Medium and long-term effects of low doses of Chlorpyrifos during the postnatal, preweaning developmental stage on sociability, dominance, gut microbiota and plasma metabolites

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Abstract

Autism spectrum disorder (ASD) is a complex neurodevelopmental pathology characterized by altered verbalizations, reduced social interaction behavior, and stereotypies. Environmental factors have been associated with its development. Some researchers have focused on pesticide exposure. Chlorpyrifos (CPF) is the most used Organophosphate. Previous developmental studies with CPF showed decreased, enhanced or no effect on social outcomes eminently in mice. The study of CPF exposure during preweaning stages on social behavior is sparse in mice and non-existent in rats. Perinatal stressors could be at the basis of ASD development, and around postnatal day 10 in the rat is equivalent to the human birthday in neurodevelopmental terms. We explored the effects of exposure to low doses (1mg/kg/mL/day) of CPF during this stage regarding: sociability, dominance gut microbiome and plasma metabolomic profile, since alterations in these systems have also been linked to ASD. There was a modest influence of CPF on social behavior in adulthood, with null effects during adolescence. Dominance and hierarchical status were not affected by exposure. Dominance status explained the significant reduction in reaction to social novelty observed on the sociability test. CPF induced a significant gut microbiome dysbiosis and triggered a hyperlipidemic, hypoglycemic/hypogluconeogenesis and a general altered cell energy production in females. These behavioral results in rats extend and complement previous studies with mice and show novel influences on gut metagenomics and plasma lipid profile and metabolomics, but do not stablish a relation between the exposure to CPF and the ASD phenotype. The effects of dominance status on reaction to social novelty have an important methodological meaning for future research on sociability.

Key words

Chlorpyrifos; Development; ASD; Sociability; Dominance; Gut microbiota; Metabolomics

Evidence of approval (Animals)

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1. Introduction

Autism spectrum disorder (ASD) is a complex neurodevelopmental pathology defined by reduced verbalizations and communication abilities, increased stereotypies and ritualistic/repetitive behaviors, and altered sociability skills (Diagnostic and statistical manual of mental disorders-$^{5th\ ed.}$, APA, American psychiatric association). 1 out of 166 children meet the diagnostic criteria for ASD (WHO, World Health Organization, 2018; DiCicco-Bloom et al. 2006).

Empirical studies regard ASD as having a high degree of heritability, and specific analyses of a multitude of genetic factors provide support for its polygenic nature (Grove et al. 2019). However, the impact of environmental factors on ASD development, progression, and severity has attracted increasing interest in recent decades with emphasis on socio-economic status, perinatal stress events and drug/xenobiotic exposure (Chaste & Leboyer 2012). Regarding the latter category, developmental exposure to various pesticides such as Carbamates and Organophosphates (OP) has been the focus of several experimental studies (Herbert MR 2010; Shelton et al. 2014). Chlorpyrifos (CPF) is the most widely used OP in recent decades. CPF is used as an insecticide, fungicide and herbicide for agricultural and industrial purposes. CPF exerts its main neurotoxicological profile by inhibiting the different Cholinesterases (ChEs) at both the Central Nervous (CNS) and systemic level (Eaton et al. 2008). However, various preclinical studies have also proposed alternative molecular targets for the neurotoxic profile of developmental CPF exposure (Burke et al. 2017).

Studies in mice have analyzed the effects of gestational (Lan et al. 2017, 2019; De Felice et al. 2014, 2015; Venerosi et al. 2010; Mullen et al. 2013), postnatal (Venerosi et al. 2008; Ricceri et al. 2003; Basaure et al., 2019) and both gestational and postnatal (Venerosi et al. 2006, 2015; Ricceri et al. 2006) exposure to CPF on social and/or ultrasound vocalization outcomes. Briefly, developmental exposure doses ranged from 1 to 6 mg/kg/day, and only a few of these studies found decreased social rates in exposed animals (Lan et al. 2017, 2019; De Felice et al. 2014; Venerosi et al. 2010), with enhanced social skills being found in other cases (Ricceri et al. 2006; Venerosi et al. 2006, 2015). The effects of CPF exposure on social and communication skills are highly dependent on the basal state of the organism, as found in KO Reeler mice with basal abnormal social traits (Mullen et al. 2013), other ASD-like strains (De Felice et al. 2015) and/or the APOE variant (Basaure et al., 2019).

Studies on late postnatal, preweaning exposure to CPF and ASD's symptomatology are sparse and focused on mice (Basaure et al., 2019; Venerosi et al. 2006, 2008; Ricceri et al. 2003, 2006). Other authors have also proposed that the human perinatal window could be an essential stage in ASD development (Getahun et al. 2017; Martinez-Morga et al. 2018). This stage has its murine equivalence at around postnatal day (PND) 10 in neurodevelopmental terms. Moreover, some essential cellular and molecular mechanisms characterize this period, such as synaptogenesis and myelinization

development as well as the peak period of maturation of vasopressin and oxytocin systems (Venerosi et al. 2006; Semple et al. 2013; Tait et al. 2009).

The relation between CNS and gut microbiome composition and the metabolomic profile of ASD patients and preclinical models is under intense research. The gut microbiota dysbiosis -the alteration of the relative abundance of different bacteria populations- associated with ASD is shown in the reviews recently published (i.e. Srikantha & Mohanjeri 2019; Fattorusso et al. 2019; Mohamadkhani A 2018; Fowlie et al. 2018). Alternatively, both fecal and systemic metabolomic studies have also revealed altered patterns in ASD patients and preclinical models (Mohamadkhani A 2018; Mussap et al. 2016; Ruggeri et al. 2014). On this way, ASD diagnosed children have been linked to both decreased (Gamma-aminobutyrate and Butyric acid) and increased (Isopropano, Glutamate, Propionic acid, amongst others) fecal metabolites (Mohamadkhani A 2018) as well as the they present alterations on different metabolites associated with mitochondrial dysfunction or amino acid metabolism in different biofluids (Mussap et al., 2016).

As observed in the ASD-associated research, CPF exposure has been also associated with alterations in both microbiota and metabolome profiles. Following this, extensive research using low doses of CPF administered at development stages induce gut dysbiosis in mice (Joly Condette et al. 2013, 2014, 2015, 2016; Reygner et al. 2016). Albeit with less intensity than the microbiome, developmental CPF exposure has also been linked to the specific alteration of various hepatic, brain, and systemic metabolites and metabolic pathways. From all the different metabolic pathways and components, CPF exposure has been linked to important alterations in metabolites that intermediate cell energy production, amino acid metabolism (Xu et al., 2015; Wang et al., 2009), as well as glucose and lipid metabolism (Wang et al., 2009), also following low doses during critical developmental stages (Slotkin et al., 2005). However, studies on the influences of exposure to CPF during postnatal, preweaning stages developmental stages on both gut microbiota and metabolic profile is essentially inexistent, with some very recent exceptions (Perez-Fernandez et al., 2019).

The aim of the present study is to explore the effects of late postnatal, preweaning exposure to low doses of CPF on 1) Social outcomes at both the medium (adolescence) and long-term (adulthood), 2) The constitution of dominance and social hierarchies, and 3) gut microbiota and systemic metabolites, including the lipid profile. The present study also included both Sexes for possible dimorphic specificities. We hypothesize that this dosage regime could decrease social rates and induce different alterations both microbiota populations and metabolites.

2. Materials and Methods

2.1. Experimental Animals

60 (30 females, half of each exposed to CPF) adolescents (PND 32-33) and 85 (41 females -19 exposed to CPF- and 44 males -22 exposed to CPF-) adult (postnatal month -PNM- 6 to 7) Wistar rats were used. The rats were born in our facilities. Briefly, full-

term pregnant mothers (n=19) arrived at our facilities and were individually caged. After 5 days of acclimation, the animals gave birth to 6-15 pups per mother (190 pups). At PND1 (birthday was set as PND0), all pups were separated from their original mothers, mixed, and randomly distributed 10 (5 females) to each mother ensuring a representative population and avoiding dam-related bias. Weaning (4 animals per cage of the same Sex) was done at PND21. For the present experiments, animals from all the 19 dams were selected to avoid the litter bias. The room was set up with a constant temperature of 22±2ºC and humidity of 50±10%, and a 12-hour cycle with lights on at 8:00h. Young animals were fed ad libitum (A04 Standard Free, Panlab), whilst adults followed a maintenance diet of 20g for males and 17g for females from PND74, in order to control weights. Water was provided ad libitum. Experimental timeline is displayed in the **Image 1**. The experimental units were, in all cases, every single animal behavioral outcome or biological samples (blood plasma or stool). The present study is included in the project ES040130002260. The various experiments were conducted in accordance with the Spanish Royal Decree 53/2013 and the European Community Directive (2010/63/EU) for animal research. The Animal Research Committee of the University of Almería gave their approval for the experiments.

