Chronic stress alters serum lipids:
effects due to "stress eating" versus metabolic changes

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Abstract

Chronic stress alters serum lipids:
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Background: Much research indicates that psychological stress can alter serum lipids, contributing to cardiovascular disease. However, this theory remains controversial. It is also unknown how this effect occurs: whether due to stress eating versus metabolic changes. This knowledge deficit impairs the understanding and clinical treatment of the increasingly ubiquitous patient experiencing stress and dyslipidemia.

Methods: We applied chronic stress to 24 LDL-receptor knockout mice for six weeks, and observed corticosterone, serum triglycerides, total and fractional cholesterol, and body weight. We used fed and fasted conditions to observe serum lipids with and without contribution from diet, as well when a highly-palatable food was available.

Results: Stressed mice consumed more highly palatable food, and gained significantly more body weight ($p=0.03617$) than did the unstressed mice. In agreement, both total cholesterol and triglycerides were significantly higher after providing sugar water, than prior to providing it ($p=0.0171$ and $p=0.0379$). We found several significant elevations in stressed mice's lipids, but these are difficult to interpret without observing larger trends, and given the habituation to the stressor that occurred.

Conclusion: These findings support our hypothesis that stress can raise serum lipids by promoting stress eating of highly-palatable food. This can promote dyslipidemia by causing weight gain and raising serum lipids, as occurred in our study. More research is needed regarding stress' ability to affect lipids in ways that are unrelated to diet.

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1. Introduction

Stress is referred to as America's number-one health problem (1). It is also an independent risk factor for the leading cause of death, cardiovascular disease (CVD)(2).

Clinicians first suspected a stress-CVD association in the 1950s, when confronted with patients whose heart diseases could not be explained by the classic non-lipid CVD risk factors (1, 3-5). This led to research implicating a "Type A coronary prone" personality, including traits such as hurriedness, anger, and hostility. Studies found that Type A men were nearly twice as likely to have heart disease than men who were not Type A (1, 6). Today, leading organizations such as the American Heart Association recognize stress as a contributing factor to heart disease risk (7).

Stress likely progresses CVD through many mechanisms: It can raise blood pressure, heart rate, inflammatory markers, and other mechanisms (8, 9). This review focuses on arguably the most controversial mechanism: Stress' potential to progress CVD by altering serum lipids. Some research indicates that psychological stress can alter lipids (10), whereas other research does not (11).

2. Literature Review

Observational Studies

Observational research on stress and lipids began in the 1950's (10). It was pioneered mainly by clinicians, who sought to explain a "hurry sickness" that they observed among their patients, whose heart diseases could not be explained by the classic non-lipid cardiovascular risk factors.

They began to study the effects of naturally-occurring stresses in subjects' lives. They found higher lipids among workers during periods of occupational stress (12-19), and among students during exams (20-27). See example in **Figure 1**. Experiencing stressful life events even related to higher total/HDL cholesterol ratios years after the events occurred (28, 29).

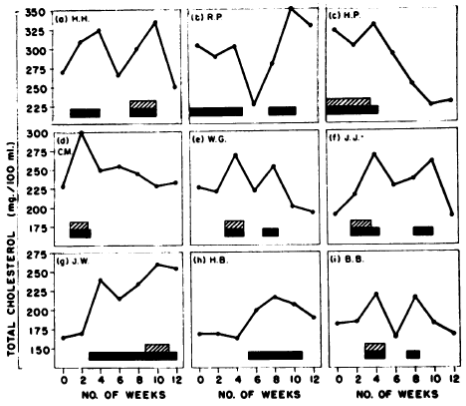


FIG. 3. Average serum cholesterol during experimental interval.

Figure 1: Cholesterol levels increase around periods of work stress (black bars) and emotional stress (shaded bars) in forty accountants (16)

Other observational studies assessed stress in cross-sectional manners, using questionnaires, psychological indices, and/or interviews. They measured stress in widely varying ways: Some gathered total stress exposure, through objective means such as workload or experiencing stressful life events; some collected subjective experiences of stress, or measured traits that may reflect stress, such as anxiety and Type A behaviors. Stress traits related to more risk-associated lipid profiles among over 3,800 subjects in the Framingham Offspring Study (30), over 72,000 women in the Nurses' Health Study (31), and other cohorts (32-40).

However, some observational studies did not find significant correlations between stress traits and lipids. Negative results were found among large cohorts of over 6,000 subjects (41-43), in other smaller cohorts (44-50), and the relationship was even inverse in other research (51-53).

Research on occupational stress and lipids has produced especially controversial findings. Measures of job strain related to more atherogenic lipid profiles, and the inverse to more protective lipid profiles, in many studies (54-65). However, several studies produced negative results (66-76), or found inverse associations (77, 78).

Of note, observational studies cannot shed much light on *how* stress affects lipids. It is impossible to determine if the lipid results of these studies are due to stress-induced metabolic changes, versus stress eating. This is because observational studies do not control, nor closely monitor, subjects' diets and/or biomarkers of stress. Free-living humans may also respond to stress by altering other lipid-affecting behaviors, such as smoking, alcohol consumption, and/or physical activity.

The inability of this type of research to discern *how* stress affects lipids, highlights the great need for a lab trial which *can* closely control and monitor subjects' food intakes and stress levels. Therefore, we conducted a lab trial with exactly these advantages, which is presented in the Primary Study section.

Experimental Trials

a. Human trials

Humans experience higher total or fractional cholesterols around laboratory stress sessions, compared to rest periods or control groups (79-88). Stress hormones even correlate directly with serum lipids, in some research (85, 89). Many biological mechanisms could explain this positive relationship between stress hormones and lipids, as discussed below in Mechanisms. Individuals deemed high stress reactors (based on stress hormone levels) even had higher cholesterol at three-year follow-up (90), as well as greater central adiposity (91).

However, some trials found that stress did not significantly alter lipids, particularly after adjusting for the reduction in plasma volume that can occur with stress (79, 92-94). This "hemoconcentration" concentrates plasma solutes such as lipids, causing them to falsely appear elevated. Still, other trials did adjust for changes in plasma volume, and at least one of the lipid results remained significant (80, 81, 86, 88, 95-97).

Several trials with positive results also fed subjects identical meals, or had them fast, suggesting that stress eating doesn't account for the entirety of stress' ability to affect lipids (80, 82, 84-88). For example, one study fed human subjects an identical meal, and observed higher postprandial lipids during stress sessions versus control sessions (see **Figure 2**)(98).

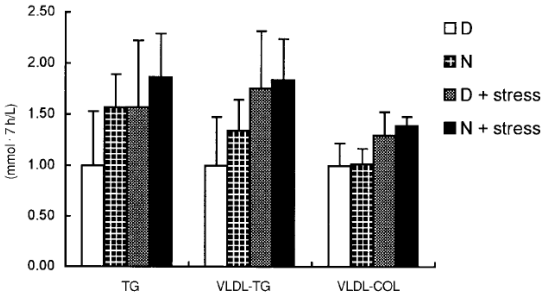


Figure 2: Postprandial lipid levels are higher during stress sessions than control sessions, at both day (D) and night (N)(98)

By fasting their subjects, these studies were able to remove the confounding effect of postprandial lipids. However, a trial is needed that applies chronic stress to both fasted and fed animals. This study could observe stress' effects on lipids with and without the effects of stress eating. Properly studying stress eating would also require providing a highly-palatable food, and tracking its consumption along with subjects' body weight.

Only a controlled laboratory trial that includes these elements would be able to observe stress' effects on lipids including and excluding the effects of stress eating. Such a study is required to identify precisely *how* stress affects lipids. We responded to this great need for research by conducting exactly such a study, as presented in the Primary Study section.

b. Primate trials

Much research on primate stress came from the Wake Forest University Primate Center, which induced stress by rearranging the animals' social groups. These studies found no significant effects on serum lipids, although the stressed primates developed significantly more cholesterol plaque lesions in their arteries (99-101). See example in **Figure 3** (100).

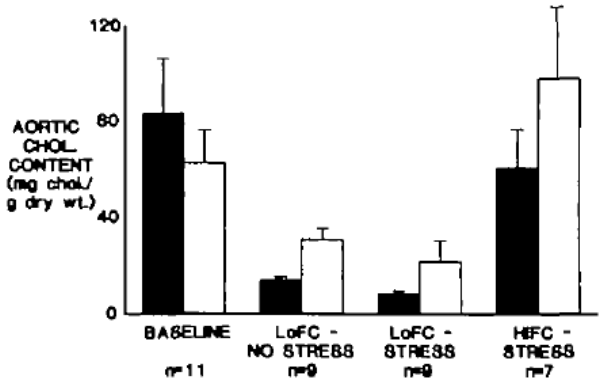


Figure 3: No significant difference in serum cholesterol between stressed and non-stressed primates fed a low-lipid (LoFC) diet. However, the stressed group developed significantly more atherosclerotic plaques (100)

While these may be negative results with regard to the stress-cholesterol association, they are positive results for the stress-CVD association. These findings support that stress likely progresses CVD through multiple mechanisms including, but not limited to, raising lipids, and that stress can progress CVD even when lipid-raising does not occur (99-101).

Alternatively, one study classified primates as high and low stress reactors, based on heart rate reaction to induced fear of capture (102). It found that high reactors had higher total cholesterol than low reactors, as well as greater intima media thickness. However, an observational study of wild baboons living in their natural habitat found that subordinate males (assumed to be more stressed) did not have significantly different total cholesterol or LDL (but had significantly lower HDL) than dominant animals (103).

These primate trials have come the closest to being able to determine exactly *how* stress affects lipids, because they included diet manipulations. However, they lack the element of fasting, which is essential to observing stress' effects on lipid metabolism that are unrelated to diet. Therefore, the design of our primary study is very similar to that of these primate trials, but with the added element of fasting.

c. Rodent trials

Rodent trials have also observed higher total or fractional cholesterols among stressed animals (104-111). Others found no significant association between stress and cholesterol (112-

115), or inverse associations (116-120). Some trials added the dimension of controllability or avoidability of the stressors: Unpredictable and uncontrollable foot shocks resulted in the greatest increases in lipids, versus shocks that could be predicted or controlled by signals or lever-pushing (114).

However, these trials similarly left much need for further research as to *how* stress affects lipids. The majority of rodent studies did not include diet or fasting manipulations, so were unable to determine if the observed effects were due to stress-induced metabolic changes versus stress eating. Therefore, we filled this need by conducting a rodent stress study that did include these elements, as presented in the Primary Study section.

3. Mechanisms by which Stress may Promote Dyslipidemia

a. Increased appetite

Stress hormones affect the hypothalamic-pituitary-adrenal (HPA) axis in a way that promotes stress eating—often of rich foods high in saturated fat and cholesterol (2, 121). Resulting chylomicrons circulate, their lipids become stored in adipose tissue, and remnants are taken up by the liver and secreted as VLDL—which in turn can be converted to downstream cholesterol-rich particles (2).

Specifically, stress hormones directly heighten the reward of eating on brain reward centers (2). High cortisol also inhibits the production of its predecessor, corticotrophin-releasing hormone, a satiety factor (91). Similarly, insulin resistance reduces the satiating effect of insulin, as observed in the over-eating, obese rodents who lack hypothalamic insulin receptors (121).

Stress may also stimulate release of appetite-increasing NPY. Theories on this mechanism include that NPY is released from sympathetic nerves activated during stress (122), and/or that a feedback loop exists between corticotrophin-releasing hormone and NPY (121).

These mechanisms are observed in observational studies where subjects consume significantly more calories and fat during periods of stress (18). Even in a laboratory setting, self-reported chronic stress related to greater caloric consumption at a buffet, lower vegetable intake, stronger food cravings, and gerater body fat masses (123). In agreement, self-reported stress eating related directly to waist circumference in a large cohort, which then related to metabolic syndrome, which raises CVD risk (124)

Other examples are the high-cortisol state of Cushing's syndrome, often accompanied overeating and obesity, and the opposite phenotype that often occurs with the low-cortisol state of Addison's disease or after adrenalectomies (2). It is important to note, however, that both humans and rodents differ in whether they respond to stress by eating more or eating less (18, 37, 47).

Of note, subjects generally stress eat highly-palatable foods more so than they do healthy, nutritious foods (122). Highly-palatable foods are usually higher in fat, cholesterol, and/or sugar, which greatly impact serum lipids. Specifically, saturated fat and cholesterol mainly contribute to the cholesterol portion of lipoproteins, whereas sugar mainly raises triglycerides (2). This latter

mechanism justifies the use of sugar water as a highly palatable food in our primary study presented below.

This strong evidence behind stress eating contributed to a hypothesis of our primary study: that stress can affect lipids by causing changes in dietary intake. This evidence also supports our inclusion of a highly-palatable food in addition to chow, and our measurement of its consumption along with mice's body weight. We also ensured to use a stress protocol that is similar to those that have successfully increased subjects' preference for sweet food, independent of hunger (112, 113, 119, 125-127).

b. Increased lipolysis

Stress hormones mobilize lipids into the circulation to fuel the "fight or flight" response (128)(9). Lipids can be oxidized for energy, and glycerol can be used for gluconeogenesis (2).

