Adulthood dietary exposure to a common pesticide leads to an obese-like phenotype and a diabetic profile in apoE3 mice

Fiona Peris-Sampedro [1, 2, 3], Maria Cabré [1, 4], Pia Basaure [1, 2, 3], Ingrid Reverte [1], José L. Domingo [3], Maria Teresa Colomina [1, 2, 3, *]

[1] Research in Neurobehaviour and Health (*NEUROLAB*), Universitat Rovira i Virgili, Tarragona, Spain

[2] Department of Psychology and Research Center for Behavioural Assessment (*CRAMC*), Universitat Rovira i Virgili, Tarragona, Spain

[3] Laboratory of Toxicology and Environmental Health, School of Medicine, *IISPV*, Universitat Rovira i Virgili, Reus, Spain

[4] Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Tarragona, Spain

*** Corresponding author:** Dr Maria Teresa Colomina, Department of Psychology and Research Center for Behaviour Assessment (CRAMC), Universitat Rovira i Virgili, Sescelades Campus, 43007 Tarragona, Spain. Telephone: (34) 977 550875; Fax. (34) 977 558088. E-mail: mariateresa.colomina@urv.cat

Abstract

Increasing evidence links the widespread exposure to organophosphate (OP) pesticides to the global epidemics of type 2 diabetes and obesity. Our recent data highlighted gene x environment interactions: mice expressing the human apolipoprotein E3 (apoE3) isoform were more prone to develop obesity than those expressing apoE2 or apoE4 upon dietary challenge with chlorpyrifos (CPF), the most used OP worldwide. Thus, we aimed to further explore the contribution of the *APOE3* genotype on the emergence of obesity and related metabolic dysfunctions upon subchronic exposure to CPF. Seven-month-old targeted replacement apoE3 and C57BL/6N male mice were orally exposed to CPF at 0 or 2 mg/kg body weight/day for 8 consecutive weeks. We examined body weight status, food and water intake, lipid and glucose homeostasis, metabolic biomarkers concentrations, insulin levels and insulin resistance, and leptin and ghrelin profiles. CPF exposure generally increased food ingestion, glucose and total cholesterol concentrations, and tended to elevate acyl ghrelin levels. Nonetheless, excess weight gain and increased leptin levels were inherent to apoE3 mice. Moreover, the propensity towards a diabetic profile was markedly higher in these animals than in C57BL/6N, as they showed a higher homeostatic model assessment for insulin resistance index and higher insulin levels. Although both genotypes were metabolically affected by CPF, the results of the present investigation revealed that apoE3 mice were the most vulnerable to developing obesity and related disturbances following CPF administration through the diet. Since the *APOE3* genotype is the most prevalent worldwide, current findings have particular implications for human health.

Keywords: Pesticide, Apolipoprotein E, Obesity, Diabetes, Leptin, Ghrelin

Abbreviations: OP, organophosphate; apoE, apolipoprotein E; CPF, chlorpyrifos; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); HOMA-IR, homeostatic model assessment for insulin resistance; ChE, cholinesterase; WHO, World Health Organization; apoE TR mouse model, apoE targeted replacement mouse model; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; AST, aspartate transaminase; ALT, alanine transaminase; ACh, acetylcholine; BChE, butyrylcholinesterase.

1. Introduction

Over the last two centuries, the human lifespan has increased markedly because the development of industrialized societies has led to an improved quality of life. In this context, individuals are constantly and unconsciously exposed to a wide range of xenobiotics, the long-term effects of which are often unknown. Despite its obvious neurotoxic effect (Eaton et al., 2008), chlorpyrifos (CPF) is still the most widely used organophosphate (OP) pesticide in Europe, for both agricultural and urban purposes. It has been classified as a potent inhibitor of both systemic and brain cholinesterases (ChE), leading to the onset of acute neurotoxic symptomatology. However, an increasing body of reports have suggested that CPF also disrupts the serotonergic neurotransmitter system (Slotkin et al., 2015), targets serine hydrolase enzymes (Quistad et al., 2006b) and interferes with the signalling of hormones, some of which – for example, insulin and leptin - are related to energy homeostasis (Lassiter and Brimijoin, 2008; Slotkin et al., 2005). In accordance, sundry investigations have shown that CPF exposure induce a broad spectrum of effects, including metabolic disturbances (Lasram et al., 2014; Peris-Sampedro et al., 2014).

Type 2 diabetes accounts for over 90% of all cases of diabetes. Sedentary lifestyle, obesity, careless dietary habits, low socioeconomic status and genetic vulnerability are well-known risk factors that contribute to its emergence (Zimmet et al., 2001). Nowadays, the prevalence of obesity and type 2 diabetes worldwide is increasing at epidemic rates. According to the World Health Organization (WHO), 13% of the adult population was obese (body mass index ≥ 30 kg/m^2) in 2014, while the predictions of the incidence of type 2 diabetes are not very encouraging, pointing to 366 million type 2 diabetes patients in 2030 (Wild et al., 2004). In the light of this trend, the risk factors commonly studied fail to explain by themselves the global boom of both diseases. Hence, "non-traditional" risk factors have been reconsidered (Arrebola et al., 2015; Howell et al., 2015). Some epidemiological evidence links general pesticide exposure (Arrebola et al., 2013, 2015; Suarez-Lopez et al., 2015) and more specifically OP exposure (Montgomery et al., 2008; Saldana et al., 2007) to a higher incidence of type 2 diabetes and related metabolic dysfunctions. Nevertheless, experimental studies are scarce. Very little research has investigated the metabolic and endocrine effects that emerge following adulthood exposure to CPF in rodents, being most studies focused on early-life exposure (Lassiter and Brimijoin, 2008; Slotkin et al., 2005). Current knowledge of adulthood exposure to CPF is limited to four studies carried out in rats. From these, two revealed a weight gain in treated subjects (Ehrich et al., 2004; Meggs and Brewer, 2007) and the other two pointed to

disturbances of both glucose and lipid metabolisms in exposed animals (Acker and Nogueira, 2012; Elsharkawy et al., 2013). In general, these protocols were based on high CPF doses.

Apolipoprotein E (apoE) is a glycoprotein mainly involved in the maintenance of plasma lipid homeostasis, and is basically synthesised in the liver, but also in the brain and adipose tissue (Frühbeck, 2004; Gee and Keller, 2005). The human *APOE* gene is polymorphic and presents three major allelic variants (*ε2*, *ε3*, *ε4*), coding for three main isoforms associated with a low-to-high prevalence following the apoE2 < apoE4 < apoE3 rank order (Corbo and Scacchi, 1999). While apoE3 is accepted as the healthy phenotype, recent experimental data have shown that it tends to be more prone to developing diet-induced obesity (Arbones-Mainar et al., 2008; Huebbe et al.0, 2014; Karagiannides et al., 2008), and more vulnerable to decabromodiphenyl ether (Reverte et al., 2013). In a recent study, we found that apoE3 mice were more vulnerable to gain excess weight upon CPF exposure than apoE2 and apoE4 mice (Peris-Sampedro et al., 2015).

