Generalization of contextual fear is sex-specifically affected by high salt intake

Short title: Salt sex-specifically influences fear generalization

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Abstract

A hallmark symptom of many anxiety disorders, and multiple neuropsychiatric disorders more broadly, is generalization of fearful responses to non-fearful stimuli. Anxiety disorders are often comorbid with cardiovascular diseases. One established, and modifiable, risk factor for cardiovascular diseases is salt intake. Yet, investigations into how excess salt consumption affects anxiety-relevant behaviors remains little explored. Moreover, no studies have yet assessed how high salt intake influences generalization of fear. Here, we used adult C57BL/6J mice of both sexes to evaluate the influence of two or six weeks of high salt consumption (4.0% NaCl), compared to controls (0.4% NaCl), on contextual fear acquisition, expression, and generalization. Further, we measured osmotic and physiological stress by quantifying serum osmolality and corticosterone levels, respectively. Consuming excess salt did not influence contextual fear acquisition nor discrimination between the context used for training and a novel, neutral context when training occurred 48 prior to testing. However, when a four week delay between training and testing was employed to induce natural fear generalization processes, we found that high salt intake selectively increases contextual fear generalization in females, but the same diet reduces contextual fear generalization in males. These sex-specific effects were independent of any changes in serum osmolality nor corticosterone levels, suggesting the behavioral shifts are a consequence of more subtle, neurophysiologic changes. This is the first evidence of salt consumption influencing contextual fear generalization, and adds information about sex-specific effects of salt that are largely missing from current literature.

Introduction

Excessive dietary salt intake occurs across nations and cultures [1,2]. While salt's practical uses include food preservation and flavor enhancement, consumption of too much salt has been associated with increased risk for cardiovascular diseases [3–6]. Indeed, average estimates of global salt intake indicate people consume 3,950 mg of sodium (the component of NaCl that has been associated with multiple negative health outcomes) [3,7,8]. When put in the context that, according to the World Health Organization, adults should consume less than 2,000 mg of sodium, this overconsumption of salt becomes quantifiably apparent [9]. It is estimated that reducing consumption of salt can attenuate risk for cardiovascular disease by ~20% [5,10], meaning dietary salt consumption has a profound physiological influence.

Globally, cardiovascular diseases and neuropsychiatric disorders, particularly disorders involving anxiety symptoms, are often comorbid [11–15] (see also reviews [16,17]). Increasing literature suggests dietary components such as excess sugar and/or fat are associated with both metabolic conditions like insulin resistance and type II diabetes, as well as with neuropsychiatric conditions (e.g., depression) and diseases (e.g., Alzheimer's) [18–22]. Trait anxiety appears largely unaffected by excess salt consumption [23–27] (reviewed in [28]). Far less understood is how excess salt intake might affect neuropsychiatric symptoms like generalized (i.e., non-specific, state) anxiety, though some researchers have begun to recognize this possibility [29,30]. We hypothesized that excessive consumption of dietary salt would enhance fear generalization in rodents, a possibility that has not yet been explored.

Fear generalization is an established neurobehavioral phenomenon that occurs across species, and studies are making considerable progress in identifying brain regions critical to this process [31–34]. These include specific subregions of the cingulate and prefrontal cortices, hippocampus, amygdala, and brainstem nuclei (e.g., locus coeruleus). Growing evidence indicates the hippocampus and amygdala are affected by excess salt consumption in rodents

66 [24,26,35–41], including: reductions in nicotinic receptor levels [35]; increased markers of
67 oxidative stress [24,26,37,41], neuronal activation [42], and neuroinflammation [42]; reduced
68 long-term potentiation [40]; and increased microvasculature leakiness [36]. Other brain regions
69 remain underexplored. This evidence, combined with human evidence of comorbidity between
70 anxiety disorders and cardiovascular diseases, supports our hypothesis – at least based upon
71 male data – that fear generalization is augmented by high salt intake. Unfortunately, both the
72 scant literature on behavioral effects of increased salt intake, and the larger body of work on the
73 neurocircuitry of fear generalization, have focused almost exclusively on male rodents. Here,
74 we included both sexes in our studies, but given the current state of the literature we could not
75 formulate data-based *a priori* hypotheses specifically regarding how female fear generalization
76 behavior might differ from male fear generalization under conditions of excess salt intake.
77

Materials and methods

Animals

Mice of both sexes on a C57BL/6J background were purchased from Jackson Laboratory (Bar Harbor, ME) and bred in-house. All mice were at least 9 weeks old prior to commencing experiments. At the start of diet manipulation, mice were singly housed to facilitate accurate quantification of diet and water consumption relative to the mouse's body weight. Prior to experiments, all mouse cages contained Nestlets (Ancare, Bellmore, NY) and huts; at the time of singly housing for diet manipulation, two additional pieces of enrichment (e.g., swing, wood block, etc.) were added to each cage. Cages contained 7090 Teklad Sani-chip bedding (Envigo, East Millstone, NJ) and were housed in a 12:12 light/dark cycle room, with lights on at 07:00, and temperature maintained at 22 ± 2°C.

Prior to diet manipulation, mice were fed LabDiet 5001 rodent laboratory chow (LabDiet, Brentwood, MO) *ad libitum*. At the time of diet manipulation, mice were pseudorandomly assigned to either a control diet (0.4% NaCl w/w; D17012, Research Diets, Inc., New Brunswick, NJ) or a high salt diet (4.0% NaCl; D17013; Fig. 1A). These diets were available *ad libitum*, and water was always available *ad libitum*. Twice weekly, measurements of diet and water consumption plus body weight were taken. Refer to Supporting Information for statistics and graphs on average water consumed per day (S15 and S19 Tables; S9 and S13 Figs.); average food consumed per day (S16 and S20 Tables; S10 and S13 Figs.); weekly body weight changes (S17 and S21 Tables; S11 and S14 Figs.); raw body weights (S18 and S22 Tables; S12 and S14 Figs.); average NaCl consumed per day (S23 and S28 Tables; S15 and S20 Figs.); average NaCl consumed as percentage of body weight (S24 and S29 Tables; S16 and S20 Figs.); weekly ratio between water:NaCl consumption (S25 and S30 Tables; S17 and S21

Figs.); average kcal consumed per day (S26 and S31 Tables; S18 and S22 Figs.); and average kcal consumed as percentage of body weight (S27 and S32 Tables; S19 and S22 Figs.).

Fig. 1. ***Study timeline and fear conditioning procedures.***
A) Mice were assigned to either a control (0.4% NaCl) or high salt (4.0% NaCl) diet for two or six weeks. Experiment 1 involved diet manipulation for two weeks, at which time mice underwent context fear training, followed 48 h later by context fear testing. Experiment 2 (grey shading) involved diet manipulation for six weeks, at which time mice underwent context fear training, followed 48 h later by context fear testing. Experiment 3 involved diet manipulation for two weeks, at which time mice underwent context fear training, followed four weeks later (while continuing the same diet manipulation) by context fear testing. B) Mice trained with a mild foot shock in the Training Context were tested 48 h (Experiments 1, 2) or four weeks (Experiment 3) later in either the Training Context or the Neutral Context. The Training Context included a metal grid floor, visible illumination, patterned background, and 70% ethanol scent. The Neutral Context included a smooth acrylic floor, infrared illumination, no background, and Windex scent. C) Control no shock mice were exposed to the Training Context, but were never administered a foot shock. These mice were tested at the same timelines for Experiments 1-3 in the Training Context, to assess for any baseline influences of diet consumption on fear behavior.

Diet manipulations lasted for two weeks (Experiment 1; Fig. 1A) or six weeks (Experiments 2 and 3; Fig. 1A). All procedures were approved by the Kent State University Institutional Animal Care and Use Committee (protocol 536 LG 22-14), and adhered to the National Research Council's Guide for the Care and Use of Laboratory Animals, 8th Ed. [43]. Every measure was taken to minimize suffering.

Experimental timelines

Experimental timelines are graphically illustrated in Fig. 1A. For Experiment 1, context fear training occurred after two weeks of diet manipulation, and context fear testing occurred 48 h after training. For Experiment 2, training occurred after six weeks of diet manipulation, and testing occurred 48 h after training. For Experiment 3, training occurred after two weeks of diet manipulation, and testing occurred four weeks after training (a standard time frame for natural development of fear generalization in mice [44]).

