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Consumption of a high n-3 polyunsaturated fatty acid diet during gradual mild physiological stress in rats

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ABSTRACT

n-3 Polyunsaturated fatty acids (n-3PUFAs) may be beneficial for anxiety and depression under stressful conditions. Studies however, typically utilise physical or sudden physiological stress, while gradual physiological stress is also relevant to human conditions. Using deoxycorticosterone acetate (DOCA) administration to induce gradual physiological stress, this study investigated the impact of n-3PUFAs under gradual physiological stress in rats. Animals (aged 2 months) (N=8–12/group) received daily injections of DOCA or vehicle and were concurrently fed a high n-3PUFA or control diet for eight weeks. Behavioural measures were taken throughout. Behavioural tests and physiological measures were conducted after six and eight weeks respectively. DOCA administration decreased plasma renin, plasma proteins and relative adrenal weight, and increased water intake, relative kidney weight, and anxiety in the open field. These findings demonstrate disruptions to the renin–angiotensin–aldosterone system, a result of mild physiological stress, that also impact on anxiety behaviours. No effects of n-3PUFAs were found.

1. Introduction

N3 long chain polyunsaturated fatty acids (n-3PUFAs) have been suggested as a possible treatment for depressive and anxiety conditions [1–3]. Various studies demonstrate beneficial effects in both humans and animals, although evidence is mixed [1–3]. In rats specifically, several studies currently suggest an effect of n-3PUFAs on anxiety- and depressive-related behaviours e.g. [4,5], although not all studies demonstrate effects or consistent effects e.g. [6,7].

The discrepancies between studies have been suggested to result from differences in stress [3,8–10]. Acute physical stress, as a result of a brightly lit or noisy test situation, and chronic physical stress, e.g. through several weeks of restraint stress, have resulted in the demonstration of greater benefits of n-3PUFAs than less stressful situations or experiences e.g. [3,8,11]. Physiological stress, as a result of injury and/or inflammation has also resulted in enhanced impacts of n-3PUFAs [12–16]. Again, however, effects of n-3PUFAs are inconsistent, and conclusions difficult to draw. Trofimuik and Braszko [17], for example, demonstrate no effects of n-3PUFA supplementation in open field and elevated plus maze tests following 2 h/day restraint stress for 21 days, Song et al. [18] demonstrate inconsistent effects of n-3PUFAs in the open field test following injections to induce inflammation, and Plamandon and Roberge [19] demonstrate no effects of n-3PUFA supplementation in open field and elevated plus maze tests following forebrain ischaemic surgery. n-3PUFA supplementation even enhanced the deleterious effects of chronic mild stress for 6 weeks in the novelty suppression of feeding test (animals fed after a longer latency) as conducted by Vancassel et al. [10]. Chalon et al. [20] also report decreased activity in the open field test following n-3PUFA supplementation in combination with restraint stress, and Carrie et al. [5] and Nakashima et al. [21] report reversed effects on anxiety behaviours (reduced exploration of the open arms of the elevated plus maze).

Studies in animals are intended to model human conditions, but human depressive and anxiety conditions are known to result not just from sudden or high levels of stress, but also from more mild but long term stressors such as changes in lifestyle or gradual failures in health e.g. [22,23]. Effects of mild long term physical stressors have been investigated using the chronic mild stress model (with mixed results for n-3PUFAs), e.g. [10], but as far as we are aware, effects in response to gradual physiological changes or gradual physiological stress, have not been investigated. Gradual physiological stress compared to sudden physiological onslaughts may more closely reflect a human situation where regulatory

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systems are gradually disrupted by changes to lifestyle [24–27], and thus warrants investigation.

Given the previous evidence that n-3PUFAs may be dependent on the level of stress to influence depression- and anxiety-relevant behaviours, this study aimed to investigate the impact of n-3PUFAs on anxiety- and depressive behaviours in rats under gradual physiological stress. The renin–angiotensin–aldosterone system is a stress-responsive system in the body, known to be influenced by mild stressors in humans and animals [28–31]. Alterations to this system may serve as a form of mild physiological stress, allowing investigation of the interactions between mild gradual physiological stress and behavioural and physiological responses to n-3PUFAs. Deoxycorticosterone acetate (DOCA) is a mineralocorticoid agonist, known to disrupt the renin–angiotensin–aldosterone system. Previous studies demonstrate increased salt appetite and increased anxiety- and depressive-behaviours in rats following DOCA administration in the absence of excess salt for consumption [32]. In the present study, we tested the hypothesis that DOCA administration would produce both depressive- and anxiety-relevant behaviours in rats, and that a diet high in n-3PUFAs would prevent these behavioural alterations.

2. Methods

2.1. Animals

Forty-two male Sprague Dawley rats (Harlan Sprague, Indianapolis, IL), aged two months and weighing 200–250 g at the start of the study were used. Animals were housed individually in a standard animal facility on a 12 h light/dark cycle (lights on at 6 a.m.), temperature 20–22 °C, with ad-libitum access to food and water. Animals were allowed to adapt to the animal facility for 12 days before study procedures commenced. All study procedures were implemented only after adaptation to the animal facility (i.e. no procedures were undertaken during weaning, or prior to birth). Use of weaned animals was intended to more closely resemble a human situation where n-3PUFA provision can be adequate during pregnancy and weaning, as a result of maternal resources. All procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by The University of Iowa Animal Care and Use Committee.