This mobilization of lipid fuels occurs in part because catecholamines increase expression of adipose triglyceride lipase and hormone sensitive lipase (2). Catecholamines also phosphorylate and activate hormone-sensitive lipase, and phosphorylate perilipin proteins, causing conformational changes that allow lipases to have contact with lipid droplets (2).

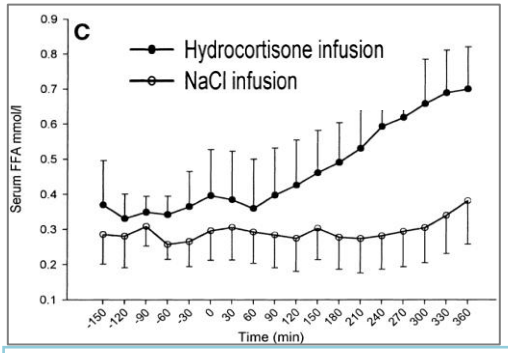


Figure 4: Serum free fatty acids increase with hydrocortisone (cortisol) infusion versus control NaCl infusion (129)

High cortisol also leads to increased serum free fatty acids (see **Figure 4**), glycerol, and whole-body lipolysis, which has been confirmed by indirect calorimetry (129). In addition, cortisol, catecholamines, and glucagon stimulate phosphatidate phosphohydrolase, leading to increased hepatic TG synthesis (130). Similarly, glucocorticoids and FFA increase activity of HMG-CoA reductase in the liver, stimulating synthesis of cholesterol (10).

Norepinephrine can also induce lipolysis by stimulating beta receptors in adipose tissue, and by decreasing insulin levels. In addition, norepinephrine decreases hepatic lipase activity, which could increase plasma levels of VLDL, IDL, and LDL (10).

In summary, liver secretion of VLDL increases during stress, due to a high supply of fatty acids. These fatty acids are supplied either by diet, via the appetite-increasing effects described previously, or by the adipose tissue, via the lipolytic mechanisms just described (130). Triglyceride-rich VLDL particles are circulated and degraded to downstream cholesterol-rich lipoproteins.

This strong evidence behind stress' ability to alter lipid metabolism supported a hypothesis of our primary study: that stress eating doesn't account for the entirety of stress' ability to affect lipids. That is, stress can affect lipids even with the effects of postprandial lipids removed. The next several mechanisms are examples of evidence that supports this hypothesis. We also ensured to incorporate fasting into our study design, in order to observe stress-induced changes in lipids without contribution from diet.

c. Insulin resistance

Cortisol, norepineprhine, and fatty acids can reduce insulin sensitivity (10, 129, 130). Cortisol specifically reduces translocation of glucose transporters (especially GLUT4) to cell membranes, and causes inefficiencies in post- insulin receptor signal transduction (131). This is advantageous during stress because it favors glucose supply to the CNS, for heightened awareness and decision making.

Insulin resistance causes less activity of lipoprotein lipase (LPL), because insulin stimulates LPL. Reduced LPL activity causes less uptake of lipoproteins into peripheral tissues, retaining lipoproteins in the blood (2, 131). For example, Cushing's syndrome patients even with normal body weight and normal rates of free fatty acid turnover still experience hyperlipidemia, attributed to their high cortisol-induced insulin resistance (132).

d. Promoting central adiposity

Despite stimulating whole-body lipolysis, glucocorticoids specifically inhibit abdominal lipolysis (129). For example, this phenomenon is observed in Cushing's syndrome, where hypercortisolemia results in distinct abdominal obesity (132). Other research shows that individuals with high cortisol reactivity have greater central adiposity (91)(15). This occurs because glucocorticoid receptors are four times more abundant in central fat than in subcutaneous fat (14, 15), and intra-abdominal has more of the enzyme that converts cortisone into cortisol (91, 122, 133).

Resulting visceral adiposity only worsens stress-induced hyperlipidemia. Highly-innervated central fat increases lipid turnover, insulin resistance, and cholesterol biosynthesis in the whole body (2). To capture this mechanism in our primary study, we ensured to track body weights.

e. Reduced LDL receptor activity

Hepatic LDL receptor activity is increased by insulin and decreased by cortisol (see **Figure 5**)(129, 134). Therefore, LDL-receptor activity is reduced in the insulin-resistant, high-cortisol state of stress. This results in less clearance of lipoproteins from the circulation, retaining more in the blood (2). For example, hyperlipidemia often accompanies the hypercortisolemia of Cushing's syndrome or glucocorticoid medication use (132).

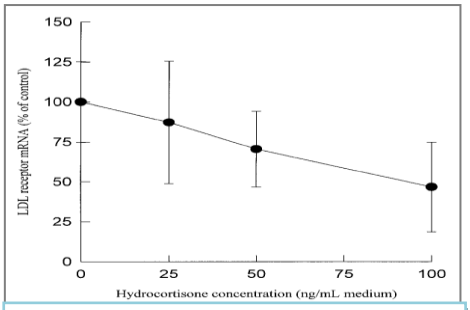


Figure 5: LDL receptor expression decreases with increasing cortisol concentration in cultured hepatic cells (134)

f. Chronic low-grade stress

The physiologic stress response was designed for acute situations (135). It mobilizes energy, quickens pulse, and raises blood pressure—delivering fuel for decision-making and

movement needed to avoid an acute danger (135). However, today's common stressors are not predators--but rather persistent states of hectic modern lives. Unfortunately, a concept of non-specificity in stress theory states that human physiology responds the same way to chronic stress as it does to an acute predator (128, 135). Therefore, the mechanisms of chronic stress persist, unresolved, at low-grade levels, with pathogenic impacts on lipid metabolism (2).

This long-term exposure to chronic stress creates a "wear and tear" on the body called an allostatic load (136). In stress disorders (anxiety, PTSD, hostile/aggressive states, and others), this allostatic load is associated with CVD, as well as chemical imbalances, disturbances in diurnal rhythms, and atrophy of brain structures (136). We designed our primary study to include such chronic, low-grade stress, related to this pathology.

4. Primary Study

Background and Significance

The body of research on stress and lipids has yielded very controversial results, as is evident in the preceding literature review. It remains unclear how much stress raises risk for dyslipidemia, if at all. Therefore, we followed this review with a primary study, to confirm that psychological stress correlates with increased cholesterol levels.

Furthermore, this literature review revealed a striking knowledge deficit: it is unknown how exactly stress may affect lipids. Stress can affect lipids through two main pathways. First, it can promote stress eating, of "healthy" and/or highly-palatable foods, thereby raising postprandial lipids. This intake may also cause weight gain, and related increased lipid turnover and insulin resistance, as discussed in Mechanisms. Second, stress can cause changes in lipid metabolism and insulin sensitivity, and these effects can occur in both the fasted and fed states.

This knowledge deficit remains because no study has yet been able to observe exactly *how* stress affects lipids. Observational studies gather an indiscernible mix of stress' effects on lipid metabolism and eating behavior, and experimental trials have thus far only included elements of fasting, but not feeding.

This lack of knowledge inhibits the care and understanding of the growing number of patients experiencing stress and dyslipidemia. For example, it is unknown if prevention and treatment should focus on stress' effect on eating behaviors, or on stress-induced metabolic changes, because it is unknown how much each route contributes to stress-lipid pathology.

Therefore, we followed this literature review with a primary study designed to fill this knowledge deficit. We have included elements in its design that will uniquely enable us to observe stress' effects on lipids with and without the effects of stress eating. Applying chronic stress to fed mice enables us to observe stress' total impact on lipids. Doing so in the fasted state allows us to observe stress' effect on lipids with the confounding effect of stress eating removed. Thus we will be able to identify the portion of stress-induced lipid changes that are attributable stress eating versus metabolic changes that are unrelated to diet.

Specific Aims and Hypotheses

Hypotheses:

1. Stress *increases* serum total cholesterol
2. Stress increases cholesterol by both promoting stress eating, thus raising postprandial lipids, and through mechanisms that are unrelated to diet, such as non-prandial metabolic changes

Aim 1. To identify the correlation between psychological stress and serum lipids, by measuring the changes in cholesterol and triglycerides in response to stress

Aim 2. Identify the portion of stress-induced lipid changes that are attributable to stress eating, and accompanying metabolic and weight changes

A. use fed conditions to observe the effects of stress on lipids

B. elucidate stress eating's effects on lipids under two conditions: ad libitum access to chow, and ad libitum access to chow and a highly-palatable food

C. observe the effects on fractional cholesterols: VLDL, LDL, and HDL

Aim 3. Identify the portion of stress-induced lipid changes that are attributable to mechanisms not related to diet

A. use fasted conditions to remove the confounding effect of postprandial lipids

B. observe the effects on lipoprotein cholesterol levels: VLDL, LDL, and HDL

Methods

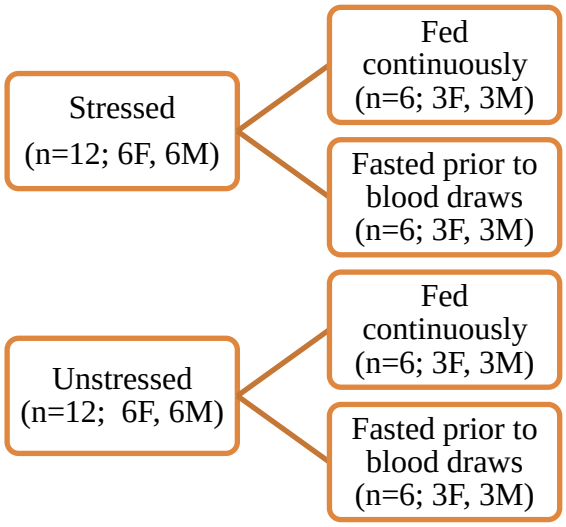
a. Animals

Experiments were carried out using 24 homozygous LDL-receptor knockout mice (Jackson Laboratory, Bar Harbor, ME, catalog #2207). Males and females (12M, 12F) were used, to observe effects on both genders. All mice were 14 weeks old at baseline, and weighed 22.6 ± 2.1g (males) and 17.4 ± 0.7g (females). They were acclimated to a Specific Pathogen Free facility, and then housed in conventional Allentown polycarbonate cages (50 cm × 18 cm × 13 cm). Conditions were quiet and controlled (temperature 25 ± 2 °C), with a 12-h light/dark cycle. Standard chow and water were available *ad libitum*, except for the diet manipulations described below. All procedures were approved by the University of Washington IACUC.

b. Treatment groups

Mice were randomly divided into stressed and non-stressed groups. Using a 2x2 factorial design, these groups were further divided into groups that were fasted prior to blood draws, versus not fasted prior to blood draws. See **Figure 6**.

Figure 6: Treatment Groups



c. Pilot study

The first two weeks of research were a pilot study, in which we tested a chronic stressor that has been used in few prior studies (137, 138): soiling stressed mice's cage bedding with the urine of competitive/aggressive mouse strains. For these initial two weeks, stressed mice's bedding was soiled with urine from male "black 6" mice (C57BL/6, Jackson Laboratory). To prevent habituation, we changed and re-soiled the bedding with different urine twice weekly.

We also performed a restraint stress test on a sub-set of mice (four mice) during the Pilot Study. This enabled us to gather a theoretical maximum corticosterone response, for comparison with the response from soiled bedding stress.

Stressed mice were also housed individually, a well-documented stressor in these innately social animals (139). Non-stressed mouse bedding was left untouched, and they were group-housed.

d. Chronic stress plan

The soiled bedding stressor tested in the Pilot Study failed to produce sufficient stress response (see Results). Therefore, we discontinued its use, and changed our stressor to the well-documented restraint stress for the remaining four weeks of research (140, 141). This stressor involved immobilization for 90 minutes 3x weekly, in well-ventilated, 50ml conical Falcon tubes (142, 143). Stress sessions occurred in a separate room, while non-stressed mice remained in

their home cages. Stressed mice remained singly housed, while non-stressed mice remained group-housed.

This chronic stress plan is similar to that used in prior research, which successfully resulted in increased corticosterone and preference for sweet food independent of hunger (112, 113, 119, 125-127). It is intentionally relatively mild: more frequent and longer stress protocols are models for depression and anhedonia--an outcome that we wanted to avoid (144). Similarly, we avoided chronic mild stress (CMS) protocols, as these have induced anhedonia and reduced sucrose preference and consumption (126, 145).

