**Title: Excess maternal salt or fructose intake programs sex-specific, stress- and fructose-sensitive hypertension in the offspring.**

**Running title: Programming of sex-specific hypertension**

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**Abstract**

**Aims:** The Western diet is typically high in salt and fructose which have pressor activity. Maternal diet can affect offspring blood pressure but the extent to which maternal intake of excess salt and fructose may influence cardiovascular function of the offspring is unknown. We sought to determine the effect of moderate maternal dietary intake of salt and/or fructose on resting and stimulated cardiovascular function of the adult male and female offspring.

**Methods and Results:** Pregnant rats were fed purified diets (+/-4% salt) and water (+/-10% fructose) before and during gestation and through lactation. Male and female offspring were weaned onto standard laboratory chow. From 9-14 weeks of age, cardiovascular parameters (basal, circadian, stimulated) were assessed continuously by radiotelemetry. Maternal salt intake rendered opposite-sex siblings with a 25 mm Hg difference in blood pressure as adults; males were hypertensive (15 mm Hg MAP), females were hypotensive (10 mm Hg MAP) above and below controls, respectively. Sex differences were unrelated to endothelial nitric-oxide activity *in vivo* but isolation-induced anxiety revealed a significantly steeper coupling between blood pressure and heart rate in salt-exposed males but not females. MAP of all offspring was refractory to salt-loading but sensitive to subsequent dietary fructose, an effect exacerbated in female offspring from fructose-fed dams. Circadian analyses of pressure in all offspring revealed higher mean set-point for heart rate and relative non-dipping of nocturnal pressure.

**Conclusions:** Increased salt and fructose in the maternal diet has lasting effects on offspring cardiovascular function that is sex-dependent and related to the offspring's stress-response axis.

**Keywords:** rat, hypertension, fructose, salt, maternal, stress

## Introduction

Ancestral man is predicted to have eaten a diet high in fibre, potassium, complex carbohydrates and protein and low in sodium, refined sugars and energy density. Typically, a paleolithic diet provided a plant-to-animal energy ratio of 1:1 with the net acid-load being alkaline[1; 2]. Analyses of the diets of modern hunter-gatherer populations support these predictions[2; 3]. Since this time, when physiological and metabolic systems were evolving, there has been a gradual transition away from this Palaeolithic diet. With the emergence of agriculture (*ca*. 7 to 5,000 years ago) through to the industrial revolution (*ca.* last 100 years), the 'Modern diet' has rapidly become low in fibre and high in sodium, simple sugars and energy density [4]. When superimposed on the Palaeolithic genotype and physiology, the modern diet has resulted in an increased incidence of non-communicable diseases (NCD), estimated to account for 60% of all deaths worldwide[5]. The economic impact of NCD is vast; $558, $237 and $33 billion in China, India and the UK, respectively[6] whilst $750 billion is spent annually in the United States for diabetes and hypertension alone[7].

Modification of diet offers an achievable and economically beneficial prevention strategy for NCD. Short-term consumption of a 'Paleolithic' diet produces significant reductions in blood pressure, cholesterol, triglyceride and insulin resistance[8]. In addition, reduced salt intake (e.g. to 3g/day) is predicted to reduce all-cause mortality in the United States by 44-92,000 individuals, saving an estimated $10-24 billion annually[9]. Reducing sugar-sweetened beverage consumption by 1 serving/day reduced systolic BP by 1.8 mmHg[10]. Earlier dietary intervention, for example to pregnant mothers or those considering pregnancy, may have added benefit as an adverse periconceptional and/or prenatal nutritional exposure has been shown to increase risk of NCD's (e.g. cardiovascular or metabolic disease) in the adult offspring[11; 12; 13] – a paradigm referred to as the developmental programming of health and disease.

The majority of developmental programming studies to recapitulate either a 'Westernized' or under/over-nourished diet in experimental models have used a low protein, or a high-fat and/or a high sugar paradigm[14; 15; 16]. In the UK, whilst higher than optimal (RNI; reference nutrient intake) intake of saturated fat is observed, high total fat intake is not. Indeed, data from the National Diet and Nutrition Survey suggests, total fat consumption is close to recommended, but that fructose and salt intake remain high[17]. In the US a similar dietary pattern of high fructose and high salt intake has been observed raising concerns about increased cardiovascular disease risk[18; 19].

The delayed programming effect of a maternal diet high in simple sugars (e.g. fructose[20]) or salt has been considered[21; 22]. Feeding sucrose to pregnant rats can influence hepatic metabolism and reduce offspring birthweight[23], and fructose-feeding during lactation renders the resultant adult offspring vulnerable to cardiometabolic risk[20]. A maternal diet high in salt is one of the few dietary challenges to repeatedly produce hypertensive offspring[21; 24]. More importantly, increased intake of salt in (or added to) food potentiates intake of simple sugars (e.g. from drinking sugar sweetened beverages)[25]. As each is known to influence cardiovascular health, it is important to consider their potential interaction experimentally. Sex-specific effects are widely observed in developmental programming studies[26], sex is an important consideration with regard to disease susceptibility,[27] and there has been recent criticism of sex-bias (in favour of males) in translational medicine

64 studies[28; 29]. It is therefore important to also consider potential sex-specific responses after
65 maternal dietary intervention with respect to offspring cardiovascular function.

66 To date, no study has considered the delayed cardiovascular consequences on adult offspring (male
67 and female) of the combined intake of fructose and salt by the dam. Excess salt in the diet increases
68 fluid intake; disappointingly, this tends to be of sugar-sweetened beverages [25]. We anticipate that
69 high maternal intake of fructose and salt renders adult offspring prone to hypertension and
70 hypersensitive to further consumption of salt or fructose. The aim of the present study was to
71 characterise the cardiovascular health of adult male and female rat offspring after maternal
72 consumption of a high salt and/or fructose diet before and during her pregnancy and for the
73 duration of her lactation. Baseline cardiovascular health of all offspring was assessed 24/7 by
74 radiotelemetry, as previously described by us after maternal salt intake [24]. Cardiovascular
75 hypersensitivity *in vivo* was assessed during four further experimental studies: 1) during sympathetic
76 activation induced by anxiety-related isolation, 2) during nitric-oxide blockade with N(G)-nitro-L-
77 arginine methyl ester (L-NAME), 3) during dietary salt- or 4) dietary fructose-loading to determine if
78 postnatal response is conditioned by prenatal exposure. During each challenge, all data recorded
79 was submitted for further non-linear regression analyses to determine potential effects on
80 cardiovascular function through the circadian cycle. Finally, offspring hearts were studied ex vivo
81 using the perfused Langendorff system to assess isolated cardiac function. For all outcome
82 measures, we have assessed cardiovascular responses in different-sex siblings.

