1
2 **Synergistic effects of low-level stress and a Western**
3 **diet on metabolic homeostasis, mood and**
4 **myocardial ischemic tolerance**
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38 **ABSTRACT**

39 **Background.** How low-level psychological stress and overnutrition interact in
40 influencing cardiometabolic disease is unclear. Mechanistic overlaps suggest
41 potential synergies, however findings are contradictory. We test whether low level
42 stress and Western diet (WD) feeding synergistically influence homeostasis, mood
43 and myocardial ischemic tolerance. **Methods.** Male C57Bl6/J mice were fed a
44 control or WD (32%/57%/11% calories from fat/carbohydrates/protein) for 12 wks,
45 with subgroups restrained for 30 min/day over the final 3 wks. Metabolism, behavior,
46 tolerance of perfused hearts to ischemia/reperfusion (I/R), and cardiac 'death
47 proteins' were assessed. **Results.** The WD resulted in insignificant trends to
48 increased body weight (+5%), glucose (+40%), insulin (+40%), triglycerides (+15%)
49 and cholesterol (+20%), and reduced leptin (-20%), while significantly reducing
50 insulin sensitivity (100% rise in HOMA-IR, $P<0.05$). Restraint did not independently
51 influence metabolism, while increasing HOMA-IR a further 50% (and resulting in
52 significant elevations in insulin and glucose to 60-90% above control) in WD mice
53 ($P<0.05$), despite blunting weight gain in control and WD mice. Angiogenesis with
54 restraint or WD was non-additive, whereas anhedonia (reduced sucrose
55 consumption) only arose with their combination. Neuroinflammation markers
56 (hippocampal TNF- α , IL-1 β) were unchanged. Myocardial I/R tolerance was unaltered
57 with stress or WD alone, while combination worsened dysfunction and oncosis (LDH
58 efflux). Apoptosis (nucleosome accumulation) and death protein expression (BAK,
59 BAX, BCL-2, RIP-1, TNF- α , cleaved caspase-3 and PARP) were unchanged.
60 **Conclusion.** Mild, anxiogenic yet cardio-metabolically 'benign' stress interacts
61 synergistically with a WD to disrupt homeostasis, promote anhedonia (independently
62 of neuroinflammation), and impair myocardial ischemic tolerance (independently of

63 apoptosis and death protein levels).

64 INTRODUCTION

65 Disturbances in mood and metabolism are strongly inter-related (9,14,64) and
66 increasingly common in both developing and developed countries. However, how
67 these common factors interact in influencing chronic disease development remains
68 to be detailed. In 2014 ~63% of Australian adults were overweight or obese (4),
69 projected to increase to >75% by 2025 (29). Chronic psychosocial stress, promoting
70 and reinforced by obesity (61), is also prevalent: a recent British survey found 3/4 of
71 >4500 surveyed suffered periods of stress in the prior year that left them
72 overwhelmed/ (55), and the 2018 Gallup Global Emotions Report (26) indicated 1/3
73 of people experienced significant stress (with negative experience rising globally
74 since 2010). Exemplifying important socioeconomic influences, the recent global
75 recession increased yearly supply of mental health medications in regions of the
76 USA, with medication use (57) and perceived stress (56) related to layoff numbers
77 (11).

78 Importantly, metabolic and mood disturbances increasingly co-exist (64),
79 reflecting growing prevalence of multimorbidity, which now affects 1/3 of all
80 Australian adults (30) and is the 'new normal' in coronary heart disease (82).
81 Longitudinal psychosocial stressors, including occupational (16,40) and other forms
82 (10,14), appear to exacerbate cardiometabolic disease, promoting obesity, insulin-
83 resistance and myocardial infarction (15,41,62,71), together with CVD mortality (27).
84 Nonetheless, how mood and metabolic disorders interact to modify cardiovascular
85 physiology and phenotype is poorly understood, and demands investigation (73).
86 How they interact in the genesis of mood and metabolic diseases is similarly unclear:
87 while emerging evidence of shared mechanistic networks and
88 metabolic/neuroendocrine sequelae (64,80) suggests mood and metabolic

disturbances may act additively or even synergistically, experimental findings are contradictory. For example, chronic stress reportedly: i) has no effect on glycemic control with high fat feeding (60); ii) worsens insulin-resistance in models of obesity (18,19,24); or iii) improves metabolic homeostasis with high-fat feeding (23) and protects against diabetes (35). These latter effects may involve stress-dependent hypophagia and weight loss (7,63,78). Similarly, the behavioral effects of chronic stress are reportedly ameliorated (23,42,52,61) or exacerbated (75,85) with high fat and/or carbohydrate diets.

In the present study we employ murine models to test the hypothesis that even mild, relatively benign levels of chronic psychological stress (brief daily restraint) and metabolic perturbation (WD feeding) may synergistically disrupt metabolic homeostasis, behavior and/or the heart's ability to withstand damage during I/R. No study has assessed how mild levels of stress and overnutrition interact in determining myocardial tolerance to ischemic or other insult.