Habituation is a major concern in stress research. However, a study using a similar chronic restraint stress protocol found no decline in corticosterone response to tube restraint over 21 days of repeated tube restraint (146). Its chronically tube-restrained rats even showed increasing basal corticosterone prior to the restrain sessions, with repeated stress sessions.

e. Diet manipulations

Half of all mice were fasted for 10h prior to weekly blood draws, while the other half received ad-lib chow for the entire study. This duration of fasting is similar to the 12h duration used in prior research to gather fasting lipid panels in LDL-R-/- mice (147). Fasting clears dietary lipids (chylomicrons) from the serum, allowing us to observe stress' effect on lipids with the confounding effect of postprandial lipids removed. In contrast, fed mice's serum includes lipoproteins from both dietary intake, and from non-prandial metabolic changes that are unrelated to intake

Furthermore, sugar water (50% w/v) was provided continuously to all mice, for the final two weeks of study. All mice continued to receive ad-lib regular chow (when not fasted) and regular drinking water during this time. As discussed in Mechanisms: Increased appetite, sugar is a highly-palatable food that mainly raises serum triglycerides (2). This manipulation is important because stress eating doesn't only involve nutritious food, but rather highly-palatable foods with greater impacts on serum lipids (121). We measured sugar water consumption by recording the volumes in bottle feeders.

As stated in our Specific Aims and Hypotheses, our main purpose for providing sugar water was to observe how the intake of a highly-palatable food contributes to serum lipids. To a lesser extent its intake could also contribute to weight gain, and related increased insulin resistance and lipid turnover (121, 131).

As discussed previously, these elements of both feeding and fasting are key to how our study will fill the knowledge deficit of *how* stress affects lipids. They will uniquely enable us to observe stress' effects on lipids with and without the effects of stress eating.

f. Timeline

		Pilot study					
		Week 1	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**
Stressed n=12 6M, 6F	Fasted prior to blood draws n=6 3M, 3F	Singly housed Soiled bedding stress	Singly housed Soiled bedding stress	Singly housed Restraint stress Sugar water	Singly housed Restraint stress Sugar water	Singly housed Restraint stress	Singly housed Restraint stress
	Fed continuously n=6 3M, 3F	Singly housed Soiled bedding stress	Singly housed Soiled bedding stress	Singly housed Restraint stress Sugar water	Singly housed Restraint stress Sugar water	Singly housed Restraint stress Sugar water	Singly housed Restraint stress Sugar water
Non-stressed n=12 6M, 6F	Fasted prior to blood draws n=6 3M, 3F	Group housed	Group housed	Group housed Sugar water	Group housed Sugar water	Group housed Sugar water	Group housed Sugar water
	Fed continuously n=6 3M, 3F	Group housed	Group housed	Group housed Sugar water	Group housed Sugar water	Group housed Sugar water	Group housed Sugar water

g. Blood collection

Blood was drawn from all mice once weekly, using the sub-mandibular method without anesthesia (148). Blood was collected into tubes with heparin (BD Microtainers), and centrifuged for 10 minutes at 2.7RPM x 0.6G. Plasma was immediately aliquoted and frozen in fresh sample tubes until needed. An terminal bleed was performed during week six, at which time mice were sacrificed by anesthesia and euthanasia injection. Livers were harvested and preserved, for potential use in further research.

h. Assays

Serum was tested with the following commercially-purchased assay kits, according to their protocols: total cholesterol (Cell Biolabs Total Cholesterol Colorimetric Assay Kit, STA-384); corticosterone (Enzo Life Sciences Competitive Corticosterone Assay Kit, ADI-900-097); and triglycerides (Cayman Chemical Serum Triglyceride Determination Kit, 10010303). FPLC was also used to fractionate the samples, and cholesterol and triglycerides were then measured in each of the fractions.

All assay samples were tested in duplicate. See Appendix X for the standard curves obtained from each assay; R^2 values ranged from 0.9817 - 0.999. Note that when corticosterone duplicates were $\geq$ 3000 pg/ml discrepant from each other, we assumed these to be in error and eliminated them from analysis (applied to 11 out of 70 total observations).

i. Statistical analysis

The University of Washington Statistics Department provided extensive guidance with our statistical analysis. Data was analyzed using the STATA software package. We compared continuous variables by using linear regression, and analyzed categorical variables by two-way ANOVA. We also used paired t-tests to determine significance of differences between group means. Results were determined significant at the level of $p<0.05$. Note that all analyses presented in Results compare equal sample sizes, except when noted otherwise.

Results

a. Pilot study

The soiled bedding stressor that we tested during the pilot study did not produce a significant stress response (see **Table 1**).

Table 1: Soiled bedding stressor did not produce a significant corticosterone response

	Week 1 Corticosterone (pg/ml)	Week 2 Corticosterone (pg/ml)
Stressed by soiled bedding	Mean: 1799.06	Mean: 1139.91
	SD: 837.04	SD: 931.58
Unstressed	Mean: 1907.14	Mean: 960.08
	SD: 1029.17	SD: 1220.95
t-test for difference between groups	p=0.1164	p=0.6974

However, we also tested the restraint stressor during the pilot study, on a sub-set of four mice. This stressor did produce a corticosterone response, which was significantly greater than the response to soiled bedding, and to no stress (see **Table 2**). Therefore, we used restraint as the stressor for the remainder of research.

Table 2: Restraint stress test did produce a corticosterone response, significantly greater than the responses to soil bedding and no stress

	Week 1 Corticosterone (pg/ml)
Stressed by restraint stress test	Mean: 9209.81
	SD: 2476.85
Stressed by soiled bedding	Mean: 1799.06
	SD: 837.04
Unstressed	Mean: 1907.14
	SD: 1029.17
t-test for difference between stress from restraint and stress from soiled bedding	p=0.00001
t-test for difference between stress from restraint and no stress	p=0.00002

b. Corticosterone

Table 3 shows corticosterone results at weeks one and six. Unfortunately, we suspect that the week six corticosterone results were greatly affected by the increased handling of the animals at week six. Mice were handled more extensively at this time because the terminal bleed was performed, requiring mice to be transported between facilities, relocated into unfamiliar paper crates, and wait for over an hour in a foreign laboratory.

This was likely very stressful for the unstressed mice especially, who had very little prior handling. This potentially explains the unstressed mice's week six corticosterone, which is paradoxically elevated from their week one corticosterone. There is little other explanation for this other than the stressful handling at week six; the unstressed mice were left undisturbed in their home cages for the entire study, except for the weekly bleeds.

It is difficult to determine if habituation occurred due to the inaccuracy in our week six corticosterone data. However, our stress protocol is similar to that which has previously successfully avoided habituation (146). Immobilization also caused a significant stress response in our pilot study, and that the efficacy of this method is well-documented (140, 141).

Table 3: Corticosterone levels at weeks one and six; suspect error due to stressful animal handling in week six

	Week 1 Corticosterone (pg/ml)	Week 6 Corticosterone (pg/ml)
Stressed	Mean: 1799.1 *stressed by soiled bedding in week 1	Mean: 5987.145
	SD: 837.0	SD: 3435.11
Unstressed	Mean: 1907.1	Mean: 6513.362
	SD: 1029.2	SD: 2035.513
t-test for difference between groups		p=0.7887

Note that the above analysis compares unequal sample sizes (7 stressed vs. 4 unstressed observations), due to removing corticosterone results that were obviously in error, as described in Methods: Assays.

Repeating this analysis using *all* corticosterone results, without removing any data due to suspected error, still shows an insignificant difference in corticosterone at week six (see **Table 4**). The error in this assay's results are apparent in **Table 4**'s large standard deviations. Error is also apparent in the unstressed mice's corticosterone, which appears enormously, unreasonably elevated over that of the stressed mice, even considering the extra stress of first-time handling that the unstressed mice experienced.

Table 4: Corticosterone levels at week six, without removing any values due to suspected error

	Week 6 Corticosterone (pg/ml)
Stressed	Mean: 9616.08
	SD: 6851.68
Unstressed	Mean: 31926.37
	SD: 53540.62
t-test for difference between groups	p=0.1857

c. Total cholesterol and triglycerides

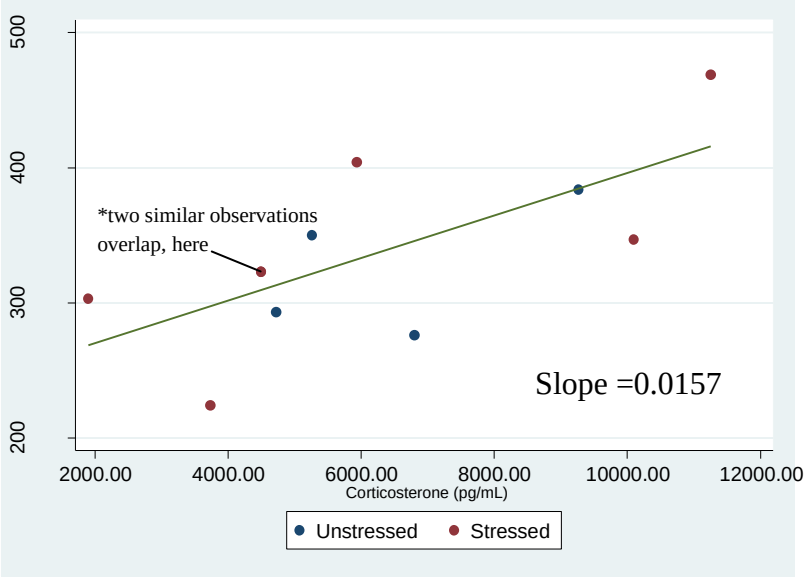
Neither total cholesterol nor triglyceride levels were significantly different, based on ANOVA analysis of samples that were pooled by gender and treatment group (see **Table 5**). Note that the Week 3 Triglycerides analysis (only) compares unequal sample sizes (9 stressed vs. 10 unstressed observations), due to inability to collect samples from some mice.

Table 5: No significant differences in fasting lipids, by week and treatment

		Week 3 Total Cholesterol	Week 3 Triglycerides	Week 4 Total Cholesterol	Week 4 Triglycerides	Week 5 Total Cholesterol	Week 5 Triglycerides	Week 6 Total Cholesterol	Week 6 Triglycerides
Stressed	Mean	219.5	63.8	165.0	61.1	284.5	81.0	275.5	71.6
	SD	43.4	10.5	39.3	8.3	43.3	37.4	36.1	22.0
Unstressed	Mean	177.0	77.5	188.5	57.6	236.5	83.8	243.5	67.9
	SD	9.2	17.1	2.9	21.6	14.4	17.2	36.1	25.7
ANOVA for differences between groups		p=0.1033	p=0.0548	p=0.2776	p=0.6435	p=0.0801	p=0.8332	p=0.4685	p=0.7349

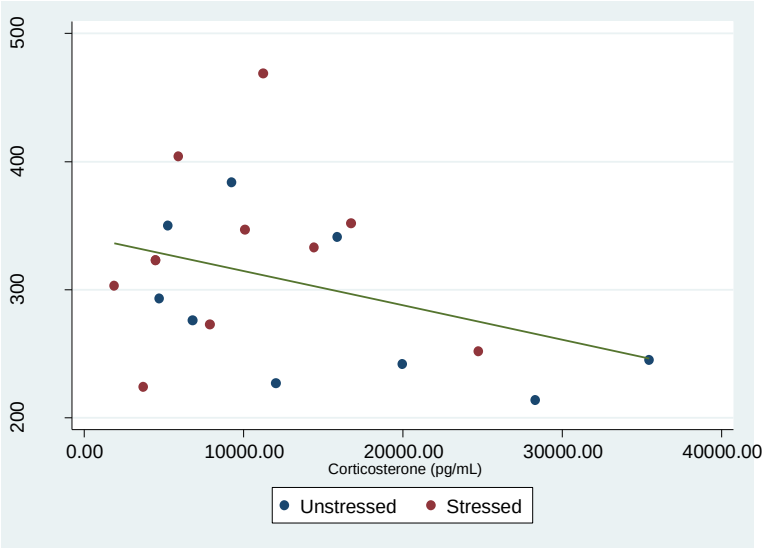
However, regression analysis of corticosterone versus individual mice's total cholesterol measures at week six showed a significant correlation (p=0.020), with a positive R^2 of 0.4683 (see **Figure 7**). Note that this analysis compares unequal sample sizes (7 stressed vs. 4 unstressed observations), due to removing corticosterone results that were obviously in error, as described in Methods: Assays.