Context fear conditioning

Females and males always underwent behavior separately. Fear conditioning took place in identical Coulbourn Instruments chambers (7 in D × 7 in W × 12 in H; Allentown, PA) composed of two opposing aluminum walls and two Plexiglas walls surrounding a stainless-steel shock grid floor. Each chamber was located within its own sound-attenuating enclosure, and had a camera mounted to the top for real-time measurements of freezing behavior (absence of all movement except that necessary for breathing) using FreezeFrame (v. 5.201, Coulburn Instruments).

Context fear training

The Training Context (Fig. 1B) consisted of the shock grid floor, a patterned background, visible illumination, and scent cue (70% ethanol). Training occurred at either the two week or six week time point (Fig. 1A). Training involved exposure to five, 1 s, 0.8 mA scrambled foot shocks pseudorandomly administered during a 6 min training session at 137, 186, 229, 285, and 324 s. Training with five foot shocks is optimal for eliciting time-dependent contextual fear generalization in rodents [44–46]. Freezing behavior was quantified as the average percent freezing across the first six 5 s bins (i.e., 30 s) that occurred immediately after the 5 s bin containing the foot shock.

151 ## Context fear testing

152 Testing occurred at either the two (Experiment 1) or six week (Experiments 2 and 3) time point
153 (Fig. 1A). Mice were tested either in the Training Context, or in a Neutral Context which
154 consisted of a smooth floor, no background, only infrared illumination, and a different scent cue
155 (Windex®, SC Johnson, Racine, WI; Fig. 1B). All mice were only ever tested in a single
156 context, and testing lasted for 10 minutes, with the average percentage freezing occurring
157 during minutes two through six being quantified and analyzed [46]. Foot shocks were never
158 administered during testing.

159 ## No shock controls

160 Control 'no shock' mice were run concurrently in every cohort of animals (Fig. 1C) for every
161 Experiment. These no shock mice were exposed to the Training Context for 'training', but no
162 foot shock was ever administered. Likewise, control no shock mice were tested in the Training
163 Context, to evaluate how diet manipulation and the Experiment timelines influenced freezing
164 behavior in the absence of any contextual fear conditioning.

165

166 # Serum measurements

167 Forty-eight hours after testing for context fear expression, mice were briefly anesthetized with
168 isoflurane then rapidly decapitated for collection of trunk blood. Trunk blood was allowed to clot
169 at room temperature for 30 min, then was spun at 3500 rpm for 1 h at 4°C. Serum was then
170 removed from tubes and aliquoted into two vials – one for measuring serum osmolality, the
171 other for measuring serum corticosterone. Serum osmolality was measured immediately using
172 an Osmo1 single-sample micro-osmometer (Advanced Instruments, Norwood, MA). Prior to
173 every serum batch, a Clinitrol™ 290 reference solution (Advanced Instruments) from a new
174 ampule was measured to confirm accurate readings. Samples for serum corticosterone were
175 stored immediately at −80°C until analysis with a corticosterone ELISA kit (Enzo Life Sciences,

Inc., Farmingdale, NY) using their small volume protocol. Serum corticosterone levels were log-transformed to account for positive skewness [27,47,48]. Log-transformed serum corticosterone levels are, for brevity, hereafter referred to as 'cort' levels.

Statistical analyses

Data were analyzed with GraphPad Prism 9.4.0 (GraphPad Software, San Diego, CA) and IBM SPSS Statistics 28.0.0.0 (IBM, Armonk, NY). Our *a priori* significance threshold was set at $p<0.05$. Analyses were made within each Experiment. Repeated measures data (diet/water consumption, salt or kcal calculations, body weight, context fear training) were analyzed using 3-way repeated measures ANOVAs within individual sexes ([repeated measure, e.g., body weight] × diet × context) and pairwise comparisons with Bonferroni correction. Greenhouse-Geisser corrections were employed for within-subjects analyses. Measurements of contextual fear expression, serum osmolality, and serum corticosterone across contexts were analyzed with a 3-way ANOVA (diet × sex × context) and Holm-Šídák's post-hocs. Control no shock fear behavior data were analyzed separately, given these were purposely included as negative controls and not intended to be analyzed in comparison to experimental groups. These no shock control data were analyzed on their own with 3-way ANOVAs (diet × sex × context) and Holm-Šídák's post-hocs, or with repeated measures 3-way ANOVAs within each sex ([repeated measure, e.g., body weight] × diet × sex) plus pairwise comparisons with Bonferroni correction, and also using Greenhouse-Geisser corrections for within-subjects analyses. All ANOVA statistics are reported in Supporting Information, in S1-S32 Tables. The criterion to exclude outliers was *a priori* assigned as being >5 standard deviations ± mean. Numbers of mice per sex/shock/diet/context condition varied, and exact numbers are present in the respective figure legends for each group of data. Broadly: no shock fear behavior n=7-9; shock fear behavior n=7-10; shock serum osmolality n=8-9; shock serum corticosterone n=7-9. Details of outliers

201 that were excluded (training baseline, n=1; no shock testing, n=1; shock testing, n=4; osmolality,
202 n=1; log-transformed serum corticosterone, n=2; water:NaCl consumption ratio across weeks,
203 n=8) are provided in S33 Table. Graphs were created with GraphPad Prism, and show the
204 mean ± the 95% confidence interval (CI), with non-repeated measures graphs also showing
205 individual data points.
206

Results

No shock controls were built into every Experiment to evaluate how consumption of a high salt (4.0% NaCl) diet, relative to the control (0.4% NaCl) diet, might affect fear behavior in the absence of contextual fear conditioning. For 'training' of no shock controls, no three-way interactions of time × sex × diet were observed in any of the three experiments, nor were two-way interactions of sex × diet nor time × diet (S1 Table). The only two-way interaction evaluated that reached the significance threshold was for a time × sex interaction in training for Experiment 3 (S1 Table), indicating that during 'training' one sex behaved differently over time than the other. In Experiments 1 and 2, no significant main effects of sex nor diet were detected, though both had significant main effects of time (S1 Table), meaning behavior of mice changed over the course of 'training'. This is to be expected when mice are initially exploring a new environment, then become complacent in the absence of any apparent threat. Pairwise comparisons indicated a few time points at which significant differences in freezing behavior between males consuming control and high salt diets were detected (S1A Fig., left and right panels), but these did not exhibit a consistent pattern, and were absent in Experiment 2 (S1A Fig., middle panel), suggesting they were not meaningful.

As with 'training', testing of no shock control mice revealed no significant interactions involving diet, and no main effect of diet (S2 Table), illustrating that diet did not impact freezing behavior in the absence of any fear conditioning. A significant interaction between Experiment × sex was observed, but Holm-Šídák's post-hoc tests revealed no significant differences across the sexes within any Experiment, nor within the sexes across any Experiments (S1B Fig.), indicating at a granular level, there were no Experiment-specific sex differences.

