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Accepted Article

Dietary milk phospholipids attenuate chronic stress-induced changes in behaviour and endocrine response across the lifespan

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Running title: Milk phospholipid intake attenuates chronic stress

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Abbreviations: HPA: hypothalamic-pituitary-adrenal; MFGM: milk fat globule membrane; BMP: buttermilk powder; PND: postnatal day; OFT: open-field test; EPM: elevated-plus maze

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Accepted Article

Abstract

Scope. Increasing scientific evidence is validating the use of dietary strategies to support and improve brain health throughout the lifespan, with tailored nutritional interventions catering for specific life stages. Dietary phospholipid supplementations in early life and adulthood have been shown to alleviate some of the behavioural consequences associated with chronic stress. This study aims to explore the protective effects of a tailored phospholipid-enriched buttermilk on behavioural and endocrine responses induced by chronic psychosocial stress in adulthood, and to compare these effects according to the life stage at which the supplementation was started.

Methods and Results. We developed a novel phospholipid-enriched dairy product and assessed social, anxiety- and depressive-like behaviours, as well as the stress response and cognitive performance following chronic psychosocial stress in C57BL/6J mice, with supplementation beginning in adulthood or early life. Milk phospholipid supplementation from birth protected adult mice against chronic stress-induced changes in endocrine response to a subsequent acute stressor and reduced innate anxiety-like behaviour in non-stressed animals. When starting in adulthood, the dietary intervention reversed the anxiety-like phenotype caused by chronic stress exposure.

Conclusion. Dairy-derived phospholipids exert differential protective effects against chronic psychosocial stress depending on the targeted life stage and duration of the dietary supplementation.

Keywords: milk phospholipids – diet – chronic stress – anxiety – hypothalamic-pituitary-adrenal axis – life stages

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Introduction

The constituents that make up our diet play a key role in human physiology and metabolism, as well as mood and mental wellbeing (1,2). This has spurred an increased interest in the use of dietary strategies to support and improve brain health throughout the lifespan (3,4). For example, specific diets have shown to impact metabolic and neurodevelopmental processes in early life (5), modulate neuroplasticity and behaviour of adolescents (6), protect against the deleterious consequences of chronic stress in adulthood (7), and reduce physiological dysfunctions related to ageing (8). Among the large panel of nutrients available, phospholipids have been shown to improve brain health and behaviour across the lifespan in preclinical and human studies (9).

Phospholipids are amphiphilic compounds representing the major components of all biological membranes (10), and they are specifically enriched in milk fat globule membrane (MFGM), a natural source of phospholipids released by milk-producing cells as a globule surrounded by a lipid trilayer (11). Recent studies with targeted dietary supplementations with phospholipids have been used in humans and rodent models, and have shown promising potential against stress-induced alterations in behaviour and HPA axis response (12–17).

Chronic stress exposure has been shown to be a key risk factor for the development of major depression in humans (18), and the comorbid deficiencies in the activity of the hypothalamic-pituitary-adrenal (HPA) axis, as indexed by circulating glucocorticoids (19). To date, no dietary strategy has demonstrated efficacy in ameliorating the deleterious consequences of chronic stress exposure in adulthood.

In the present study, we explore the protective effects of two distinct dairy-derived phospholipid-enriched diets from early-life through to adulthood, or from adulthood onward, against behavioural and endocrine alterations induced by chronic psychosocial stress in adulthood. We selected these specific ages to match two important periods of life in mammals. At 8 weeks old, mice are

considered to reach full adulthood; while after post-natal day 0, it represents the window of first exposition to the environment. Using these ages allow us to answer the question whether phospholipids, as important nutritional components, can improve brain health in the short term (as provided only in adulthood), or in the long term (as provided from early life onwards). Specifically, we assessed their anxiety-like and depressive-like behaviour, as well as their social and cognitive performances. We found that supplementation with dairy-derived phospholipids from early life prevented the chronic psychosocial stress-induced alterations in the activity of the HPA axis as measured with the plasma corticosterone levels. In addition, we showed that when given in adulthood, milk phospholipids reversed the anxiety-like behaviour due to chronic stress exposure. Our results demonstrate a positive impact of milk phospholipid supplementation on chronic stress-related symptomology, with different effects depending on when the nutritional supplementation commenced.

Methods

Phospholipid enrichment of buttermilk powder substrate into diets

Prior to the beginning of the study we prepared a phospholipid-enriched dairy product. Spray dried buttermilk powder (BMP) was provided by Cremo SA (Fribourg, Switzerland) with compositional analysis by standard IDF protocols determining 5.29% fat, 33.7% protein and 45% carbohydrate, with a starting phospholipid content determined as 1.39%. Starting concentrations of individual phospholipids are recorded in **Table S1**. The phospholipid content of the BMP substrate and phospholipid-enriched fractions were measured by quantitative ^{31}P-NMR spectroscopy using a Bruker Avance III HD 500 MHz unit (Bruker, Massachusetts, USA) and carried out by Spectral Services (Köln, Germany). To concentrate the phospholipid content, protein and carbohydrate were removed by enzyme hydrolysis and ultrafiltration, as previously described (25, 27) with adaptations detailed in

the supplementary data. Digestive enzymes Alcalase® 2.4L (endoproteinase from Bacillus licheniformis) and Flavourzyme® 500L (endoproteinase and exopeptidase from Aspergillus oryzae) were obtained from Novozymes (Bagsværd, Denmark). Citric acid and NaOH were obtained from Sigma-Aldrich. The final concentration of the phospholipid-enriched BMP was 11.8% (Table S2). As a reference control, the commercially available product Lipamine M20 (Lecico GmbH; 16-21%) was used. The phospholipid composition of this product has been previously reported (40). Both powders were added to the diet at the manufacturing stage (ssniff Spezialdiäten GmbH, Germany) to yield a control chow, an APC_PL chow (supplemented with 3g of phospholipid-enriched BMP per 100g of chow), and a Lipamine chow (supplemented with 2.22 g of Lipamine per 100g of chow) (Table S2). Taking body weight into consideration, animals consumed approximately 450 mg/kg daily of dairy phospholipid supplementation of either Lipamine or APC-PL, which was chosen and in line with previous studies conducted in mice (17,20).

Animals

All animal protocols were approved by the Animal Experimentation Ethics Committee at University College Cork and by the Health Products Regulatory Authority of Ireland, in accordance with EU Directive 2010/63/EU. We assessed the impact of dietary supplementation, across 2 distinct administration periods, on behaviour and host physiology in male C57BL/6J mice (Envigo U. K.). In the adulthood study, the diet was first administered when the animals reached 8 weeks of age and was maintained throughout the stress protocol and behavioural testing, which began at weeks 11 and 14 respectively. In the early life study, the diet was administered first to the dams immediately after delivery and then to the offspring once they were weaned at 21 days post-delivery **(Figure 1A)**. Similarly, the diet was maintained throughout the stress protocol and behaviour. For the adulthood study, on the day of arrival, all mice were single housed and randomly put under the specific diets and kept under standard conditions (21 ± 1^{o}C, 55 ± 10% humidity), on a 12-h light/dark cycle (lights on at 07:00 h). In the early life study, we used adult males and females C57BL/6J from Envigo, U. K.

for breeding. After delivering their litter, the mothers were randomly put under specific diets and only the male pups were kept for the early life study. At 8 weeks old, all animals were singly housed and kept under the respective diets. In both studies, the body weight of animals and the food consumption were monitored weekly.