MATERIALS AND METHODS

Animals. Young adult male C57Bl6/J mice aged 7-8 weeks were sourced from the *Animal Resources Centre* (Canning Vale, Western Australia, Australia). All animal work was approved and performed in accordance with the guidelines of the Animal Ethics Committee of Griffith University (MSC/07/17) and the Australian code of practice for the care and use of animals for scientific purposes. Mice had *ad lib* access to water and food throughout all studies, were maintained in an artificial 12-hour day/night lighting cycle at a constant temperature of 21°C (40% humidity).

Dietary intervention. Mice were housed 4-5 per cage and ear punched for

identification. After a minimum 1 week acclimatization period mice (to ~9 weeks of age) mice weighed 22.2 ± 0.3 g and were randomly assigned to either a control diet (Meat Free Rat and Mouse Cubes, Specialty Feeds, Western Australia) or a WD ($n=16$ for each). To model low-level dietary 'stress' equivalent to the human WD (35% calories from fat/49% from carbohydrate/16% from protein in the US population [83]), without inducing overt obesogenesis, we selected a diet in which fat, carbohydrates and protein provide 32%, 57% and 11% of calories, respectively. This moderate fat diet is predicted to have minor effects on body weight, total calorie intake and energy expenditures over 10-12 weeks (33), distinct from high fat (45-60% of calories) diets employed in obesity studies (3,6,19,22,23,43,66,74,79). Experimental data confirmed predicted outcomes (**Figs 1-4**).

The WD feed consisted of 125 g of animal fat, 140 g of sugar and two 397 g tins of condensed milk, added to 800 g of powdered rodent chow (see supplementary **Table S1** for nutritional analyses: (<http://doi.org/10.17605/OSF.IO/AQ2XF>). Fresh food was provided daily with serving sizes pre-determined at 15 g per mouse/day for the obesogenic groups (ensuring uninterrupted access to fresh food). After 9 weeks, subgroups ($n=8$ each) were subjected to 3 weeks of daily restraint stress. The 4 experimental groups thus comprised: control diet (C); Western diet (WD); control diet + restraint (C+R); and WD + restraint (WD+R). Glucose tolerance tests (GTTs), open field tests (OFT) and sucrose preference tests (SPT) were undertaken at 8 and 12 weeks to assess metabolic and behavioral changes in control and WD mice, pre- and post-stress.

The mice studied thus span an age range that corresponds to ages of onset and progression of type 2 diabetes and mood disorders in humans: from 8-9 weeks (early adult, ≤ 20 years of age in humans) with progression over a 12-week period

(~10 human years). This is relevant to the range over which type 2 diabetes emerges in humans, together with the approximate period from initial metabolic dysregulation to disease diagnosis (67), and is similarly relevant to mood and anxiety disorders which exhibit median age of onset in the early to mid 20's and peak incidence from mid-late adolescence to early 40's (38,48).

Mild stress - brief daily restraint. Our goal was to model a very mild level of chronic stress, insufficient to independently disturb metabolic state or induce a depression phenotype (ie. 'sub-clinical'). The C57Bl/6 mouse studied here is more resilient than other strains, for example the stress sensitive Balb/c mice, which exhibit exaggerated inflammation and corticosterone reactivity, and less effective neurochemical normalization with repeated stress (12,54,77). Since predictable homotypic stress is less aversive than heterotypic stress, with repeated restraint less likely to induce depression as opposed to unpredictable stress (88), we selected a chronic restraint model (37,60,63). Restraint episodes, from as little as 15 min to a protracted 8 hrs, transiently increase corticosterone in mice (28,34), with baseline concentrations ultimately recovering to control levels with chronically repeated restraint (7,12,78), distinct from sustained elevations with heterotypic stressors (28). We therefore selected a relatively brief 30 min duration of restraint. Since behavioral impacts depend strongly on number of restraint episodes rather than total 'volume' of restraint, requiring ≥ 14 days to emerge (39), we opted for a 21 day duration. Thus, commencing in experimental week 9, groups of control and WD mice were subjected to 30 min restraint in acrylic cylinder animal holders. Data (**Figs 1-3**) confirm absence of metabolic disruption or a depressive outcome (no change in anhedonia - hallmark of depression; [49]), while supporting significant stress in terms of both suppressed weight gain and behavioral hallmarks of chronic aversive stress

(angiogenesis in control diet mice, reduced sucrose consumption in WD mice).

Metabolic assessment. Mice were weighed weekly, and GTTs performed at 8 and 12 weeks to assess glucose homeostasis. Mice were subjected to a 4 hour morning fast before acquiring tail-tip blood for fasting glucose analysis using an Accu-check II glucometer (Roche Diagnostics, Castle Hill, Australia). A 20% glucose bolus (2 g/kg) was then administered intraperitoneally (1) and blood glucose levels documented at 15, 30, 60, 90, 120 and 180 minutes post-injection. Total area under glucose curves (AUC) was calculated as a measure of glucose tolerance.

