diencephalon moderate numbers of the AChE-positive cells were noted, mostly observed in the preoptic and pretectal nuclei. In the mesencephalon (Fig. 1b) small round shaped cells positive to AchE histochemistry were observed in the torus longitudinalis (tlo). In contrast, optic tectum (TeO) included many stained fibers (Fig. 1c) but only few cell somata. Interestingly, the hypothalamus included high densities of AChE-positive fibers, mainly in the dorsal hypothalamic area (dHA) and the diffuse nucleus of the inferior lobe (DIF), but not positive cell

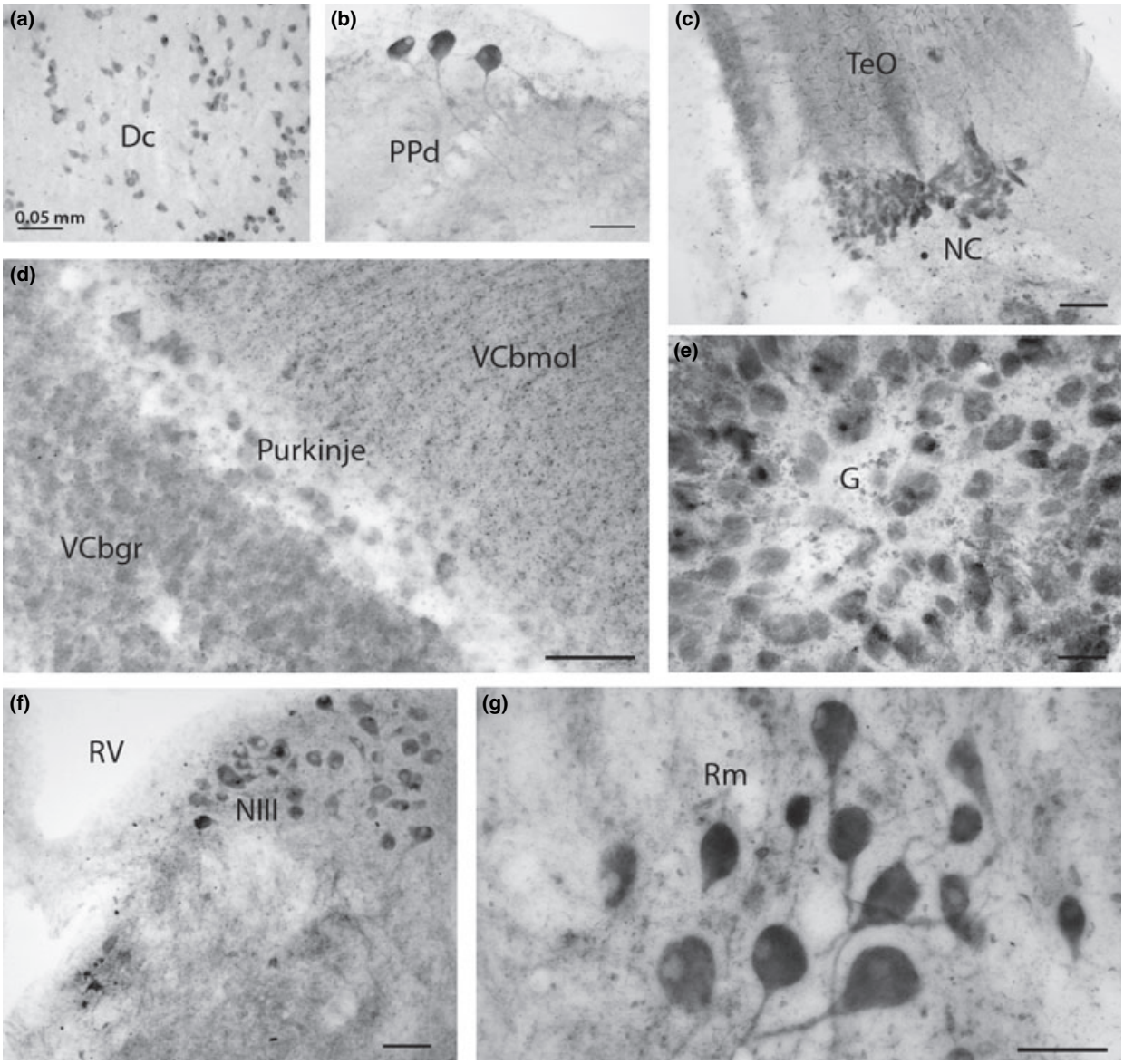

Figure 2 Pattern of the NADPH-diaphorase (NADPH-d) labeling of the adult *Sparus aurata* brain. (a) NADPH-d positive small cells in the central zone of the dorsal telencephalic area (Dc). (b) Stained cells of the dorsal periventricular pretectal nucleus (PPd). (c) Stained cells and fibers by NADPH-d histochemistry of the optic tectum (TeO) and cortical nucleus (NC) of the gilthead sea-bream mesencephalon. (d) NADPH-d expression in the Purkinje cell layer of the valvula cerebellum and in the fibers of the molecular layer of the valvula cerebellum (VCbmol). (e) High magnification image showing the NADPH-d positive neurons of the glumerular nucleus (G). (f) Section showing the NADPH-d expression in the cells and axons of the oculomotor nucleus (NIII). (g) High magnification image showing NADPH-d positive cell somata and their axons of the medial raphe nucleus (Rm), with the characteristic lack of NADPH-d staining in their nuclei, 170 × 160 mm (300 × 300 DPI)

somata. AChE staining was observed in Purkinje cells of the ganglionic cell layer and granule cells of the cerebellum (Fig. 1d). The glumerular nucleus (G) was observed to have large AChE-positive neurons (Fig. 1e). All main subdivisions of the medulla oblongata including the widespread distribution of small, medium and large with long axons AChE-positive cells were observed. Specifically, the reticular formation with the raphe nuclei (Fig. 1f) and the motor nuclei of the cranial nerves (Fig. 1g) included large cells densely stained by AChE. The AchE distribution in sea-bream brain is in agreement with previous studies in other teleost fish species (Contestabile & Zannoni 1975; Pérez *et al.* 2000; Clemente *et al.* 2004).