For the social defeat sessions, 45 male CD1 mice were purchased from Envigo, U. K. (9 weeks old at the beginning of the study) and screened for their aggressive behaviour prior to the first stress day. For two consecutive days, a conspecific mouse was introduced in the cage of the tested CD1 and the delay for the first attack to occur was measured. The CD1 with shortest attack latencies (n=41) were ranked as the most aggressive and were selected for the social defeat procedure. The 4 less aggressive CD1 are kept for the social interaction test.

Chronic psychosocial stress procedure

At 11 weeks of age, half of the animals were submitted to the chronic psychosocial stress paradigm, and this was continued daily until the culls at week 17. The procedure was carried out as previously described using intermittent bouts of social overcrowding and the psychosocial social defeat as stressors (21–24) and is detailed in the Supplementary Data.

Behavioural tests

Early life behaviour: To determine the effects of milk phospholipid supplementation during early life, males pups were assessed for anxiety-like behaviour at postnatal day 9 (PND) using ultrasonic vocalizations (USV). Male pups were separated from their mother and kept on a heat pad for 10 minutes (30-33°C). They were placed into a cage with an ultrasonic wave detector overhead for 3 minutes and were then returned to their mother. The number of USV was scored using the software SASLAB32 from Avisoft Bioacoustics, as an index of anxiety-like behaviour. At PND10, maternal attachment was tested using the maternal odour preference protocol. A cage separated in three parts with wire mesh separators contained the bedding from their own or another mother in the sides, and a clean (neutral) bedding in the middle. After 10 minutes of isolation on a heat pad, pups

were placed on the neutral bedding for one minute. Then the separators were removed and the animals were free to explore for two minutes. The session was recorded and the time spent on each bedding was later manually scored. The discriminatory preference for their mother's bedding is used as an index of social development.

At 14 weeks of age, all animals went through a battery of behavioural tests **(Figure 1B)**. Before all behavioural procedures, animals were acclimatized to the testing room for 1 hour, and all tests were carried out between 10:00 and 16:00 h. The protocols of the behavioural tests are further detailed in the Supplementary Data.

Social interaction test: After three weeks of chronic psychosocial stress, the social interaction test was performed at day 24 **(Figure 1B)** as previously described (23,24).

3-chamber social interaction test: At day 25 **(Figure 1B)**, the social behaviour was assessed in the 3-chamber social interaction test, following a published protocol (23,24).

Marble burying test: At day 26 **(Figure 1B)**, the marble burying test was performed as previously described (24) to assess neophobia, an anxiety-like behaviour to new objects (25).

Stress-induced hyperthermia: At day 28, the stress response was assessed using the stress-induced hyperthermia **(Figure 1B)** using a published protocol (24).

Open-field test (OFT): On day 32 **(Figure 1B)**, the response to a novel stressful environment and the locomotor activity were assessed as previously described (24).

Novel object recognition: On days 33 and 34 **(Figure 1B)**, the recognition memory was tested in the previous open-field using two different protocols depending on the study. For the early life study, an additional trial was added 1h30 after the acquisition phase. For each trial, we kept the same familiar object and two different novel objects were used (after 1h30 and 24h). Each trial lasted for 5 minutes. For the adulthood study, mice were placed in presence of two identical objects. 24 hours

after, mice were allowed to explore the familiar and a novel object. Each trial lasted 10 minutes. The time spent exploring each object in each trial was manually scored by a blinded experimenter. The discrimination index was calculated as follow: (time exploring the novel object – time exploring the familiar object) / (time exploring both objects).

Elevated-plus maze (EPM): To assess the anxiety-like behaviour, the EPM was used on day 35 **(Figure 1B)**, as previously described (24).

Forced swim test: To measure the antidepressant-like property of a phospholipid-enriched diet, the forced swim test was performed on day 35 for half of animals and day 36 for the other half **(Figure 1B)**, following a protocol published elsewhere (24).

Plasma collection

Blood from each animal was sampled by tail-tip 5 minutes before and 15, 45 and 90 minutes after the beginning of the swims, following a published method (24). The whole blood was taken using an EDTA-containing capillary, deposited in an Eppendorf and centrifuged for 15 minutes at 3,500 g, 4°C. Plasma was collected and stored at −80°C for later analysis.

Corticosterone quantification in plasma

Corticosterone quantification of plasma samples obtained in the forced swim test was performed using a corticosterone ELISA (Enzo Life Sciences, ADI-901-097), and was carried out using duplicates and according to the manufacturer's guidelines. The dilution factor of the samples was 1:40 except for the baseline timepoints (1:20), as they are expected to contain less corticosterone. Samples did not undergo any additional freeze-thaw cycles. The absorbance was read with a plate reader (Synergy HT, BioTek Instruments, Inc.) at 405 nm. For each assays, the sensitivity was around 25%, and intra- and inter-assay precisions were <15%. Duplicates with ≥ 15% coefficient of variability were re-analysed.

Statistics

All data were assessed for normality using the Shapiro-Wilk test. Outliers were removed according the results of the ROUT method (5%) using Graphpad Prism 6 (GraphPad Software Inc.). The following statistics were done with SPSS 26 (IBM Corp). The data related to the body weight and food intake were analysed using General Linear Model on repeated measures, followed by Fisher's least significant difference (LSD) post-hoc test. For all the other variables, normally distributed data were analysed using a one-way or a 2way ANOVA, followed by Fisher's LSD post-hoc test. Non-parametric datasets were analysed using the Kruskal-Wallis test, followed by the Mann-Whitney U test. A p value <0.05 was deemed significant.

Results

Phospholipid enrichment of buttermilk powder

Enrichment using the BMP substrate yielded an 8.5-fold increase in lipid content from 5.29% to 45.08% total lipid on a dry matter basis in the 50 kDa retentate (**Table 1**). Accordingly, total phospholipid content also increased 8.5-fold from 1.39% to 11.84%. Carbohydrate content decreased from 45% in the starting BMP substrate to 0.7% in the 50 kDa retentate, while protein content was reduced from 33.73% in the BMP substrate to 32.1% in the 50 kDa retentate. The content of individual phospholipids is recorded in **Table S2** (40).