Behavioral assessment. The OFT and SPT were conducted on all 32 mice in weeks 8 and 12, assessing baseline behavior and the influences of WD feeding $\pm$ restraint stress. A single trained investigator undertook all animal handling and assessment to acclimate animals prior to study, and limit confounding stress during the prolonged experimental periods. Observation of mice within the OFT arena tests exploratory behavior in a foreign environment (37,84). The test arena was a 56 cm x 56 cm box with 4x4 marked grid and a center square. The test was started when a mouse was placed in the center square. All behavior and movement was recorded for 20 minutes using a digital camera for subsequent analysis. Parameters assessed included line crossings (overall locomotor activity), center square entries and duration (exploratory behaviors) and grooming. Behavioral outcomes were normalized to individual values measured prior to the experimental protocol.

The reward-based SPT identifies animal preference for 1% sucrose vs. water, assessing anhedonia (reduced pleasure-seeking from enjoyable activities) which is considered a hallmark of depression (49). Individual mice were placed in isolated cages with 2 bottles containing water over a 24 hour acclimatization period. Bottles were then refilled, one with water and the other with 1% sucrose solution, and

intakes measured over 24 hours.

Cardiac function and ischemic tolerance. Mice were anesthetized with 60 mg/kg sodium pentobarbital at the end of experiments, blood collected and hearts excised and mounted on a Langendorff perfusion system (65,70). Hearts were perfused in a non-recirculating manner with a modified Krebs-Henseleit buffer (119 mM NaCl, 11 mM glucose, 22 mM NaHCO₃, 4.7 mM KCl, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 1.2mM EDTA, 0.5 mM and 2.5 mM CaCl₂) bubbled with 95% O₂/5% CO₂ at 37°C (pH 7.4). To measure contractile function, a fluid-filled plastic film balloon was inserted into the left ventricle and inflated to yield an end-diastolic pressure (EDP) of 5 mmHg during stabilization. An ultrasonic flow-probe in the aortic perfusion line, connected to a T206 flow meter (Transonic Systems Inc., Ithaca, NY, USA), measured coronary flow rate. Peak left ventricular systolic, end-diastolic and developed (systolic - diastolic) pressures, heart rate, coronary flow and +/-dP/dt were continuously monitored at 1 KHz using a 4 channel MacLab system (AD Instruments Pty Ltd., Castle Hill, Australia) connected to an Apple iMac computer. A thermal probe connected to a 3-channel Physitemp TH-8 digital thermometer monitored the temperature of the perfusate and surrounding water bath (Physitemp Instruments Inc, Clifton, NJ, USA) (65,70). Hearts were stabilized for 20 min and then switched to ventricular pacing at 7 Hz using an SD9 stimulator (Grass Instruments, Quincy, MA) for 10 min prior to induction of ischemia. Global normothermic ischemia was induced for 25 min, followed by 45 min of aerobic reperfusion. Hearts were snap-frozen in liquid N₂ for later analysis.

Hippocampal TNF- α and Il-1b. At the time of heart extraction, brains were rapidly removed and placed into ice cold PBS. Hippocampal regions were dissected free under magnification, frozen in liquid N₂ and stored at -80°C until analysis.

Thawed tissue was homogenized in cold lysis solution (Bio-Plex® Cell Lysis kit; Bio-Rad Laboratories, Gladesville NSW, Australia) containing 2 mM phenylmethylsulfonyl fluoride. Tissue homogenates were sonicated and centrifuged at 4,500 g for 5 min, supernatants collected and protein concentrations determined using a BCA assay (Bio-Rad Laboratories). Sample volumes were adjusted to normalize protein and hippocampal IL-1 β and TNF- α contents were assessed using a Bio-Plex magnetic bead-based assay (Bio-Rad Laboratories), according to manufacturer's instructions. For comparison purposes, IL-1 β and TNF- α levels were normalized to values for control diet mice.

Biochemical analyses of blood and coronary effluent. Blood samples taken at sacrifice were used for immediate glucose determinations. Serum collected at sacrifice and coronary effluent samples collected during reperfusion were stored at -80°C until later analysis. Serum triglyceride determinations were performed using a triglyceride quantification colorimetric kit (Biovision Inc. Milpitas, California, USA). Serum leptin was assayed via ELISA according to manufacturer instructions (CSB-E04650m-96T; Cusabio, College Park, MD, USA), and total cholesterol content via colorimetric/fluorescent assay kit (#ab65390; Abcam, Melbourne, VIC, Australia). Insulin levels were determined using an ELISA kit (Crystal Chem, Downer Grove, Illinois, USA). Insulin resistance was calculated from fasting serum glucose and insulin values using the homeostasis model assessment of insulin resistance (HOMA-IR), as in prior rodent studies (4,19,20,23,60,66,74,85):

$$\text{HOMA-IR} = [\text{mg/dL Glucose} \times \mu\text{U/ml Insulin}] / 405.$$

In perfused heart studies, total myocardial efflux of LDH throughout the entire post-ischemic period was measured as a biomarker of cell death. This measure correlates strongly with infarct assessment via TTC staining in this murine heart

model (65). Total effluent throughout reperfusion was collected on ice before analysis using a LDH cytotoxicity assay kit (Abcam, Melbourne, VIC, Australia). Efflux of LDH was normalized to heart mass (mU/g).

