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Abstract   It is crucial to fully understand nutrition in the American lobster, *Homarus americanus*, given the large interest in hatchery rearing for enhancement. Dietary studies have demonstrated that gross brain development as well as American lobster health and survival is greatly influenced by diet, and in particular the amount and type of omega-3 fatty acids present in it. We conducted an assessment of how two important fatty acids, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, influence a lobster's neurogenesis, growth, and survival. To evaluate the specific effects of EPA and DHA, lobsters were fed experimental casein-based diets that varied in the source of oil. The control diet had flax oil (no EPA or DHA), whereas experimental diets were rich in EPA, DHA, or an even mix of the EPA and DHA oils. Lobsters fed the diets containing DHA oil developed fewer brain cells than those lobsters not fed this oil. Lobsters fed DHA oil also had a slightly, but not statistically significant longer survival time compared with lobsters fed the other diets. The decreased neurogenesis in the DHA-enriched diets points to the need to understand regulation of nerve cell formation in lobsters, and the functional implications of neurogenesis. Because of the large effects of diet on physiology and health, nutrition will play a critical role in further development of lobster enhancement and aquaculture projects.

Keywords   aquaculture; brain; docosahexaenoic acid; eicosapentaenoic acid; enhancement; nutrition

INTRODUCTION

Long chain polyunsaturated fatty acids (LC-PUFAs) are critical for healthy brain development and function. They make up 20% of the brain's dry weight, highlighting their potential importance for brain function, and have roles in membrane structure and cytokine regulation (Hibbeln et al. 2006). In humans, research suggests that abnormalities in fatty acid metabolism may play a part in a range of neurodevelopmental and psychiatric disorders (Hallahan & Garland 2005; Hibbeln et al. 2006). For example, several studies support a connection between dietary intake of omega-3 fatty acids and the prevalence of depressive illnesses (Horrobin 2002; Tanskanen et al. 2001). The omega-3 fatty acids EPA (eicosapentaenoic acid, $20{:}5\omega3$) and DHA (docosahexaenoic acid, $22{:}6\omega3$) are of particular importance (Masuda 2003). These molecules must be obtained from dietary sources because although there is some small amount of chain elongation from the omega-3 parent ALA (α-linolenic acid, $18{:}3\omega3$), this ability varies among animals, but typically represents only a small percentage of the total LC-PUFAs in the brain (Arts et al. 2001).

Marine food chains are among the richest sources of EPA and DHA (Arts et al. 2001). Previous studies on American lobster (*Homarus americanus*, Milne Edwards 1837) have found them to be both dietarily and mechanistically reliant on LC-PUFAs (Tlusty et al. 2005; Beltz et al. 2007). Tlusty et al. (2005) observed higher survival and growth rates in lobsters fed *Artemia* enriched with *Spirulina* or omega-3 fatty acids compared with those fed unenriched *Artemia*. This dietary supplementation was subsequently linked to a change in the level and temporal pattern of neuronal proliferation in the brains of lobsters (Beltz et al. 2007). Previously, lobsters fed unenriched *Spirulina* were observed to have a circadian pattern of neurogenesis, with a peak in neuronal proliferation at dusk, and the lowest level of proliferation at dawn (Goergen et al. 2002). However, when comparing lobsters fed unenriched versus *Spirulina*-enriched *Artemia*, those fed the *Spirulina*-enriched diet had more neuronal proliferations and did not exhibit the circadian pattern observed in lobsters fed unenriched *Artemia* (Beltz et al. 2007). The loss of the circadian pattern was attributed to a large increase in the basal level of neurogenesis in lobsters fed the *Spirulina*-enriched *Artemia*, whereas the level of neuronal proliferation at dusk increased only slightly (Beltz et al. 2007). The circadian phenomenon was therefore masked in lobsters fed the enriched diet.

One difficulty with the studies of Tlusty et al. (2005) and Beltz et al. (2007) was that the diets were commercially prepared, and the specific fatty acids included were not controlled. Beltz et al. (2007) postulated that it was either the ratio of omega-3 to omega-6 fatty acids, or the level of ALA that was primarily driving the differences in neurogenesis and survival. However, further discrimination of a causal relationship was not possible because ALA, EPA and DHA all varied among the diets (Beltz et al. 2007). Thus, we wanted to investigate dietary influences on neurogenesis in American lobster using laboratory prepared diets and controlling all forms of omega-3 fatty acids. Here we report on the level of neuronal proliferation in the brain of lobsters, and the growth and survival of lobsters fed a diet devoid of long chain omega-3 fatty acids compared with lobsters fed a diet high in EPA, DHA, or a 50:50 by volume mix of EPA and DHA oils.