Milk phospholipid supplementation affects body weight and food consumption

As expected, time had a significant effect on body weight (early life: $F_{(8,424)}=187.585$, $p=0.000$; **Figure 2A-B** – adulthood: $F_{(8,424)}=276.972$, $p=0.000$; **Figure 2E-F**) and food consumption (early life: $F_{(7,357)}=11.022$, $p=0.000$; **Figure 2C-D** – adulthood: $F_{(7,371)}=4.266$, $p=0.000$; **Figure 2G-H**) in both studies. In the early life study, phospholipids (APC_PL and Lipamine) significantly increased the body weight of non-stressed mice (**Figure 2A**), whereas chronic stress seemed to prevent this increase (**Figure 2B**) (stress effect $F_{(1,53)}=2.655$, $p=0.109$; diet effect: $F_{(2,53)}= 13.636$, $p=0.000$; interaction:

192 $F_{(2,53)}$= 2.563, p=0.087). APC_PL also increased the food intake, but only until mice were 11 weeks of
193 age (stress effect $F_{(1,51)}$=0.363, p=0.549; diet effect: $F_{(2,51)}$=5.058, p=0.010; interaction: $F_{(2,51)}$=0.334,
194 p=0.718; **Figure 2C-D**). In the adulthood study, phospholipid-enriched diets increased the body
195 weight of non-stressed (**Figure 2E**) and stressed mice (stress effect $F_{(1,53)}$=1.566, p=0.216; diet effect:
196 $F_{(2,53)}$=16.121, p=0.000; interaction: $F_{(2,53)}$=0.093, p=0.911; **Figure 2F**). APC_PL also increased the food
197 intake of non-stressed mice until 12 weeks of age (**Figure 2G**), and following chronic stress exposure
198 (stress effect $F_{(1,53)}$=0.737, p=0.394; diet effect: $F_{(2,53)}$=19.714, p=0.000; interaction: $F_{(2,53)}$=1.513,
199 p=0.230; **Figure 2H**).

200 **Milk phospholipid supplementation reduces the anxiety-like behaviour**

201 In the early life study, mice were submitted to two behavioural tests at PND9 and 10. Pups from
202 mothers fed with phospholipid-enriched diets emitted less USV than pups from mothers receiving
203 the control diet (One-way ANOVA: $F_{(2,59)}$=5.056, p=0.010; **Figure 3A)**. The following day, the
204 attachment to the mother was tested. Pups that did not move from the neutral bedding (where they
205 were put at the beginning of the test) were removed from the analysis. We did not find any
206 difference between groups in the time spent in the bedding of their own or from another mother
207 (Kruskal Wallis H=3.458, p=0.177; Kruskal Wallis H=3.578, p=0.167; **Table 2**).

208 In the OFT, the anxiety-like behaviour was assessed by measuring the time spent and the number of
209 entries in the center of the arena. In the early life study, the 2way ANOVA revealed an overall effect
210 of the stress on the time spent in the center (stress $F_{(1,56)}$=5.182, p=0.027; diet $F_{(2,56)}$=7.578, p=0.001;
211 interaction $F_{(2,56)}$=1.292, p=0.284), but the post-hoc test failed to highlight any significant difference
212 between groups **(Table 2)**. Nevertheless, chronic stress exposure significantly decreased the number
213 of entries in the center of the open-field and this was not reversed by the diets (stress $F_{(1,58)}$=16.719,
214 p=0.000; diet $F_{(2,58)}$=1.526, p=0.227; interaction $F_{(2,58)}$=0.367, p=0.695; **Figure 3B**). In the adulthood
215 study, the time spent in the center remained unchanged (stress $F_{(1,58)}$=2.662, p=0.109; diet
216 $F_{(2,58)}$=0.107, p=0.899; interaction $F_{(2,58)}$=0.212, p=0.810; **Table 3**). However, chronic stress exposure

significantly decreased the number of entries in the center (stress $F_{(1,58)}$=10.639, p=0.002; diet $F_{(2,58)}$=0.127, p=0.881; interaction $F_{(2,58)}$=0.123, p=0.884; **Figure 3D**). The behaviour of stressed mice fed with the dairy phospholipids did not differ from the non-stressed ones **(Figure 3D)**, suggesting a protective effect of phospholipid against stress-induced anxiety-like behaviour measured in the OFT.

In the early life study, chronic stress exposure decreased the time spent and the number of entries in the open arms of the EPM (time: stress $F_{(1,57)}$=38.870, p=0.000; diet $F_{(2,57)}$=4.218, p=0.020; interaction $F_{(2,57)}$=0.171, p=0.843; **Figure 3C** – entries: Kruskal Wallis H=20,125, p=0.001; **Table 2**), and diet did not change these alterations. Moreover, non-stressed mice receiving APC_PL spent more time in the open arms than controls **(Figure 3C)**, demonstrating that APC_PL taken since early life could exert an anxiolytic activity in non-stressed animals. Chronic stress also altered these measures in the adulthood study (time: stress $F_{(1,57)}$=10.453, p=0.002; diet $F_{(2,57)}$=6.560, p=0.003; interaction $F_{(2,57)}$=2.490, p=0.093; **Figure 3E** – entries: stress $F_{(1,57)}$=38.074, p=0.000, diet $F_{(2,57)}$=0.218, p=0.805; interaction $F_{(2,57)}$=0.857, p=0.430; **Table 3**). Stressed mice under APC_PL supplementation tended to spend more time in open arms than those receiving the control diet (p=0.056, **Figure 3E**), showing that APC_PL could attenuate the chronic stress-induced anxiety-like behaviour.

The marble burying test was also used to explore the anxiety-like behaviour. In the early life study, there was a significant effect of the stress (Kruskal Wallis H=26,514, p=0.000). Indeed, stressed animals buried more marbles than non-stressed mice of each respective diet regardless of diet **(Table 2)**. In the adulthood study, the Kruskal Wallis test revealed significant differences between groups (Kruskal Wallis H=12.333, p=0.031). In non-stressed animals, there was an increase in the number of marbles buried by animals receiving Lipamine **(Table 3)**. In stressed animals, only APC_PL significantly increased the number of buried marbles, compared to stressed animals receiving the control diet **(Table 3)**.

Milk phospholipid supplementation does not affect social behaviour and recognition memory

In the social interaction test, there was no effect of stress or diet on the social interaction ratio (early life study: stress $F_{(1,58)}$=0.583, p=0.449; diet $F_{(2,58)}$=0.572, p=0.568; interaction $F_{(2,58)}$=0.215, p=0.807; **Table 2** – adulthood: Kruskal Wallis H=3.877, p=0.567; **Table 3**) and on the social avoidance (early life study: stress $F_{(1,57)}$=1.489, p=0.228; diet $F_{(2,57)}$=0.006, p=0.994; interaction $F_{(2,57)}$=1.123, p=0.333; **Table 2** – adulthood: stress $F_{(1,54)}$=15.481, p=0.000; diet $F_{(2,54)}$=2.871, p=0.066; interaction $F_{(2,54)}$=0.621, p=0.541; **Table 3**).

In the three-chamber test, there was no difference in sociability between groups (early life study: Kruskal Wallis H=11.045, p=0.005; **Table 2** – adulthood: stress $F_{(1,58)}$=0.008, p=0.930; diet $F_{(2,58)}$=0.828, p=0.443; interaction $F_{(2,58)}$=2.321, p=0.108; **Table 3**). All mice spent more time interacting with the mouse rather the object, as revealed by a 1-sample t-test comparing each mean to 50% ($p<0.001$; where 50% means the experimental mouse spent the same amount of time interacting with the object or the conspecific). Again, there was no difference in social novelty between groups (early life study: stress $F_{(1,59)}$=0.022, p=0.882; diet $F_{(2,59)}$=0.597, p=0.554; interaction $F_{(2,59)}$=0.325, p=0.724; **Table 2** – adulthood: Kruskal Wallis H=13.998, p=0.016; **Table 3**).

