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# Early life stress exacerbates the obesogenic and anxiogenic effects of a Western diet without worsening cardiac ischaemic tolerance in male mice

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**Original Article**

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## Abstract

Early life stress (ELS) and a Western diet (WD) promote mood and cardiovascular disorders, however, how these risks interact in disease pathogenesis is unclear. We assessed effects of ELS with or without a subsequent WD on behaviour, cardiometabolic risk factors, and cardiac function/ischaemic tolerance in male mice. Fifty-six new-born male C57BL/6J mice were randomly allocated to a control group (CON) undisturbed before weaning, or to maternal separation (3h/day) and early (postnatal day 17) weaning (MSEW). Mice consumed standard rodent chow (CON, $n = 14$; MSEW, $n = 15$) or WD chow (WD, $n = 19$; MSEW + WD, $n = 19$) from week 8 to 24. Fasted blood was sampled and open field test and elevated plus maze (EPM) tests undertaken at 7, 15, and 23 weeks of age, with hearts excised at 24 weeks for Langendorff perfusion (evaluating pre- and post-ischaemic function). MSEW alone transiently increased open field activity at 7 weeks; body weight and serum triglycerides at 4 and 7 weeks, respectively; and final blood glucose levels and insulin resistance at 23 weeks. WD increased insulin resistance and body weight gain, the latter potentiated by MSEW. MSEW + WD was anxiogenic, reducing EPM open arm activity *vs.* WD alone. Although MSEW had modest metabolic effects and did not influence cardiac function or ischaemic tolerance in lean mice, it exacerbated weight gain and anxiogenesis, and improved ischaemic tolerance in WD fed animals. MSEW-induced increases in body weight (obesity) in WD fed animals in the absence of changes in insulin resistance may have protected the hearts of these mice.

## Introduction

Early life stress (ELS) or adversity is an important determinant of health and disease risk later in life.[1–5] Such links underpin the Developmental Origins of the Health and Disease theory.[6] which is an extension of Barker's thrifty phenotype hypothesis that also relates to maternal or prenatal determinants. Subsequent studies identify important influences of both pre- and early post-natal environments.[7]

The role of adverse early life experiences such as abuse and neglect in promoting different adult diseases is well appreciated.[1–5,8] Stressful or traumatic events during developmental stages of life have profound negative consequences in both the short- and long-term,[9] with compelling evidence ELS increases risks of cardiovascular disease[1,4,5,10,11] and behavioural disorders, including: major depressive disorder (MDD),[9,12–14] post-traumatic stress (PTSD), bipolar, and generalised anxiety disorders.[15–21] Nonetheless, pathophysiological mechanisms linking ELS to co-morbid mood, metabolic, and cardiovascular disorders remain to be detailed. Importantly, how ELS interacts with and influences dietary or metabolic disease risk factors is unclear.

Both ELS and metabolic disease risks/disorders are strongly linked and prevalent.[3,8,10] For example, a 2020 government report indicated that the incidence of child maltreatment in the USA was increasing, with almost 700,000 *reported* cases in 2018 – of these ~60% involved neglect.[22] It has been documented that victims of such maltreatment – specifically neglect – are more likely to be overweight or obese in later life.[23]

Approximately 60%–70% of the population of developed countries such as Australia, the United Kingdom, and the USA are overweight or obese.[24,25] Since obesity is a key risk-factor for cardiovascular disease[26] and myocardial infarction,[27] together with type 2 diabetes[28] and mood disorders,[29] these comorbidities incur enormous health and socio-economic costs on society. Indeed, MDD and ischaemic heart disease – both linked to stress and dietary factors – have been identified as leading chronic disease burdens in recent years.[30–33] Precisely how these two major risk factors interact in chronic disease development requires further interrogation.

Based on recent studies linking weight gain[34–36] to shifts in cognition, hippocampal glucocorticoid signalling, and affective state,[36] we hypothesised that ELS and an obesogenic Western diet (WD) would positively interact in driving metabolic, behavioural, and myocardial risks or abnormalities. We investigate how ELS induced with maternal separation and early weaning (MSEW) influences cardiometabolic risk factors, behaviour/affective state, and myocardial function and ischaemic tolerance in euglycaemic lean, and insulin resistant obese, male mice.

## Methods

### Animal ethics and study design

Fifteen pregnant (embryonic day 15–18) female C57BL/6J mice were sourced from the Animal Resources Centre (ARC, Perth, Western Australia). Mice were housed in Green Line GM500 individually ventilated cages stored in DGM racks (Tecniplast S.p.A, Varese, Italy), under an artificial 12-hour day-night light cycle (7:00 a.m. – 7:00 p.m.) at 21°C (40% humidity) and had ad *libitum* access to water and standard rodent chow. Researchers were necessarily unblinded for the control and MSEW groups allocation, however the perfusionist was blinded to group allocations throughout the study. The sample size chosen for this study was guided by power analyses for Langendorff perfusion outcomes of left ventricular developed pressure (LVDP) recovery. The authors assert that all procedures contributing to this work comply with the ethical standards of the *Australian code of practice for the care and use of animals for scientific purposes* and was approved by the Animal Ethics Committee of Griffith University (MSC/02/19).

#### Animal groups

Pregnant female mice were monitored daily to confirm birth date of pups. Mouse pups were randomly assigned to two groups: offspring subject to daily MSEW (MSEW, $n = 34$); or offspring left undisturbed until weaning (CON, $n = 33$). At eight weeks of age, only male mice from each group were randomly assigned to two diet sub-groups; a diet representative of the modern Western diet (WD, $n = 19$; MSEW + WD, $n = 19$) or standard rodent chow (CON, $n = 14$; MSEW, $n = 15$) (Fig. 1).

### Maternal separation and early weaning

Pregnant female mice produced litters of 1–7 pups, with day of birth defined as postnatal day 0. Pups were subjected to daily maternal separation in which the dams were removed from the cages for 3 h between 9:00 a.m. and 12:00 p.m. from postnatal day 2. Separated offspring cages were placed on heat mats set to ~36°C whilst the dam was moved to a separate cage for the 3 h separation. At postnatal day 17, the dam was removed, and offspring prematurely weaned onto a soft standard rodent chow. Control mice were weaned 4 days later on postnatal day 21 (Fig. 2). Maternal separation and early weaning independently manifest increased anxiety-like behaviour and elevations in corticosterone levels in mice.[37] Metabolic changes have also been reported, including hyperglycaemia and insulin resistance.[38]

### Dietary composition and macronutrient distribution

Mice were provided *ad libitum* access to either a control diet (Irradiated Rat and Mouse Cubes, Specialty Feeds, Glen Forrest, Western Australia) or a high fat, high sugar obesogenic diet representative of the WD for 16 weeks (Table 1). Previous studies in our laboratory show this obesogenic diet causes increases in body weight, visceral fat accumulation, insulin resistance, and myocardial sensitivity to ischaemia-reperfusion injury in C57BL/J mice.[39,40]

**Table 1.** Nutritional composition of animal diets

| | Control diet | Western diet | Fold-change |
|---|---|---|---|
| Energy (kJ) | 1420 | 2135 | 1.50 |
| Energy (kcal) | 339.38 | 510.35 | 1.50 |
| Protein (g) | 23.00 | 18.39 | 0.80 |
| Fat-Total(g) | 12.00 | 20.95 | 1.75 |
| -Saturated (g) | 2.03 | 9.50 | 4.67 |
| -Other (g) | 9.97 | 11.46 | 1.15 |
| Carbohydrates (g) | 65.00 | 60.76 | 0.93 |
| -Sugar (g) | n/a | 23.11 | n/a |
| Sodium | 20.00 | 23.63 | 1.18 |

Values expressed per 100 g dry weight.