NADPH-diaphorase labeling, characterized by cytoplasmic labeling leaving the nucleus unlabeled, similar to previous report (Villani & Guarnieri 1995), was observed in several brain areas of the adult gilthead sea bream. In the telencephalon (Fig. 2a), while NADPH-diaphorase positive neurons were found in all telencephalic areas, in the dorsal telencephalon (pallium), the number of stained cells was higher than that observed in the ventral telencephalic areas (subpullium). Most of these cells were found to be round shaped and small-sized cells had no stained axons. In the preoptic, and pretectal areas (Fig. 2b) few large labeled neurons with long axons were observed. The hypothalamic areas of the brain of sea bream were observed to have some scattered heavily stained medium sized neurons. In the mesencephalon, few round-shaped small neurons were found in the optic tectum (TeO), in contrast to high numbers of medium strong stained cells revealed by NADPH-diaphorase histochemistry in the cortical nucleus (NC; Fig. 2c). The rombencephalon and the cerebellum included a high density of NADPH-diaphorase stained neurons. In the cerebellum, faint NADPH-diaphorase activity was observed in the Purkinje cells of the ganglionic cell layer and in the cells of granular layer (Fig. 2d), but strongly labeled fibers were found in the molecular layer. The motor nuclei neurons and the fusiform neurons of the reticular formation were heavily stained as shown by their characteristic morphology (Fig. 2e–g). These findings are in agreement with previous studies in teleost fish species showing the neuronal NOS-like enzymes by means of NADPH-diaphorase histochemistry and NOS immunohistochemistry (Holmqvist *et al.* 1994, 2000; Arevalo *et al.* 1995; Bruning *et al.* 1995; Villani & Guarnieri 1995; Bordieri *et al.* 2005).