Cognitive performance was assessed using the novel object recognition test under two different protocols. In the early life study, the memory of the animals was tested 1h30 and 24h after the acquisition phase. No effect of the stress or the diet was found significant at 1h30 (stress $F_{(1,53)}$=0.001, p=0.974; diet $F_{(2,53)}$=1.212, p=0.306; interaction $F_{(2,53)}$=0.278, p=0.758) and 24h (stress $F_{(1,53)}$= 1.317, p=0.256; diet $F_{(2,53)}$=0.400, p=0.672; interaction $F_{(2,53)}$=1.916, p=0.157) **(Table 2)**. In adulthood, the recognition memory was tested 24h after the acquisition phase, and the cognitive performances of the mice were similar between groups (stress $F_{(1,53)}$= 0.042, p=0.838; diet $F_{(2,53)}$=2.733, p=0.074; interaction $F_{(2,53)}$=0.438, p=0.648) **(Table 3)**.

Milk phospholipid supplementation affects locomotor activity

The locomotor activity was monitored in the open-field apparatus. A 2way ANOVA showed significant differences in locomotor activity between groups in both studies (early life: stress

267 $F_{(1,58)}$=12.342, p=0.001; diet $F_{(2,58)}$=5.648, p=0.006; interaction $F_{(2,58)}$=0.304, p=0.739; **Table 2** –
268 adulthood: stress $F_{(1,56)}$=13.740, p=0.001; diet $F_{(2,56)}$=3.276, p=0.046; interaction $F_{(2,56)}$=0.893,
269 p=0.416; **Table 3**). In the early life study, the locomotor activity was reduced by APC_PL in both non-
270 stressed and stressed mice. In addition, stress and Lipamine had an additive effect in reducing the
271 locomotor activity **(Table 2)**. When the dietary intervention started in adulthood, mice submitted to
272 chronic stress and fed with APC_PL had reduced locomotor activity **(Table 3)**.

273 **Milk phospholipid supplementation does not affect depressive-like behaviour**

274 The forced swim test was used to assess the depressive-like behaviour. Phospholipid
275 supplementation in early life had no effect on the immobility time (Kruskal Wallis H=9.596, p=0.088)
276 **(Table 2)**. Nevertheless, in the adulthood study, there was an overall effect of the stress (stress
277 $F_{(1,56)}$=7.214, p=0.010; diet $F_{(2,56)}$=1.473, p=0.239; interaction $F_{(2,56)}$=0.153, p=0.858). Post-hoc analysis
278 did not reveal any significant difference between groups **(Table 3)**.

279 **Milk phospholipid supplementation alleviates the stress response**

280 To assess the stress response, we performed the stress-induced hyperthermia test. In the early life
281 study, exposure to chronic stress decreased this measure (Kruskal Wallis H=17,709, p=0.003) **(Table**
282 **2)**, and this was not reversed by any phospholipid supplementation. In the adulthood study, there
283 was a significant effect of the stress on stress-induced hyperthermia (Kruskal Wallis H=19.418,
284 p=0.002; **Table 3**). In addition, APC_PL reduced the variation in body temperature following the
285 acute stressor, showing a potential protective effect of this diet **(Table 3)**.

286 The stress response was also studied by measuring the corticosterone plasma levels before and after
287 the forced swim test, representing an acute stressor **(Figure 4)**. In the early life study, chronic stress
288 increased the levels of plasma corticosterone in basal conditions and this was reversed by
289 phospholipid intake (stress $F_{(1,51)}$=16.287, p=0.000; diet $F_{(2,51)}$=1.447, p=0.246; interaction
290 $F_{(2,51)}$=1.796, p=0.177; **Figure 4A**). The suppressive effect of dietary supplementation with
291 phospholipids on stress-related plasma corticosterone levels were still present 15 (stress

292 $F_{(1,52)}$=6.940, p=0.001; diet $F_{(2,52)}$=2.374, p=0.104; interaction $F_{(2,52)}$=2.880, p=0.066) and 45 minutes
293 after the beginning of the swims (stress $F_{(1,46)}$=7.499, p=0.009; diet $F_{(2,46)}$=0.831, p=0.443; interaction
294 $F_{(2,46)}$=2.323, p=0.111). After 90 minutes, the corticosterone levels went back to control for all groups
295 (stress $F_{(1,51)}$=7.905, p=0.007; diet $F_{(2,51)}$=1.287, p=0.286; interaction $F_{(2,51)}$=0.268, p=0.766; **Figure 4B**).
296 In the adulthood study, chronic stress exposure also altered the levels of plasma corticosterone in
297 basal conditions (stress $F_{(1,45)}$=15.534, p=0.000; diet $F_{(2,45)}$=2.745, p=0.076; interaction $F_{(2,45)}$=0.063,
298 p=0.939; **Figure 4C**), 15 minutes (stress $F_{(1,44)}$=56.405, p=0.000; diet $F_{(2,44)}$=0.362, p=0.699; interaction
299 $F_{(2,44)}$=1.234, p=0.302) and 45 minutes (stress $F_{(1,41)}$=19.990, p=0.000; diet $F_{(2,41)}$=1.024, p=0.369;
300 interaction $F_{(2,41)}$=4.143, p=0.024) after the swims **(Figure 4D)**. After 90 minutes, all corticosterone
301 levels went back to control (stress $F_{(1,41)}$=3.371, p=0.075; diet $F_{(2,41)}$=0.159, p=0.854; interaction
302 $F_{(2,41)}$=0.075, p=0.928; **Figure 4D**). No effects of diets were detected.

303

304 Discussion

305 In this study we demonstrate that dietary phospholipids derived from milk have differential
306 beneficial effects on alterations induced by chronic stress exposure in adulthood; depending on the
307 life stage targeted or the duration of the supplementation. More precisely, when a phospholipid-
308 enriched diet is administered from early life and throughout the lifespan, it prevented chronic stress-
309 induced increases in corticosterone plasma levels in response to an acute stressor. When the dietary
310 intervention started in adulthood, it reversed the anxiety-like behaviour due to chronic psychosocial
311 stress.

312 We also compared two different phospholipid enriched diets, one consisting of an in house prepared
313 buttermilk enriched with phospholipids (26), and one containing a commercially available
314 concentrate of milk phospholipids (Lipamine), both resulting in a similar daily phospholipid
315 consumption (450 mg/kg). As phospholipids are a type of fat, they induced an increase in body

316 weight in non-stressed animals, without affecting their food consumption. Interestingly, chronic
317 stress prevented this increase in animals receiving phospholipids from early life, in line with previous
318 findings from our laboratory (21,27). When taken in adulthood, the dairy-derived phospholipids
319 increased both body weight and food consumption in stressed mice. This finding is in accordance
320 with previous studies showing that chronic social stress can induce hyperphagia in subordinate mice
321 (23,28,29). Our data suggest that starting the dietary intervention in early life is critical to obtain the
322 protective effect against stress-induced hyperphagia. Although it would have provided important
323 dietary insights for our study, we decided to not weigh the animals before weaning. Indeed, handling
324 animals at this life stage could have generated excessive stress in both dams and pups.