Acquisition of contextual fear resulted in no significant three- nor two-way interactions in any of the three Experiments within either sex (S3-S5 Tables). Likewise, no main effects of Context nor diet were detected (S3-S5 Tables), meaning diet condition did not affect fear learning, and

232 there were no inherent differences in mice to be tested in one Context or the other. In fact, the
233 only significant main effect was that of time, detected across all three Experiments (S3-S5
234 Tables). This illustrates all mice that underwent contextual fear conditioning with foot shocks
235 present during training exhibited increasing levels of freezing (i.e., fear behavior) over the
236 course of the training session (Fig. 2). Pairwise comparisons between male mice to be tested in
237 the Neutral Context indicated that, in Experiment 2, mice consuming high salt exhibited elevated
238 freezing levels following the second and third of the five training foot shocks, relative to mice
239 consuming control diet (Fig. 2B, right panel). All other acquisition curves lacked significant
240 differences across diets and future testing contexts (Fig. 2), indicating that a high salt diet does
241 not impact acquisition of contextual fear after two (Experiments 1, 3) or six (Experiment 2)
242 weeks of consumption.
243

244 Fig. 2. ***Context fear training across Experiments***.
245 Mice assigned to 0.4% NaCl represented by blue symbols, mice assigned to 4.0% NaCl
246 represented by red symbols; mice to be tested in Training Context represented by squares and
247 solid lines, mice to be tested in Neutral Context represented by circles and dotted lines. All
248 mice were trained in the Training Context with five, pseudorandomized mild (1 s, 0.8 mA) foot
249 shocks during a 6 min training session. Percent freezing during training of mice in A)
250 Experiment 1, B) Experiment 2 (grey shading), and C) Experiment 3. Baseline percent freezing
251 averaged across the first two minutes of the training session and plotted along x-axis as 0 time
252 point. Average percent freezing for each 30 sec period following each of the five mild foot
253 shocks are plotted along x-axis as Post-shock Periods 1-5. Experiment 1: 0.4% NaCl females
254 Training Context, n=7; 0.4% NaCl females Neutral Context, n=9; 4.0% NaCl females Training
255 Context, n=8; 4.0% NaCl females Neutral Context, n=9; 0.4% NaCl males Training Context,
256 n=8; 0.4% NaCl males Neutral Context, n=9; 4.0% NaCl males Training Context, n=7; 4.0%

NaCl males Neutral Context, n=8. Experiment 2: 0.4% NaCl females Training Context, n=9; 0.4% NaCl females Neutral Context, n=8; 4.0% NaCl females Training Context, n=8; 4.0% NaCl females Neutral Context, n=8; 0.4% NaCl males Training Context, n=9; 0.4% NaCl males Neutral Context, n=8; 4.0% NaCl males Training Context, n=8; 4.0% NaCl males Neutral Context, n=10. Experiment 3: 0.4% NaCl females Training Context, n=8; 0.4% NaCl females Neutral Context, n=9; 4.0% NaCl females Training Context, n=9; 4.0% NaCl females Neutral Context, n=8; 0.4% NaCl males Training Context, n=7; 0.4% NaCl males Neutral Context, n=8; 4.0% NaCl males Training Context, n=8; 4.0% NaCl males Neutral Context, n=8. Data are graphed as mean ± 95% confidence interval. ǂindicates $p<0.05$ difference between males to be tested in Neutral Context and consuming 0.4% and 4.0% NaCl diets at indicated time points.

Unlike context fear training, where diet was without significant effect on acquisition, high salt diet consumption did significantly affect context fear expression. Moreover, these dietary effects were often sex-selective. Across all three Experiments, there were no significant three-way interactions, nor were there any significant diet × context interactions (S7 Table). However, sex-specific fear behaviors between contexts were significant for Experiments 2 and 3, and fear behaviors across diet and sex combinations were significant for Experiment 3 (S7 Table). Further, diet alone had a significant effect in Experiment 2, and sex and context independently had significant effects on fear behavior in Experiment 1 (S7 Table). In other words, diet was without a significant effect in Experiment 1, but diet did influence the outcomes of Experiments 2 and 3, and for all three Experiments, sex had a significant – and mostly interactional – impact as well.

Holm-Šídák's post-hoc testing helped indicate directionality of high salt consumption's effects on contextual fear expression and generalization across sexes for the three different Experiment timelines employed. Experiment 1 involved training mice after two weeks of diet manipulation,

282 and testing 48 h after training. The expected low (average ~20% or less) freezing levels in the
283 Neutral Context, with higher (average ~40% or more) freezing levels in the Training Context,
284 were observed across nearly all the sex and diet combinations (Fig. 3A). A non-significant trend
285 was noted for contextual fear expression in the Training Context between females and males
286 consuming the high salt diet (p=0.0542; Fig. 3A). Excepting this, Experiment 1 suggests that
287 contextual fear expression after two weeks of high salt diet consumption is unaffected across
288 contexts and sexes.
289

290 Fig. 3. ***Context fear expression across contexts and Experiments***.
291 Mice assigned to 0.4% NaCl represented by blue symbols, mice assigned to 4.0% NaCl
292 represented by red symbols; mice tested in Training Context represented by squares and solid
293 lines, mice tested in Neutral Context represented by circles and dotted lines. Percent freezing
294 during minutes two through six of the 10 min testing session are graphed for all mice. Testing
295 occurred A) 48 h after training in Experiment 1, during which mice underwent two weeks of diet
296 manipulation; B) 48 h after training in Experiment 2 (grey shading), during which mice
297 underwent six weeks of diet manipulation; and C) four weeks after training in Experiment 3,
298 during which mice underwent six total weeks of diet manipulation (training occurred after two
299 weeks of diet manipulation). Experiment 1: 0.4% NaCl females Training Context, n=7; 0.4%
300 NaCl females Neutral Context, n=9; 4.0% NaCl females Training Context, n=8; 4.0% NaCl
301 females Neutral Context, n=9; 0.4% NaCl males Training Context, n=8; 0.4% NaCl males
302 Neutral Context, n=9; 4.0% NaCl males Training Context, n=7; 4.0% NaCl males Neutral
303 Context, n=8. Experiment 2: 0.4% NaCl females Training Context, n=9; 0.4% NaCl females
304 Neutral Context, n=8; 4.0% NaCl females Training Context, n=8; 4.0% NaCl females Neutral
305 Context, n=8; 0.4% NaCl males Training Context, n=9; 0.4% NaCl males Neutral Context, n=8;
306 4.0% NaCl males Training Context, n=8; 4.0% NaCl males Neutral Context, n=10. Experiment

3: 0.4% NaCl females Training Context, n=8; 0.4% NaCl females Neutral Context, n=9; 4.0% NaCl females Training Context, n=9; 4.0% NaCl females Neutral Context, n=8; 0.4% NaCl males Training Context, n=7; 0.4% NaCl males Neutral Context, n=8; 4.0% NaCl males Training Context, n=8; 4.0% NaCl males Neutral Context, n=8. Data are graphed as mean ± 95% confidence interval. *$p<0.05$, **$p<0.01$, ***$p<0.001$ indicate difference between Training Context and Testing Context within same sex and diet. $^{+++}$indicates $p<0.001$ difference between females and males on same diet and tested in same context.

Experiment 2 involved training mice after six weeks of diet manipulation, then testing 48 h following training. Similar to Experiment 1, contextual fear expression was largely unaffected by diet or sex, with one exception. Male mice consuming the control diet exhibited reduced contextual fear expression in the Training Context as compared to female mice consuming the control diet (Fig. 3B); this sex difference in Training Context fear expression was absent in mice of both sexes consuming the high salt diet. As anticipated when testing for contextual fear expression relatively soon after training (in this case, 48 h), freezing behavior averaging ≤ 20% indicated that mice did not exhibit fear behavior in the Neutral Context (see S1 Fig. for no shock comparisons).

Experiment 3 evaluated how high salt intake affects contextual fear generalization by training mice after two weeks of diet manipulation, then testing for contextual fear expression four weeks later (i.e., after 6 wks of diet manipulation). When doing this, contextual fear generalization (freezing ~40% or more in Neutral Context) was observed in all but males consuming a high salt diet (Fig. 3C). In fact, contextual fear expression in females consuming a high salt diet tested in the Neutral Context was so high that it was statistically indifferent from females consuming either diet and tested in the Training Context (Fig. 3C). On the contrary, males tested in the Neutral Context after consuming high salt exhibited significantly lower contextual fear than

332 females tested in the Neutral Context after consuming high salt (Fig. 3C). This indicates a
333 bidirectional, sex-selective effect of high salt consumption on contextual fear generalization.
334 Regardless of diet condition, all mice had *ad libitum* access to drinking water. Nonetheless, we
335 evaluated if serum osmolality was affected across diet condition, sex, or testing Context in mice
336 trained with foot shocks, as well as across diet, sex, and Experiment in control no shock mice.
337 In control no shock mice, no three-way interactions were observed, but a significant two-way
338 interaction where serum osmolality across the sexes differed between Experiments (S11 Table).
339 Despite this interaction, Holm-Šídák's post-hoc testing revealed no significant directional
340 differences when the data were examined between sexes within each Experiment for control no
341 shock mice. Analyses of mice trained with foot shocks revealed zero three- or two-way
342 interactions, and no significant main effects (S12 Table). Serum osmolality was not affected by
343 diet, neither in control no shock mice nor in fear conditioned mice. Taken together, these
344 findings suggest serum osmolality was unaltered in all mice, and thus the behavioral changes
345 observed were not the consequence of osmotic stress. For food, water, salt, and kcal
346 consumption data, plus body weight records, readers are referred to the Supporting Information.
347