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## Materials and Methods

***Ethics:*** Animal procedures were carried out under license and in accordance with the Home Office animals (Scientific Procedures) Act 1986 and approved by the local animal welfare and ethical review board of the University of Nottingham.

***Diet design:*** In brief, Sprague Dawley dams (190-200g; 8-10 weeks of age) were kept in a temperature (20-22$^{o}$C) and humidity (55-65%) controlled environment and subjected to a 12 hour light/dark cycle (0700-1900h). Rats were randomly assigned to one of 4 treatment (diet) groups; 1) Control diet (CD; n=6), fed purified standard chow (TD.08164; Teklad Harlan, Maddison. WI.) and tap water; 2) Salt diet (SD; n=6), fed purified standard chow with 4% NaCl added (TD.08162 Teklad Harlan, Maddison WI.) and tap water; 3) Fructose diet (FD; n=6), fed purified standard chow (TD.08164) and tap water with 10% fructose (Sigma-Aldrich, UK) added; 4) Fructose/Salt diet (FSD; n=6), fed purified salt diet (TD.08162) and tap water with 10% fructose added. Diet composition has been published previously [30]. All rats were fed the experimental diets *ad libitum* for at least 28 days prior to conception and throughout gestation and lactation.

***Radiotelemetry and baseline cardiovascular recording:*** At 9 weeks of age, one male and one female offspring from each litter were surgically instrumented for radiotelemetric recording of blood pressure (TA11PA-C40; DSI, St-Paul, MN USA) from the descending abdominal aorta as described previously[31]. In brief, the rats were fully anaesthetised (fentanyl citrate; Sublimaze, Janssen-Cilag and medetomidine hydrochloride; Domitor, Pfizer, UK; 300 µg/kg of each *i.p.*), for probe implantation (TA11PA-C40; DSI, St-Paul, MN USA). Anaesthesia was reversed (Antisedan, Pfizer UK; 1 mg/kg) and analgesia administered (buprenorphine; Buprecare, Animalcare UK; 0.02 mg/kg *s.c.*) together with a long-acting antibiotic (Amoxycare LA; 0.05 ml *i.m.*). All 24 rats that underwent surgery completed the study and all were subsequently housed with a same-sex sibling to minimize stress. Cardiovascular variables were recorded (Dataquest GOLD v4.02; DSI, St-Pauls MN USA) at intervals (x2 15 sec periods per 15 minutes) during a 5-7 day recovery and baseline period and during cardiovascular challenges which each lasted for further 5-7 day periods. Male and female siblings were recorded simultaneously, each with a same-sex cage mate present at all times, but challenges were conducted in a random order. At the end of all experiments, rats were euthanized in a sealed chamber using a rising concentration of $CO_2$, followed by cervical dislocation after confirmation of cardiac arrest.

***Radiotelemetry and stimulated cardiovascular recording***: CV challenge 1) *Isolation-induced anxiety*, after a recovery period, the untelemetered sibling was removed from the cage for a 24 h period and blood pressure and heart rate recorded continuously (i.e. x2 15sec periods per minute; 2880 datapoints in total). Thereafter, siblings were reunited and recording continued at intervals. With 5-7 day recovery and wash-out periods between each challenge telemetered rats were subjected to three further experimental studies in a randomised fashion, each lasting 5-days with a further 2-days recording during recovery: CV challenge 2) *Nitric-oxide blockade*, the drinking water was substituted for fresh water with $N_{(G)}$-nitro-L- arginine methyl ester (L-NAME) dissolved at a concentration of 150 µg ml$^{-1}$ (equivalent to 4.1 mg L-NAME·day$^{-1}$); CV challenge 3) *Salt-loading*, standard chow was substituted for purified chow with 4% NaCl (TD.08162 Harlan) and CV challenge 4) *Fructose-loading*, the drinking water was substituted for fresh water with 10% fructose solution.

***The Isolated Heart (Langendorff) preparation:*** One male and one female offspring from each control or salt-exposed dams (offspring of fructose-fed dams were not included) were randomly selected, anaesthetised (3% isofluorane in 2L $min^{-1}$ $O_2$) and killed by cervical dislocation. Within 90 seconds, the heart was excised and cannulated via the aorta to Langendorff perfusion apparatus (AD Instruments, Oxford, UK) and reverse-perfused with Krebs Henseleit buffer (118mM NaCl, 4.7mM KCl, 1.2 mM $KH_2PO_4$, 1.2mM $MgSO_4$, 25 mM $NaHCO_3$, 11mM glucose and 1.25 mM $CaCl_2$ pH 7.4 bubbled with 95%/5% $O_2/CO_2$). Perfusion was maintained at a constant pressure of 60 mmHg, with perfusate warmed to 37.4°C, and the heart immersed in a water jacketed temperature controlled glass chamber set at 37.4°C therefore ensuring normothermia throughout the perfusion protocol. Contractile function (left ventricular developed pressure) was determined by an intravascular balloon, adjusted to an end diastolic pressure of 5-10mmHg. Data were recorded for a 30min baseline period after 15-30 min stabilisation via transducers (Senso-Nor 844, AD Instruments) using the Powerlab Acquisition System (AD Instruments).

***Statistics:*** The study was designed with a 2 (±fructose) x 2 (±salt) factorial structure and was analyzed by a General Linear Model (GLM) approach for normally distributed data or after log-transformation for a skewed error distribution (Genstat v16, VSNi, UK). All data are presented as means ± SEM or s.e.d. (standard error of the differences between comparisons, for a more conservative estimate of the contrast variance). Whilst $P \leq 0.050$ was accepted as indicating statistical significance, values of *P* from 0.06-0.09 are also presented to indicate effects falling close to the arbitrary significance boundaries. Using one male or female offspring per litter per determination avoids complicating the statistical model with shared intra-litter variance. For offspring cardiovascular analyses, data were either tested as summary measures (e.g. hourly means of blood pressure) or, for circadian analyses, by incorporating all recorded cardiovascular data (e.g. 2880 datapoints per animal; 14,400-17,280 datapoints per group [n=5-6 animals of each sex] into a non-linear regression model fitting a Fourier-curve ($Y=\alpha+ß sin(2\pi(X+\varepsilon)/w)$ to derive four parameters $\alpha$, set-point; $ß$, amplitude; $w$, wavelength and $\varepsilon$, offset, which were analysed by GLM.

