Credit author statement

A.C performed the experiment and data analyses; F.B developed and designed the experiment of the supplementary data; A.C, C.B, T.K developed and designed the experiment and data analyses; A.C, C.B, T.K wrote the manuscript; A.C, C.B, T.K, F.B proofread the manuscript.

Alteration of predatory behaviour and growth in juvenile cuttlefish by fluoxetine and venlafaxine

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Authors contribution

A.C performed the experiment and data analyses; F.B developed and designed the experiment of the supplementary data; A.C, C.B, T.K developed and designed the experiment and data analyses; A.C, C.B, T.K wrote the manuscript; A.C, C.B, T.K, F.B proofread the manuscript.

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Keywords: antidepressant; monoamines; cephalopod; *Sepia officinalis*; food intake; development; maturation

Highlights

- **Very low ng·L^{-1} concentrations of antidepressants impaired predatory behaviour**
- **Cumulated fluoxetine and venlafaxine at 5 ng·L^{-1} each decreased feeding motivation**
- **Cumulated fluoxetine and venlafaxine at 5 ng·L^{-1} each decreased successful prey capture**
- **Cumulated fluoxetine and venlafaxine at 5 ng·L^{-1} each decreased growth**

Abstract

Antidepressants in coastal waters may affect ontogeny of predatory behaviour in cuttlefish, which may, as a result, affect growth of newly-hatched cuttlefish. We investigated the effects of two of the most

prescribed antidepressants, fluoxetine (FLX) and venlafaxine (VEN) in environmentally realistic concentrations on the predatory behaviour of hatchlings of *Sepia officinalis*. Newly-hatched cuttlefish were exposed from 1 hour (*i.e.*, day 1) to 5 days after hatching to either FLX alone (5 $ng \cdot L^{-1}$) combined with VEN (2.5 $ng \cdot L^{-1}$ or 5 $ng \cdot L^{-1}$ each) to simulate an environmentally realistic exposure scenario. Their predatory behaviour was analysed through several parameters: prey detection, feeding motivation and success in catching the prey. All parameters improved in control animals over the first five days. The combination of FLX and VEN at 5 $ng \cdot L^{-1}$ each altered the predatory behaviour of the hatchlings by increasing the latency before attacking the prey, *i.e.*, reducing feeding motivation, as well as by reducing the number of successful attacks. The changes in predatory behaviour tended to reduce food intake and affected growth significantly at 28 days post-hatching. Exposures to either FLX at 5 $ng \cdot L^{-1}$ or FLX and VEN in mixture at 2.5 $ng \cdot L^{-1}$ each tended to produce similar effects, even though they were not statistically significant. It is likely that the antidepressants affect maturation of the predatory behaviour and/or learning processes associated with the development of this behaviour. The slightest delay in maturation processes may have detrimental consequences for growth and population fitness.

1. Introduction

Cuttlefish are active predators capable of catching diverse types of prey (Hanlon and Messenger, 2018), such as small crustaceans and molluscs, but also fishes. *Sepia officinalis* becomes an efficient predator over time by maturation of its nervous system and by learning whilst still in the egg and, notably, beginning with hatching (Darmaillacq et al., 2008; Dickel et al., 1997). Indeed, hatchlings do not benefit from parental care because the parents die before their offspring hatch. Juvenile cuttlefish, therefore, have to forage independently (Hanlon and Messenger, 2018). To ensure their survival, they have to learn quickly how to hunt efficiently, as newly-hatched cuttlefish can subsist without feeding only for a few days by relying on the remainders of their internal yolk reserve (Dickel et al., 1997). To avoid any nutrient deficiency, they usually begin to hunt before their yolk is entirely depleted (Wells, 1958). The critical nature of these first days post hatching is underscored by their cuttlebone, which becomes positively

buoyant if juveniles do not eat by the fifth day after hatching. As a matter of consequence, the juveniles become unable to hunt and they quickly perish (Boletzky, 1975; O'Brien et al., 2017). Furthermore, newly-hatched cuttlefish will have to grow quickly to become efficient and less endangered predators as growth is positively correlated to food intake. Reduced food intake and growth during the initial life stage may persist at later life stages and throughout lifetime (Koueta and Boucaud-Camou, 1999).

Juvenile cuttlefish have to adapt their hunting method depending on the prey type (Hanlon and Messenger, 2018). With a crab in sight, cuttlefish will apprehend their prey by jumping from behind with partially open arms and without using their tentacles (Duval et al., 1984). On the contrary, *S. officinalis* will attack a shrimp by ejecting the two tentacles equipped with large suckers, to capture the prey and bring it to the arms and mouth (Messenger, 1977). It appears that juvenile cuttlefish have a preference for shrimps as first prey after hatching over crabs or young fish (Darmaillacq et al., 2006; Guibé et al., 2012). To accomplish this hunting technique, newly-hatched cuttlefish need to (1) detect their prey, (2) assess the distance between themselves and their prey, (3) and eject their tentacles in the right direction and fast enough for successful seizure. These technical parameters need to be improved by maturing and learning over the first few hours to days after hatching.

Behaviour in many species is regulated by the nervous system and influenced by many neurotransmitters. Amongst them, serotonin (5-HT), norepinephrine (NE) and dopamine (DA) are monoamines known to be involved in in several behaviours (Weiger, 1997), such as locomotor activity (Pavlova, 2001), aggressive behaviour (Huber et al., 1997), as well as predatory behaviour and food intake (He et al., 2018; Palovcik et al., 1982; Wang et al., 2002). Consequently, any change in the levels of these neurotransmitters may result in modifications of diverse behaviours and bear the possibility of cognitive impairments during development. Therefore, environmental chemicals that interfere with the serotoninergic, as well as the noradrenergic and dopaminergic system of non-target organisms, could disturb behavioural traits in aquatic organisms such as cuttlefish. A group of pharmaceuticals, the antidepressants of the family of selective serotonin reuptake inhibitors (SSRI) and serotonin-norepinephrine reuptake inhibitors (SNRI),

could be of particular concern, as they interact with the pre-synaptic membrane transporters that help regulating neurotransmitter levels in the synaptic cleft. Because of their continuous release into the aquatic environments worldwide *via* wastewater effluents, these pharmaceuticals may be considered pseudo-persistent micropollutants (Bueno et al., 2012; Nielsen and Gøtzsche, 2011; OECD Health Statistics 2019 - OECD). Indeed, antidepressants are persistently detected and quantified at low $ng \cdot L^{-1}$ concentrations in surface waters (Bueno et al., 2012; Meador et al., 2016; Metcalfe et al., 2010; Paíga et al., 2016; Rúa-Gómez and Püttmann, 2012a, 2012b). Our study combines two of the most prescribed antidepressants, Prozac© and Effexor©, and their respective active molecules fluoxetine (FLX) and venlafaxine (VEN) (Fong and Ford, 2014). The antidepressants FLX and VEN have been typically detected at the lower $ng \cdot L^{-1}$ range in aquatic environments (Kwon and Armbrust, 2006) and were found at 0.3 and 1.3 $ng \cdot L^{-1}$, respectively, in the coastal waters of Northwestern France (Minguez et al., 2016). Therefore, frequently reported low $ng \cdot L^{-1}$ concentrations of single antidepressants do not effectively reflect the true load of these pharmaceuticals, as several antidepressants are released simultaneously into the water bodies and add to higher total concentrations (Klosterhaus et al., 2013; Meador et al., 2016; Richmond et al., 2016). Despite their low toxicity and trace amounts, FLX and VEN released into the aquatic environment may affect particularly sensitive behavioural traits in non-target organisms, such as hatchlings of cuttlefish. Indeed, antidepressants have been shown to induce behavioural impairments in various aquatic species, such as changes in maturing/learning capacities (Chabenat et al., 2019; Di Poi et al., 2013), camouflage (Bidel et al., 2016a; Di Poi et al., 2014), locomotor activity (Barry, 2013; Guler and Ford, 2010; Mesquita et al., 2011; Tan et al., 2020) or reproductive behaviour (Campos et al., 2016; Fong, 1998). Predatory behaviour was also affected by the two psychoactive drugs in several aquatic species, such as striped bass (Bisesi et al., 2016, 2014; Gaworecki and Klaine, 2008), fathead minnow (Stanley et al., 2007; Weinberger and Klaper, 2014), goldfish (Forsatkar et al., 2014; Mennigen et al., 2010), or shore crab (Peters et al., 2017).

