



Dietary fatty acids modulate cortisol concentrations and social dominance during social confrontations in adolescent male guinea pigs

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ABSTRACT

The hypothalamic-pituitary-adrenal (HPA)-axis and related glucocorticoid concentrations regulate physiology and behavior, which can be modulated by nutritional conditions, particularly by the dietary fatty acid composition. Omega-3 polyunsaturated fatty acids (PUFAs) have been shown to promote hypothalamic-pituitary-adrenal (HPA)-axis functions, whereas saturated fatty acids (SFAs) in general produce adverse effects and even increase baseline glucocorticoid concentrations. Glucocorticoids (e.g. cortisol) were further documented to modulate the establishment of dominance relationships, while the involvement of dietary fatty acids remains understudied. This study focused on different effects of PUFAs and SFAs on cortisol concentrations and social dominance in male guinea pigs. Three groups of animals were maintained on diets high in PUFAs (10 % w/w walnut oil), SFAs (10 % w/w coconut fat), or on an untreated control diet starting already prenatally. During adolescence, at an age of 60, 90, and 120 days, each individual's saliva cortisol concentrations and hierarchy index (calculated by initiated and received agonistic behavior) were measured during basal group housing conditions and stressful social confrontations with unfamiliar individuals of the other groups. SFA males showed highest baseline cortisol concentrations, lowest cortisol responses to social confrontations, and became subdominant. PUFA and control males showed significant cortisol responses. However, while control males became dominant during social confrontations, the hierarchy index in PUFA males decreased with age. Individual hierarchy indices during consecutive social confrontations revealed a high consistency. The findings presented here indicate that dietary fatty acids differently affect HPA-axis functions and social dominance but the underlying mechanisms remain to be determined.

1. Introduction

Glucocorticoids such as cortisol are released by the adrenal glands as a result of hypothalamic-pituitary-adrenal (HPA)-axis activity and are strongly involved in the regulation of energy balance and homeostasis. Various effects on physiological, metabolic, and behavioral functions enable to cope with stressful and energetically demanding conditions and, therefore, glucocorticoids are important mediators and indicators of physiological stress responses (Chrousos, 2009; Sapolsky et al., 2000). HPA-axis functions and glucocorticoid concentrations, however, can be strongly affected by nutritional conditions, particularly by the dietary fatty acid composition. High-fat diets in general and specifically saturated fatty acids (SFAs) increase the risk for obesity and cause HPA-axis dysfunctions, which resulted in increased glucocorticoid concentrations under non-stimulated conditions and decreased glucocorticoid

responses to HPA-axis stimulation in rats and pigs (Hryhorczuk et al., 2017; Lomax et al., 2013). Additionally, depressive and anxiety-like behaviors and cognitive impairments emerge, which are not only linked to effects of dietary SFAs but also to HPA-axis dysfunctions and chronically increased glucocorticoid concentrations in general (Chrousos, 2009; Décarie-Spain et al., 2018; Ferraz et al., 2011). In contrast, dietary intake of essential polyunsaturated fatty acids (PUFAs), in particular omega-3 (n-3) PUFAs, diminished glucocorticoid concentrations and counteracted behavioral impairments due to chronic stress in rats (Ferraz et al., 2011; Hennebelle et al., 2012). Moreover, diets high in n-3 PUFAs seem to stimulate HPA-axis activity in response to acute stressors (Caroprese et al., 2014) and, therefore, possibly facilitate adequate physiological stress responses.

The n-3 and omega-6 (n-6) PUFAs eicosapentaenoic acid (20:5 n-3), docosahexaenoic acid (22:6 n-3), and arachidonic acid (20:4 n-6), which

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can either be directly obtained through the diet or synthesized via their respective dietary precursor alpha-linolenic acid (18:3 n-3) and linoleic acid (18:2 n-6), are important neuronal and metabolic components (Bazinet and Layé, 2014). Increased neuronal n-3 PUFA concentrations positively contribute to monoaminergic neurotransmission (Chalon, 2006), which also affects HPA-axis functions (Chen and Su, 2013), and prevents social impairments (De Theije et al., 2015). N-3 PUFAs are furthermore suggested to decrease aggressive behaviors through neuronal influences (DeMar et al., 2006). However, by enabling adequate cortisol responses to stressful social confrontations with unfamiliar individuals, a diet enriched in walnut oil high in n-3 and n-6 PUFAs even increased aggressive behavior in guinea pigs, which resulted in higher social ranks. In contrast, dietary supplementation of SFA-rich coconut fat increased cortisol concentrations under basal conditions, decreased cortisol responses to social confrontations, and resulted in subdominant positions (Nemeth et al., 2016a). These results indicate that different effects of PUFAs and SFAs on glucocorticoid concentrations under basal social housing conditions and in response to social challenges could play a role in the establishment of social systems.

Initially high levels of aggressiveness are essential to establish dominance relationships, but low levels thereafter indicate a stable social hierarchy, which applies similarly to social rodent species such as rats (Mikics et al., 2007) and guinea pigs (Wallner and Dittami, 2003). Furthermore, increased glucocorticoid concentrations can facilitate aggressiveness in response to social challenges (Mikics et al., 2007), which may impact on social hierarchies. Findings in rats revealed that the outcome of a first encounter is not linked to glucocorticoid concentrations, but a long-lasting establishment of dominance relationships is indeed affected (Timmer and Sandi, 2010). Such long-lasting social organizational effects of glucocorticoids definitely represent a highly interesting concept with regard to dietary fatty acids and could promote the understanding of how these nutrients can affect behavior and social living via HPA-axis functions.

This study was designed to determine effects of dietary PUFAs and SFAs, supplemented through walnut oil and coconut fat, respectively, as permanent part of the animals' environmental conditions on cortisol concentrations and social dominance in response to social confrontations with unfamiliar conspecifics in adolescent male domestic guinea pigs. Experiments were carried out in a repeated measures design and each male was confronted with the same individuals at an age of 60, 90, and 120 days. This enabled determining possible effects of dietary fatty acids on cortisol concentrations and social dominance linked to the animals' age and/or on the consistency of such effects during this early ontogenetic period. We hypothesized that increasing cortisol concentrations during social confrontations positively affect social dominance especially in PUFA males, while SFA males become subdominant due to a reduced cortisol responsiveness. Moreover, these different effects were expected to be detectable already at early ages and to become more pronounced with increasing age.

2. Methods

2.1. Ethical statement

Experiments were performed in accordance with EU Directive 2010/63/EU for animal experiments and the national laws for animal keeping and experimentation. The study has been examined and approved by the internal board on animal ethics and experimentation of the Faculty of Life Sciences of the University of Vienna (# 2014-005) and the Austrian Federal Ministry of Science and Research (BMWF-66.006/0024-II/3b/2013).