When compared with matched caffeine-treated animals no differences in AChE nor NADPH-diaphorase labeling were found. Quantification of labeled cells allowed statistical comparison between the number of AChE and NADPH-d positive profiles in adult gilthead sea-bream brain areas (Fig. 3). In all of the examined areas, no statistical significant differences were observed. These findings are interesting because caffeine is known to be a mixed adenosine alpha receptor antagonist and prolonged consumption of high doses of caffeine caused changes in the activity of cholinergic neurones in rat cortical neurons (Corradetti *et al.* 1986). In agreement, adenosine acting on brain adenosine G-coupled receptors (Fredholm *et al.* 2001) is suggested to inhibit acetylcholine release from cholinergic cortical neurons (Materi *et al.* 2000), while caffeine is known to increase acetylcholine release, exerting central amplification of cholinergic action (Carter *et al.* 1995; Ghelardini *et al.* 1997). In addition, a previous study has shown that caffeine-induced increases in

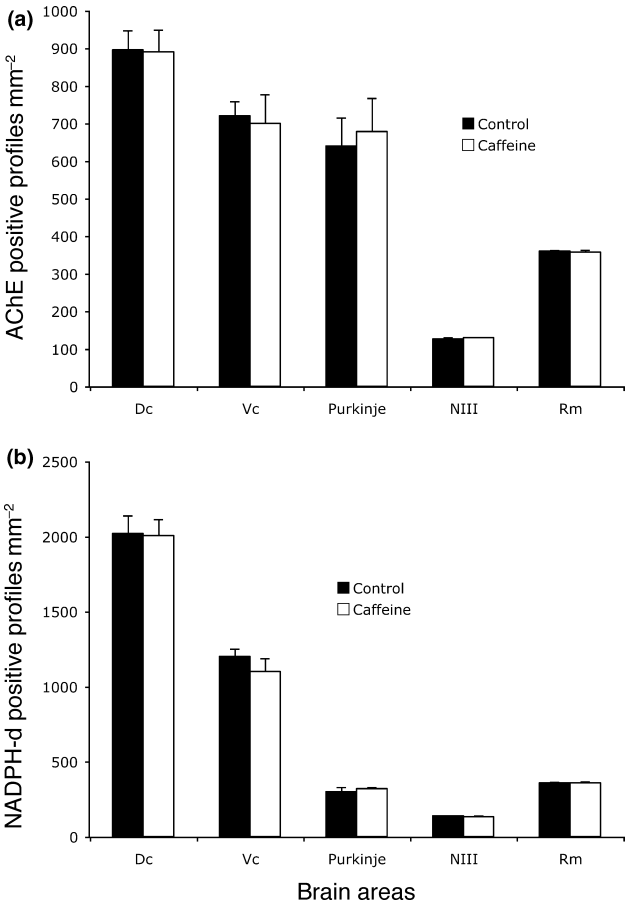

Figure 3 Number of AChE (a) and NADPH-diaphorase (b) positive cell profiles (mean ± SEM) per unit area mm^2 of 40 μm thick sections in central zone of the dorsal telencephalic area (Dc), in the central nucleus of the ventral thelencephalic area (Vc), in the Purkinje cell layer of the valvula cerebellum, in the oculomotor nucleus (NIII), and in the medial raphe nucleus (Rm) of the adult *Sparus aurata* brain, measured in control and caffeine-treated individuals, 118 × 175 mm (600 × 600 DPI)

locomotor activity were possibly modulated by NO in mice (Kayir & Uzbay 2004). Chronic ingestion of caffeine equivalent to about 100 mg kg^{-1} day^{-1} in mice caused a wide range of biochemical alterations in the central nervous system, specifically increasing in the density of brain adenosine, cholinergic muscarinic, and nicotinic receptors (Shi *et al.* 1993). However, in the present study, caffeine-treatment failed to induce any changes in the histochemical pattern and density of cerebral cholinergic and cholinoceptive neurons or NADPH-diaphorase positive neurons. One explanation might be related to the dose of caffeine treatment, speculating that a different dose may be more effective. Alternatively, it is possible that caffeine potentates selectively the activity of the enzymes under study, without affecting the density or the morphology of the expressing cells and fibers. Further studies are necessary to establish a relationship between caffeine and adenosine receptors as well as with acetylcholine and NO activity, as suggested for mammals.

