Flaxseed oil as omega-3 polyunsaturated fatty acid source modulates cortisol concentrations and social dominance in male and female guinea pigs

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**A B S T R A C T**

Flaxseed oil is an excellent source of the essential omega-3 polyunsaturated fatty acid (PUFA) alpha-linolenic acid (ALA). Omega-3 PUFAs are important neuronal components and can counteract aggressive, depressive, and anxiety-like behavior, reduce glucocorticoid (e.g. cortisol) concentrations under chronic stress but also increase acute glucocorticoid responses. As glucocorticoids per se and glucocorticoid responsiveness can modulate the establishment of dominance hierarchies, we investigated if flaxseed oil high in ALA can promote social dominance through effects on glucocorticoid concentrations. Two male and two female groups of domestic guinea pigs (n = 9 per group) were maintained on a control or a 5% (w/w) flaxseed oil diet for four weeks. Social behaviors, hierarchy indices, locomotion, and saliva cortisol concentrations were determined during basal group housing conditions and stressful social confrontations with unfamiliar individuals of the other groups. Flaxseed groups had increased basal cortisol concentrations and showed no cortisol increase during social confrontations. Cortisol concentrations in control groups significantly increased during social confrontations. However, flaxseed males became dominant irrespective of cortisol concentrations. In females, the opposite was detected, namely a higher dominant status in control compared to flaxseed females. Open-field- and dark-light-tests for anxiety-like behavior revealed no pronounced differences, but flaxseed males showed the highest locomotor activity. Flaxseed oil as an ALA source sex-specifically promoted social dominance irrespective of cortisol concentrations and responses. The underlying neuronal mechanisms remain to be determined, but a sex-specific energetic advantage may have accounted for this effect.

1. Introduction

Nutritional conditions are an important determinant of neurophysiological and mental health by affecting the structure and function of the central nervous system (Bourre, 2006). Certain plant-based oils such as flaxseed (Linum usitatissimum) oil have attracted attention in this context because of high concentrations of the dietary essential omega-3 polyunsaturated fatty acid (n-3 PUFA) alpha-linolenic acid (ALA, 18:3 n-3) (Santos et al., 2020; Singh et al., 2011). Dietary ALA represents the precursor for the long-chain PUFAs eicosapentaenoic acid (20:5 n-3) and docosahexaenoic acid (22:6 n-3) (Sprecher, 2000). Long-chain PUFAs are important components of neuronal membrane phospholipids and determine the membranes physical and physiological properties (Hishikawa et al., 2017). Although the conversion efficiency seems to be low, adequate ALA intake through flaxseed oil significantly contributed to neuronal long-chain n-3 PUFA concentrations in rats (Barcelo-Coblijn et al., 2005).

Research in the last decades revealed a major involvement of dietary n-3 PUFAs in preventing mood disorders such as depression, anxiety, and aggressiveness (Chen and Su, 2013; DeMar Jr et al., 2006; Ferraz et al., 2011; Gajos and Beaver, 2016). These neurobehavioral influences are strongly related to altered neurotransmitter signaling in the
GABAergic, serotonergic, or dopaminergic system (Chalon, 2006; Chen and Su, 2013). This also includes effects on the regulation of the hypothalamic-pituitary-adrenal (HPA)-axis and its control of glucocorticoid (e.g. cortisol) concentrations (Chen and Su, 2013; Larrieu et al., 2016). Glucocorticoids modulate physiological and behavioral responses to energetically demanding and stressful challenges (Romero et al., 2009; Sapolsky et al., 2000), but long-term exposure to increased glucocorticoid concentrations can impair mood and behavior (Lehmann et al., 2013). In this context, n-3 PUFAs have been shown to counteract increased glucocorticoid concentrations and related depressive and anxiety-like behavior in chronically stressed rats and mice (Ferraz et al., 2011; Larrieu et al., 2014).

In addition to anti-stress effects of n-3 PUFAs, studies in sheep supplemented with flaxseed oil revealed a stronger increase in glucocorticoid concentrations in response to acute physical stressors and energetic demands (Caroprese et al., 2012, 2014). This corresponds to in vitro findings showing that PUFAs possibly stimulate acute HPA-axis activation (Katoh et al., 2004). From a behavioral perspective, these influences are of major interest because HPA-axis and glucocorticoid responsiveness play a significant role in behavioral responses to social challenges. Studies in rats, for example, revealed that individuals with a high glucocorticoid responsiveness to stress display more aggressive and anxiety-like behavior (Walker et al., 2017). Glucocorticoids per se can modulate the establishment of dominance hierarchies, possibly indicating effects on memory regarding social relationships (Timmer and Sandl, 2010). Recent findings in guinea pigs also suggest that acute glucocorticoid responses to social confrontations are important to achieve a dominant position (Nemeth et al., 2016a, 2021), a process that was positively affected by dietary PUFA supplementation (Nemeth et al., 2016a).