The apoE targeted replacement (TR) mouse model was originally created by Sullivan et al (1997). These animals have a C57BL/6N background but their murine *apoE* gene has been replaced by one of the three most prevalent human *APOE* alleles. Thus, apoE TR mice differ from C57BL/6N in that they carry and express functional human apoE isoforms at physiological levels. It has been established that this expression does not alter any known endogenous regulatory sequence (Sullivan et al., 1997), being the subsequent phenotype in mice similar to that found in humans (Hauser et al., 2011).

Based on our previous results and from evidence gathered in the literature, the main objectives of the current investigation were: a) to provide greater insight into the metabolic disturbances, ranging from hormonal imbalance to disturbed eating behaviour, as a result of CPF exposure, and b) to investigate how the human *ε3* allele might favour their emergence. For these purposes, the metabolic profile of both apoE3 and C57BL/6N male mice were assessed and compared after an 8-week period of oral exposure to CPF.

2. Material and methods

2.1 Chemicals

CPF (*O*,*O*-diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate, purity 99.5%) was supplied by Sigma-Aldrich (Seelze, Germany). Standard rodent chow (Panlab, Barcelona, Spain) was supplemented with CPF at a concentration intended to deliver a dose of 2 mg/kg body

weight/day, based on the results of our recent study (Peris-Sampedro et al., 2015). The protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) was also purchased from Sigma-Aldrich.

2.2 Animal care

Seven-month-old apoE TR male mice and C57BL/6N male mice were used. Mice homozygous for the human *ε3* allele were obtained from Taconic (Taconic Europe, Lille Skensved, Denmark), and C57BL/6N mice were purchased from Charles River (Charles River France, L'Arbresle, France). After a quarantine period, the animals were properly housed in plastic cages containing 2-3 individuals in an environmentally controlled room equipped with a 12-h light-dark automatic light cycle (light: 08:00 - 20:00 h), a temperature of 22 ± 2ºC, and a relative humidity of 50% ± 10%. Mice were allowed access to food and fresh water *ad libitum* and given a standard chow diet (Panlab, Barcelona, Spain) before the experiment started. The use of animals and the experimental protocol design were supervised and approved by the Animal Care and Use Committee of the Rovira i Virgili University (Tarragona, Spain). Likewise, efforts were made to alleviate animal suffering as established by the Spanish Royal Decree 53/2013 and and the European Communities Council Directive (86/609/EEC).

2.3 Treatment protocol

The animals were weighed and then distributed into four experimental groups (n = 10/group): control apoE3, control C57BL/6N, CPF-exposed apoE3, and CPF-exposed C57BL/6N. Mice were fed either a standard or a CPF-supplemented rodent chow (2 mg/kg body weight/day) for 8 consecutive weeks, and were checked for cholinergic signs twice a week. After the treatment period, animals were subjected to a 3-hour fast before being anesthetized with carbon dioxide and euthanized by cardiac puncture. Blood was immediately collected into 500 µL tubes containing EDTA (BD Microtainer®, Plymouth, United Kingdom), and centrifuged at 3000 rpm for 20 min at 4ºC to obtain plasma, which was aliquoted and stored at -80 ºC.

2.4 Plasma cholinesterase activity

Plasma ChE activity was evaluated as an indicator of the systemic CPF effect (Eaton et al., 2008). It was determined spectrophotometrically using a commercial available kit, as

recommended by the supplier. Briefly, the cholinesterase enzyme hydrolyses butyrylthiocholine to give thiocholine and butyrate. The reaction between thiocholine and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) produces 2–nitro–5-mercaptobenzoate, a yellow compound which can be measured at 405 nm. The enzymatic activity of exposed animals was calculated on the basis of the activity value of the control mice, and represented as a percentage.

2.5 Body weight status and food and water consumption

The body weight status of the mice was recorded weekly over the treatment period. Food intake was estimated on a daily basis for a 7-day period by subtracting the uneaten pellets at the end of the week from the total amount of food given at the beginning. To obtain a more accurate value, we made sure there was no leftover food scattered around the cage. The average daily food consumption obtained was divided by the number of animals in the cage. Water intake was estimated in the same way.

2.6 Analysis of metabolic biomarkers

Plasma concentrations of total cholesterol, triglycerides, albumin and creatinine, as well as total activity levels of aspartate (AST) and alanine (ALT) transaminases were determined after 3 h of food withdrawal as biomarkers of metabolic state in both control and CPF-fed mice. They were determined with commercially available kits supplied by QCA (Química Analítica Clínica S.A., QCA, Amposta, Spain). Briefly, every absorbance measurement was carried out in duplicate according to the manufacturer's instructions at a constant temperature of 37ºC with a semiautomatic COBAS MIRA analyser (Hoffman-La Roche & Co., Basel, Switzerland). Before sacrifice, fasting glucose was measured by tail bleeding using a handheld glucometer (Accu-check Performa, Roche Diagnostics, Sant Cugat del Vallès, Spain). Each parameter was expressed in the international system of units (SI).

2.7 Measurement of insulin sensitivity

Insulin sensitivity was estimated by determining fasting plasma insulin levels and by computing an insulin resistance score: the homeostatic model assessment for insulin resistance (HOMA-IR). Plasma insulin levels were assessed in duplicate with a commercially available ELISA mouse kit supplied by Merck Millipore (Darmstadt, Germany), following the

manufacturer's instructions, and were expressed in SI. Insulin resistance was estimated on the basis of both fasting glucose and fasting insulin values, using the HOMA-IR index first described by Matthews et al. 1985, as follows: HOMA-IR = (fasting insulin x fasting glucose) / 22.5, where insulin and glucose concentrations were expressed in mU/L and SI, respectively. The conversion factor used for insulin was 1 mU/L = 6 pmol/L, which was based on the first international standard for insulin issued by the WHO in 1987 (Vølund, 1993).