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References

Adrio, F., Anadon, R. & Rodriguez-Moldes, I. (2000) Distribution of choline acetyltransferase (ChAT) immunoreactivity in the central nervous system of a chondrostean, the Siberian sturgeon (*Acipenser baeri*). *J. Comp. Neurol.*, **426,** 602–621.

Arevalo, R., Alonso, J.R., Garcia-Ojeda, E., Brinon, J.G., Crespo, C. & Aijon, J. (1995) NADPH-diaphorase in the central nervous system of the tench (*Tinca tinca* L., 1758). *J. Comp. Neurol.*, **352,** 398–420.

Bayne, D.R., Dunseth, D. & Ramirios, C.G. (1976) Supplemental feeds containing coffee pulp for rearing tilapia in Central America. *Aquaculture*, **7,** 133–146.

Bordieri, L., di Patti Bonaccorsi, M.C., Miele, R. & Cioni, C. (2005) Partial cloning of neuronal nitric oxide synthase (nNOS) cDNA and regional distribution of nNOS mRNA in the central nervous system of the Nile tilapia *Oreochromis niloticus*. *Mol. Brain Res.*, **142,** 123–133.

Bruning, G., Katzbach, R. & Mayer, B. (1995) Histochemical and immunocytochemical localization of nitric oxide synthase in the central nervous system of the goldfish, *Carassius auratus*. *J. Comp. Neurol.*, **358,** 353–382.

Burgess, J.W. (1981) Chronic exposure to caffeine during early development modifies spatial behavior in juvenile jewel fish schools. *Pharmacol. Biochem. Behav.*, **17,** 137–140.

Burgess, J.W. & Monachello, M.P. (1983) Chronic exposure to caffeine during early development increases dendritic spine and branch formation in midbrain of optic tectum. *Brain Res.*, **282,** 123–129.

Butcher, L.L. (1983) Acetylcholinesterase histochemistry. In: *Methods in Chemical Neuroanatomy. Handbook for Chemical Neuroanatomy*, **Vol. 1** (Björklund, A. & Hökfelt, T. eds), pp. 1–49. Elsevier, Amsterdam, Netherlands.

Cabezas, M.T., Estrada, E., Murillo, B., Gonzalez, J.M. & Bressani, R. (1976) Coffee hulls and pulp. XII. Effect of storage of coffee pulp on its nutritive value for calves. *Arch. Latinoamerican Nutr.*, **26,** 203–215.

Carter, A.J., O'Connor, W.T., Carter, M.J. & Ungerstedt, U. (1995) Caffeine enhances acetylcholine release in the hippocampus *in vivo* by a selective interaction with adenosine A1 receptors. *Pharmacol. Exp. Ther.*, **273,** 637–642.

Chao, N.H., Yang, C.P., Tsai, H.P., Liang, W.H. & Liao, I.C. (1993) Studies on chromosome manipulation in cyprinid loach (*Misgurnus anguillicaudatus*), common carp (*Cyprinus carpio*), and small abalone (*Haliotis diversicolor*). *Special Issue Biol. Int.*, **28,** 72–80.

Christensen, M.S. (1981) Preliminary tests on the suitability of coffee pulp in the diets of common carp (*Cyprinus carpio* L.) and catfish (*Clarias mossambicus* Peters). *Aquaculture*, **25,** 235–242.

Clemente, D., Porteros, Á., Weruaga, E., Alonso, J.R., Arenzana, F.J., Aijón, J. & Arévalo, R. (2004) Cholinergic elements in the zebrafish central nervous system: histochemical and immunohistochemical analysis. *J. Comp. Neurol.*, **474,** 75–107.