Social dominance has rarely been addressed with regard to the physiological and behavioral influences of dietary n-3 PUFAs. By modulating HPA-axis functions, glucocorticoid concentrations, and social behavior, n-3 PUFAs may ultimately affect social dominance. We therefore investigated the effects of flaxseed oil high in ALA on cortisol concentrations, social behavior, social dominance, locomotion, and anxiety-like behavior in male and female domestic guinea pigs. The analyses were performed in established social groups and during social confrontations between unfamiliar individuals, representing a stressful social challenge due to competition for dominant positions (Nemeth et al., 2016a; Wallner and Dittami, 2003). We hypothesized that flaxseed oil supplementation results in a stronger cortisol increase during social confrontations, thereby promoting social dominance, but counteracts anxiety-like behavior. Effects on social dominance were mainly expected in males due to stronger pronounced social hierarchies compared to females.

2. Methods

2.1. Ethical statement

Animal keeping and experimentation were performed in accordance with EU Directive 2010/63/EU for animal experiments and national laws. The study has been examined and approved by the board on animal ethics and experimentation of the Faculty of Life Sciences of the University of Vienna (Nr. 2018-026) and the Austrian Federal Ministry of Education, Science and Research (BMBWF-66.006/0010-V/3b/2019).

2.2. Guinea pigs and housing conditions

All domestic guinea pigs (18 males and 18 females aged 18 ± 4 months) used in this study were bred at the department's animal care facility. They were descendants of a heterogeneous and multicolored stock, which was established in 2012 and is regularly mixed with new guinea pigs from local breeders. Prior to the experiment, guinea pigs were randomly allocated to single-sexed groups of nine individuals, resulting in two male and two female groups. Each group was housed in equally equipped and environmentally enriched enclosures (2.4 m × 2 m) built of laminated chipboard; the floor was covered with bedding material. The daily provided feed consisted of guinea pig pellets (ssniff V2233, ssniff Spezialdiäten GmbH, Soest, Germany) and hay. Each male group received 315 g pellets per day (35 g per individual) and each female group 225 g (25 g per individual), which was determined beforehand to reach the sex-specific requirements. Additionally, each group was provided with 100 g hay per day, which has a low nutritional value, but should promote tooth abrasion and support intestinal activity. Water was available ad libitum. Ambient conditions, including a 12 h/12 h light-dark cycle with lights on at 07:00 a.m., a temperature of 20 ± 2 °C, and a humidity of 50 ± 5%, were maintained throughout the experiment.

2.3. Experimental procedures

2.3.1. Experimental diets

For the experiment, the guinea pig pellets of one randomly chosen male and female group was enriched with 5% (w/w) flaxseed oil high in the n-3 PUFA ALA. Feed mixtures were freshly prepared each week. Due to individual higher caloric density, the daily provided flaxseed oil-enriched pellets were adjusted to 283.5 g (31.5 g per individual) for the male flaxseed group and to 202.5 g (22.5 g per individual) for the female flaxseed group. The pellets of the other groups remained unchanged (control groups); for nutrient and fatty acid composition of the diets see Table 1. This should ensure isocaloric food amounts for male and female groups, respectively. All guinea pigs immediately accepted the diets and no rejections were observed. Nevertheless, they were regularly weighed to ensure adequate food intake. Throughout the experiment described in the following, males were heavier than females, but no differences in body mass were detected between diet groups (control males: 1083 ± 65 g; flaxseed males: 1073 ± 49 g; control females: 944 ± 26 g; flaxseed females: 944 ± 48 g). Feeding was always carried out between 09:00 a.m. and 12:00 p.m. The food was always recognized to be fully consumed by 07:00 a.m. on the following day. The groups were maintained on the respective diets for four weeks before behavioral tests were performed, but the feeding regime was maintained throughout testing.

2.3.2. Group housing

After four weeks on the feeding regimes, the social groups were video recorded for 30 min from the bird’s-eye view on three consecutive days always starting at 09:00 a.m. This was done to later analyze each guinea pig’s behavior in their social groups (group housing condition, GH). All

| Fatty acids [%] | Control diet | Flaxseed diet |
|----------------|--------------|---------------|
| 14:0           | 0.01         | 0.02          |
| 16:0           | 0.54         | 0.94          |
| 18:0           | 0.09         | 0.16          |
| 20:0           | 0.01         | 0.02          |
| 16:1           | 0.02         | 0.07          |
| 18:1 (n-9)     | 0.52         | 1.62          |
| 18:2 (n-6)     | 1.63         | 2.29          |
| 18:3 (n-3)     | 0.30         | 2.78          |