## Results

***Maternal food intake:*** At conception, food intake was similar in rats fed salt diet but marginally reduced in those with fructose-sweetened water available (CD, 10.3±1.0; SD, 10.9±0.9; FD, 9.36±0.8; 7.02±0.9 g/day; $P_{fructose}$ = 0.01). Food intake increased with advancing gestational age: by day 20 gestation (term ~day 21), rats were eating approximately double the quantity at conception and those rats with fructose-sweetened water available were still consuming marginally less food (CD, 22.6±2.2; SD, 21.8±2.0; FD, 18.2±1.9; 16.6±1.9 g/day; $P_{fructose}$ = 0.02). Nevertheless, using the AIN-93G formulation and despite a marginal reduction in food intake in those rats with fructose available, the diets (TD.08164 and TD.08162) still met macro- and micronutrient requirements for pregnant rats [32].

***Resting cardiovascular status of adult offspring:*** Prenatal exposure to salt-diet (SD) significantly increased blood pressure in male offspring; systolic, mean and diastolic pressures being 15 mmHg higher than age-matched dietary controls (CD; Table 1, Figure 1a). In contrast, female siblings tended to be hypotensive; systolic, mean and diastolic pressures being 10 mmHg lower than dietary controls (Table 1, Figure 1b). Circadian analyses of pressure and heart rate, incorporating all measured datapoints for each animal within each diet group, suggested less dipping of nocturnal heart rate in male offspring exposed *in utero* to high maternal salt (Figure 1c) and in female offspring exposed *in utero* to high maternal fructose (Figure 1d). The latter, additionally, exhibited less dipping of nocturnal blood pressure (Figure 1e). Such effects, despite no excessive dietary intake post-natally, suggests long-term programming of cardiovascular sensitivity and reactivity in the offspring. We then tested this hypothesis in a number of experiments:

***Stimulated cardiovascular responses – isolation-induced stress:*** Immediately upon removal of their sibling from the cage, the single-housed telemetered offspring exhibited a robust cardiovascular response (Figure 2a-d). Despite differing baselines, the magnitude of the change in pressure and heart rate were similar between dietary groups, but when the slopes of the relationship between paired values were analyzed, the male, but not female, offspring of dams fed salt-diet exhibited a significantly steeper response: calculated slopes (mean, 95% confidence interval) for male offspring were: CD, 3.26 (3.02-3.49); FD, 2.81 (2.63-2.99); SD, 5.36 (5.17-5.55); FSD, 5.38 (5.15-5.60) beats $min^{-1}$ $mmHg^{-1}$, P<0.001; and for female offspring: CD, 4.77 (4.59-5.08); FD, 4.26 (3.91-4.60); SD, 4.47 (4.25-4.69); FSD, 3.30 (3.12-3.48) beats $min^{-1}$ $mmHg^{-1}$ (Figure 2e,f). In short, the male offspring of dams fed a high-salt diet are hypertensive, with greater short-term cardiovascular reactivity to anxiety-related stimuli that leads on in the long-term to less-dipping of heart rate at night. We then assessed whether such a phenotype was underpinned by programmed cardiovascular changes in a) the periphery, by examining cardiovascular function on a background of tonic endothelial nitric oxide blockade and b) the heart, by using the langendorff technique in isolated hearts.

a) ***Stimulated cardiovascular responses – nitric-oxide blockade:*** Upon consumption of L-NAME mean arterial pressure increased significantly in both sexes of all groups (Figure 3a,b), with the magnitude of change (i.e. increase from baseline) being similar between groups and sexes (pooled estimate, 43.3±2.6 mm Hg). The oscillation in heart rate increased with duration of L-NAME treatment in both males and females i.e. the ß-coefficient increased from 37.1±3.1 (day 1-2) to 50.1±3.0 beats/min (day 4-5) for males and females alike (Figure 3c,d). Despite L-NAME

treatment, circadian analyses indicated heart rate to remain elevated in male, but not female, offspring of salt-fed dams (352 vs. 337 ± 2.1 beats/min; $P$<0.001; Figure 3e). In addition, the reduced dipping of heart rate at night in the male offspring from salt-loaded dams was retained (Figure 3e). Similarly, adult female, but not male, offspring of fructose-fed dams, retained higher average heart rates: 384 vs. 362 ± 2.1 beats/min (Figure 3f). Programmed sex-specific pathways in the adult offspring, that independently influence adult cardiovascular control after maternal salt or fructose loading, were therefore beginning to emerge: for males, maternal high salt diet renders them reactive to further cardiovascular stressors as adults; for females, maternal high fructose has a similar effect. Each was apparently independent of endothelial NOx status.

***b) Adult offspring isolated heart function at 8 weeks of age:*** With hearts mounted on the langendorff apparatus, heart rate was higher (P=<0.001) in the female offspring of dams fed salt diet (males, 312 *vs.* 308; females, 310 *vs.* 330 beats/min for CD *vs.* SD, respectively) but left ventricular developed pressure (males, 39 [17-45] *vs.* 39 [35-53]; females, 51[46-56] *vs.* 48[39-50] mm Hg for medians [IQR] of CD *vs.* SD, respectively) and the maximal positive derivative of the rate of change in developed pressure (+dp/dt) were not different between groups (males, 1076 [609-1449] *vs.* 1617 [1481-1670]; females, 1448 [1271-1700] *vs.* 1568 [1475-1744] mm Hg for medians[IQR] of CD *vs.* SD, respectively).

Without any obvious programmed alteration to tonic endothelial (nitric-oxide) activity or cardiac function we next tested whether male and female offspring were rendered differentially reactive to the same inducing dietary stimulus in their mothers.

***Stimulated cardiovascular responses – salt-sensitivity:*** There was little measurable effect of high-salt intake on cardiovascular status in the male and female offspring of all dietary groups. Circadian analyses did indicate, however, that with salt-loading the offspring of fructose-exposed dams exhibited significantly blunted nocturnal dipping of pressure (ß-coefficient males; 3.9 *vs.* 5.3 ±0.4 mmHg; F=3.8; $P_{light*salt}$=0.001) and heart rate (ß-coefficient males, 39.5 *vs.* 45.3 ±2.1 mmHg; F=6.3; $P_{light*fructose}$=0.001; females, 37.1 *vs.* 41.9 ±1.7 mmHg; F=2.5; $P_{light*fructose}$=0.01).

***Stimulated cardiovascular responses – fructose-sensitivity:*** Consumption of fructose *per se* had little cardiovascular effect in control offspring (CD effect size, 1.0±2.3 mm Hg). In male offspring from salt-loaded dams, high fructose intake elicited a significant pressor response (SD effect size, 6±2.6 mm Hg; P=0.002), that was greater in male offspring from fructose-loaded dams (FD effect size, 8.1±2.6 mmHg; Figure 4a). For female offspring, high fructose intake increased pulse pressure (effect size, +5.2±3.1 mm Hg; P=0.005), but this effect was 2-fold greater if their dams had also been fructose-loaded (FD effect size, 10.3±3.1 mm Hg; Figure 4b). Heart rate varied with the light/dark cycle, as in the unchallenged state, but was not overly influenced by 5-days fructose consumption (Figure 4c,d). In male rats, previously exposed to maternal salt-loading, the increase in heart-rate from day-to-night as the rats became active was diminished (a change of 54 beats/min *vs.* 60 beats/min ± 5; $P_{salt}$<0.005).