### Behavioural analyses

Mouse behaviour was assessed using the open field test (OFT) and elevated plus maze (EPM) at 7, 15, and 23 weeks – these tests measure anxiety and exploratory behaviours in mice.[41,42] Mice were placed in the study room (away from arenas) for 30 min to acclimate. A recovery period of 48 h was interposed between OFTs and EPMs, to limit potential influences of the first test on behaviour in the subsequent test.

#### Open field test

The arena was 70 × 70 × 36 cm (L × W × H). Mice were individually placed in the centre square and video-recorded for 20 min then placed back in its cage and the arena thoroughly cleaned with 80% ethanol between tests. The following behavioural parameters were measured: total distance travelled, average speed, number of entries into the centre square, elapsed time while in the centre square, and elapsed time in the peripheral zone. Locomotor behaviour can be measured by distance and average speed. Animals experiencing anxiety will present with less time and fewer entries into the centre square.

#### Elevated plus maze

The maze was 100 × 100 × 50 cm (L × W × H). The EPM was performed in accordance with the established methodology.[42] Mice were individually placed in the centre of the maze (where the open and closed arms meet) and video-recorded for 5 min. The following markers of anxiety-like behaviour were quantified: ratio of entries into open arms/closed arms; ratio of time elapsed while in the open arms/closed arms; and number of head dips over the edge of an open arm. Mice experiencing elevated levels of anxiety present with less time and fewer entries into open arms of the maze. Head dipping can be considered as directed exploration and reflect decreased levels of anxiety.

### Cardiometabolic risk measurements

*Body mass:* Total body weight was measured weekly using a laboratory scale (A&D Weighing GX-2000 precision scale, A&D

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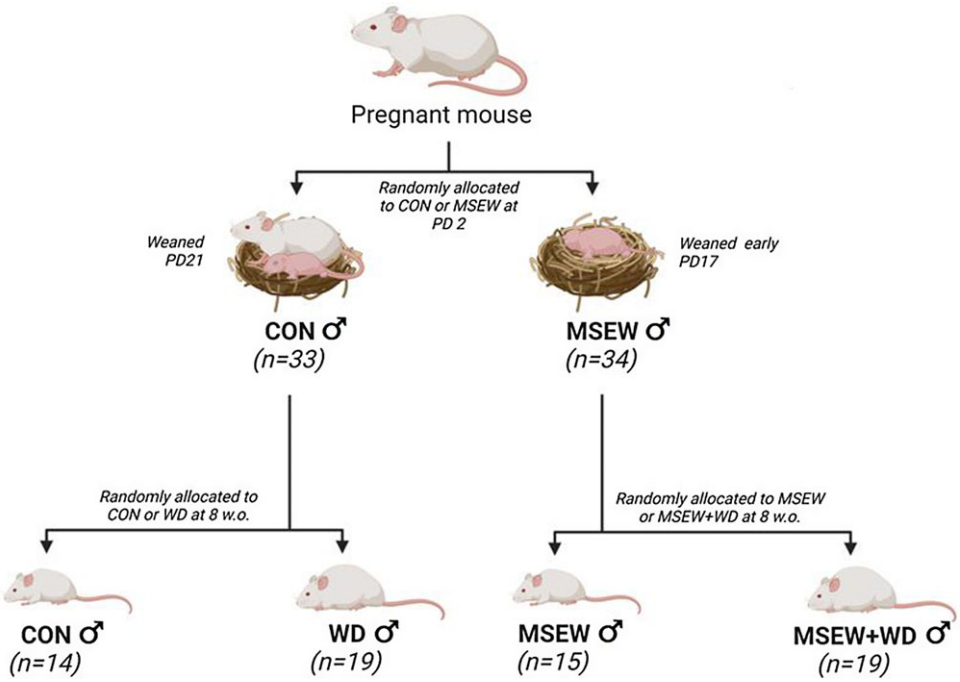


**Figure 1.** Experimental groups. PD – postnatal day, w.o. – weeks old, CON – control group (standard rodent chow + standard development), MSEW – maternal separation group (standard rodent chow + maternal separation and early weaning), WD – Western diet group (fed a simulated WD). Made with BioRender.

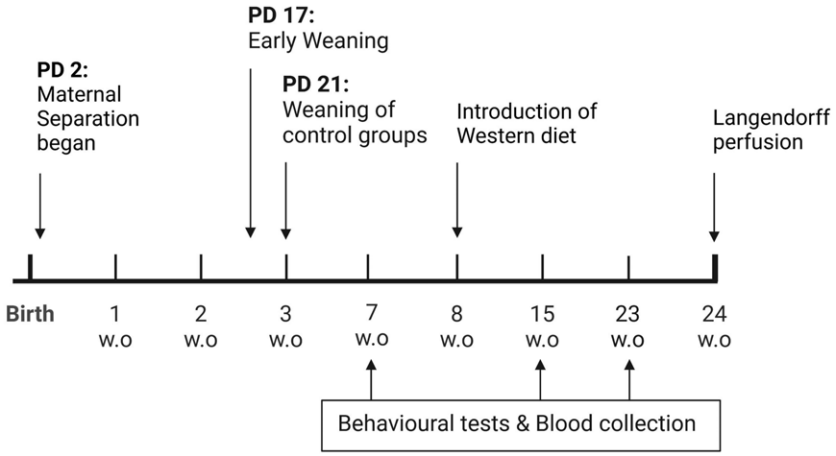


**Figure 2.** Experimental timeline. PD – postnatal day, w.o. – weeks old. Made with BioRender.

Australasia Pty. Ltd, Adelaide, South Australia) from 4 weeks of age until euthanasia.

*Serum lipids, glucose, and insulin* At 7, 15, and 23 weeks, mice were fasted for 4 h before acquiring blood via tail bleeds. Tails were numbed with Lignocaine and Prilocaine, each at 2.5% w/w. Fasted blood glucose levels were measured using a glucometer (Accu-chek Performa glucometer; Roche, Indianapolis, USA). Whole blood was stored on ice for 55 ± 5 min before centrifugation at 1000 g for 10 min. Serum was collected and stored at −80°C until analysis. Randomly selected sub-sets of serum samples were subsequently quantified for triglyceride and insulin levels using enzyme-linked immunosorbent assays (ELISA) under manufacturer's instructions (Triglyceride Quantification Colorimetric/Fluorometric Kit, BioVision, California, USA; Ultra-Sensitive Mouse Insulin ELISA Kit, Crystal Chem, Illinois, USA). To estimate insulin resistance, insulin and fasting blood glucose levels were used to calculate the HOMeostatic Assessment of Insulin Resistance (HOMA-IR) and the Quantitative Insulin-sensitivity ChecK Index (QUICKI).

### Serum corticosterone

Whole blood was collected at 23 weeks, between 8:30 and 10:30 am, and centrifuged for serum collection (as previously mentioned), with serum stored at −80°C. A randomly selected sub-set of serum samples were analysed via ELISA, according to manufacturer's instructions (Corticosterone ELISA kit, Enzo Life Sciences, New York, USA).