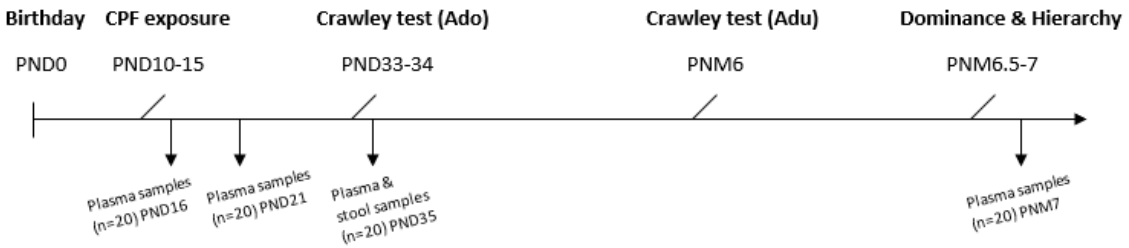

Image 1. Experimental design. A total of 185 rats were used. Half of them females. Half of each sex were randomly allocated to CPF exposure, and the remaining to vehicle condition from PND10 to 15. The social and social novelty behavior of both adolescent -Ado- (PND33-34, n= 60) and adult -Adu- (PNM 6) in a modified Crawley test. Dominance (n= 85) and established hierarchies (n= 72) of the adult rats were also evaluated. Plasma samples were obtained at PND16, 21, 35 and PNM7 (n= 20 at each time, half females, half of each sex exposed to CPF) for metabolomic analyses. Stool samples (n= 20, half females, half of each sex exposed to CPF) were also taken at PND35 for metagenomic analyses.

2.2. Neurotoxic agent

CPF (Fluka Analytical, purity of 99.9%) was administered by forced oral administration from PND10 to PND15 inclusive, between 12h-13.30h. Half of the animals of each Sex from each dam were randomly assigned to CPF or vehicle (corn oil) exposure. This period was chosen because some critical neurological mechanisms take place during this time, such as the peak of oxytocin and vasopressin hormones, and the development of myelinization; PND10 is approximately the day of birth in humans, and is thus considered a good model for perinatal influences on health for translational purposes (Venerosi et al. 2006; Semple et al. 2013; Tait et al. 2009). 1 mg/kg/ml/day of CPF was chosen and diluted in Corn Oil, which is widely used due to its facilitatory absorption

properties (Timchalk et al. 2002). For the control condition, vehicle was used at the same volume.

2.3. Behavioral tasks

2.3.1. Crawley's sociability test both adolescence (18-19 days after exposure) and adulthood (5 and a half months after exposure)

Description of the paradigm. In order to avoid some possible limitations of the traditional three-chambered Crawley's test paradigm, we designed the procedure without walls (Supplementary Image 1). Sociability testing was conducted in an open field (75x75cm) and the chambers were digitally created without physical barriers. We made these 2 changes (size and lack of physical barriers) as these conditions created a more unrestrained exploratory environment for the animals. Distances between the center of the strangers' walls and the limits of "contact" and "approximation" (equivalent to the chamber in traditional paradigms) zones were set following pilot studies in both adolescent and adult rats separately. The dependent variables analyzed were defined as two categories: 1) Motor control, with total distance (cm), time in movement (seconds), mean velocity (cm/seconds), and rearing (frequencies) and 2) Sociability and reaction to social novelty indexes, using the total time [for social (Time S1 - Time empty)/(Time S1 + Time empty) and reaction to novelty (Time S2 - Time S1)/(Time S2 + Time S1)], as described in previous reports (Baronio et al. 2015), in approximation and contact zones, as well as the time of sniffing behavior for the active exploration of the animal. The design of the digital arena and the recording of the outcome were both conducted using Ethovision 3.1. (Noldus).

Behavioral procedure. As with the classical three-chambered Crawley's test, the protocol was divided into three different phases: 1) Habituation. The experimental animal had 5 minutes of free exploration, 2) Sociability. Stranger 1 was placed at a corner of the apparatus, isolated from the experimental rat but being allowed both visual and odor contact for 10 minutes, and 3) Reaction to Social Novelty. Stranger 2 was placed on the opposite corner, maintaining the Stranger 1 in its location, and forcing a social choice situation for a further 10 minutes.

Animals (all 60 adolescents and 85 adults) were driven to the experimental room one day before the procedure for a 1-hour session of room acclimation. On the experimental days, the animals were driven to the room one hour before the procedure. The classical phases previously described were then completed for each animal. The cleaning protocol was carried out with ethanol (70%) between animals and Clidox (1:5:1) between cages. Odd series were developed by males and even by females. Treatment condition was also balanced throughout the day for time of day control. Room temperature and humidity were set as the normal housing parameters and under dim-light conditions between 9h-14h. Both Strangers 1 and 2 were completely unknown to the experimental animal.

2.3.2. The tube test: Social dominance and hierarchical status (6 – and a half months after exposure)

Description of the paradigm. Two classical tube tests were conducted using opaque PVC tubes. For males, the tube was 100 cm in length and 7 cm in diameter. For females, the tube was 85 cm length and 5.5 cm in diameter. These measures were chosen for two reasons: 1) The tube should be of sufficient length to force the "dominant" rat to push for an acceptable time/distance criteria, whilst the "submissive" animal should have time to react, and 2) The diameter should be wide enough to allow the animals to move back and forth but narrow enough to prevent them from turning on their own axis. A small longitudinal aperture was made to the upper part of the tubes in order to control the localization of the animals. Three gates (2 at the tube external segments and another in the center) were designed to limit free movement and proper disposition before the "fights". The criteria for winning a match was defined as the opponent placing 4 paws out of the tube in its initial external box. The dependent variable analyzed was the percentage of wins of each animal. The tube test was used in order to study both the direct dominance (unknown animals) and the well-established hierarchies (animals from the same home-cage).

Behavioral procedure. The animals were moved to the experimental room 6 days before the start for paradigm habituation and training. At the beginning, some rats started to get into the tube and move back and forth following some gentle pressure by the experimenter. This was followed by 5 consecutive days of reinforced straight-run behavior. Briefly, a few pellets were placed in the final segment of the tube and the opposite external box. Most of the animals quickly learned to go straight to the opposite direction as this reinforcement schedule was counterbalanced (all animals were reinforced for moving in both directions).

On experimental days, animals were driven to the experimental room 1 hour before the start. Firstly, we assessed dominance (direct dominance experiment). Each rat was faced with 3 different unknown rats of the same Sex, similar weight, but from a different dam, different home cage (completely unknown) and opposite Treatment condition. Experimental animals' order was counter-balanced 1 exposed followed by one control and so on. Each animal "fought" 3 consecutive times against the same animal. Following this, a rest period g was set for the next fight (between 30-45 minutes). This created a total of 9 matches for each rat. All of the 85 adult rats completed this protocol.

Although the first dominance test was designed to study the direct influences of CPF on dominance, it does not provide us with information on well-established hierarchies, transitivity, and paradigm validation. Given that this information is critical for studying the validation of the paradigm throughout transitivity (when animal A beats B, and B beats C, A must beat C), we proceeded to analyze pre-established hierarchies (dominance and situation in the hierarchy of the rats from the same home cage) (well-established hierarchies experiment). Because of this, we only used animals from n=4 home cages. The total sample in this case was 72 rats (36 females -16 exposed to CPF- and 36 males -19 exposed to CPF). Each animal "fought" against the other three co-habitants 3 times each, thus creating a final number of 9 matches for each animal. Room

temperature, humidity, light conditions, and experimental hours were as described previously.

2.4. Molecular analyses

2.4.1. Sacrifice protocol

Two days after completion of the behavioral procedures, a representative subsample of 5 animals per group (randomly selected) were sacrificed, with 16, 21, 35 days old and 7 months of age. Briefly, rats were sacrificed by fast decapitation and blood was collected into a PYREX tube covered with 2,2',2",2"'-(Ethane-1,2-diyldinitrilo) tetraacetic acid (EDTA). While one of the experimenters processed the blood samples for plasma extraction (3500 rpm for 20 minutes at 4ºC), another took stool samples from the whole gut, and these were then flash frozen. All the samples were then stored at -80ºC until use.