2.2. General study design

Following adaptation to the animal facility, all rats were subjected to gradual physiological stress vs. no stress, and concurrently received a high n-3PUFA diet vs. a control diet (minimal n-3PUFAs), and effects on behaviour and physiology were measured. Gradual physiological stress was induced using administration of DOCA in the absence of excess salt for consumption. Animals were treated with DOCA and given ad-libitum access to allocated high n-3PUFA or control diets for a period of eight weeks. Effects of n-3PUFA supplementation for this duration have previously been demonstrated e.g. [4,33]. The combination of DOCA treatment and n-3PUFA provision resulted in the maintenance of four groups of animals throughout the study: (a) VC – vehicle treatment/control diet ($N=8$); (b) Vn3 – vehicle treatment/n-3PUFA diet ($N=8$); (c) DC – DOCA treatment/control diet ($N=12$); and (d) Dn3 – DOCA treatment/n-3PUFA diet ($N=12$).

2.3. DOCA administration

Animals were either treated with: DOCA (10 mg/kg suspended in 1 ml/kg safflower oil); or vehicle only (1 ml/kg safflower oil). Subcutaneous injections were given daily from 10.00–12.00 a.m. All animals were also handled daily following injections. Salt provision was maintained at standard levels (based on usual physiological requirements) for all animals [34] (Table 1).

2.4. n-3PUFA supplementation

Concurrently with DOCA administration, half of the animals in each group were fed either: an n-3PUFA supplemented diet (Research Diets, Inc., New Brunswick, US); or an n-3PUFA deficient diet (control) (Research Diets, Inc., New Brunswick, US). Diets were based on the AIN-93G growing rodents diet [34], and were identical in all aspects except fat source and n-3PUFA provision (Table 1). The levels of n-3PUFA provided have previously been found to impact on behavioural tests as used here e.g. [4]. Diets were stored in airtight containers and provided fresh every other day to prevent decomposition due to exposure to the atmosphere. N-3PUFA supplementation as opposed to deprivation was used to more closely resemble a human situation, and concurrent n-3PUFA provision was used to mimic a prevention as opposed to a treatment scenario.

Table 1
Composition of control and n-3PUFA diets.

Nutrients		Control	n-3PUFA
Protein (g/100 g food)		19.8	19.8
CHO (g/100 g food)		66.8	66.8
Fat (g/100 g food)		4.9	4.9
Fat source	Safflower oil (mg/100 g food)	25	25
	High oleic safflower oil (mg/100 g food)	25	
	Menhaden oil (mg/100 g food)		25
Fatty acid provision	Saturated (%fat)	8.1	18.4
	Monounsaturated (%fat)	45.3	17.7
	Polyunsaturated (%fat)	46.5	63.5
	Linoleic acid (%fat)	46.5	42.6
	Alpha-Linolenic acid (%fat)	0.1	0.9
	Eicosapentaenoic acid (%fat)	0	7.5
	Docosahexaenoic acid (%fat)	0	5.4
	Total n-3PUFAs (%fat)	0.1	17.0
	Total n-3PUFAs (g/100 g food)	0.003	0.82
	Total n-6PUFAs (g/100 g food)	2.32	2.08
Salt (g/100 g food)		2.59	2.59

2.5. Outcome measures

Effects of DOCA administration and n-3PUFAs were investigated using regular measures of water intake, food intake, body weight and sucrose intakes; measures of behaviour at six weeks in open field, elevated plus maze and forced swim tests; and physiological measures of plasma sodium, haematocrits, proteins, osmolality, plasma renin and organ weight (heart, lungs, kidneys, adrenals) in relation to body weight, at eight weeks.

2.5.1. Water intake, food intake, body weight

Food and water intakes were measured on random days (2–4 days/week) throughout the study by weighing food and water containers. Body weights were measured weekly using a standard animal balance.

2.5.2. Sucrose intake

Sucrose intake tests were used as an operational index of anhedonia [32,35]. Animals were presented with access to a 2% sucrose solution (2 g sucrose/100 g water) for one hour, from 8:00–9:00 a.m., and amount consumed in that hour was measured. Animals were adapted to the hourly exposure in days 3–7 of their initial adaptation to the animal facility, and baseline readings were taken on days 10 and 11 of adaptation. Sucrose intake testing was conducted weekly on two consecutive days as occurred at baseline. Access to food and water were not restricted during sucrose intake tests.