2.2. Animals

All domestic guinea pigs (*Cavia aperea f. porcellus*) used in this study were descendants of a heterogeneous and multicolored stock of animals

maintained at the department's animal care facility. Prior to the experiments, 30 male and 30 female adult animals were randomly allocated to single-sexed social groups of ten individuals. Each group's enclosure (2 m × 1.6 m) was built of laminated chipboard, the floor was covered with standard woodchip bedding material, and several shelters were provided. A temperature of 20 ± 2 °C, a humidity of 50 ± 5 %, and a 12/12 h light-dark cycle (lights on at 07:00 a.m.) were maintained throughout the study.

Each male and female group was randomly assigned to one of three different experimental diets as previously described (Nemeth et al., 2018). A daily amount of 300 g guinea pig pellets (ssniff V-2233, ssniff Spezialdiäten GmbH, Soest, Germany) was provided per social group. This was left untreated for the control groups or enriched with 10 % (w/w) walnut oil (PUFA groups) or 10 % (w/w) coconut fat (SFA groups). Walnut oil contains 62 % linoleic acid (n-6) and 12 % alpha-linolenic acid (n-3), yielding an n-6:n-3 ratio of approximately 5:1, which is suggested to represent an optimal PUFA supply with regard to optimizing tissue n-3 PUFA concentrations (see e.g. Blank et al., 2002). Coconut fat contains 92 % SFAs, including 48 % lauric acid (12:0), 22 % myristic acid (14:0), 17 % palmitic acid (16:0), and 5 % stearic acid (18:0). This resulted in different fat contents compared to the control group (% of total food; control group: 3.3 %, PUFA group: 12.9 %, SFA group: 13.0 %) and fatty acid compositions (% of total food; control group: 0.67 % SFAs, 1.98 % PUFAs; PUFA group: 1.54 % SFAs, 9.27 % PUFAs; SFA group: 9.30 % SFAs, 2.33 % PUFAs). For a detailed analysis of total dietary fatty acid composition and effects on plasma fatty acid patterns with regard to the same dietary regimes see Nemeth et al. (2018). The group-specific diets were provided in several food bowls. Additionally, 50 g of hay was provided daily per group, which has almost no nutritional value but promotes abrasion of the teeth and supports intestinal activity. Water was available ad libitum in several drinking bottles.

Animals were maintained on the diets for 100 days in order to ensure maximum fatty acid uptake and integration into metabolic pathways and tissues. Groups did not differ in body mass, neither in advance to the feeding procedure (two-way analysis of variance: $F_{5,57} = 1.01$, $p = 0.42$) nor after the 100-day supplementation period (two-way analysis of variance: $F_{5,51} = 1.89$, $p = 0.11$; control males: 899 ± 23 g; control females: 918 ± 19 g; PUFA males: 962 ± 28 g; PUFA females: 907 ± 50 g; SFA males: 910 ± 31 g; SFA females: 834 ± 16 g). Males and females of the same diet group were then mated and separated again as soon as the first females were detected to be pregnant. For each diet group, two mating groups of five males and five females each, which were randomly chosen, were formed. A total number of 69 pups were born and housed together with their mother and the other females and pups belonging to the same diet group. Males, which represent the focus animals of this study, were separated from females at an age of 20–30 days and integrated into new single-sexed social groups containing only offspring of the same diet group. Male groups were maintained in their group constellation (control: n = 14, PUFA: n = 14, SFA: n = 13) and continuously fed with the respective diet as outlined above from birth throughout the study.

2.3. Experimental procedures

At an age of 60, 90, and 120 days, each animal's behavior was analyzed in its established social group (group housing) and during social confrontations with unfamiliar individuals of the other groups. Saliva samples were collected during both situations in order to measure saliva cortisol concentrations. Animals of all diet groups were born within one month and thus were not exactly of the same age, but the birth dates were equally distributed among groups. Nonetheless, in order to ensure that only similarly aged individuals were confronted with each other, the procedures described in the following were performed whenever a triplet of animals (one animal per diet group) reached a mean age of 60 (± 5), 90 (± 5), and 120 (± 5) days,

respectively.

The procedure always started at 09:00 a.m. with video recordings of the established social groups (group housing condition). For this, all shelters were removed from the enclosures in order to ensure visibility of all animals. As this also had to be done each day for cleaning purposes, animals were habituated to this situation. A whole group was video recorded for 30 min with a camera located directly above the enclosure and fixed at the ceiling. However, only the behavior of the respective focus animals that had reached an age of 60, 90, or 120 days was analyzed afterwards. Directly after video recordings at 09:30 a.m., saliva samples were collected from the same animals. For this, a standard cotton bud was inserted into the animal's mouth and saliva collected for approximately one minute. During this time span, a possible HPA-axis response caused by any handling stress would not come to bear and, moreover, cortisol concentrations are not affected by this procedure even in case of repeated samplings (Fenske, 1997). Cotton buds containing saliva were transferred to a micro centrifuge tube and always centrifuged immediately after the sampling procedure (14,000 rpm, 10 min); pure saliva was stored at -20°C until later analysis. Cortisol measurements in saliva highly correlate with measurements in plasma (Nemeth et al., 2016b) and were chosen because of the minimal invasiveness of this method, which enables repeated and multiple samplings within a short time span.

At 09:00 a.m. on the following day, the same focus animals were subjected to social confrontations with unfamiliar individuals of the other diet groups (social confrontation condition), involving three males per setup. Social confrontations were performed in squared arenas (1 m^2), which were built of laminated fiberboard and the floors were covered with standard woodchip bedding material. No food or water was provided during this situation. Social confrontations were carried out in a separate test room but under the same ambient conditions as outlined above. Animals were video recorded for 30 min and a saliva sample was collected directly thereafter. All animals were then returned to their social group.

Social confrontations represent a highly stressful situation for male guinea pigs and they show reliable and repeatable cortisol responses to this situation (Nemeth et al., 2016b). Previous studies also revealed that animals show highest behavioral frequencies during the first 30 min of social confrontations (Nemeth et al., 2014; Wallner and Dittami, 2003) and establish dominance relationships through agonistic interactions (Nemeth et al., 2016a). Therefore, the experimental procedure aimed to analyze the animals' acute cortisol and behavioral responses to this social stressor compared to and dependent on the initial condition during group housing. Moreover, repeated analyses at mean ages of 60, 90, and 120 days were carried out to determine possible age-effects (e.g., at which age any possible fatty acid effects emerge) and/or the consistency of such effects (e.g., variation of cortisol concentrations and social relationships across consecutive social confrontations) during adolescence. The same individuals were confronted with each other at the respective ages in order to determine the consistency and any long-lasting effects.

