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

Jasmin N. Beaver, Brady L. Weber, Matthew T. Ford, Anna E. Anello, Kaden M. Ruffin, Sarah K. Kassis, T. Lee Gilman*

Department of Psychological Sciences, Brain Health Research Institute, & Healthy Communities Research Institute

Kent State University, Kent, OH, 44242, USA

*Corresponding Author

T. Lee Gilman, Ph.D.

lgilman1@kent.edu

209 Kent Hall Addition

600 Hilltop Dr.

Department of Psychological Sciences

Kent State University

Kent, OH 44242

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]. Far less understood is how excess salt intake might affect neuropsychiatric symptoms like generalized (i.e., non-specific) anxiety, though some researchers have begun to recognize this possibility[23,24]. 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[25–28]. 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[29–37], while other brain regions remain underexplored. This evidence, combined with human

evidence of comorbidity between anxiety disorders and cardiovascular diseases, supports our hypothesis – at least based upon male data – that fear generalization is augmented by high salt intake. Unfortunately, both the scant literature on behavioral effects of increased salt intake, and the larger body of work on the neurocircuitry of fear generalization, have focused almost exclusively on male rodents. Here, we included both sexes in our studies, but given the current state of the literature we could not formulate data-based *a priori* hypotheses specifically regarding how female fear generalization behavior might differ from male fear generalization under conditions of excess salt intake.

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; Figure 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 Supplemental Material for statistics and graphs on average water consumed per day (Supplemental Tables S1, S5; Supplemental Figures S1, S5A-C); average food consumed per day (Supplemental Tables S2, S6; Supplemental Figures S2, S5D-F); weekly body weight changes (Supplemental Tables S3, S7; Supplemental Figures S3, S6D-F); raw body weights (Supplemental Tables S4, S8; Supplemental Figures S4, S6A-C); average NaCl consumed per day (Supplemental Tables S9, S14; Supplemental Figures S7, S12A-C); average NaCl consumed as percentage of body weight (Supplemental Tables S10, S15; Supplemental Figures S8, S12D-F); weekly ratio between water:NaCl consumption (Supplemental Tables S11, S16; Supplemental Figures S9,

S13); average kcal consumed per day (Supplemental Tables S12, S17; Supplemental Figures S10, S14A-C); and average kcal consumed as percentage of body weight (Supplemental Tables S13, S18; Supplemental Figures S11, S14D-F).

Diet manipulations lasted for two weeks (Experiment 1; Figure 1A) or six weeks (Experiments 2 and 3; Figure 1A). All procedures were approved by the Kent State University Institutional Animal Care and Use Committee, and adhered to the National Research Council's Guide for the Care and Use of Laboratory Animals, 8th Ed.[38].

Experimental timelines

Experimental timelines are graphically illustrated in Figure 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[39]).

Figure 1

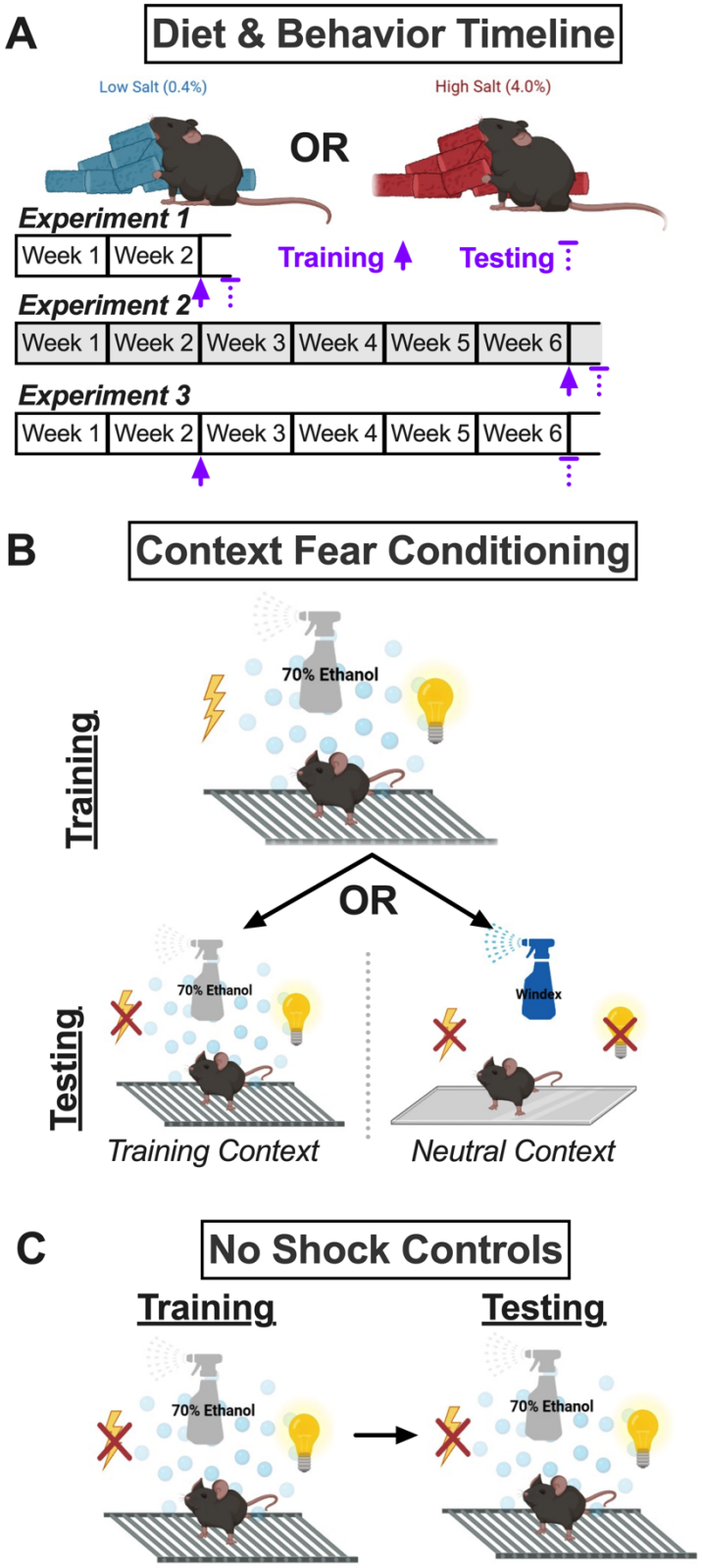
A
Diet & Behavior Timeline
Low Salt (0.4%)
High Salt (4.0%)
OR
Experiment 1
Week 1
Week 2
Training
Testing
Experiment 2
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6
Experiment 3
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6
B
Context Fear Conditioning
Training
70% Ethanol
OR
Testing
70% Ethanol
Windex
Training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