Myocardial nucleosome content and death protein expression. Ventricular lysates from post-ischemic hearts were assayed for nucleosome content (histone-complexed DNA fragments) as a marker of apoptosis, and expression of cell death proteins via western immunoblot analysis. Tissue was homogenized in a glass dounce with 1.0 mL of ice-cold lysis buffer (Kinexus buffer containing 20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 40 mM β -glycerophosphate, 20 mM NaPP and 1% Triton X-100, together with protease and phosphatase inhibitors: 1 mM PMSF, 10 μ M leupeptin, 3 mM benzamidine, 5 μ M pepstatin A, 1 mM NaO). The homogenate was centrifuged at 600 g for 10 min at 4°C to remove cellular debris. Protein content of the lysate was assayed via Pierce™ BCA Protein assay (Thermo Fisher), and samples diluted to ~1 μ g/ μ L protein, aliquoted and stored at -80°C until immunoblot analyses.

For assessment of apoptosis, post-ischemic nucleosome content was assayed via ELISA according to manufacturer instructions (Roche Cell Death Detection ELISAPLUS, Sigma-Aldrich Pty Ltd, North Ryde NSW). Assay results were normalized to content in hearts from un-stressed control diet mice. For immunoblotting, lysate samples were mixed with equal volumes of 2x loading dye containing 5% β -mercaptoethanol, and denatured at 95°C for 5 min before cooling on ice. A total of 30 μ g protein/sample was loaded into hand-cast 10% acrylamide gels and separated at 150 V for 60 min. Proteins were transferred to a polyvinylidene difluoride fluorescent membrane at a constant 75 V for 1.5 hrs, and blocked with Odyssey fish serum for an additional 2 hrs at room temperature. Transferred proteins

264 were incubated in primary antibody (Bax, Cell Signaling #2772, 1:1000; Bak, Cell
265 Signaling #D4E4, 1:1000; Bcl-2, Cell Signaling #3498, 1:1000; PARP, Cell Signaling
266 #9532, 1:1000; cleaved caspase-3, Cell Signaling, #9661, 1:1000; TNF α , Cell
267 Signaling, #2775, 1:1000; RIP, Santa Cruz, sc-133102, 1:1000; GAPDH, Santa Cruz
268 sc-32233, 1:5000) for 18 hrs at 4°C with gentle rocking. Membranes were washed 4
269 times in TBST (5 min each) and again in TBS for 5 min before incubation with
270 secondary antibodies (LI-COR IRDye® 680RD, donkey anti-mouse: #925-68072; or
271 goat anti-rabbit: #925-68071, 1:30,000) at room temperature in the dark. Membranes
272 were then washed 4 times in TBST (5 min each) and in TBS for 5 min, before drying
273 overnight between paper towels in the dark. Membranes were visualized on a LI-
274 COR Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE USA) with
275 densitometric data normalized to GAPDH in each sample.

276 *Statistical analyses.* Data are presented as means $\pm$ standard error of the
277 mean (SEM). Outliers were identified using the Grubbs test, and normality of data
278 distributions tested by Shapiro-Wilk test. As a result, fasting insulin was transformed
279 ([insulin]⁻²), guided to Box-Cox analysis. Gaussian distribution of the transformed
280 data was confirmed. A 2x2 ANOVA was employed followed by Tukeys post-hoc test
281 for multiple comparisons (using GraphPad Prism 7). A *P* value <0.05 was
282 considered indicative of statistical significance in all tests.

RESULTS

Metabolic phenotype. The WD alone induced very mild metabolic disruption, with only insulin sensitivity significantly modified. (**Figs 1-2**). The WD mice exhibited insignificant (+5%) weight gain compared with control diet mice after 12 weeks feeding (**Fig 1A**). Circulating levels of glucose, insulin, triglycerides, cholesterol, and leptin were not significantly altered (**Fig. 1**), however the WD did reduce insulin sensitivity, estimated from HOMA-IR (**Fig. 2A**). Nonetheless, glucose tolerance was not significantly altered (**Fig. 2B,C**), with GTTs undertaken after 8 weeks of WD feeding also showing no change at this earlier time (supplementary **Fig. S1**: <http://doi.org/10.17605/OSF.IO/AQ2XF>). Spontaneous locomotor activity, reflective of active energy expenditure, also appeared unaltered by the diet (**Fig. 3A**). The mild stress protocol similarly failed to independently influence metabolic parameters in control diet animals, although weight gain over the 3 wk stress period was significantly reduced (**Figs. 1 and 2**).

Despite no effects in control diet mice, restraint stress significantly worsened metabolic homeostasis in WD mice, resulting in a further 35% rise in insulin to levels significantly higher than control ($P < 0.05$ vs. control), and a 50% rise in HOMA-IR beyond levels in WD animals ($P < 0.05$ vs. WD) (**Figs 1 and 2A**). These effects appear synergistic: the rise in insulin with WD+R (+26 μ U/ml, or +90%) was 3-fold greater than the sum of independent responses (+12 μ U/ml or +42% in WD, and -4 μ U/ml or -14% in C+R); and the rise in HOMA-IR (+23, or +197%) was 2-fold greater than the sum of independent responses (+11 or +94% in WD, and +1 or +8% in C+R). Fasting glucose was significantly elevated in WD+R mice, though did not differ from values in WD or C+R mice (**Fig. 1**). Interestingly, triglyceride levels in