Figure 7: Significant direct correlation (p=0.020) between total cholesterol and corticosterone at week six



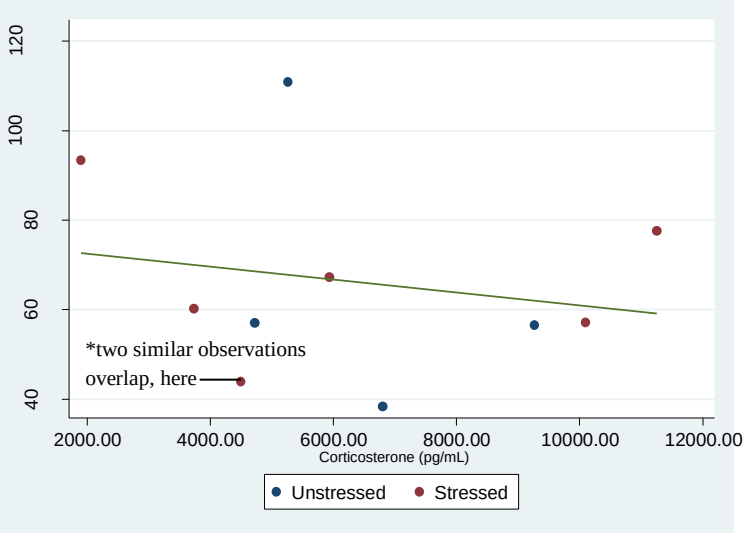
Note that this analysis compares unequal sample sizes (7 stressed vs. 4 unstressed observations), due to removing corticosterone results that were obviously in error, as described in Methods: Assays. Repeating this analysis using *all* corticosterone results, without removing any data due to suspected error, renders this correlation insignificant (p=0.114) (see **Figure 8**).

Figure 8: Comparing total cholesterol with *all* corticosterone values, without removing any values due to suspected error, renders the correlation insignificant (p=0.114)



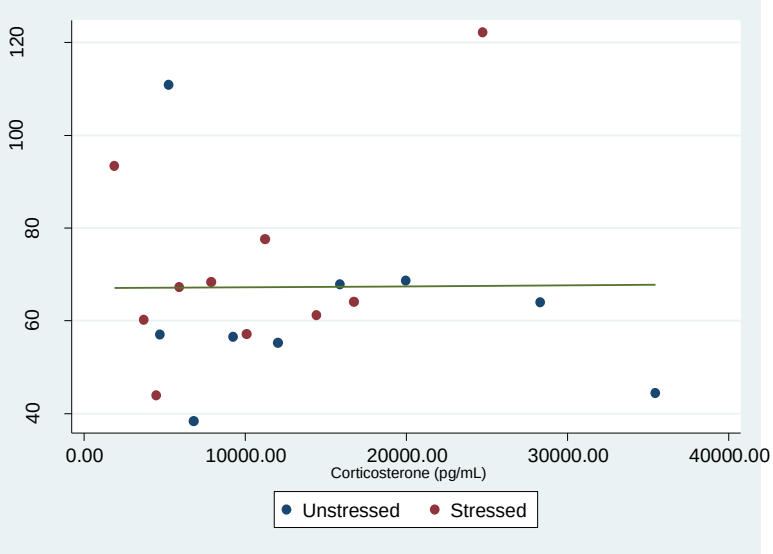
Regression analysis of corticosterone versus individual mice's triglycerides at week six showed remained insignificant (see **Figure 9**).

Figure 9: Insignificant (p=0.589) correlation between triglycerides and corticosterone at week six



Repeating this analysis using *all* corticosterone results, without removing any data due to suspected error, still shows an insignificant correlation (p=0.971) (see **Figure 10**).

Figure 10: Comparing triglycerides with *all* corticosterone values, without removing any values due to suspected error, still shows an insignificant correlation (p=0.971)



d. Fractional cholesterol and triglycerides

Please see **Figures 11-17** for box plots of fractional lipids by gender and treatment group, also visually depicted in FPLC output.

We ran t-tests at the 95% confidence level to analyze differences in lipid content of specific lipoprotein fractions, between groups. Results are summarized here:

Table 6: Differences in lipid content of lipoprotein fractions, significant results highlighted

Cholesterol in VLDL	Unstressed males	Vs	Stressed males	p=0.8011
	Unstressed females	Vs	Stressed females	p=0.1310
Cholesterol in LDL	Unstressed males	Vs	Stressed males	p=0.1785
	Unstressed females	Vs	Stressed females	p=0.0168*
Cholesterol in HDL	Unstressed males	Vs	Stressed males	p=0.1141
	Unstressed females	Vs	Stressed females	p=0.1253
Triglycerides in VLDL	Unstressed males	Vs	Stressed males	p=0.4259
	Unstressed females	Vs	Stressed females	p=0.5690
Triglycerides in LDL	Unstressed males	Vs	Stressed males	p=0.0003*
	Unstressed females	Vs	Stressed females	p=0.3984
*=p<0.05				

The only significant differences involve the LDL fraction: Stressed females had significantly more cholesterol in their LDL than did unstressed females, and stressed males had significantly more triglyceride in their LDL than did unstressed males.

Figure 11: Cholesterol content of VLDL fraction: no significant results

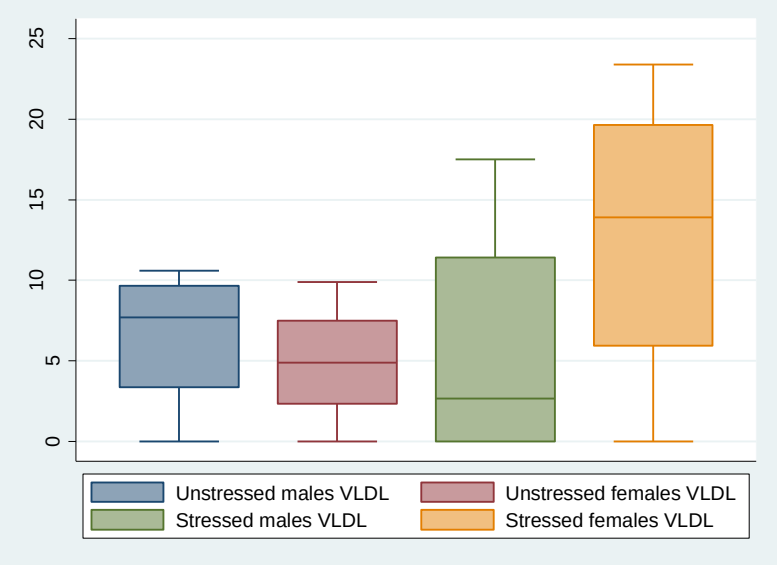


Figure 12: Cholesterol content of LDL fraction: stressed females significantly greater

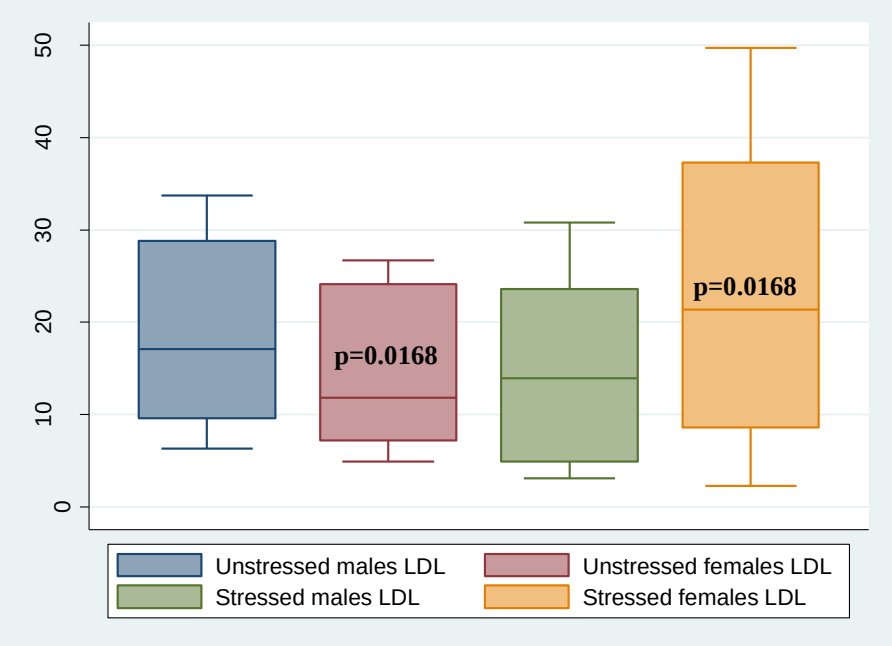


Figure 13: Cholesterol content of HDL fraction: no significant results

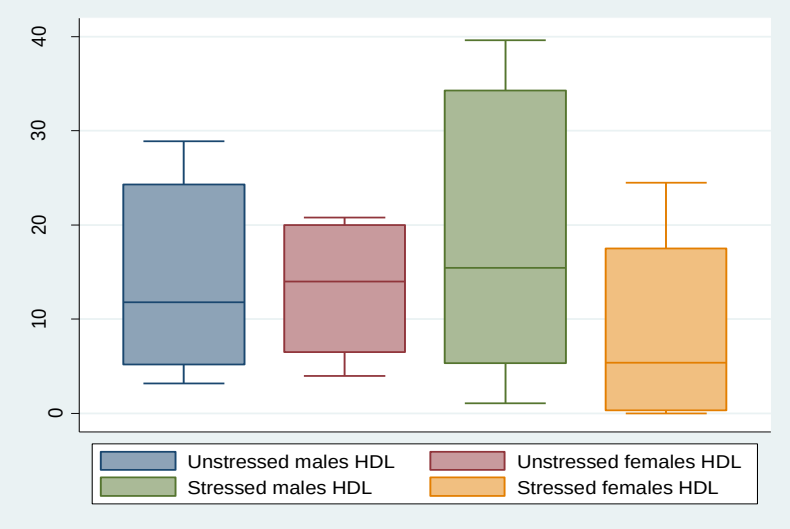


Figure 14: FPLC of cholesterol fractions, by gender

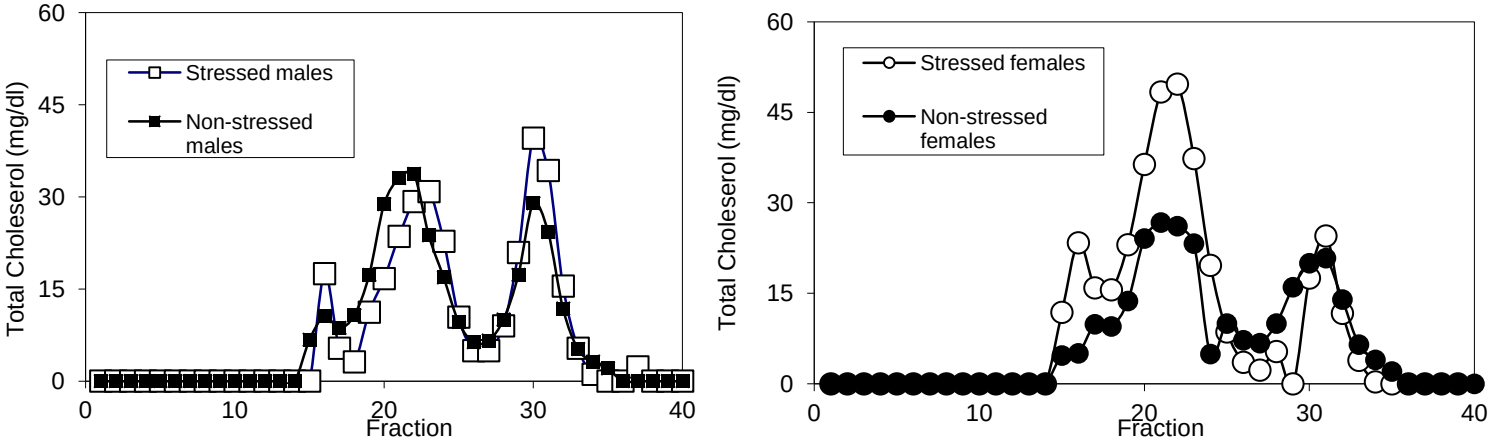


Figure 15: Triglyceride content of VLDL fraction: no significant results

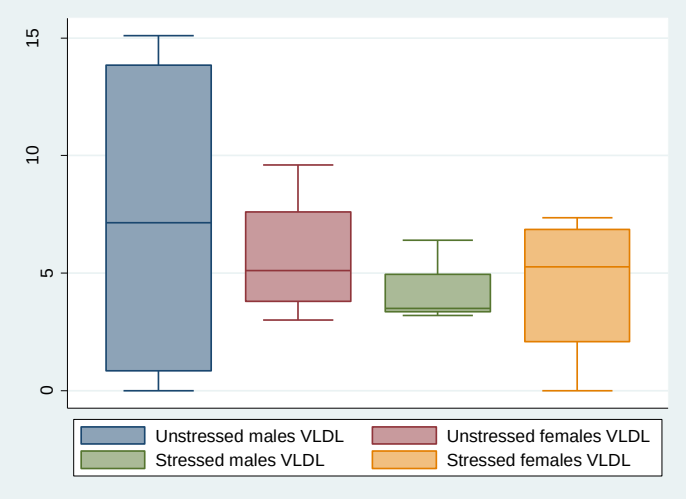


Figure 16: Triglyceride content of LDL fraction: stressed males significantly greater