2.4.2. Gut microbiota composition (20 days after exposure)

The total stool was removed from -80ºC and quickly mixed (Heidolph RZR1) in order to obtain a proper representation of the whole microbiome. 100mg was taken and the genomic DNA (gDNA) isolation was conducted following the company's instructions (PureLinkTM Microbiome DNA purification kit). Samples were stored at -80ºC and later analyzed in an external laboratory by blind -to both sex and treatment- technicians (STABvida, Portugal). The quality of the samples was checked with gel electrophoresis (1% agarose gel) and a Phred quality score was recorded at each amplification cycle. gDNA was quantified by fluorometry (Qubit). Following this, the 16S rRNA V3 and V4 regions were amplified, the library was completed following the Illumina 16S Metagenomic Library preparation, and sequencing (250bp paired-end sequencing reads) was conducted in the MISeq reagent Kit v2 in the Illumina MiSeq platform (for a deep revision on Illumina systems, please see Garrido-Cárdenas et al. 2017). Finally, initial Pass Filtered sequence reads were classified with Illumina 16S metagenomics workflow at the different taxonomic levels. The secondary dependent variables analyzed in the present study were: 1. Species diversity, analyzed by Shannon Species Diversity Classification (Index used to characterize species diversity in a specific community) and 2. Total number of detected species. The main dependent variable was the relative abundance (percentage of bacteria from the whole microbiome that belongs to a specific family or category) at genus and species taxonomic category. The relative abundance of some of the most important bacteria from the phylum level were also analyzed as some of them (i.e. Firmicutes and Bacteroidetes) have been systematically linked to ASD and other developmental pathologies. A total of 20 animals (10 females, half of each sex exposed to CPF) were randomly selected for this analysis, with 35 days of age.

2.4.3. Plasma NMR metabolomomics (24h, 6 days, 35 days and 6 and a half months after exposure)

All chemical reagents used were of analytical grade. D_2O (99.9%) was purchased from Eurisotop and NaCl was purchased from Sigma Aldrich. The samples were prepared

according to Beckonert, et al. (2007) with some modifications. Briefly, 200 µL of blood plasma was mixed with 400 µL of D_2O containing 0.9% NaCl. The resulting mixture was centrifuged for 5 min at 13500 rpm. 500 µL of supernatant was transferred into an oven-dried 5 mm NMR tube for the analysis.

^{1}H NMR spectra of serum samples were obtained at 600 MHz on a Bruker Avance III HD 600 spectrometer, equipped with a 5 mm QCI quadruple resonance pulse field gradient cryoprobe and a thermostat-controlled sample case with 24 positions. The water-suppressed Carr-Purcell-Meibom-Gill (CPMG) pulse sequence with a total spin echo delay of 96ms was used to attenuate broad signals from lipoprotein or protein signals. All samples were measured at 293 ± 0.1 K, without rotation and using 16 dummy scans prior to the 180 acquired scans. The spectrometer transmitter was locked to D_2O frequency using a mixture of H_2O−D_2O (9:1). Acquisition parameters were set as follows: size of fid = 32K, spectral width = 22.0 ppm, acquisition time = 1.24 s, relaxation delay = 3 s, number of loops = 120, spin-echo delay = 400 µs, line broadening = 0.3 Hz, receiver gain = 50.8. All spectra were automatically phased, baseline-corrected, and calibrated to the anomeric proton signal of glucose at δ_H 5.23 ppm. Acquisition and processing of NMR spectra were carried out by the TOPSPIN software (version 3.6).

All NMR spectra were phased, baseline corrected, and then data reduced to 250 integrated regions of equal width of 0.04 ppm corresponding to the region of δ_H 0.5 to 10.5 using the Amix 3.9.4 software (Bruker Biospin GmbH). The regions of δ_H 5.18 to 4.34 ppm, of δ_H 3.70 to 3.46 and of δ_H 3.34 to 3.02 ppm were excluded from the bucketing process to remove artifacts of residual water and EDTA resonances. The area for each segmented region of chemical shift (bucket) was calculated, and the integral values contributed to an intensity distribution of the whole spectrum. Scaling the intensity of individual peaks to the total intensity recorded in the defined regions reduced any significant concentration differences from individual animals. Bucket tables were imported into the SIMCA-P software version 14.0 (Umetrics) for multivariate statistical analysis. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) models were scaled to pareto and unit variance, respectively. 80 rat plasma samples from four sampling time points (20 rats per time point, 10 females, half of each sex exposed to CPF) PND16, 21, 35 and 209 -PNM7- were analyzed by ^{1}H NMR spectroscopy.

2.5. Statistical analyses

For social behavior, repeated measures analysis of variance (ANOVA) for both contact and approximation zones were conducted with the within-subject factor of *Index* (two levels, social Index, and novelty Index) and the between-subject factors of *Sex* and *Treatment* (CPF and control). For locomotor control, individual two-way ANOVAs at each phase were conducted with these factors. For dominance and hierarchy status, the average number of won fights per animal (as a percentage) was analyzed using a further two-way ANOVA. For the influence of dominance on social behavior, the previously

indicated ANOVA was conducted but adding the third-factor *Dominance* derived from the dominance experiment (unknown rivals). For gut microbiota composition, Shannon species diversity Index, percentage of successful reads at each taxonomic level, the relative abundance of the 5 most important bacteria at phylum level as well as the relative abundance of each bacteria at genus were analyzed with individual two-way ANOVAs. When significant at the genus level, a Multivariate ANOVA was also conducted on the significant genus by taking all the species that comprised the specific genus. When significant, down-stream univariate analyses were carried out in order to identify which specific species accounted for the significant effect of genus. For all of these analyses, *post hoc* pair-wise comparisons were chosen. All the analyses were conducted with SPSS v25. Statistical significance was set at $p \leq 0.05$. The data are represented in terms of means and SEM in the various figures and tables. For metabolomics analysis, Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) modeling, these were carried out using SIMCA-P.

3. Results

A subsample of 5 animals per group from this cohort was used for ChEs and AChE activity analysis. CPF exposure did not significatively inhibit total ChE (Perez-Fernandez et al., 2019) and AChE (unpublished results) activity at the frontal cortex 24 hours following the final exposure. There were no significant differences between animals in terms of ocular opening or weight during development. Furthermore, weight was not affected by CPF exposure throughout the life span (data not shown). The data of all the 60 adolescent and 85 adult rats were included in the final statistical analyses for sociability, locomotor, and direct dominance (vs. unknown rivals of the opposite treatment group). In the case of gut microbiome, 19/20 animals' stool samples were finally included in the statistical analysis because the gDNA sample of one female exposed to CPF had not enough quality to be processed by the external laboratory. In the case of the metabolomic analyses, plasma sample from all the **80** animals were included.

3.1. Behavioral outcomes

3.1.1. CPF & Sociability

Adult rats generally decreased their sociability in the reaction to social novelty phase, showing a significant *Index x Sex x Treatment* interaction in the approximation zone [$F(1,81)= 5.564$, $p= 0.021$] (Figure 2a). Post hoc analysis revealed that both control females and exposed males strongly decreased their reaction to novelty exploration in relation to their own rates at the social stage ($p= 0.004$ and 0.005, respectively), something that was not observed in their exposed (females) and control (males) counterparts ($p= 0.937$ and 0.351, respectively). There were not further significant effects concerning *Sex*, *Treatment* or their interaction neither in adults nor adolescent rats in approximation, contact or sniffing behavior (**Figure 1a, b and c and Figure 2b and c**).

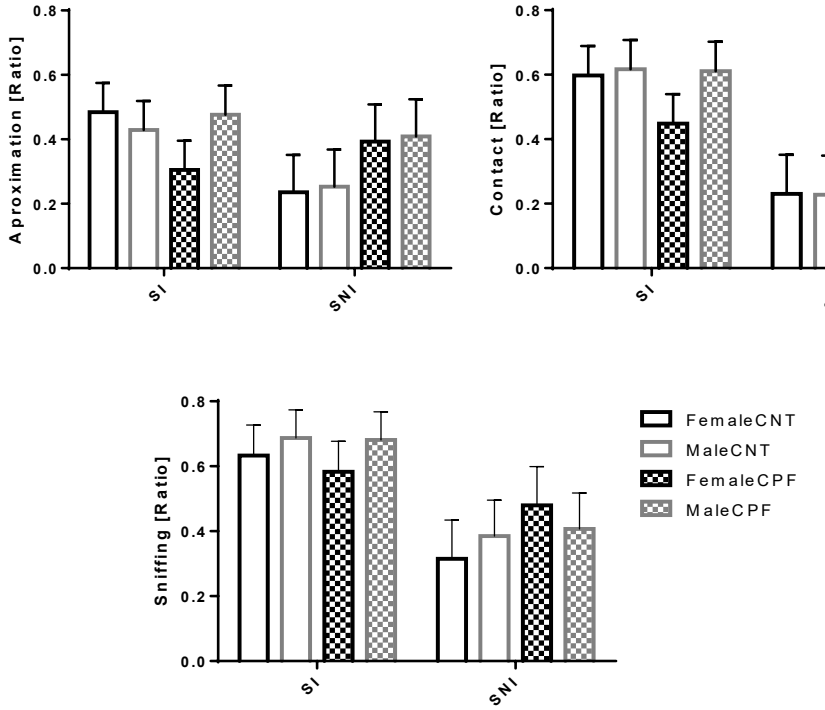

Figure 1. CPF influences on sociability during adolescence. Sociability (SI) and reaction to social novelty (SNI) indexes in approximation zone (a, up-left), contact zone (b, up-right) and sniffing behavior (down) in adolescent rats. Data are expressed by means and SEM.