WD+R mice were lower than with restraint or a WD alone (though not differing from control), while plasma cholesterol and leptin did not significantly differ across groups (**Fig. 1**). These detrimental metabolic outcomes arose despite restraint dependent inhibition of weight gain over the 3 wk protocol in WD mice, although final body weights remained slightly higher in WD+R vs. C (but not WD or C+R) mice (**Fig. 1**). Glucose clearance curves from GTTs did not statistically differ (see supplementary **Fig.S2**: <http://doi.org/10.17605/OSF.IO/AQ2XF>) with AUC unaltered across groups (**Fig. 2B**). However there was a tendency to elevated glucose at later times in the WD+R group (delayed glucose removal). Supporting impaired/delayed glucose uptake, circulating levels at 180 min post glucose challenge remained significantly elevated in the WD+R group compared with C or C+R mice (**Fig. 2C**).

Behavioral changes. Mild stress and a WD were independently anxiogenic: locomotor activity (line crossings) was unaltered (**Fig. 3A**), while each individually decreased center square entries (**Fig. 3B**), and restraint stress additionally reduced center square duration (**Fig. 3C**). Grooming time was unchanged (data not shown). No shifts in OFT behaviors (or sucrose preference) were evident at an earlier 8 week time point (see supplementary **Fig. S3**: <http://doi.org/10.17605/OSF.IO/AQ2XF>). While sucrose intake was not altered by the WD or restraint alone, there was evidence of reduced sucrose consumption (indicative of anhedonia, a hallmark of depression) with stress in WD mice (**Fig. 3D**), equivalent to a decline to <65% of that in control mice. Despite significant behavioral changes, there was no evidence of neuroinflammation, based on stability of hippocampal TNF- α and IL-1b levels (**Fig. 4**).

Myocardial ischemic tolerance and death protein expression. Baseline contractile function and coronary flow in perfused hearts were comparable across groups (**Table 1**). Interestingly, while heart weight was not modified by either the WD

or restraint stress alone, their combination was associated with a higher heart weight (+13% vs. control) (**Table 1**). However, the heart:body weight ratio was largely unchanged (insignificant +4% vs. control). Post-ischemic LDH efflux, a marker of oncotic death, was unaltered with either the WD or restraint stress alone (**Fig. 5A**). However, stress synergistically elevated efflux in the hearts of WD mice (~4-fold greater than the sum of independent effects of stress and a WD). Conversely, post-ischemic nucleosome content was unaltered by the WD and/or restraint (**Fig. 5B**). Similar to LDH efflux, post-ischemic contractile dysfunction (indicated by reduced pressure development and increased EDP) was not significantly worsened with the WD or stress alone (**Figs. 5C and D**). However functional outcomes were substantially impaired with mild stress in WD mice (**Fig. 5**).

Consistent with lack of change in nucleosome content, neither the WD and/or restraint modified expression of total or cleaved apoptotic and necroptotic proteins in post-ischemic myocardium (when both forms of death are executed) (**Figs. 6 and 7**).

DISCUSSION

The present study demonstrates that low levels of psychological and dietary or metabolic stress may synergistically disrupt markers of metabolic homeostasis, mood and myocardial ischemic tolerance. Behavioral changes were not associated with shifts in neuroinflammatory mediators (IL-1b, TNF- α), and the latter cardiac outcome appears independent of post-ischemic apoptosis and death protein expression. While confirming our hypothesis of detrimental interactions between low levels of increasingly common (frequently co-existent) risk factors, the molecular mechanistic underpinnings of these significant metabolic, behavioral and myocardial

357 outcomes await further study.

358 *Metabolic influences of mild restraint stress and a WD.* As predicted from prior
359 characterization of diet effects in C57Bl/6 mice (33), the moderate fat diet was not
360 substantially obesogenic and was associated with a 40% rise in fasting insulin that
361 did not achieve significance. However, WD feeding significantly reduced insulin
362 sensitivity. Greater impacts of the WD on insulin control vs. glucose levels agrees
363 with evidence they are governed by differing factors (81), and with observed
364 disruption of insulin levels/signaling without detectable changes in glucose with high
365 fat feeding in mice and humans (17). Although restraint alone was without effect,
366 synergistic disruption of homeostasis was evident in WD+R mice: elevations in
367 insulin and HOMA-IR were 2- to 3-fold greater than the sum of independent effects
368 of a WD and stress, with glucose levels also significantly elevated with WD+R.

369 The additive or synergistic impairment of metabolism with the WD+R agrees
370 with reports that stress worsens glucose homeostasis in animals fed a hypercaloric
371 diet (18), and evidence combined effects of stress and diet may exceed the sum of
372 their independent effects (19,24,74). The present data suggest a combination of
373 chronic low-level stress and a WD promotes an early or pre diabetic state,
374 significantly increasing blood glucose and insulin levels beyond those in C mice, and
375 increasing insulin-resistance (HOMA-IR) above levels in all other groups. This
376 contrasts reportedly protective effects of chronic stress on glucose homeostasis and
377 development of diabetes (7,35,63), attributable in part to hypophagia and weight
378 loss. Evidence of an inhibitory influence of stress on triglyceride levels in WD mice
379 was unexpected, though agrees with de Sousa Rodrigues and colleagues, who also
380 observed a moderate fall in triglycerides with combined stress and WD despite

worsened glucose homeostasis (19).