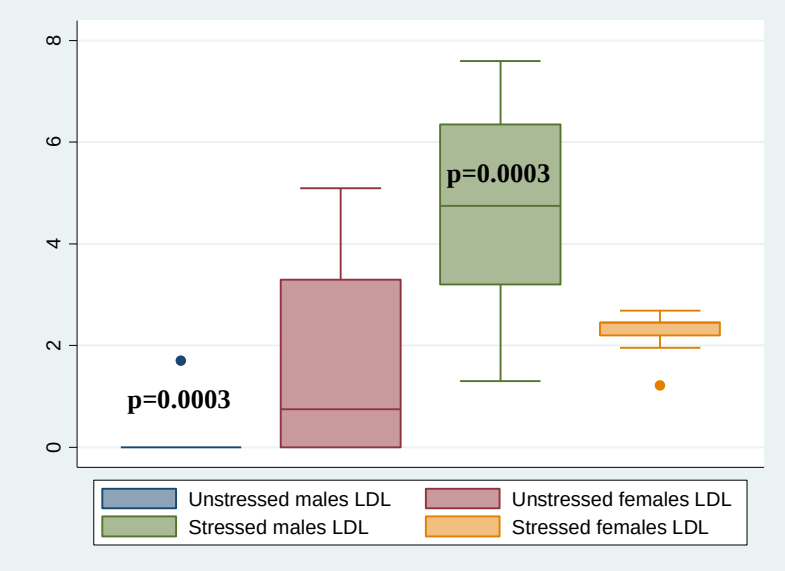
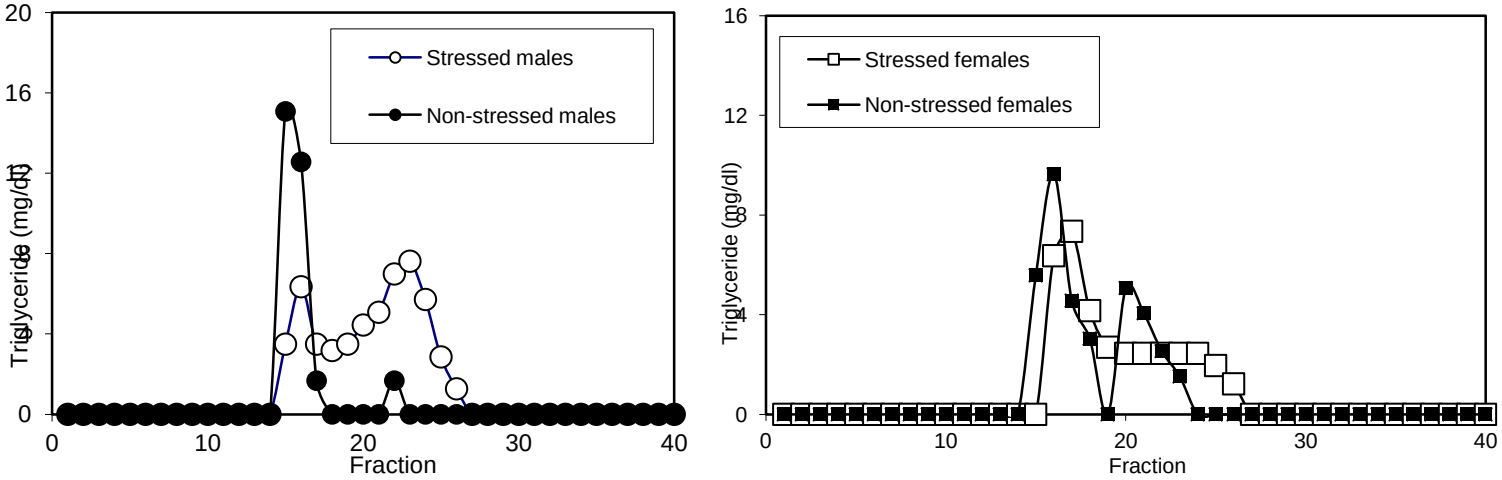


Figure 17: FPLC output for triglyceride fractions, by gender



e. Sugar water consumption

Sugar water consumption is shown in **Figure 18**. Stressed mice consumed 42.4 ± 15.0 ml sugar water over the two weeks of providing it, and unstressed mice consumed 40.0 ml ± 13.9 ml. Regression analysis showed that week six total cholesterol and triglycerides did not significantly correlate with sugar water consumption (p=0.301 and p=0.105, respectively). Nor did sugar water consumption relate to the stress vs. unstressed manipulation (p=0.7027). Note

that the triglycerides before providing sugar water analysis (only) compares unequal sample sizes (9 stressed vs. 10 unstressed observations), due to inability to collect samples from some mice.

However, we suspect that our measurements of sugar water consumption were low in accuracy (see Limitations). A more reliable indicator of intake, t-tests showed that both total cholesterol and triglycerides after providing sugar water were significantly greater than total cholesterol and triglycerides prior to providing sugar water for two weeks (see **Table 7**).

Figure 18: Sugar water consumption

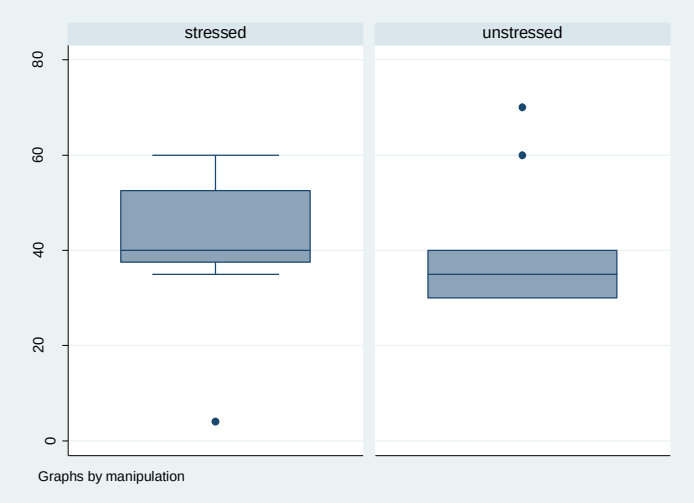


Table 7: Triglycerides and total cholesterol after providing sugar water were significantly greater than prior to providing sugar water

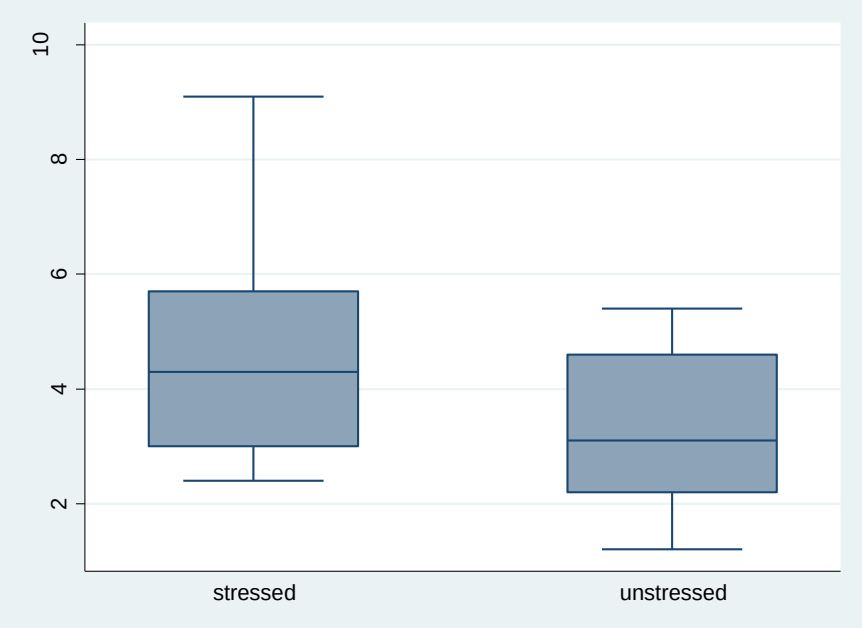
Triglycerides before providing sugar water	Mean: 130.46 mg/dl
	SD: 23.6 mg/dl
Triglycerides after providing sugar water	Mean: 152.10 mg/dl
	SD: 43.3 mg/dl
t-test for difference between before and after sugar water	p=0.0379
Total cholesterol before providing sugar water	Mean: 375.0 mg/dl
	SD: 11.3 mg/dl
Total cholesterol after providing sugar water	Mean: 532.5 mg/dl
	SD: 80.6 mg/dl
t-test for difference between before and after sugar water	p=0.0171

f. Body weight

Stressed mice gained 4.8 ± 2.3 grams over the six weeks of research, and unstressed mice gained 3.2 ± 1.4 grams. This difference in body weight gain was statistically significant (p=0.03617). **Figure 19** shows that stressed mice had greater weight gain over time (slope of

curve = 1.57 grams gained per week, on average) than did the unstressed mice (slope of curve = 0.885 grams gained per week, on average).

Figure 19: Stressed mice gained significantly more weight (p=0.03617)



Conclusion

Most notably, the stressed mice in our study gained significantly more weight than did the unstressed mice. This is a reliable indicator that the stressed mice did consume more sugar water, although our sugar water measurements are likely of low accuracy. Similarly, both total cholesterol and triglycerides were significantly higher after providing sugar water, than prior to providing it.

These findings support our hypothesis that stress promotes appetite and stress eating of highly-palatable food, which is backed by a large body of evidence (2, 18, 91). Increased food intake, especially of highly-palatabe food, can raise serum lipids, as we observed in significantly greater lipid measures after providing sugar water. Resulting body weight gain can also promote dyslipidemia (2).

It would be imprudent to draw conclusions from our FPLC data at this time, without observing larger trends. It is important to note, however, that many prior studies have failed to detect changes in lipids, although stress caused concurrent changes that promote dyslipidemia or atherosclerosis. For example, most primate studies detected no changes in lipids, although stressed primates had significantly greater atherosclerotic lesions (99-101). More research is needed regarding stress' ability to affect lipids beyond promoting stress eating and related elevated postprandial lipids, and weight gain and its consequences.

Therefore, the most notable finding from the current research is confirmation of our hypothesis that stress can affect lipids by promoting stress eating. Stressed mice consumed more highly-palatable food than did unstressed mice, which resulted in significantly more body weight gain, and significantly greater triglycerides and total cholesterol after providing sugar water.

Limitations

First, the results of our corticosterone assay gave very large standard deviations - even greater than the means, in some cases. As described in Methods: Assays, we removed corticosterone results that were obviously in error. This alarmingly applied to 11 out of 70 total observations, and led to comparing unequal sample sizes during our analysis, in the instances noted in Results.

Several factors could account for the error in our corticosterone assay. Technical error could be a cause, given that this very complex competitive binding assay was performed by a technician with no prior experience. In fact, technical error is apparent in that many samples' duplicate results were very discrepant from each other. Still, other aspects could account for the large variability in individual stress responses: the duration of handling each animal (stressful) during the procedure, gender differences, differences in prior exposure to stress/handling, and other factors.

In addition, we were on occasion simply unable to obtain samples from mice: Three mice died prior to the end of the study due to colony-acquired dermatitis, potentially collecting too much blood, and unknown causes, or we were unable to collect samples via the submandiublar method. This also contributes to comparing unequal sample sizes during our analysis, in the instances noted in Results. (All other analyses in Results do compare equal sample sizes.)

It's also likely that our week six corticosterone measures are inaccurate. Mice were handled more extensively at this time because the terminal bleed was performed, requiring mice to be transported between facilities, relocated into unfamiliar paper crates, and wait for over an hour in a foreign laboratory. This likely contributed to the unstressed mice's week six corticosterone being significantly elevated from their week one corticosterone. The week six transport was the first time the unstressed mice were significantly handled, which could have caused significant stress.

An additional limitation is that our measurements of sugar water consumption are likely of little accuracy. We suspect that the bottle feeders leaked and spilled some of the solution, and that staff may have replaced the bottles, unaware of our plans for measurement. Therefore, please see body weight gain and the lipid measurements as more valid indicators of food consumption.

Related, another drawback is that mice's fasting lipids tend to increase as they gain weight. We did not have a way to ensure equal weight gain between the stressed and unstressed groups, which would be ideal for truly ruling out the effects of stress eating and related weight gain.

Finally, the soiled bedding stressor that we tested during the pilot study may have failed because the "black 6" strain may be too closely related to our LDL-R knockout mice. A stronger response may be observed with urine from more foreign mouse strains, or mouse predators, as has been done previously (137, 138).

Need for further research

The results from the current study add to the evidence that stress can affect lipids by promoting stress eating of highly-palatable food. More research is needed, however, on stress' ability to affect lipids in ways that are unrelated to diet.

It appears to be quite tricky to observe stress' effects on lipids: Many studies have found no significant effect on lipids, despite stress concurrently causing other changes that are well-documented to promote dyslipidemia and atherosclerosis. A possible interpretation of this is that research with negative results did not sufficiently stress its subjects. It is possible that stress only significantly affects lipids when the stress is sufficiently severe, above a certain threshold. For example, this could explain primate studies' negative results when using social group rearrangement as the stressor (18, 99-101), versus significant positive results when using the acute stress of capture as the stressor (102).