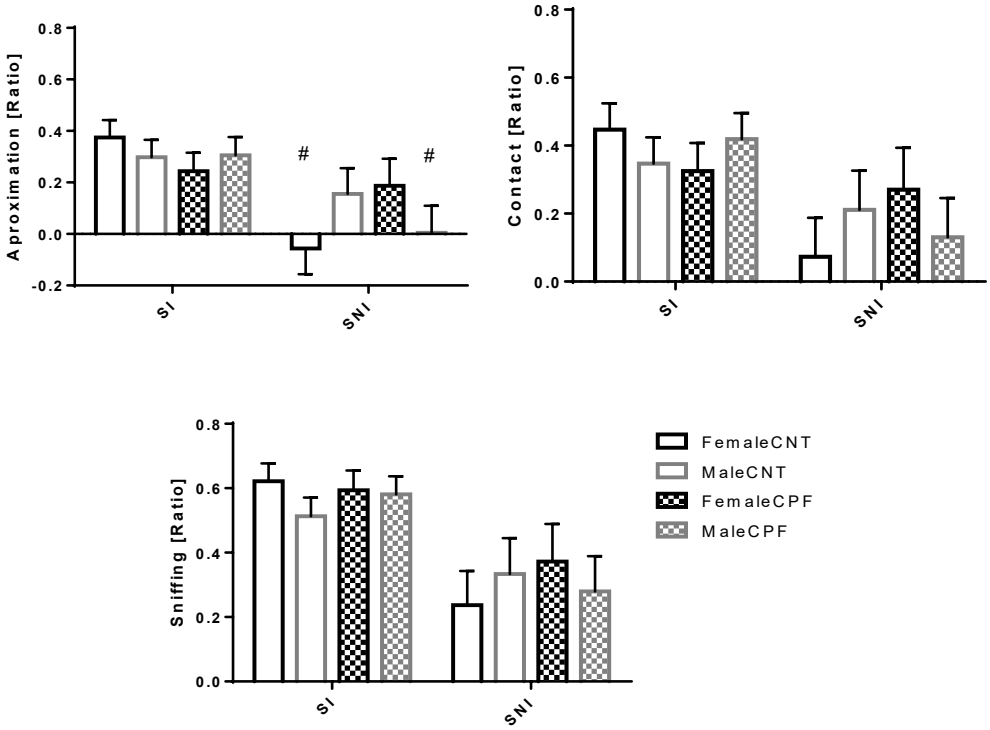

Figure 2. CPF influences on sociability during adulthood. Sociability (SI) and reaction to social novelty (SNI) indexes in approximation zone (a, up-left), contact zone (b, up-right) and sniffing behavior (down) in adult rats. Data are expressed by means and SEM. # means significant differences ($p<0.05$) in SNI from the respective SI values.

3.1.2.CPF & Dominance.

When given a tube test with direct matches between CPF and unknown control animals, CPF rats had a similar percentage of victories compared to control animals (**Figure 3**). No significant effects were found for either *Treatment* or the *Sex x Treatment* interaction. A parallel analysis of well-established social hierarchies to study the validity of the test revealed high rates of transitivity of females (92%) but only moderate rates for males (64%). Similarly, a specific analysis of these well-established hierarchies also revealed no significant effects of either *Treatment* or the *Sex x Treatment* interaction (Supplementary Figure 1). Interestingly, when comparing both models (dominance with unknown and hierarchy with well-known animals) CPF exposed males showed enhanced dominance when faced with a known rat (Supplementary Figure 2). However, the ANOVA revealed only a marginally significant *Sex x Treatment* interaction. Since not all the animals assessed by Crawley's test were included in the hierarchy analysis (only cages of 4 animals were included), the final dominance status was extracted from the direct dominance test.

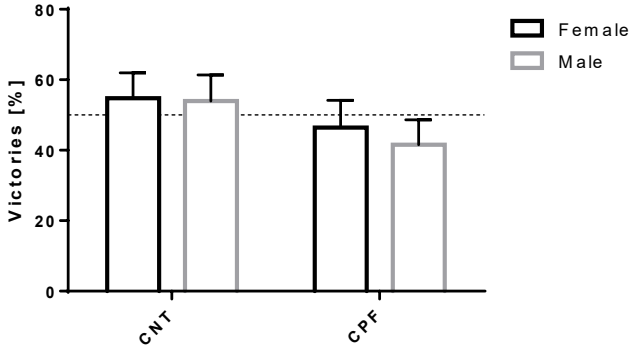

Figure 3. Dominance Test. Percentage of victories after 9 matches versus unknown animals. Data are expressed by means and SEM.

3.1.3.Reaction to social novelty & Dominance

Adult rats were labeled as dominant or non-dominant (submissive) when their percentage of victories against unknown animals was > (dominant) or < (non-dominant) 50%. This factor was introduced in the reaction to social novelty analysis. The significant decrease of the reaction to social novelty in adulthood was completely explained by dominance status, where dominant animals did not react to the novelty (**Figure 4a, b and c**). the rmANOVA showed significant main effects of *Dominance* [$F(1,77)= 7.040$, $p= 0.010$; $F(1,77)= 6.308$, $p= 0.014$; $F(1,73)=10.239$, $p= 0.002$ for approximation and contact zones and sniffing behavior, respectively] and the *Index x Dominance* interaction [$F(1,77)= 8.025$, $p= 0.006$; $F(1,77)= 7.983$, $p= 0.006$; $F(1,73=13.781$, $p< 0.001$)]. Post hoc analysis revealed that both dominant and submissive rats had similar sociability indexes during the social phase. However, the dominant rats drastically reduced their reaction to social novelty Index compared with the non-dominant rats ($p= 0.001$; $p= 0.002$; $p<0.001$ for contact and approximation zones and sniffing behavior, respectively) as well as their own index profile during the

social phase ($p< 0.001$ both zones and sniffing behavior). No further significant differences were found concerning the exposure condition, *Sex* or any other interaction.

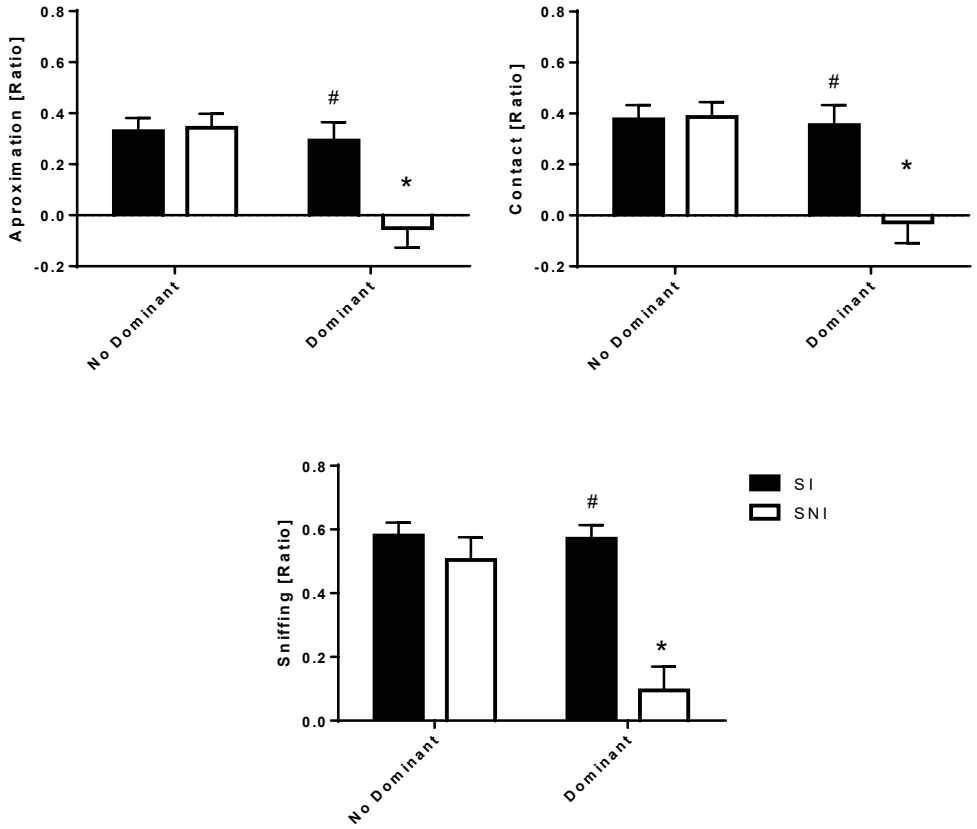

Figure 4. Influences of dominance on the social traits. Influences of dominance status on sociability (SI) and reaction to social novelty (SNI) indexes in approximation zone (a, up-left) and contact zone (b, up-right) **and sniffing behavior (c, down)** in adult rats. Data are expressed by means and SEM. * means significant differences ($p<0.05$) between dominant and no dominant rats in SNI. # means significant differences ($p<0.05$) between both indexes in dominant animals.