***Heart rate variability (HRV):*** During all challenges HRV was calculated. HRV exhibited marked circadian and ultradian patterns under control conditions which was unaffected by L-NAME treatment, salt-loading or high intake of fructose (Figure 5a-f). However, notably, regardless of the challenge HRV distinctly peaked at 20.00h in all groups (Figure 5 a-f).

## Discussion

The adverse metabolic consequences of increased consumption of extrinsic sugars in particular fructose, has been widely reported [33; 34; 35; 36]. Only one study in mice[37] and one in rats[38] have described the cardiovascular effects of additional dietary salt on cardiovascular function; none have considered their interaction when fed to pregnant dams and, subsequently, to their offspring. In the current study, we reveal some clear circadian and sex-specific effects of high maternal intake of salt or fructose on cardiovascular physiology in the adult offspring. Two independent sex-specific phenotypes emerge that are retained despite no significant consumption of salt or fructose postnatally; maternal salt-loading has distinct and marked hypertensive effects on male offspring, maternal fructose-loading appears to have greater cardiovascular effects on female offspring. Importantly, for fructose in particular, these effects in the female offspring are exacerbated by further fructose intake, as would naturally tend to occur in human populations.

The adverse cardiovascular effects of increased consumption of salt have long been recognized[39]; for fructose, the deleterious consequences are the subject of much recent debate[40]. Taking an evidence-base approach, however, would favour the hypothesis that increased consumption of fructose after the introduction of high fructose corn syrup and sugar-sweetened beverages has had a negative impact on cardiovascular health[35; 41]. When considering impact of diet on health (including offspring health) then relativity is all important; early hominids evolved eating approximately ≈0.25 g/day salt and no more than 2% energy/day from simple sugars. Current estimated average consumption is 8-12 g/day salt and 18-25% energy/day from simple sugars. Relative to our ancestral diet, during which our physiology was moulded over many thousands of years, the current average diet represents a considerable physiological burden. In the context of developmental programming, in which maternal malnutrition may influence fetal development to result in adaptations that become deleterious in a westernised nutritional environment, then it is unsurprising that such a physiological burden is not without effect. Using an animal model to recapitulate a westernised dietary pattern, the current study illustrates how this burden may translate to the offspring, and how these responses are sex- and nutrient-specific. For salt-loaded dams, effect size in sibling offspring is ≈25 mm Hg (males are hypertensive [≈15mmHg above controls], females hypotensive [≈10mmHg below controls]). Such large sex-specific effect size are rarely, observed [42; 43].

Sex-specific effects are often observed within the developmental programming paradigm [44] but, to our knowledge, none as marked as in the current study. This study was designed to illustrate potential sex-specific, delayed developmental effects but not interrogate potential mechanisms should they arise. For example, whilst a number of models have inferred sex-specific effects of programming by adopting the relatively crude approach of gonad removal, a more appropriate intervention would be to use highly specific and reversible sex-hormone antagonists longitudinally. Some excellent recent studies that have shown programming of a sex-specific cardiovascular phenotype (such as increased blood pressure in male but not female offspring) have identified an absence of estrogen in males as a causal factor (42, 43); in effect, estrogen acts as a 'pro-survival factor' mitigating (perhaps epigenetically) the adverse consequences of a nutritionally-poor developmental environment until concentrations decline in middle-age and morbidity and mortality rates (e.g. for cardiovascular outcomes) in females begin to rise – the basis for estrogen replacement therapy (44). However, being genetically male or female and interacting differently with the

immediate (e.g. intrauterine) environment could be important; for example, periconceptional exposure to a maternal methyl deficient diet for only 6 days (day 0 to day 6 gestation) revealed significant sex-specific differential DNA methylation of CpG islands in the fetal livers at day 90 gestation i.e. of the altered loci as a result of the dietary treatment, 53% were specific to male and only 12% specific to female (15).

Programmed alterations of cardiovascular control in salt-exposed offspring appears independent of tonic endothelial nitric oxide; if this were the case then L-NAME treatment should have revealed differences in short-term responses (i.e. the magnitude of increase in first 8-12 hours) or long-term regulation. However, a simple procedure to induce temporal anxiety – removing the cage-mate for a 24h period – does reveal marked differences in male, salt-exposed offspring. This has two important consequences; first, generation of curves of the coupling between pressure and heart rate at this time indicates that salt-exposed hypertensive male offspring, but not non-hypertensive female siblings, have a greater rate of rise of heart rate per unit pressure relative to female salt-exposed offspring. This suggests a centrally-mediated alteration at the level of the brain or peripheral autonomic nervous system and/or an effect on cardiac function. The latter can be ruled out, as *ex vivo* cardiac function, as shown by the langendorff preparation was not significantly different. Furthermore, we have previously shown, that the offspring of salt-loaded dams have altered set-points for osmolar regulation – a phenotype indicative of alterations at the level of the brain [(24)]. Additionally, the data clearly indicate that measurements of resting blood pressure in telemetered rats should always be conducted with same-sex sibling cage mates in order to achieve a true 'resting or ambulatory reading'; single-housed rats are easily stressed which has a marked negative impact on resting cardiovascular variables.

For the first time, we provide evidence that increased maternal fructose consumption has important effects on adult offspring cardiovascular control. Resting blood pressure was unaltered by increased maternal fructose intake but the circadian oscillation in pressure and heart rate was significantly blunted, reflective of a 'non-dipping' nocturnal pattern – previously identified as a significant risk factor for later cardiovascular disease[(45)]. This finding is intriguing considering the limited exposure to fructose; none had consumed any fructose since they were weaned at 3 weeks of age. A number of studies have previously reported a pressor effect of fructose either given acutely, using high doses (66% of total energy intake [(46)]) or chronically (using lower doses [(35)]) and others reporting no effects [(47)]. Furthermore, our data suggest that maternal diet renders offspring (in particular female offspring) with a residual, increased sensitivity to further fructose intake. Mean arterial or pulse pressure in male and female offspring increased significantly more in prenatally fructose-exposed groups relative to control animals. Given that chronic L-NAME treatment did not reveal any difference in fructose-exposed groups suggests no residual involvement of tonic nitric-oxide activity. A recent study demonstrated an altered pattern of vascular smooth muscle prostanoid release may be a contributing factor to fructose-induced vascular sensitivity [(48)], but equally up-regulation of other vasoconstrictor, anti-natriuretic or diminished vasodilatory pathways may be causal. We have measured a number of fructose-induced advanced glycation end-products such as fructosamine (an indicator of fructose-induced protein glycosylation), uric acid and glucose and found no difference in the basal state to account for alterations in fructose-sensitivity. Acute fructose ingestion has been shown to increase blood pressure, likely through an effect on cardiac sympathetic sensitivity [(49)]. The current study illustrates that the effects of fructose ingestion after being exposed *in utero* to a

maternal diet high in fructose have a distinct sex-specific bias, with females being more fructose-sensitive.