348 Fig. 4. ***Serum osmolality in context fear conditioned mice across Experiments***.
349 Mice assigned to 0.4% NaCl represented by blue symbols, mice assigned to 4.0% NaCl
350 represented by red symbols; mice tested in Training Context represented by squares and solid
351 lines, mice tested in Neutral Context represented by circles and dotted lines. Serum osmolality
352 in context fear conditioned mice from A) Experiment 1, B) Experiment 2 (grey shading), and C)
353 Experiment 3. Experiment 1: 0.4% NaCl females Training Context, n=8; 0.4% NaCl females
354 Neutral Context, n=9; 4.0% NaCl females Training Context, n=8; 4.0% NaCl females Neutral
355 Context, n=9; 0.4% NaCl males Training Context, n=8; 0.4% NaCl males Neutral Context, n=9;
356 4.0% NaCl males Training Context, n=8; 4.0% NaCl males Neutral Context, n=8. Experiment 2:

0.4% NaCl females Training Context, n=9; 0.4% NaCl females Neutral Context, n=9; 4.0% NaCl females Training Context, n=8; 4.0% NaCl females Neutral Context, n=8; 0.4% NaCl males Training Context, n=9; 0.4% NaCl males Neutral Context, n=9; 4.0% NaCl males Training Context, n=9; 4.0% NaCl males Neutral Context, n=9. Experiment 3: 0.4% NaCl females Training Context, n=8; 0.4% NaCl females Neutral Context, n=9; 4.0% NaCl females Training Context, n=9; 4.0% NaCl females Neutral Context, n=8; 0.4% NaCl males Training Context, n=7; 0.4% NaCl males Neutral Context, n=8; 4.0% NaCl males Training Context, n=7; 4.0% NaCl males Neutral Context, n=7. Data are graphed as mean ± 95% confidence interval.

In addition to serum osmolality, serum corticosterone levels were also measured in all mice 48 h after completion of context fear testing. Similar to serum osmolality, no three-way interactions were detected in log-transformed serum corticosterone (cort) levels of no shock control mice, but we did find a significant interaction between sexes across Experiments (S13 Table). Unlike with serum osmolality, Holm-Šídák's post-hoc tests revealed directional, diet-specific differences in cort levels within sexes across Experiments, and across sexes within an Experiment (S6 Fig.). Specifically, control-diet consuming females had lower cort levels in Experiment 2 relative to Experiment 1 (p=0.0423). Similarly, high salt-consuming males had lower cort levels in Experiment 3 relative to Experiment 1 (p=0.0109). Within Experiment 3, females consuming high salt had significantly higher cort levels relative to high salt males (p=0.0143). Considering these changes were observed in mice that were not exposed to a foot shock at any point, these data suggest that six weeks of high salt consumption could sex-selectively reduce cort levels in males, but not females. However, the absence of a significant difference (p=0.2369) between cort levels of high salt males between Experiments 1 and 2 challenges this interpretation.

Cort levels in mice that underwent context fear conditioning with foot shocks had no significant three- nor two-way significant interactions (S14 Table). Sex alone had a significant effect on cort levels in Experiments 1 and 3, whereas context specifically influenced cort levels in

383 Experiment 2. Drilling down to directional differences, there were largely no significant
384 differences detected by Holm-Šídák's post-hoc tests (Fig. 5A, 5B). The one exception was that
385 in Experiment 3, males had lower cort levels than females (p=0.0183), specifically between
386 those consuming control diet and tested in the Training Context (Fig. 5C). Similar, but non-
387 significant, trends were noted between females and males tested in the Neutral Context
388 consuming control diet (p=0.0549) or consuming high salt (p=0.0859; Fig. 5C) for Experiment 3.
389 Generally, cort levels did not consistently vary as a result of sex, diet, testing context, nor their
390 interaction, indicating that shifts in circulating corticosterone are not responsible for the
391 observed changes in contextual fear expression nor contextual fear generalization. Circulating
392 cort levels were largely not correlated with contextual fear expression, except in females
393 consuming high salt and tested in the Training Context in Experiment 3 (p=0.039; see S23 Fig.).
394

395 Fig. 5. ***Log-transformed serum corticosterone levels in fear conditioned mice across***
396 ***Experiments***.
397 Mice assigned to 0.4% NaCl represented by blue symbols, mice assigned to 4.0% NaCl
398 represented by red symbols; mice tested in Training Context represented by squares and solid
399 lines, mice tested in Neutral Context represented by circles and dotted lines. Log-transformed
400 serum corticosterone levels in context fear conditioned mice in A) Experiment 1, B) Experiment
401 2 (grey shading), and C) Experiment 3. Experiment 1: 0.4% NaCl females Training Context,
402 n=6; 0.4% NaCl females Neutral Context, n=7; 4.0% NaCl females Training Context, n=7; 4.0%
403 NaCl females Neutral Context, n=7; 0.4% NaCl males Training Context, n=8; 0.4% NaCl males
404 Neutral Context, n=9; 4.0% NaCl males Training Context, n=8; 4.0% NaCl males Neutral
405 Context, n=8. Experiment 2: 0.4% NaCl females Training Context, n=8; 0.4% NaCl females
406 Neutral Context, n=9; 4.0% NaCl females Training Context, n=7; 4.0% NaCl females Neutral
407 Context, n=7; 0.4% NaCl males Training Context, n=9; 0.4% NaCl males Neutral Context, n=9;

4.0% NaCl males Training Context, n=8; 4.0% NaCl males Neutral Context, n=10. Experiment 3: 0.4% NaCl females Training Context, n=7; 0.4% NaCl females Neutral Context, n=8; 4.0% NaCl females Training Context, n=8; 4.0% NaCl females Neutral Context, n=7; 0.4% NaCl males Training Context, n=8; 0.4% NaCl males Neutral Context, n=8; 4.0% NaCl males Training Context, n=8; 4.0% NaCl males Neutral Context, n=7. Data are graphed as mean ± 95% confidence interval. ǂindicates $p<0.05$ difference between females and males on same diet and tested in same context.

Discussion

This is the first investigation into how context fear training, and subsequent expression and generalization, are affected across the sexes by a high salt diet. Our findings suggest that a relatively short (two week) period of high salt consumption might reduce expression of recently learned context fear in males, whereas a longer (six week) consumption period of high salt prevents decreases in expression of recently learned context fear in males. Moreover, context fear generalization was enhanced in females, but attenuated in males, by consumption of a high salt diet. Despite being contrary to our hypothesis regarding males, these data indicate that excess salt consumption affects behavior in a sex-selective manner. Further, these behavioral changes occurred independent of any changes in serum osmolality, and likewise largely did not map onto serum corticosterone levels, indicating that neither osmotic nor physiologic stress states were responsible.