3.1.4.CPF & Locomotor activity.

Four variables were studied in order to rule out the possibility that "social" differences could be due to motor alterations following CPF exposure. In this regard, total time of movement, distance traveled, mean velocity and rearing frequencies did not produce significant main effects of *Treatment* or a *Sex x Treatment* interaction at any phase (habituation, social, and novelty phases) for either adolescent or adult rats (**Table 1 and 2**).

	Female control	Male control	Female CPF	Male CPF	Two-way ANOVA *Treatment*	Two-way ANOVA *Sex x Treatment*
Phase 1. Habituation						

Time in movement	108.1 ± 3.84	129.6 ± 3.87	114.7 ± 4.76	127.2 ± 4.5	F(1,56)= 0.242, p= 0.624	F(1,56)= 1.126, p= 0.293
Distance traveled	3232.8 ± 236.85	2849.4 ± 67.73	3200.5 ± 162.84	3078.6 ± 107.71	F(1,56)= 0.393, p= 0.534	F(1,56)= 0.692, p= 0.409
Mean velocity	11.5 ± 0.77	10 ± 0.31	11.6 ± 0.52	11.1 ± 0.41	F(1,56)= 1.288, p= 0.261	F(1,56)= 0.803, p= 0.374
Rearing frequency	58.3 ± 3.78	58.3 ± 4.38	61.9 ± 3.82	67.3 ± 3.43	F(1,56)= 2.655, p= 0.109	F(1,56)= 0.488, p= 0.488
	Phase 2. Social interaction					
Time in movement	201.2 ± 11.67	208.1 ± 14.10	207.3 ± 17	202.2 ± 8.59	F(1,56)< 0.001, p= 0.995	F(1,56)= 0.210, p= 0.649
Distance Traveled	3613.5 ± 671.25	2571.5 ± 99.86	3168.2 ± 303.12	2689.3 ± 165.89	F(1,56)= 0.185, p= 0.669	F(1,56)= 0.547, p= 0.463
Mean velocity	6.3 ± 1.10	4.4 ± 0.18	5.6 ± 0.57	4.6 ± 0.29	F(1,56)= 0.133, p= 0.717	F(1,56)= 0.486, p= 0.489
Rearing frequency	77.6 ± 6.81	71.9 ± 6.30	77.2 ± 9.12	64.9 ± 5.33	F(1,56)= 0.282, p= 0.597	F(1,56)= 0.225, p= 0.637
	Phase 3. Reaction to social novelty					
Time in movement	201.2 ± 9.42	209.2 ± 26.87	170.6 ± 16.67	221 ± 23.67	F(1,56)= 0.215, p= 0.645	F(1,56)= 1.090, p= 0.301
Distance Traveled	2914.7 ± 566.17	2081.8 ± 170.31	2182.8 ± 257.96	2346.8 ± 241.14	F(1,56)= 0.460, p= 0.501	F(1,56)= 2.095, p= 0.153
Mean velocity	5.2 ± 0.92	3.7 ± 0.31	4 ± 0.53	4.3 ± 0.43	F(1,56)= 0.176, p= 0.676	F(1,56)= 2.393, p= 0.127
Rearing frequency	72.9 ± 6.92	64.3 ± 9.17	65 ± 8.58	69.4 ± 9.72	F(1,56)= 0.027, p= 0.869	F(1,56)= 0.563, p= 0.456

Table 1. CPF exposure on locomotor activity (adolescence). Locomotor activity of 4 different outcomes in every phase of the Crawley's test in adolescent rats. Data are expressed by means and SEM. Two-way ANOVA results from *Treatment* and *Sex x Treatment* are defined.

	Female control	Male control	Female CPF	Male CPF	Two-way ANOVA *Treatment*	Two-way ANOVA *Sex x Treatment*
	Phase 1. Habituation					
Time in movement	160.3 ± 3.76	154.5 ± 4.63	167.3 ± 4.81	154.9 ± 5.93	F(1,81)= 0.560, p= 0.456	F(1,81)= 0.462, p= 0.499
Distance Traveled	2691.8 ± 87.7	2318.8 ± 80.74	3131.7 ± 279.64	2347.2 ± 68.85	F(1,81)= 2.650, p= 0.107	F(1,81)= 2.046, p= 0.156
Mean velocity	9 ± 0.30	7.7 ± 0.27	10.5 ± 0.93	7.8 ± 0.23	F(1,81)= 2.672, p= 0.106	F(1,81)= 2.040, p= 0.157
Rearing frequency	52.5 ± 3.58	41.2 ± 2.24	53.7 ± 3.46	42 ± 2.74	F(1,81)= 0.108, p= 0.744	F(1,81)= 0.009, p= 0.927
	Phase 2. Social interaction					
Time in movement	215.1 ± 15.47	183 ± 13.93	220.8 ± 10.91	160.2 ± 16.63	F(1,81)= 0.333, p= 0.566	F(1,81)= 0.929, p= 0.338
Distance Traveled	3156.4 ± 220.36	2391.1 ± 138.24	3775.1 ± 424.35	2212.8 ± 181	F(1,81)= 0.765, p= 0.384	F(1,81)= 2.504, p= 0.117
Mean velocity	5.3 ± 0.37	4 ± 0.23	6.3 ± 0.71	3.7 ± 0.30	F(1,81)= 0.753, p= 0.388	F(1,81)= 2.506, p= 0.117
Rearing frequency	59 ± 5.90	42.1 ± 3.90	60.6 ± 4.6	34.5 ± 3.81	F(1,81)= 0.425, p= 0.516	F(1,81)= 0.981, p= 0.325
	Phase 3. Reaction to social novelty					
Time in movement	148.8 ± 16.70	128.2 ± 20.06	160.6 ± 12.65	140 ± 19.85	F(1,81)= 0.434, p= 0.512	F(1,81)< 0.001, p= 0.997
Distance Traveled	2356.2 ± 272.34	1528.6 ± 140.62	3050.7 ± 665.10	1978.3 ± 279.41	F(1,81)= 2.402, p= 0.125	F(1,81)= 0.110, p= 0.741
Mean velocity	4 ± 0.46	2.6 ± 0.24	5.1 ± 1.11	3.3 ± 0.47	F(1,81)= 2,348, p= 0.107	F(1,81)= 0.100, p= 0.753
Rearing frequency	41.7 ± 6.39	26.2 ± 4.20	41 ± 4.49	31 ± 4.85	F(1,81)= 0.151, p= 0.699	F(1,81)= 0.292, p= 0.590

Table 2. CPF exposure on locomotor activity (adulthood). Locomotor activity of 4 different outcomes in every phase of the Crawley's test in adult rats. Data are expressed by means and SEM. Two-way ANOVA results from *Treatment* and *Sex x Treatment* are defined.

3.2. Molecular outcomes

3.2.1. CPF & Gut microbiota composition

The analysis of total number of species and the Shannon species diversity did not reveal significant effects of CPF exposure for *Treatment* or a *Sex x Treatment* interaction. A significant effect of *Sex* was found in Shannon's species diversity with higher rates in male rats [F(1,15)= 4.861, p= 0.043]. The analysis of the percentage of the passed filters successful reads at each taxonomic category revealed no significant effects of *Treatment* or a *Sex x Treatment* interaction. No significant differences were found for any bacteria at phylum (**Figure 5**).