325 Since the body weight of mice was susceptible to change by dairy-derived phospholipids, we
326 measured the distance moved in the OFT, to measure the locomotor activity of adult animals. It was
327 shown that 3 weeks of stress altered the locomotor activity of mice, but this was no longer observed
328 after 6 weeks, suggesting an adaptation to stress on this behaviour (27). Accordingly, our results
329 revealed no effect of stress on locomotor activity of mice receiving the control chow after 5 weeks of
330 stress. However, phospholipids taken from birth decreased this parameter, while when taken in
331 adulthood, this occurred only in stressed mice.

332 Phospholipid metabolism has previously been linked to the pathophysiology of anxiety disorders
333 (30,31). Our data clearly demonstrated a positive effect of milk phospholipid supplementation on
334 anxiety-related behaviours. When the supplementation started in early life, the dairy-derived
335 phospholipids reduced the number of USV in pups transiently separated from their mothers. When
336 these animals reach adulthood, milk phospholipid-enriched diets increased the time spent in the
337 open arms in the EPM in non-stressed conditions. As previously described, anxiolytic treatments are
338 expected to reduce the anxiety-like behaviour of rodents and increase their time spent in the open
339 arms (32,33). Our findings suggest for the first time that milk phospholipids exert an anxiolytic-like
340 effect in control mice. When the dietary supplementation started in adulthood, we showed that

341 unlike Lipamine, APC_PL could specifically prevent the anxiety-like phenotype induced by chronic
342 stress exposure. As animals received the same overall concentration of dietary milk phospholipids,
343 APC_PL might be more efficient in modulating anxiety-like behaviour (Figure S2). This finding could
344 be related to the individual phospholipid composition of both powders. Indeed, there is a plethora of
345 types of phospholipids that could lead to different effects depending on their relative amount. In our
346 study, this was the only difference we found between the effects of the two different phospholipid-
347 enriched diets. Previous work determined that phospholipid supplementation for two months in
348 adulthood did not reverse the anxiety-like behaviour induced by a developmental deficiency in α-
349 linolenic acid (34). Therefore, our results may be specific to behavioural impairments caused by
350 chronic stress exposure, differences in stress susceptibility or be due to different compositions,
351 relative amounts and bioavailability of the phospholipids. The reason why this beneficial effect on
352 behaviour is not observed in animals receiving the supplementation in early stages of life remains
353 unclear. Overall, we demonstrate a protective effect of milk phospholipids on stress-induced
354 physiology and behavior, but further studies are warranted to investigate precise molecular
355 mechanism.

356 Our behavioural data suggest that the beneficial effects of milk phospholipids may be specific to the
357 anxiety-like phenotype. Indeed, we found no effect of chronic intake of phospholipids in the
358 immobility time during the forced swim test, which is used to screen antidepressant-like properties
359 of molecules (35). To date, only two studies used the forced swim test to explore the effects of
360 phospholipids on depressive-like behaviour in mice (17,20). In the first one, authors showed that 7
361 days of oral administration of phosphatidylcholine reduced the immobility time of animals
362 submitted to 3 days of restraint stress (17). The short-term efficacy of the treatment may be related
363 to the duration of the treatment itself, or to the compound administered. Indeed, the
364 supplementation was enriched in phosphatidylcholine alone, rather than in complex phospholipids
365 found in milk. Another study found that a mixture of phospholipids taken for six weeks, could
366 reverse immobility time in the forced swim test in female mice (20). Memory performances were

previously shown to be promoted by phospholipid-enriched diets (9). In particular, mice receiving a phospholipid supplementation from PND16 to 44 had better recognition memory in the novel object recognition task in adulthood (36). It is noteworthy that our protocol was different in comparison. Indeed, mice were allowed to explore the familiar objects in two trials before the recognition phase, itself occurring only 4 hours after the familiarization (36). Our data revealed no beneficial effect of phospholipids on recognition memory. It is possible that protocols of novel object recognition test that we used were not optimal to highlight the potential beneficial effects on memory of the phospholipid supplementations. In addition, phospholipid-enriched diets did not show an effect on social behaviour in mice, independently to when the intervention started. To our knowledge, no study has explored the effects of phospholipids on social behaviour. Social behaviour engages specific mechanisms that could be independent from phospholipid pathways, although this needs to be confirmed in further studies.

We also assessed the endocrine responses to acute stress following the intake of phospholipid-enriched milk diets. Chronic psychosocial stress is known to enhance basal levels of corticosterone in the plasma as well as its elevation after an exposure to an acute stress, reflecting an hyperactivity of the HPA axis (24). A blunted cortisol response to stress due to phospholipid supplementation in healthy adult was proposed in human studies (12,37) but it is unknown if phospholipids could have beneficial effects on endocrine response to stress in chronically stressed individuals. Here, we brought evidence supporting this idea, since dairy-derived phospholipid supplementation starting at birth and continuing across the lifespan prevented the hyperactivity of the HPA axis in stressed mice. More importantly, this was not observed when animals received the supplementation in adulthood. When starting at adolescence, dietary supplementation with MFGM could normalize the HPA axis response to restraint stress in a rat model of maternal separation (38). Although this study used an early life stress instead of an adulthood stress, it suggests that intervention in adolescence could be enough to preserve HPA axis integrity. Early life and adolescence are critical neurodevelopmental periods influenced by nutrition (39,40). The phospholipid supplementation may act on the

development of stress pathways that would explain the protective effect they have on corticosterone response to stress. This hypothesis is supported by the fact that this is not seen in mice receiving the supplementation in adulthood when the stress pathways are mature. Future investigations should assess the effects of phospholipid supplementation on the postnatal development of the HPA axis. In addition, different mixtures of phospholipids should be investigated to discriminate the beneficial effects of dietary phospholipids in stress, and further studies are warranted. Another important consideration is the duration of the dietary intervention and further investigate the effect of dietary phospholipids on critical life stages, including adolescence versus early life.