Previous studies assessing how excess salt intake affects context fear behavior exclusively used male rats [25] or male mice [26]. These studies both used higher salt diets of 8.0% NaCl, compared to the 4.0% NaCl diet used here [25,26]. Both teams reported attenuated context fear expression, indexed by freezing behavior. This freezing behavior was measured during testing that occurred in the Training Context either 24 h [26], or both 2 and 24 h [25], after training. Their observations differ from ours, which did not observe significant effects of high salt diet on contextual fear expression in the Training Context. A similarity between the present study and that of Ge and colleagues is the absence of any diet influence on fear acquisition to the auditory cue [26], or in our case, to the context. However, this raises an important caveat regarding both prior investigations - rodents were trained using a cued fear paradigm with an auditory tone [25,26], rather than training the rodents as the present study did with a traditional context fear conditioning protocol that lacks discrete cues. These different training procedures engage distinct brain regions [49,50], and likewise elicit distinguishable patterns of neuronal

442 activation [51–53]. Thus, in the present study we assessed contextual fear expression
443 specifically following contextual fear conditioning, rather than cued fear conditioning.
444 Generalization of contextual fear is a hallmark symptom of many anxiety disorders, and is a
445 conserved cross-species neurobehavioral phenomenon that is useful for understanding how
446 neurophysiological changes influence behaviors [32,54]. The majority of contextual fear
447 generalization investigations in rodents have been performed in males [33], though studies have
448 recently begun to include females. Such sex-inclusive studies indicate that rodents of both
449 sexes can and do exhibit generalized contextual fear, at timepoints both days [55] and weeks
450 [56] after context fear training. Sex differences in the influence of various contextual modalities
451 have been reported (see review [33]), and in our study contextual differences included tactile,
452 olfactory, and visual modalities. We observed robust discrimination between contexts across
453 the sexes when training was temporally proximal to testing, as well as convincing evidence of
454 contextual fear generalization in both sexes consuming control diet when testing occurred four
455 weeks following training. In contrast to our hypothesis that high salt intake would enhance
456 contextual fear generalization in males, we instead found an attenuation of this measure in
457 males, but an enhancement in females. Given the near absence of investigations into how
458 excess salt intake affects female behaviors (see review [28]), plus the relatively scant literature
459 on contextual fear generalization in females (see review [33]), we did not have enough
460 information to formulate an *a priori* hypothesis for females consuming high salt. Some
461 researchers have suggested excess salt intake exacerbates neuropsychiatric symptoms and
462 conditions [29,30]. Our data support this relationship, at least in females. However, the
463 relationship between high salt consumption and anxiety-relevant behaviors was not supported in
464 our male mice, and in fact our data suggest an inverse relationship. Given frequent comorbidity
465 between cardiovascular diseases and pathological anxiety [11–15] (see also reviews [16,17]),
466 and the adverse impact of overconsumption of salt on cardiovascular disease risk [5,10], this
467 finding in males was surprising. Nonetheless, it may be that in males, high salt-mediated

468 cardiovascular pathology is necessary for emergence of increased anxiety-related behaviors,
469 whereas in females excess salt is sufficient. This would parallel findings indicating greater
470 metabolic disruptions in men relative to women with comorbid obesity and mental health
471 disorders [57–59] (see review [60]), greater incidence of depression and anxiety in men versus
472 women with multiple sclerosis [61], and increased incidence of a biomarker of cardiovascular
473 disease in men versus women with depression [62]. However, some literature suggests this is
474 not a consistent finding across disease conditions [63].
475 Along these lines, we evaluated if mice consuming excess salt exhibited peripheral indicators
476 of osmotic (serum osmolality) or physiological (serum corticosterone) stress. Changes in these
477 measures could suggest an overall disrupted state of homeostasis that might confound
478 contextual fear learning and/or expression. For example, osmotic stress can reduce sensitivity
479 to pain [64]. Though the shock level (0.8 mA) and duration (1 sec) that we use are intended to
480 only elicit discomfort/distress, it remains possible that osmotic stress could skew sensory
481 perception of this brief aversive experience. Similarly, endogenous corticosterone levels can
482 influence temporal development of contextual fear generalization as well as the persistence of
483 contextual discrimination [65]. The absence of any sex-, diet-, or context-specific differences in
484 serum osmolality indicates that osmotic stress was not present in our experimental animals. No
485 significant correlations between cort levels and fear expression in the Neutral Context were
486 detected for either sex in Experiment 3. Indeed, of all the mice trained in the Training Context
487 with foot shocks, only Experiment 3 females consuming high salt and tested in the Training
488 Context displayed a significant (negative) correlation between context fear expression and cort
489 levels (S23 Fig.). The absence of any significant correlations between cort levels and any other
490 sex/diet/context combination across Experiments indicates that cort levels overall probably did
491 not confound observed context fear expression and generalization.

492 # Limitations

493 Limitations of this study include singly housing of mice to allow for tracking of individual
494 food/water/salt/kcal consumption and changes in body weight, meaning that mice were
495 excluded from social stimulation. Some data suggests behavioral responses to single housing
496 differs across the sexes in mice and rats (see review [66]), so this might have influenced sex-
497 specific behaviors observed in the present study. However, a meta-analysis of 293 male and
498 female mouse studies indicates that singly housing mice reduces coefficients of variation in trait
499 variability by 37% in both sexes [67], suggesting our housing condition may have reduced the
500 variability of our data. Diet manipulations were relatively short (two or six weeks), restricted to
501 non-breeding adults, and contained no other accompanying unhealthy dietary components (e.g.,
502 low fiber, high fat). Only a single shock level and context fear conditioning paradigm was used,
503 and mice were only tested in a single context. Thus, for future studies it will be important to
504 evaluate behavior in group housed mice, assess longer diet manipulation periods and include
505 additional unhealthy dietary components, and study how consumption of excess salt by younger
506 mice affects their fear behaviors. Testing of behavior during the natural activity period of mice
507 would be informative, as would assessing generalization and concurrent cort levels during
508 different stages of the circadian cycle [68].

509 Conclusions

510 Our study provides foundational evidence of sex-selective consequences of excess salt intake
511 on context fear expression and generalization. Additional studies will be essential to flesh out
512 these initial findings and determine what brain cell populations are most impacted by excess salt
513 intake, and how the effects of high salt might be mitigated. One logical starting point is
514 evaluation of microglia, given converging evidence that both fear processing [69,70] and high
515 salt intake [42,71,72] affect microglial activation. Moreover, oral administration of the brain-
516 penetrant anti-inflammatory drug minocycline can mitigate microglial activation and behavioral
517 changes in rodents consuming high salt [42], and the same drug attenuated fear measures in

518 human participants [73]. Future studies should also assess how excess salt intake affects cued
519 fear expression and discrimination, and in so doing begin identifying how specific limbic brain
520 regions might differentially be affected in their functionality and/or connectivity following
521 extended periods of excess salt consumption. The evidence presented here, in conjunction with
522 past literature focused on salt's effects on behaviors outside of the fear domain(reviewed in
523 [28]), indicates that salt is an underappreciated, non-caloric influence on conserved mammalian
524 behaviors.

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Supporting Information

Tables

S1 Table. Three-way repeated measures ANOVAs on context fear 'training' for control no shock mice across Experiments.

S2 Table. Three-way ANOVAs on context fear testing of control no shock mice across Experiments.

S3 Table. Three-way repeated measures ANOVAs on context fear training for context fear conditioned mice of both sexes in Experiment 1.

S4 Table. Three-way repeated measures ANOVAs on context fear training for context fear conditioned mice of both sexes in Experiment 2.

S5 Table. Three-way repeated measures ANOVAs on context fear training for context fear conditioned mice of both sexes in Experiment 3.

S6 Table. Three-way repeated measures ANOVAs on full 10 min time course of context fear testing for control no shock mice across Experiments.

S7 Table. Three-way ANOVAs on context fear expression (min 2-6) for context fear conditioned mice across Experiment.

S8 Table. Three-way repeated measures ANOVAs on full 10 min time course of context fear testing for mice of both sexes in Experiment 1.

S9 Table. Three-way repeated measures ANOVAs on full 10 min time course of context fear testing for mice of both sexes in Experiment 2.

S10 Table. Three-way repeated measures ANOVAs on full 10 min time course of context fear testing for mice of both sexes in Experiment 3.

S11 Table. Three-way ANOVAs on serum osmolality in control no shock mice across Experiments.

S12 Table. Three-way ANOVAs on serum osmolality in context fear conditioned mice across Experiments.

S13. Three-way ANOVAs on log-transformed serum corticosterone levels in control no shock mice across Experiments.

S14 Table. Three-way ANOVAs on log-transformed serum corticosterone in context fear conditioned mice across Experiments.

S15 Table. Three-way repeated measures ANOVAs on weekly average water consumption per day for context fear conditioned mice across Experiments.

S16 Table. Three-way repeated measures ANOVAs on weekly average food consumption per day for context fear conditioned mice across Experiments.

S17 Table. Three-way repeated measures ANOVAs on weekly body weight change for context fear conditioned mice across Experiments.

S18 Table. Three-way repeated measures ANOVAs on twice weekly body weight measurements of context fear conditioned mice across Experiments.

S19 Table. Three-way repeated measures ANOVAs on weekly average water consumption per day for control no shock mice across Experiments.