Previous studies revealed that the dietary regimes as applied here can differently affect body mass gain (Nemeth et al., 2018). In this study, groups did not differ in body mass at an age of 60 days (analysis of variance: $F_{2,38} = 1.91$, $p = 0.16$; control: $590 \pm 19\text{ g}$, PUFA: $575 \pm 14\text{ g}$, SFA: $547 \pm 14\text{ g}$) or 90 days (analysis of variance: $F_{2,38} = 1.04$, $p = 0.36$; control: $725 \pm 17\text{ g}$, PUFA: $751 \pm 14\text{ g}$, SFA: $725 \pm 13\text{ g}$), but PUFA males were heavier than the others at an age of 120 days (analysis of variance: $F_{2,36} = 7.86$, $p = 0.001$; control: $801 \pm 16\text{ g}$, PUFA: $890 \pm 15\text{ g}$, SFA: $818 \pm 21\text{ g}$). In order to determine if this difference in body mass had any effect on saliva cortisol concentrations and behavior, it was considered in the statistical analyses.

2.4. Behavioral analyses

Videos were analyzed using The Observer XT 10 (Noldus,

Wageningen, the Netherlands). Frequencies of initiated (active) and received (passive) agonistic behaviors were analyzed as described by Rood (1972) for guinea pigs using continuous recording (Martin and Bateson, 2007). Agonistic behaviors included displacing, chasing, biting, fighting, rumba rumble, stand threat, kick back, and mounting. Initially, sociopositive behaviors (huddling, social grooming, nose-nose contact, naso-anal sniffing) were also considered in the analyses, but they revealed no pronounced differences between groups, ages, or the social conditions, and were thus not included. Furthermore, the major aim was to determine social dominance as a measure of a successful behavioral response to social confrontations. Therefore, only agonistic behaviors were used for further analyses. The proportion of won agonistic interactions on total agonistic interactions an individual was involved in is usually used to calculate a social rank index in guinea pigs (Coulon, 1975). Social dominance in guinea pigs is usually associated with increased aggressive behavior, especially in the beginning of social confrontations when dominance relationships are established (Nemeth et al., 2014; Wallner and Dittami, 2003). In order to include as many observed behaviors as possible in the determination of an individual's hierarchy index, the index was calculated by: (total initiated agonistic behaviors) / (total initiated and received agonistic behaviors). This yielded values between 0 and 1: values closer to 1 result from more initiated agonistic behaviors and indicate dominance, while values closer to 0 result from more received agonistic behavior and indicate subdominance.

2.5. Saliva cortisol analyses

Saliva cortisol concentrations were analyzed by a biotin-streptavidin enzyme-immunoassay using a cortisol-specific antibody (Palme and Möstl, 1997). Cortisol measurements in saliva were previously shown to reliably indicate HPA-axis responses with strong relations to plasma levels in guinea pigs (Fenske, 1997; Nemeth et al., 2016b). Based on preceding measurements of pooled samples to determine the 50 % steroid binding, single samples were diluted 1:50 and cortisol concentrations (ng/mL) were measured in $10\ \mu\text{l}$ inputs. All analyses were run in duplicates. The intra- and inter coefficients of variance were 10.29 % and 4.56 %, respectively.

2.6. Statistical analyses

Statistics were performed using R 3.5.2. (R Core Team, 2018) and the additional packages 'nlme' (Pinheiro et al., 2017) for performing linear mixed models (LMM) and 'phia' (De Rosario-Martinez, 2015) for post-hoc analyses. Saliva cortisol concentrations and the hierarchy index were analyzed by applying two different LMMs:

Model 1. The conditions (group housing and social confrontations) were treated as repeated measurements, thereby allowing direct comparisons of both conditions. These models included 'group' (control, PUFA, SFA), 'age' (60d, 90d, 120d), and 'condition' (group housing, social confrontation) as fixed effects.

Model 2. Measurements during social confrontations were analyzed adjusted for group housing conditions, thereby allowing (1) determining effects of initial conditions during group housing on measurements during social confrontations and (2) a post-hoc analysis of predicted values for social confrontations (statistically adjusted for group housing conditions), which represent "baseline-corrected" values. These models included 'group' and 'age' as fixed effects as well as the respective group housing measurement as covariate. Initially, also group housing measurements of the other individuals during social confrontations (the opponents) were considered as additional covariates, but they had no effect at all. For a more detailed analysis of social confrontations, post-hoc interaction analyses with Bonferroni corrections were carried out on significant factor interaction and main effects to consider not only differences in the intercept when covariates are included in the models but also differences with regard to mean covariate effects. For further

analysis of the hierarchy index during social confrontations, the cortisol response was calculated by the natural logarithm of cortisol concentrations during social confrontations divided by cortisol concentrations during group housing and included as additional predictor. This was done to determine if the cortisol response affected the hierarchy index during social confrontations.

For all analyses, the factor levels "group control", "age 60d", and "condition GH" were set at the intercept and the other groups, ages, and condition were analyzed in contrast to this and, therefore, all statistics in the result section represent comparisons to the intercept unless otherwise stated. Individual ID was included as random effect to correct for repeated measurements. Initially, mother ID was also included to correct for relatedness, but this proved to have no effect and was not further considered. Full models always started with all possible interactions, but were then fitted (removal of non-relevant interactions and main effects) based on the Akaike information criterion (AIC). This revealed that the initially included covariate 'body mass' showed no significant effects on saliva cortisol concentrations and the hierarchy index during group housing and social confrontations, which was therefore excluded from statistical analyses (effect of body mass at last appearance in the model: saliva cortisol: $F_{1,155} = 3.46$, $p = 0.06$; hierarchy index: $F_{1,147} = 3.42$, $p = 0.07$).

Linear models were applied to determine the consistency in saliva cortisol concentrations, the cortisol response, and hierarchy index during consecutive social confrontations. Models included the respective parameter measured during social confrontations at an age of 90d or 120d as response variable and the same parameter measured at the previous confrontation at an age of 60d or 90d, respectively, as predictor.

Initially, also 'group' was included as predictor, but no significant interactions were detected and it was therefore excluded from these analyses.