Overall, this study highlights differential beneficial effects of milk phospholipids in stress response depending on when the intervention started. We decided to target the early life, a period of most rapid brain development, and adulthood, during which stress-related disorders may appear following chronic stress exposure. In early life, dietary interventions can have long-lasting consequences (41). Here, we showed that supplementation with milk phospholipids during the early life period led to a significant reduction of the anxiety-like behaviour in pups and non-stressed animals, and to a protective effect on the reactivity of the HPA axis in adulthood. In parallel, when this intervention occurred in adulthood, APC_PL specifically reversed the anxiety-like phenotype induced by chronic psychosocial stress. This clearly demonstrated that the life stage during which the dietary phospholipid intervention begins is critical in the modulation of behaviour and endocrine output regulated by the HPA axis. These findings demonstrate the promising potential of diet as a targetable and tangible dietary intervention strategy to counteract the deleterious effects of chronic psychological stress. Most notably, this study also highlights the importance to consider critical developmental time windows in the context of stress response and dietary interventions. Future studies should investigate the mechanism of dairy-derived phospholipids and its associated health benefits at specific life stages and considering sex differences. Given the limited number of papers investigating the effects of dietary phospholipids and the multitude of phospholipids available, much

419 further work is needed to disentangle the mechanistic effects of each family of phospholipids,
420 duration of exposure and effect at different life stages. In conclusion, we have generated and
421 analysed a novel phospholipid-enriched buttermilk, which can be used to further investigate
422 mechanistic effects of dairy-derived phospholipids in stress response and stress-related diseases.
423 Moreover, the novel phospholipid-enriched buttermilk, described here, has promising potential as a
424 commercially attractive ingredient with advantageous potential to allow fortification of innovative
425 nutritional strategies to promote brain health across the lifespan.

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Statement of authors' contributions. MS conducted the experimental work, the data analysis and initial writing of the manuscript. FD and AM equally contributed to the manuscript. FD played a significant role in conducting the in vivo work. AM performed and optimised the phospholipid-enrichment of the buttermilk and subsequent analysis and characterizations of the final product. PF assisted with the in vivo work. KR contributed to the data analysis. HS, JFC, TGD, and CS were responsible for the conception and the design of the study. Interpretation of data analysis was performed by MS, KR, JFC and HS. TGD, JFC, CS, PS, KR and BR contributed to the manuscript and its revisions. HS performed final revisions of the manuscript. All authors approve the final version of the manuscript and agree to be accountable for all aspects of the work.

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Table 1: Compositional analysis of phospholipid-enriched buttermilk (APC_PL) powder

Component	BMP substrate	Phospholipid-enriched 50kDa retentate	Low-fat 50kDa permeate
Protein	33.7%	32.1%	24.21%
Total Fat (including total PL)	5.29%	45.08%	1.8%
(Total PL)	(1.39%)	(11.84%)	(0.4%)
Carbohydrate	45%	0.7%	69.8%
Ash	7.33%	15.4%	2.04%
Moisture	3.32%	2.32%	1.66%

Table 2: Behavioural measurements in the early life study. * represents the significant differences compared to the control diet with the same stress treatment (diet effect); # represents the significant differences compared to the respective non stress group of the same diet (stress effect). N. A.: not applicable. *p<0.05; **p<0.01; # p<0.05; ## p<0.01; ### p<0.001

	Non-stressed Control	Non-stressed APC_PL	Non-stressed Lipamine	Stressed Control	Stressed APC_PL
lizations (USV)					
V	112.78±21.61	84.90±16.56*	58.22±8.37**	N. A.	N. A.
own mother bedding (%)	32.50±8.01	44.92±10.60	60.28±12.55	N. A.	N. A.
other mother bedding (%)	5.14±1.49	2.50±1.05	0.93±0.49	N. A.	N. A.
n test					
tio (%)	118.18±6.89	110.54±3.95	105.15±4.52	109.36±6.10	104.20±7.70
the corners (s)	18.49±2.92	14.20±2.38	14.15±1.97	10.60±1.36	15.24±4.52
al interaction test					
me mouse versus object (%)	71.81±4.21	66.33±1.71	63.85±3.99	74.16±2.88	74.22±0.91##
me new versus familiar mouse (%)	53.98±5.07	50.15±4.15	60.98±5.28	58.03±4.50	58.67±2.94
test					
arbles buried	14.10±1.28	13.38±1.43	14.50±0.73	18.11±0.39##	17.56±0.41##
hyperthermia					
ody temperature (Δ)	1.56±0.15	1.35±0.18	1.46±0.14	0.84±0.12###	0.90±0.15#
nter (s)	135.45±6.64	149.69±17.67	173.87±24.71	103.18±13.12	107.08±15.28# 1
ed (cm)	3866.41±298.12	3225.19±271.35*	3468.95±206.02	3474.46±183.99	2644.85±155.46** 26
cognition					
n index 1h30	-0.06±0.087	0.07±0.09	0.17±0.10	0.03±0.17	-0.01±0.11
n index 24h	0.06±0.06	0.07±0.13	0.26±0.11	0.18±0.15	-0.04±0.10
naze					
tries in open arms	4.10±0.86	5.38±0.78	5.13±0.83	1.78±0.66#	1.89±0.39##
st					
ne (s)	86.50±6.99	80.13±9.81	79.88±5.99	88.67±6.71	104.78±6.38

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Table 3: Behavioural measurements in the adulthood study. * represents the significant differences compared to the control diet with the same stress treatment (diet effect); # represents the significant differences compared to the respective non stress group of the same diet (stress effect). *p<0.05; **p<0.01; # p<0.05; ## p<0.01; ### p<0.001

	Non-stressed Control	Non-stressed APC_PL	Non-stressed Lipamine	Stressed Control	Stressed APC_PL	Stressed Lipamine
	125.32±32.94	81.46±15.57	116.01±14.58	143.23±41.66	87.95±22.24	89.85±13.98
	2.67±0.57	3.06±0.62	3.84±1.20	1.56±0.51	3.20±0.62	1.13±0.36$^{###}$
	62.30±4.50	62.84±3.29	74.35±2.18	70.15±5.41	66.86±2.58	63.02±3.55
	63.43±4.40	60.08±5.18	54.06±3.42	60.31±6.07	57.62±2.60	71.73±3.73$^{##}$
	11.17±2.40	14.75±2.93	16.00±1.18*	14.25±1.36	16.60±1.17*	15.17±1.22
	1.28±0.21	1.68±0.17	1.31±0.10	1.04±0.10	0.66±0.05**$^{##}$	1.03±0.14
	71.99±13.30	56.70±14.67	42.37±10.89	47.55±10.36	63.47±17.31	56.75±21.82
	3769.08±345.70	3871.40±396.38	3533.22±78.06	3365.68±177.87	2940.08±287.83*$^{#}$	3081.82±181.33
	0.04±0.09	0.25±0.06	0.00±0.08	0.11±0.08	0.18±0.08	0.05±0.11
	12.00±1.53	10.75±2.32	11.57±2.47	4.00±0.78$^{###}$	4.80±1.20$^{##}$	5.33±1.73$^{###}$
	84.42±6.99	75.91±8.01	79.88±6.00	95.27±8.16	101.40±6.63	101.86±7.66

Figure legends

Figure 1. Experimental design of the early life and adulthood studies.
A: In the early life study, the mothers were put under specific diets the day they gave birth, and the animals were kept under the specific diet after weaning. Animals were submitted to 2 behavioural tests: the isolation-induced ultrasonic vocalizations and the attachment to the mother, at postnatal day (P) 9 and 10 respectively. In the adulthood study, animals were put under the specific diets at 8 weeks old. In both studies, animals were single housed at 8 weeks old (w), and half of them were submitted to the chronic psychosocial stress protocol when they were 11 weeks old until the end of the experiments. All mice underwent a battery of behavioural tests starting at 14 weeks old and were culled at 16 weeks old.
B: Timeline of the battery of behavioural tests. Day (D) 0 is the first day of psychosocial stress. SIT: social interaction test, 3CSIT: 3-chamber social interaction test, MB: marble burying test, SIH: stress-induced hyperthermia, OFT: open-field test, NOR: novel object recognition, EPM: elevated-plus maze, FST: force swim test.