Table 1 Nutrient and fatty acid composition of the experimental diets.
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3.8 females (control male
flaxseed male
2016a). On the first day after group housing measurements, single-sex
competition for dominant positions during this setup (Nemeth et al.,
physiological and behavioral stress responses in guinea pigs because of
environmental conditions should have resulted in an increased number
2.3.3. Social confrontations
Social confrontations with unfamiliar individuals reliably elicit
physiological and behavioral stress responses in guinea pigs because of
competition for dominant positions during this setup (Nemeth et al.,
2016a). On the first day after group housing measurements, single-sex
social confrontations (SC 1) were performed involving two males or
two females (control male + flaxseed male or control female + flaxseed
female). Three days afterwards, mixed-sex social confrontations (SC 2)
were performed involving two males and two females (control male +
flaxseed male + control female + flaxseed female). Single-sex confronta-
tion pairs remained the same for mixed-sex social confrontations. The
idea of performing two social confrontations under different social
environmental conditions should have resulted in an increased number
of behavioral interactions during SC 2, thereby enabling to determine
the consistency of dominance relationships established during SC 1. In
order to ensure that individuals with a similar hierarchy rank were
confronted with each other, video recordings of the group housing
condition were preliminarily analyzed by applying ad libitum sampling
(Martin and Bateson, 2007): focusing on initiated and received agonistic
behaviors, a preliminary hierarchy index was calculated for each indi-
vidual by the proportion of initiated agonistic behavior on total
agonistic interactions an individual was involved in. Based on this
analysis, only high, medium, or low ranking guinea pigs were con-
fronted with each other (the hierarchy index was analyzed in more
detail afterwards, see Section 2.4.1).
Social confrontations were performed at 09:00 a.m. in nine parallel
setups of squared arenas (1 m²) built of laminated fiberboard; the floor
was covered with bedding material. The parallel setups ensured that the
whole social groups were subjected to social confrontations at the same
time. The respective individuals were transferred to the arenas and
video recorded from the bird’s-eye view for 30 min to later analyze their
behavior. Afterwards, all individuals were weighed and saliva samples
were collected for analyses of saliva cortisol concentrations. These
concentrations were used to determine acute physiological stress re-
sponses to social confrontations. After these measurements, all guinea
pigs were returned to their social groups.

2.3.4. Open-field and dark-light tests
One day after mixed-sex social confrontations a standard open-field
test was performed and on the next day a dark-light test was carried out.
Both tests are used to measure exploratory behavior and anxiety in
guinea pigs (Zipser et al., 2013); the implementation of the tests and the
analyzed parameters were slightly modified. For both tests, each indi-
vidual was singly transferred to a squared arena (1 m²) built of lami-
nated fiberboard; the floor was covered with bedding material. For the
dark-light test, a single round shelter (diameter 25 cm, height 20 cm)
with a 12 cm × 12 cm entry was placed in a corner with the entry
pointing to the center of the arena. In both tests, an individual was
placed into a corner of the arena, which was the corner opposite to the
shelter in the dark-light test. Both tests were performed for 10 min and
were video recorded from the bird’s-eye view. All tests were performed
between 09:00 a.m. and 12:00 a.m. Bedding material was exchanged
and the whole arena was cleaned after each test.

2.4. Analyses

2.4.1. Behavioral analyses
Video recordings were analyzed using the open source Behavioral
Observation Research Interactive Software BORIS (Friard and Gamba,
2016).

Frequencies (total number per 30 min) of initiated and received so-
cial and sexual behaviors were analyzed for group housing and social
confrontations by applying continuous recording (Martin and Bateson,
2007). Behaviors were categorized in sociopositive behavior (nose-nose,
social grooming, ano-genital sniffing), aggressive behavior (head-thrust,
attack, chase, bite, tooth chatter, stand threat, fight), dominance
behavior (rumba rumble, mounting, displacement [retreat of the
opponent]), and (in case of mixed-sex social confrontations) sexual
behavior (ano-genital sniffing, chin-rump-follow, rumba rumble,
mounting/copulation); behaviors mainly followed previous descriptions
by Rood (1972). Additionally, locomotion was analyzed as total dura-
tion per 30 min. Rumba rumble and mounting are male-specific sexual
behaviors, but they are also shown by dominant males towards sub-
ordinates (Machatschke et al., 2008) and were either coded as dominant
or sexual behavior depending on the sex of the receiver. For group
housing, behavioral analyses were performed separately for each of the
three consecutive days and were then averaged per individual.

Initiated and received dominance behavior were used to calculate a
hierarchy index for each individual by dividing total initiated dominant
behavior by the sum of total initiated and received dominant behavior.
This follows previous attempts to measure social dominance in guinea
pigs (Nemeth et al., 2020), which is usually based on the proportion of
won fights (Coulon, 1975; Zipser et al., 2013). However, several guinea
pigs (especially females) did not show any dominance behavior during
social confrontations resulting in a low sample size for this analysis. We
therefore performed a linear mixed model in R (R Core Team, 2020)
using package “nlme” (Pinheiro et al., 2020) to determine correlations
between initiated and received sociopositive, aggressive, and dominant
behaviors shown during agonistic encounters and the hierarchy index.
This analysis revealed significant positive effects of all initiated behav-
iors and significant negative effects of all received behaviors (R² =
0.48). We therefore re-calculated the hierarchy index based on all be-
haviors (total initiated behavior / (total initiated behavior + total
received behavior)) shown during agonistic encounters, which highly
correlated with the initial hierarchy index (R² = 0.75) and, moreover,
yielded values for each individual and social condition. This calculation,
based on total initiated and received behaviors, relied on a higher
number of behaviors and provided an adequate measure of an in-
dividual’s hierarchy index. During mixed-sex social confrontations,
the hierarchy index was calculated based on interactions within sexes only.