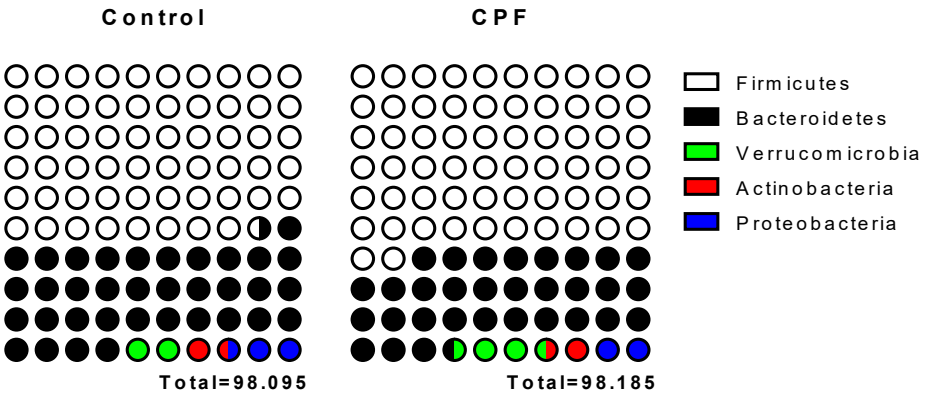

Figure 5. Influences of CPF exposure on gut microbiome (phylum). Relative abundance of the 5 most important bacteria in the phylum taxonomic category in both control and CPF animals.

Otherwise, CPF induced an important dysbiosis at the taxonomic categories of genus and species (**Tables 3 and 4**). At the genus level, CPF exposure generally reduced the relative abundance of most of the significant bacteria (13 out of 16). Of these, Moryella, Slackia, Aggregibacter, and Caldicellulisiruptor were the most abundant and thus the most important in terms of their influence on general microbiome functioning. Although Moryella showed no significant differences at post hoc analysis, the relative abundance of Slackia and Aggregibacter was found to increase as a result of CPF exposure. Regarding the *Sex x Treatment* interaction, most of the significant effects were derived from the decreased relative abundance of the various bacteria in exposed males (Rhodosphirilum, Actinobaculum, and Phascolarctobacterium) and females (Amaricocus, Chondromyces, and Zhouia) compared with their respective controls.

Genus	Factor	Two-way ANOVA	Outcome (Post Hoc)	Relative Abundance (‰)
Moryella	S*T	F(1,15)= 5.401, p= 0.035	N.S.	28.02662711
Slackia	T	F(1,15)= 4.644, p= 0.048	+	23.70090868
Aggregatibacter	T	F(1,15)= 4.592, p= 0.049	+	1.37064989
Caldicellulosiruptor	T	F(1,15)= 12.676, p= 0.003	-	1.06289442
Rodhosphirillum	T	F(1,15)= 9.446, p= 0.008	-	0.98866895
	S*T	F(1,15)= 6.257, p= 0.024	CNT/M + CNT/F; CNT/M + CPF/M	0.98866895
Neorickettsia	T	F(1,15)= 10.576, p= 0.005	-	0.70328963
Mycoplasma	T	F(1,15)= 5.971, p= 0.027	-	0.63817684
Thiomonas	T	F(1,15)= 5.227, p= 0.037	-	0.32625821
Helicobacter	T	F(1,15)= 12.413, p= 0.003	-	0.206281
Methylobacterium	T	F(1,15)= 7.908, p= 0.013	-	0.18126758
Ehrlichia	T	F(1,15)= 7.450, p= 0.016	-	0.15871684
Rhodobacter	T	F(1,15)= ,5.011 p= 0.041	-	0.15044579
Saccaropolyspora	T	F(1,15)= 10.667, p= 0.005	-	0.14010784
Carboxydocella	T	F(1,15)= 4.871, p= 0.043	+	0.13514595
Chlorobaculum	T	F(1,15)= 4.722, p= 0.046	-	0.10745932
Amaricoccus	S*T	F(1,15)= 8.098 , p= 0.012	CNT/M – CNT/F; CNT/F + CPF/F	0.10648653
Zhouia	S*T	F(1,15)= 5.796, p= 0.029	CNT/M – CNT/F; CNT/F + CPF/F	0.08071653
Chondromyces	S*T	F(1,15)= 9.394, p= 0.008	CPF/M + CPF/F; CNT/F + CPF/F	0.04413932
Jeotgalicoccus	T	F(1,15)= 8.994, p= 0.009	-	0.02541116
Phascolarctobacterium	S*T	F(1,15)= 9.533, p= 0.008	CNT/M + CNT/F; CNT/M + CPF/M	0.01606011
Leptothrix	T	F(1,15)= 7.194, p= 0.017	-	0.01539174
Actinobaculum	S*T	F(1,15)= 6.557, p= 0.022	CNT/M + CNT/F; CNT/M + CPF/M	0.01164579
Ferrimicrobium	S*T	F(1,15)= 4.521, p= 0.05	N.S.	0.00972205

Table 3. CPF influences on gut microbiome (Genus). Significantly altered bacteria at the genus taxonomic category both *Treatment* and *Sex x Treatment* interaction. + and – means higher or lower relative abundance in CPF exposed rats. CNT/M= control male, CNT/F= control female, CPF/M= exposed male and CPF/F= exposed female. N.S. means no significant differences at post hoc. Bacteria is scheduled based on the relative abundance average (‰).

The relative abundance of 9 bacteria was significantly altered by CPF exposure and 8 were affected in a sex-dimorphic manner at the species level. The relative abundance of all except Carboxydocella Ferrireducens was reduced in the CPF group. In terms of Sex influences on CPF exposure, we found decreased Actinobacculum Suis and Phascolarctobacterium Suiccinatutens in CPF exposed males and Chondromyces Pediculatus and Zhouia Amyolitica in exposed females. Interestingly, CPF exposure increased the relative abundance of unspecific Mycoplasma in males compared with controls.

Genus	Specie	Factor	Multivariate ANOVA	Two-way ANOVA	Outcome (Post Hoc)	Relative Abundance (‰)
Moryella	Indoligenes	S*T	F(2,14)= 4.530, p= 0.030	F(1,15)= 5.411, p= 0.034	N.S.	28.0179673
Caldicellulosiruptor	Uns.	T	F(2,14)= 5.924, p= 0.014	F(1,15)= 12.688, p= 0.003	-	1.050833
Neorickettsia	Helminthoeca	T	N.A.	F(1,15)= 10.576, p= 0.005	-	0.70328963
Methylobacterium	Uns.	T	F(2,14)= 7.442, p= 0.006	F(1,15)= 11.046, p= 0.005	-	0.17451895
Ehrlichia	Ovina	T	N.A.	F(1,15)= 7.450, p= 0.016	-	0.15871684
Mycoplasma	Edwardii	T	F(9,7)= 3.788, p= 0.046	F(1,15)= 15.887, p= 0.001	-	0.15467358
Carboxydocella	Ferrireducens	T	N.A.	F(1,15)= 4.871, p= 0.043	+	0.13514595
Helicobacter	Suncus	T	F(2,14)= 6.849, p= 0.008	F(1,15)= 8.244, p= 0.012	-	0.10467958
Saccaropolyspora	Uns.	T	F(3,13)= 7.320, p= 0.004	F(1,15)= 8.671, p= 0.010	-	0.08702563
Zhouia	Amylolitica	S*T	N.A.	F(1,15)= 5.796, p= 0.029	CNT/M – CNT/F; CNT/F + CPF/F	0.08071653
	Uns.	S*T	F(9,7)= 4.809, p= 0.025	F(1,15)= 4.798, p= 0.045	CNT/M – CPF/M	0.06806442
Chondromyces	Pediculatus	S*T	N.A.	F(1,15)= 9.394, p= 0.008	CPF/M + CPF/F; CNT/F + CPF/F	0.04413932
	Haemominutum	S*T	N.A.	F(1,15)= 5.641, p= 0.031	N.S.	0.02638758
Phascolarctobacterium	Succinatutens	S*T	N.A.	F(1,15)= 9.533, p= 0.008	CNT/M + CNT/F; CNT/M + CPF/M	0.01606011
Leptothrix	Discophora	T	N.A.	F(1,15)= 7.194, p= 0.017	-	0.01539174
Actinobaculum	Suis	S*T	N.A.	F(1,15)= 6.557, p= 0.022	CNT/M + CNT/F; CNT/M + CPF/M	0.01164579
Ferrimicrobium	Acidifilum	S*T	N.A.	F(1,15)= 4.521, p= 0.05	N.S.	0.00972205

Table 4. CPF influences on gut microbiome (Species). Significantly altered bacteria at species taxonomic category both *Treatment* and *Sex x Treatment* interaction. An initial multivariate ANOVA, when significant, lead to a two-way ANOVA and, when significant, post hoc analyses were carried out. + and – means higher or lower relative abundance in CPF exposed rats. CNT/M= control male, CNT/F= control female, CPF/M= exposed male and CPF/F= exposed female. N.S. means no significant differences at post hoc. N.A. means non-applicable. Bacteria is scheduled based on the relative abundance average (‰).