In hopes of identifying this threshold of stress severity needed to affect lipids, we have included a summary of stress-lipid research in Appendix III. It informs researchers of what stressors have successfully impacted serum lipids in prior research, and thus are theoretically above a threshold of stress severity that is need to do so. Ideally Appendix III will guide researchers in choosing effective stressors which are severe enough to affect lipids, enabling them to detect a stress-lipids association in their studies. This improvement in research will help produce results that further expand our knowledge of this important topic.

It is also important to note that cortisol levels are related to diurnal cycles, and thus vary at times of day. Cortisol is generally highest upon wakening, and declines over the waking hours (149). Much of the research included in the Literature Review did not specify if cortisol measures were taken at the same time of day for each subject, consistently. This highlights the need to acknowledge diurnal cycles' effects on cortisol in future research, and ensure to check cortisol at consistent times of day.

Some technical improvements can be made in future studies that can afford greater time and financial costs. Care must be taken to minimize stressful animal handling, as this can affect the corticosterone outcome being studied. This is especially tricky at time-intensive terminal bleeds, and this was a source of error in our study. Elaborate rodent feeders are also available, which allow for very accurate measurement of food consumption. Assays can also be best tested in triplicate. Finally, it is worth revisiting the soiled bedding stressor in future research, using urine from more foreign mouse strains or from mouse predators.

5. Therapies to Reduce Stress

a. Individual stress screenings

Individuals vary greatly in both their psychological and physiological reactivity to stress. For example, cortisol relates directly to waist-to-hip ratio in "high reactor" individuals (see **Figure 20**)(85, 91) who also have higher cholesterol (90). One trial even found that Type A subjects' cholesterol increased following a stress task, whereas Type B subjects' cholesterol decreased (89).

Similarly, some studies show that lipids relate differently to *individual* stress traits, whose effects are lost in umbrella terms such as "Type A" (30, 85). Other research has found differences in stress reactivity based on age, BMI, occupation, area of residence, and even family history of heart disease.

It may be helpful for individuals to know what type of reactor they are—what specific stressors they react to most (150). This will help steer individuals to the stress-management therapies most beneficial to them. Many screening tools exist and can be self-administered, including the Framingham Type A surveys, Spielberg's State Trait Anxiety Questionnaires, the Jenkins activity survey, and others (135).

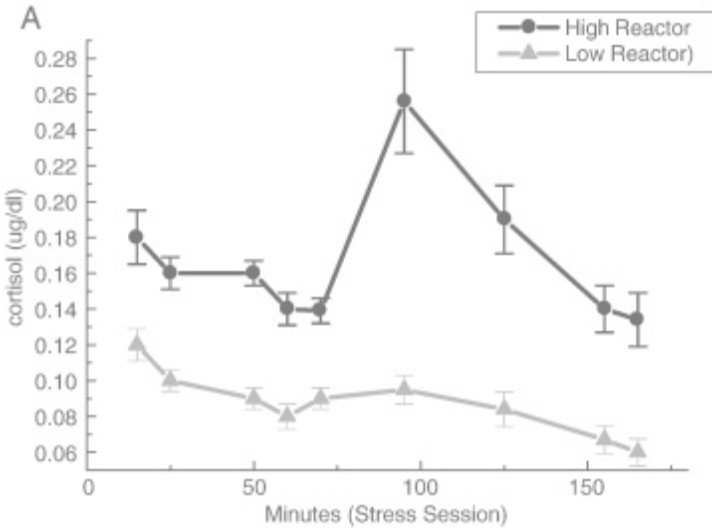


Figure 20: The cortisol response curve is shifted up in high reactors, showing a more obesogenic response to stress in some individuals (91)

b. Physical activity

Modern-day stressors are not predators to run from, so the physical activity component is uncoupled from the stress response (135). Exercise may use some of the mobilize lipid, preventing it from remaining in the circulation, causing metabolic changes, and being stored viscerally (91, 131). For example, stressed rats that were allowed to exercise had lower cortisol and cholesterol than stressed, inactive rats (117). Activity may even relieve psychological stress, and serve as an alternative mood-booster to stress eating (91, 131, 135).

c. Stress management techniques

Helpful strategies include Yoga, anger management, anxiety counseling, and biofeedback (135). Stress management training results in decreased total and LDL cholesterol in CVD patients randomly assigned to the behavior modification versus those assigned to usual care--independent of changes in diet (151). For example, Dr. M. Friedman (who coined "Type A") would prescribe his patients lengthy books to read, to "drive in the slow lane," and to "leave your watch at home" (135).

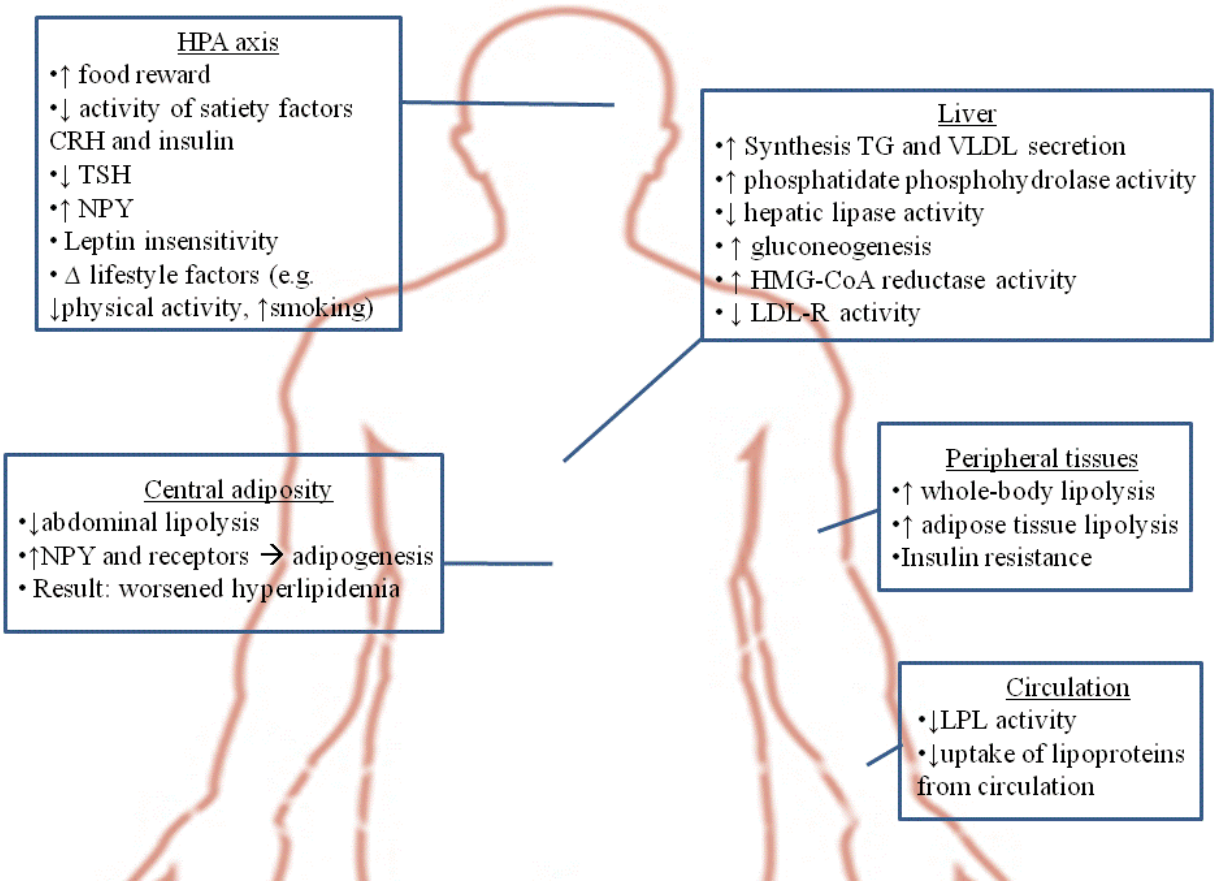
d. Improved occupational settings

Surveys confirm that occupational pressures are a leading source of modern stress (152). Top job stressors include anxiety-producing management styles, vague or changing job descriptions, concerns about employment, discrimination, violence, physical and verbal abuse, and others (152). Ideally, increased recognition of stress as a viable health risk will increase measures to reduce occupational stress on the part of employers.

e. Parenting interventions

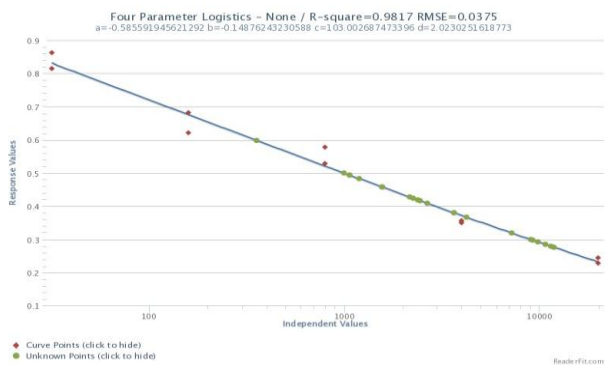
Type A behavior runs in families—in part due to genetics, and in part due to Type A parents modeling this behavior to children, as indicated by studies on twins (135). The Type A profile is even observed in toddlers who work quickly, and are impatient and aggressive. Many parenting books advise parents on how to avoid modeling Type A behavior.

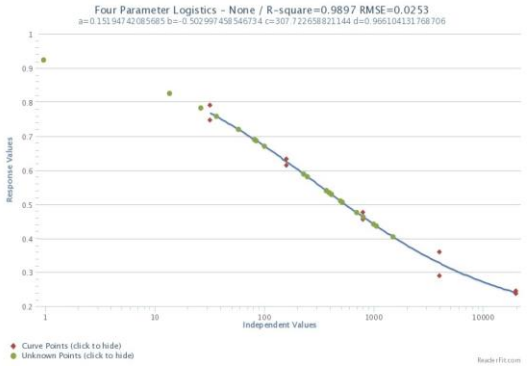
Appendix I: Proposed mechanisms by which stress promotes dyslipidemia