Behaviors analyzed in the open-field test were the total time of
locomotion and total time in the center (at least 25 cm distance from the
arena’s walls). For the dark-light test, the total time of locomotion, the
time until the first full entrance into the shelter, and total time in the
shelter were recorded.

2.4.2. Saliva sampling and cortisol analyses
Saliva samples were collected using standard cotton buds, which
were inserted into the mouth and moved around gently for 1 min. Cotton
buds were then centrifuged (10,000 rpm, 5 min) and pure saliva was
stored at −20 °C. Saliva cortisol concentrations were analyzed by
colorimetric immunoassay (IBL Hamburg GmbH; Hamburg,
Germany). Samples were diluted 1:10 and analyzed following the
manufacturer’s instructions. All analyses were run in duplicates. Intra
and inter coefficients of variance of control samples were 5.7% and
9.1%, respectively. The biological validity of saliva cortisol analyses in
guinea pigs was demonstrated previously (Nemeth et al., 2016b).
For further analyses, the cortisol response for each individual and social
confrontation was calculated based on the natural logarithm of social
confrontation cortisol divided by group housing cortisol. This yielded
positive values in case of increased cortisol concentrations and negative values in case of decreased cortisol concentrations during the respective social confrontation, standardized on the logarithmic scale.

2.4.3. Statistical analyses
Statistics were performed using R 4.0.3 (R Core Team, 2020). All behavioral and physiological data collected during group housing and social confrontations were analyzed by linear mixed models (LMMs) using package “lme4” (Pinheiro et al., 2020). LMMs included diet (control, flaxseed), sex (male, females), condition (GH: group housing, SC 1: single-sex social confrontations, SC 2: mixed-sex social confrontations), and their interaction as fixed effects and individual ID as random effect to correct for repeated measurements. LMMs were chosen because of repeated measurements using the same individuals for each condition. This represents a more restrictive analysis (e.g. compared to separate analyses for each condition) and should yield more robust effects. Moreover, LMMs allowed post-hoc repeated measurement analyses, which was important to determine cortisol responses to social confrontations based on basal group housing measurements. With regard to behavioral analyses, this was not analyzed, because behavioral responses to social confrontations are indicated rather by the frequency of displayed behaviors per se than by changes compared to group housing, especially when considering the different preconditions for the social setups (e.g. effects of different number of males and/or females as interaction partners on behavioral frequencies).

To analyze the relationships between saliva cortisol concentrations and the hierarchy index, two further analysis steps were performed: 1) the hierarchy index was included as covariate in the LMM-analysis of neither an individual’s body mass (F\( ^{1,32} = 9.817, p = 0.004; \) fitted model R\(^2 = 0.32\)). Post-hoc analyses revealed more locomotion during group housing (GH) in flaxseed males compared to control males (t = 3.257, p = 0.011, d = 1.63) (Fig. 1A). Flaxseed males also showed more locomotion than flaxseed females during both social confrontations (SC 1: t = 5.173, p < 0.001, d = 2.59; SC 2: t = 3.268, p = 0.010, d = 1.63; all other pairwise comparisons p > 0.077) (Fig. 1A).

Sociopositive behavior was significantly affected by the interaction of sex and condition (F\( _{2,66} = 6.882, p = 0.002; \) fitted model R\(^2 = 0.35\)). Females in general showed more sociopositive behavior compared to males during GH (t \(= 3.902, p < 0.001, d = 1.34\) and during SC 1 (t = 2.750, p = 0.001, d = 0.94) (Fig. 1B). Analyzing only intrasexual interactions during SC 2 revealed more sociopositive behavior in flaxseed males compared to control males (t = 2.103, p = 0.043, d = 1.05; all other pairwise comparisons p > 0.092). Sociopositive behavior during SC 2 was mainly directed towards the other sex (overall: 75 ± 3%).

Aggressive behavior was significantly affected by interactions of diet and condition (F\( _{2,64} = 4.347, p = 0.017 \) as well as sex and condition (F\( _{2,64} = 7.771, p = 0.001; \) full model R\(^2 = 0.30\)). Males in general showed more aggressive behavior than females during GH (t = 2.416, p = 0.022, d = 0.83) and SC 1 (t = 2.209, p = 0.035, d = 0.76) (Fig. 1C). During SC 2, control females showed significantly more aggressive behavior than control males (t = 4.004, p = 0.001, d = 2.00) and flaxseed females (t = 4.633, p < 0.001, d = 2.32; all other pairwise comparisons p > 0.564) (Fig. 1C). Intrasexual interactions during SC 2 similarly revealed a difference in aggressive behavior only in females (males: t = 1.845, p = 0.092, d = 0.75). A high proportion of aggressive behavior in all groups, but especially in females, was directed towards the other sex (control males: 48 ± 10%, flaxseed males: 73 ± 8%, control females: 87 ± 4%, flaxseed females: 70 ± 13%).