3.2.2. CPF & Metabolomic and lipid profile

Chemical shifts, signal multiplicities and coupling constants of the metabolites identified in plasma samples are detailed in Supplementary Table 1. Metabolites mainly belong to the following classes: amino acids, lipids, nucleic acids derivatives, organic acids, and carbohydrates. Metabolite identification was achieved thanks to the BBIOREFCODE 2 database from Bruker, public NMR databases such as COLMAR (Bingol et al. 2014)) and HMDB (Wishart et al. 2018) and the literature (MacIntrye et al. 2010; Wang et al. 2009, 2012; Stringer et al. 2011).

The PCA was conducted on the ^{1}H NMR data for visualizing major trends in high-throughput datasets. PCA scores plot that groups similar samples based on the input data, and PCA loadings plot that indicates which spectral areas (or buckets) contribute more to the variation between groups were generated. The PCA scores and loadings plots for the NMR spectra at the four sampling time points are shown in Supplementary Figure 3 a and b, respectively. Supplementary Figure 3a displays a clear discrimination of rat plasma samples into four groups based on the aging process, regardless of the administration of the CPF, with PC1 and PC2 explaining 58.3% and 11.3% of the total variance, respectively. Supplementary Figure 3b shows the discriminant buckets that correlate with the aging process in the whole set of rat plasma samples analyzed. Rat plasma samples from PNM7, because of the aging process, presented higher amounts of lactic acid, fatty acids (FA), unsaturated fatty acids (UFA), glucose, low and very low-density lipoproteins (LDL and VLDL).

PCA was then applied to the NMR data for each sampling time point. Additionally, the effect of CPF exposure was assessed for male or female rats (Supplementary Figure 4a-d). Analyzing the PCA score plots, no difference was observed between exposed and control groups, except for female rats at PNM7. Variable importance in projection (VIP) scores from a partial least squares discriminant analysis (PLS-DA) was obtained to select the most discriminant variables responsible for the differences among the exposed and control samples from female rats at PNM7. The PLS-DA scores plot and VIP-scores are shown in **Figure 6a and b**, respectively. **Figure 6a** shows that plasma samples from female rats at PNM7 can be distinguished into two groups due to the administration of CPF. The PLS-DA model was validated by the permutation test. VIP's value expresses the contribution of the individual variables in the definition of the F-latent vector model. Due to the normalization applied in the definition of the VIP, discriminant buckets showing values above 1 were considered to contribute significantly to the discrimination (**Figure 6b**).

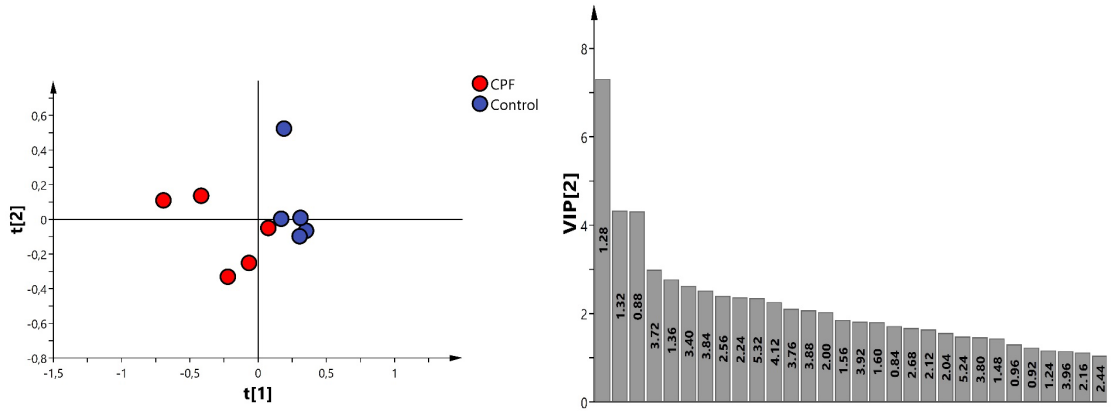

Figure 6. Influences of CPF exposure on metabolic profile. a, left) PLS-DA scores plot generated from 1 H CPMG NMR data of plasma samples obtained from female rats belonging to the CPF-exposed group (red), and the control group (blue), at sampling point t4 (PNM7) and b, right) Variable importance in projection (VIP) scores plot obtained from the PLS-DA analysis (Var ID buckets), displaying the variables that most contribute to the discrimination observed between CPF-exposed and control plasma samples in female rats at sample point t4.

The metabolites whose NMR signals are contained in these buckets of largest VIP coefficients, and therefore contributing more significantly to the discrimination, are shown in **Table 5**. In terms of the female rat metabolome, CPF exposure produced an increase of LDL/VDL, N-acetylglycoprotein (NAc), FA and UFA at PNM7. However, the administration of CPF reduced the levels of glucose, the organic acids citrate and lactate, and the amino acids glutamine, alanine, leucine, and serine.

Bucket	Metabolite	Outcome
1.36, 1.28, 1.24, 0.92 - 0.84	LDL/VLDL	+
4.12; 1.32	Lactate	-
5.24, 3.92 - 3.72, 3.40	Glucose	-
2.68, 2.56	Citrate	-
2.24, 2.04, 2.00, 1.60, 1.56	FA	+
2.04	NAc	+
5.32	UFA	+
2.44, 2.16, 2.12	Glutamine	-
1.48	Alanine	-
0.96	Leucine	-
3.96	Serine	-

Table 5. CPF exposure influences on plasma metabolites. Metabolites that were shown to increase (+) or decrease (-) on plasma samples of female rats after CPF treatment at sampling point t4 (PNM7).

4. Discussion

1 mg/kg of CPF for 6 consecutive days at the late postnatal preweaning developmental stage induced a modest alteration in the reaction to social novelty in adulthood by both enhancing (females) and decreasing (males) novelty exploring indices in relation to their respective social scores. It induced medium-term effects on gut dysbiosis and a hyperlipidemic, hypoglycemic/hypogluconeogenesis profile in adult females. This is the first time that these behavioral and molecular findings have been linked to this exposure protocol. We also found an important implication of dominance status on reaction to social novelty behavior, with important implications in future uses of the Crawley test. All these effects were found at doses without systemic toxicity and unrelated to ChEs inhibition in the CNS (Perez-Fernandez et al. 2019).

4.1. Influences of CPF exposure on behavioral outcomes

Exposure did not affect sociability or reaction to social novelty skills in adolescent rats, but exposed animals increased (females) and decreased (males) their reaction to social novelty indexes in relation to the social scores in adulthood. Both control females and exposed males significantly decreased their novelty rates from the social phase. CPF exposure blocked this in females, producing a similar behavior in both phases. The subtle reduction at the novelty phase found in male controls was stronger in exposed males, although given that the differences between groups at each phase did not reach significance, this conclusion must be treated with caution. In fact, the weakness of this data is confirmed by the lack of significant effects of the exposure condition in the closer interactions (time in contact zone), active exploratory behavior (sniffing) included.

Adult control females did not react to social novelty when the approximation zone was analyzed. This unexpected behavior could reflect some degree of attachment to the familiar rat, a general lack of reaction to novelty, or both. However, the influence of the modulations done to the Crawley paradigm (open field version) in the present study could also be responsible of this behavioral pattern, although the normal behavior shown during adolescence could point towards other explanations. In fact, the active exploratory sniffing of the control females was closer to the expectations, although they still being the group with the lower reaction rate. Interestingly, CPF exposure blocked this effect in females. Previous studies have found enhanced sociability or reaction to social novelty following gestational CPF exposure in females using different paradigms of social investigation as well as ultrasound vocalizations (De Felice et al., 2014; Venerosi et al., 2006). Venerosi et al. (2006) found that this effect following gestational exposure was blocked by re-exposing the animals during the preweaning stage. To our special interest, Ricceri et al. (2003) found that low doses of CPF from PND11 to 14 enhanced social behaviors in both sexes and aggressive responses in males in late adolescence. These authors found that CPF exposure also increased the rate of reaction to novelty. This decreased "fear" in response to novelty could be partially explained by anxiety modulation following CPF exposure during this period (Ricceri et al. 2006). This latter study also found that preweaning exposure to low doses of CPF increased both maternal behaviors in females and aggression in males and CPF postnatal exposure influence depends on previous gestation administration. Increased maternal responses in female rats following preweaning CPF exposure were also found, along with decreased anxiety in females (Venerosi et al. 2008).