2.5.3. Open field

The open field test was used as an operational index of anxiety-relevant and exploratory behaviours [3]. Our open field consists of a 1 m × 1 m black plastic square surrounded by 38 cm high walls, the base of which is divided by markings into 9 equal squares of approx. 33 cm^2, eight around the edge of the square and one in the centre. Animals were placed in the centre of the central square at the start of the test and allowed to move freely for a 5 min period. Tests were conducted under bright lighting for half of the animals and dim lighting for half of the animals (evenly distributed between groups), from 9 a.m. to 2 p.m., and equipment was cleaned with 70% ethanol solution between animals. Bright lighting was used to enhance the stressful nature of the test [3]. Number of lines crossed (between squares), number of central lines crossed (to enter/leave the central square), time in the central square at the start, total time in the central square and number of rears were recorded. Line crosses were defined as a cross by all four paws, time in the central square was determined by the position of the two ears.

2.5.4. Elevated plus maze

The elevated plus maze was used as an operational index of anxiety-relevant and exploratory behaviours [3]. The elevated plus maze consists of 2 open arms (50 cm × 10 cm) and 2 closed arms (50 cm × 10 cm), separated by a 10 cm × 10 cm central area. The closed arms are surrounded by 48 cm high walls. The entire maze is made of black plastic and elevated 50 cm from the floor. Animals were placed in the central area facing an open arm, and allowed to move freely in the maze for 5 min. Tests were conducted under dim lighting from 9 a.m. to 2 p.m., three days after the open field test was conducted. Equipment was cleaned with 70% ethanol solution between animals. Number of entries into open and closed arms, and amount of time spent in open and closed arms were recorded. Entry into an arm was defined by presence in the arm of the two forepaws, and time spent in an arm was defined by location of the two ears. Bright and dim lighting was not used in the elevated plus maze to avoid confounding as a result of lighting condition in the open field test.

2.5.5. Forced swim test

The forced swim test was used as an operational index of behavioural despair [3]. The forced swim test uses a transparent plastic cylinder (15 cm diameter) filled to a depth of 30 cm with tap water of 23–25 °C, from which no escape is possible. Animals were placed in the water and allowed to move freely for 5 min. The forced swim test is traditionally conducted using 2 trials – one of 15 min followed by a further trial of 5 min 24 h later, to result in a learned helplessness in the animal and subsequent despair [3]. Only one trial was used to demonstrate effects of anhedonia, as opposed to effects of learning [36]. Only one trial has previously been demonstrated as sufficient to demonstrate anhedonia [36], and has been suggested as a more accurate reflection of emotion in the absence of learning [36]. The impact of n-3PUFAs on learning has been suggested e.g. [14,17]. Tests were conducted three days after the elevated plus maze was completed, under dim lighting from 9 a.m. to 3 p.m. Water was changed after every animal. Time spent actively trying to escape from the start to the first period of immobility, and total time spent actively trying to escape were recorded. Active escape movements included swimming (active forward movement on the surface of the water), diving (active forward movement under the water) and climbing (active movement with the forepaws towards the walls of the cylinder). Immobility was defined as minimal movement sufficient only to keep the animal from drowning. Swimming, diving, and climbing were summed to provide one index of active escape activity; and immobility was used as the operational index of behavioural despair, according to previous tests of validity [3].

For the open field, elevated plus maze, and the forced swim tests, animals were tested in a random order within blocks of 10–12 animals with roughly even numbers from all experimental groups per block, and even numbers under bright and dim conditions during the open field test. All animals undertook the open field test (either in bright or dim lighting), then the elevated plus maze test, then the forced swim test. Each animal only took each test once. All tests were conducted as recommended [3,36]. All measures were coded independently by two researchers using video recordings taken from directly above. Discrepancies between researchers of more than 5% were recoded until agreement within 5% was reached.

2.5.6. Plasma measures

At the end of the study, trunk blood was collected following decapitation and placed into a tube containing an anti-coagulant. Blood was centrifuged at 4 °C at 3500 rpm for 15 min to obtain plasma, and stored at −80 °C. Plasma osmolality was determined using a freezing-point osmometer (Model 5004, Precision Systems, Natick, MA); plasma sodium concentrations were measured in a Na^+/K^+ analyser with ion-specific electrodes (Nova 1. Nova Biomedical, Walthan, MA). Plasma proteins (total proteins) were determined by refractometer (National Instrument, Baltimore, MD). Plasma renin activity was measured using commercially available kits. All procedures were conducted according to previously published methodology [28].

2.5.7. Organ weight

Following decapitation, heart, lungs, kidneys and adrenals were harvested and weighed in relation to body weight.

2.6. Analyses

Data were analysed for differences dependent on DOCA administration (groups V vs. D), diet (groups C vs. n3) and interactions

between these, using repeated measures ANOVA or univariate ANOVA as appropriate. Data from the open field test, elevated plus maze test and forced swim test were analysed by MANOVA (one per test). Analyses on data from the open field test also investigated differences dependent on lighting condition (bright vs. dim) and all associated interactions as above. Parametric tests were used following assessment of the data to ensure that assumptions were not violated.

3. Results

3.1. Water intakes, food intake and body weight

DOCA-treated animals consumed significantly more water than vehicle animals over time ($F(11,418)=3.15$, $p<0.01$), but no effects were found dependent on diet (largest $F(11, 363)=0.68$, $p=0.62$). Significant increases in body weight ($F(7,266)=808.76$, $p<0.01$), and decreases in food intake ($F(11,363)=41.92$, $p<0.01$) were found over time, but no differences were found dependent on DOCA administration or diet (largest $F(11,363)=1.36$, $p=0.19$). Water intakes, food intakes and body weight are provided in Fig. 1.