S20 Table. Three-way repeated measures ANOVAs on weekly average food consumption per day for control no shock mice across Experiments.

S21 Table. Three-way repeated measures ANOVAs on weekly body weight changes for control no shock mice across Experiments.

S22 Table. Three-way repeated measures ANOVAs on twice weekly body weights of control no shock mice across Experiments.

S23 Table. Three-way repeated measures ANOVAs on weekly average NaCl consumed per day by context fear conditioned mice across Experiments.

S24 Table. Three-way repeated measures ANOVAs on weekly average NaCl consumed as a percentage of body weight in context fear conditioned mice across Experiments.

S25 Table. Three-way repeated measures ANOVAs on weekly ratio of water to NaCl consumed in context fear conditioned mice across Experiments.

827

828 **S26 Table. Three-way repeated measures ANOVAs on weekly average kcal consumed**
829 **per day by context fear conditioned mice across Experiments.**

830

831 **S27 Table. Three-way repeated measures ANOVAs on weekly kcal consumed as a**
832 **percentage of body weight in context fear conditioned mice across Experiments.**

833

834 **S28 Table. Three-way repeated measures ANOVAs on weekly average NaCl consumed**
835 **per day by control no shock mice across Experiments.**

836

837 **S29 Table. Three-way repeated measures ANOVAs on weekly NaCl consumed as a**
838 **percentage of body weight by control no shock mice across Experiments.**

839

840 **S30 Table. Three-way repeated measures ANOVAs on weekly water to NaCl ratio**
841 **consumed by control no shock mice across Experiments.**

842

843 **S31 Table. Three-way repeated measures ANOVAs on weekly average kcal consumed**
844 **per day by control no shock mice across Experiments.**

845

846 **S32 Table. Three-way repeated measures ANOVAs on weekly average kcal consumed as**
847 **a percentage of body weight by control no shock mice across Experiments.**

848

849 **S33 Table. Details of confirmed outliers (> 5 standard deviations (SD) ± average of data**
850 **set sans suspected outlier) within respective data sets. Outliers are marked with red**
851 **font.**

852 # Figures

853 **S1 Fig. Fear behavior in no shock control mice across Experiments.**

854

855 **S2 Fig. Fear behavior in no shock control mice across Experiments with shortened Y**

856 **axes.**

857

858 **S3 Figure. Time course of context fear expression testing in no shock groups**.

859

860 **S4 Fig. Time course of context fear expression testing in shock groups**.

861

862 **S5 Fig. Serum osmolality in no shock control mice across Experiments**.

863

864 **S6 Fig. Log-transformed serum corticosterone levels of no shock control mice across**

865 **Experiments**.

866

867 **S7 Fig. Raw (pre-transformed) serum corticosterone levels of no shock control mice**

868 **across Experiments**.

869

870 **S8 Fig. Raw (pre-transformed) serum corticosterone levels in fear conditioned mice**

871 **across Experiments**.

872

873 **S9 Fig. Average water consumed per day by context fear conditioned mice across**

874 **Experiments.**

875

S10 Fig. Average food consumed per day by context fear conditioned mice across Experiments.

S11 Fig. Weekly body weight change in context fear conditioned mice across Experiments.

S12 Fig. Body weight of context fear conditioned mice across Experiments.

S13 Fig. Average water and food consumed per day by control no shock mice across Experiments.

S14 Fig. Body weights and body weight changes in control no shock mice across Experiments.

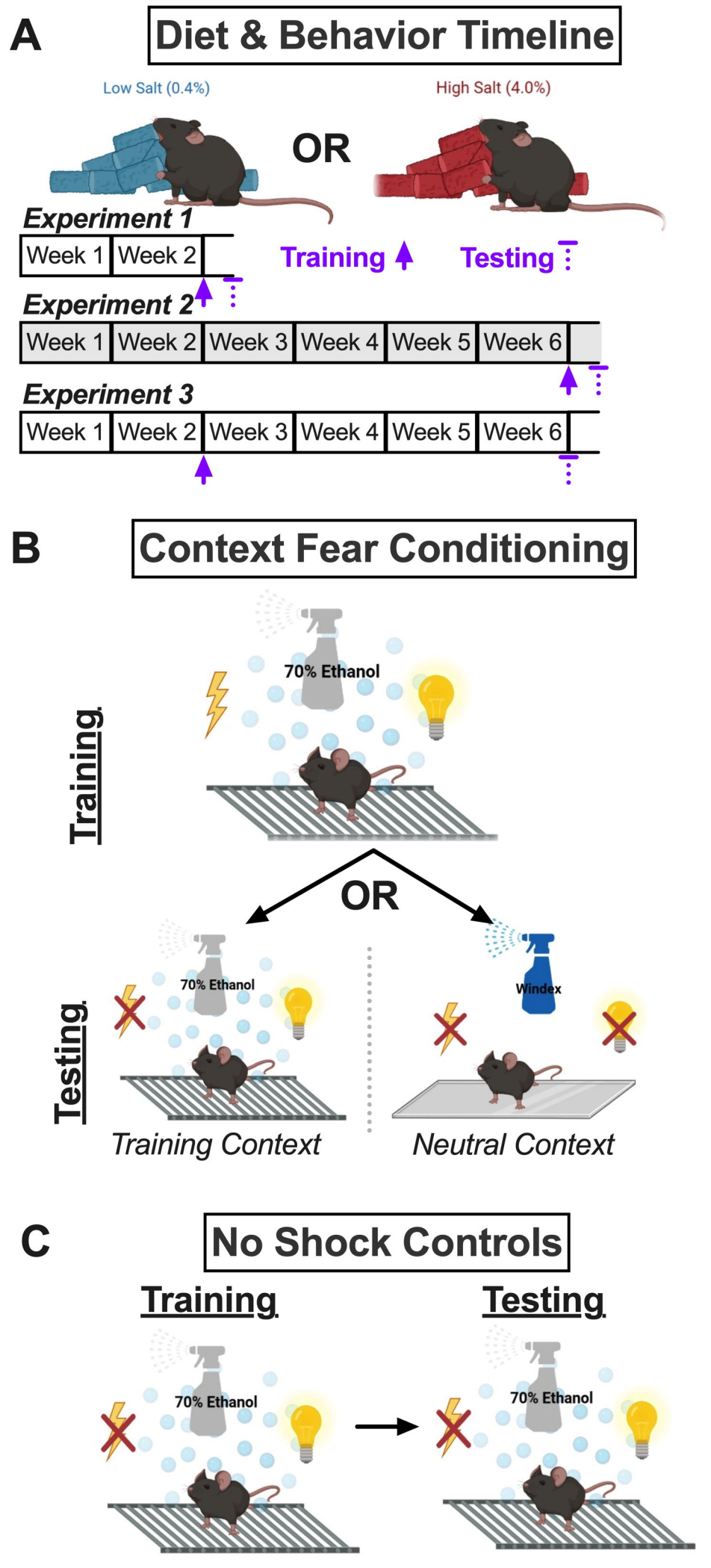
S15 Fig. Average NaCl consumed per day by context fear conditioned mice across Experiments.