Dominance behavior was significantly affected by the interactions of diet and sex (F\( _{1,32} = 17.393, p < 0.001\), diet and condition (F\( _{2,66} = 3.956, p = 0.024\), and sex and condition (F\( _{2,66} = 6.306, p = 0.003\); fitted model R\(^2 = 0.42\)). Males in general showed more dominance behavior compared to females during GH (t = 2.417, p = 0.022, d = 0.81) and SC 1 (t = 2.529, p < 0.001, d = 1.75) (Fig. 1D). Dominance behavior was also higher in flaxseed males compared to control males during SC 1 (t = 3.302, p = 0.010, d = 1.40) and higher in control females compared to flaxseed females during SC 2 (t = 4.321, p < 0.001, d = 1.84; all other pairwise comparisons p > 0.304) (Fig. 1D).

A significant three-way interaction of diet, sex, and condition was detected with regard to the hierarchy index (F\( _{2,64} = 6.247, p = 0.003; \) full model R\(^2 = 0.39\)). During both social confrontations, flaxseed males had significantly higher hierarchy indices (SC 1: p = 0.011, d = 2.62; SC 2: p = 0.036, d = 2.12) (Fig. 1E). In females, the opposite was detected during SC 2, namely a higher hierarchy index in control females (p = 0.003, d = 2.99) (Fig. 1E).

The frequency of sexual behavior shown towards females during SC 2 did not differ between control and flaxseed males (t = 0.236, p = 0.817, d = 0.12; control: 17.1 ± 7.7, flaxseed: 20.1 ± 10.1). Received sexual behavior also did not differ between control and flaxseed females (t = 1.128, p = 0.284, d = 0.56; control: 23.4 ± 9.6, flaxseed: 11.7 ± 4.0).

3.2. Saliva cortisol concentrations
A significant interaction effect of diet and condition was detected on 3 sum of squares. The criterion of significance was set at p ≤ 0.05. All values represent means and standard errors of the mean.

3. Results

3.1. Behavior
The duration of locomotion was significantly affected by the interaction of sex and the social condition (F\( _{2,66} = 7.189, p = 0.002\)) and the interaction of diet and sex (F\( _{1,32} = 9.817, p = 0.004; \) fitted model R\(^2 = 0.32\)). Post-hoc analyses revealed more locomotion during group housing (GH) in flaxseed males compared to control males (t = 3.257, p = 0.011, d = 1.63) (Fig. 1A). Flaxseed males also showed more locomotion than flaxseed females during both social confrontations (SC 1: t = 5.173, p < 0.001, d = 2.59; SC 2: t = 3.268, p = 0.010, d = 1.63; all other pairwise comparisons p > 0.077) (Fig. 1A).
saliva cortisol concentrations ($F_{2,66} = 7.458, p = 0.001$), while an interaction of sex and condition missed the criterion of significance marginally ($F_{2,66} = 2.745, p = 0.072$; fitted model $R^2 = 0.29$). During GH, cortisol concentrations were in general higher in flaxseed-supplemented groups compared to control groups ($t = 4.113, p < 0.001, d = 1.41$) and higher in males than in females ($t = 2.259, p = 0.031, d = 0.77$) (Fig. 2). No differences were detected during social confrontations (always $p > 0.237$).

Compared to GH, both control groups showed increased saliva cortisol concentrations after SC 1 (males: $t = 2.564, p = 0.025, d = 1.28$; females: $t = 3.514, p = 0.002, d = 1.76$) and SC 2 (males: $t = 3.273, p = 0.003, d = 1.64$; females: $t = 5.942, p < 0.001, d = 2.97$) (Fig. 2). In contrast, cortisol concentrations in both flaxseed groups showed no differences after SC 1 and SC 2 compared to GH (always $p > 0.121$).

3.3. Relationships between hierarchy index and saliva cortisol concentrations

Including the hierarchy index as covariate in the analysis of saliva cortisol concentrations yielded significant interactions of the index with diet and condition ($F_{2,58} = 6.893, p = 0.002$) and with diet and sex ($F_{1,58} = 4.205, p = 0.045$; fitted model $R^2 = 0.45$). This analysis revealed a negative relationship between the hierarchy index and cortisol concentrations during GH in control groups ($p < 0.001$, effect size $= 4.48$) and a positive relationship only in control males during SC 1 ($p = 0.013$, effect size $= 2.47$) (Fig. 3A). No further effects were detected (always $p > 0.166$) (Fig. 3B). In general, higher cortisol concentrations during GH resulted in lower cortisol responses during social confrontations ($F_{1,33} = 150.355, p < 0.001$, effect size $= 12.26$) (Fig. 3C).
3.4. Open-field and dark-light performance

The duration of locomotion in the open-field test revealed a significant interaction of diet and sex ($F_{1,32} = 10.622, p = 0.003$; full model $R^2 = 0.41$). Flaxseed males showed more locomotion compared to flaxseed females ($t = 5.208, p < 0.001, d = 2.60$; all other pairwise comparisons $p > 0.174$) (Fig. 4A). The time in the center of the open-field only showed a tendency to be higher in males compared to females ($F_{1,34} = 3.565, p = 0.068, d = 0.65$; fitted model $R^2 = 0.07$) (Fig. 4B).