Circulating leptin was not significantly modified with the WD or stress protocols. Reported effects of stress on leptin remain mixed, including evidence it can decrease (23,50) or increases leptin (85,87) in animals on control diets, reduce leptin elevations with high fat feeding (23,87), or exert limited effects relative to dietary influences (51). Lack of significant change in leptin may reflect minor weight gain in this model (<5% vs. control), coupled with declining insulin sensitivity. Since insulin promotes leptin synthesis and release (53), reduced insulin sensitivity with the WD $\pm$ stress (**Fig. 1**) may constrain leptin changes. These data also indicate the fall in insulin sensitivity with the WD, and the added impact of restraint, are unrelated to leptin resistance (which is characterized by chronically elevated leptin). Leptin resistance implicated in metabolic disorders (58) and depression (64,80) may require more extreme or prolonged levels of overnutrition and stress than the mild levels specifically modelled here.

Suppression of weight gain over the 3 wk restraint protocol is consistent with clinically relevant stress, and indicates that metabolic disruption with restraint in WD mice is not a result of increased weight gain. While hypophagia and weight loss are characteristic of chronic stress (6,18,19,39,47,78), differential weight gain in low fat vs. weight loss in high fat fed mice has been reported (23), together with stress dependent hyperphagia and weight gain in both control and high fat diet animals (74). The latter study also reported synergistic impairment of insulin sensitivity, in agreement with our observations, however changes here are independent of weight gain.

Behavioral influences of mild restraint stress and a WD. Chronic restraint and

a WD were independently anxiogenic, consistent with behavioral outcomes in other models of chronic stress (22,37,87), and of obesity, metabolic syndrome and diabetes (43,69). Significant anxiogenesis coupled with impaired weight gain confirm chronic stress with the restraint protocol. Anxiogenic outcomes were not additive, and appeared moderately reduced in WD+R mice, contrasting effects of chronic stress in models of diabetes (23), or of high fat (87) or carbohydrate (75) diets in models of stress. However, there is also support for anxiolysis with palatable high fat/high carbohydrate diets (42,52,61), which may explain the less pronounced behavioral outcome in the WD+R group. Distinct from anxiety behavior, a synergistic anhedonia was evident: neither stress nor WD independently influenced sucrose consumption, while combination reduced sucrose intake to <65% of control levels. The former observation agrees with evidence repeated restraint (homotypic) stress is unlikely to independently induce depression, whereas chronic unpredictable (heterotypic) stress does (88). Features of depression may emerge in a body weight dependent manner (32) in highly obesogenic models that exhibit more than double the relative weight gain observed here (79,87). However, the WD regime induced a minor (~5%) increase in weight, and restraint countered weight gain while reducing sucrose preference. These data suggest low-level stress may promote depressive behavior in WD fed animals, whereas even more severe levels of restraint fail to induce depression in otherwise healthy animals (88).

These observations are consistent with involvement of metabolic disruption in the pathogenesis of depression (23,75,87), and with our proposed psycho-immune-neuroendocrine (PINE) network model (64,80) in which depression, obesity, diabetes and coronary heart disease are to be mechanistically interlinked. A commonality of mechanisms implicated in both glycemic dysregulation and anhedonia, for example

(80), is congruent with augmented metabolic and behavioral effects of combined stress and a WD here. In humans, melancholic and non-atypical non-melancholic depression are associated with weight loss, while atypical depression is linked to weight gain (64) and greater levels of inflammatory cytokines (44). The combined outcomes in WD+R mice, including evidence of emergent depressive behavior (anhedonia) without weight gain or shifts in hippocampal TNF- α or IL-1b, thus appear somewhat consistent with features of melancholic/non-atypical depression. Stability of cytokine levels agrees with recent reports of little to no change in hippocampal mRNAs for these and other inflammatory mediators (and no change in TNF- α protein) in C57Bl/6 mice exposed to chronic restraint (54,77). Such observations suggest changes in hippocampal TNF- α or IL-1b are not essential to disordered mood with low level stress and/or WD feeding, or that other cytokines (or overall inflammatory status rather than individual mediators) are involved. In this regard, a clinical trial of TNF- α targeted infliximab revealed no overall effects in treatment-resistant depression, though there was evidence of some benefit in those with particularly high inflammatory state (68). This is congruent with reduced depressive behaviors with anti-inflammatory treatment in subjects with inflammatory disorders (36), whereas benefits in depression per se are less clear.