3.2. Sucrose intakes

Sucrose intakes also increased over time ($F(7,266)=12.04$, $p<0.01$), but no effects of DOCA treatment or n-3PUFAs were found (largest $F(7,266)=1.48$, $p=0.17$). Sucrose intakes are displayed in Fig. 2.

3.3. Open field, elevated plus maze, forced swim tests

Results from all three tests are displayed in Figs. 3–5. Significant effects were found only in the open field test. In brightly lit conditions, rats performed significantly less crosses of the central lines ($F(1,34)=4.30$, $p=0.05$), line crosses in total ($F(1,34)=10.27$, $p<0.01$), and significantly less rears than in dimly lit conditions ($F(1,34)=11.03$, $p<0.01$). DOCA-treated rats also performed significantly less line crosses in total compared to vehicle-treated rats, regardless of lighting ($F(1,34)=4.79$, $p=0.04$). No effects of n-3PUFA treatment were found (largest $F(1,34)=2.28$, $p=0.14$). There were no significant effects in the elevated plus maze test (largest $F(1,41)=1.17$, $p=0.29$), or the forced swim test (largest $F(1,41)=1.67$, $p=0.20$), but there was a trend towards a difference between DOCA- and vehicle-treated animals across both measures of activity in the forced swim test ($F(2,60)=2.37$, $p=0.09$), where DOCA-treated animals spent less time in escape activity than vehicle-treated animals.

3.4. Plasma renin, sodium, haematocrits, proteins, osmolality and organ weight

Plasma and organ weight measures are given in Table 2. Plasma renin and plasma proteins were significantly lower in DOCA-treated animals compared to vehicle ($F(1,21)=4.84$, $p=0.04$; $F(1,19)=32.34$, $p<0.01$ respectively). Plasma sodium, haematocrits and osmolality, did not differ between DOCA treated and untreated animals (largest $F(1,23)=1.54$, $p=0.23$). Relative weights of hearts and lungs also did not differ (largest $F(1,24)=0.19$, $p=0.67$), but weights of kidneys were significantly higher and weights of adrenals were significantly lower in relation to