Finally, the current study clearly illustrates that moderate over-consumption of salt and/or fructose by dams during pregnancy and lactation is able, in the offspring, to recapitulate many of the known pathophysiological effects of these micronutrients despite little exposure of the offspring to these diets. This has marked implication for non-communicable disease in western populations. Continued intake of refined, low nutritional-quality diets in the next generation, following maternal over-consumption, has the potential to vertically transmit adverse health outcomes through generations. Reversal of this trend is going to require preventative action prior to birth and as a result will also take generations to effect a response. Given the implications for human populations we would also strongly endorse recent commentaries and initiatives to reduce both the quantity of salt[50] and fructose[18] consumed as part of the modern Western diet.

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**Conflict of Interest:** none declared

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**Figure Legends**

**Figure 1.** *Circadian analyses of pressure and heart rate in adult male and female offspring from dams fed fructose or salt*. Circadian variation in mean arterial pressure (MAP; A,B) and heart rate (C,D,E) derived from Fourier curves in adult male and female offspring of dams fed 1) control diet and water *ad libitum* (CD, n=6 males/females), 2) control diet and 10% fructose in water *ad libitum* (FD, n=5 males/females), 3) 4% salt diet and water *ad libitum* (SD, n=5 males/females) and 4) 4% salt diet and 10% fructose in water *ad libitum* (FSD, n=5 males/females). Fourier plots represent predicted mean regression curve for each group (Genstat v16; VSNi Ltd). Digital time is 00.00am = 0.0000 and 23h.59min.59sec = 0.9999.

**Figure 2.** *Mean arterial pressure (A,B), heart rate, (C,D) and slopes of the relationship (E,F) between mean arterial pressure and heart rate in male and female offspring at ≈10 weeks of age from dams fed fructose and/or salt*. Data are (○) control diet and water *ad libitum* (n=6), (▲) control diet and 10% fructose in water *ad libitum* (n=5), (Δ) 4% salt diet and water *ad libitum* (n=5), (●) 4% salt diet and 10% fructose in water *ad libitum* (n=5) for males and females. Data were measured continuously (i.e. sampled at 2 outputs per minute) by telemetry for a 1h baseline period and subsequently for 2 hours after removal of their sibling from the cage. Regression lines were generated in Graphpad Prism 5.0.

**Figure 3.** *Mean arterial pressure (A,B), heart rate (C,D) and Fourier curves (E,F) for circadian variation in heart rate in response to L-NAME in the male and female offspring of dams fed fructose and/or salt*. Data are (○) control diet and water *ad libitum* (n=6), (▲) control diet and 10% fructose in water *ad libitum* (n=5), (Δ) 4% salt diet and water *ad libitum* (n=5), (●) 4% salt diet and 10% fructose in water *ad libitum* (n=5) for males and females. Data were measured intermittently (for 30secs every 15mins for 7 days) by telemetry and hourly means calculated as a summary measure of the cardiovascular response. Data were analysed within sex by General Linear Mixed Model (Genstat v13). NS, non-significant. L-NAME was provided in the drinking water (150µg $ml^{-1}$).

**Figure 4.** *Mean arterial pressure (A), pulse pressure (B) and summary measures of heart rate (C,D) during fructose ingestion in the male and female offspring of dams fed fructose and/or salt*. Data are (○) control diet and water *ad libitum* (n=6), (▲) control diet and 10% fructose in water *ad libitum* (n=5), (Δ) 4% salt diet and water *ad libitum* (n=5), (●) 4% salt diet and 10% fructose in water *ad libitum* (n=5) for males and females. Data were measured intermittently (for 30secs every 15mins for 7 days) by telemetry and hourly means calculated as a summary measure of the cardiovascular response. Data were analysed within sex by General Linear Mixed Model (Genstat v13). NS, non-significant. Fructose was provided in the drinking water (10% solution).

**Figure 5.** Heart rate variability (HRV) in male and female offspring from dams fed (○) control diet and water *ad libitum* (n=6), (▲) control diet and 10% fructose in water *ad libitum* (n=5), (Δ) 4% salt diet and water *ad libitum* (n=5), (●) 4% salt diet and 10% fructose in water *ad libitum* (n=5) for males and females during 5 days of (a,b) L-NAME treatment, (c,d) 4% salt-loading and (e,f) 10% fructose in drinking water. Heart rate was derived from the radio telemetric pressure pulse and recorded intermittently (for 30secs every 15mins) for the duration [7 days] of each nutritional challenge. HRV was calculated as the variance ($SD^2$) in heart rate for each hour of recording. Data were highly

positively skewed and were therefore analysed by General Linear Mixed Model with a gamma error distribution and logarithm-link function; back-transformed means are presented (Genstat v16).

**Table 1. Summary measures analysis of r*esting cardiovascular status of adult male and female offspring from dams consuming salt and/or fructose***

| *Daytime (resting) cardiovascular parameters of adult male and female offspring* | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | salt | | | *P* value | | |
| **Male offspring** | **fructose** | **no** | **yes** | **s.e.d.** | **Fructose** | **Salt** | **Fr*S** |
| Systolic pressure (mm Hg) | **no** | 128 | 142 | 2.8 | NS | 0.03 | 0.001 |
| | **yes** | 134 | 131 | | | | |
| Mean arterial pressure (mm Hg) | **no** | 106 | 121 | 3.0 | NS | 0.004 | 0.003 |
| | **yes** | 110 | 110 | | | | |
| Diastolic pressure (mm Hg) | **no** | 88 | 103 | 3.3 | 0.07 | 0.004 | 0.01 |
| | **yes** | 90 | 91 | | | | |
| Pulse pressure (mm Hg) | **no** | 40.4 | 39.2 | 3.0 | NS | NS | NS |
| | **yes** | 43.7 | 40.3 | | | | |
| Heart rate (beats/min) | **no** | 410 | 394 | 8 | 0.06 | NS | NS |
| | **yes** | 417 | 419 | | | | |
| **Female offspring** | | | | | | | |
| Systolic pressure (mm Hg) | **no** | 132 | 122 | 5.3 | NS | NS | NS |
| | **yes** | 129 | 127 | | | | |
| Mean arterial pressure (mm Hg) | **no** | 112 | 102 | 4.2 | NS | 0.07 | NS |
| | **yes** | 110 | 109 | | | | |
| Diastolic pressure (mm Hg) | **no** | 94 | 85 | 4.1 | NS | 0.08 | NS |
| | **yes** | 92 | 91 | | | | |
| Pulse pressure (mm Hg) | **no** | 38.0 | 36.7 | 3.7 | NS | NS | NS |
| | **yes** | 36.7 | 36.7 | | | | |
| Heart rate (beats/min) | **no** | 382 | 366 | 8.7 | 0.04 | NS | NS |
| | **yes** | 394 | 389 | | | | |