2.8 Quantification of plasma leptin, total ghrelin and acyl ghrelin levels

Plasma leptin levels were determined in order to provide further insight into the body weight status, while acyl and total ghrelin levels were assessed to evaluate more in depth feeding behaviour. The concentration of plasma leptin was measured in duplicate with a mouse ELISA kit provided by Merck Millipore (Darmstadt, Germany), as recommended by the supplier, and was expressed in SI. Prior to storage, plasma aliquots intended for the determination of total ghrelin and acyl ghrelin levels were supplemented with AEBSF in order to prevent hormone degradation by proteases. At this point, both ghrelin statuses were also evaluated in duplicated with commercially available mouse ELISA kit from Merck Millipore. Acyl ghrelin and total ghrelin levels were expressed in pg/mL and ng/mL, respectively.

2.9 Statistical analysis

Data were analysed with the SPSS statistical package (version 20.0), and reported as mean values ± SE. Two-way analysis of variance (ANOVA) were performed to establish the contribution of both the CPF and *APOE* genetic background to the inhibition of plasma ChE, food and water consumption, metabolic biomarkers, hormones profiles and HOMA-IR index values. Throughout the 8-week experiment the body weight profile was studied by two-way repeated-measures ANOVA with the period of time as the within-subject factor. Tukey's *post hoc* test was used for multiple comparisons. A correlation analysis, determined by linear regression, was performed to assess the relationship between body weight and circulating levels of leptin. Statistical significance was set at $p < 0.05$.

3. Results

3.1 Plasma cholinesterase inhibition and signs of toxicity

During the experimental period, we noticed no apparent signs of cholinergic toxicity in any group. Assessed under fasting conditions at the end of the treatment, plasma ChE activity dropped to 32.12 % in CPF-exposed mice.

3.2 CPF triggers body weight gain in apoE3 mice and increases food intake

Prior to the exposure period, *post hoc* analyses showed no significant differences in initial body weight between groups. The exposure led to gradual weight gain throughout the experiment [$F_{7,39} = 13.662$, $p < 0.001$]. Thus, CPF-exposed mice showed higher body weights than their respective controls. A triple interaction (time x treatment x genotype) was noted during the experiment [$F_{7,39} = 2.355$, $p = 0.048$], and the genotype tended to have an overall effect [$F_{1,39} = 3.128$, $p = 0.085$]. Highest propensity to the CPF obesogenic effect was observed in apoE3 mice [$F_{1,19} = 5.077$, $p = 0.037$], which weighed more than their control counterparts from the fourth week until the end of the treatment (Fig. 1A). In contrast, we found only an upward trend in exposed C57BL/6N animals [$F_{1,19} = 3.443$, $p = 0.080$] (Fig. 1B). While water consumption was not altered over the experiment, CPF exposure increased food intake [$F_{1,39} = 124.361$, $p < 0.001$] (Table 1).

--Insert Figure 1 over here--

--Insert Table 1 over here--

3.3 CPF increases total cholesterol and fasting glucose levels

The levels of metabolic biomarkers are set out in Table 2. With regards to plasma lipids, CPF exposure generally increased total cholesterol levels [$F_{1,39} = 4.736$, $p = 0.036$]. There were no significant differences in plasma triglycerides between groups. The genotype affected plasma creatinine levels differently [$F_{1,26} = 10.989$, $p = 0.003$], having apoE3 mice higher concentrations than C57BL/6N animals. As expected, plasma albumin levels and both ALT and AST activities were unaltered between genotypes and were statistically indistinguishable among groups, indicating that there was no deterioration of renal and hepatic functions upon dietary CPF exposure. Both the genotype [$F_{1,39} = 6.214$, $p = 0.017$] and the treatment [$F_{1,39} = 4.893$, $p = 0.033$] altered fasting glucose concentration. The highest levels of glucose were inherent to the *ε3* allele carriers and the CPF-treated mice on the other hand.

--Insert Table 2 over here--

3.4 Insulin levels and insulin resistance are higher in CPF-exposed apoE3 mice

Hyperglycemia emerged after prolonged exposure to CPF. To determine if these high levels of fasting glucose were related to higher rates of insulin resistance, both fasting insulin levels (Fig. 2A) and HOMA-IR (Fig. 2B) were assessed. Both the genotype [$F_{1,34} = 17.010$, $p < 0.001$] and the treatment [$F_{1,34} = 11.112$, $p = 0.002$] influenced insulin levels, and a genotype x treatment interaction was found [$F_{1,34} = 4.337$, $p = 0.046$]. Data from both genotypes were then studied separately. Reanalyses showed that CPF exposure increased fasting plasma insulin levels in both apoE3 [$F_{1,17} = 8.143$, $p = 0.011$] and C57BL/6N mice [$F_{1,16} = 8.892$, $p = 0.009$]. Strikingly, however, *post hoc* testing revealed that exposed apoE3 mice were more sensitive to CPF as their insulin levels were 54.62% higher than those found in their treated counterparts (Fig. 2A).

--Insert Figure 2 over here--

Both the genotype [$F_{1,34} = 11.808$, $p = 0.002$] and the treatment [$F_{1,34} = 10.477$, $p = 0.003$] were found to have an overall effect on HOMA-IR. A significant interaction between genotype and treatment [$F_{1,34} = 4.245$, $p = 0.048$] was also found. Further analysis confirmed an overall effect of the treatment on HOMA-IR in both apoE3 [$F_{1,17} = 7.868$, $p = 0.013$] and C57BL/6N mice [$F_{1,16} = 6.382$, $p = 0.023$], which indicates that CPF exposure leads to higher rates of insulin resistance. Likewise, *post hoc* analyses highlighted the propensity of *ε3* allele carriers to develop insulin resistance as their HOMA-IR values were 59.79% higher than those of C57BL/6N mice exposed to CPF (Fig. 2B).

3.5 Leptin levels of apoE3 mice are increased by CPF

Both the genotype [$F_{1,33} = 11.655$, $p = 0.002$] and the treatment [$F_{1,33} = 11.037$, $p = 0.002$] showed overall effects on leptin levels (Fig. 3A). In fact, only apoE3 mice were found to have significantly elevated leptin concentration after CPF exposure [$F_{1,16} = 9.356$, $p = 0.008$], suggesting greater amounts of fat depots in these subjects. In addition, we studied the relationship between body weight status at the end of the treatment and circulating levels of

leptin, determined in plasma after a 3-h fast period (Fig. 3B). Body weight and leptin were strongly correlated ($r^2 = 0.729$, $p < 0.001$). The linear regression indicated that for each gram of weight gain, leptin levels increased in 1.827 µg/L.

--Insert Figure 3 over here--

3.6 CPF tends to increase acyl ghrelin levels

Repeated exposure to CPF tended to increase acyl ghrelin levels [$F_{1,26} = 3.775$, $p = 0.064$], which could explain the elevated rates of food intake found in the treated animals (Fig. 4A). Furthermore, total ghrelin levels were dependent upon genotype [$F_{1,28} = 4.328$, $p = 0.048$]: C57BL/6N mice appeared to have higher concentrations than apoE3 (Fig. 4B).