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### Cardiac function and ischaemic tolerance in Langendorff perfused hearts

Cardiac function and intrinsic tolerance to ischaemia-reperfusion were assessed using a Langendorff heart perfusion model detailed by us previously,[39,40,43] At 24 weeks, mice were anaesthetised (intraperitoneal sodium pentobarbital injection, 60 mg $kg^{-1}$). A surgical plane of anaesthesia was confirmed by assessing pedal withdrawal and tail pinch reflexes at 5-min intervals. Mice were euthanised by anaesthesia with pentobarbitone followed by thoracotomy and rapid excision of the heart into ice-cold Krebs Buffer, before Langendorff perfusion. The aorta was retrogradely perfused with modified Krebs-Henseleit buffer, gassed with 95% $O_2$–5% $CO_2$, maintained at 37°C (pH 7.4) and containing (in mM): 119 NaCl, 11 glucose, 22 $NaHCO_3$, 4.7 KCl, 1.2 $MgCl_2$, 1.2 $KH_2PO_4$, 1.2 EDTA, and 2.5 $CaCl_2$. A fluid-filled balloon constructed from polyvinyl chloride film and connected to a pressure transducer for contractile assessment was placed in the left ventricle via an incision in the atrial appendage and inflated to an end-diastolic pressure (EDP) of 3-5 mmHg. Hearts were then immersed in perfusate in a water–jacketed bath at 37°C. Temperature of perfusate was continuously monitored with a thermal probe connected to a Physitemp TH-8 digital thermometer (Phyisitemp Instruments Inc., Clifton, NJ, USA). Coronary flow was measured using an ultrasonic flow-probe proximal to the aortic cannula and connected to a T206 flowmeter (Transonic Systems Inc., Ithaca, NY, USA). A 4-channel MacLab system (AD instruments Pty Ltd, Castle Hill, Australia) relayed to an Apple iMac collected and processed systolic pressure, end-diastolic pressure (EDP), coronary flow (CF), heart rate (HR), and the positive ($+dP/dt$) and negative ($-dP/dt$) differentials of pressure change over time, reflecting inotropic and lusitropic states.

After a 15 min equilibration period, hearts were assessed for normoxic (baseline) function for 10 min while paced at 420 beats.$min^{-1}$ using an SD9 stimulator (Grass Instruments, Quincy, MA, ISA). Hearts with abnormal function (functional criteria outlined by us previously[43]) were excluded from analysis. To initiate normothermic global ischaemia coronary perfusion was stopped for 25 min. Coronary flow was recommenced for 40 min, inducing aerobic reperfusion. Final post-ischaemic functional recoveries were assessed after 45 min reperfusion. Measures include HR, CF, EDP, systolic pressure, LVDP and $+dP/dT$, $-dP/dT$.

### Statistical analyses

Statistical analyses were performed using GraphPad Prism version 9.5.1 for Windows (GraphPad Software, La Jolla California, USA). Shapiro-Wilks test determined all data were normally distributed. Two-way ANOVA with Sidak's post-hoc test was used to assess differences between two groups with multiple time-points. Unpaired *t*-test with Welch's correction was used to assess differences between two groups at a single time-point. All tests adhere to an alpha value of 0.05, notation was made where a *P*-value achieved <0.01, 0.001, or 0.0001.

## Results

### Body weight and weight gain

*Body weight before WD feeding:* Pups were not weighed for the first 4 weeks. At the end of 4 weeks., MSEW animals exhibited significantly higher body weights (by ~10%) than control littermates ($p < 0.001$, Fig. 3). However, body weight subsequently normalised across the two groups from 5 weeks.

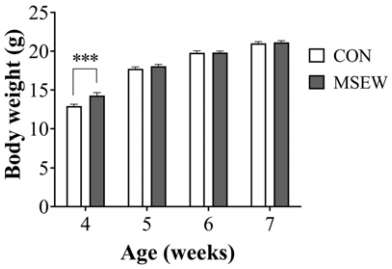


**Figure 3.** Body weight from 4 weeks to 8 weeks (prior to initiation of WD feeding in the WD subgroup). ***$p < 0.001$. Data presented as Mean ± SEM.
Abbreviations: CON, Control ($n = 33$); MSEW, Maternal separation and early weaning ($n = 34$).

*Body weight after WD feeding:* Transition to a WD markedly increased body weight compared with control diet mice (Fig. 4) – final weights in WD groups ranged from 40 to 45 g compared with up to 30 g in control diet animals. The pattern of weight gain in control diet mice was unaltered by MSEW (Fig. 4A). In contrast, MSEW significantly increased weight gain in WD fed mice ($p < 0.001$). This obesogenic effect of MSEW was evident within 4 weeks of WD feeding (Fig. 4B).

### Circulating glucose, insulin, triglyceride, and corticosterone levels

*Blood biochemistry before WD feeding:* Insulin levels, HOMA-IR, and QUICKI values were similar in CON and MSEW animals prior to introduction of the WD, although MSEW reduced fasted blood glucose at 7 weeks (Table 2). Serum triglyceride concentrations were increased at 7 weeks in MSEW *vs*. CON mice ($p < 0.05$), while fasted serum corticosterone levels were unaltered by MSEW (Table 2). An age-dependent fall in corticosterone was evident across all groups by 23 weeks, with levels <1500 pg/mL (Fig. 3).

*Blood biochemistry after WD feeding:* An initial MSEW-dependent reduction in blood glucose was lost over time, with a significant elevation in MSEW *vs*. CON mice evident at 23 weeks (8.9 ± 0.4 *vs.* 7.8 ± 0.3 mmol/L, $p < 0.05$; Fig. 5A). There was also evidence of emerging insulin insensitivity at 23 weeks (Fig. 5B), as indicated by lower QUICKI values in MSEW mice ($p < 0.05$). Conversely, fasted blood glucose and insulin levels, and insulin sensitivity were unaltered by MSEW in WD mice (Fig. 5). Fasted serum corticosterone levels were unaltered by MSEW in both control and WD groups (Fig. 5F).

### Behavioural responses

*OFT outcomes:* MSEW increased open field activity at 7 weeks (prior to diet changes). Distance (9406 ± 207 cm), speed travelled (7.9 ± 0.2 cm/s) and centre square entries (34 ± 2) increased significantly with MSEW compared to CON mice (8207 ± 247 cm, 6.9 ± 0.2 cm/s and 27 ± 2, respectively, $p < 0.05$; Fig. 6). Despite increased entries, time spent in the centre square was unchanged (Fig. 6C), consistent with a general increase in locomotor activity rather than select change in thigmotaxis. At 15 and 23 weeks there were no detectable differences in behaviour between groups, with MSEW not influencing final behaviour at 23 weeks in either CON or WD mice (Fig. 7).

*EPM outcomes:* No differences in EPM measures were evident between groups at 7 and 15 weeks (Fig. 8). At 23 weeks, most EPM

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**Table 2.** Fasted blood biochemistry and serum analyses at 7 weeks

| | BGL (mmol/L) | Serum insulin (μU/mL) | HOMA-IR | QUICKI | Serum triglycerides (nmol/μL) | Serum corticosterone (pg/mL) |
|---|---|---|---|---|---|---|
| **CON** | 9.9 ± 0.3 *(n = 15)* | 7.5 ± 0.4 *(n = 15)* | 3.4 ± 0.2 *(n = 15)* | 0.321 ± 0.005 *(n = 15)* | 4.3 ± 0.1 *(n = 14)* | 2503 ± 362 *(n = 6)* |
| **MSEW** | 9.0 ± 0.5* *(n = 14)* | 9.0 ± 1.0 *(n = 14)* | 3.6 ± 0.4 *(n = 14)* | 0.321 ± 0.002 *(n = 14)* | 5.0 ± 0.3* *(n = 12)* | 2816 ± 435 *(n = 9)* |

After MSEW exposure and before WD introduction. Data expressed as Mean ± SEM, *$p < 0.05$. BGL – blood glucose level, HOMA-IR – homeostatic assessment of insulin resistance, QUICKI – quantitative insulin-sensitivity check index, CON – Control, MSEW – Maternal separation and early weaning.