Table 1 Proximate analysis (% basis) of the four diets used, the total number of fatty acid species in each diet, and their fatty acid composition expressed as % of the total fatty acid content (mg g^{-1} diet). Only fatty acids constituting 5% or more of the total fatty acids of any one diet are listed. The diets were a control made with flax oil (C), or were made with fish oil high in docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), or a 50:50 by volume mix of the latter two oils (EPA:DHA).

		Diet			
		C	DHA	EPA	EPA:DHA
	Moisture	43.30	51.37	46.54	49.13
	Protein	47.30	38.6	43.9	40.60
	Fat	5.39	4.31	4.68	4.57
	Fiber	0.69	1.89	0.54	0.55
	Ash	2.69	2.34	2.28	2.77
Fatty acid profile					
Total no.		16	25	27	28
Palmitic	16:0	13.26	11.77	11.36	11.57
Stearic	18:0	5.05	3.47	5.10	4.28
Oleic	18:1ω9	16.52	7.61	11.39	9.41
Linoleic	18:2ω6	29.43	24.72	25.27	25.17
a-Linolenic (ALA)	18:3ω3	31.88	3.57	3.50	3.32
Eicosapentaenoic (EPA)	20:5ω3	0.11	5.45	26.93	16.54
Docosahexaenoic (DHA)	22:6ω3	0.00	25.86	3.41	14.25
Sum of remaining		3.74	17.55	13.04	15.47
Σ ω3		32.00	39.79	36.37	37.91
Σ ω6		29.43	25.51	27.48	26.65
Total (mg g^{-1} diet)		3.21	2.32	2.54	2.49

MATERIALS AND METHODS

The diets used in this study were modified after a formulated research diet (Conklin 1995). Casein (vitamin free, 80.6% of dry ingredients) was the base item, with 5.6% soy lecithin, 2.8% kelp meal, 2.8% vitamin mix, 2.8% bone meal, 2.2% *Spirulina*, 1.7% mineral mix, and 1.3% astaxanthin (Naturrose™). 100g of this dry mix was mixed with 78 ml prepared gelatin (Knox™), with 5 ml of oil. The oil for the control (C) diet was flax oil (high in ALA) and, for the experimental diets, oil that was high in EPA (MEG-3 Ocean Nutrition, Nova Scotia, Canada, diet EPA), DHA (MEG-3 03/55 EE, Ocean Nutrition, Nova Scotia, Canada, diet DHA), or a 50:50 by volume mix of EPA and DHA (diet EPA/DHA). Fatty acids content of the diets were analysed by the New Jersey Feed Laboratory (Trenton, United States) using the Bligh & Dyer (1959) method, then methylation of the lipids (AOAC 2006, 969.33) and analysis by gas chromatography (AOAC 2006, 991.39) (Table 1).

This study was conducted as two sequential experiments, with the first being an assessment of levels of neurogenesis, and the second being the assessment of the survival and growth of the lobsters fed the four different diets. Lobsters for each experiment were hatched from the eggs of four females held at the New England Aquarium (Boston, United States). The resultant juveniles were held at 17°C in a fibreglass seawater tray (193 cm □ 18 cm □ 2 cm) that was part of a larger 1705 litre recirculation system (10% water renewal daily) with a light/dark cycle of 12:12 h. The lobsters were all held individually within the tray in 4.5 cm diameter mesh containers. For the neurogenesis experiment, 80 stage V lobsters were maintained on the flax oil diet. Upon moulting to stage VI, they were randomly divided onto one of the four dietary treatments. The lobsters were then held on the experimental diets for 0, 14, or 21 days.