--Insert Figure 4 over here--

4. Discussion

The present study aimed to fully explore the metabolic effects of a subchronic dietary exposure to CPF, as well as to assess whether the human *APOE3* genotype could exacerbate their emergence. The metabolic disturbances arising out of the 8-week treatment period were evaluated in both apoE3 and C57BL/6N adult male mice and then compared. Specifically, body weight status, food intake, lipid and glucose homeostasis, metabolic biomarker concentrations, insulin levels and insulin resistance, and leptin and ghrelin profiles were investigated. Our results indicated that repeated dietary doses of CPF, devoid of signs of cholinergic toxicity, induced metabolic alterations in both genotypes. Nevertheless, this study shed novel and significant evidence supporting the vulnerability of human *ε3* carriers to the development of obesity and related metabolic disturbances in response to CPF. Indeed, although CPF broadly increased food intake, weight gain and higher plasma leptin levels were inherent to CPF-fed apoE3 mice. Furthermore, both exposed groups exhibited hyperinsulinemia and displayed insulin resistance, but these effects were more prominent in the *ε3* carriers. Total cholesterol and fasting plasma glucose levels increased overall in treated animals. CPF exposure also tended to increase plasma acyl ghrelin levels.

Despite the growing body of epidemiological data linking pesticide exposure with increased incidence of obesity (Kim et al., 2015), little attention has been paid to the contribution of CPF.

To the best of our knowledge, only three experimental studies have revealed weight gain after early-life (Lassiter and Brimijoin, 2008) or adulthood exposures to CPF (Ehrich et al., 2004; Meggs and Brewer, 2007). The present investigation indicated that repeated exposure to CPF induced a weight gain in both apoE3 and C57BL/6N mice. Nevertheless, this increase was faster and steeper in *ε3* carriers, in agreement with our previous study (Peris-Sampedro et al., 2015). The present results also revealed an increase in leptin levels in exposed-apoE3 animals. Moreover, body weight status at the end of the treatment period and circulating leptin levels were strongly correlated. The concentration of leptin has been positively correlated with the percentage of body fat in humans (Harris, 2000). This hormone operates as a satiety signal, inhibiting food intake and promoting energy expenditure (Pan et al., 2014). Nonetheless, its levels have been found to be elevated in obese individuals (Harris, 2000). The increase in leptin levels described here suggests that CPF-treated apoE3 mice had higher amounts of body fat, thus indicating that CPF exposure could be increasing their adiposity. Despite no significant changes in leptin levels, Lassiter and Brimijoin (2008) argued that the excess weight gain observed after developmental exposure to CPF could be due to an increased adiposity in rats. Accordingly, Meggs and Brewer (2007) found that subcutaneous exposure to 5 mg/kg/day CPF for 3 months increased adipose tissue in rats. Therefore, adipose tissue is expected to be a potential target for CPF, which is highly lipophilic in nature.

In recent years several studies have suggested that the *APOE3* genotype contributes to the development of diet-induced obesity (Arbones-Mainar et al., 2008; Karagiannides et al., 2008). ApoE3 mice subjected to a western-type diet were phenotypically more obese than apoE4 mice, while their total and subcutaneous amounts of fat also increased (Arbones-Mainar et al., 2008). In agreement with this, Huebbe et al. (2014) reported that apoE3 mice were heavier than apoE4 not only when they were on a high-fat diet, but also on a low-fat diet. When these authors explored the mechanisms by which the *APOE3* genotype could be contributing to increased fat depots, they suggested that the *ε3* carriers were more efficient at harvesting dietary energy (Huebbe et al., 2014). In the light of the above, the combination of apoE3 isoform expression with CPF exposure would provoke an additive effect, and greater body weight would be expected in exposed apoE3 mice. The present results are consistent with the genotype-dependent weight gain observed in our previous study (Peris-Sampedro et al., 2015), raising the issue of whether the human *ε3* allele could be promoting fat accumulation and, subsequently, favouring an obese-like phenotype after CPF exposure.

Increasing epidemiological and experimental evidence suggests that OPs disrupt glucose metabolism and cause insulin resistance, leading to type 2 diabetes (Lasram et al., 2014).

Nevertheless, the data are sometimes contradictory and fail to define how they trigger them. Furthermore, only few studies have investigated whether adulthood exposure to CPF can contribute to the onset of insulin resistance or type 2 diabetes, the most frequently studied OPs being malathion and diazinon (Lasram et al., 2014). In the current investigation, repeated exposure to CPF induced moderate fasting hyperglycemia 8 weeks after the treatment started. In this context, only two studies have explored the role of CPF in disturbing glucose homeostasis throughout adulthood (Acker and Nogueira, 2012; Elsharkawy et al., 2013). Despite differences in experimental protocols, our data are in agreement with those reported by these studies, which found an increase in glucose levels in both Wistar and Sprague-Dawley adult male rats after a single acute dose of 50 mg/kg CPF (Acker and Nogueira, 2012) and following a 3 month-period of oral exposure to CPF at 30 mg/kg body weight (Elsharkawy et al., 2013). The mechanisms by which OPs exert their hyperglycemic function are the subject of intense debate. One of the most widely accepted is that they disrupt the gluconeogenesis and glycogenolysis pathways in the liver, but the findings are rather varied. The work conducted by Acker and Nogueira (2012) revealed increased activities of both tyrosine aminotransferase and glucose-6-phosphatase enzymes, pointing to enhanced CPF-related liver glucose production. However, they found an increase in hepatic glycogen levels, but not a decrease, which indicates that this route is not associated with hyperglycemia upon CPF exposure. A possible explanation for the elevated glucose levels observed in response to CPF is its cholinergic disrupting effect. It is well-known that acetylcholine (ACh) elicits the release of adrenaline and noradrenaline in the adrenal medulla (Butterworth and Mann, 1957). Indeed, it has been suggested that ChE inhibitors exacerbate this ACh-induced catecholamine release (Akiyama et al., 2003), which could trigger transient hyperglycemia by decreasing insulin-stimulated translocation of glucose transporters to the plasma membrane (Mulder et al., 2005). Ultimately, the excessive release of catecholamines could lead to insulin resistance (Ziegler et al., 2012).