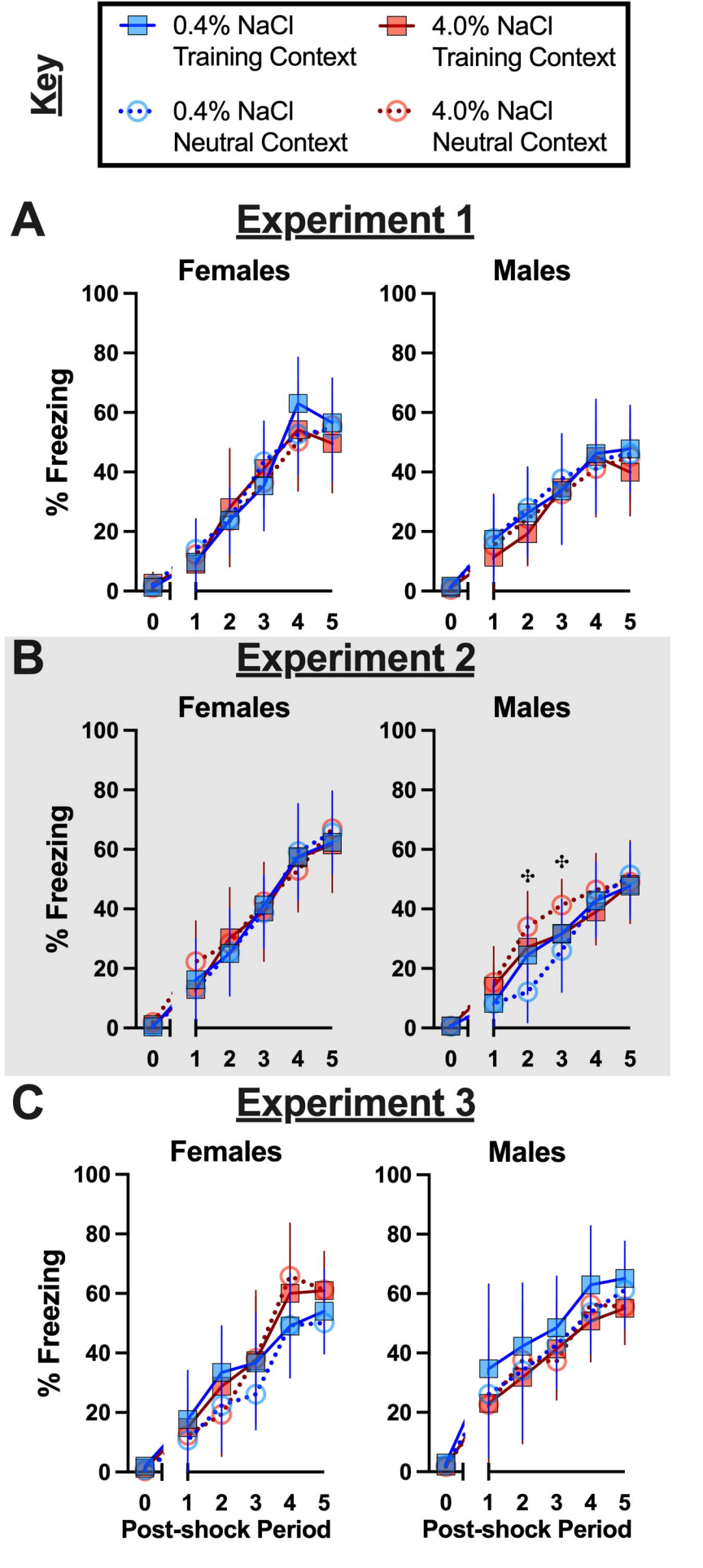
S16 Fig. NaCl consumed as a percentage of body weight by context fear conditioned mice across Experiments.

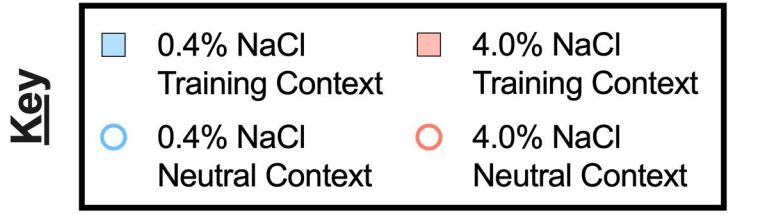
S17 Fig. Water to NaCl consumed ratio for context fear conditioned mice across Experiments.

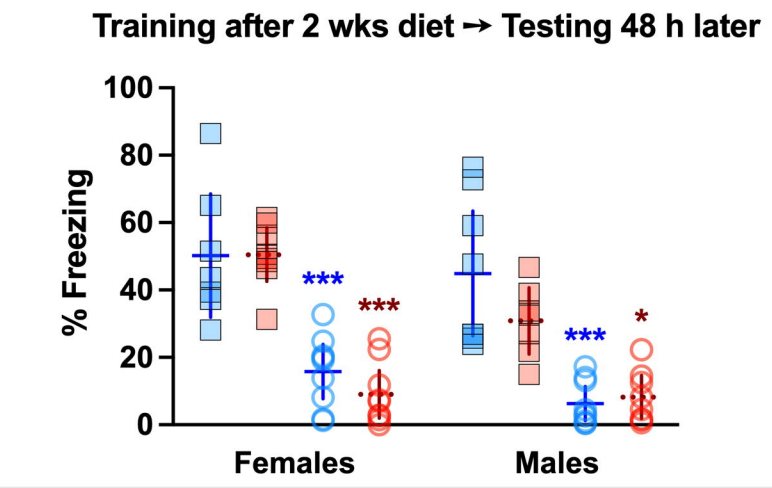
S18 Fig. Average kcal consumed per day by context fear conditioned mice across Experiments.

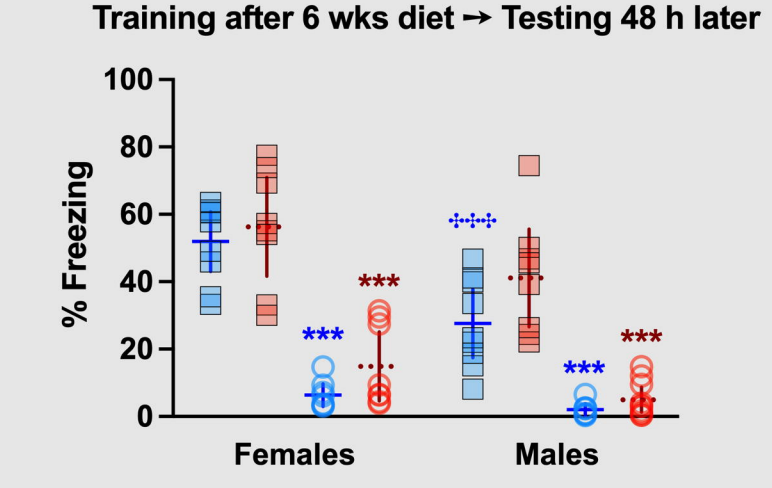
902 **S19 Fig. Consumed kcal as a percentage of body weight by context fear conditioned**
903 **mice across Experiments.**
904
905 **S20 Fig. Average NaCl consumed per day and NaCl consumed as a percentage of body**
906 **weight by control no shock mice across Experiments.**
907
908 **S21 Figure. Ratio of water to NaCl consumed by control no shock mice across**
909 **Experiments.**
910
911 **S22 Fig. Average kcal consumed per day and kcal consumed as a percentage of body**
912 **weight by control no shock mice across Experiments.**
913
914 **S23 Fig. Correlations between context fear expression and log-transformed serum**
915 **corticosterone levels across contexts and Experiments.**
916
917 **S24 Figure. Correlations between context fear expression and log-transformed serum**
918 **corticosterone levels in control no shock mice across Experiments.**

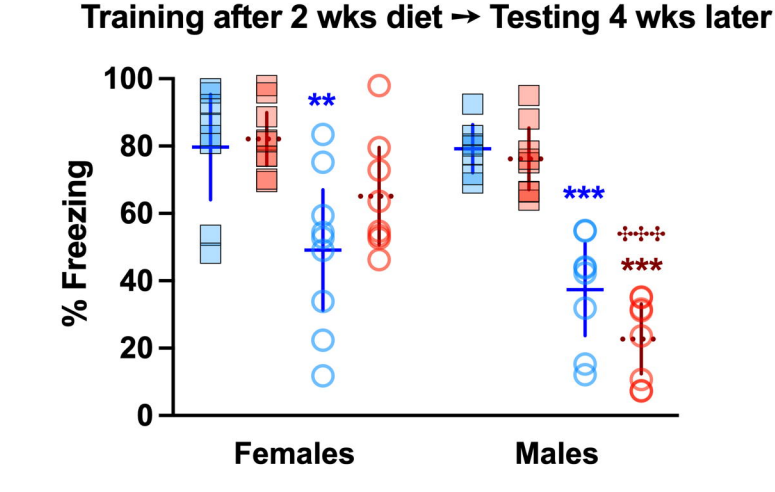

A
Diet & Behavior Timeline
Low Salt (0.4%)
High Salt (4.0%)
OR
Experiment 1
Week 1
Week 2
Training
Testing
Experiment 2
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6
Experiment 3
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6
B
Context Fear Conditioning
Training
70% Ethanol
OR
Testing
70% Ethanol
Windex
Training Context
Neutral Context
C
No Shock Controls
Training
Testing
70% Ethanol
70% Ethanol