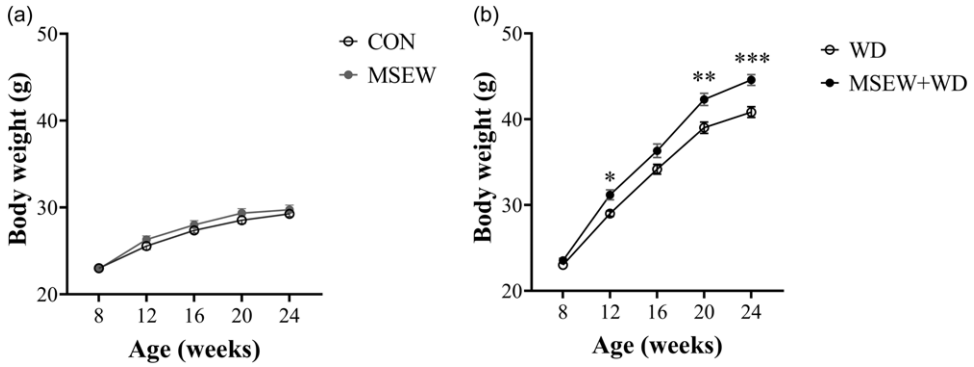


**Figure 4.** Body weight from 8 weeks to 24 weeks (after initiation of WD feeding in the WD subgroup). **$p < 0.01$, ***$p < 0.001$. Data presented as mean ± SEM. Abbreviations: CON, Control ($n = 14$); MSEW, Maternal separation and early weaning ($n = 15$), WD, Western diet ($n = 19$), MSEW + WD ($n = 19$).

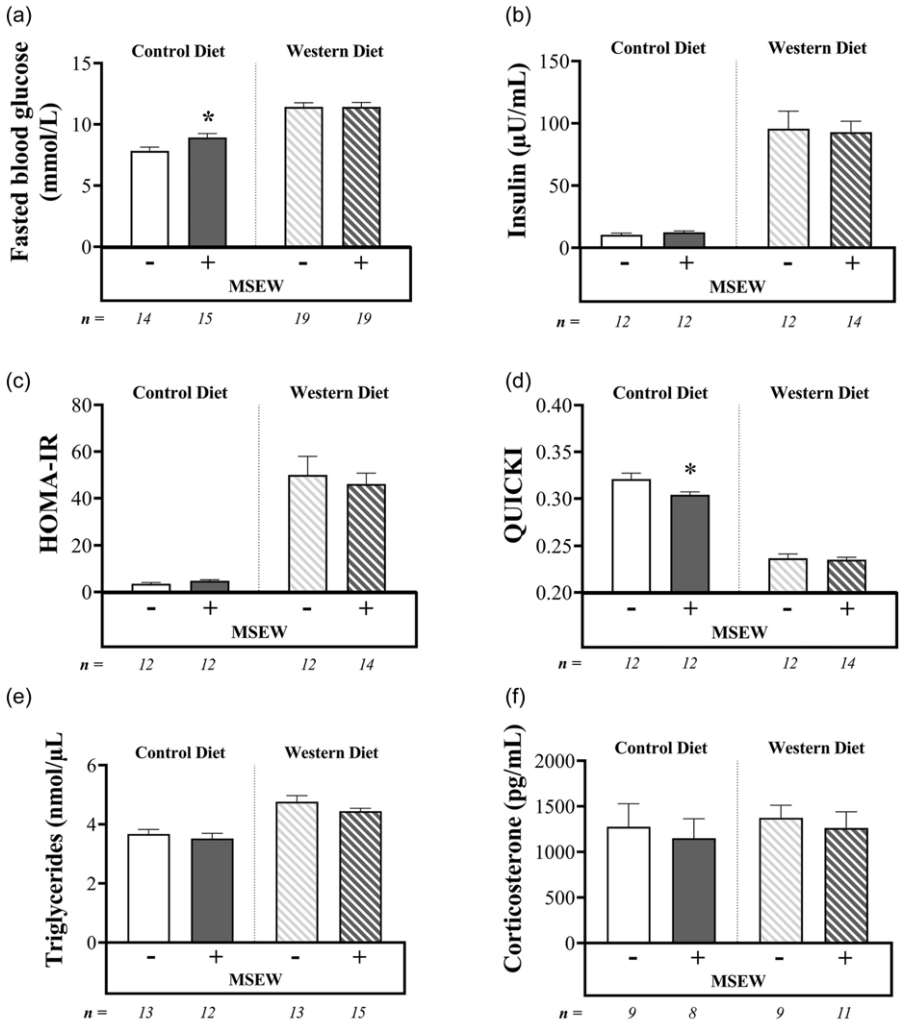


**Figure 5.** Blood biochemistry and serum analyses (at 23 weeks). Data presented as Mean ± SEM. *$p < 0.05$. BGL – blood glucose levels, HOMA-IR – homeostatic assessment of insulin resistance, QUICKI – quantitative insulin-sensitivity check index, CON – Control, MSEW – Maternal separation and early weaning,, WD – Western diet.

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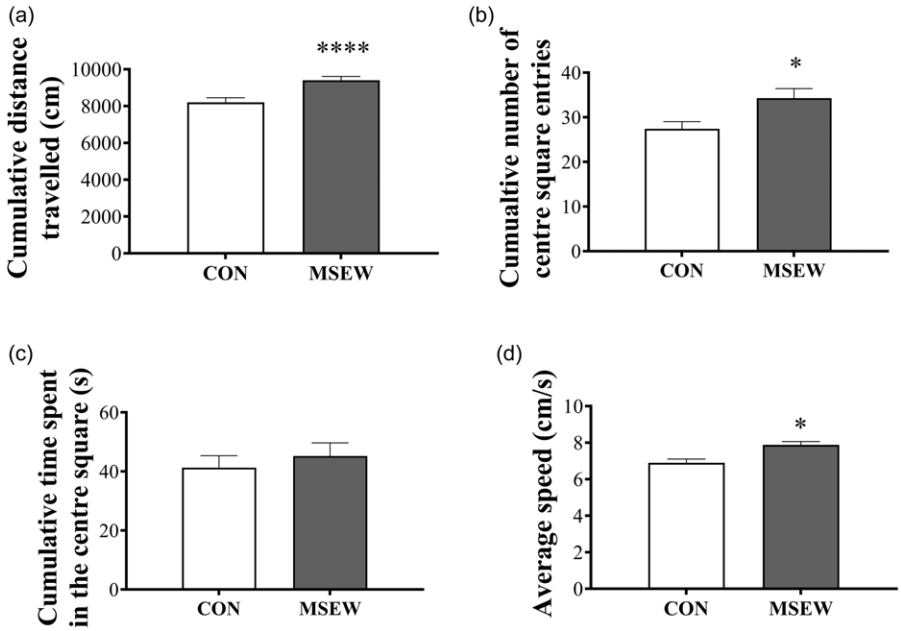


**Figure 6.** Open field test outcomes (at 7 weeks, pre-dietary intervention) (a) Distance travelled after 20 min. (b) Number of centre square entries. (c) Duration in the centre square (seconds). (d) Average movement speed. Data presented as Mean ± SEM. *$p < 0.05$, ****$p < 0.0001$. CON – Control ($n = 26$). MSEW – Maternal separation early weaning ($n = 32$).