**Table 1**. Blood pressures and heart rate were derived from radiotelemetric signals and reflect average values during the 'resting' period (i.e. day-time; 7am to 7pm) over a 7-day period. Data are means with standard error of the difference (s.e.d) for the comparison from n=5-6 male or females per dietary group (n=5-6 dams per dietary group). Data were analysed by 2 (salt, yes/no) × 2 (fructose, yes/no) factorial ANOVA within each sex (Genstat v13). Statistical significance was accepted at P<0.05. Fr*S; interaction of fructose*salt

531 Figure 1

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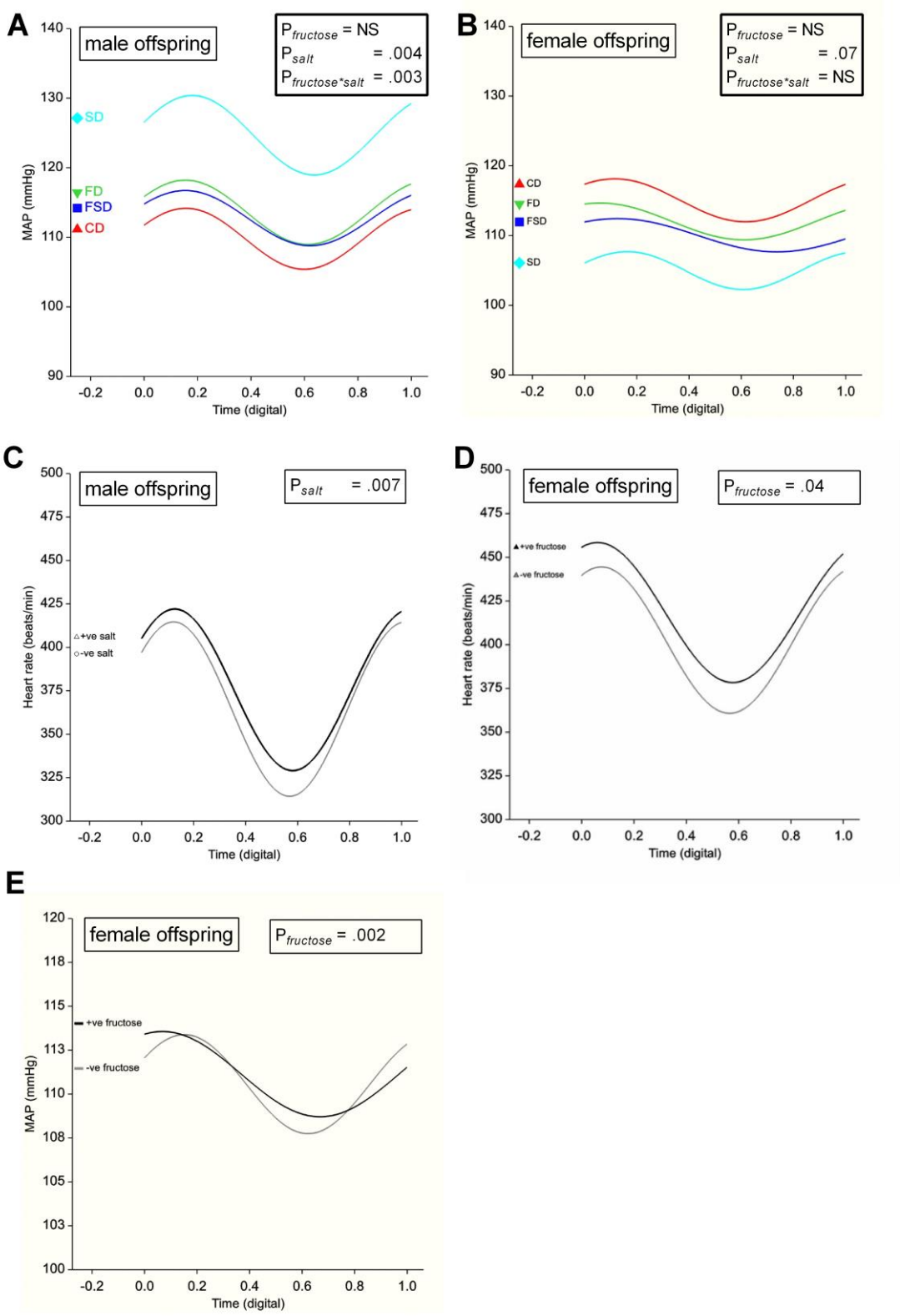