Figure 2. Body weight and food intake over time.
Body weight (A-B) and daily food intake (C-D) of non-stressed and stressed mice from the early life study. Phospholipids (APC_PL and Lipamine) increased the body weight in non-stressed mice of the early life study (A), and chronic stress prevented this increase in body weight in stressed animals (B). Phospholipids (APC_PL) increased the food intake until non-stressed (C) and stressed (D) mice were 11 weeks old.
Body weight (E-F) and daily food intake (G-H) of non-stressed and stressed mice from the adulthood study. Phospholipids (APC_PL and Lipamine) increased the body weight in non-stressed (E) and stressed (F) mice. In non-stressed mice phospholipids (APC_PL) also increased the food intake until 12 weeks of age (G), and this was observed across the lifespan in stressed animals (H).
Data are expressed as mean ± SEM (n=7-12 per group). * represent the significant differences compared to the control diet with the same stress treatment (diet effect), *p<0.05; **p<0.01; ***p<0.001.

Figure 3. Effects of a phospholipid-enriched diet on anxiety-like behaviour.
(A) Isolation-induced ultrasonic vocalization (USV). Diets enriched in phospholipids (APC_PL and Lipamine) decreased the number of USV.
Number of entries in the center in the open-field test (B) and time spent in open arms of the elevated-plus maze (C) in the early life study. There was no effect of intake of phospholipid from early life on the anxiety-like behaviour induced by chronic psychosocial stress.
Number of entries in the center in the open-field test (D) and time spent in open arms of the elevated-plus maze (E) in the adulthood study. Phospholipid enriched diets (APC_PL) taken in adulthood could reverse the anxiety-like behaviour induced by chronic psychosocial stress.
Data are expressed as mean ± SEM (n=7-12 per group). * represent the significant differences compared to the control diet with the same stress treatment (diet effect), # represent the significant differences

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Accepted Article

compared to the respective non-stressed group of the same diet (stress effect). *p<0.05; **p<0.01; # p<0.05; ## p<0.01; ### p<0.001.

Figure 4. Hypothalamic-pituitary-adrenal axis response after exposure to an acute stress.
Basal corticosterone plasma levels are altered in chronically stressed animals (A & B), and this was reversed by a phospholipid dietary supplementation (APC_PL and Lipamine) since in early life (A) but not in adulthood (C).
The corticosterone release following the forced swim test (acute stress) was altered by chronic psychosocial stress in both studies. A diet enriched in phospholipids (APC_PL and Lipamine) could protect against this alteration when taken since birth (B) but not in adulthood (D).
Data are expressed as mean ± SEM (n=6-11 per group). * represent the significant differences compared to the control diet with the same stress treatment (diet effect), # represent the significant differences compared to the respective non stress group of the same diet (stress effect). *p<0.05; **p<0.01; # p<0.05; ## p<0.01; ### p<0.001.

Figure 1

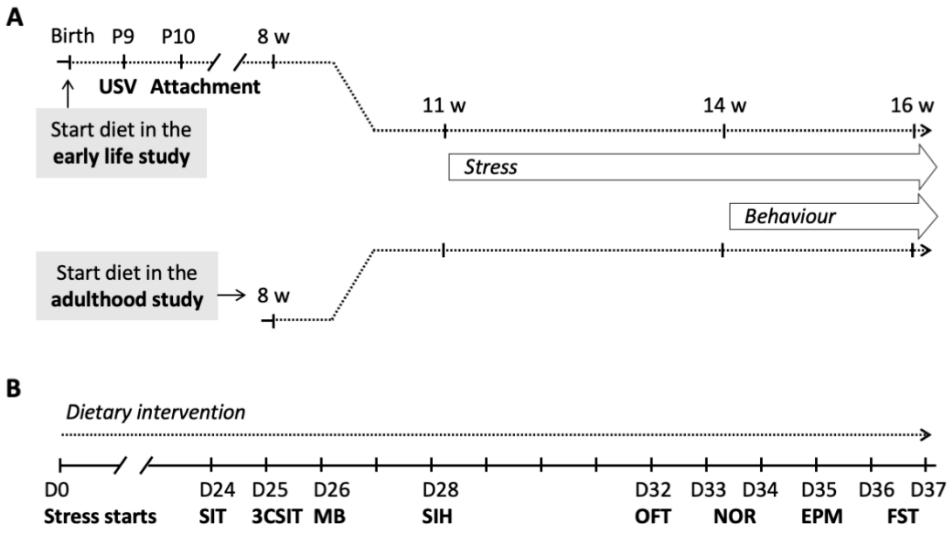

A
Birth
P9
P10
8 w
USV
Attachment
Start diet in the
early life study
11 w
14 w
16 w
Stress
Behaviour
Start diet in the
adulthood study
8 w
B
Dietary intervention
D0
Stress starts
D24
SIT
D25
3CSIT
D26
MB
D28
SIH
D32
OFT
D33
NOR
D34
D35
EPM
D36
FST
D37