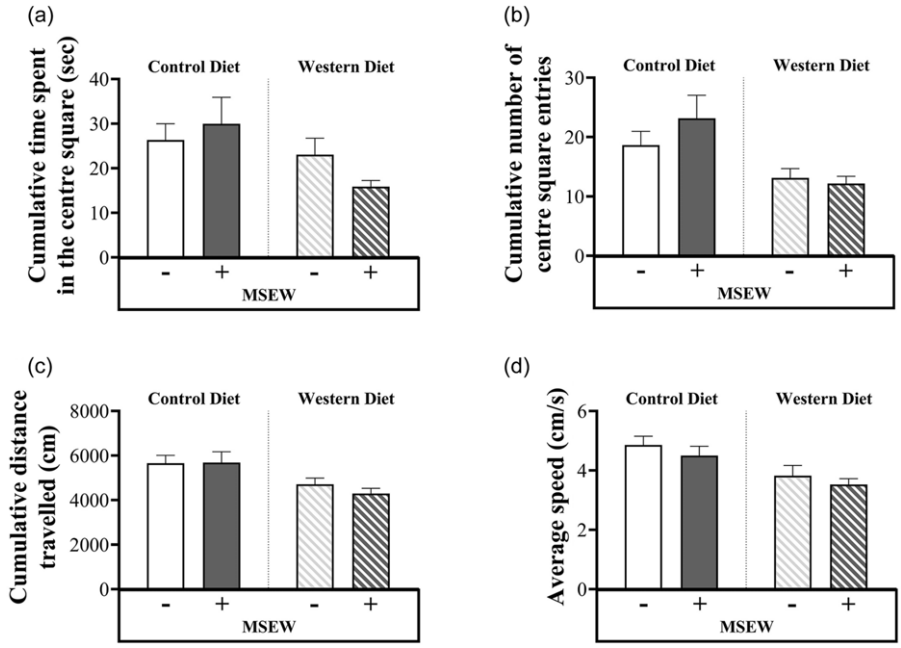


**Figure 7.** Open field tests outcomes (at 23 weeks) (a) Time duration spent in the centre square. (b) Number of centre square entries. (c) Distance travelled after 20 min (d) Average speed. Data presented as Mean ± SEM. CON – Control (**$n = 14$**), WD – Western diet (**$n = 17$**), MSEW – Maternally separated early weaning ($n = 12$), MSEW + WD ($n = 17$).

measures trended towards lower values in MSEW + WD mice, including fewer open arm entries than CON animals ($p < 0.05$, Fig. 9A). The ratio of open:closed arm entries was reduced by MSEW specifically in WD mice (MSEW + WD: 0.23 ± 0.05 *vs.* WD: 0.47 ± 0.07, $p < 0.01$, Fig 9B). All other EPM measures were not significantly modified (Fig 9).

### *Heart weights and pre- and post-ischaemic cardiac function*

*Heart weights:* Final heart weights, expressed as whole dry weight or as a ratio to body weight, were unaltered by MSEW in both the control diet and WD mice (Fig. 10A & B).

*Pre-ischaemic and post-ischaemic cardiac function:* pre-ischaemic function was unaltered across groups, including comparable LV pressure development, d*P*/d*t*, and coronary flows (Table 3). Similarly, no significant differences in post-ischaemic function were detected with MSEW alone, though MSEW significantly improved contractility (MSEW + WD: 3198 ± 171 *vs.* WD: 2723 ± 110 mmHg/s, Fig. 11E) and recovery of left ventricular developed pressure (MSEW + WD: 61.7 ± 2.9 *vs.* WD: 51.9 ± 2.5%, Fig. 11B) in WD-fed groups. Ischaemic contracture development, including peak contracture, time to onset of, and peak contracture, was also comparable across groups (Fig. 12).

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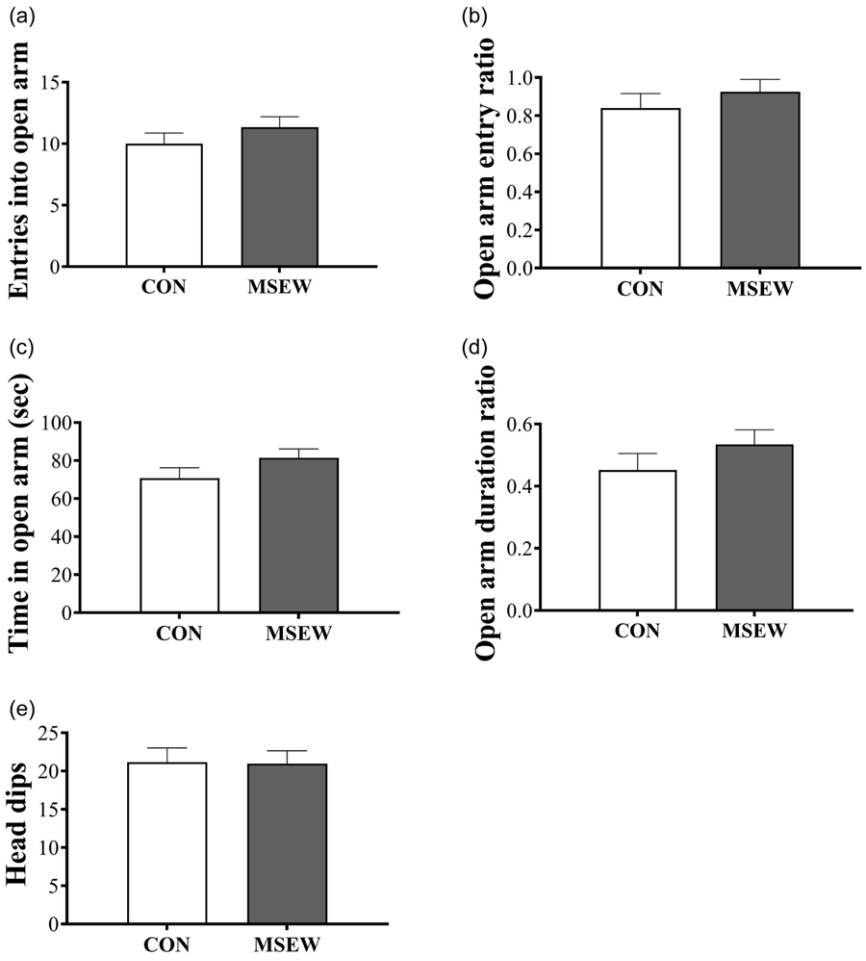


**Figure 8.** Elevated plus maze outcomes (at 7 weeks). (a) Number of entries to the open arm after 5 min. (b) A ratio of open arm entries to closed arm entries. (c) Time duration spent on the open arm. (d) A ratio of time spent on the open arm over the time spent on the close arm. (E) Number of head dips performed. Data presented as Mean±SEM. CON – Control ($n = 31$), MSEW – Maternal separation early weaning ($n = 29$).

### *Markers of ischaemic injury*

Total coronary effluent LDH levels after 40 minutes reperfusion were higher in WD fed animals (WD, $n = 6$: 7.03 ± 0.98 IU/g; MSEW + WD, $n = 8$: 7.07 ± 1.31 IU/g) than animals fed standard rodent chow (CON, $n = 6$: 4.60 ± 0.71 IU/g, MSEW, $n = 6$: 3.47 ± 0.62 IU/g) however MSEW did not significantly increase coronary effluent LDH levels in either the lean or obese mice.

## Discussion

Understanding how ELS impacts behaviour, metabolism, and cardiac health can enable and underpin improved approaches to managing mental and cardiovascular health of youth suffering increased allostatic loads as a result of early life adversity. The data presented here shows that ELS may have effects on health in later life, dependent upon diet. As expected, the WD has largely increased body weight and caused insulin resistance, and hypertriglyceridaemia. However, this study focused on how ELS interacted with a WD rather than known effects of the WD itself. Early effects of ELS include increased locomotor activity and shifts in blood glucose and triglycerides shortly after ELS induction, followed by the later emergence of hyperglycaemia in animals on standard rodent chow but not the WD. Importantly, ELS/MSEW potentiated the influences of a WD on body weight gain and anxiety-like behaviour but not insulin resistance in adult mice. Paradoxically, these adverse body weight and behavioural changes induced by ELS in WD fed animals, were associated with improved post-ischaemic functional outcomes in these obese, insulin resistant mice.