Cephalopods have the most developed brain amongst all molluscs, allowing for a complex behavioural repertoire (Hanlon and Messenger, 2018). Because of their elevated cognitive capacities (Darmaillacq et

al., 2006; Dickel et al., 1997; Mather and Dickel, 2017), which mature during their first life stages, they are particularly sensitive models to study the potential effects of psychotropic substances on animal behaviour. Amongst the various behavioural traits displayed by juvenile cuttlefish, predatory behaviour after hatching is fundamental for growth and survival of the hatchlings. Any modification of foraging that reduces predation success due to pollutants present in the shallow coastal waters, where female cuttlefish lay their eggs, could affect population recruitment. Moreover, a fundamentally impaired predatory behaviour following antidepressant exposure may reduce hunting success and, consequently, food intake. Reduced food intake, in turn, is likely to have an impact on other physiological or morphological aspects, such as reduced growth (Koueta and Boucaud-Camou, 1999). Earlier studies showed that embryos of *S. officinalis* do indeed bioaccumulate pollutants such as heavy metals or the antidepressant FLX (Bidel et al., 2016a; Lacoue-Labarthe et al., 2016) and reported effects of antidepressants on juvenile cuttlefish. For instance, Di Poi *et al.* (2013) demonstrated alterations of cognitive abilities of cuttlefish hatchlings and Bidel *et al.* (2016) as well as Di Poi *et al.* (2014) found that camouflage was affected. Although studies on the effects of single molecules, such as FLX and VEN, on predatory behaviour exist (Gaworecki and Klaine, 2008; Mennigen et al., 2010; Stanley et al., 2007), few studies investigated the effects of cumulated antidepressants on this particular behaviour (Bisesi et al., 2016) and no study investigated their effects at a very early life stage, essential for growth and population survival. Yet, cumulated concentrations of major antidepressants constitute a more realistic environmental scenario than testing single antidepressants. Therefore, this study assesses the impact of two antidepressants at cumulated low $ng \cdot L^{-1}$ concentrations, combining a SSRI (FLX) and a SNRI (VEN) on predatory behaviour and growth — two fundamental ecological parameters— in highly sensitive hatchlings of *S.officinalis.*

2. Material and methods

2.1. Animal rearing and experimental conditions

Eggs of wild cuttlefish (*Sepia officinalis*) were collected in the English Channel (off the coast of Luc-sur-mer, France) at different locations and several times to ensure genetic variability of eggs laid by different

females. They were randomly mixed and kept until hatching in filtered natural sea water in an open-loop system under a natural summer photoperiod (16h light:8h dark) in a climate chamber (15±1°C) at the 'Centre de Recherches en Environnement Côtier' (CREC) of the University of Caen Normandy at Luc-sur-mer, France. Egg laying days were unknown and spread out over time. Thus, eight hatchlings were collected daily and isolated as explained below until a total of 60 cuttlefish between the 11th and 19th of July 2017 was reached.

At hatching, cuttlefish were transferred immediately to glass beakers (Ø 11 cm, height 6 cm; one animal per beaker, henceforth designated 'home tanks'), assigned randomly to four experimental groups and exposed individually (n=15 per group) from their very first hours post-hatching to either (1) carbon-filtered seawater (control), or (2) 5 ng·L^{-1} FLX alone (FLX5), or (3) 2.5 ng·L^{-1} FLX and 2.5 ng·L^{-1} VEN in mixture (FLXVEN2.5) as well as (4) 5 ng·L^{-1} FLX and 5 ng·L^{-1} VEN in mixture (FLXVEN5). Cuttlefish were reared over a period of 28 days in a semi-static system supplied with 250 mL carbon-filtered seawater (FSW) to assess the effects of antidepressant exposure on growth. Temperature was maintained at 15±1°C and the photoperiod was set 16h light:8h dark. Constant conditions during the exposures, *i.e.*, waterborne antidepressant concentrations, salinity of 35±1 PSU, O_2-levels not less than 80%, as well as nitrite and nitrate levels within acceptable levels ($[NO_2^-]_{max}$=0.05 mg·L^{-1}, $[NO_3^-]_{max}$=0.15 mg·L^{-1}), were maintained by transferring each animal daily to a new home tank filled with renewed FSW and spiked with the respective antidepressant concentrations. Cuttlefish were fed once a day during the predation test with a sand shrimp (*Crangon crangon*). All shrimps were adapted to the size of the cuttlefish to be properly caught and eaten, *i.e.*, a shrimps' length of about 3-6 mm relative to a dorsal mantle length of the juvenile cuttlefish of 1 cm or less. After the predation tests were terminated at day 5, the animals were exposed and fed as before with one shrimp per day over 28 days of exposure to assess the growth of the animals. No mortality was observed during the test duration, *i.e.*, 28 days.

Animal hatchery and animal experiments were carried out in accordance with the guidelines of the EU Directive 2010/63/EU for animal experiments (Protocol approved by the French National Ethical Committee for Animal Experimentation, Agreement number: 035121.02).

2.2. Chemical contamination

A set of stock solutions at 10 $\mu g \cdot L^{-1}$ of either FLX hydrochloride (CAS 56296-78-7, Sigma Aldrich, St. Louis, USA) or VEN hydrochloride (CAS 99300-78-4, Sigma Aldrich) was prepared in distilled water and kept at -80°C until use. For daily water renewals of the cuttlefish home tanks, stock solutions were diluted in FSW to the appropriate final concentrations. Preliminary studies confirmed that the actual concentrations after 24 hours were not less than 75% of the nominal concentration. Waterborne FLX and VEN concentrations were chosen to approximate measured surface water concentrations quantified along the Northwestern coast of France, *i.e.*, 0.3 and 1.3 $ng \cdot L^{-1}$ for FLX and VEN respectively (Minguez et al., 2016). The FLX5-treatment was used as a reference to compare the results of the present study to those of previous studies, as FLX represents the model antidepressant, which was first SSRI released into the environment and has therefore been used in many studies over the last decades to study its effects and similar antidepressants on physiological and behavioural responses in aquatic animals. In comparison, the effects of VEN alone at 5 $ng \cdot L^{-1}$ (VEN5) have been assessed in a separate experiment with slightly different settings, the results of which can be found in the supplementary data.

2.3. Predation test

2.3.1. Experimental setup

Predatory behaviour of each individual cuttlefish was assessed at five time points: from 1 day post-hatching (dph) corresponding to an exposure of 1h in contaminated FSW until 5 dph (Fig. 1) using the predation test described in the following. Recordings were conducted at the same hours (between 9 and 11am) to avoid any influence of circadian rhythms on feeding behaviour and to maintain equal exposure durations between the tests, *i.e.*, 24 hours. Each day, cuttlefish were moved within their home tanks (to

avoid additional stress) inside a custom-made black tent for predation test recording, in order to avoid any external stimuli. A circular LED lamp (Neewer®, Guangdong, China) served as light source. The lamp was adjusted in an oblique position 60 cm above the tanks with the inner part of the tent being lined with white fabrics so as to reflect the light in order to avoid direct light on cuttlefish. The camera (Legria HF R506, Canon Camera Co. Inc., Tokyo, Japan) was installed on a stand adjacent to the lamp. Four cuttlefish, one from each of the conditions, passed the test simultaneously (Fig. 2). Opaque walls separated the tanks in order to avoid visual inter-individual influence (*e.g.*, mimic) (Fig. 2). The test recording began when a shrimp was inserted in the centre of the home tank with the cuttlefish tending to remain close to the tank's walls. The video recording lasted 10 min, after which each animal was returned to its experimental exposure zone. If the cuttlefish did not eat the shrimp within the 10 min test period, the shrimp was removed from the tank and excluded from the common batch to avoid any cross contamination or habituation from the shrimp. The experimental setup for VEN5 is described in the methods to the supplementary data.