In the dark-light test, the total duration of locomotion was significantly higher in males than in females ($F_{1,33} = 8.814, p = 0.006, d = 1.02$; fitted model $R^2 = 0.20$) (Fig. 4C). The time until the first full entrance into the shelter was generally lower in females than in males ($F_{1,30} = 11.688, p = 0.002, d = 1.17$; fitted model $R^2 = 0.26$; control males: $92 \pm 36$ s, flaxseed males: $155 \pm 68$ s, control females: $20 \pm 10$ s, flaxseed females: $24 \pm 6$ s) and the total time in the shelter was higher in females compared to males ($F_{1,34} = 13.106, p = 0.001, d = 1.24$; fitted model $R^2 = 0.26$) (Fig. 4D).

4. Discussion

The results presented here suggest sex-specific positive effects of cortisol responses and of flaxseed oil high in n-3 PUFAs on social dominance. However, in contrast to our hypothesis and to our previous findings (see Nemeth et al., 2016a), these effects were not associated. Unexpectedly, flaxseed oil supplementation resulted in increased cortisol concentrations during group housing compared to control groups. This decreased relative cortisol responses during social
confrontations. Elevated glucocorticoid concentrations due to the circadian rhythm or chronic stress can decrease glucocorticoid responses to acute stressors (Harris et al., 2012; Rich and Romero, 2005; Romero et al., 2009). Artificially induced stress before a first social encounter can also result in subdominance and induce memory formation for the established social relationship (Cordero and Sandi, 2007). Corresponding to these effects, saliva cortisol responses were inversely related to basal cortisol concentrations and positively affected individual hierarchy indices during social confrontations in control males. Importantly, however, a sex-specific contribution of flaxseed oil to social dominance was independent of cortisol concentrations and responsiveness but apparently hindered control males from becoming dominant. In contrast, a negative effect of flaxseed oil supplementation was detected in females, finally resulting in a subdominant status. Anxiety-like behaviors in the open-field and dark-light tests determined by the time spent in the center and the shelter, respectively, were not affected by flaxseed oil supplementation. Nevertheless, a higher locomotor activity in flaxseed males throughout and differences in the social organization could explain the detected effects on cortisol concentrations and, furthermore, the sex-specific dominance outcomes during social confrontations.

Analysis of social behaviors during group housing revealed no differences between diet groups, which could have explained the increased cortisol concentrations in flaxseed groups. Generally higher basal cortisol concentrations in males compared to females were presumably related to more aggressive and dominance behavior in order to maintain social hierarchies. Although the establishment of social hierarchies and attaining the dominant position is related to high levels of aggressiveness in rodents (Mikics et al., 2007; Wallner and Dittami, 2003), no differences in aggressive behavior between male diet groups were detected during social confrontations as well. Reduced aggressive behaviors due to n-3 PUFAs (see e.g., Gajos and Beaver, 2016) and a stimulation of aggressiveness by increased cortisol responsiveness (see e.g., Walker et al., 2017) could have resulted in equal frequencies of aggressive behavior. Social dominance of flaxseed males was therefore not based on aggressive behavior but on their assertiveness, as reflected in increased dominance behavior. In contrast, the final dominant status of control females was indeed related to increased aggressiveness, which could have also been facilitated by their cortisol responses.

In addition to a sex-specific effect of flaxseed oil on social dominance, flaxseed males showed highest locomotor activity throughout the study. Findings in rats revealed a male-specific relationship between low n-3 long-chain PUFA content of the brain and decreased locomotor activity (Levant et al., 2006). The authors concluded that this sex difference reflected a lower sensitivity of neuronal functions to altered n-3 PUFA levels in females. Accordingly, increased ALA availability could have sex-specifically promoted locomotor activity in the present study. A higher activity can increase glucocorticoid concentrations and circulating glucose concentrations as acute energy source (Contarteze et al., 2008). Findings in guinea pigs further suggest that a relatively high percentage of dietary ALA is beta-oxidized (Fu and Sinclair, 2000). Moreover, lower plasma n-3 PUFA levels in males after PUFA-supplementation may indicate higher oxidation rates compared to females (Nemeth et al., 2016). An increased dietary ALA intake could have therefore sex-specifically promoted energy turnover. This was perhaps reflected in highest locomotor activity and cortisol concentrations in flaxseed males. If such sex-specific effects may have also contributed to social rank attainment remains to be determined.