### *Early life stress influences WD–induced behavioural changes*

Childhood maltreatment, including parental neglect/abuse, poverty, neighbourhood violence, and bullying, may interact in promoting both mood and cardiometabolic disorders.[15–19] Maternal separation also promotes mood disorders[42,44–46] and is employed in animal models to mimic early life adversity.[47,48] We used MSEW to mimic ELS, previously shown to elicit sustained anxiety-like behaviour in C57BL/6J mice.[37] Although mice subjected to MSEW did not exhibit early anxiety-like behaviour at 7 weeks (when locomotor behaviour in the open field appeared to be increased), significant anxiety-like behaviour was evident at 23 weeks. Both anxiogenic and anxiolytic outcomes have been documented with MSEW,[37,44,45] however, this is the first study to reveal a transition from early hyperactivity to anxious behaviour later in life, albeit in mice fed a WD.

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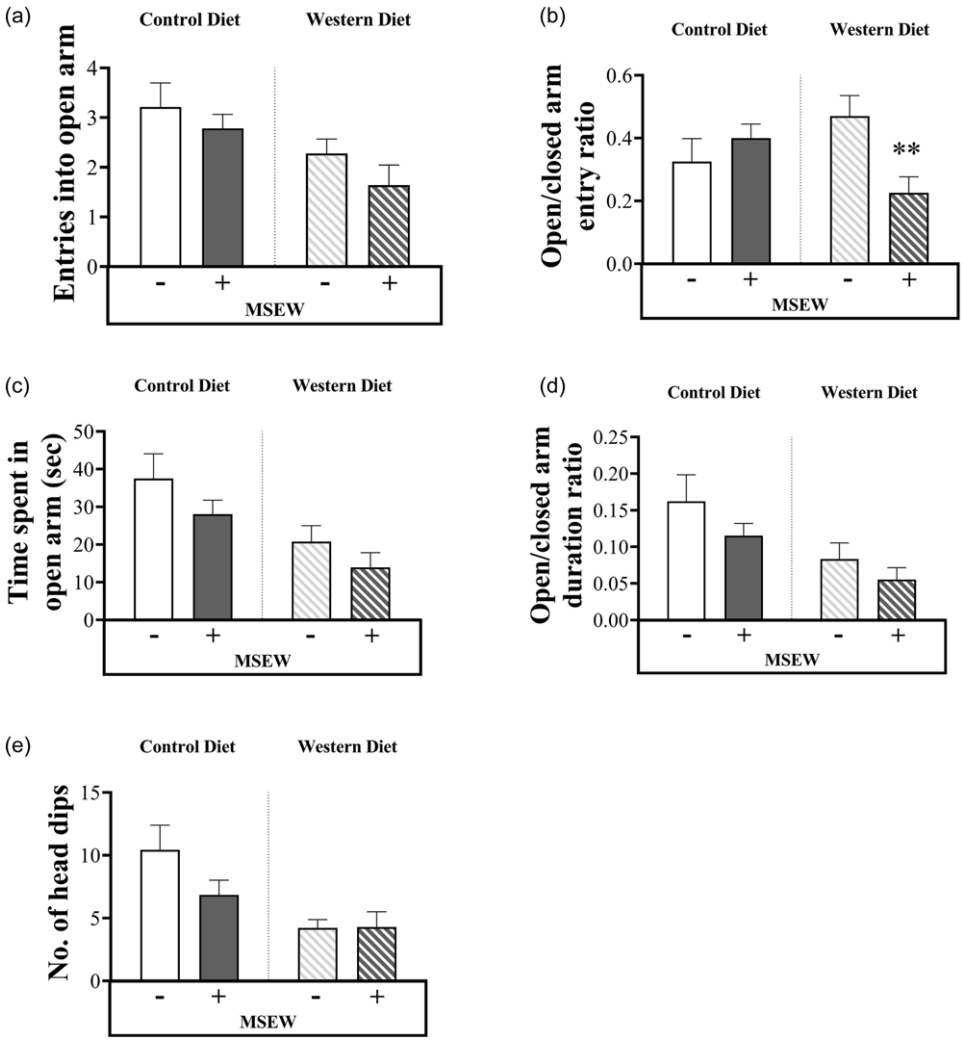


**Figure 9.** Elevated plus maze outcomes (at 23 weeks). (a) Number of entries to the open arm after 5 min. (b) A ratio of open arm entries to closed arm entries. (c) Time duration spent on the open arm. (d) A ratio of time spent on the open arm over the time spent on the close arm. (e) Number of head dips performed. Data presented as Mean ± SEM. $^{**}p < 0.01$, CON – Control ($n = 10$), MSEW – Maternal separation early weaning ($n = 14$). WD – Western diet ($n = 18$), MSEW + WD ($n = 14$).

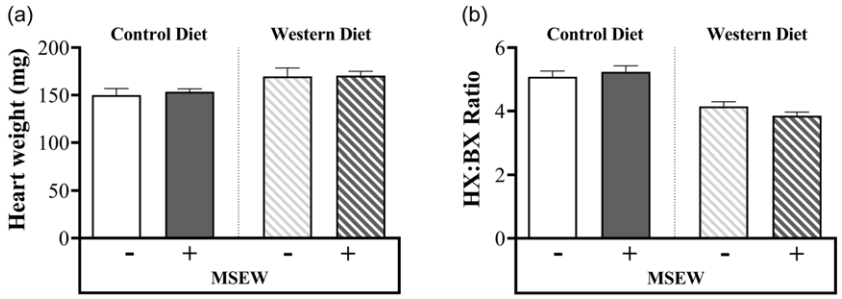


**Figure 10.** Heart weights. (a) Heart weight. (b) HX:BX - heart weight/ body weight ratio. Data presented as Mean ± SEM, CON – Control ($n = 6$), MSEW – Maternal separation early weaning ($n = 6$), WD – Western diet ($n = 6$), MSEW + WD ($n = 12$).

While C57BL/6J mice subjected to 4–8 h of maternal separation per day in the study of George *et al.* (2020) also increased locomotor activity in the open field, this was associated with increased time in the closed arm and fewer entries into the open arm of the EPM.[37] Here, MSEW alone did not worsen anxiety like-behaviour in otherwise healthy animals (only in WD mice). A potential factor contributing to these differing is animal age. George *et al.* (2020) assessed behaviour at postnatal day 65 (~9 weeks) compared to 15 and 23 weeks here.[37] A systematic review and meta-analysis indicates that maternal separation may

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**Table 3.** Baseline perfusion measurements

| | Heart rate (bpm) | End-diastolic pressure (mmHg) | Systolic pressure (mmHg) | LVDP (mmHg) | Coronary flow (ml/min) | Coronary flow/g (ml/min/g) | +dP/dT (mmHg/s) | -dP/dT (mmHg/s) |
|---|---|---|---|---|---|---|---|---|
| **CON** | 418 ± 2 | 3.3 ± 0.8 | 112 ± 11 | 109 ± 11 | 2.3 ± 0.3 | 13.5 ± 1.5 | 4062 ± 382 | −2217 ± 369 |
| **MSEW** | 418 ± 2 | 3.7 ± 0.9 | 124 ± 7 | 122 ± 9 | 3.3 ± 0.6 | 21.5 ± 5.7 | 4745 ± 316 | −2700 ± 364 |
| **WD** | 421 ± 2 | 3.4 ± 0.7 | 141 ± 6 | 138 ± 6 | 6.0 ± 1.1 | 36.8 ± 7.5 | 4899 ± 145 | −2310 ± 267 |
| **MSEW + WD** | 417 ± 1 | 4.3 ± 0.5 | 136 ± 4 | 132 ± 4 | 4.7 ± 0.7 | 27.8 ± 4.5 | 5224 ± 200 | −3103 ± 323 |

LVDP = left ventricular developed pressure, dP/dT = change in pressure / change in time. Data presented as Mean ± SEM, CON - control ($n = 6$), MSEW - Maternal separation early weaning ($n = 6$), WD - western diet ($n = 6$), MSEW + WD ($n = 12$).