Model assumptions were checked by applying Shapiro-Wilk and Levene's tests and by visual inspections of model residuals. In order to obtain normal distribution of residuals, cortisol concentrations were transformed by applying the natural logarithm. Due to heteroscedasticity, models for cortisol concentrations were corrected for different variance structures in the highest interaction. All model statistics are based on type 3 sum of squares. The significance level was set at $p \leq 0.05$ with two-sided testing. Power analyses using library 'pwr' (Champely, 2017) revealed that the given sample sizes and degrees of freedoms in each analysis were adequate to extract large effect sizes (Cohen's $f^2 > 0.35$) at a statistical power of 80 %. All results are presented as means with standard errors and covariate effects as slopes with standard errors.

3. Results

3.1. Saliva cortisol concentrations

Saliva cortisol concentrations were significantly affected by group interacting with age and group interacting with condition. Moreover, a significant interaction of age and condition was detected (Fig. 1A, Table 1 model 1). Saliva cortisol concentrations did not differ in the PUFA ($p = 0.24$) and SFA ($p = 0.60$) groups compared to the control group during group housing at an age of 60d (Table 2 model 1). Concentrations did not change in the control group from 60d to 90d of age

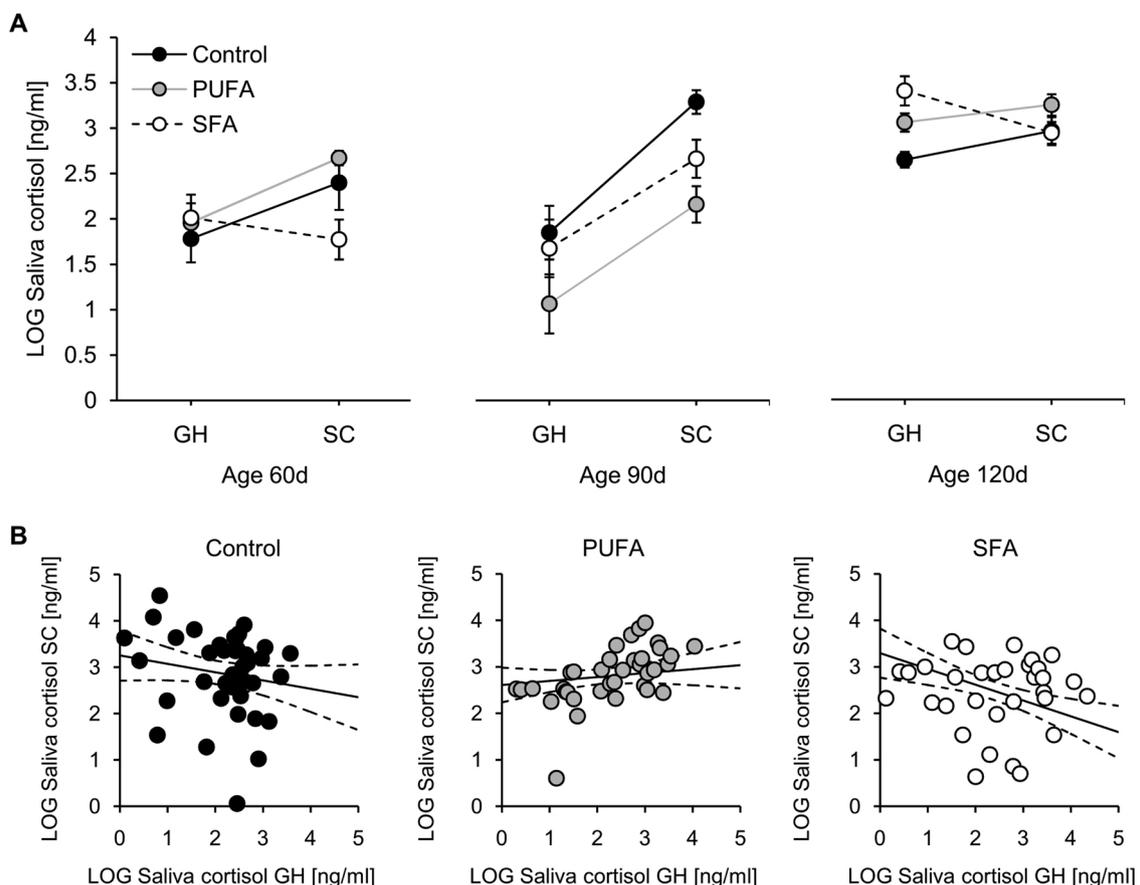


Fig. 1. Effects of dietary fatty acids on saliva cortisol concentrations (log transformed). (A) Saliva cortisol concentrations for male guinea pigs maintained on a control, polyunsaturated fatty acid (PUFA), or saturated fatty acid (SFA) diet during group housing (GH) and social confrontations (SC) at an age of 60, 90, and 120 days. For statistics see Tables 1 and 2. (B) Effects of saliva cortisol concentrations measured during group housing (GH) on saliva cortisol concentrations measured during social confrontations (SC) in male guinea pigs maintained on a control, polyunsaturated fatty acid (PUFA), or saturated fatty acid (SFA) diet (corrected for repeated measurements). Control: -0.18 ± 0.12 , n.s.; PUFA: 0.09 ± 0.15 , n.s.; SFA: -0.34 ± 0.16 , $p \leq 0.05$.

Table 1

Linear mixed model statistics (fitted ANOVA tables) for analysis of saliva cortisol concentrations (log transformed) and the hierarchy index.

Response variable	Model	Predictor	df	F-value	p-value		
Saliva cortisol	model 1	group	2,38	0.75	0.48		
		age	2,179	11.83	< 0.001		
		condition	1,179	9.56	0.002		
		group : age	4,179	9.30	< 0.001		
		group : condition	2,179	7.37	0.001		
		age : condition	2,179	12.59	< 0.001		
		group : age : condition	n.a.	n.a.	n.a.		
	model 2	group	2,38	0.09	0.91		
		age	2,60	4.54	0.02		
		LOG cortisol GH	1,60	2.17	0.15		
		group : age	4,60	5.74	0.001		
		group : LOG cortisol GH	2,60	4.95	0.01		
		age : LOG cortisol GH	n.a.	n.a.	n.a.		
		group : age : LOG cortisol GH	n.a.	n.a.	n.a.		
		Hierarchy index	model 1	group	2,38	1.09	0.35
				age	n.a.	n.a.	n.a.
				condition	1,168	7.38	0.007
group : age	n.a.			n.a.	n.a.		
group : condition	2,168			4.84	0.009		
age : condition	n.a.			n.a.	n.a.		
group : age : condition	n.a.			n.a.	n.a.		
model 2	group		2,38	1.64	0.21		
	age		2,43	3.82	0.03		
	hierarchy index GH		1,43	1.98	0.17		
model 2	group : age	4,43	2.83	0.04			
	group : hierarchy index GH	n.a.	n.a.	n.a.			
	age : hierarchy index GH	n.a.	n.a.	n.a.			
	group : age : hierarchy index GH	n.a.	n.a.	n.a.			
	group : age : hierarchy index GH	n.a.	n.a.	n.a.			

model 1: group housing and social confrontations as repeated measurements; model 2: social confrontations in response to group housing (GH). Models were fitted based on the Akaike information criterion. n.a.: not available as this predictor dropped out of the model during model fitting.