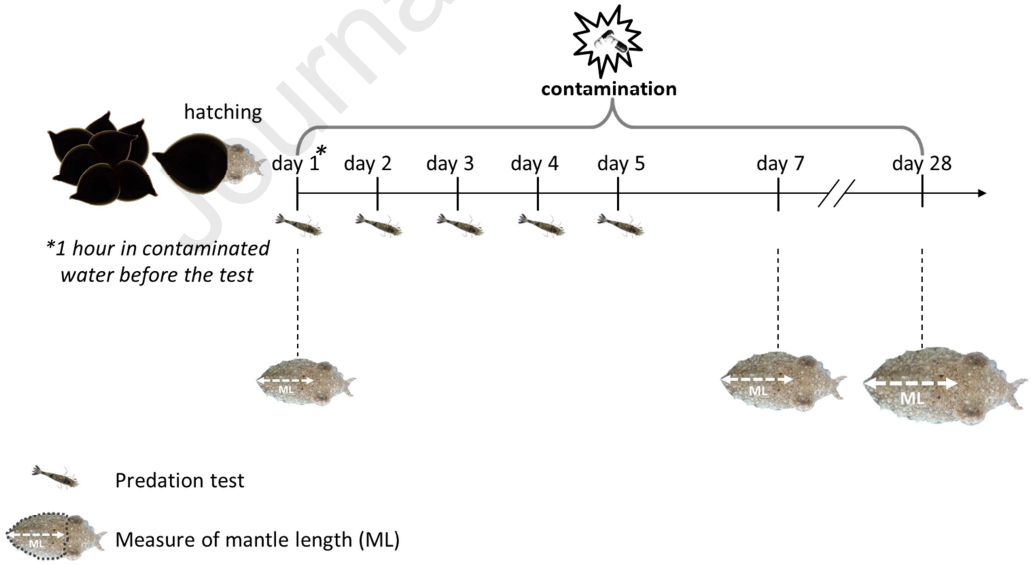


Figure 1. *Timeline of the experiments. The day of hatching, the cuttlefish were placed in contaminated water one hour before the first test and the exposure lasted 28 days. Each cuttlefish passed the predation*

test each day for 5 days. Growth was monitored by measuring mantle length (ML) at three time points, i.e., day 1, 7 and 28 post-hatching.

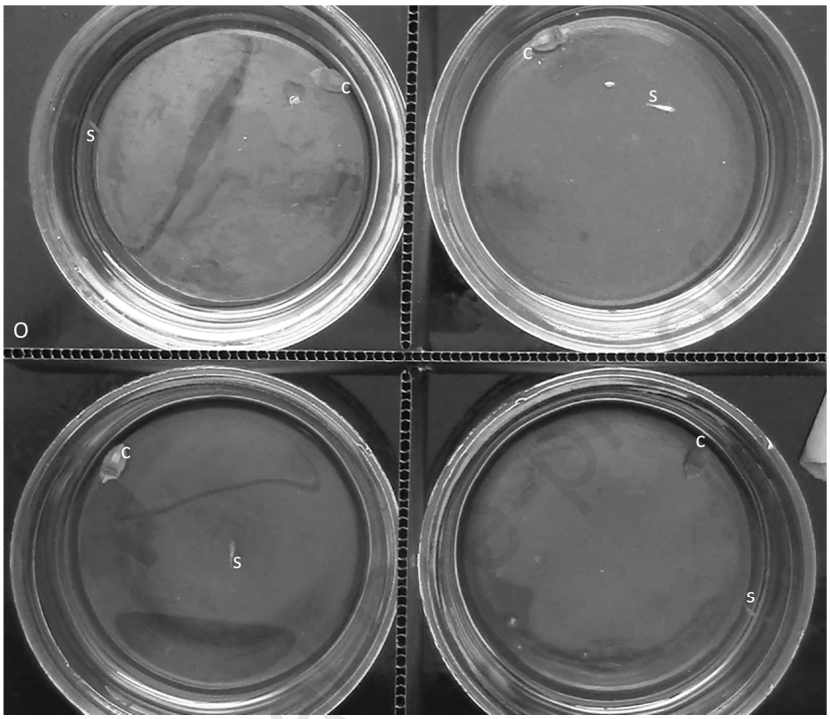


Figure 2. *Experimental set-up of the predation test. Cuttlefish passed the test within their home tanks. c: cuttlefish; s: shrimp; O: opaque walls.*

2.3.2. Behavioural analyses

Video-recordings were analysed manually *a posteriori*. Predatory behaviour of juvenile cuttlefish was assessed on the basis of (i) whether the prey was actually detected by the cuttlefish, (ii) the cuttlefish showed feeding motivation, *i.e.*, attacked the prey it detected and (iii) feeding behaviour, which was defined as a successful attack and eating the prey.

Prey detection was estimated through six different criteria that were either associated with the presence of the prey, or with the behaviour of cuttlefish. Prey-associated criteria, which accounted for likely detection of the shrimp by the cuttlefish, were assessed as: (i) the shrimp was moving in the beaker, (ii) the shrimp was less than 3 cm away from the cuttlefish, (iii) the shrimp was in the cuttlefish's field of

vision, which covers a total of 320°. At least two of these prey-associated criteria had to be observed simultaneously in order to accept a prey as detected, even if no obvious behaviour of the cuttlefish towards the shrimp could be observed. Predator-associated criteria, which confirmed that shrimp were detected by the cuttlefish were assessed as: (i) the cuttlefish positioned itself towards the prey, (ii) the cuttlefish attempted an attack, but missed the prey (*i.e.*, miscalculated the distance), (iii) the cuttlefish attempted an attack, but the prey escaped. If either of these predator-associated criteria, alone or combined with any other prey- or predator-associated criteria occurred, prey detection could be established with certainty. Eventually, prey detection was expressed as the percentage of one of the following categories relative to the total number of observations: firstly, cuttlefish that successfully detected, attacked and ate the shrimp (detection + success), secondly, cuttlefish that detected the prey but did not perform a successful attack (detection + no success), and thirdly, cuttlefish that did not detect the shrimp (no detection).

For *feeding motivation*, two indicators were established:

(i) The latency before the first attack corresponded to the time between the shrimp's entry into the home tank and the first time the cuttlefish ejected its tentacles towards the shrimp. If the animals did not attack, the maximum latency of 600 s was assigned.

(ii) The number of attempts to catch the prey until the cuttlefish succeeded to seize the shrimp, *i.e.*, accomplishing a successful attack, or until the 10 min test ended (removal of the shrimp).

Feeding behaviour was determined by the number of successful attacks, *i.e.*, the shrimp were attacked and eaten by the cuttlefish within the 10 min test, for each of the treatment groups at each time point.

Behavioural endpoints for VEN5 alone differed to some extent from the above-described analyses and are explained in the methods to the **supplementary data**.

2.3.3. Food intake

Food intake was determined as the total of shrimps eaten by each cuttlefish over the first five dph, hence the maximum number being five, *i.e.*, one per predation test and time point.