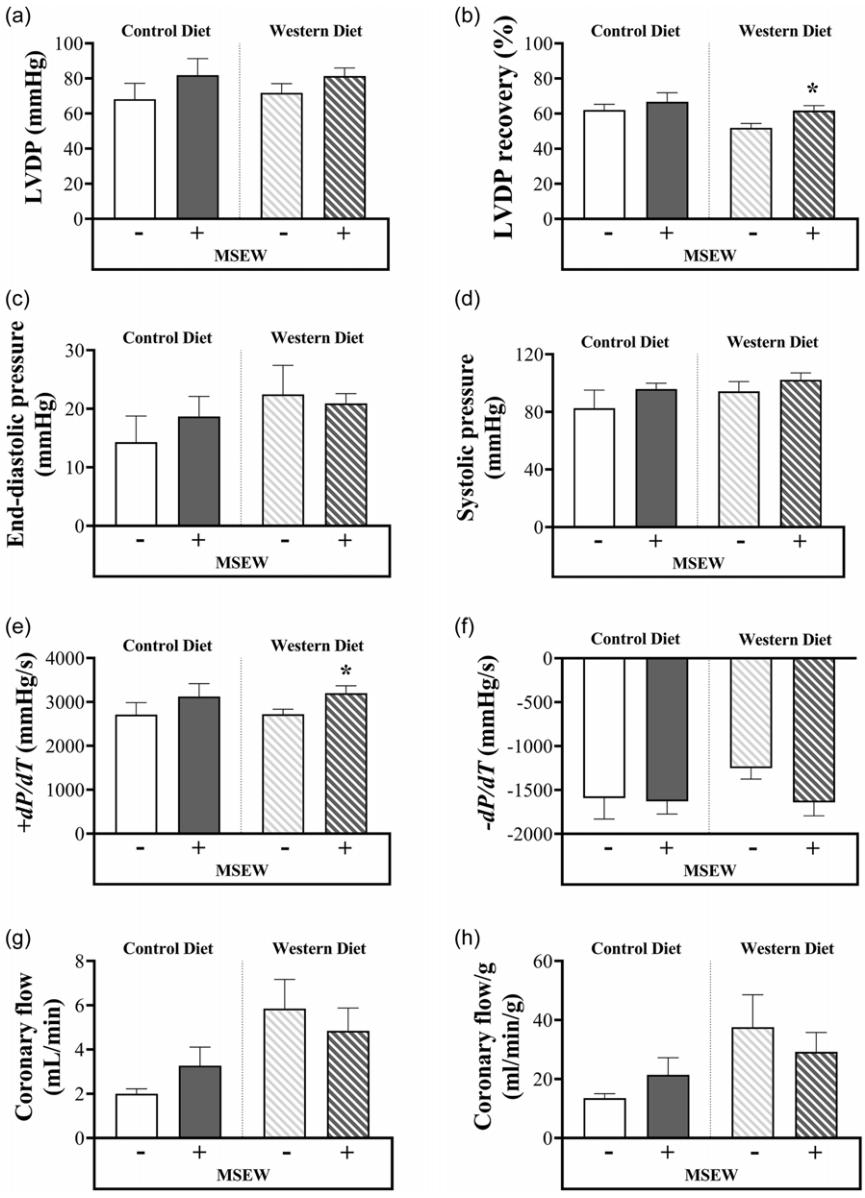


**Figure 11.** Post-ischaemic functional recoveries in perfused hearts. At 40 m reperfusion: (a) LVDP, (b) LVDP recovery, (c) End-diastolic pressure, (d) Systolic pressure, (e) Positive change in pressure over time, (f) Negative change in pressure over time, (g) Coronary flow, (h) Coronary flow per gram of heart weight. CON – control, MSEW – Maternal separation and early weaning, WD – Western diet. $N = 6$ for CON, MSEW, and WD. $N = 12$ for MSEW + WD. Data presented as Mean ± SEM. *$p < 0.05$.

increase defensive but not exploratory behaviours.[47] As with other reviews, it was concluded that lack of standardisation in maternal separation protocols underpins heterogenous study outcomes.[49,50]

Previous studies also demonstrate strong associations between obesity and mood disorders,[39,51,52] with diet or genetically induced obesity anxiogenic in rodents.[53–58] Our results corroborate these

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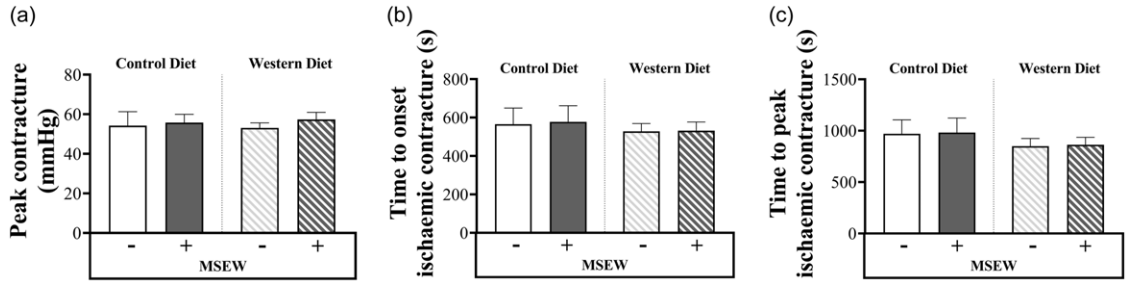


**Figure 12.** Rate and extent of ischaemic contracture in perfused hearts. (a) Peak contracture. (b & c) Time to contracture and time to peak contracture. CON – control, MSEW – maternal separation, WD – western diet. $N = 6$ for CON, MSEW, and WD. $N = 12$ for MSEW + WD. Data presented at Mean ± SEM.

associations and support a synergistic effect of MSEW and a WD on anxiogenesis. Highly variable and sometimes opposing interactions between stress and WD feeding have been reported, for example a palatable cafeteria diet may ameliorate anxious behaviour induced by maternal separation.[59] Palatability may itself be a complicating factor, with sweet and/or fatty foods potentially countering affective disturbances through reward circuits.[59,60] The cafeteria diet itself differs considerably from the WD here, in terms of both macro- and micro-nutrient composition. Studies of later life stress similarly report variable outcomes, including additive or synergistic impacts of chronic stress and a WD[39,61] *vs.* counteracting effects.[59,62–65] The current data support a potentially synergistic influence of ELS on WD-dependent anxiogenesis.