At the end of the dietary periods, to assess the degree of neuronal proliferation, the substitute nucleoside 5-bromo-2'-deoxyuridine (BrdU) was used to label neuronal precursors in the S phase. Six to seven lobsters per diet-time period were incubated for 6 h at room temperature, in a 2 mg/ml BrdU/seawater bath, where the substituted nucleoside 5-bromo-2'-deoxyuridine (BrdU) was used to label brain cells in the S phase. No lobster mortality occurred during this step. Brains were dissected from the lobsters under cold lobster saline (462 mM NaCl, 16 mM KCl, 34 mM CaCl$_2$, 17 mM MgCl$_2$, 11 mM α-D(+)-glucose, and 10 mM HEPES buffer; pH 7.4)
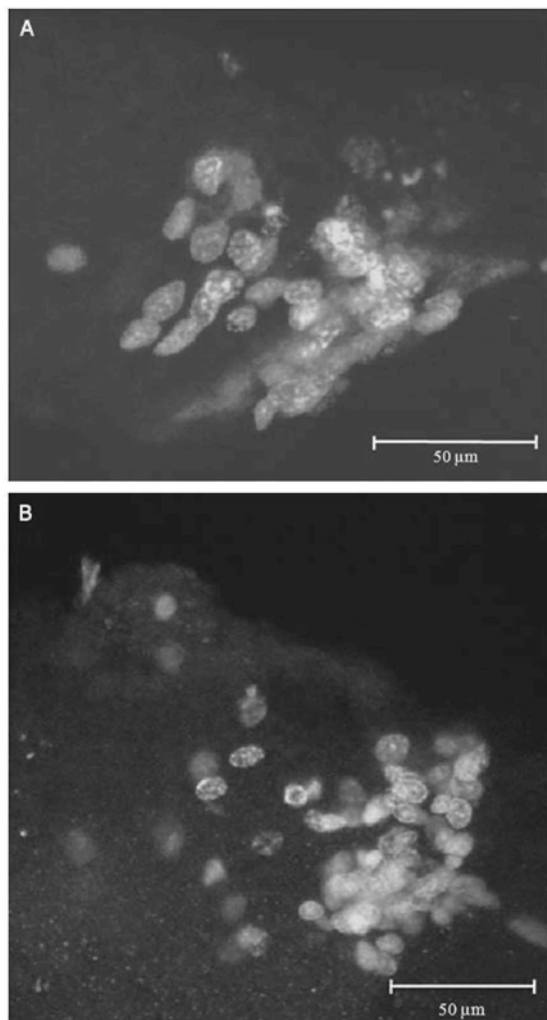

Fig. 1 Image of 1 μm thin section showing the BrdU-labelled nuclei in the lateral proliferation zones in the brain of moult stage VI lobsters. Cell clusters are from juvenile lobsters fed the **A**, control diet for 21 days, and **B**, eicosapentaenoic acid enriched diet for 21 days, after moulting in stage V.

and fixed for 12 h at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The brains were desheathed after 4–5 h to eliminate proliferating cells located in the perineurium. Preparations were rinsed in PB containing 0.3% Triton X-100 (PBTx) for 1.5 h and then incubated in 2 N HCl for 20 min. The brains were rinsed in several baths of PBTx, incubated for 150 min at room temperature in a mouse anti-BrdU primary antibody (1:50; Invitrogen Corp, Carlsbad, United States), rinsed again in a series of PBTx

baths, incubated overnight at 4°C in a goat anti-mouse antibody conjugated to the fluorophore Alexa 488 (1:50; Invitrogen Corp, Carlsbad, United States), and then rinsed for 1.5 h in PB in a series of baths before mounting in Gelmount™ (Biømeda, Foster City, United States) as whole mounts.

Specimens were viewed using a Leica TCS SP confocal microscope. Optical sections were taken at intervals of 1 μm and saved as three-dimensional stacks (Fig. 1). From these images, labelled cells in the lateral proliferation zone (LPZ) on each side of the lobster midbrain were counted for all the lobsters within test series. Only cells within the cluster were counted. They were defined by their 3D position within the cluster, by the spherical shape and by the presence of fluorescence within the cell nucleus, while omitting jagged or elongated cells that could be either migratory cells or bits of torn brain sheath. Fluorescence was observed by eye directly on the computer screen. A transparent film was attached to the screen and all spherical-shaped cells within the cluster with visual staining were drawn with a fine permanent marker on a transparent film to avoid multiple counts. The drawings were made while the laser went through all 1 μm layers of the clusters. It was assumed that no two cells were of exactly the same size and in the same position, so the cells that appeared to be of identical size and position in a series of consecutive layers were counted only once. The counts were done blind, selecting the mounted slides at random in the dark microscopy laboratory. Reading the label of the slide and labelling each image were done after the cluster was counted, and the true identification was established after all the samples were counted. Because a constant time was used for the BrdU immersion, and cells hold onto the stain after exiting the S phase, any variation in the number of cells stained by the BrdU are indicative of the total number of cells going through the S phase (Goergen et al. 2002).