Myocardial influences of mild restraint stress and a WD. Data reveal for the first time synergistic effects of low level stress and a WD on myocardial I/R tolerance. Despite no independent impacts, combination of a WD and restraint stress markedly worsened post-ischemic dysfunction and a marker of oncotic death. Absence of an independent effect of restraint is consistent with reports ischemic intolerance and exaggerated myocyte death arise with levels of chronic stress sufficient to induce depression (31). Here, cardiac ischemic intolerance only

455 emerges in animals evidencing anhedonia - a hallmark of depression. Unaltered or
456 improved I/R tolerance with the WD also agrees with our observation of conserved
457 cardiac outcomes in moderately obese but insulin-sensitive rats (20). Other studies
458 provide evidence of cardioprotection in different models of obesity (22,66,76),
459 whereas hearts from obese insulin-resistant rats (21,85) and mice (3) appear
460 sensitized to I/R injury. These collective findings suggest a primary role for insulin
461 sensitivity in I/R tolerance, with evidence of a threshold sensitivity here: I/R tolerance
462 markedly declined with a 3-fold rise in HOMA-IR, yet was unchanged with a 2-fold
463 increase. Synergistic suppression of I/R tolerance appears to be independent of
464 post-ischemic apoptosis (nucleosome accumulation), and expression of apoptotic
465 proteins, the necroptosis mediator RIP-1 and the death ligand TNF- α . Rather, the
466 select increase in intracellular enzyme (LDH) efflux supports exaggerated oncotic
467 death, which may in turn contribute to worsened contractile dysfunction. The basis of
468 this novel effect awaits clarification, though there is some support for shifts in
469 survival kinase signaling and cardiac structure with chronic stress (31). Whereas
470 death protein expression and apoptosis are not modified here, shifts in survival
471 kinase signaling patterns with either diet (20) or stress (31) may participate, and
472 warrant future study. Similarly, myocardial morphological and ultrastructural
473 influences of stress and a WD are worthy of investigation, with evidence here of an
474 increased heart weight in WD+R vs. C mice, and of myocardial remodeling/fibrosis
475 with more severe chronic stress in prior studies (31). Hypertrophy has been linked to
476 reduced I-R tolerance, though this may reflect the impacts of cardiac
477 dysfunction/failure rather than of heart mass *per se* (25). Indeed, physiological forms
478 of cardiac hypertrophy are associated with preserved or improved I-R outcomes
479 (8,86). As heart:body weight ratio was unchanged, the increased heart weight in

WD+R mice is congruent with a greater body weight rather than hypertrophy. Nonetheless, analysis of cardiac ultrastructure may be informative.

Study Limitations. Several limitations and constraints are worth noting. First, to assess myocardial injury we have not directly measured infarct size, as we sought to also examine relevant protein expression and nucleosome levels. We thus assess multiple post-ischemic end-points, encompassing contractile dysfunction, nucleosome content (caspase-dependent DNA fragmentation) and total LDH efflux (sarcolemmal disruption/oncotic death). The latter is a common measure of viability or death, which we show correlates strongly with infarct size in this experimental model (65). Secondly, due to institutional ethics constraints, we were unable to fast mice beyond 4 hrs (or overnight). Although hepatic glycogen may be more effectively depleted with overnight fasting, this also increases insulin-stimulated glucose uptake in mice, thus morning fasting is useful in assessing insulin responses in a more physiological state (5). However, it is possible the 4 hr fast may mask subtle changes in glucose levels and GTT in WD±R mice. Third, we use a surrogate measure of insulin sensitivity that is widely employed in murine studies of dietary obesity (3,19,23,66,74), rather than the euglycemic-hyperinsulinemic clamp method: unavoidable stress and technical challenges in mice render the latter approach an inappropriate added intervention in this study of low-level stress. The surrogate HOMA-IR (and the directly related quantitative insulin-sensitivity check index) correlates with euglycemic-hyperinsulinemic clamp measures of insulin resistance in humans (72), monkeys (46), rats and mice (45,59), and has also been validated in both pregnant and non-pregnant female and insulin-resistant male rats (2,13). While a useful surrogate when the euglycemic-hyperinsulinemic clamp method is prohibited, we nonetheless note that correlation with clamp measures is weaker in

rodents vs. humans and monkeys (reflecting in part technical difficulties in small animal models). Finally, we did not acquire data on food consumption, which would be useful in assessing potential roles of feeding behavior (hyper/hypophagia) vs. shifts in 'metabolic efficiency' (weight gain/calorie intake) in responses to WD and/or stress protocols. Nonetheless, our data indicate that the metabolic dysfunction with the WD $\pm$ stress is not a result of increased weight gain.

Conclusions. The combined effects of co-morbid disorders - multimorbidity - are poorly defined and warrant further study (73). The present data reveal detrimental effects of mild disturbances in mood and metabolism that are not necessarily predictable from individual influences. Mild restraint stress and a WD synergistically disrupt metabolic homeostasis and mood, and reveal a heretofore unappreciated interaction in influencing myocardial phenotype. The latter effect appears independent of apoptosis and expression of pro-apoptotic and necroptotic proteins. While confirming our primary hypothesis, the mechanistic underpinnings of the different metabolic, behavioral and myocardial outcomes revealed await further delineation. Nonetheless, these data indicate that low levels of prevalent lifestyle factors (mild stress and a WD) may strongly interact to worsen both coronary heart disease risk (eg. via hyperinsulinemia, insulin-resistance, hyperglycemia) and the hearts intrinsic resistance to associated ischemia.