The group housing situation further revealed a negative relationship between cortisol concentrations and hierarchy indices in control groups, while no such effect was detected in flaxseed groups. Increased basal cortisol concentrations in flaxseed groups are therefore mainly attributable to more dominant individuals. Previous studies in guinea pigs revealed no relation between cortisol concentrations and dominance positions (Sachser et al., 1998; Zipser et al., 2013). The group housing situation in control groups therefore represents an exceptional finding. A possible explanation could be a different social organization in control and flaxseed groups. In this context, it was recently suggested that PUFA supplementation can modulate social hierarchies and rank-specifically affect cortisol and testosterone concentrations (Nemeth et al., 2020). Interestingly, flaxseed oil has been shown to increase testosterone concentrations through steroidogenesis in the bovine species Bos frontalis (Perumal et al., 2019) and in rams (Li et al., 2017). Although testosterone was not investigated in this study, it can decrease cortisol responsiveness to stress in guinea pigs (Lürzel et al., 2010). A different social organization in flaxseed groups related to altered endocrine functions could have affected basal cortisol concentrations and responses to social confrontations. Despite the fact that only one experimental group per sex and diet was investigated here, the outlined considerations call for examining the basal group housing conditions to better understand cortisol and behavioral responses to social confrontations. Nevertheless, social group-specific influences cannot be excluded and therefore the social group housing condition represents a clear limitation with regard to the results. Future studies should therefore be carried out in multiple social groups per dietary treatment and include higher sample sizes in order to draw reliable conclusions regarding effects on the social organization.

Irrespective of the above-mentioned possible explanations (e.g., increased energy turnover and locomotion, different social organization and limitations), flaxseed oil supplementation clearly increased basal saliva cortisol concentrations, thereby decreasing cortisol responses during social confrontations. This is in strong contrast to previous findings for flaxseed supplementation, which revealed decreased glucocorticoid concentrations under long-term stress (Meneses et al., 2019) but even increased glucocorticoid responses to acute stressors (Caroprese et al., 2014). While long-term effects of n-3 PUFAs on HPA-
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Appendix A. Model statistics for full and fitted models

| Response            | Predictor               | Statistics                  | Full model | Fitted model |
|---------------------|-------------------------|-----------------------------|------------|--------------|
|                     |                         |                             | df        | F            | p            | df | F            | p            |
| Locomotion          | Diet                    | 1,32                        | 0.081      | 0.778        | 1,32         | 0.039        | 0.844 |
|                     | Sex                     | 1,32                        | 2.465      | 0.126        | 1,32         | 5.713        | 0.023 |
|                     | Condition               | 2,64                        | 1,300      | 0.280        | 2,66         | 2,157        | 0.124 |
|                     | Diet: sex               | 1,32                        | 2.038      | 0.163        | 1,32         | 9.817        | 0.004 |
|                     | Diet: condition         | 2,64                        | 2.892      | 0.063        | 2,66         | 2,527        | 0.088 |
|                     | Sex: condition          | 2,64                        | 3.620      | 0.032        | 2,66         | 7.189        | 0.002 |
|                     | Diet: sex: condition    | 2,64                        | 0.959      | 0.389        |              |              |      |
|                     | Diet                    | 1,32                        | 0.078      | 0.782        |              |              |      |
|                     | Sex                     | 1,32                        | 8.520      | 0.006        | 1,34         | 15.224       | <0.001 |
|                     | Condition               | 2,64                        | 2.793      | 0.069        | 2,68         | 2.511        | 0.089 |
|                     | Diet: sex               | 1,32                        | 0.054      | 0.818        |              |              |      |
|                     | Diet: condition         | 2,64                        | 2.032      | 0.139        |              |              |      |
|                     | Sex: condition          | 2,64                        | 1.307      | 0.278        | 2,68         | 6.882        | 0.002 |
| Sociopositive       | Diet: sex: condition    | 2,64                        | 1.087      | 0.343        |              |              |      |
|                     | Diet                    | 1,32                        | 0.657      | 0.424        |              |              |      |
|                     | Sex                     | 1,32                        | 0.930      | 0.342        |              |              |      |
| Aggressive          | Condition               | 2,64                        | 6.885      | 0.002        |              |              |      |

(continued on next page)
### Response Predictor Statistics

|                        | Full model | Fitted model |
|------------------------|------------|--------------|
|                        | df | F  | p   | df | F  | p   |
| Diet: sex             | 1,32 | 1.108 | 0.301 |     |     |     |
| Diet: condition       | 2,64 | 4.347 | 0.017 |     |     |     |
| Sex: condition        | 2,64 | 7.771 | 0.001 |     |     |     |
| Diet: sex: condition  | 2,64 | 2.518 | 0.089 |     |     |     |
| Diet                  | 1,32 | 1.140 | 0.294 | 1,32 | 5.833 | 0.022 |
| Sex                   | 1,32 | 0.759 | 0.390 | 1,32 | 0.007 | 0.935 |
| Condition             | 2,64 | 13.890 | <0.001 | 2,66 | 14.266 | <0.001 |
| Diet: sex             | 1,32 | 1.442 | 0.239 | 1,32 | 17.393 | <0.001 |
| Diet: condition       | 2,64 | 4.340 | 0.017 | 2,66 | 3.956 | 0.024 |
| Sex: condition        | 2,64 | 5.380 | 0.007 | 2,66 | 6.306 | 0.003 |

#### Dominant

|                        | Diet: sex: condition | Diet | Sex | Condition |
|------------------------|-----------------------|------|-----|-----------|
| Diet                   | 2,64                 | 1,32 | 0.328 | 0.571 |
| Sex                    | 1,32                 | 0.189 | 0.666 |
| Condition              | 2,64                 | 5.441 | 0.007 |
| Diet: sex              | 1,32                 | 0.416 | 0.524 |
| Diet: condition        | 2,64                 | 7.139 | 0.002 |
| Sex: condition         | 2,64                 | 3.460 | 0.037 |