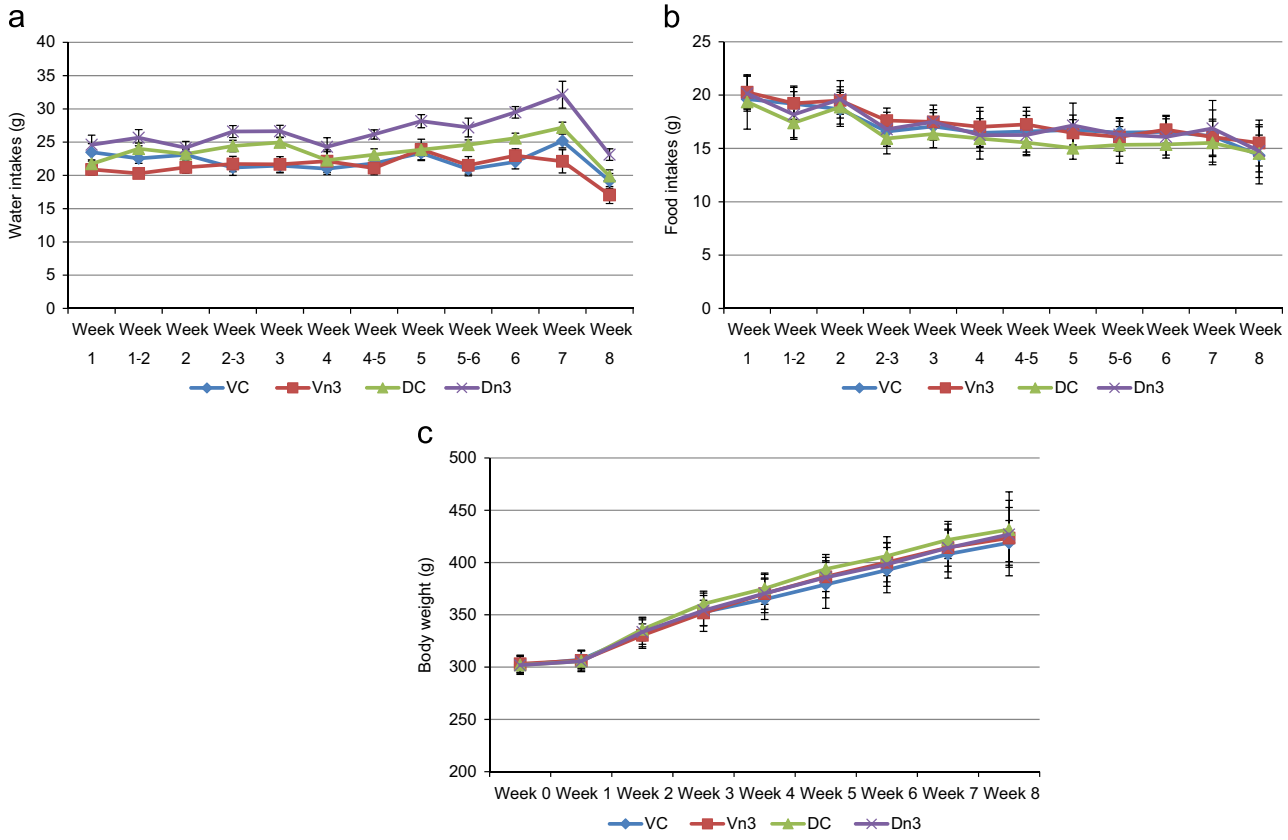

Fig. 1. Mean ± standard error water intakes/day (a), food intakes/day (b) and body weight (c) each week in VC (vehicle injections/control diet, $N=8$, diamond marker); Vn3 (vehicle injections/n-3PUFA diet, $N=8$, square marker); DC (DOCA injections/control diet, $N=12$, triangular marker); and Dn3 (DOCA injections/n-3PUFA diet, $N=12$, cross marker).

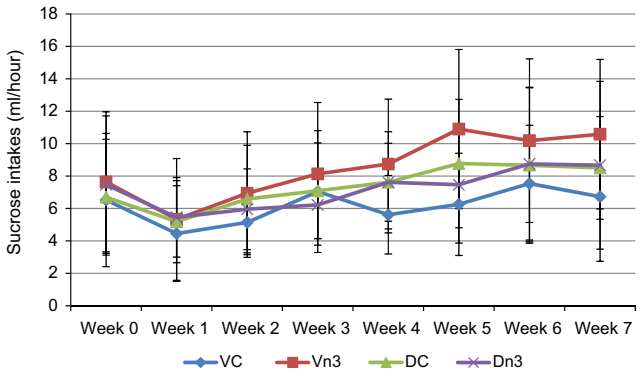

Fig. 2. Sucrose solution consumed (ml/h) per week (mean ± standard error) in VC (vehicle injections/control diet, $N=8$, diamond marker); Vn3 (vehicle injections/n-3PUFA diet, $N=8$, square marker); DC (DOCA injections/control diet, $N=12$, triangular marker); and Dn3 (DOCA injections/n-3PUFA diet, $N=12$, cross marker).

body weight in DOCA-treated animals compared to vehicle (smallest $F(1,27)=4.56$, $p=0.04$). No differences were found dependent on diet (largest $F(1,23)=2.03$, $p=0.17$).

4. Discussion

This study investigated the impact of a high n-3PUFA diet on anxiety- and depressive behaviours in rats, during concurrent DOCA administration. Results indicate that: (1) chronic DOCA administration (in the absence of excess salt) increased water intake, increased the relative weight of kidneys, decreased plasma renin and plasma proteins, and decreased the relative weight of the adrenals; (2) chronic DOCA administration increased anxiety-related behaviours; and (3) n-3PUFAs did not impact on any of the measures employed.