524 ***Perspectives and Significance***

525 Cardiovascular, metabolic and mood disorders share common risk factors and
526 increasingly co-exist in multimorbidity. Determining chronic disease risk and
527 understanding progression and outcomes requires an appreciation of potential
528 additivity or synergism among metabolic and mood related risk factors, particularly
529 those that may otherwise appear relatively 'benign'. The current findings highlight the
530 importance of even low level disturbances in mood and metabolism on disease
531 development and sequelae, evidence fundamental linkages between metabolic,
532 mood and cardiovascular disorders, and reveal the high sensitivity of myocardial
533 ischemic tolerance to mild and modifiable shifts in lifestyle factors.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

E.F.D.T., F.T., and J.P.H. conceived and designed research; E.F.D.T., F.T., D.O., T.G., T.H., L.W., J.N.P., and J.P.H. performed experiments; E.F.D.T., FT, DO, T.G., T.H., and L.W. analyzed data; E.F.D.T., A.C., J.N.P., N.J.C.S., and J.P.H. interpreted results of experiments; E.F.D.T. prepared figures; E.F.D.T., N.J.C.S., and J.P.H. edited and revised manuscript; E.F.D.T., F.T., A.C., D.O., T.G., T.H., L.W., J.N.P., N.J.C.S., and J.P.H. approved final version of manuscript.

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TABLES

Table 1. Heart Weights, and Baseline Coronary Flow and Ventricular Pressure Development in Perfused Hearts

	C	WD	C+R	WD+R
Heart Weight (g)	155±6	160±5	164±7	175±5 *
Heart:Body Weight Ratio	5.62±0.10	5.67±0.10	5.85±0.09	5.87±0.08
Coronary Flow (ml/min/g)	18.9±1.2	22.4±3.2	20.2±2.4	22.3±2.6
LV Pressure Development (mmHg)	109±8	118±14	110±3	122±5

Heart weight, heart:body weight ratios, and baseline coronary flow and left ventricular (LV) pressure development in perfused hearts (paced at 420 bpm) from mice consuming a control (C) or WD ± 3 wks chronic restraint stress (R). Means±SEM ($n=8$ /group). *, $P<0.05$ vs. C.

FIGURE LEGENDS

Fig.1. Body weights and circulating metabolic factors in mice consuming a control (C) or WD $\pm$ 3 wks mild restraint stress (R). Data are shown for: **A)** final body weight, and **B)** weight change over the final 3 weeks (period of restraint stress); **C)** non-fasted blood glucose; **D)** fasted blood glucose; **E)** fasted insulin; **F)** triglycerides; **G)** total cholesterol; and **H)** leptin. Bars represent the mean $\pm$ SEM ($n=7-8$ /group). *, $P<0.05$.

Fig. 2. Insulin sensitivity indicated by HOMA-IR (**A**), together with area under the curve for glucose tolerance tests (GTTs) (**B**), and blood glucose levels at 180 min post glucose challenge (**C**) in mice consuming a control (C) or WD $\pm$ 3 wks mild restraint stress (R). Means $\pm$ SEM ($n=8$ /group); *, $P<0.05$.

Fig. 3. Behavioral outcomes in mice consuming a control (C) or WD $\pm$ 3 wks mild restraint stress (R). Data shown for: **A)** line crossings (locomotor activity); **B)** center square entries (exploratory behavior); and **C)** duration in the center square in open field tests; and **D)** water and sucrose intake. Means $\pm$ SEM ($n=8$ /group); *, $P<0.05$.

Fig. 4. Hippocampal pro-inflammatory cytokines in mice consuming a control (C) or WD $\pm$ 3 wks mild restraint stress (R). Data shown for: **A)** TNF- α ; and **B)** IL-1 β . Means $\pm$ SEM ($n=5-8$ /group). No significant differences in levels were detected.

883

884 **Fig. 5.** Responses to I/R in perfused hearts from mice consuming a control (C) or
885 WD $\pm$ 3 wks mild restraint stress (R). Data are shown for: **A)** total post-ischemic LDH
886 efflux (marker of oncotic death); **B)** cardiac nucleosome content (marker of
887 apoptosis); **C)** post-ischemic recovery of left ventricular (LV) pressure development;
888 and **D)** post-ischemic recovery of left ventricular (LV) end-diastolic pressure.
889 Means $\pm$ SEM ($n=8$ /group); *, $P<0.05$.

890

891 **Fig. 6.** Expression of proteins influencing apoptosis in post-ischemic myocardium
892 from mice consuming a control (C) or WD $\pm$ 3 wks mild restraint stress (R). Data are
893 shown for TNF- α , BCL-2, BAX and cleaved caspase-3. Representative protein blots
894 are also shown. Means $\pm$ SEM ($n=8$ /group). No statistically significant expression
895 differences were detected between groups ($P>0.05$ for all comparisons).

896

897 **Fig. 7.** Expression of proteins influencing necroptosis and apoptosis in post-
898 ischemic myocardium from mice consuming a control (C) or WD $\pm$ 3 wks mild
899 restraint stress (R). Data are shown for cleaved PARP, RIP-1 and BAK.
900 Representative protein blots are also shown. Means $\pm$ SEM ($n=8$ /group). No
901 statistically significant expression differences were detected between groups ($P>0.05$
902 for all comparisons).

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