The growth and survival experiment was conducted by selecting 120 sibling lobsters that had moulted to stage IV the day before. These were measured (carapace length, 0.1 mm) and weighed (0.01 g) and then randomly divided and placed on one of the four experimental diets. They were counted and fed daily for 52 days. Carapace length and lobster weight were recorded initially, after every moult, and at the end of the experiment.

Neurogenesis data were first analysed as a two-way repeated measures ANOVA (Quinn & Keough 2002) with the individual lobster being the repeated measure, and the factors being brain side and "diet-day treatment". There were a total of 9 diet-day treatments in this study, the four diets and two sample periods after the lobsters were switched onto the diets (14 and 21 days), as well as a initial control set (0 days on the diet). Pending no significant individual brain-side effect, and a significant diet-day treatment effect, the individual values for both brain sides were averaged, and the data were reanalysed as a two-way ANOVA of diet (C, EPA, DHA, and DHA/EPA) and day (14, 21). Survival data for all three experiments were analysed using a log-rank Kaplan-Meier (Gehan-Breslow statistic) survival analysis with multiple comparisons performed with the Holm-Sidak method (SigmaStat 3.1, Systat, Richmond, United States) (Quinn & Keough 2002). Dietary difference in the survival time was calculated using a one-way ANOVA on square-root transformed data with multiple comparisons performed post-hoc with the Tukey test (Quinn & Keough 2002). Percentage change in length and weight were calculated for the first moult on the experimental diet. Values were ln (x+1) transformed and then differences between diet treatments were evaluated using a one-way ANOVA.

RESULTS

Survival rates for lobsters on the DHA diets seemed to be higher between Day 10 and 40, but the diets did not significantly influence survival of lobsters. The shape of the survival curves was not statistically different across diets (Gehan-Breslow statistic = 1.89, d.f. = 3, $P > 0.50$, Fig. 2). A pooled survival value for all lobsters was 47.5%, which was similar between the lobsters of the growth and the neurogenesis experiments. The average day of mortality was significantly different (square-root transformed values, one-way ANOVA, $F_{3,59}$ = 3.44, $P < 0.05$, power$_{\alpha = 0.05}$ = 0.573), although no paired comparisons between diet treatments were statistically significant (Tukey test, $q < 3.58$, $P > 0.05$). The trend in day of mortality matched the visually apparent difference in the survival data (Fig. 2), with average time to mortality being lowest in the C and EPA treatments (mean ± 1 SE: 15.7 ± 3.6, 16.9 ± 4.2 days, respectively), and higher in the DHA and EPA:DHA treatments (27.9 ± 4.6, 30.9 ± 4.3 days, respectively).

Diet did not influence growth, as percentage increase in length or weight of the first moult on each diet ($F_{3,34}$ = 0.99, $P > 0.4$; $F_{3,34}$ = 0.62, $P > 0.6$, respectively, Table 2). The increase in length
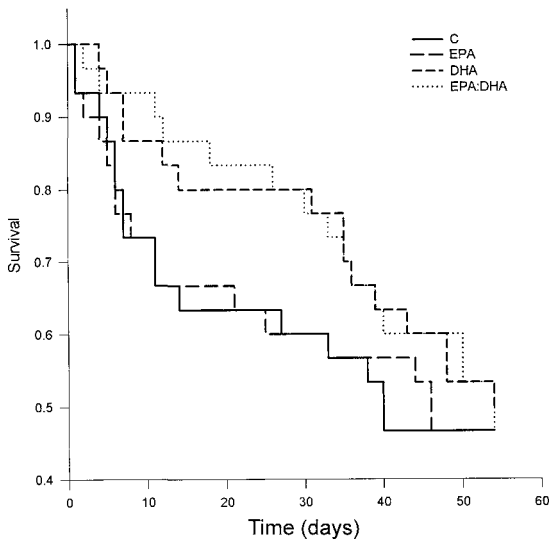
Fig. 2 Time of survival of cohorts of 30 lobsters fed one of four experimental diets differing in the source of fatty acids. The diets were a control made with flax oil (C), or were made with fish oil high in docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), or a 50:50 by volume mix of the latter two oils (EPA:DHA).
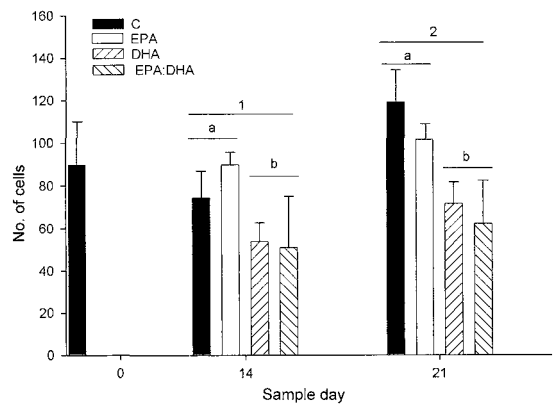
Fig. 3 Average number of BrdU+ nuclei (+1 SE, $n = 6$ per treatment) of neuronal precursor cells in hemibrains of American lobster, *Homarus americanus*, fed one of four experimental diets for 14 or 21 days. The diets were a control made with flax oil (C), or were made with fish oil high in docosahexaenoic acid (DHA), eicosapentae-noic acid (EPA), or a 50:50 by volume mix of the latter two oils (EPA:DHA). Letters refer to statistically similar main effects for diet, and numbers refer to statistically similar main effects for sample day (two-way ANOVA, $P < 0.05$).