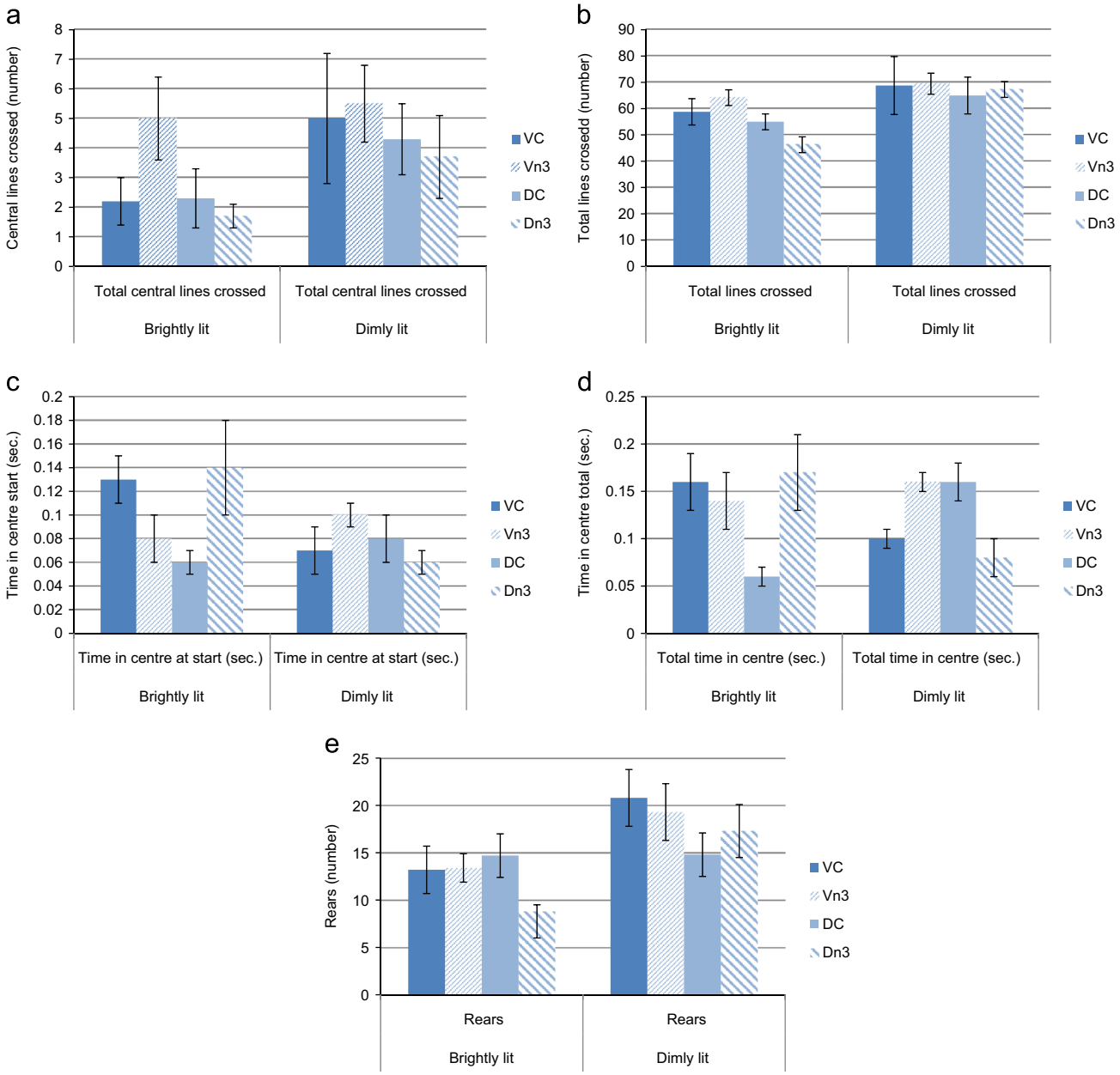

Fig. 3. Outcomes (mean ± standard error) from the Open Field Test for VC (vehicle injections/control diet, $N=8$); Vn3 (vehicle injections/n-3PUFAdiet, $N=8$); DC (DOCA injections/control diet, $N=12$); and Dn3 (DOCA injections/n-3PUFA diet, $N=12$) groups.

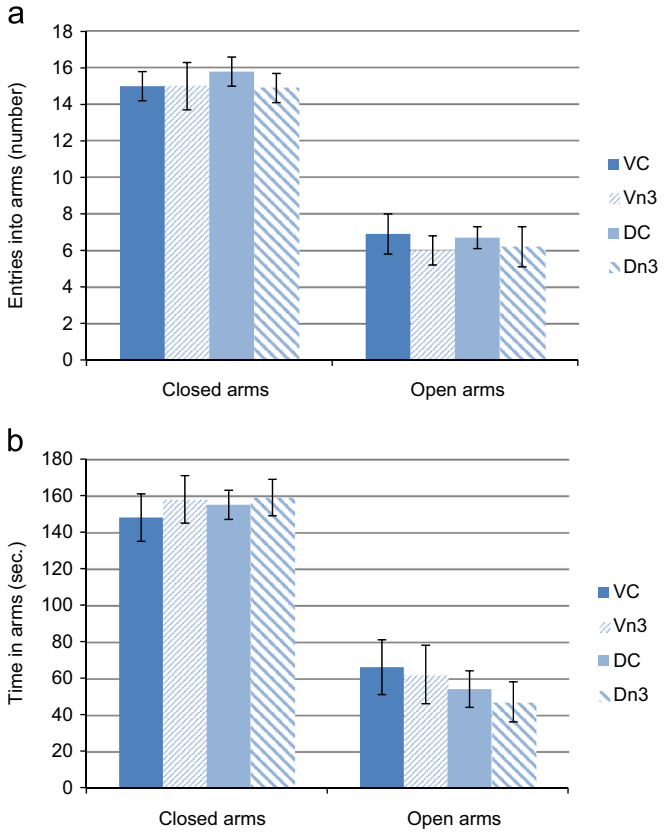

Fig. 4. Outcomes (mean ± standard error) from the Elevated Plus Maze Test for VC (vehicle injections/control diet, $N=8$); Vn3 (vehicle injections/n-3PUFAdiet, $N=8$); DC (DOCA injections/control diet, $N=12$); and Dn3 (DOCA injections/n-3PUFA diet, $N=12$) groups.

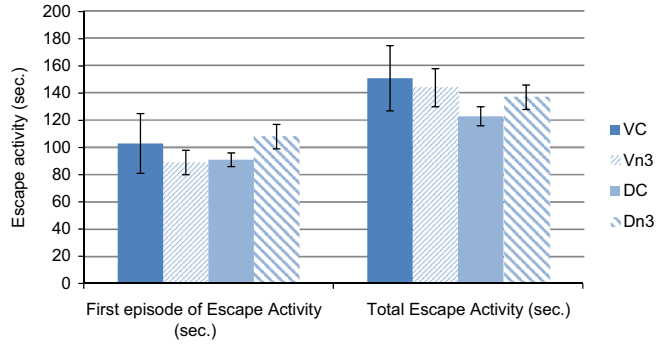

Fig. 5. Outcomes (mean ± standard error) from the Forced Swim Test for VC (vehicle injections/control diet, $N=8$); Vn3 (vehicle injections/n-3PUFAdiet, $N=8$); DC (DOCA injections/control diet, $N=12$); and Dn3 (DOCA injections/n-3PUFA diet, $N=12$) groups.