($p = 0.77$), but they decreased in the PUFA group ($p < 0.001$). However, a general increase afterwards at an age of 120d ($p < 0.001$) was even stronger in the SFA group ($p = 0.05$) resulting in highest cortisol concentrations. During social confrontations, cortisol concentrations were significantly increased in the control group ($p = 0.002$). Cortisol concentrations in the PUFA group did not differ to the control group ($p = 0.62$) during social confrontations, but they remained lower in the SFA group ($p = 0.001$). Overall, the cortisol increase during social confrontations was strongest pronounced at an age of 90d ($p = 0.007$), but lowest at an age of 120d ($p = 0.05$) (Fig. 1A, Table 2 model 1).

Analyzing saliva cortisol concentrations during social confrontations adjusted for concentrations during group housing yielded further group-specific effects (Table 1 model 2). Concentrations measured during group housing had no pronounced effect on those measured during social confrontations in the control group ($p = 0.15$). Although the effects detected in the PUFA and SFA groups did not differ to the control group (PUFA: $p = 0.08$, SFA: $p = 0.33$) (Table 2 model 2), a group-specific post-hoc analysis revealed that higher cortisol concentrations during group housing were related to lower concentrations during social confrontations in the SFA group ($p = 0.001$) (Fig. 1B). Moreover, this analysis yielded a significant group:age interaction on saliva cortisol concentrations measured during social confrontations (Table 1 model

Table 2

Fitted model coefficients for analysis of saliva cortisol concentrations (log transformed).

Model	Coefficient	Value	sem	t-value	p-value
model 1	Intercept (group Control, age 60d, condition GH)	1.75	0.23	7.71	< 0.001
	group PUFA	0.31	0.26	1.20	0.24
	group SFA	0.16	0.29	0.54	0.60
	age 90d	0.09	0.31	0.29	0.77
	age 120d	0.90	0.23	3.91	< 0.001
	condition SC	0.70	0.23	3.09	0.002
	group PUFA : age 90d	-1.28	0.32	-4.03	< 0.001
	group SFA : age 90d	-0.12	0.35	-0.34	0.74
	group PUFA : age 120d	0.09	0.26	0.37	0.72
	group SFA : age 120d	0.59	0.29	2.02	0.05
	group PUFA : condition SC	-0.10	0.19	-0.50	0.62
	group SFA : condition SC	-0.75	0.21	-3.51	0.001
	model 2	Intercept (group Control, age 60d, LOG cortisol GH = 0)	2.69	0.39	6.92
group PUFA		-0.18	0.43	-0.42	0.67
group SFA		-0.15	0.50	-0.30	0.77
age 90d		1.03	0.35	2.96	0.005
age 120d		0.75	0.37	2.00	0.05
LOG cortisol GH		-0.18	0.12	-1.47	0.15
group PUFA : age 90d		-1.23	0.43	-2.88	0.006
group SFA : age 90d		-0.22	0.45	-0.49	0.63
group PUFA : age 120d		-0.27	0.41	-0.65	0.52
group SFA : age 120d		0.74	0.46	1.59	0.12
group PUFA : LOG cortisol GH		0.27	0.15	1.78	0.08
group SFA : LOG cortisol GH		-0.16	0.16	-0.98	0.33

model 1: group housing and social confrontations (SC) as repeated measurements; model 2: social confrontations in response to group housing (GH). Models were fitted based on the Akaike information criterion.

2). Post-hoc tests corrected for the above-mentioned effects of group housing cortisol concentrations revealed significantly higher cortisol concentrations during social confrontations in the PUFA group compared to the SFA group at an age of 60d ($p = 0.001$). Cortisol concentrations were highest in the control group compared to the other groups at an age of 90d ($p = 0.01$), while no differences were detected during social confrontations at an age of 120d ($p = 1$ for all further pairwise comparisons) (Fig. 1A).

3.2. Hierarchy index

The hierarchy index was significantly affected by a group:condition interaction, while no age-effects were detected in this analysis (Fig. 2A, Table 1 model 1). All groups showed a similar hierarchy index during group housing (PUFA vs. Control: $p = 0.62$, SFA vs. Control: $p = 0.15$) (Table 3 model 1). However, the index was significantly increased in the control group during social confrontations compared to group housing ($p = 0.01$). No such effect was detected in the PUFA and SFA groups, which resulted in a significantly lower hierarchy index in both groups (PUFA: $p = 0.02$, SFA: $p = 0.005$) compared to the control group during social confrontations (Fig. 2A, Table 3 model 1).

In the second analysis, the hierarchy index during social confrontations was not affected by the index measured during group housing ($p = 0.17$), which applied equally to all groups (Fig. 2B, Table 1 model 2). Furthermore, this analysis revealed an interaction effect of group and age on the hierarchy index during social confrontations (Table 1 model 2). While the hierarchy index during social confrontations increased in the control group with age (90d: $p = 0.04$, 120d: $p = 0.01$), the opposite was found in the PUFA group, namely a decreasing hierarchy index with age (90d: $p = 0.03$, 120d: $p = 0.002$). The SFA group showed the same index during social confrontations at all ages (90d: $p = 0.15$, 120d:

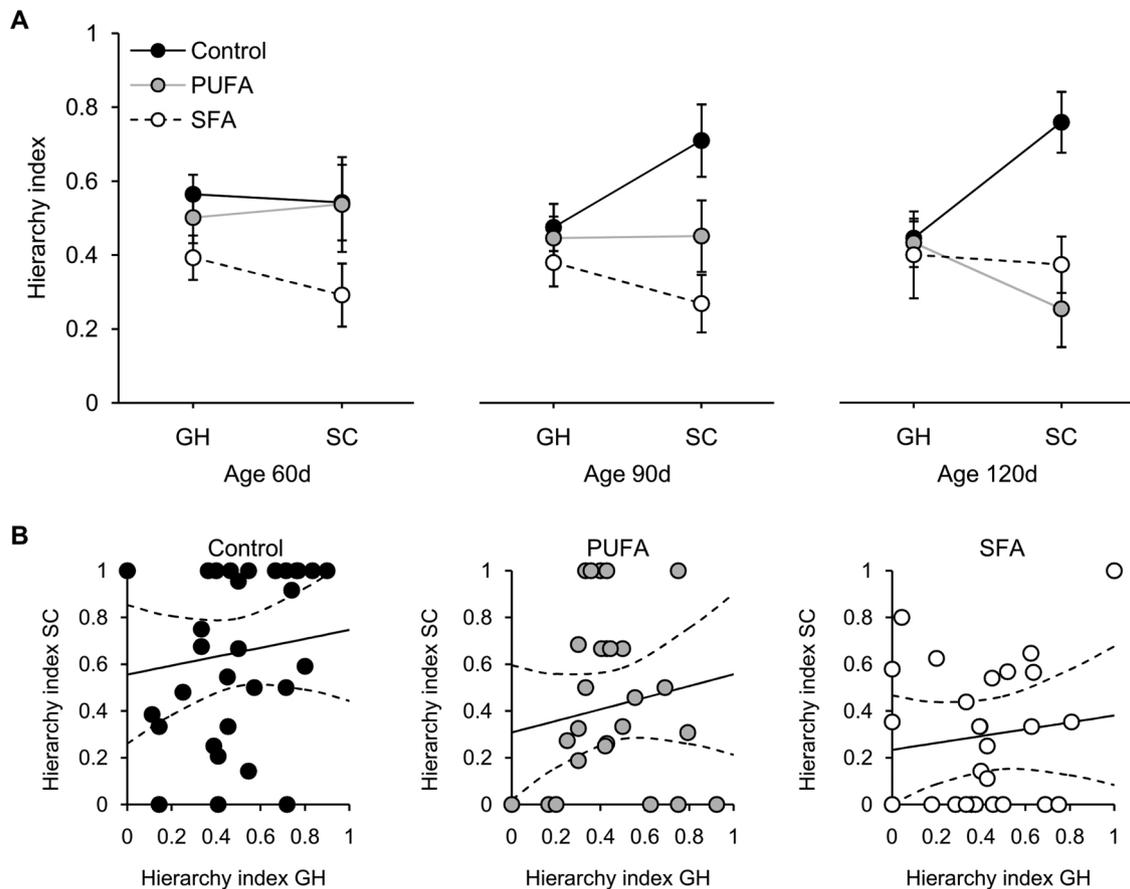


Fig. 2. Effects of dietary fatty acids on the hierarchy index. (A) Hierarchy index of male guinea pigs maintained on a control, polyunsaturated fatty acid (PUFA), or saturated fatty acid (SFA) diet during group housing (GH) and social confrontations (SC) at an age of 60, 90, and 120 days. For statistics see Tables 1 and 3. (B) Effects of the hierarchy index measured during group housing (GH) on the hierarchy index measured during social confrontations (SC) in male guinea pigs maintained on a control, polyunsaturated fatty acid (PUFA), or saturated fatty acid (SFA) diet (corrected for repeated measurements). Control: n.s., PUFA: n.s., SFA: n.s.

Table 3
Fitted model coefficients for analysis of the hierarchy index.

Model	Coefficient	Value	sem	t-value	p-value
model 1	Intercept (group Control, condition GH)	0.50	0.05	9.99	<
	group PUFA	-0.04	0.07	-0.50	0.62
	group SFA	-0.11	0.07	-1.46	0.15
	condition SC	0.17	0.06	2.72	0.01
	group PUFA : condition SC	-0.21	0.09	-2.44	0.02
	group SFA : condition SC	-0.25	0.09	-2.86	0.005
model 2	Intercept (group Control, age 60d, hierarchy index GH = 0)	0.38	0.13	2.97	0.005
	group PUFA	0.07	0.14	0.51	0.62
	group SFA	-0.18	0.14	-1.26	0.22
	age 90d	0.22	0.10	2.09	0.04
	age 120d	0.29	0.11	2.60	0.01
	hierarchy index GH	0.20	0.14	1.41	0.17
	group PUFA : age 90d	-0.36	0.16	-2.29	0.03
	group SFA : age 90d	-0.22	0.15	-1.46	0.15
	group PUFA : age 120d	-0.51	0.16	-3.22	0.002
	group SFA : age 120d	-0.25	0.17	-1.49	0.15

model 1: group housing and social confrontations (SC) as repeated measurements; model 2: social confrontations in response to group housing (GH). Models were fitted based on the Akaike information criterion.

$p = 0.15$) (Fig. 2A, Table 3 model 2). Furthermore, post-hoc tests corrected for group housing conditions revealed that the SFA group had a lower index during social confrontations compared to the control group at an age of 90d ($p = 0.02$) and 120d ($p = 0.03$) and also the PUFA

group exhibited a lower index compared to the control group at an age of 120d ($p = 0.01$; $p > 0.2$ for all further pairwise comparisons) (Fig. 2A).

3.3. Effect of cortisol response on hierarchy index

Including the cortisol response in the analysis of the hierarchy index during social confrontations yielded a significant interaction of group, age, and the cortisol response ($F_{4,29} = 3.13, p = 0.03$). The cortisol response positively affected the hierarchy index during social confrontations in the PUFA group at an age of 60d ($0.32 \pm 0.10, p = 0.001$) and in the control group at an age of 120d ($0.30 \pm 0.12, p = 0.02$); no further significant effects were detected (data not shown). Analyzing the opposite, namely that the hierarchy index might have affected the cortisol response, revealed no significant three-way interaction ($F_{4,29} = 2.03, p = 0.12$; no age- and/or group-specific effects) and no main effect of the hierarchy index ($F_{1,41} = 0.92, p = 0.34$) after removal of non-significant interactions.

3.4. Individual consistency during consecutive social confrontations

Individual saliva cortisol concentrations measured during social confrontations at an age of 60 days had no effects on those measured at an age of 90 days ($F_{1,37} = 0.13, p = 0.72$). Similarly, concentrations at an age of 90 days had no effects on cortisol concentrations at an age of 120 days ($F_{1,35} = 0, p = 1$). Also with regard to the cortisol response, no relations were detected (90d ~ 60d: $F_{1,29} = 0.02, p = 0.88$; 120d ~ 90d: $F_{1,28} = 1.99, p = 0.17$) (data not shown).

Individual hierarchy indices determined during social confrontations

at an age of 60 days and 90 days positively affected hierarchy indices during the subsequent confrontations at an age of 90 days and 120 days, respectively (90d ~ 60d: $F_{1, 23} = 5.96$, $p = 0.02$; 120d ~ 90d: $F_{1, 19} = 6.55$, $p = 0.02$) (Fig. 3).