#### Hierarchy index

|                        | Diet: sex: condition | Diet | Sex | Condition |
|------------------------|-----------------------|------|-----|-----------|
| Diet                   | 2,64                 | 1,32 | 8.629 | 0.006 |
| Sex                    | 1,32                 | 0.759 | 0.390 |
| Condition              | 2,64                 | 13.079 | <0.001 |
| Diet: sex              | 1,32                 | 3.101 | 0.089 |
| Diet: condition        | 2,64                 | 2.341 | 0.042 |
| Sex: condition         | 2,64                 | 1.596 | 0.211 |

#### Saliva cortisol 1

|                        | Diet: sex: condition | Diet | Sex | Condition |
|------------------------|-----------------------|------|-----|-----------|
| Diet                   | 2,64                 | 1,32 | 5.127 | 0.003 |
| Sex                    | 1,32                 | 0.711 | 0.405 |
| Condition              | 2,64                 | 11.834 | 0.001 |
| Diet: sex              | 1,32                 | 3.101 | 0.089 |
| Diet: condition        | 2,64                 | 2.783 | 0.071 |
| Sex: condition         | 2,64                 | 1.881 | 0.176 |
| Condition              | 2,64                 | 3.642 | 0.033 |
| Diet: sex: condition   | 2,64                 | 0.392 | 0.678 |
| Diet: sex: condition   | 2,64                 | 3.458 | 0.068 |
| Diet: condition: HI    | 2,64                 | 6.662 | 0.003 |

#### Saliva cortisol 2

|                        | Diet: sex: condition | Diet | Sex | Condition |
|------------------------|-----------------------|------|-----|-----------|
| Diet                   | 2,54                 | 1,32 | 0.469 | 0.498 |
| Sex                    | 1,32                 | 8.108 | 0.008 |
| Condition              | 2,54                 | 8.842 | 0.015 |
| Diet: sex              | 1,32                 | 3.101 | 0.088 |
| Diet: condition        | 2,54                 | 2.783 | 0.071 |
| Sex: condition         | 2,54                 | 1.881 | 0.176 |
| Condition              | 2,54                 | 3.642 | 0.033 |
| Diet: sex: condition   | 2,54                 | 0.392 | 0.678 |
| Diet: sex: condition   | 2,54                 | 3.458 | 0.068 |
| Diet: condition: HI    | 2,54                 | 6.662 | 0.003 |

#### Hierarchy index SC

|                        | Diet: sex: condition | Diet | Sex | Condition |
|------------------------|-----------------------|------|-----|-----------|
| Diet                   | 2,54                 | 1,32 | 0.469 | 0.498 |
| Sex                    | 1,32                 | 8.108 | 0.008 |
| Condition              | 2,54                 | 8.842 | 0.015 |
| Diet: sex              | 1,32                 | 3.101 | 0.088 |
| Diet: condition        | 2,54                 | 2.783 | 0.071 |
| Sex: condition         | 2,54                 | 1.881 | 0.176 |
| Condition              | 2,54                 | 3.642 | 0.033 |
| Diet: sex: condition   | 2,54                 | 0.392 | 0.678 |
| Diet: sex: condition   | 2,54                 | 3.458 | 0.068 |
| Diet: condition: HI    | 2,54                 | 6.662 | 0.003 |

#### Cortisol response

|                        | Diet: sex: condition | Diet | Sex | Condition |
|------------------------|-----------------------|------|-----|-----------|
| Diet                   | 1,28                 | 0.049 | 0.826 |
| Sex                    | 1,28                 | 8.108 | 0.008 |
| Condition              | 1,28                 | 17.779 | 0.000 |
| Diet: sex              | 1,28                 | 0.037 | 0.850 |
| Diet: condition        | 1,28                 | 0.256 | 0.617 |
| Diet: cortisol GH      | 1,28                 | 0.175 | 0.679 |
| Sex: condition         | 1,28                 | 0.131 | 0.720 |
| Sex: cortisol GH       | 1,28                 | 0.000 | 0.994 |
| Condition: cortisol GH | 1,28                 | 0.161 | 0.691 |
| Diet: sex: condition   | 1,28                 | 0.400 | 0.532 |
| Diet: cortisol GH      | 1,28                 | 0.003 | 0.955 |
| Diet: cortisol GH      | 1,28                 | 0.394 | 0.535 |

#### OF Locomotion

|                        | Diet: sex: condition | Diet | Sex | Condition |
|------------------------|-----------------------|------|-----|-----------|
| Diet                   | 1,32                 | 10.622 | 0.003 |
| Sex                    | 1,32                 | 6.287 | 0.017 |
| Condition              | 1,32                 | 0.358 | 0.554 |

#### OF Center

|                        | Diet: sex: condition | Diet | Sex | Condition |
|------------------------|-----------------------|------|-----|-----------|
| Diet                   | 1,32                 | 2.143 | 0.153 |

(continued on next page)
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