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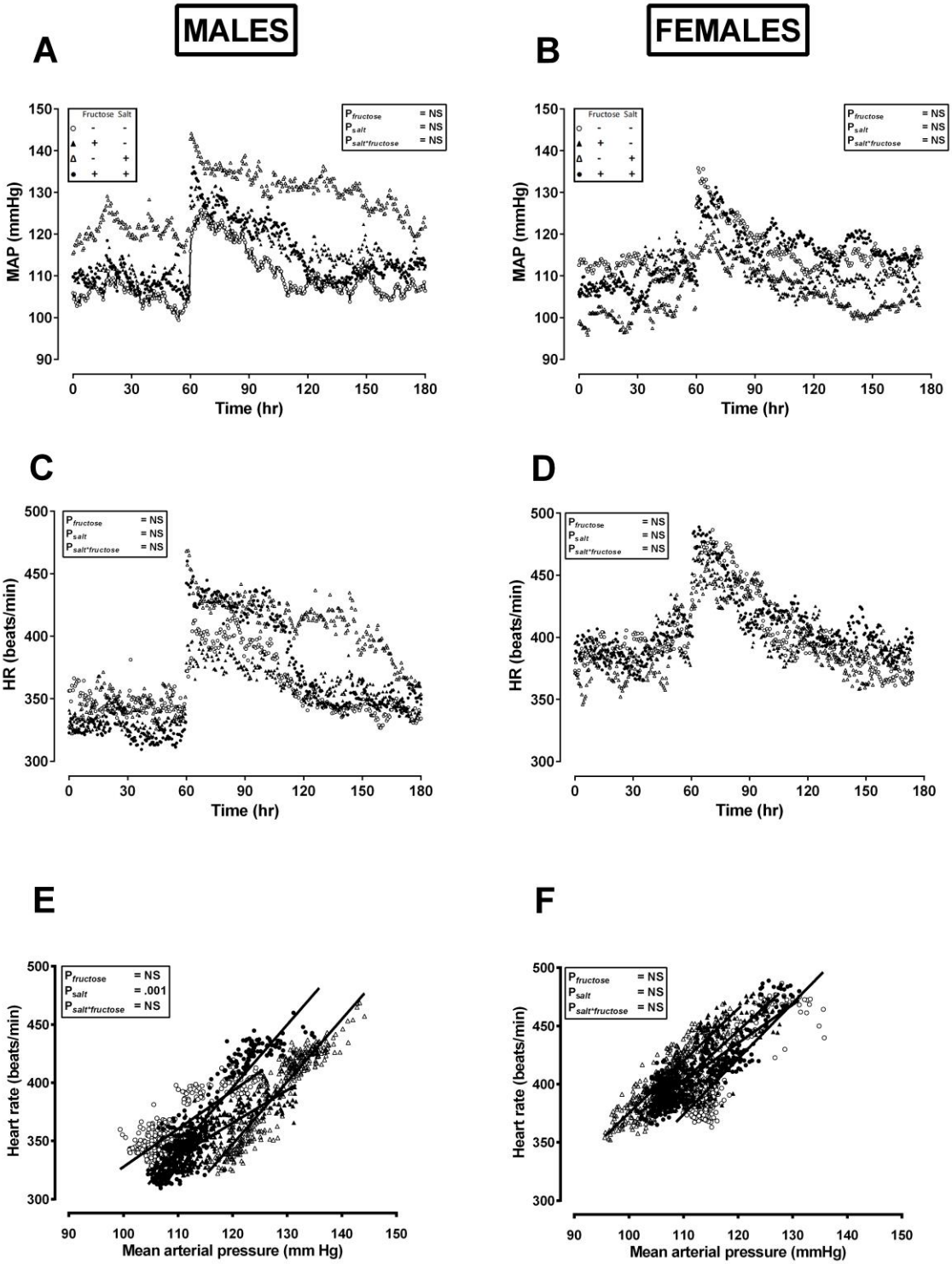


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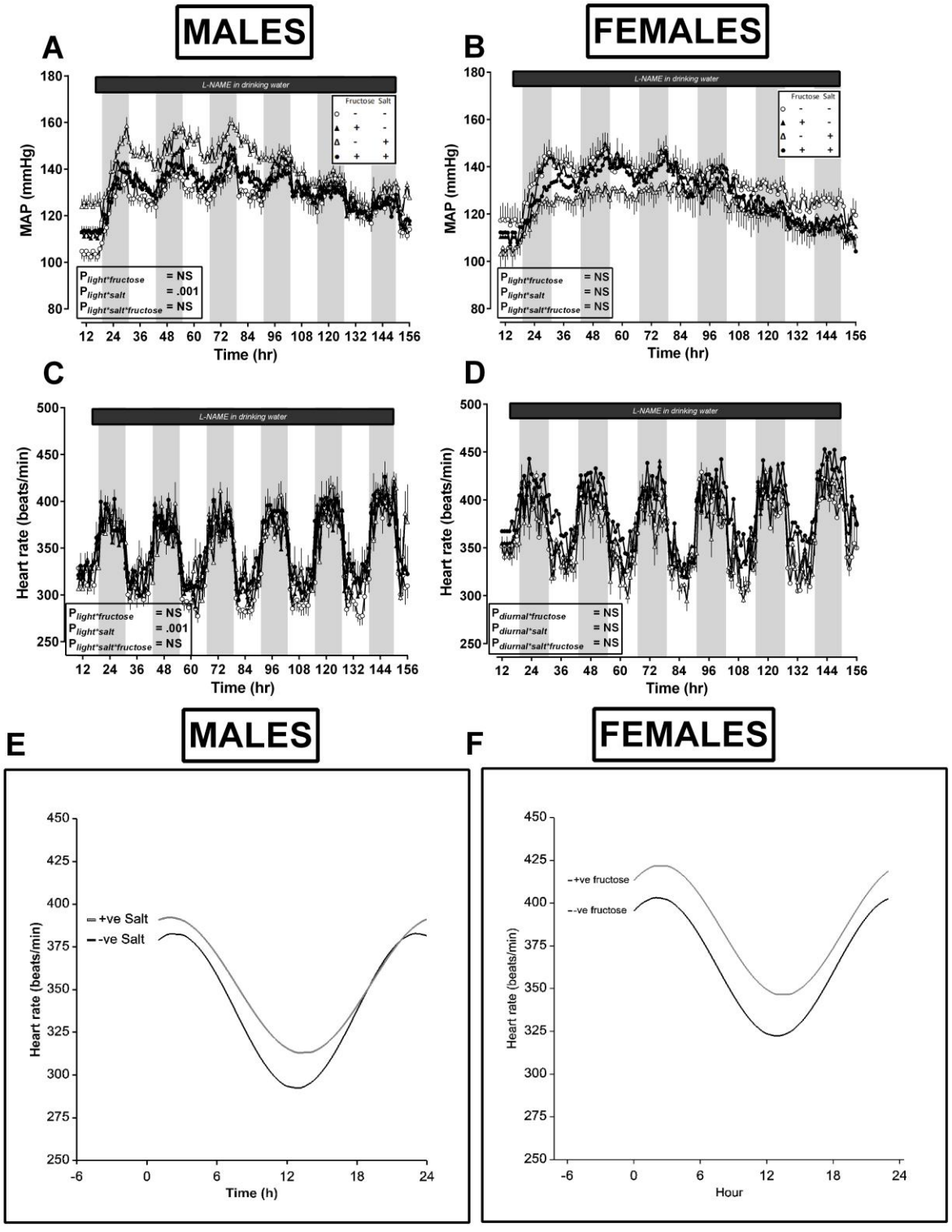