2.4. Growth

Growth was evaluated by measuring the mantle length (ML) of the cuttlefish from the dorsal end to back of the eyes on still pictures (Fig. 1) at 1, 7 and 28 dph, until significant size differences became apparent between groups. Each animal's ML was measured in triplicate, *i.e.,* on three different pictures using ImageJ© (Bethesda, Maryland, USA), for each age and the mean of the three values was taken as its ML. To guarantee that ML was randomly distributed and cuttlefish started from the same growth distribution for all treatment groups, ML on day 1 was compared across all groups. That being the case, an analysis across all groups and ages was performed, as described in the statistical analyses, to reveal any difference between treatments and ages.

2.5. Statistical analysis

All statistical tests were performed using R Studio (Version 1.1.453, RStudio Inc., Boston, USA, http://www.r-project.org). As data did not meet the assumptions of normality and homogeneity of variance, we used nonparametric permutation test analyses of data from factorial experiments (function ezPerm; "ez" package). The number of iterations was set to 1000. We used mixed within-and-between Ss designs (*i.e.*, repeated factors: age; independent factor: experimental groups) to analyse feeding behaviour (*i.e.,* successful attacks), feeding motivation (*i.e.,* latency before the first strike; number of attempts to catch the prey) and growth (*i.e.*, mantle length) at each age. Prey detection was analysed with Friedman test (function friedman_test; "coin" package) on raw data. Additionally, a Kruskal-Wallis test was performed for growth in order to verify the equal distribution of ML among cuttlefish at 1 dph (function kruskal_test; "coin" package). For all types of analysis, if the null hypothesis was rejected ($p \leq 0.05$), post-hoc permutation tests were performed to highlight any differences between experimental groups and between ages, using the "RVAidememoire" package (function pairwise.perm.t.test). We adjusted the false discovery rate using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

3. Results

3.1. Predation test

Prey detection did neither differ significantly among treatment groups (Friedman test: p=0.413), nor among ages (Friedman test: p=0.698) (Fig. 2). Each group had a very high detection rate, *i.e.*, more than 87.5% of the animals detected the prey regardless of treatment group or age.

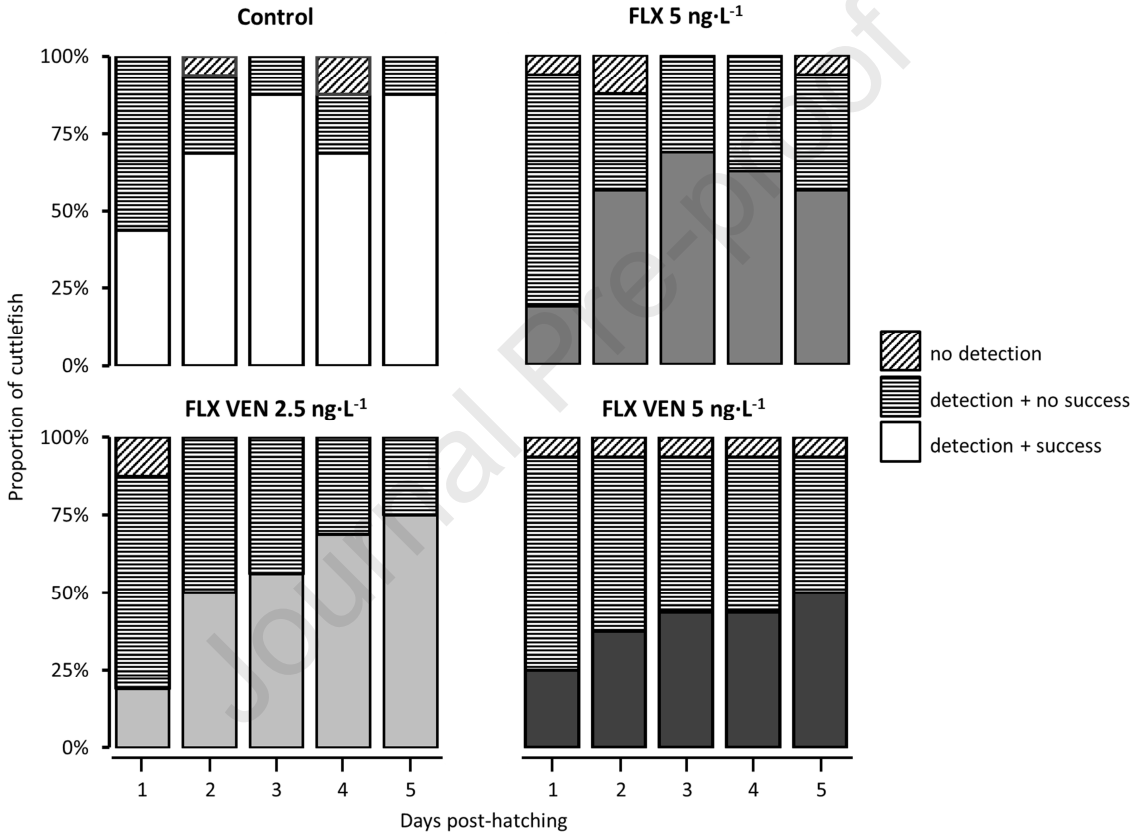
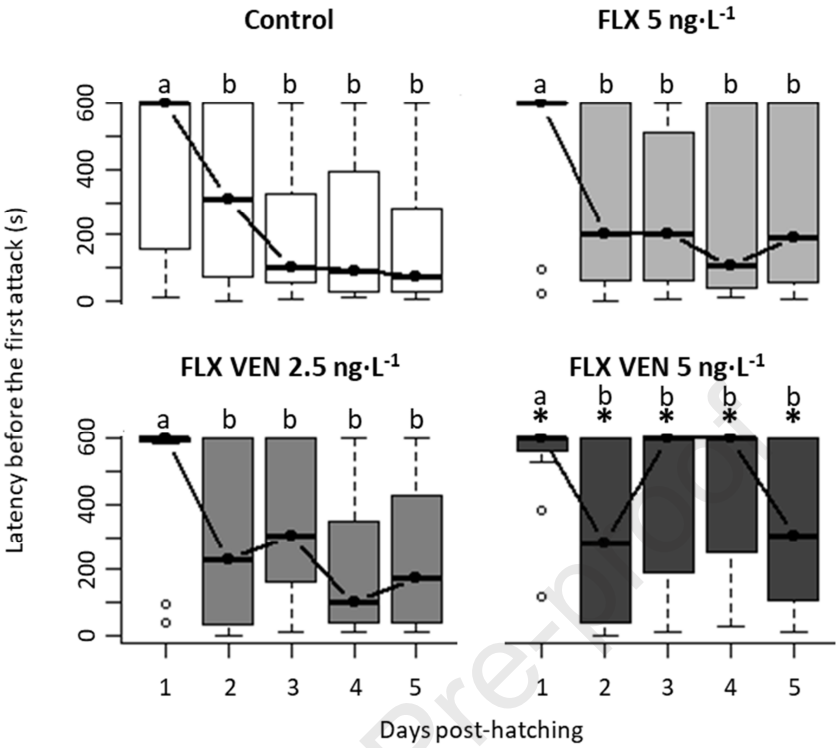


Figure 2. *Prey detection during a 10 min predation test by juvenile cuttlefish exposed to four conditions: control (non-exposed group), fluoxetine (FLX) at 5 ng·L^{-1}, fluoxetine and venlafaxine in combination (FLX VEN) at 2.5 ng·L^{-1} or 5 ng·L^{-1} each. Predation test was conducted at five time points of exposure (1, 2, 3, 4 and 5 days post-hatching). Friedman test showed no significant difference in detection in any group at any age (n=15 per treatment group).*

Latency before the first attack was significantly different among groups (permutation test across all groups: p=0.040). Pairwise comparisons showed that the latency was significantly greater in the FLXVEN5 than in the control group (post-hoc permutation test: p=0.024; Fig. 3). A greater latency was also found for VEN5 (Wilcoxon-Mann Withney test: p=0.008) (Fig. S1, supplementary data). Even if no significant difference did clearly emerge between the FLX5 and the FLXVEN2.5, respectively and the FLXVEN5 group, their latencies tended to be lower than the latter (post-hoc permutation test: FLX5-FLXVEN5, p=0.072; FLXVEN2.5-FLXVEN5, p=0.072). Similar trends were observed between the control group and either of the FLX5 and FLXVEN2.5 groups (post-hoc permutation test: control-FLX5, p=0.075; control-FLXVEN2.5, p=0.089).