Both direct dominance and hierarchy status were unaffected by CPF exposure, but the influence of dominance status on reaction to social novelty rates was relevant, as the animals that showed a dominant profile showed a reduced reaction to social novelty, non-observed in submissive rats. This is the first time that dominance status has been demonstrated to be an essential factor in the regulation of the reaction to social novelty. However, Jupp et al. (2016) found the opposite effect but in terms of reaction to a novel environment, where dominant rats did show higher rates of motricity. Nevertheless, this observation of a decrease in social skills in dominant animals could be linked to the findings of a two other studies with monkeys (Czoty et al. 2010; Riddick et al. 2009). From our perspective, the opposite results found in Jupp et al. (2006) versus these two studies with monkeys and the present work with rats could be the nature of the stimulus and are not necessary contradictory. That is, from an ethological perspective, it could have sense that dominant animals are prone to extensively explore novel environments but avoid interaction with novel stimuli. Thus, the present study would extend this last category to the social dimension. However, the dominance status in the present study was obtained exclusively from the tube test, which also suppose a limitation for the final conclusions.

4.2. Influences of CPF exposure on molecular outcomes

CPF induced an important dysbiosis in both genus and species levels, showing the influence of CPF exposure on multitude bacteria, which has never before been linked to this OP. Exposure to CPF has been linked to an increase in different bacteria at family, genus and species levels (Joly Condette et al. 2013, 2015; Fang et al. 2018; Zhao et al. 2016; Reygner et al. 2016). However, we failed to confirm these modulations following the preweaning exposure protocol described here. The specific bacteria population affected following this exposure protocol differently evolved by age, as showed in recent reports using samples extracted 6 months after exposure (Perez-Fernandez et al., 2019). Both Slackia (increased) at the genus and unspecific Caldicellulosiruptor (decreased) at species levels were the most important affected bacteria. Slackia bacteria have recently been associated with hyperlipidemia in colorectal cancer patients (Han et al. 2019), along with this type of cancer in dogs (Herstad et al. 2018). However, this association has not been systematically found (Kasai et al. 2016). Furthermore, enriched Slackia exigua species was also found in other types of cancer (Coker et al. 2018). Caldicellulosiruptor is an anaerobic, Gram-positive bacterium known for its ability to degrade complex carbohydrates such as cellulose (Ozdemir et al. 2012). However, its implication for health and behavior regulation is unknown as well as its interaction with CPF, OPs or any other inexistent xenobiotics. Regarding the remaining significant, less abundant bacteria found in the present study, there are no known implications for health, and evidence for their associations with CPF exposure is sparse and, in most the cases, non-existent.

CPF also induced hyperlipidemic (increased plasma LDL, VLDL, fatty acids and unsaturated fatty acids levels), hypoglycemic/ hypogluconeogenesis (decreased plasma glucose levels), and altered amino acid profiles in adult (PNM7) female rats, indicating a clear sex-dimorphic effect with females as the most vulnerable population target in terms of metabolites and the lipid system. Hyperlipidemia is a commonly found metabolic profile following OP exposure (Elsharkawy et al. 2013). As previously indicated, we only found one developmental study that showed this profile (Slotkin et al. 2005). The authors exposed animals during the early postnatal window (PND1 to 4) at the same dose that we used here but they did not find altered glucose at the serum level, and lipid alteration was focused on males. Thus, our study is the first developmental model that describes long-term lipidic and glucose alterations following early CPF exposure in female rats. In relation to CPF exposure during adulthood, middle-high doses of acute CPF in adult rats increased general levels of triglycerides, low-density lipoprotein, as well as decreased high-density lipoproteins (Acker & Nogueira 2012). Interestingly, ASD patients have also been linked to abnormal lipid metabolism (Tierney et al. 2006; Shedlock et al. 2016).

Lipid metabolism is influenced by glucose levels and vice versa (Parhofer KG 2015). Our hypoglycemia/hypogluconeogenesis pattern found in females contrasts with the most common hyperglycemic/gluconeogenesis profile following hepatic alterations (i.e. glycogen synthase modulation) following OP exposure (Rahimi & Abdollahi 2007; Acker & Nogueira 2012). Interestingly, a temporal decrease in serum levels of glucose

and total triglycerides has recently been found in young adults following chronic exposure to low doses of CPF (0.3mg/kg/day) in fat-enriched diets (Fang et al. 2018). This could also be the consequence of a hypocorticosteronemia process, since the opposite has been linked to a hyperglycemic profile in previous studies (i.e. Acker & Nogueira 2012). Other authors found that a ketogenic diet (rich in lipids but poor in carbohydrates) enhanced various ASD-like behaviors in asocial mice, including sociability (Ruskin et al. 2017). Only females that followed the ketogenic feeding regime exhibited a significant preference for Stranger 2. Ruskin's study and the present work support the notion that lower levels of glucose and higher levels of lipids are linked to an enhanced reaction to social novelty in female rats. Indeed, low glycemic diets have also been linked to an enhancement of autistic behaviors in ASD mice models (Currais et al. 2016).

This hypoglycemia/ hypogluconeogenesis is congruent with the general alteration of energy production by basic elements such as lactate, alanine, and citrate (Cori, Cahill and Krebs cycles, respectively). Decreases in alanine can also generate a general reduction in hepatic production of glucose (Felig P 1973). Citrate is a product derived from the first reaction in the Krebs cycle, which converts oxaloacetate and acetyl CoA into citrate and CoA. Interestingly, the downregulation of ATP citrate synthase (which regulates this process) was previously found in a mixture of CPF and nickel (Boatti et al. 2012). Thus, the reduction of lactate, glucose, and citrate in exposed females is compatible with a general decrease in energy production. Different enzymes associated with the Krebs cycle were hypoactive following CPF exposure and cold stress application, supporting the notion of a decreased cellular metabolic rate at the CNS (Basha & Poojary 2014).

Wang et al. (2009) examined the effects of CPF exposure on blood (serum) metabolites at low doses for male rats and found decreased levels of lactate, alanine, increased levels of NAc and no influence on glucose and different amino acids in exposed females. However, they found decreased levels of LDL/VLDL and increased glutamine levels in animals exposed to CPF. Both the present results and those of Wang's study suggest that low doses of CPF exposure have the potential risk of inducing neurotoxicity by disturbing cellular energy production and fatty acid metabolism, and in our case, this could be particularly true for females.

5. Conclusions and future guidelines

Taking all together, sub-chronic exposure to low doses of CPF during the late postnatal, preweaning developmental stage does not alter social behavior during adolescence and only modestly modulates adult reactions to social novelty, with no effects on dominance status. Thus, the results regarding social behavior are limited and inconclusive, making it difficult to associate this exposure time and dosage with ASD. However, this exposure protocol altered gut microbiome composition at both genus and species taxonomic levels (two weeks after exposure) and induced a long-term (six and a half months after exposure) hyperlipidemic and hypoglycemic/ hypogluconeogenesis profile

with an apparent general decrease in cell energy production. In addition to CPF exposure, the present research also reveals a novel role for dominance in the reaction to social novelty. Although most of these results are novel and congruent with those in the existing literature, further research is needed in order to clarify the specific mechanisms underlying the alterations observed here, particularly for metabolite-related outcomes.

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Conflict of interests

The authors declare no conflict of interest.

Authors' declaration

All the authors have read the manuscript, agree that this work is ready for submission, and accept responsibility for its contents.

Authors' contribution

All the authors have contributed to this study. Mr. Cristian Perez-Fernandez completed the animal care, exposure protocol, behavioral tasks, gDNA extraction from stool samples, sacrifice protocol, statistical analyses and wrote the first version of the manuscript. Mr. Miguel Morales-Navas helped in all the behavioral tasks and statistical analyses as well as revised and improved the quality of the manuscript. Dr. José Miguel Aguilera-Saez, Dra. Ana Cristina Abreu and the professor Ignacio Fernández carried out (experimental procedure, data analyses, and figures and tables' design) the plasma metabolomic experiments and also revised the manuscript until its current form. Dra. Laia Guardia-Escote and Dr. José Antonio Garrido-Cárdenas helped in gut microbiome conceptualization and analysis as well as improved the quality of the manuscript. The professor Maria Teresa Colomina, Dra. Estela Giménez and the professor Fernando Sánchez-Santed set the original experimental design/conceptualization and hypothesis, supervised the experimental protocols and improved and create the final version of the manuscript.

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