4. Discussion

Male guinea pigs respond to social confrontations with unfamiliar individuals by showing high levels of aggressiveness and an increase in cortisol concentrations. The establishment of a social hierarchy via agonistic behavior can be observed within the first 30 min (Nemeth et al., 2016a). With regard to this short time span, cortisol increases can be assumed to represent acute stress responses. Although especially n-3 PUFAs are known for their "anti-stress" effects (Ferraz et al., 2011; Song et al., 2003), n-3 and n-6 PUFAs similarly increase the corticotropin-releasing hormone (CRH)-induced release of adrenocorticotrophic hormone (ACTH) from pituitary cells in vitro, whereas SFAs (e.g., lauric acid, palmitic acid, stearic acid) decrease the release of ACTH (Katoh et al., 2004). As ACTH stimulates glucocorticoid-release by the adrenal cortex, this indicates that PUFAs can promote acute HPA-axis activation and SFAs exert suppressive effects. SFAs were found to decrease cortisol responses to social confrontations in the study presented here, which perfectly fits to the in vitro findings. However, cortisol responses in PUFA males did not differ to control males and, therefore, PUFAs had no stimulating effects on cortisol responses in this study. Nevertheless, dietary fatty acid effects and general differences in acute cortisol responses with age could have modified behavioral responses to social confrontations and possibly contributed to the establishment of social dominance relationships.

SFA males showed increased cortisol concentrations during group housing at an age of 120 days and overall lowest cortisol responses during social confrontations. Moreover, an impaired HPA-axis as indicated by lowest cortisol responses in this group was found on the individual level: a negative relationship between cortisol concentrations during group housing and social confrontations indicates that higher baseline cortisol concentrations resulted in lower stimulated concentrations. Increased tissue SFA concentrations (e.g., myristic acid, palmitic acid, and stearic acid) may increase glucocorticoid concentrations by upregulating 11 β -hydroxysteroid-dehydrogenase type1 (Petrus et al., 2015; Vara Prasad et al., 2010), the enzyme which catalyzes the conversion of inactive cortisone to cortisol. Additionally, a high-SFA diet linked to increased glucocorticoid concentrations has been shown to result in a downregulation of genes expressing mineralocorticoid and glucocorticoid receptors as well as CRH in the hypothalamus. As these genes are involved in the negative feedback control of the HPA-axis and

glucocorticoid production, this effect indicates suppressed HPA-axis activity and could represent a neuronal adaptation to increased glucocorticoid concentrations in order to prevent negative long-term effects (Hryhorczuk et al., 2017). Glucocorticoids, however, stimulate gluconeogenesis and glucose represents an acute energy source for muscles and the brain (Andrews and Walker, 1999). A suppression of glucocorticoid release could thus have impaired physiological and behavioral responses to social confrontations, which represent energetically demanding situations in guinea pigs (Nemeth et al., 2014). In addition to the significantly decreased cortisol response in SFA males, a low hierarchy index during social confrontations indicates a subdominant status and suggests physiological and behavioral maladaptation.

In contrast, PUFA and control males showed increasing cortisol concentrations in responses to social confrontations, which corresponds to previous findings (Nemeth et al., 2016a). Moreover, cortisol concentrations measured during group housing and social confrontations were unrelated in these groups. This implies that acute cortisol responses were not affected by circulating cortisol concentrations and indicates adequate HPA-axis functions in both groups. Findings in rats revealed that a high glucocorticoid responsiveness related to an altered expression of receptors controlling HPA-axis functions facilitates aggressiveness (Walker et al., 2017). This could increase the probability of becoming dominant. Cortisol responses in PUFA males positively affected the individual social hierarchy index during social confrontations at an age of 60 days, while the same effect was detected in control males at an age of 120 days, indicating that cortisol responses could affect the establishment of dominance relationships. N-3 PUFA deficiency has been shown to negatively affect HPA-axis functions through glucocorticoid receptor signaling pathways in the prefrontal cortex (Larrieu et al., 2014, 2016), a brain area that is suggested to play an important role with regard to social dominance (Wang et al., 2011). Recent findings also revealed that the downregulation of glucocorticoid receptors in the nucleus accumbens increased the probability of becoming dominant (Papilloud et al., 2020). These studies highlight the mesolimbic system with regard to social dominance. Nevertheless, the general role PUFAs play in the neuronal control of social dominance remains to be determined, but adequate n-3 PUFA supply could be relevant. A positive effect of additional n-3 PUFA supplementation may even be questioned, because in contrast to control males, which also faced no n-3 PUFA deficiency, PUFA males never became clearly dominant: their hierarchy index was similar to control males at an age of 60 days and even decreased with age thereafter.

Males compete for dominance positions and the related access to food and mating partners (Holekamp and Strauss, 2016). A higher dominance position is also related to increased reproductive success in

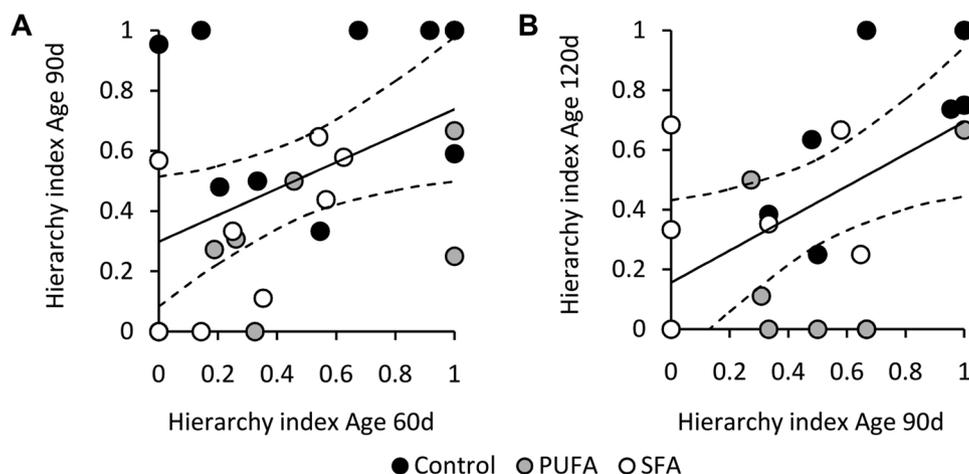


Fig. 3. Relationships between individual hierarchy indices during consecutive social confrontations. (A) Age 60 days and 90 days. 0.439 ± 0.180 , $p \leq 0.05$. (B) Age 90 days and 120 days. 0.538 ± 0.210 , $p \leq 0.05$.