Importantly, latency evolved with age (permutation test across all groups: p<0.001). Indeed, pairwise comparisons showed that latency decreased drastically between the first day after hatching and day 2 (post-hoc permutation test: d1-d2 p=0.005), followed by a more moderate decrease between day 2 and day 3 as well as day 4 and day 5, which were, however, statistically non-significant (post-hoc permutation test: p>0.05). This pattern is easily observed in the control group (Fig. 3). Within the treatment groups, latency also decreased very rapidly between day 1 and day 2, but developed less steadily in the FLX5 and, notably, in the FLXVEN2.5 groups or even oscillated from day 2 to day 5 in the FLXVEN5 group (Fig. 3). Remarkably, in all treatment groups, animals rarely attacked one hour after hatching, *i.e.*, at day 1, but attacks by hatchlings after 1 hour were more frequent in the control group.



277

278 **Figure 3.** *Latency before the first attack towards the prey, a sand shrimp (*Crangon crangon*), over a 10*
279 *min predation test. Newly-hatched cuttlefish were tested at five time points of exposure, i.e., 1, 2, 3, 4 and*
280 *5 days post-hatching. Four groups of cuttlefish were exposed to either fluoxetine (FLX) at 5 ng·L^{-1}, or*
281 *fluoxetine and venlafaxine in combination (FLX VEN) at 2.5 ng·L^{-1}, or 5 ng·L^{-1} each, with non-exposed as*
282 *controls. Boxplots show medians (horizontal bars), upper and lower interquartile ranges (boxes) as well*
283 *as highest and lowest values (whiskers); circles represent outliers. Post-hoc permutation tests adjusted*
284 *with FDR correction (Benjamini-Hochberg procedure) were performed for inter-group and inter-age*
285 *comparisons: a and b indicate significant differences among ages within groups ($p<0.05$); * shows*
286 *significant differences among the exposed groups and the control group ($p<0.05$; n=15 per treatment*
287 *group)*

288 *The number of attempts made to attack* was not significantly different among groups (permutation test
289 across all groups: p=0.359), but it was significantly different among ages (post-hoc permutation test:
290 p=0.001; Fig. 4). Pairwise comparison confirmed that cuttlefish from each group attacked more often at

day 5 than during the first 4 dph (post-hoc permutation test: p=0.04). Even if the results did not show any statistical difference, it may be noticed that gradually, more and more cuttlefish attacked their prey each day, no matter what the treatment conditions were (post-hoc permutation test: d1-d3, p=0.08; d1-d4, p=0.08). Indeed, the same pattern was seen in successful attacks, with an increasing proportion of cuttlefish attacking their prey each dph (Fig. 5).

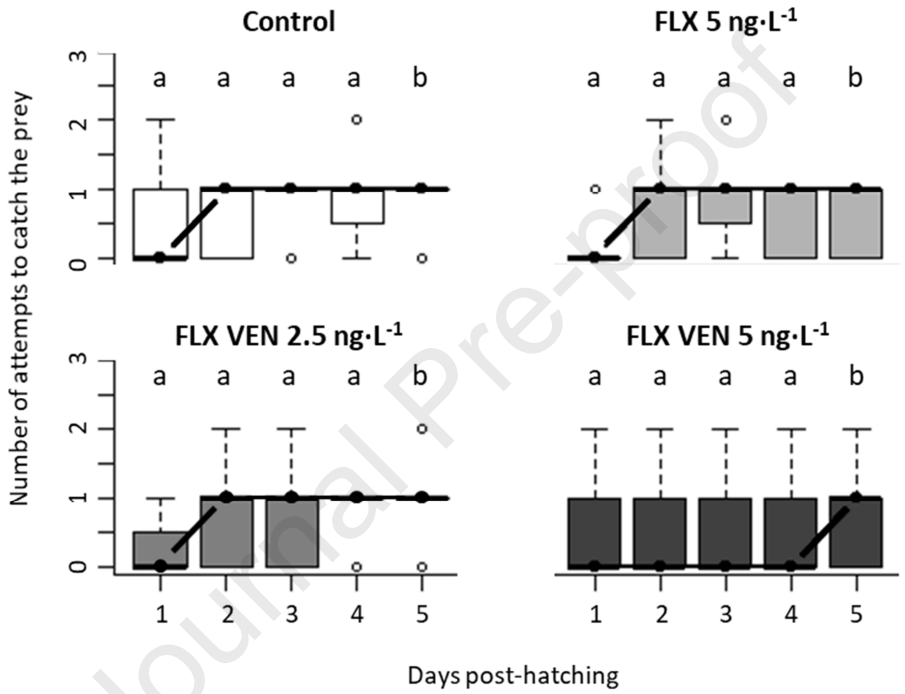


Figure 4. *Number of attempts made to catch the prey, a sand shrimp (*Crangon crangon*), over a 10 min predation test. Newly-hatched cuttlefish were tested at five time points of exposure, i.e., 1, 2, 3, 4 and 5 days post-hatching. Four groups of cuttlefish were exposed to either fluoxetine (FLX) at 5 ng·L^{-1}, or fluoxetine and venlafaxine in combination (FLX VEN) at 2.5ng·L^{-1}, or 5 ng·L^{-1} each, with non-exposed animals as controls. Boxplots show medians (horizontal bars), upper and lower interquartile ranges (boxes) as well as highest and lowest values (whiskers); circles represent outliers. Post-hoc permutation tests adjusted with FDR correction (Benjamini-Hochberg procedure) were performed for inter-group and inter-age comparisons: a and b indicate significant differences among ages (p<0.05; n=15 per treatment group)*

306 *Successful attacks* were significantly different among groups (permutation test across all groups:
307 p=0.018). Whereas, the control group showed the highest overall rate of successful attacks (except for d4,
308 Fig. 5), the FLXVEN5 group had the lowest rate of successful attacks (post-hoc permutation test:
309 FLXVEN-control, p=0.024). The FLX5 and FLXVEN2.5 tended to have a lower rate of successful
310 attacks, as compared to the control group, but these differences did not attain statistical significance (post-
311 hoc permutation test: FLX5-control, p=0.084; FLXVEN2.5-control, p=0.084). The lack of statistical
312 significance may be explained by a high intra-group variability within FLX5 and FLXVEN2.5 groups.

313 As with the latency, the rate of successful attacks differed among ages (permutation test across all groups:
314 p<0.001). In fact, pairwise comparisons showed an increase in successful attacks with age between d1 and
315 d2, d3, d4, d5 (post-hoc permutation test: d1-d2, p=0.015; d1-d3, d1-d4 and d1-d5, p=0.007, respectively)
316 for each group. Although this statistically significant increase with age was obtained for all groups, the
317 FLXVEN5 treatment stood out as the group with the lowest progressive increase (at d5: 56.25% against
318 87.5% for control; 62.5% for FLX5; 75% for FLXVEN2.5). In the experiment that tested VEN alone, the
319 success rate was lowered by 20 percentage points as compared to the control (VEN5=76.66% ± 13.19%
320 SEM against 96.15 ± 3.84% SEM for control; for details see supplementary data). This resulted in
321 significantly lower proportion of cuttlefish that ate a shrimp within the 20 min test in animals exposed to
322 VEN alone at 5 $ng \cdot L^{-1}$ (Fisher's exact test: p<0.001) (Fig. S2, supplementary data), suggesting a reduction
323 of food intake when exposed to VEN5.