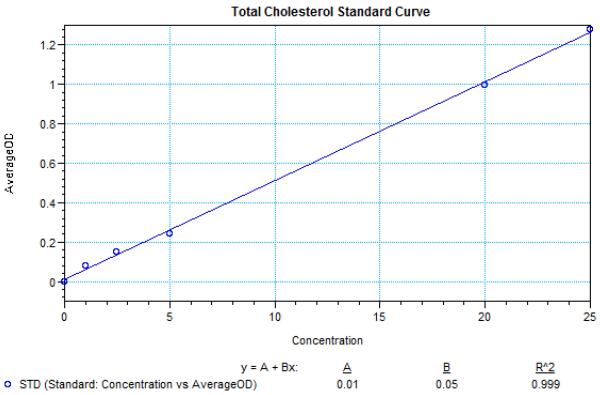


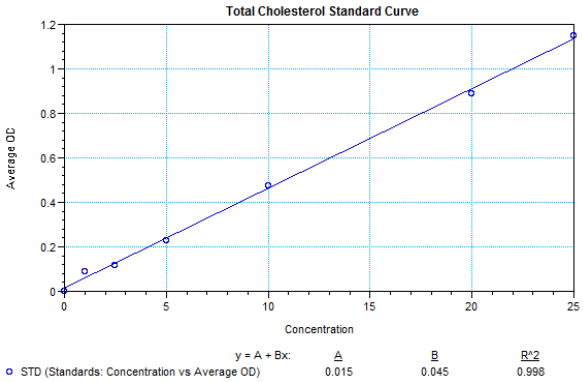
Appendix II: Assay standard curves

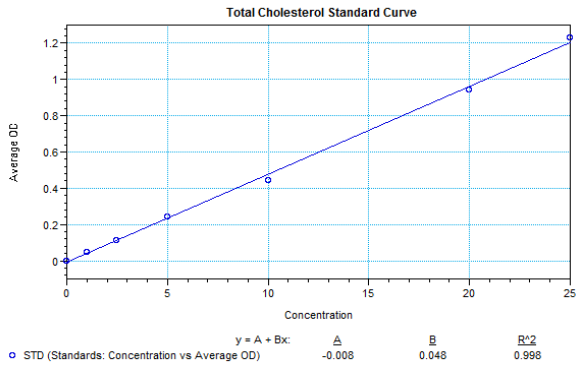
R^2 values range from 0.9817 - 0.999

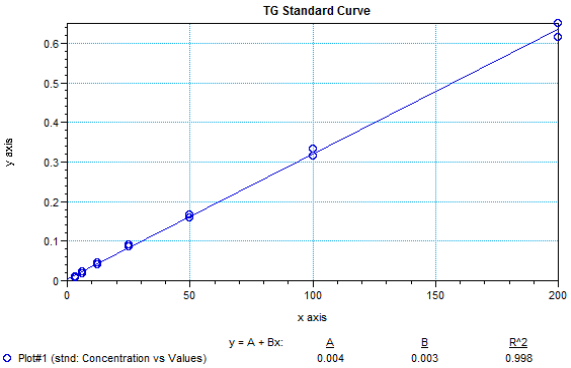


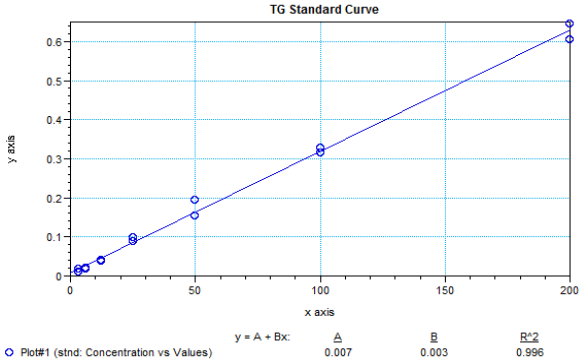












Appendix III: Summary of Stress-Lipid Research

Clinical Trials							
Human Trials							
Reference	**Subjects**	**Stressor**	**Duration**	**Diet**	**Results**	**Results corrected for hemoconce-ntration?**	**Impact on lipids**
Arnetz BB, Fjellner B, Eneroth P 1985	20 men	Color word conflict task and math	Single session	N/A: Single lab session	No change in TC cholesterol or TG	No (not stated)	--
Arnetz BB & Fjellner B 1986	20 male medical students and employees	Color word conflict task and math	Single session	N/A: Single lab session	Increase in TG	No (not stated)	↑
Bachen EA, Muldoon MF, Matthews KA, et al 2002	52 men	Cognitive tasks, some subjects given Labetalol to reduce response	Single session	N/A: Single lab session	FFAs, TG, TC, and LDL rose during stress	Yes: FFA and TG results remained significant	↑
Bacon SL, Ring C, Lip GYH, et al 2004	51 men	Mental arithmetic, cycling on an ergometer	Single session	N/A: Single lab session	TC and LDL rose during stress	Yes: rendered the observed lipid increases insignificant	--
Davis MC & Matthews KA 1990	35 female smokers	Speech task	Single session	Lab session followed overnight fast	Stress increased in FFAs, TC, LDL, and TG	No (not stated)	↑
Finney ML, Stoney CM, Engebretson TO 2002	46 men	Speech task	Single session	Lab session followed 12-hr fast	Stress increased TG	Yes: results remained significant	↑
Fredrikson M, Blumenthal JA 1992	73 Type A men	Mental arithmetic task, type A questionnaire	Multiple sessions	Lab session followed 14-hr fast	Cortisol and norepineprhine correlated directly with TG and TC/HDL ratio	No (not stated)	↑
Le Fur C, Romon M, Lebel P, et al 1999	14 subjects	Computer mental stress task	Hourly for seven hours following meal	Subjects consumed identical meals	Postprandial lipids not significantly affected by stress	No	--
Matthews KA, Davis MC, Stoney CM 1991	52 healthy men and women	Mirror tracing and Stroop task	Single session	N/A: Single lab session	Increases in TC, LDL, and TG	No (not stated)	↑
McCann BS, Magee MS, Broyles FC, et al 1995	32 men	Stroop colorword interference task, mental arithmetic, epinephrine infusion	Multiple sessions	Subjects fasted prior to sessions	FFAs, TG, TC, and LDL rose during stress or epinephrine infusion	Yes: results remained sinificant for FFA and TG increases during epinephrine infusion only	↑
Muldoon MF, Bachen EA,	26 men	Stroop test and mental arithmetic	Two sessions	Not stated	Stress increased TC	Yes: rendered the observed	--

Manuck SB, et al 1992						lipid increases insignificant	
Muldoon MF, Herbert TB, Patterson SM, et al 1995	44 subjects	Frustrating cognitive task	Single session	Not stated	Stress increased TC and LDL	Yes: rendered the observed lipid increases insignificant	--
Patterson SM, Gottdiener JS, Hecht G, et al 1993	18 men	Challenging mental arithmetic	Single session	Subjects fasted 12 hrs prior	Stress increased TC and LDL	Yes: rendered the observed lipid increases insignificant	--
Patterson SM, Matthews KA, Allen MT, et al 1995	17 women	Mirror image tracing, Stroop Color–Word Interference Test, speech task	Single session	Subjects fasted 12 hrs prior	Stress increased FFA, TC, TG, and LDL	Yes: FFA results remained significant	↑
Perez-Parada J, Jhangri GS, Lara N, et al 2007	18 men and women with panic disorder	Pentagastrin injection-induced panic attacks	Single session	Subjects fasted 12 hrs prior to injections	Delayed (24 h) increase in LDL following panic attacks in men	No (not stated)	↑
Roy MP, Kirschbaum C, Steptoe A 2001	82 men	Mental arithmetic, speech task,Daily Stress Inventory (DSI), others	Single session	Subjects fasted overnight	High stress reactors (determined by cortisol levels) had greater LDL and TC/HDL ratio	No (not stated)	↑
Spence JD, Manuck SB, Munoz C, et al 1990	40 subjects	Mental arithmetic and mirror tracing	Single session	Not stated	Stress increased TC and TG	No (not stated)	↑
Steptoe A, Brydon L 2005	199 men and women	Stress tasks: color-word interference and mirror tracing	Single session	Not fasted, but abstained from caffeine	Tasks caused increases in TC and LDL; individual reactivity to stress predicted TC/HDL ratio at 3-yr follow-up	No	↑
Stoney CM, Matthews KA, McDonald RH, et al 1988	41 subjects	Serial subtraction task, a videotaped speech task, and a self-evaluation task	Single session	Not stated	Elevated LDL during stress tasks	Not stated	↑
Stoney CM, Owens JF, Matthews KA 1990	15 women	Social evaluative speech task (2 min preparation, 3 min speech); Mental arithmetic (6 min); Isometric exercise (2.5 min)	Single session	Not stated	Increase in LDL during stress	Not stated	↑
Stoney CM, Niaura R, Bausserman L 1997	18 men	Stress tasks such as speech task and serial subtraction task	Two sessions, an average of 16 months apart	Subjects fasted for 12 hrs prior, could have orange juice	Higher TC, TG, and LDL following stress tasks	Yes: results remained significant for speech task	↑
Stoney CM & Hughes JW 1999	37 men	Speech task	Single session	Subjects fasted for 12 hrs prior, could have orange juice	TC, TG, and LDL increased during stress, more pronounced reactivity among men with	Yes: results remained significant	↑

					family history of myocardial infarction		
Suarez EC, Williams RB, Jr., Kuhn CM, et al 1991	24 subjects	Mental arithmetic stress tasks, Cook and Medley Hostility scale	Single session	Not stated	Stress reactivity (determined by epinephrine, norepineprhine, and cortisol) related to higher TC and LDL following stress in Type A subjects; Type B subjects had inverse cholesterol responses to stress	Not stated	↑
Stoney CM, Niaura R, Bausserman L, et al 1999	100 pilots, mostly men	Positive and Negative Affect Scale, Cohen 14-item Perceived Stress Scale, lab stress tasks and questionnaires	Single session	Subjects fasted, fed a meal during session/s	Higher TC and LDL during both chronic and acute stress	No (not stated)	↑
Wirtz PH, Ehlert U, Bartschi C, et al 2009	45 men	Public speaking and mental arithmetic	Single session	Subjects fasted for 2 hrs prior	Norepinephrine predicted TC and LDL	Yes: results remained significant	↑
Primate Trials							
Reference	**Subjects**	**Intervention**	**Duration**	**Diet**	**Results**		**Impact on lipids**
Kaplan JR, Manuck SB, Adams MR, et al 1993	83 male monkeys with atherosclerosis	Social group rearrangement	42 months	Cholesterol-supplemented, then either high- or low-lipid diet	Cholesterols were not significantly different between groups; stressed animals had greater IMT and arterial lesions	No (not stated)	--
Kaplan JR, Manuck SB, Clarkson TB, et al 1983	29 male cynomolgus monkeys	Social group rearrangement	21 months	Low cholesterol and low saturated fat	Cholesterols were not significantly different between groups; stressed animals had greater IMT and arterial lesions	No (not stated)	--
Manuck SB, Adams MR, McCaffery JM, et al 1997	20 estrogen-deficient female monkeys	Display of a "monkey glove" evoking threat of capture or handling	30 months	Moderately atherogenic, 40% calories from fat	High stress reactors (determined by heart rate) had higher TC and greater IMT	No (not stated)	↑
Other Animal Trials							
Reference	**Subjects**	**Intervention**	**Duration**	**Diet**	**Results**		**Impact on lipids**
Andersson IJ, Jiang YY, Davidge ST 2009	Pregnant female apo-E deficient mice	Restraint stress	Five days	Standard chow	Cholesterol not different between offspring of stressed and unstressed mice	No (not stated)	--

Berger DF, Starzec JJ, Mason EB, et al 1980	84 male rats	Operant conditioning boxes including shocks	30 days	Cholesterol- and fat-supplemented diet	No signficant TC difference between unsignaled, shocked rats and unshocked rats	No (not stated)	--
Brennan FX, Grahn RE, Watkins LR, et al 2003	27 male rats	Shocks, yoked or escape sessions	Single session	Standard chow	Stressed rats had higher TC, LDL, and VLDL	No (not stated)	↑
Bryant HU, Kuta CC, Story JA 1988	Female mice	Immobilization	28-day stress schedule	Cholesterol- and cholic-acid-supplemented diet	Stress increased TC	No (not stated)	↑
Degordoa JCR, Santafe J, Domenech JS, et al 1994	124 rats	Immobilization	Single session	Standard chow	Higher TG, TC, and LDL in stressed rats	Yes: results remained significant for males only	↑
Hershock D, Vogel WH 1989	Male rats	Immobilization	Single session	Standard chow, cholesterol-supplemented, and fat-supplemented	FFAs and TG affected by stress depending on diet; TGs increased during stress in fasted rats. No significant cholesterol response	No (not stated)	↑
Huang YS, Mills DE, Ward RP 1990	Rats	Isolation	Two weeks	Fat-supplemented (omega-6)	No significant lipid response	No (not stated)	--
Lavin-Palmieri M, Sanchez-Serrano D 1984	Male mice	Social isolation	12-13 weeks	Standard chow	Lower TC in stressed groups	No (not stated)	↓
Manting L, Haihong Z, Jing L, et al 2011	24 male rats	Swimming in ice water, vibration, others	55 days	Standard chow or high-fat diet	Stressed group had higher TC, TG, and liver TG	No (not stated)	↑
Mumma JO, Thaxton JP, Vizzier-Thaxton Y, et al 2006	Single Comb White Leghorn hens	ACTH infusion	7 dats	Standard diet	Stressed group had higher TC	No (not stated)	↑
Pare WP, Rothfeld B, Isom KE, et al 1973	24 male rats	Foot shocks	Two, eight, and 30 days	cholesterol-supplemented	Higher TC in various body compartments of stressed animals after two, eight, and 30 days	No (not stated)	↑
Ricart-Jane D, Rodriguez-Sureda V, Benavides A, et al 2002	Male rats	Immobilization	Two periods of four-five days	Standard chow	Acute and chronic stress reduced VLDL	No (not stated)	↓
Servatius RJ, Ottenweller	18 male rats	Immobilization and shocks	One day or three days	Standard chow	Higher TC in stressed animals	No (not stated)	↑

JE, Gross JL, et al 1993							
Starzec JJ, Berger DF, Hesse R 1983	72 male rats	Operant conditioning boxes including shocks	30 days	Cholesterol and fat-supplemented	Stressed animals had lower aortic cholesterol than non-stressed animals	No (not stated)	↓
Tsopanakis C, Kotsarellis D, Dontas I 1988	Rabbits	Cold stress	9 week study period	Standard or cholesterol-supplemented	TC and TG decreased in stressed rabbits	No (not stated)	↓
Yehuda S, Rabinovitz S, Carasso RL, et al 2000	40 male rats	Forced swimming or cortisol infusion	Single session	Supplemented with n-3 and n-6 fatty acids	Higher TC in stressed rats	No (not stated)	↑