Table 2 Initial, final, and the percentage change per day between the first and second moult of carapace length and weight of laboratory-reared lobsters fed one of four experimental diets. Values are average (upper) and 95% CI (lower). The diets were a control made with flax oil (C), or were made with fish oil high in docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), or a 50:50 by volume mix of the latter two oils (EPA:DHA).

Diet	n	Carapace length (mm)			Weight (g)		
		Initial	Final	Δ % day^{-1}	Initial	Final	Δ % day^{-1}
C	9	4.91	5.41	0.36	0.06	0.08	0.96
		0.34	0.40	0.19	0.02	0.02	0.64
EPA	8	4.98	5.35	0.29	0.06	0.07	0.73
		0.33	0.33	0.12	0.01	0.02	0.57
DHA	13	4.79	5.13	0.35	0.05	0.08	1.98
		0.16	0.21	0.34	0.01	0.04	0.17
EPA:DHA	8	5.19	5.51	0.27	0.07	0.08	0.72
		0.42	0.32	0.20	0.01	0.02	0.22

during the first moult averaged over all diets was $7.79 \pm 0.85\%$ which corresponds to an increase of $0.32 \pm 0.11\%$ day^{-1}. The increase in weight averaged $29.3 \pm 8.2\%$, or $1.10 \pm 0.23\%$ day^{-1}. All of the surviving lobsters fed the DHA and DHA:EPA diets respectively, moulted once, whereas 62.5% and 43.8% of the surviving lobsters fed C and EPA diets, respectively, moulted once. The number of days to moult on average ranged from 25 to 29. Considering

animals that moulted twice, only 73%, 56%, 31%, and 25% of the lobsters fed diets DHA, DHA:EPA, C, and EPA, respectively, moulted twice.

The diet-day treatment had a significant effect on the number of BrdU-labelled cells in the brain of American lobster (two-way repeated measures ANOVA, $F_{8,47} = 7.83, P < 0.001$), whereas the lobsters did not demonstrate any laterality in neurogenesis ($F_{1,33} = 0.49, P > 0.4$), and there was no diet-day □

side interaction ($F_{8,33} = 0.32, P > 0.9$). The subsequent diet □ day analysis of averaged values per individual indicated that both diet (two-way ANOVA, $F_{3,45} = 16.87, P < 0.001$) and day ($F_{1,45} = 16.84, P < 0.001$) exhibited significant treatment effects, with no statistically significant interaction ($F_{3,45} = 2.45, P > 0.08$, Fig. 3). Within the diet treatments, lobsters fed C and EPA diets had 50% more cells labelled with BrdU than those on the DHA and EPA:DHA diets (Fig. 3). More cells were also labelled 21 days after switching feeds as compared with 14 days. Within the control diet, the 0 day sample exhibited 89.7 ± 10.3 labelled cells, whereas 14-day and 21-day samples had 74.3 ± 17.1 and 119.1 ± 20.6 new cells, respectively.