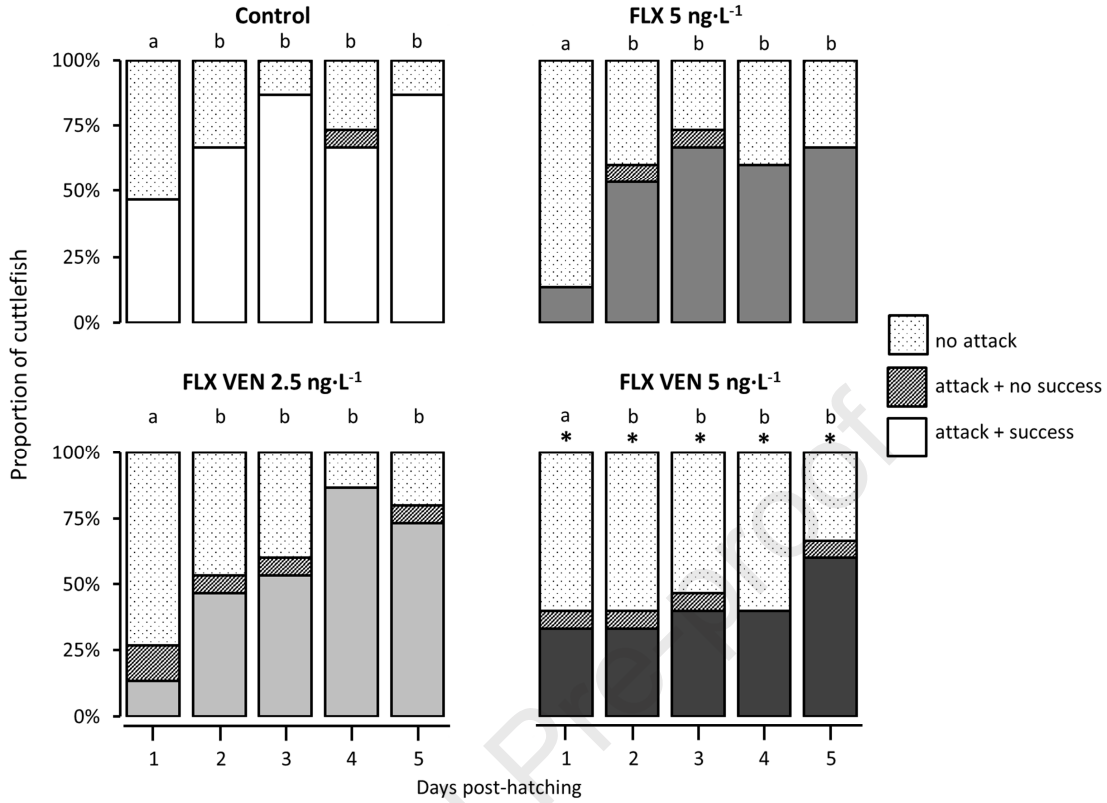


Figure 5. *Proportion of cuttlefish which did (i) attack the prey with success, did (ii) attack the prey without success, or did (iii) not attack the prey at all over a 10 min predation test. Four groups of cuttlefish were exposed to either fluoxetine (FLX) at 5 ng·L^{-1}, or fluoxetine and venlafaxine in combination (FLX VEN) at 2.5 ng·L^{-1}, or 5 ng·L^{-1} each, with non-exposed animals as controls. Post-hoc permutation tests (n=15 per treatment group) adjusted with FDR correction (Benjamini-Hochberg procedure) were performed for inter-groups and inter-age comparisons: a and b indicate significant differences among ages within groups (p<0.05); * shows significant differences between the control group and either of the exposed groups (p<0.05)*

The food intake, i.e., number of shrimps eaten throughout the first five dph was not significantly different among groups, even though a trend may be noticed (Kruskal-Wallis test, p=0.068). Cuttlefish from the control group ate in average 3.5±1.1 shrimps during the first five dph, whereas cuttlefish exposed to

antidepressants such as FLX5, FLXVEN2.5 and FLXVEN5 groups ate in average 2.6±1.4, 2.7±1.3, 2.1±1.8 shrimps, respectively.

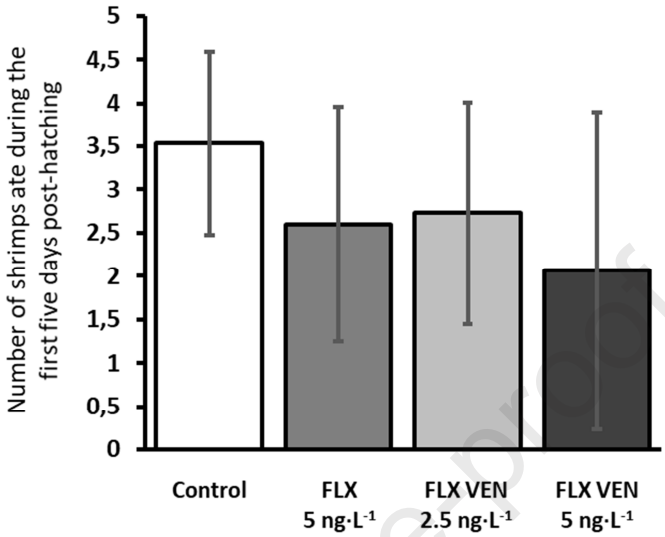


Figure 6. *Food intake represented by the number of shrimps eaten during the first five days post-hatching by cuttlefish exposed or not (control) to either fluoxetine (FLX) at 5 ng·L^{-1}, or fluoxetine and venlafaxine in combination (FLX VEN) at 2.5 ng·L^{-1}, or 5 ng·L^{-1} each. Means ± SEM; n=15 per treatment group.*

3.5. Growth

On the first dph, the size-distribution of MLs of cuttlefish was statistically equal (Kruskal-Wallis test, p=0.662) among all groups. Indeed, at 1 dph all groups were exhibiting similar mean MLs (±SEM): 8.17±0.66 mm for the controls, 8.30±0.86 mm for FLX5, 8.18±0.73 mm for FLXVEN2.5 and 7.88±1.17 mm FLXVEN5. By the end of the experiment, at 28 dph, the ML of cuttlefish had significantly increased at each time point, *i.e.*, 1, 7 and 28 dph (permutation test: p<0.001; post-hoc permutation test: p=0.002 between each of the time points). There was no obvious difference between the four groups in growth of ML at any age (permutation test: p=0.217). Nevertheless, it may be noticed that the mean ML at 28 dph of the FLXVEN5 group appears to be slightly lower than in the control group: 13.10±1.44 mm against 13.97±0.82 mm, respectively. Indeed, a post-hoc permutation test between FLXVEN5 and the control

group demonstrated a significant difference (post-hoc permutation test: p=0.032), highlighting a shorter ML.