Key
0.4% NaCl Training Context
4.0% NaCl Training Context
0.4% NaCl Neutral Context
4.0% NaCl Neutral Context
A
Experiment 1
Females
Males
% Freezing
100
80
60
40
20
0
0 1 2 3 4 5
B
Experiment 2
Females
Males
% Freezing
C
Experiment 3
Females
Males
% Freezing
Post-shock Period
Post-shock Period

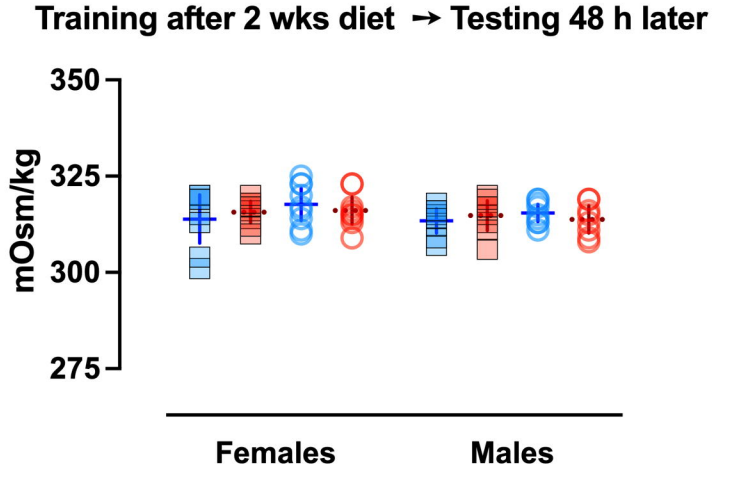

Key
0.4% NaCl Training Context
4.0% NaCl Training Context
0.4% NaCl Neutral Context
4.0% NaCl Neutral Context


A
Experiment 1
Training after 2 wks diet → Testing 48 h later
% Freezing
100
80
60
40
20
0
***
***
***
*
Females
Males

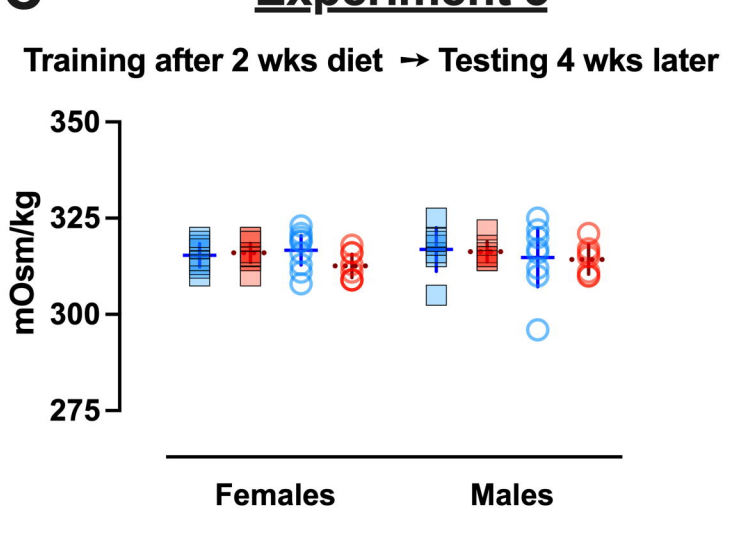

B
Experiment 2
Training after 6 wks diet → Testing 48 h later
% Freezing
100
80
60
40
20
0
***
***
***
***
Females
Males

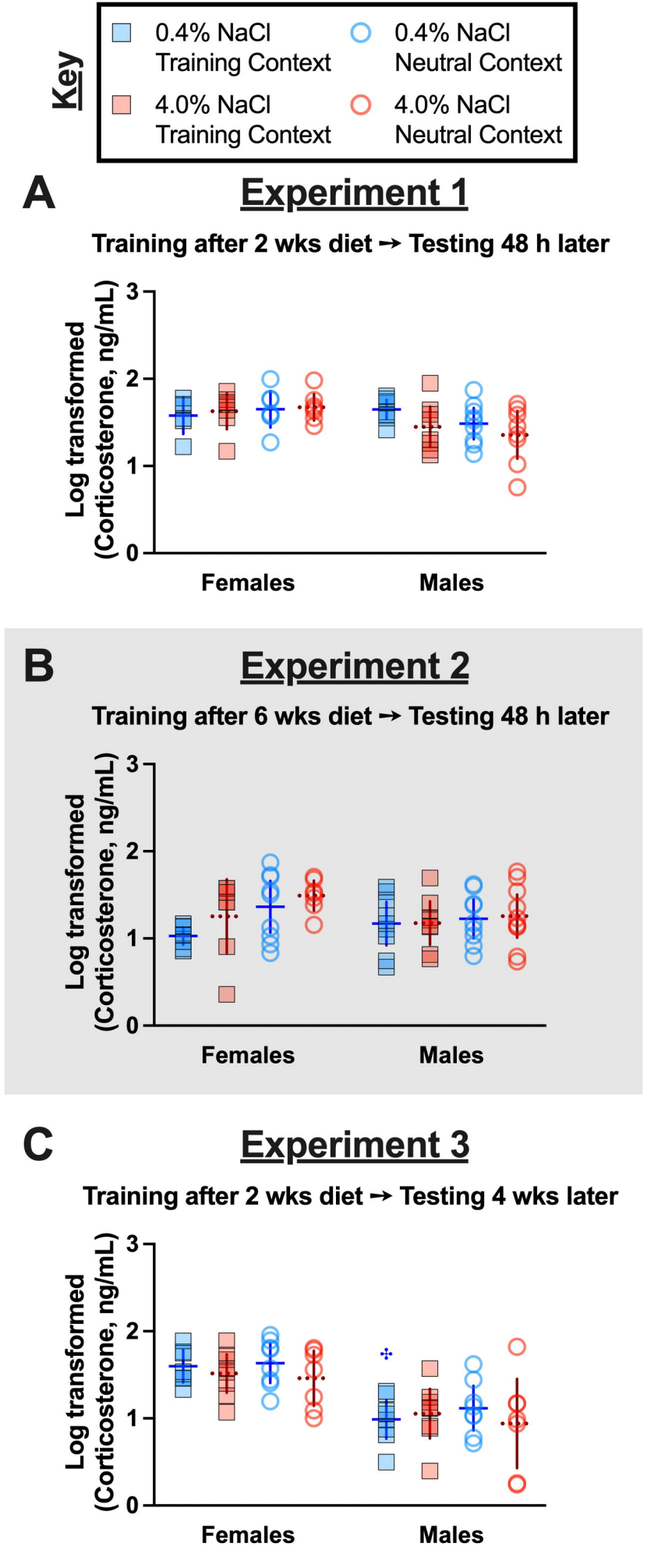

C
Experiment 3
Training after 2 wks diet → Testing 4 wks later
% Freezing
100
80
60
40
20
0
**
***
***
Females
Males

Key
0.4% NaCl Training Context
0.4% NaCl Neutral Context
4.0% NaCl Training Context
4.0% NaCl Neutral Context


A
Experiment 1
Training after 2 wks diet → Testing 48 h later
mOsm/kg
350
325
300
275
Females
Males

B
Experiment 2
Training after 6 wks diet → Testing 48 h later
mOsm/kg
350
325
300
275
Females
Males


C
Experiment 3
Training after 2 wks diet → Testing 4 wks later
mOsm/kg
350
325
300
275
Females
Males


Key
0.4% NaCl Training Context
0.4% NaCl Neutral Context
4.0% NaCl Training Context
4.0% NaCl Neutral Context
A
Experiment 1
Training after 2 wks diet → Testing 48 h later
Log transformed (Corticosterone, ng/mL)
0
1
2
3
Females
Males
B
Experiment 2
Training after 6 wks diet → Testing 48 h later
Log transformed (Corticosterone, ng/mL)
0
1
2
3
Females
Males
C
Experiment 3
Training after 2 wks diet → Testing 4 wks later
Log transformed (Corticosterone, ng/mL)
0
1
2
3
Females
Males