Increases in water intake, increases in the relative weight of the kidneys, and decreases in plasma renin and plasma proteins in response to DOCA administration in the absence of excess salt are consistent with previous studies. These effects have been reported elsewhere as a result of DOCA and aldosterone administration [26,27], and demonstrate disruptions to the renin–angiotensin–aldosterone system [29–31]. The present findings contribute to these previous results, demonstrating that the administration of DOCA for 8 weeks is sufficient to disrupt the renin–angiotensin–aldosterone system. These disruptions mimic those that may be achieved in humans as a result of mild stressors, such as daily stressors, lifestyle changes or failures in health [24–27].

In addition to physiological disruptions to the renin–angiotensin–aldosterone system, DOCA administration resulted in anxiety-relevant behaviours in the open field test, and in trends towards depression-related behaviours in the forced swim test. Brightly lit conditions also resulted in increased anxiety-related behaviours. Anxiety-related effects as a result of a bright test situation have previously been demonstrated, and demonstrate the validity of the tests used here [3]. Effects in relation to DOCA administration suggest that disruptions to the renin–angiotensin–aldosterone system via gradual DOCA administration influence anxiety- and depression-relevant behaviours in rats. Behavioural alterations are likely to have resulted from a gradual adaption to the physiological disruption caused by the DOCA administration [26,27], and in combination with the physiological disruptions in hormone and organ weights, suggest that DOCA administration may be a useful protocol for inducing and investigating mild gradual physiological stress. The present findings are consistent with previous data indicating that mild stressors contribute to depressive and particularly anxiety disorders in humans [22,23]. We are unclear why anxiety-relevant behaviours were not observed in the elevated plus maze test. We can only suggest that following our protocol, where the open field test field was conducted first and in stressful conditions for half the animals, the open field test was more anxiety-provoking than the elevated plus maze test.

Contrary to our hypotheses, no effects of n-3PUFAs were found in the present study. These findings suggest no effects of n-3PUFAs on either behavioural or physiological measures under conditions of mild gradual physiological stress. The current data are consistent with previous studies indicating an absence of effects of n-3PUFAs under non-stressful conditions. Many studies using stress and no-stress comparisons report effects of n-3PUFAs under stressful conditions, but not in situations of no stress. Song et al. demonstrate reduced anxiety-related effects in the open field [14] and elevated plus maze tests [14,15] following a high n-3PUFA diet under IL-1β administration, but not following saline administration. Kozak et al. [12] report reduced behavioural effects of induced inflammation in n-3PUFA treated animals, but no effects of n-3PUFAs in control animals (no induced inflammation), and Harauma & Moriguchi [8] report beneficial effects of n-3PUFA supplementation in response to isolation but not in animals housed in groups. Studies employing only stressful conditions also tend to report beneficial effects of n-3PUFAs. Ferraz et al. [11], for example, report reduced effects of restraint stress following n-3PUFA supplementation. Studies employing only no-stress conditions typically report no effects of n-3PUFAs. Without deliberately increasing stress, Carlezon et al. [4], Coluccia et al. [37] and Ferraz et al. [7] report no effects of n-3PUFA supplementation on activity in the open field, Belzung et al. [6] found no effects in ambulatory activity or anxiety-related activity in the elevated plus maze, and Shalbudina et al. [38] found no effects in the forced swim test. Both Carlezon et al. [4] and Ferraz et al. [7] however, do report effects of n-3PUFAs in the forced swim test, and others report these same effects e.g. [33,36]. The forced swim test, however, is stressful for animals in itself [3], and thus may demonstrate effects more under acute stress than under no stress. Other studies though have also found beneficial effects of n-3PUFAs in other tests under stress-free conditions e.g. [36], but these studies are relatively few. A lack of effects of n-3PUFAs in our study thus may again reflect the mild and gradual nature of our physiological stressor, and provide further support for the suggestion that n-3PUFAs may only impact on depression and anxiety in situations of high stress.

Stress and n-3PUFAs are most plausibly linked via the presence of n-3PUFAs in the cell membrane to result in the production of anti-inflammatory eicosanoids [39,40]. Evidence of effects of n-3PUFAs in situations of high stress, but not in situations of mild

Table 2
Outcomes (mean ± standard deviation) from plasma and organ analyses for all four groups of animals.