Contestabile, A. & Zannoni, N. (1975) Histochemical localization of acetylcholinesterase in the cerebellum and optic tectum of four freshwater teleosts. *Histochemistry*, **45,** 279–288.

Corradetti, R., Pedata, F., Pepeu, G. & Vannucchi, M.G. (1986) Chronic caffeine treatment reduces caffeine but not adenosine effects on cortical acetylcholine release. *Br. J. Pharmacol.*, **88,** 671–676.

Dermon, C.R. & Stamatakis, B. (1994) Laminar pattern of NADPH-diaphorase activity in the developing avian cerebellum. *Neuroreport*, **5,** 1941–1945.

Donkoh, A., Atuahene, C.C., Kese, A.G. & Mensah-Asante, B. (1988) The nutritional value of dried coffee pulp (DCP) in broiler chickens' diets. *Anim. Feed Sci. Technol.*, **22,** 139–146.

Dulloo, A.G., Geissler, C.A., Horton, T., Collins, A. & Miller, D.S. (1989) Normal caffeine consumption: influence on thermogenesis and daily energy expenditure in lean and postobese human volunteers. *Am. J. Clin. Nutr.*, **49,** 44–50.

Dulloo, A.G., Seydoux, J. & Girardier, L. (1991) Peripheral mechanisms of thermogenesis induced by ephedrine and caffeine in brown adipose tissue. *Int. J. Obes.*, **15,** 317–326.

Durand, P., Wada, K.T. & Komaru, A. (1990) Triploidy induction by caffeine-heat shock treatments in the Japanese pearl oyster *Pinctada fucata martensii*. *Nip. Sui. Gak.*, **56,** 1423–1425.

Ekström, P. (1987) Distribution of choline acetyltransferase-immunoreactive neurons in the brain of a cyprinid teleost (*Phoxinus phoxinus* L.). *J. Comp. Neurol.*, **256,** 494–515.

Fagbenro, O.A. & Arowosoge, I.A. (1991) Growth response and nutrient digestability by *Clarias isheriensis* (Sydenham, 1980) fed varying levels of dietary coffee pulp as replacement for maize in low-cost diets. *Biores. Technol.*, **37,** 253–258.

Fite, K.V. & Wang, S.R. (1986) Microiontophoresis and single-unit analysis of cholinergic drugs in the optic tectum of frog. *Brain Behav. Evol.*, **28,** 198–206.

Frank, M.E., Bouverat, B.P., MacKinnon, B.I. & Hettinger, T.P. (2004) The distinctiveness of ionic and nonionic bitter stimuli. *Physiol. Behav*., **80,** 421–431.

Fredholm, B.B., Ijzerman, A.P., Jacobson, K.A., Klotz, K.N. & Linden, J. (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev*., **53,** 527–552.

Gagne, F., Blaise, C. & Andre, C. (2006) Occurrence of pharmaceutical products in a municipal effluent and toxicity to rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Ecotoxicol. Environ. Saf*., **64,** 329–336.

Gardiner, N.S. & Whiteley, C.G. (1985) The interaction and inhibition of muscle lactate dehydrogenase by the alkaloid caffeine. *Biochem. Biophys. Res. Commun*., **127,** 1057–1065.

Garrett, E.E. & Griffiths, R.R. (1997) The role of dopamine in the behavioral effects of caffeine in animals and humans. *Pharmacol. Biochem. Behav*., **57,** 533–541.

Ghelardini, C., Galeotti, N. & Bartolini, A. (1997) Caffeine induces central cholinergic analgesia *Naunyn Schmiedebergs. Arch. Pharmacol*., **356,** 590–595.

Holmqvist, I., Ostholm, T., Alm, P. & Ekström, P. (1994) Nitric oxide synthase in the brain of a teleost. *Neurosci. Lett*., **171,** 205–208.

Holmqvist, B., Ellingsen, B., Alm, P., Forsell, J., Oyan, A.M., Goksøyr, A., Fjose, A. & Seo, H.C. (2000) Identification and distribution of nitric oxide synthase in the brain of adult zebrafish. *Neurosci. Lett*., **292,** 119–122.