male guinea pigs (Mutwill et al., 2019). In a previous study on dietary fatty acid effects in adult guinea pigs, social confrontations were performed for 48 h and included females and (necessarily because of this long time-span) food (Nemeth et al., 2016a). This setup resulted in a clear dominant status in PUFA males from the beginning. Although PUFA males showed cortisol responses to social confrontations in the present study, behavioral responses were perhaps actively suppressed because no resources were provided to compete for. Such an effect would perhaps involve cognitive influences of n-3 PUFAs (Luchtman and Song, 2013), which could have enabled these animals to immediately assess the situation resulting in an adjustment of their behavior. Even the diminishing effects of n-3 PUFAs on aggressiveness (see e.g. Gajos and Beaver, 2016) could have affected the behavioral responses of PUFA males with age and finally reduced their hierarchy index. This is strongly corroborated by a low frequency of initiated agonistic behavior especially at an age of 120 days (data not shown), where some animals were even not involved in any social interactions. Unfortunately, this resulted in a decreasing number of agonistic interactions for determination of hierarchy indices with regard to statistical analyses across age, and thus could have also affected the results (note: sample size for hierarchy index during social confrontations was always ≥ 8 per group and age). Taking age not into account would have yielded a significantly lower hierarchy index in SFA compared to control males ($p = 0.006$) but only a tendency for such an effect in PUFA males ($p = 0.083$).

Comparing our previous study (Nemeth et al., 2016a), which was carried out in adult individuals, and the present results may also raise the question if dietary fatty acid effects are age-specific. While positive effects of PUFAs on social dominance may be assumed during adulthood, the PUFA diet, including both n-3 and n-6, could have also produced negative long-term effects during adolescence, which finally resulted in subdominance similarly to the SFA diet in this study. Although the fat content of the fatty acid diets was far below a high-fat diet (e.g. compare to Décarie-Spain et al., 2018), high fat intake is well known to impair neuroendocrine functions and (social) behavior (Sullivan et al., 2015). This would suggest that a long-term increased dietary fat content irrespective of the fatty acid composition negatively affects social behavior and social dominance independent of HPA-axis functions. Nevertheless, as social confrontations represent energetically demanding situations, reduced aggressive behavior could have also saved energy and perhaps explain the highest body mass gain during adolescence as previously shown with regard to dietary PUFAs despite increasing cortisol concentrations (Nemeth et al., 2018).

Increasing cortisol concentrations during adolescence until adulthood, as found in all groups here, represents a common pattern in adolescent guinea pigs (Schöpper et al., 2012) and is presumably linked to structural growth and the establishment of the social system during this period. At an age of 120 days, when baseline cortisol concentrations were highest in all groups and indicated high HPA-axis activity especially in SFA males, lowest relative cortisol responses (e.g., change in cortisol from group housing to social confrontations) were detected. Chronically and/or naturally increased glucocorticoid concentrations are in general suggested to limit additional acute HPA-axis and glucocorticoid responses to stressors (Harris et al., 2012; Romero et al., 2009), because the maximum adrenocortical secretory capacity may have been reached. This effect is assumed to be modulated through mineralocorticoid and glucocorticoid receptors, which show different affinities for glucocorticoids and modulate the natural (e.g. circadian) and stress-induced HPA-axis activity, respectively (De Kloet et al., 2000; Harris et al., 2013). Increasing cortisol concentrations during adolescence could have diminished relative responses to social confrontations in all groups at an age of 120 days and an impaired HPA-axis would have exacerbated this in SFA males even resulting in a downregulation. A simple habituation to the repeated social confrontations can be excluded, because previous findings revealed that this social stressor reliably elicits cortisol responses in guinea pigs even when performed on consecutive days (Nemeth et al., 2016b).

Interestingly, at an age of 90 days, when relative cortisol responses were highest overall, the first clear dominance relationships between groups could be observed. Although the results presented here do not provide clear evidence for an overall causal relationship between cortisol responses and social dominance, dominance relationships became even more obvious afterwards at an age of 120 days. Significant findings in rats showed that increased glucocorticoid secretions before or after a first social encounter between two individuals resulted in a long-term establishment of social dominance or subordination, respectively (Timmer and Sandi, 2010; Weger et al., 2018). Glucocorticoids are known to play an important role for memory consolidation involving brain areas such as the amygdala or hippocampus as part of the meso- limbic system (Schwabe et al., 2012). This could also apply to memory for social relationships. Animals in our study definitely had to re-establish a dominance hierarchy and achieve their social rank during each social confrontation. However, highest cortisol responses at an age of 90 days could have contributed to a long-term establishment of dominance relationships and especially to subdominance in PUFA and SFA males. The positive effect of the cortisol response on the hierarchy index in control males at an age of 120 days could additionally indicate that an increase in cortisol concentrations was still important to re-establish the previous dominance hierarchy. An individual's and his opponents' group housing hierarchy indices had no effects on the newly established social hierarchy and confirms previous findings (Nemeth et al., 2016a). Accordingly, even subdominant individuals had an opportunity to become dominant during social confrontations. However, although statistical analyses helped to minimize possible genetic influences on the results (note: mother ID had no effects), any possible influence of the individual's fathers (e.g. genetic predispositions) on the results cannot fully be excluded. Future studies on dietary fatty acid effects should therefore consider specific maternal and paternal influences.

In this study we could show that cortisol responses to social confrontations are overall limited by dietary SFAs and elevated baseline cortisol concentrations, demonstrating a decreased relative HPA-axis reactivity with regard to both conditions. Accordingly, HPA-axis and corresponding cortisol responses to social confrontations could have played a role for the establishment of social hierarchies but a causal relationship and an involvement of dietary fatty acids, particularly n-3 PUFAs, in such an effect remains to be determined. The fact that individuals re-established dominance relationships during social confrontations each 30 days highlights the consistency of dietary fatty acid effects on social dominance, or more precisely subdominance. It may be questioned if these effects include memory for social relationships, which would argue for an involvement of the well-known cognitive influences of dietary fatty acids (Ferraz et al., 2011) and glucocorticoids (Lupien et al., 2002). Nevertheless, the findings presented here confirm the importance of balanced dietary PUFA and SFA intake for HPA-axis functions and demonstrate effects on social behavior and social life.

Data availability

The datasets generated and/or analyzed during this study are available from the corresponding author on reasonable request.

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CRediT authorship contribution statement

Matthias Nemeth: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing - original draft, Writing - review & editing. **Daniela Schuster:** Data curation, Formal analysis, Investigation,

Validation, Writing - review & editing. **Eva Millesi:** Conceptualization, Methodology, Resources, Software, Supervision, Writing - review & editing. **Bernard Wallner:** Conceptualization, Methodology, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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