OPs are known to generally alter lipid metabolism (Lasram et al., 2014). Just as found for glucose, repeated exposure to CPF increased total cholesterol levels in mice 8 weeks after the treatment started. Likewise, Elsharkawy et al. (2013) reported elevated plasma cholesterol levels following subchronic oral exposure to CPF in rats. They related this increase to liver cell damage, which was verified by light microscopic examination. While our exposure paradigm did not affect triglyceride concentrations, Elsharkawy et al. (2013) found reduced triglyceride levels in CPF-treated rats, which were also explained in terms of liver damage. Intrinsic mechanisms of CPF to promote hypercholesterolemia have not been yet disclosed. However,

it is worth pointing out that CPF has been shown to target such key enzymes related to lipid metabolism as monoacylglycerol lipase and fatty acid amide hydrolase, among others (Quistad et al., 2006a).

Without underestimating the importance of exposure to other OPs for insulin resistance and type 2 diabetes outcomes (Lasram et al., 2014), the present study combining adulthood CPF exposure and human apoE3 isoform expression in mice appears to have no specific precedent. Repeated exposure to CPF led to increased insulin levels and the higher HOMA-IR values pointed to the development of insulin resistance, in both apoE3 and C57BL/6N mice. Hyperinsulinemia is considered to be indicative of insulin resistance, as well as a predictor of developing type 2 diabetes. Interestingly, although the insulin pathway was notably disturbed in both genotypes following CPF exposure, the effect was greater in apoE3 mice. Visceral obesity has been associated with insulin resistance (Yamashita et al., 1996). Indeed, it has been shown that leptin plays a role in modulating insulin action and sensitivity, and has been related to the emergence of insulin resistance and subsequent type 2 diabetes (Söderberg et al., 2007). Taking into account that the *APOE3* genotype appears to more efficiently harvest dietary energy through fat deposition, we hypothesize that apoE3 isoform expression aggravates insulin resistance and subsequent type 2 diabetes following CPF exposure.

Contrary to what might be expected, high levels of leptin in obesity fail to inhibit food intake (Harris, 2000). Our results indicated that repeated exposure to CPF generally increased food ingestion. In line with these findings, we recently found that CPF exposure tended to alter feeding behaviour (Peris-Sampedro et al., 2015). In relation to this, the role of ghrelin deserves special mention. It is an orexigenic hormone, secreted by the stomach, which stimulates food intake. Two forms of ghrelin coexist in the blood: acyl ghrelin and des-acyl ghrelin. However, only the acylated form has been shown to bind the growth hormone secretagogue receptor. The inactivation of acyl ghrelin into the deacylated form depends on hydrolization by the butyrylcholinesterase (BChE) enzyme (De Vriese et al., 2004). It is well-established that CPF inhibits both systemic and brain ChE enzymes. Accordingly, inhibition of BChE by CPF would be expected to increase acyl ghrelin levels, thereby leading to increased food intake. In support of this, we found that acyl ghrelin levels increased, although not significantly, after repeated exposure to CPF. Likewise, there is increasing evidence to suggest that acyl ghrelin could be a modulator of glucose homeostasis, and its elevated circulating levels could also be a triggering factor for type 2 diabetes (Huang et al., 2014). This finding shed novel information about how CPF exposure, in terms of BChE inhibition, would elicit type 2 diabetes outcome.

In conclusion, the results of the present study show that repeated exposure to the pesticide CPF can considerably disrupt not only glucose and lipid homeostasis but also feeding behaviour in adult male mice. Together with recent results (Peris-Sampedro et al., 2015), the current data provide enough evidence to suggest that human apoE3 isoform expression increases vulnerability to developing obesity and related metabolic dysfunctions after CPF exposure. Although not conclusive, the results of this study suggest that CPF has hormonal targets, such as leptin or ghrelin, on which, to date, little has been reported in the scientific literature. The CPF dose used in our experiment, although free from cholinergic symptoms, is relatively high when compared with the dose that would be expected for typical non-occupational exposures. Therefore, further research is required to provide new insights into what doses would be exempt from metabolic effects. Given the wide distribution of the apoE3 phenotype worldwide, as well as the ubiquitous use of CPF, it is worth asking whether the combination of the two factors is contributing to the global incidence of obesity and type 2 diabetes.

Conflict of interest

The authors declare that there is no conflict of interest associated with their contribution to this manuscript.

Acknowledgements

This work was supported by the Ministry of the Economy and Competitiveness (MINECO, Spain) (grant number PSI2010-21743-C02-01) and the Commission for Universities and Research of the Department of Innovation, Universities and Enterprise of the *Generalitat de Catalunya* (grant number 2013 FI_B00170).

The authors would like to thank Amparo Aguilar, Esperanza Chernichero and Juan València (Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili), for their skilful technical support with animal care.

References

Acker, C.I., Nogueira, C.W., 2012. Chlorpyrifos acute exposure induces hyperglycemia and hyperlipidemia in rats. Chemosphere 89, 602–8.

Akiyama, T., Yamazaki, T., Mori, H., Sunagawa, K., 2003. Inhibition of cholinesterase elicits muscarinic receptor-mediated synaptic transmission in the rat adrenal medulla. Auton. Neurosci. 107, 65–73.

Arbones-Mainar, J.M., Johnson, L.A., Altenburg, M.K., Maeda, N., 2008. Differential modulation of diet-induced obesity and adipocyte functionality by human apolipoprotein E3 and E4 in mice. Int. J. Obes. (Lond). 32, 1595–605.

Arrebola, J.P., Pumarega, J., Gasull, M., Fernandez, M.F., Martin-Olmedo, P., Molina-Molina, J.M., Fernández-Rodríguez, M., Porta, M., Olea, N., 2013. Adipose tissue concentrations

of persistent organic polluants and prevalence of type 2 diabetes in adults from Southern Spain. Environ. Res. 122, 31-7.

Arrebola, J.P., González-Jiménez, A., Fornieles-González, C., Artacho-Cordón, F., Olea, N., Escobar-Jiménez, F., Fernández-Soto, M.L., 2015. Relationship between serum concentrations of persistent organic pollutants and markers of insulin resistance in a cohort of women with a history of gestational diabetes mellitus. Environ. Res. 136, 435–40.

Butterworth, K.R., Mann, M., 1957. The release of adrenaline and noradrenaline from the adrenal gland of the cat by acetylcholine. Br. J. Pharmacol. Chemother. 12, 422–6.

Corbo, R.M., Scacchi, R., 1999. Apolipoprotein E (APOE) allele distribution in the world. Is APOE*4 a "thrifty" allele? Ann. Hum. Genet. 63, 301–10.

De Vriese, C., Gregoire, F., Lema-Kisoka, R., Waelbroeck, M., Robberecht, P., Delporte, C., 2004. Ghrelin degradation by serum and tissue homogenates: identification of the cleavage sites. Endocrinology 145, 4997–5005.