Observational Studies

Reference	Subjects	Stress index	Results	Impact on lipids
Albert CM, Chae CU, Rexrode KM, et al 2005	72,359 female nurses	Phobic anxiety scale of Crown-Crisp index	Phobic anxiety related to diagnosed high cholesterol	↑
Alfredsson L, Hammar N, Fransson E, et al 2002	10,382 men and women	Job Strain: Questionnaire, Karasek's demand-control model	Job strain (low decision latitude and high demands) related to lower TC	↓
Biersner RJ, McHugh WB, Rahe RH 1981	13 male softball players	Skill level (low skill assumed to mean higher stress)	Higher TC in less-skilled (higher-stress players)	↑
Biersner RJ, McHugh WB, Rahe RH 1984	26 male divers in Navy training course	Mood Questionnaire, diving experience	Less diving experience (higher-stress) related to higher TC	↑
Bijlani RL, Gandhi BM, Tandon BN 1983	Eight medical students	Periods of stress (around exams)	TC and LDL increased around exams	↑
Bijlani RL, Sud S, Gandhi BM, et al 1986	Eight male medical students	Periods of stress (around exams)	TC and LDL increased around exams; repeated measures did not replicate	↑
Chaput LA, Adams SH, Simon JA, et al 2002	792 women	Cook-Medley Hostility scale	Hostility directly related to TG	↑
Chikani V, Reding D, Gunderson P, et al 2004	1,500 women	Framingham questionnaires, Spielberger Trait anger-reaction sub-scale	Anger-reaction scores directly correlated with TC, TG, and LDL	↑
Chikani V, Reding D, Gunderson P, et al 2005	1,500 women	Karasek Job Content Questionnaires (JCQ)	Job demand related to higher TG in farm residents and higher TC in non-farm residents	↑
Clark DA, Arnold EL, Foulds EL, Jr., et al 1975	150 young men	Stressful periods in Air Force Academy training	TC increased during stressful periods	↑
Eaker ED, Sullivan LM, Kelly-Hayes M, et al 2004	3,873 men and women	Bortner Rating Scale, Framingham scales, Spielberger Trait-Anger, Cook-Medley Hostility	Hostility related to higher total/HDL ratio in men; anger related to higher total/HDL ratio in women	↑
Evolahti A, Hultcrantz M, Collins A 2009	107 women	Job Content Questionnaire and psychological interview	Job strain related to higher LDL/HDL and TC/HDL ratio	↑
Flynn MA, Anderson A, Rutledge M, Nolph GB, Krause G, Ellersieck MR 1984	26 medical students	Periods of stress (around exams)	No change in lipids	--
Fornari C, Ferrario M, Menni C, et al 2007	5,695 men and women	Job Content Questionnaire (JCQ)	Job strain related to higher TC in women only	↑

Francis KT 1979	20 male and female students	Stressful periods (around exams), State-Trait Anxiety Inventory, Multiple Affect Adjective Check List	TC and LDL increased during periods of academic stress	↑
Friedman M, Rosenman RH, Carroll V 1958	162 executives	In-person interview	TC increased during exams	↑
Gill JJ, Price VA, Friedman M, et al 1985	118 officer students	Notification of selection for military line command	Increase in TC	↑
Goldstein DS, Dionne R, Sweet J, et al 1982	21 dental surgery patients	Baseline to pre-op waiting period of several days	No change in any lipids	--
Greenlund KJ, Liu K, Knox S, et al 1995	2,665 men and women	Job Content Questionnaire	No significant associations	--
Grundy SM, Griffin AC 1959	50 male students	Periods of stress (around exams)	TC increased during exams	↑
Hamer M, Molloy GJ, Stamatakis E 2008	6,576 men and women	General Health Questionnaire	Psychological distress not significantly related to lipids	--
Haynes SG, Levine S, Scotch N, et al 1978	1,822 men and women	Framingham Type A scale	Insignifant associations between Type A behavior and cholesterol	--
Hendrix WH, Ovalle NK 2nd, Troxler RG 1985	370 hospital and Department of Defense empolyees	Stress Assessment Package instrument	Insignificant effect on HDL/TC ratio	--
Heslop P, Smith GD, Carroll D, et al 2001	6,832 mostly men	Reeder Stress Inventory	Perceived stress not significantly related to cholesterol after adjusting for socio-economic position	--
Jonsson D, Johansson S, Rosengren A, et al 2003	1,413 women	Questionnaire	TC lower in women at higher stress levels	↓
Kang MG, Koh SB, Cha BS, et al 2004	169 male workers	Job Content Questionnaire	No significant differences in lipids between higher-job strain and lower-job strain groups	--
Kang MG, Koh SB, Cha BS, et al 2005	152 men	Karasek's Job Strain Model, Job Content Questionnaire	Lower decision latitude related to higher TC and TG	↑
Kirkeby OJ, Risoe C, Kirkeby K 1984	9 female medical students	Periods of stress (around exams)	Increase in TC	↑
Kivimäki M, Leino-Arjas P, Luukkonen R, et al 2002	812 employees	Questionnaire	Job strain directly related to TC at five-year follow-up	↑
Kobayashi Y, Hirose T, Tada Y, et al 2005	1401 female Japanese workers	Job Content Questionnaire	No significant associations between job strain measures and TC, TG, or LDL	
Kulenović AD, Kučukalić A, Maleč D 2008	100 veterans; half with PTSD	Life Stressor List, Manchester Short Assessment of Quality of Life Scale, and Folkman-Lazarus Coping Strategies Questionnaire	Higher lipids in veterans with PTSD	↑
LeBlanc J, Ducharme MB 2005	20 men and women	Big-Five Inventory	Extraversion related to higher TC; neuroticism related to lower TC	↑ or ↓ depending on trait
Lundberg U, Fredrikson M, Wallin L, et al 1989	60 men and women	Physiologic markers of stress while home, work, and laboratory stress	TC and LDL directly related to BP in all conditions in men; TC, TG, and LDL directly related to epinephrine and norepinephrine during laboratory stress in women	↑
Lundberg U, Hedman M, Melin B, et al 1989	60 men and women	Videotaped Structured Interview (VSI)	Type A and time urgency positively correlated with TC in men; hostility positively correlated with TC in	↑

			women	
McCann BS, Benjamin GA, Wilkinson CW, et al 1999	173 lawyers	Daily stress inventory and periods of occupational stress	Apo B and TG higher in periods of high workload	↑
McCann BS, Benjamin GAH, Wilkinson CW, et al 1996	40 male and female students	Periods of stress (around exams), self-reports of stress and workload	LDL increased during exams	↑
McCann BS, Warnick GR, Knopp RH 1990	14 men and women	Subjective estimates of stress and workload, objective measure of workload	Perceived workload related to higher TC	↑
Melamed S 1994	941 employed women	Israeli PERI life event scale, Zung's self-rating anxiety and depression scales, an emotional reactivity scale	Emotional reactivity related to higher TC/HDL ratio	↑
Netterstrom B, Danborg L, Olesen H 1988	35 medical students	Periods of stress (around exams)	No significant influence on TC	--
Niaura R, Herbert PN, Saritelli AL, et al 1991	20 tax accountants and 40 students	Periods of occupational stress (tax season for accountants, exams for students)	No significant relation between stress and TC	--
O'Donnell L, O'Meara N, Owens D, et al 1987	13 male and female students	Periods of stress (around exams)	Higher TC and LDL around exams; no change in VLDL or TG	↑
Orth-Gomer K & Unden AL 1990	150 male workers	Tension/pressure and overtime ratings	No significant effect on TC or TG	--
Persson R, Ørbæk P, Kecklund G, et al 2006	100 construction workers; half working 40 hrs/wk and half working 84 hrs/wk	Work hours	Decreased TC in the 84hr workweek group, progressively decreasing over 7 days	↓
Peter R, Alfredsson L, Hammar N, et al 1998	4,958 men and women	Questionnaire and Likert scale	High effort-reward ratio related to increased TC and TC/HDL ratio in men; high intrinsic effort related to higher LDL in women	↑
Pieper C, LaCroix AZ, Karasek RA 1989	NHANES I (n=1937), NHANES II (n=2925), NHES (n=2291), WCGS (n=3023), EHS (n=2379)	Various survey instruments	Decision latitude positively related to TC, no effect for psychological demands, in NHANES I and II. No effect of decision latitude or psychological demands on TC in NHES. Negative relationship between decision latitude and total cholesterol, and no effect for psychological demands in WCGS. Negative relationship between decision latitude and TC. No effect for psychological demands in EHS.	↑ and --
Pincomb GA, Lovallo WR, Passey RB, et al 1987	20 medical students	Periods of stress (around exams)	No change in TC or TG	--
Rahe RH, Ryman DH, Biersner RJ 1976	51 underwater demolition trainees	Stressful periods during training	Higher TC during stress periods	↑
Räikkönen K, Lassila R, Keltikangas-Järvinen L, et al 1995	69 men	Maastricht Questionnaire and Depressive Behavior Survey Schedule	No significant relation between VE (index of chronic perceived stress) and TG	--
Reed DM, LaCroix AZ, Karasek RA 1989	8,006 men of Japanese ancestry living in Hawaii	Questionnaire	No effect on TC	--

Riese H, Van Doornen LJP, Houtman ILD, et al 2000	165 female nurses	Job Content Questionnaire	No significant relation between job strain and lipids	--
Rod NH, Gronbaek M, Schnohr P, et al 2009	7,066 women and men	Questionnaire	Stress not significantly related to TC at baseline or follow-up	--
Rose G, Kumlin L, Dimberg L, et al 2006	926 male and female workers	Psychological General Well-being Inventory (PGWB)	Experiencing a strongly stressful life event at baseline related to higher TC/HDL ratio at five-yr follow-up	↑
Rosengren A, Tibblin G, Wilhelmsen L 1991	6,935 men	Questionnaire	No significant differences in lipids between higher-stress and lower-stress groups	--
Rosenman RH, Brand RJ, Jenkins D et al 1975	257 men	Questionnaire	The type A behavior pattern was strongly related to the CHD incidence, and this association could not be explained by association of behavior pattern with any single predictive risk factor such as TC or TG	--
Ryff CD, Love GD, Urry HL, et al 2006	135 women	Questionnaire	No significant relation between anger or anxiety alone and lipids	--
Sapolsky RM, Mott GE 1987	Male baboons living in natural habitat	Subordinate males assumed to be stressed	No significant associations	--
Siegrist J, Matschinger H, Cremer P 1988	254 German workers	Self-reported job strain measures	Occupational instability, work demand, and job insecurity interaction related to higher LDL/HDL ratio and LDL	↑
Siegrist J, Peter R, Cremer P, et al 1997	179 male managers	Interview and questionnaire	Higher LDL with high job effort and low reward	↑
Smoak BL, Norton JP, Ferguson EW 1990	44 Navy trainees	Week of intense training	Decrease in TC and LDL	↓
Sorensen G, Pirie P, Folsom A 1985	2500 employed men and women	Work hours, deadlines, occu- pational mobil- ity. Reported stress symptoms.	No associations with TC	--
Su CT 2001	964 male and female workers	Karasek's job strain model	No consistent association between job strain and TC	--
Schwartz SM, Schmitt EP, Ketterer MW et al	76 male students	Cook-Medley Hostility scale, Symptom Checklist 90-Revised	Lower TC predicted by positive symptom total	↓
Theorell T, Hamsten A, de Faire U 1987	116 male myocardial infarction survivors	Subjective ratings of job strain	TC correlated positively only with demands divided by influence. TG and LDL/HDL ratio uncorreated with any rating.	↑
Trevisan M, Tsong Y, Stamler J, et al 1983	416 coronary-prone men	Self-report	TC higher with report of tension versus no tension	↑
Trevisan M, Celentano E, Meucci C, et al 1986	192 factory workers	Exposure to 6.8 (Richter scale) earthquake	Increased TC and TG in subjects exposed to earthquake, not in those unexposed. Lipid elevations remained in exposed workers two months after the earthquake.	↑
Tsuboi H, Tatsumi A, Yamamoto K, et al 2006	381 female nurses	Brief Job Stress Questionnaire	High job strain subjects had lower LDL and VLDL	↓
Tsutsumi A, Kayaba K, Ishikawa S, et al 2003	6,929 men and women	Karasek job demand-control questionnaire	Higher psychological demands associated with higher TC and total/HDL ratio in men	↑
van Doornen LJ, van Blokland R 1987	52 students	Periods of stress (around exams)	TC higher on exam day	↑

Wattoo FH, Memon MS, Memon AN, et al 2008	80 female teachers and housewives	Likert scale questionnaire	Housewives (higher stress) had higher levels of TC, TG, and LDL cholesterol	↑
Webster IW, Porritt DW, Brennan PJ 1983	262 prison officers	Health and psychological survey	Prison officers more likely to have elevated TG but not TC	↑
Weidner G, Hutt J, Connor SL, et al 1992	64 children from 64 families in Family Heart Study	Family stress measued by Family Environment Scale	Family conflict directly related to TC/HDL ratio in boys only	↑
Weidner G, Boughal T, Connor SL, et al 1997	682 men and women	Jenkins Activity Survey, Karasek job strain model	No significant associations	--
Xu W, Hang J, Gao W, et al 2011	544 men and women	Effort-reward imbalance model	Effort, overcommitment, and effort-reward imbalance related to higher TG and LDL; reward related to lower TG and LDL	↑
Yamamoto K, Okazaki A, Ohmori S 2011	1,499 workers	IMPS stress score	Stress score associated with increased TG among women	↑

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