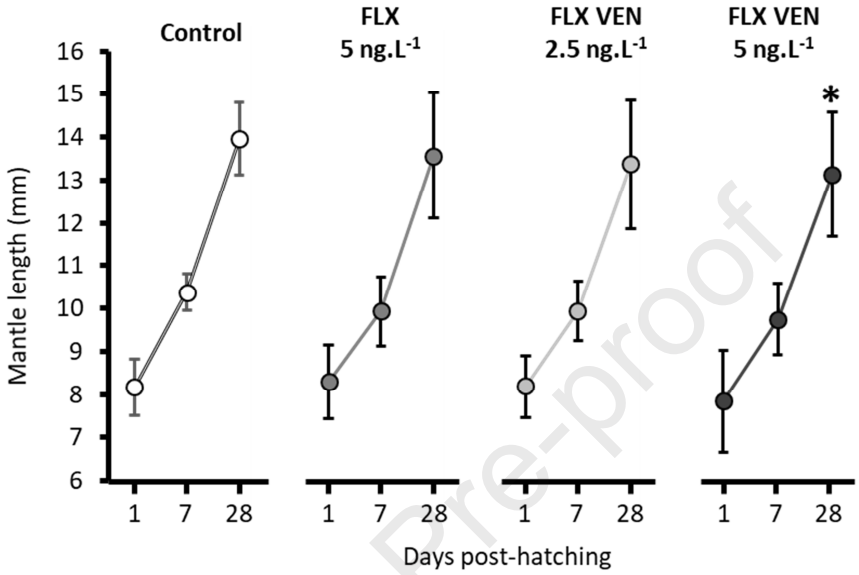


Figure 7. *Growth as assessed by mantle length over three consecutive time points (1, 7, 28 days post-hatching) of cuttlefish exposed to fluoxetine (FLX) alone at 5 ng·L^{-1}, or fluoxetine and venlafaxine in combination (FLX VEN) at 2.5 ng·L^{-1}, or 5 ng·L^{-1} each, with non-exposed animals as controls. Means ± SEM; n=15 per treatment group. Post-hoc permutation tests adjusted with FDR correction (Benjamini-Hochberg procedure) were performed for inter-group: * shows significant differences between the control group and FLXVEN 5 ng·L^{-1} (p<0.05)*

4. Discussion

The first unconditional step of hunting is to detect the prey. At hatching, cuttlefish are still maturing their visual acuity, which begins to develop already at late egg stages (Romagny et al., 2012), enabling them to detect and to gauge their environment (O'Brien et al., 2017). Before attacking, the cuttlefish needs to evaluate the distance to the prey correctly, if the attack shall be successful. Most of the juvenile cuttlefish showed a stable detection of the shrimp from day 1 to day 5, no matter the treatment group. These results

suggest that the presence of neither FLX alone, nor the combined exposure with VEN impacted their visual acuity and ability to detect the prey. A previous study from Hedgespeth *et al.* (2014) using the SSRI sertraline showed a decrease of the detection rate after eight days of exposure, thus, a decrease of feeding rate in juvenile Eurasian perch, *Perca fluviatilis*. But this impaired detection rate could only be demonstrated at much higher concentrations of 89 and 300 $\mu g \cdot L^{-1}$, whereas at the lowest concentration of 0.12 $\mu g \cdot L^{-1}$ no significant decrease in detection was observed. This corroborates our findings that the capacity to detect a potential food source is unlikely to be impacted by antidepressants at low environmental concentrations, as was the case with a ten times lower concentration in our experiments with juvenile cuttlefish. Hence, any reduction in feeding activity would necessarily derive from effects other than prey detection.

Maturation of predatory behaviour is essential for newly-hatched cuttlefish since they rely on themselves to satisfy their food requirements. To hunt prey efficiently and successfully, they have to attack their prey fast to avoid possible escapes, once the prey is detected. This necessity of improving their feeding behaviour within the first five days after hatching is corroborated by their high feeding motivation, which is characterised by a progressive decrease in the latency before the first attack as well as an increasing number of attacks over this critical period. The attacks observed in the control group were faster and more frequent with time, and highlight a continuously increasing feeding motivation as well as a sensory-motor maturation that is likely to reflect the neural maturation and learning process (Dickel et al., 2000, 1997; Messenger, 1977). The exposure to the antidepressants FLX and VEN may, therefore, have either delayed the maturation process of the predatory behaviour, the feeding motivation and/or affected learning processes. Indeed, cuttlefish exposed to a combination of FLX and VEN at 5 $ng \cdot L^{-1}$ each showed a much greater latency to attack their prey for the first time than the control group during the first five days of exposure. When testing VEN at 5 $ng \cdot L^{-1}$ alone a greater latency before the first attack compared to the control could also be observed ($p=0.008$) (Fig. S1). Similarly, cuttlefish exposed to FLX alone at 5 $ng \cdot L^{-1}$ and FLX and VEN at 2.5 $ng \cdot L^{-1}$, although not displaying a significantly prolonged latency, did neither

show the same clear decrease of latency as observed in the controls. This may be indicative of a similar, but weaker delay in maturation of predatory behaviour, as for the FLX and VEN at 5 ng·L^{-1} group. These results are consistent with earlier studies exposing *S. officinalis* to antidepressants. Di Poi *et al.* (2013) demonstrated that FLX was likely to affect memory processing, as perinatal exposure to 1 ng·L^{-1} significantly altered acquisition and retention performances in a passive avoidance learning test. In a previous study, we also observed a delay in maturation processes associated with burying behaviour in juvenile cuttlefish when exposed to FLX and VEN at 2.5 ng·L^{-1} each (Chabenat et al., 2019). Investigations of the brain structures involved in learning, *i.e.*, the vertical lobe and the optic lobes, showed that FLX and VEN induced a decrease in cell proliferation. The results obtained by Bidel *et al.* (2016a, 2016b) suggest that a decreased cell proliferation may explain the delay in maturation of predatory behaviour in juvenile cuttlefish (Dickel et al., 1997).

As in our experiments the feeding motivation increased with age, the latency before the first attack decreased, whilst the number of attacks generally increased. Despite, day-by-day group comparisons did not turn out statistically significant differences, because of high individual variation in each of the groups, the group exposed to FLX and VEN at 5 ng·L^{-1} maintained a lower number of attacks over the entire five-day exposure period, suggesting a low feeding motivation in this treatment group. In humans, one of the common side-effects related to SSRIs and SNRIs is the loss of appetite ("Antidepressants - Side effects," 2018; Capasso and Milano, 2008; Santarsieri and Schwartz, 2015). Furthermore, FLX and VEN are also prescribed for binge-eating disorders and *Bulimia nervosa* because they are considered as appetite suppressants (Carter et al., 2003). In rats, a decrease of food intake is also observed after an exposure with SSRIs or SNRIs (de Oliveira et al., 2004; Jackson et al., 1997; Russ and Ackerman, 1988).

After attacking, which in some cases may need to be repeated several times, the prey has to be seized, and secured by the cuttlefish's tentacles and arms to be eaten. Over time, newly-hatched cuttlefish become more and more successful in catching their prey and, as a matter of consequence, are able to eat each day. Cuttlefish exposed to FLX and VEN at 5 ng·L^{-1} presented the lowest increase of successful attacks over

the five days of exposure, meaning that only a few cuttlefish of this group have actually eaten every day. This resulted in a lower food intake over the five-day period compared to the control group. On the other hand, the group exposed to VEN at 5 $ng \cdot L^{-1}$ presented a significantly lower proportion of cuttlefish with successful attacks than the control group (**supplementary data**). As observed with the latency before the first strike, the two other groups exposed to FLX alone at 5 $ng \cdot L^{-1}$, or in mixture with VEN at 2.5 $ng \cdot L^{-1}$ displayed a similar tendency towards less successful attacks and lower food intake than the control. This suggests that the generally low number of attacks in juvenile cuttlefish exposed to antidepressants is likely to be related to an altered maturation of predatory behaviour, because this behaviour was improved with age in the control group. A previous study by Melnyk-Lamont *et al.* (2014) showed that after seven days of exposure to VEN at 1 $\mu g \cdot L^{-1}$, rainbow trout presented a reduced total feed intake per day. Overall, reduced feeding behaviour and food intake in several animal species agree with the known side-effects of SSRIs and SNRIs on human nutrition. Whether they have similar underlying mechanisms remains, however, to be demonstrated. The low improvement of the efficiency of the predatory behaviour may also be related to learning impairments. This and earlier studies of our laboratory consistently point to possible effects of SSRIs and/or SNRIs on learning processes in cuttlefish (Bidel, 2015; Chabenat et al., 2019; Di Poi et al., 2013).