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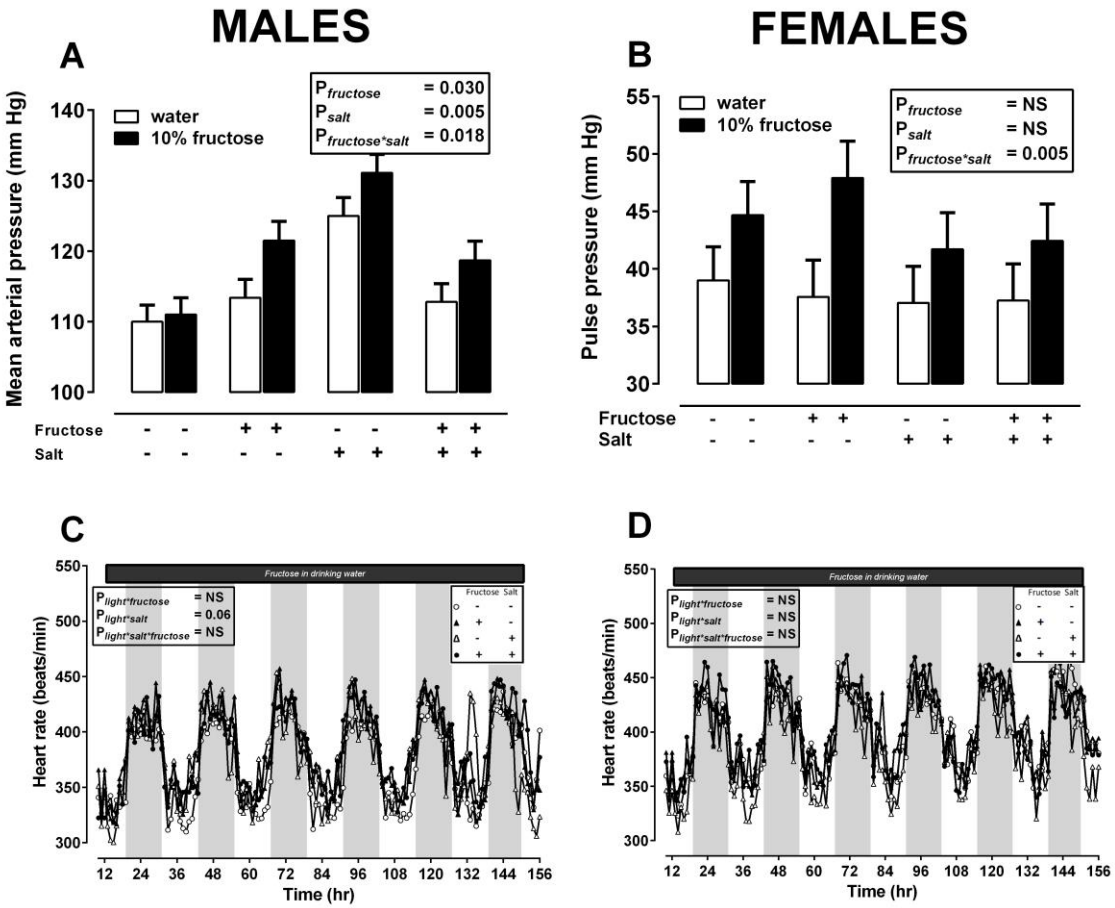
538 Figure 4

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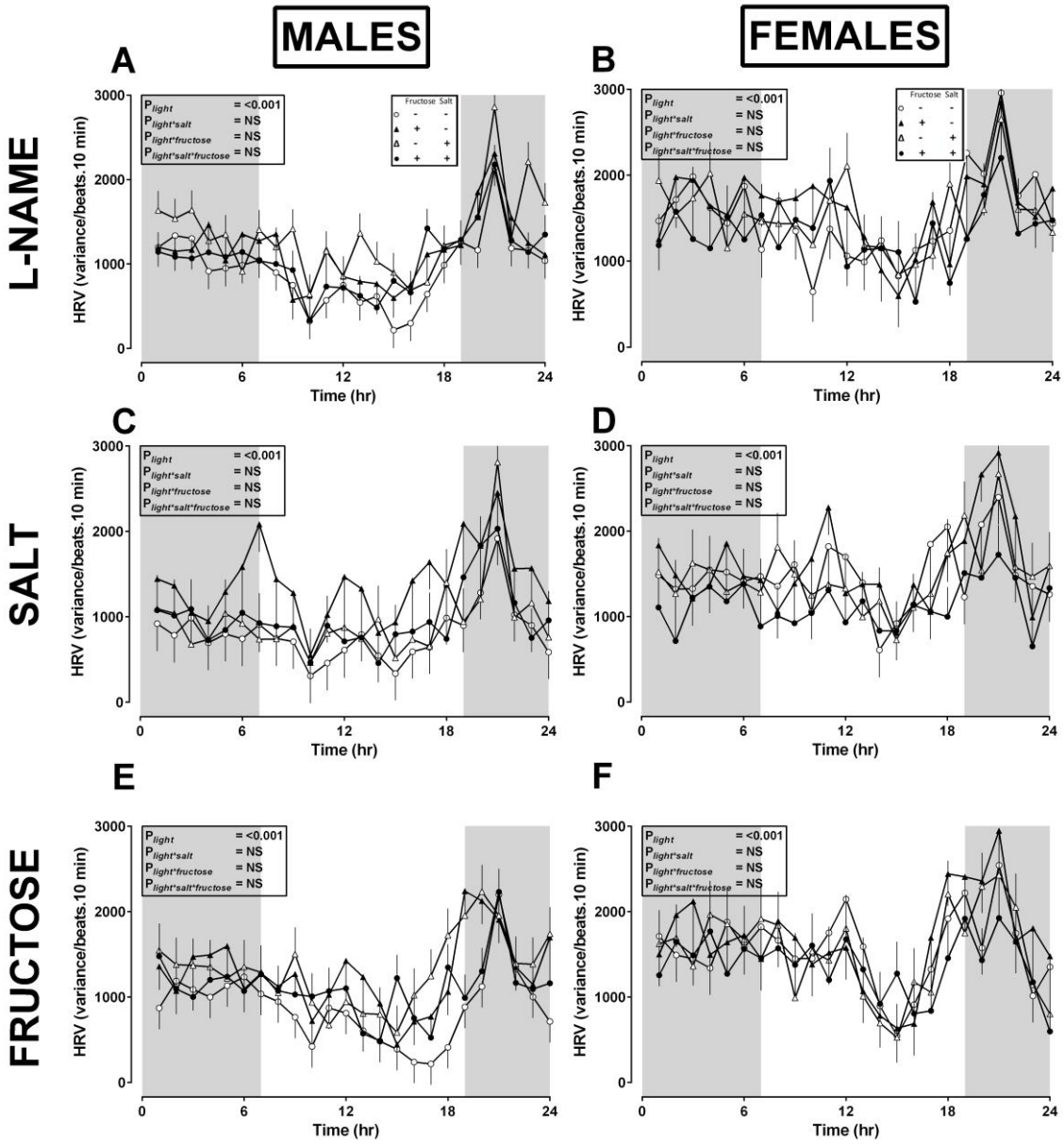
541 Figure 5

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