Jadhao, A.G. & Malz, C.R. (2004) Nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity in the brain of a cichlid fish, with remarkable findings in the entopeduncular nucleus: a histochemical study. *J. Chem. Neuroanat*., **27,** 75–86.

Kasumyan, A.O. & Døving, K.B. (2003) Taste preferences in fishes. *Fish Fish*, **4,** 289–347.

Kayir, H. & Uzbay, I.T. (2004) Evidence for the role of nitric oxide in caffeine-induced locomotor activity in mice. *J. Psychopharmacol*., **172,** 11–15.

King, W.M. & Schmidt, J.T. (1991) The long latency component of retinotectal transmission: enhancement by stimulation of isthmic nucleus or tectobulbar tract and block by nicotinic cholinergic antagonists. *J. Neurosci*., **40,** 701–712.

Kobayashi-Hattori, K., Mogi, A., Matsumoto, Y. & Takita, T. (2005) Effect of caffeine on the body fat and lipid metabolism of rats fed on a high-fat diet. *Biosci. Biotechnol. Biochem*., **69,** 2219–2223.

Kogure, A., Sakane, N., Takakura, Y., Umekawa, T., Yoshioka, K., Nishino, H., Yamamoto, T., Kawada, T., Yoshikawa, T. & Yoshida, T. (2002) Effects of caffeine on the uncoupling protein family in obese yellow KK mice. *Clin. Exp. Pharmacol. Physiol*., **29,** 391–394.

Lamb, C. & Finger, T.E. (1995) Gustatory control of feeding behavior in goldfish. *Physiol. Behav*., **57,** 483–488.

Lopez-Garcia, E., van Dam, R.M., Rajpathak, S., Willett, W.C., Manson, J.A.E. & Hu, F.B. (2006) Changes in caffeine intake and long-term weight change in men and women. *Am. J. Clin. Nutr*., **83,** 674–680.

Mackie, A.M. (1982) Identification of the gustatory feeding stimulants. In: *Chemoreception in Fishes* (Hara, T.J. ed.), pp. 275–291. Elsevier Scientific Publishing Co., Amsterdam, Netherlands.

Materi, L.M., Rasmusson, D.D. & Semba, K. (2000) Inhibition of synoptically evoked cortical acetylcholine release by adenosine: An *in vivo* microdialysis study in the rat. *J. Neurosci*., **97,** 219–226.

Mazzafera, P. (2002) Degratation of caffeine by microorganisms and potential use of decaffeinated coffee husk and pulp in animal feeding. *Sci. Agric*., **59,** 815–821.

Molist, P., Maslam, S., Velzing, E. & Roberts, B.L. (1993) The organization of cholinergic neurons in the mesencephalon of the eel, *Anguilla anguilla*, as determined by choline acetyltransferase immunohistochemistry and acetylcholinesterase enzyme histochemistry. *Cell Tissue Res*., **271,** 555–566.

Moreau, Y., Arredondo, J.L., Perraud-Gaime, I. & Roussos, S. (2003) Dietary utilisation of protein and energy from fresh and ensiled coffee pulp by the Nile tilapia *Oreochromis ninoticus*. *Braz. Arch. Biol. Technol*., **46,** 223–231.

Oliva-Teles, A. (2000) Recent advances in European sea bass and gilthead sea bream nutrition. *Aquacul. Inter*., **8,** 477–492.

Pérez, S.E., Yanez, J., Marin, O., Anadon, R., Gonzalez, A. & Rodriguez-Moldes, I. (2000) Distribution of choline acetyltransferase (ChAT) immunoreactivity in the brain of the adult trout and tract-tracing observations on the connections of the nuclei of the isthmus. *J. Comp. Neurol*., **428,** 450–474.

Scarpa, J., Toro, J.E. & Wada, K.T. (1994) Direct comparison of six methods to induce triploidy in bivalves. *Aquaculture*, **119,** 119–133.