Group[a]		VC	Vn3	DC	Dn3
Plasma	Sodium (mmol/l)	146.4 ± 5.2	141.0 ± 10.5	144.2 ± 3.2	142.2 ± 9.1
	Haematocrits (%)	40.8 ± 5.1	41.1 ± 10.9	43.0 ± 4.0	37.7 ± 4.1
	Plasma proteins(g/dl)[b]	**6.2 ± 0.5**	**6.9 ± 1.1**	**6.2 ± 0.2**	**5.9 ± 0.4**
	Osmolality (mmol/l)	312.4 ± 4.8)	320.0 ± 8.4	313.2 ± 11.8	309.2 ± 12.0
	Renin (ng/ml)[b]	**6.5 ± 1.3**	**4.4 ± 4.1**	**0.7 ± 0.9**	**0.7 ± 1.1**
Organ/body weight	Heart ($\times 10^3$)	3.1 ± 0.2	3.1 ± 0.3	3.1 ± 0.2	3.1 ± 0.1
	Lungs ($\times 10^3$)	3.9 ± 0.2	4.5 ± 0.5	4.2 ± 0.2	4.3 ± 0.8
	Kidneys ($\times 10^3$)[b]	**6.3 ± 0.6**	**6.5 ± 0.7**	**7.1 ± 0.6**	**6.8 ± 0.2**
	Adrenals ($\times 10^3$)[b]	**0.2 ± 0.04**	**0.2 ± 0.1**	**0.1 ± 0.04**	**0.2 ± 0.1**

[a] VC – vehicle injections/control diet ($N=5$), Vn3 – vehicle injections/n-3PUFA diet ($N=7$), DC – DOCA injections/control diet ($N=10$), Dn3 – DOCA injections/n-3PUFA diet ($N=6$).
[b] Significant differences within the row between DOCA treated and vehicle treated animals.

stress (as used here), further suggests that n-3PUFAs may only be of impact/importance during major or sudden disruptions to inflammatory systems, or at a major or sudden onset of inflammatory conditions. Of relevance to human conditions, n-3PUFAs then may be of benefit where depression and/or anxiety have resulted from major or sudden disruptions, such as bereavement or significant life changes, but may be less beneficial in situations of prolonged low levels of disruption, such as mild daily stressors or gradual lifestyle changes.

The suggestion of limited effects of n-3PUFAs in the absence of high stress mirrors current thinking in work with humans. Several studies conducted on humans now report limited or no effects of n-3PUFAs in individuals with either no or mild symptoms of anxiety, depression or stress e.g. [41], although possible effects are suggested in those suffering from more severe symptoms e.g. see [2]. Further work on the distinction between those conditions that may be affected and those conditions that are unlikely to be affected by n-3PUFAs is clearly required.

Limited methodological reasons may explain our lack of effects of n-3PUFAs, although these are unlikely. Previous studies suggest that the dose and duration of supplementation used here was adequate for effects to be demonstrated e.g. [4,7,33], and the number of animals and number of measures used also suggest likely demonstration of effects, should such effects exist [33]. The age of the animals at the start of the experimental procedures, the absence of procedures earlier than this (e.g. during weaning, pregnancy, or in dams), and the duration of exposure to the diet may also have limited our chances of finding effects, but similar procedures have produced effects in other studies e.g. [4,33]. Effects as a result of bright lighting demonstrate the validity of the procedures used [3], and strengthen the findings in relation to DOCA administration and n-3PUFAs. Greater exposure and earlier exposure to n-3PUFAs, however may also be of interest. Our protocol was intended to most closely reflect possible human conditions as a result of mild physiological stress where earlier intervention may have been unlikely and exposure is unlikely to be sustained in the absence of demonstrable effects [42], but we appreciate the value of early intervention and longer exposure for demonstrating more mechanistic effects. Our one trial forced swim test may have reduced the chances of demonstrating effects here, but only one trial has previously demonstrated effects of anhedonia [36], and has been suggested as a more accurate reflection of emotion in the absence of learning [36]. The absence of measures of plasma or tissue levels of n-3PUFAs is a limitation, however previous studies have demonstrated that daily dietary consumption of n-3PUFAs as provided is sufficient to increase brain n-3PUFA levels. Chalon et al. [20] used a diet where n-3PUFAs provided 5% of 6 g fats (compared to our 17% of 5 g fats) and demonstrate increased n3PUFA concentrations in several brain regions (although the diets in this study were started in dams two weeks prior to mating). Huang et al. [33] used a feeding protocol similar to ours, where n3PUFAs provided 36% of 4 g fats to demonstrate significant differences in brain and RBC membrane n-3PUFA levels and effects in behavioural tests, and the behavioural effects reported by Carlezon [4] were also obtained using a dose of n-3PUFAs only slightly higher (0.72 mg/kg body weight/d) than that used in our study (0.57 g/kg body weight/d).

In conclusion, this is the first study of which we are aware, that investigates the impact of n-3PUFAs under mild gradual physiological stress. Our findings suggest that DOCA administration in the absence of excess salt results in significant disruptions of the renin–angiotensin–aldosterone system, as well as anxiety behaviours in rats. Our findings also demonstrate no effects of n-3PUFAs under conditions of mild stress. Further studies will contribute to our understanding of the potential benefits of n-3PUFAs under different levels of stress, and can therefore aid in the development of novel treatments for individuals with depressive or anxiety disorders.

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