Eaton, D.L., Daroff, R.B., Autrup, H., Bridges, J., Buffler, P., Costa, L.G., Coyle, J., McKhann, G., Mobley, W.C., Nadel, L., Neubert, D., Schulte-Hermann, R., Spencer, P.S., 2008. Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. Crit. Rev. Toxicol. 38 Suppl 2, 1–125.

Ehrich, M., Hancock, S., Ward, D., Holladay, S., Pung, T., Flory, L., Hinckley, J., Jortner, B.S., 2004. Neurologic and immunologic effects of exposure to corticosterone, chlorpyrifos, and multiple doses of tri-ortho-tolyl phosphate over a 28-day period in rats. J. Toxicol. Environ. Health. A 67, 431–57.

Elsharkawy, E.E., Yahia, D., El-Nisr, N.A., 2013. Sub-chronic exposure to chlorpyrifos induces hematological, metabolic disorders and oxidative stress in rat: attenuation by glutathione. Environ. Toxicol. Pharmacol. 35, 218–27.

Frühbeck, G., 2004. The adipose tissue as a source of vasoactive factors. Curr. Med. Chem. Cardiovasc. Hematol. Agents 2, 197–208.

Gee, J.R., Keller, J.N., 2005. Astrocytes: regulation of brain homeostasis via apolipoprotein E. Int. J. Biochem. Cell Biol. 37, 1145–50.

Harris, R.B., 2000. Leptin--much more than a satiety signal. Annu. Rev. Nutr. 20, 45–75.

Hauser, P.S., Narayanaswami, V., Ryan, R.O., 2011. Apolipoprotein E: from lipid transport to neurobiology. Prog. Lipid Res. 50, 62–74.

Howell, G.E., Mulligan, C., Meek, E., Chambers, J.E., 2015. Effect of chronic p,p'-dichlorodiphenyldichloroethylene (DDE) exposure on high fat diet-induced alterations in glucose and lipid metabolism in male C57BL/6H mice. Toxicology 328, 112–22.

Huang, L., Tong, Y., Zhang, F., Yang, Q., Li, D., Xie, S., Li, Y., Cao, H., Tang, L., Zhang, X., Tong, N., 2014. Increased acyl ghrelin but decreased total ghrelin and unacyl ghrelin in

Chinese Han people with impaired fasting glucose combined with impaired glucose tolerance. Peptides 60, 86–94.

Huebbe, P., Dose, J., Schloesser, A., Campbell, G., Glüer, C.-C., Gupta, Y., Ibrahim, S., Minihane, A.-M., Baines, J.F., Nebel, A., Rimbach, G., 2015. Apolipoprotein E (APOE) genotype regulates body weight and fatty acid utilization- studies in gene targeted replacement mice. Mol. Nutr. Food Res 59(2), 334-43.

Karagiannides, I., Abdou, R., Tzortzopoulou, A., Voshol, P.J., Kypreos, K.E., 2008. Apolipoprotein E predisposes to obesity and related metabolic dysfunctions in mice. FEBS J. 275, 4796–809.

Kim, S.-A., Kim, K.-S., Lee, Y.-M., Jacobs, D.R., Lee, D.-H., 2015. Associations of organochlorine pesticides and polychlorinated biphenyls with total, cardiovascular, and cancer mortality in elders with differing fat mass. Environ. Res. 138, 1–7.

Lasram, M.M., Dhouib, I.B., Annabi, A., El Fazaa, S., Gharbi, N., 2014. A review on the molecular mechanisms involved in insulin resistance induced by organophosphorus pesticides. Toxicology 322, 1–13.

Lassiter, T.L., Brimijoin, S., 2008. Rats gain excess weight after developmental exposure to the organophosphorothionate pesticide, chlorpyrifos. Neurotoxicol. Teratol. 30, 125–30.

Matthews, D.R., Hosker, J.P., Rudenski, A.S., Naylor, B.A., Treacher, D.F., Turner, R.C., 1985. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28, 412–9.

Meggs, W.J., Brewer, K.L., 2007. Weight gain associated with chronic exposure to chlorpyrifos in rats. J. Med. Toxicol. 3, 89–93.

Montgomery, M.P., Kamel, F., Saldana, T.M., Alavanja, M.C.R., Sandler, D.P., 2008. Incident diabetes and pesticide exposure among licensed pesticide applicators: Agricultural Health Study, 1993-2003. Am. J. Epidemiol. 167, 1235–46.

Mulder, A.H., Tack, C.J., Olthaar, A.J., Smits, P., Sweep, F.C.G.J., Bosch, R.R., 2005. Adrenergic receptor stimulation attenuates insulin-stimulated glucose uptake in 3T3-L1 adipocytes by inhibiting GLUT4 translocation. Am. J. Physiol. Endocrinol. Metab. 289, E627–33.

Pan, H., Guo, J., Su, Z., 2014. Advances in understanding the interrelations between leptin resistance and obesity. Physiol. Behav. 130, 157–69.

Peris-Sampedro, F., Basaure, P., Reverte, I., Cabré, M., Domingo, J.L., Colomina, M.T., 2015. Chronic exposure to chlorpyrifos triggered body weight increase and memory impairment depending on human apoE polymorphisms in a targeted replacement mouse model. Physiol. Behav. 144, 37-45.

Peris-Sampedro, F., Salazar, J.G., Cabré, M., Reverte, I., Domingo, J.L., Sánchez-Santed, F., Colomina, M.T., 2014. Impaired retention in AβPP Swedish mice six months after oral exposure to chlorpyrifos. Food Chem. Toxicol. 72, 289–94.

Quistad, G.B., Klintenberg, R., Caboni, P., Liang, S.N., Casida, J.E., 2006a. Monoacylglycerol lipase inhibition by organophosphorus compounds leads to elevation of brain 2-arachidonoylglycerol and the associated hypomotility in mice. Toxicol. Appl. Pharmacol. 211, 78–83.

Quistad, G.B., Liang, S.N., Fisher, K.J., Nomura, D.K., Casida, J.E., 2006b. Each lipase has a unique sensitivity profile for organophosphorus inhibitors. Toxicol. Sci. 91, 166–72.

Reverte, I., Klein, A.B., Domingo, J.L., Colomina, M.T., 2013. Long term effects of murine postnatal exposure to decabromodiphenyl ether (BDE-209) on learning and memory are dependent upon APOE polymorphism and age. Neurotoxicol. Teratol. 40, 17–27.