Figure 2

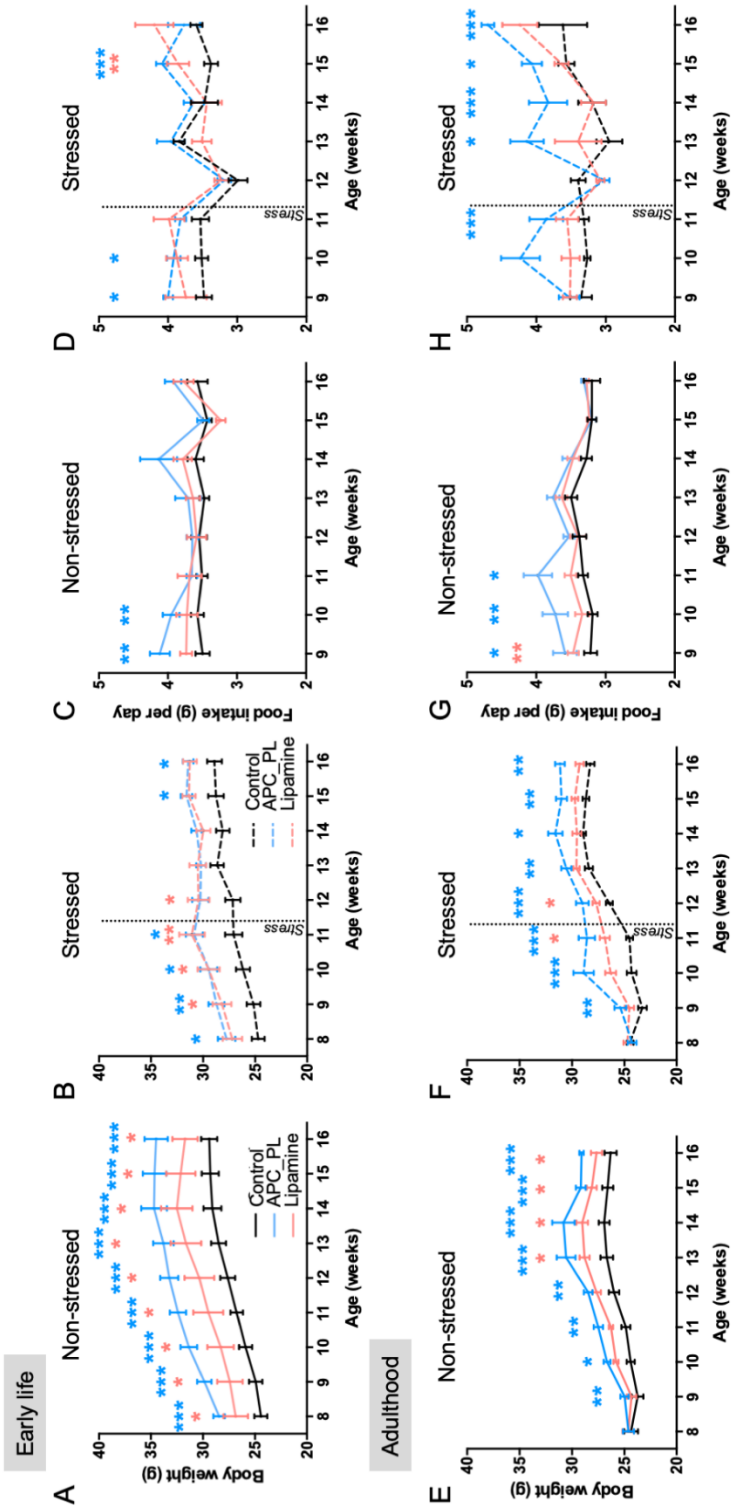

Early life
Adulthood
A
B
C
D
E
F
G
H
Non-stressed
Stressed
Body weight (g)
Food intake (g) per day
Age (weeks)
Stress
Control
APC_PL
Lipamine

Figure 3

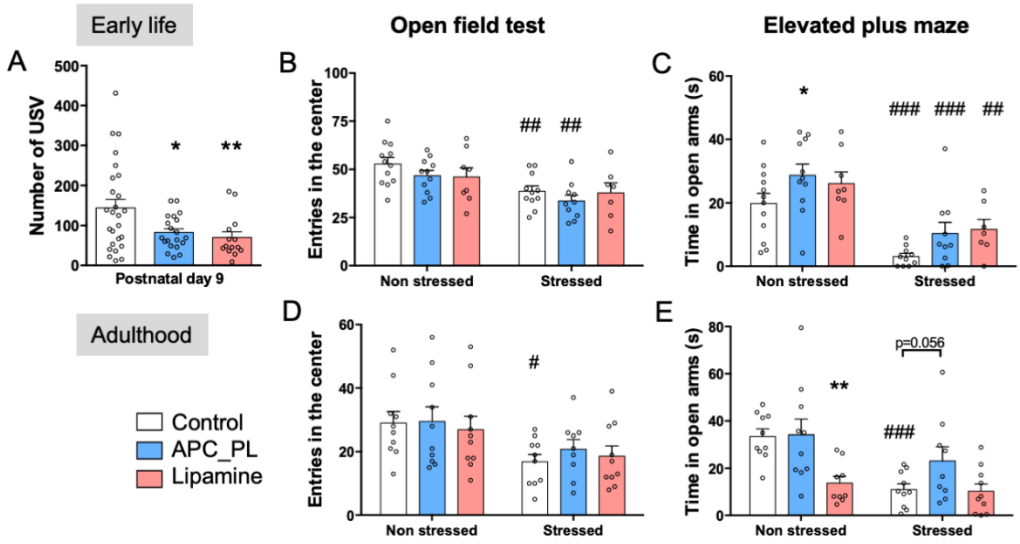
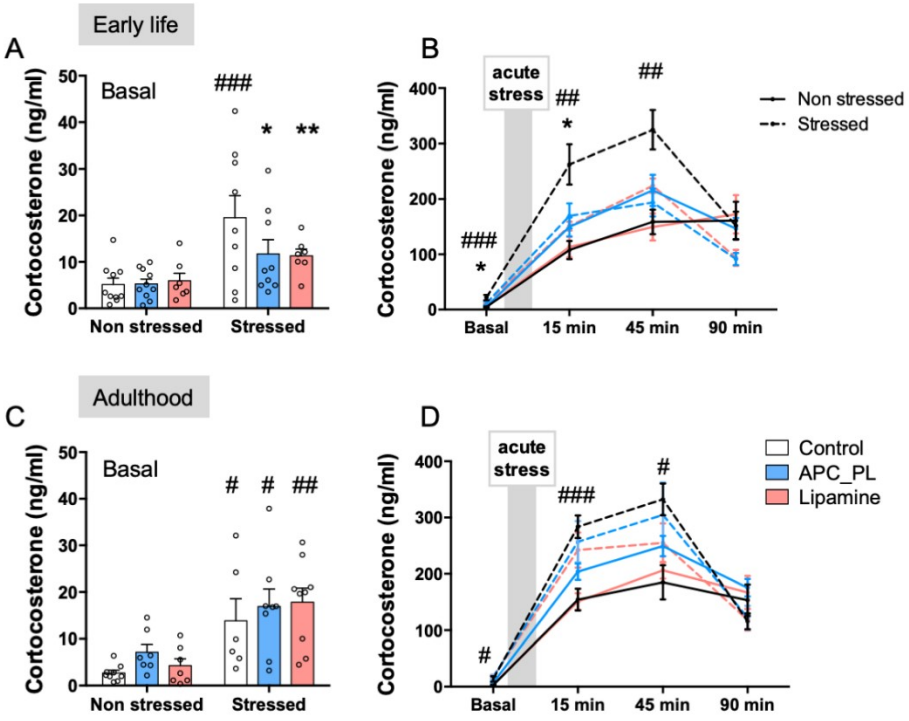

Early life
Open field test
Elevated plus maze
A
B
C
Number of USV
Postnatal day 9
Entries in the center
Non stressed
Stressed
Time in open arms (s)
Adulthood
D
E
Control
APC_PL
Lipamine
p=0.056

Figure 4


A
Early life
Basal
Cortocosterone (ng/ml)
Non stressed
Stressed
B
acute stress
Non stressed
Stressed
Basal
15 min
45 min
90 min
C
Adulthood
D
Control
APC_PL
Lipamine

Dairy-derived phospholipids exert differential protective effects against chronic psychosocial stress in C57BL/6J mice depending on the targeted life stage and duration of the dietary supplementation. Milk phospholipid supplementation from birth protected adult mice against chronic stress-induced changes in endocrine response to a subsequent acute stressor and reduced innate anxiety-like behaviour in non-stressed animals. Specifically, corticosterone plasma levels following chronic stress were reduced by phospholipid intake when supplementation started in early life. When starting in adulthood, the dietary intervention reversed the anxiety-like phenotype caused by chronic stress exposure.