Schober, A., Malz, C.R. & Meyer, D.L. (1993) Enzymehistochemical demonstration of nitric oxide synthase in the diencephalons of the rainbow trout (*Oncorhyncus mickiss*). *Neurosci. Lett*., **151,** 67–70.

Serrano, F.S.J., Lopez, J.L.S. & Martin, L.O.G. (1995) Caffeine inhibition of glycogen phosphorylase from *Mytilus galloprovincialis* mantle tissue. *Int. J. Biochem. Cell Biol*., **27,** 911–916.

Shi, D., Nikodijevic, O., Jacobson, K.A. & Daly, J.W. (1993) Chronic caffeine alters the density of adenosine, adrenergic, cholinergic, GABA, and serotonin receptors and calcium channels in mouse brain. *Cell. Mol. Neurobiol*., **13,** 247–261.

Sorimachi, M., Yamagami, K. & Nishimura, S. (1992) A muscarinic receptor agonist mobilizes Ca2 + from caffeine and inositol-1, 4, 5-trisphosphate-sensitive Ca^{2+} stores in cat adrenal chromaffin cells. *Brain Res*., **571,** 154–158.

Ulloa, R.J.B. & Verreth, J.A.J. (2003) Growth of *Oreochromis aureus* fed with diets containing graded levels of coffee pulp and reared in two culture systems. *Aquaculture*, **217,** 275–283.

Ulloa, R., Juan, B. & Verreth, J.A.J. (2002) Growth, feed utilization and nutrient digestibility in tilapia fingerlings (*Oreochromis aureus* Steindachner) fed diets containing bacteria-treated coffee pulp. *Aquacult. Res*., **33,** 189–195.

Ulloa, R.J.B., Verreth, J.A.J., Amato, S. & Huisman, E.A. (2003) Biological treatment affect the chemical composition of coffee pulp. *Biores. Technol*., **89,** 267–274.

Villani, L. (1999) Development of NADPH-diaphorase activity in the central nervous system of the cichlid fish, *Tilapia mariae*. *Brain Behav. Evol*., **54,** 147–158.

Villani, L. & Guarnieri, T. (1995) Localization of NADPH-diaphorase in the goldfish brain. *Brain Res*., **679,** 261–266.

Villani, L., Battistini, S., Bissoli, R. & Contestabile, A. (1987) Cholinergic projections in the telencephalo-habenulo-interpeduncular system of the goldfish. *Neurosci. Lett*., **77,** 263–268.

Villani, L., Guarnieri, T. & Zironi, I. (1994) Choline acetyltransferase and NADPH-diaphorase localization in the goldfish habenulo-interpeduncular system. *Neurosci. Lett*., **173,** 67–70.

Wong, C.J.H., Johnson, J.D., Yunker, W.K. & Chang, J.P. (2001) Caffeine stores and dopamine differentially require Ca^{2+} channels in goldfish somatotropes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **280,** R494–R503.

Woolf, N.J. (1991) Cholinergic systems in mammalian brain and spinal cord. *Prog. Neurobiol.*, **37,** 475–524.

Wullimann, M.F. & Roth, G. (1992) Is the nucleus corticalis of teleosts a new cholinergic central nervous system for vertebrates? *Neuroreport*, **3,** 33–35.

Yamamoto, S., Sugawara, Y. & Nomura, T. (1990) Chemical and thermal control of triploid production in Pacific oysters and mussels, with regard to controlling meiotic maturation. In: *Advances in Invertebrate Reproduction 5* (Hoshi, M. & Yamashita, O. eds), pp. 455–460. Elsevier, Amsterdam, Netherlands.

Zikopoulos, B. & Dermon, C.R. (2005) Comparative anatomy of α2 and βadrenoceptors in the adult and developing brain of the marine teleost the red porgy (*Pagrus pagrus*, Sparidae): [3H]clonidine and [3H]dihydroalprenolol quantitative autoradiography and receptor subtypes immunohistochemistry. *J. Comp. Neurol.*, **489,** 217–240.