Saldana, T.M., Basso, O., Hoppin, J.A., Baird, D.D., Knott, C., Blair, A., Alavanja, M.C.R., Sandler, D.P., 2007. Pesticide exposure and self-reported gestational diabetes mellitus in the Agricultural Health Study. Diabetes Care 30, 529–34.

Slotkin, T.A., Brown, K.K., Seidler, F.J., 2005. Developmental exposure of rats to chlorpyrifos elicits sex-selective hyperlipidemia and hyperinsulinemia in adulthood. Environ. Health Perspect. 113, 1291–4.

Slotkin, T.A., Skavicus, S., Levin, E.D., Seidler, F.J., 2015. Prenatal nicotine changes the response to postnatal chlorpyrifos: Interactions targeting serotonergic synaptic function and cognition. Brain Res. Bull. 111C, 84–96.

Söderberg, S., Zimmet, P., Tuomilehto, J., Chitson, P., Gareeboo, H., Alberti, K.G.M.M., Shaw, J.E., 2007. Leptin predicts the development of diabetes in Mauritian men, but not women: a population-based study. Int. J. Obes. (Lond). 31, 1126–33.

Suarez-Lopez, J.R., Lee, D.-H., Porta, M., Steffes, M.W., Jacobs, D.R., 2015. Persistent organic pollutants in young adults and changes in glucose related metabolism over a 23-year follow-up. Environ. Res. 137, 485–94.

Sullivan, P.M., Mezdour, H., Aratani, Y., Knouff, C., Najib, J., Reddick, R.L., Quarfordt, S.H., Maeda, N., 1997. Targeted replacement of the mouse apolipoprotein E gene with the common human APOE3 allele enhances diet-induced hypercholesterolemia and atherosclerosis. J. Biol. Chem. 272, 17972–80.

Vølund, A., 1993. Conversion of insulin units to SI units. Am. J. Clin. Nutr. 58, 714–5.

Wild, S., Roglic, G., Green, A., Sicree, R., King, H., 2004. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 27, 1047–53.

Yamashita, S., Nakamura, T., Shimomura, I., Nishida, M., Yoshida, S., Kotani, K., Kameda-Takemuara, K., Tokunaga, K., Matsuzawa, Y., 1996. Insulin resistance and body fat distribution. Diabetes Care 19, 287–91.

Ziegler, M.G., Elayan, H., Milic, M., Sun, P., Gharaibeh, M., 2012. Epinephrine and the metabolic syndrome. Curr. Hypertens. Rep. 14, 1–7.

Zimmet, P., Alberti, K.G., Shaw, J., 2001. Global and societal implications of the diabetes epidemic. Nature 414, 782–7.

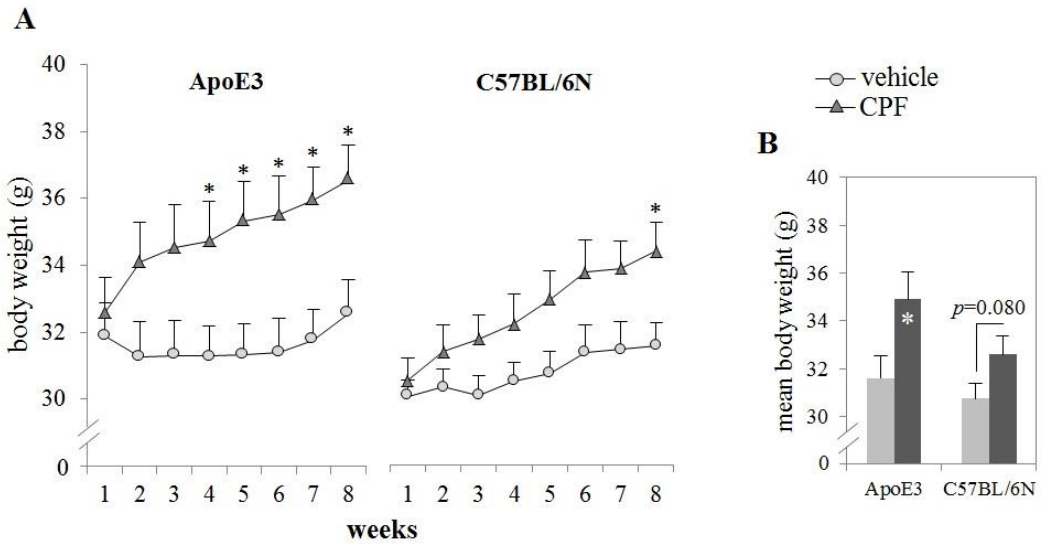

Figure 1. The body weight progression was recorded weekly over the 8-week treatment period to evaluate the obesogenic effect of subchronic oral adulthood exposure to chlorpyrifos in both apoE3 and C57BL/6N male mice (A). The mean body weight of the experimental period was also depicted for each group (B). *Asterisks* indicate significant differences between CPF-exposed mice and their corresponding control group ($p < 0.05$).

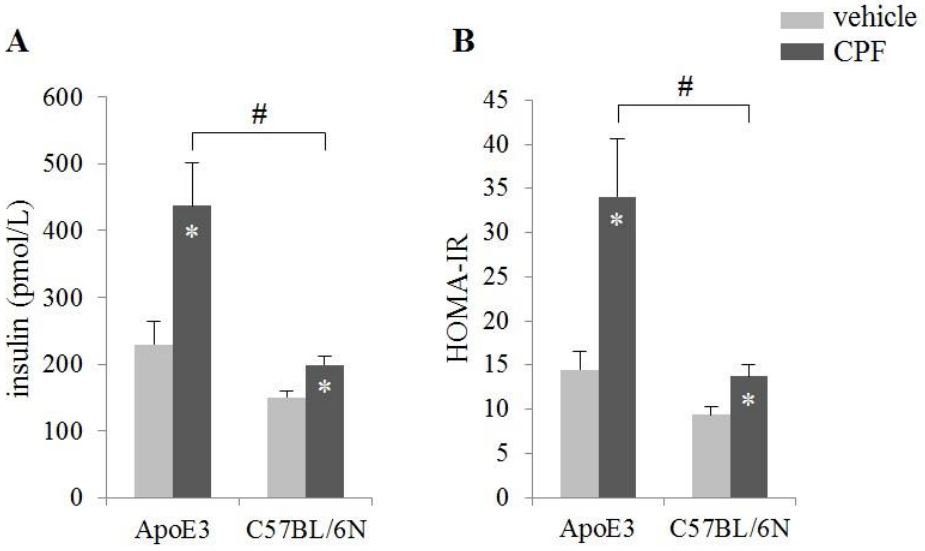

Figure 2. The fasting plasma insulin levels (A) and the estimation of insulin resistance, which was based on the HOMA-IR index (B), were evaluated to estimate insulin sensitivity after

subchronic oral adulthood exposure to chlorpyrifos in both apoE3 and C57BL/6N male mice. *Asterisks* indicate significant differences between CPF-exposed mice and their corresponding control group ($p < 0.05$), while the symbol # indicates significant differences between genotypes on the same treatment ($p < 0.05$).