DISCUSSION

Lobster brains, and the overall health and survival of the animal can be greatly affected by their diet (Tlusty et al. 2005, 2008; Beltz et al. 2007). The results in this study demonstrated clear experimental evidence that controlling the LC-PUFA content of the diet affected neurogenesis of early benthic phase American lobster. Although there were no statistically significant differences in growth or survival, the lobsters that were fed diets enhanced with the fatty acid DHA (diets DHA and EPA:DHA) tended to have slightly longer survival times, and moulted more often than those fed the C or EPA diets. It is difficult to directly compare these results to those of previous studies (Tlusty et al. 2005; Beltz et al. 2007) because of a change in the source of protein and lipids in the experimental diets. Casein was used within this set of experiments to eliminate extraneous LC-PUFA that can occur when using fish meal based diets. Furthermore, consistent with the design controlling fatty acid composition, the diets of the current experiments also had a narrower suite of fatty acids. Because of these differences, the lobsters in these experiments had equivalent survival, but lower growth than lobsters fed a more traditional *Artemia* diet (Tlusty et al. 2005). Because of the lower growth, it is likely these diets were not optimal for the lobsters, but because survival did not change, they likely were not deficient in any significant manner.

With the four diets considered here, those lobster fed the DHA and EPA:DHA diets had a slight increase in performance, including a greater incidence of moulting. However, these lobsters also exhibited fewer BrdU labelled cells in their brains than lobsters fed diets with lower amounts of this fatty acid. Currently, the benefit of increased or decreased neurogenesis is being debated in the literature (Scharfman & Hen 2007), as different studies can have equivocal results. Thus, the observation in the present study that DHA decreased neurogenesis is consistent with previous studies. Kawakita et al. (2006) observed a decrease in cell proliferation with DHA supplementation in cultures of embryonic rat neuronal stem cells. This result may be related to DHA promoting cell cycle exit and subsequent cell differentiation (Insua et al. 2003). Thus a key feature of lobster neurogenesis to be assessed is the relationship between cell proliferation, cell maturation, and cell longevity.

In addition, there are likely to be multiple factors regulating neurogenesis, and different factors may up- or down-regulate it depending on the initial state. The numbers of BrdU labelled cells increased between the two sampling periods. At these two sampling times, the lobsters were also at different moult stages. Thus, as the lobsters grew, their neurogenic capacity also changed. Long chain omega-3 fatty acids are required throughout the entire body, not just in the brain (Arts et al. 2001). Therefore, further research on neurogenesis in lobsters needs to account for the total animal's lipid budget, as well as the relationship between the lipid budget in the various stages of the moult cycle. DHA and EPA supplementation aids crustaceans in survival and growth, and in ameliorating stress responses associated with osmotic fluctuation, temperature, and water quality (reviewed in Sui et al. 2007). The low growth represented within this study may be indicative of a stress response in which the lobsters used dietary fatty acids differently than unstressed and/or fast-growing lobsters.

The studies of neurogenesis in rodents demonstrate that increased neurogenesis in the adult brain is correlated with improved abilities in learning and memory functions (Horrobin 2002). Increased exercise, enriched environment, and increased space all correlate with more neurogenesis (Masuda 2003). Therefore, before any conclusion regarding the down-regulation of neurogenesis by lobsters fed a diet high in DHA, the functional implications of the down-regulation need to be addressed. The first assessment would be if the brains cells of lobsters fed the DHA diets differentiate more quickly than of those on diets low in DHA. The second would be how the diets used here influence the neural functions of the lobsters. Except for a short-term experiment, correlating living conditions to cell proliferation and

investigating behaviour in lobsters (van der Meeren et al. 2007), to our knowledge there have been no long-term attempts to experimentally manipulate the total number of brain cells in a lobster, and then to assess any cognitive, behavioural, or sensory function. However, the results of the experiments presented here, as well as that of Beltz et al. (2007) indicate that lobster brains can be manipulated by diet, and it appears that the amount and species of omega-3 fatty acids influence neurogenesis. Although the exact mechanisms by which neurogenesis is affected needs further assessment and examination, the finding that neurogenesis is modified by diet should be examined by enhancement hatcheries as a factor to consider in the success of released animals. The ultimate goal of an enhancement programme is to produce animals that can survive to breed to recruit to the fishery upon release. Much of this survival is dependent upon appropriate behavioural functions including spatial learning, and memory regarding neighbours, traits that have been demonstrated (in organisms other than lobsters) to be influenced by the omega-3 composition of the diet (Masuda 2003). Overall, increasing the quality of diets is cost effective from a survival and growth perspective (Tlusty et al. 2005), and may have additional benefits for growing animals that can better function in a natural environment.

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