These effects of SSRI/SNRI antidepressants on behavioural traits in juvenile cuttlefish might be related to changes in the cerebral levels of monoamine neurotransmitters, but differently from what could be expected. In cuttlefish, SSRIs and SNRIs seem to act rather on DA and NE than on 5-HT. Especially VEN seems to modulate the NE system (Bidel et al., 2016a), whereas FLX seems to affect the DA system (Bidel et al., 2016b; Di Poi et al., 2014) of cuttlefish in a yet unknown manner. Indeed, both neurotransmitters have a role in feeding behaviour and both are involved in food intake in fish and mammals (He et al., 2018; Kulczykowska and Vázquez, 2010; Wang et al., 2002; Wellman, 2000). Although not established in the present study, the modifications of monoamine levels in the brain of cuttlefish resulting from the exposure to environmental concentrations of SSRIs and SNRIs confirmed by

443 earlier studies could provide an explanation for the lower feeding motivation and feeding success, thus
444 lower food intake of juvenile cuttlefish exposed to antidepressants.

445 While feeding was reduced during the first days of the cuttlefish life, growth was significantly altered by
446 the presence of antidepressants FLX and VEN at 5 $ng \cdot L^{-1}$. A previous study from Di Poi *et al.* (2014)
447 could not demonstrate effects of FLX at 1 or 100 $ng \cdot L^{-1}$ on growth in juvenile cuttlefish over two weeks
448 after hatching. Here at 28 dph, *i.e.*, after 4 weeks of exposure, the data indicated an effect of combined
449 FLX and VEN towards reduced growth. Several studies pointed to deleterious effects of FLX on growth in
450 different species. Juvenile guppies, for instance, showed decreased growth after a chronic exposure of 35
451 days to 30 ng $FLX \cdot L^{-1}$ (Pelli and Connaughton, 2015). Tadpoles of *Xenopus laevis* exposed to FLX at 10
452 $ng \cdot L^{-1}$ for a period of 70 days exhibited a reduced growth, probably linked to a decreased food intake
453 (Conners et al., 2009). The amphipod *Hyalella azteca* displayed reduced growth after an exposure to FLX
454 at 33 $\mu g \cdot L^{-1}$ for 15 days (Péry et al., 2008), while Brooks *et al.* (2003) determined the lowest observed
455 effect concentration for growth to be 5.6 $mg \cdot kg^{-1}$ in the same species. In the rotifer *Brachionus koreanus*,
456 growth was reduced after exposure to FLX at 750 to 1000 $\mu g \cdot L^{-1}$ after only three days of exposure (Byeon
457 et al., 2020). In the polychaete *Capitella teleta*, however, no effect was observed after 18 days of exposure
458 to FLX at 0.001, 0.03, 0.3 or 3.3 $\mu g \cdot g^{-1}$ dry weight of spiked sediment. Only one study, to the best of our
459 knowledge, demonstrated a stimulation of growth by exposure to FLX: in crayfish, exposure to FLX at
460 500 $\mu g \cdot L^{-1}$ enhanced growth, resulting in a greater carapace length of post-moult animals (Tierney et al.,
461 2016). Concerning VEN, no effects on growth could be demonstrated to date. For instance, 0.88 to 80
462 $\mu g \cdot L^{-1}$ VEN did not affect growth of fathead minnows (Parrott and Metcalfe, 2017). Similarly, no effect
463 on growth was observed in zebrafish or xenopus embryos after short term exposure to VEN, *i.e.*,144 hours
464 post-fertilization at 0.3 $\mu g \cdot L^{-1}$ and 48 hours at 3 $mg \cdot L^{-1}$, respectively (Sehonova et al., 2018).
465 Consequently, the literature indicates a greater impact of FLX on growth than VEN. Notwithstanding,
466 quite high concentrations of FLX were generally necessary to negatively affect growth in most of the
467 studies. In our study, a significant decrease of feeding motivation and feeding behaviour in cuttlefish

caused by an early exposure to antidepressants at low environmental concentrations, *i.e.*, 5 ng·L^{-1} FLX and VEN each, resulted in a trend towards decreased food intake and diminished growth.

5. Conclusion

In view of the reportedly low ng·L^{-1} concentrations of single antidepressants in estuarine and coastal waters more distant from wastewater treatment plants or pharmaceutical industries (Birch et al., 2015; Klosterhaus et al., 2013; Meador et al., 2016; Minguez et al., 2016), it may be questioned if aquatic animals are at risk by the contamination of the water bodies with antidepressants. The increased use and diversity of antidepressants (Nielsen and Gøtzsche, 2011) are likely to result in cumulative concentrations that are higher than those reported for single antidepressants. The combined concentrations at a cumulated 10 ng·L^{-1} of two major antidepressants were able to alter predatory behaviour in hatchlings of cuttlefish by reducing feeding motivation through increased latency before the first attack of the prey and the number of successful attacks. The lack of feeding motivation led to a lower food intake and consequently, lower growth. Exposures to either FLX at 5 ng·L^{-1} (and VEN at 5 ng·L^{-1}) or FLX and VEN in mixture at 2.5 ng·L^{-1} each tended to reduce feeding motivation and successful attacks alike, but to a lesser extent. Importantly, the continuous decrease in latency before the first attack and the increase in the number of attacks observed in the controls suggest that the hatchlings considerably improve predatory behaviour within the first days after hatching. This improvement may derive from neural maturation as well as from experience and learning. One may, therefore, assume that the antidepressant exposure affects these maturation and learning processes. Whichever way the antidepressants affect feeding behaviour, whether they act on the neuronal system or on the levels of neurotransmitters, or both, the significant reduction in feeding behaviour, a trait that is fundamental for survival, shows that the generally very low ng·L^{-1} concentrations of antidepressants in the environment give reason for concern. This holds true particularly if sensitive stages of animals with advanced cognitive abilities are exposed to residues of the varying antidepressants in their aquatic environment. Even a minor delay in the maturation and learning processes

492 related to predatory behaviour or a reduced growth can potentially reduce fitness of populations
493 confronted with the accumulated antidepressant pollution of the aquatic environments.

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501 **Authors contribution**

502 A.C performed the experiment and data analyses; F.B developed and designed the experiment of the
503 supplementary data; A.C, C.B, T.K developed and designed the experiment and data analyses; A.C, C.B,
504 T.K wrote the manuscript; A.C, C.B, T.K, F.B proofread the manuscript.

505 **Notes**

506 The authors declare no competing interest.

507 **Abbreviations**

508 5-HT, 5-hydroxytryptamine (serotonin); DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; NE,
509 norepinephrine; FLX, fluoxetine; VEN, venlafaxine; SSRI, Selective Serotonin Reuptake Inhibitor; SNRI,
510 Serotonin, Norepinephrine Reuptake Inhibitor

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Highlights

- **Very low ng·L^{-1} concentrations of antidepressants impaired predatory behaviour**
- **Cumulated fluoxetine and venlafaxine at 5 ng·L^{-1} each decreased feeding motivation**
- **Cumulated fluoxetine and venlafaxine at 5 ng·L^{-1} each decreased successful prey capture**
- **Cumulated fluoxetine and venlafaxine at 5 ng·L^{-1} each decreased growth**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.