### *Early life stress increases susceptibility to WD-induced weight gain*

We have previously shown that the WD increases body weight and insulin resistance in rodents[39,40,66–68] though we have not focussed on the WD–induced changes in this study but rather the interplay of such changes in the presence of ELS. There is evidence ELS predicts obesity in adulthood,[69] consistent with the marked increase in WD-induced weight gain in mice subjected to MSEW. Murphy et al' (2017) assessed cardiometabolic risk in male rats fed a high–fat diet and observed no changes to body weight.[70] The present study uniquely also used early weaning while Murphy et al' (2017) did not, indicating that early weaning may play an important role in the exacerbated weight gain observed here. Although the mechanism for ELS dependent weight gain has not been established, humans exposed to ELS exhibit a greater preference for calorie dense foods.[71] and an association between ELS and food addiction has been identified in individuals with high BMI.[72] Similarly, exposure of rats to MSEW increases consumption of palatable foods in later-life.[73] Although we did not document food intakes in mice studied here, body weight gains documented in the WD fed animals suggest a possible increased intake of our calorie dense food in MSEW animals. An increase in corticotropic releasing hormone and corticosterone predicted with MSEW, may also promote compulsive eating of palatable foods.[74] We only assessed resting corticosterone levels at the end of the study (20 weeks after MSEW) and found no significant differences between groups. These observations are however in agreement with reports indicating that resting corticosterone levels normalise quickly post stress.[75] A 48 h rest period was given between the EPM and blood collection and may explain why MSEW + WD animals exhibited greater anxiety responses in the EPM when compared to WD animals despite similar resting corticosterone levels. It would be useful to assess corticosterone reactivity to acute stressors, which may be augmented by ELS.[76] Our current data suggest an increased vulnerability to WD feeding may be an important mechanism linking ELS to both mood and cardiometabolic disorders.

The basis of the early increase in body weight immediately after weening is unknown, though a recent study reports that offspring of dams subjected to prenatal stress exhibit a preference for sweetened milk consumption at postnatal day 3.[77] Additionally, ELS may increase preference for obesogenic[78] and palatable comfort foods.[78,79] While the stressors differ, it is possible maternal stress induced by the MSEW may increase pup suckling and milk consumption (together with later consumption of palatable foods).

### *Early life stress promotes hyperglycaemia and insulin resistance in later life*

It is well established that calorie-rich diets induce insulin resistance in humans and rodents.[40,66,67,80–82] Interestingly, while MSEW alone disturbed glucose homeostasis, it did not exacerbate WD-dependent changes. The modest hyperglycaemia with MSEW was associated with a reduced QUICKI, though not HOMA-IR ($p = 0.075$). These findings align with literature regarding ELS and long-term impairment of glycaemic control.[3,8] While speculative, MSEW induced hyperglycaemia may be a consequence of prolonged effects of MSEW on the HPA-axis. We cannot exclude a possible role for broadly increased food consumption in MSEW dependent hyperglycaemia, consistent with early elevations in plasma triglycerides and body weight that may reflect increased post-weening feeding.[83] This in turn may involve ELS related elevations in CRH that promote compulsive palatable food consumption.[74]

### *Cardiac influences of diet and ELS*

Heart weight changes may match or lag behind body weight gain in uncomplicated[84,85] or short-term obesity,[86] while hypertension and other changes with chronic, severe obesity can induce pathological hypertrophic growth.[87–89] Interestingly, MSEW + WD had no effect on heart:body weight ratio, despite a WD-dependent increase in body weight.

Neither the WD nor MSEW modified baseline heart function in obese or lean mice. To the best of our knowledge, this study is the first to assess the combined effects of MSEW and an obesogenic WD on myocardial ischaemic tolerance. Although not statistically interrogated in the current study, it seems the WD caused the anticipated increase in insulin resistance and a marginal decrease in LVDP recovery in the hearts from these obese animals when compared to CON diet animals. While we predicted adverse effects of ELS and a WD on myocardial ischaemic tolerance, consistent

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with synergistic effects of chronic stress and a WD in adult mice,[39,90,91] MSEW appeared to paradoxically improve post-ischaemic contractile function recoveries in WD-fed mice. Previous studies in our laboratory indicate that insulin resistance is an important determinant of myocardial sensitivity to ischaemia/reperfusion injury,[68,92,93] with a significant 'threshold' insulin resistance that may not be reached here required for emergence of such adverse effects.[66] In one of our studies, obesity in aged insulin–insensitive rats protected the ischaemic heart.[66] While the MSEW + WD animals in the current study were more obese than the WD animals, the WD and MSEW + WD animals had similar levels of insulin resistance which may explain why we saw improved post ischaemic outcomes in the MSEW + WD animals. Increases in body weight without changes in insulin resistance may improve reperfusion injury salvage kinase/nitric oxide synthase signalling and reduce ischaemia and reperfusion injury.[66]

### *Limitations of the present study*

One limitation of the present study is that we did not assess corticosterone levels in weanlings immediately after the MSEW, when stress hormone levels are likely elevated. This was due to the fragility of the pups and our inability to obtain adequate blood sample volumes for the corticosterone assay in weanlings. Another limitation is the absence of females: while practical constraints limited capacity to assess outcomes in both sexes, such analysis is necessary in future work. The stress-dependence of both mood and cardiovascular disorders differs between the sexes, with women more susceptible to mood disturbances[94,95] and stress-related coronary ischaemia.[96] A recent study has reported that compared to people who report no adverse experiences, females and males that experienced more than two adverse childhood experiences were 4-times and 3.4-times more likely to develop mood disorders, respectively.[97] Murphy et al. (2017) assessed cardiometabolic risk in response to ELS protocols with a subsequent high–fat diet intervention in male and female animals. In their study, male rats were unaffected by ELS, but female rats were susceptible to increased weight gain and reduced insulin sensitivity. Metabolic changes induced by ELS were abolished with a corticosterone synthase inhibitor indicating metabolic impairments were potentially corticosterone driven in female rats. Murphy et al's findings support what is seen in humans where females appear to be more susceptible to stress-related illness. Conversely, Ho et al. (2016) observed increased aortic superoxide production in male mice, but not females and concluded that endothelial dysfunction may contribute to cardiovascular risk in ELS sufferers.[98] Thus, while the present findings implicate a sensitising effect of MSEW on WD dependent anxiogenesis and obesogenesis in males with paradoxical improvements to ischaemic tolerance, it is likely that the female mice may respond differently.[70]

Finally, analysis of food consumption would improve interpretation of the body weight changes observed. Consumption of palatable foods post-stress protocols, including maternal separation have been documented and confirmed hyperphagia would have provided clarity on ELS sustained effects on WD-dependent weight gain, immediate post-MSEW weight gain and hypertriglyceridemia, and later-life MSEW induced hyperglycaemia.

## Concluding remarks

The present study demonstrates that stress in early life may increase fasting glucose and insulin insensitivity in later life, and significantly enhances vulnerability to WD-induced obesity and anxiety in adult male mice. Such outcomes confirm the importance of early life adversity or stress in determining mood and cardiometabolic disease risks in adult life. While these data are consistent with an increased risk of cardiometabolic disease in later life, MSEW paradoxically improved cardiac ischaemic tolerance in obese mice, albeit a modest effect. Future studies should address the sex dependence of this pro-disease effect, and whether additional behavioural or cardiometabolic outcomes emerge later in life, for example influencing ageing dependent deterioration/dysfunction.

**Data availability statement.** All data is available from the corresponding author upon reasonable request.

**Acknowledgements.** All contributors are authors of this paper.

**Author contribution.** KR, EFDT, and JPH contributed to the conception and design, acquisition and interpretation of data, drafting, and editing of the manuscript. KR, TAG, TH, KH, SN, DAR, and JNP contributed to the acquisition, analysis, and interpretation of data, reviewed and edited the manuscript. KR, EFDT, and JPH are the guarantors of this work.

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**Competing interests.** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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