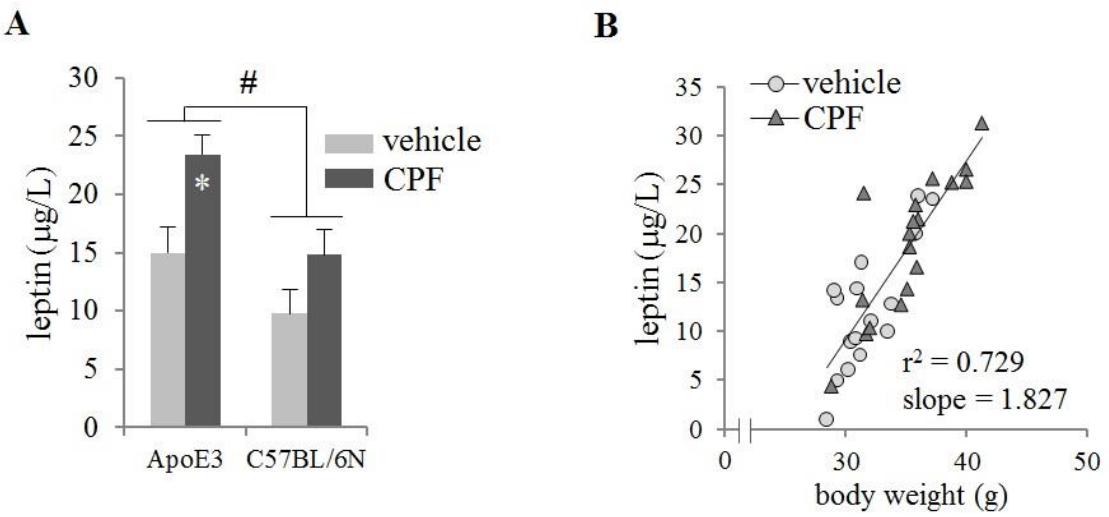

Figure 3. Effect of subchronic oral adulthood exposure to chlorpyrifos on plasma levels of leptin (A). The correlation of body weight status at the end of the 8-week treatment period and circulating plasma levels of leptin was also depicted (B). *Asterisks* indicate significant differences between CPF-exposed mice and their corresponding control group ($p < 0.05$), while the symbol # indicates significant differences between genotypes ($p < 0.05$).

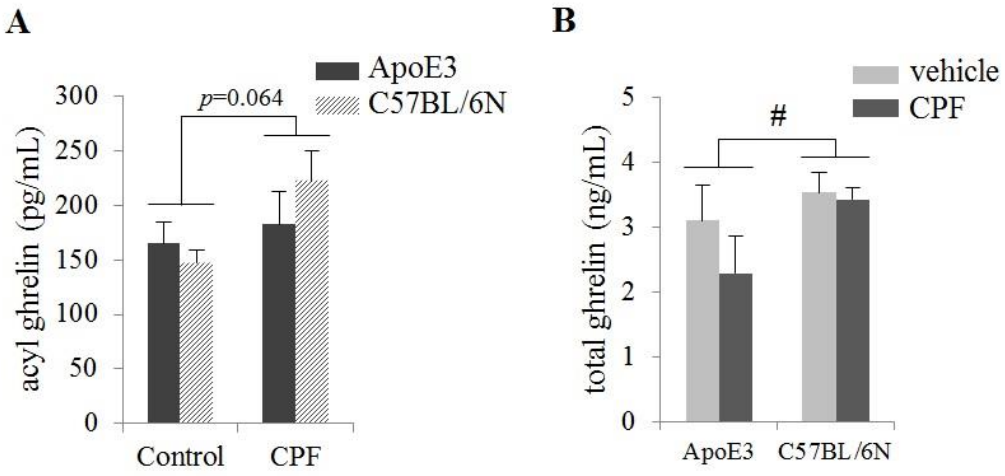

Figure 4. Effect of subchronic oral adulthood exposure to chlorpyrifos on plasma levels of both acyl ghrelin (A) and total ghrelin (B). The symbol # indicates significant differences between genotypes ($p < 0.05$).

Table 1. Mean daily intake of food and water in both apoE3 and C57BL/6N mice [a]

	Food intake (g)		Water intake (g)	
	Control	CPF	Control	CPF
ApoE3	2.65 ± 0.06	3.13 ± 0.07*	2.08 ± 0.04	2.06 ± 0.07
C57BL/6N	2.62 ± 0.03	3.20 ± 0.01*	2.12 ± 0.04	2.19 ± 0.05

[a] Statistically different changes versus the corresponding control group are indicated as * $p < 0.05$

Table 2 Plasma concentration of metabolic biomarkers in both apoE3 and C57BL/6N mice [a]

	ApoE3		C57BL/6N		Overall effects	
	Control	CPF	Control	CPF	Treatment	Genotype
FG (mmol/L)	9.07 ± 0.46	10.42 ± 0.63	8.10 ± 0.41	8.94 ± 0.45	**p = 0.033**	**p = 0.017**
Cholesterol (mmol/L)	4.14 ± 0.12	5.65 ± 0.86[†]	4.07 ± 0.13	4.48 ± 0.13*	**p = 0.036**	p = 0.167
Triglycerides (mmol/L)	1.41 ± 0.05	1.91 ± 0.35	1.59 ± 0.06	1.63 ± 0.11	p = 0.155	p = 0.782
Albumin (g/L)	41.96 ± 0.93	40.68 ± 0.71	41.46 ± 0.79	40.89 ± 0.58	p = 0.233	p = 0.850
Creatinine (µmol/L)	35.36 ± 0.00	35.36 ± 4.56	24.31 ± 3.24	25.05 ± 3.55	p = 0.910	**p = 0.003**
ALT (U/L)	10.52 ± 8.35	23.75 ± 12.12	16.77 ± 8.17	27.40 ± 9.20	p = 0.294	p = 0.658
AST (U/L)	109.94 ± 20.54	74.70 ± 17.84	91.05 ± 15.06	102.12 ± 22.97	p = 0.533	p = 0.825

[a] Statistically different changes versus the corresponding control group are indicated as * $p < 0.05$. The symbol [†] indicates a tendency ($p = 0.097$) versus the corresponding control group. FG, fasting glucose.