Figure 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.

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 (Figure 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 (Figure 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. 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.

Context fear testing

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

No shock controls

Control ‘no shock’ mice were run concurrently in every cohort of animals (Figure 1C) for every Experiment. These no shock mice were exposed to the Training Context for ‘training’, but no foot shock was ever administered. Likewise, no shock control mice were tested in the Training

Context, to evaluate how diet manipulation and the Experiment timelines influenced freezing behavior in the absence of any contextual fear conditioning.

Serum measurements

Forty-eight hours after testing for context fear expression, mice were briefly anesthetized with isoflurane then rapidly decapitated for collection of trunk blood. Trunk blood was allowed to clot at room temperature for 30 min, then was spun at 3500 rpm for 1 h at 4°C. Serum was then removed from tubes and aliquoted into two vials – one for measuring serum osmolality, the other for measuring serum corticosterone. Serum osmolality was measured immediately using an Osmo1 single-sample micro-osmometer (Advanced Instruments, Norwood, MA). Prior to every serum batch, a Clinitrol™ 290 reference solution (Advanced Instruments) from a new ampule was measured to confirm accurate readings. Samples for serum corticosterone were 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[40–42]. 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. 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. The criterion to exclude outliers was *a priori* assigned as being >5 standard deviations ± mean. Details of outliers that were excluded (training baseline, n=1; no shock testing, n=1; shock testing, n=4; osmolality, n=1; log-transformed serum corticosterone, n=2; water:NaCl consumption ratio across weeks, n=8) are provided in Supplemental Table 19. Graphs were created with GraphPad Prism, and show the mean ± the 95% confidence interval (CI), with non-repeated measures graphs also showing individual data points.

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 (Table 1A-C). The only two-way interaction evaluated that reached the significance threshold was for a time × sex interaction in training for Experiment 3 (Table 1C). In Experiments 1 and 2, no significant main effects of sex nor diet were detected, though both had significant main effects of time (Table 1A, 1B). Pairwise comparisons indicated a few time points at which significant differences in freezing behavior between males consuming control and high salt diets were detected (Figure 2A, left and right panels), but these did not exhibit a consistent pattern, and were absent in Experiment 2 (Figure 2A, middle panel).

As with 'training', testing of no shock control mice revealed no significant interactions between Experiment × sex × diet, no two-way interactions between Experiment × diet or sex × diet, and no main effect of diet (Table 2). 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 (Figure 2B). Overall, consumption of a high salt diet did not elicit any significant changes in baseline fear behavior in the absence of any contextual fear conditioning, neither during 'training' nor testing (Figure 2).

Table 1. Three-way repeated measures ANOVAs on context fear 'training' for control no shock mice across Experiments.

Table 1A

Experiment 1	**No Shock – Context Fear Training**
Sex	F(1,30)=0.036 p=0.850 partial η^2=0.001
Diet	F(1,30)=1.003 p=0.325 partial η^2=0.032
Time	F(2.92,87.68)=3.688 **p=0.016** partial η^2=**0.109**
Time × Sex	F(2.92,87.68)=2.055 p=0.114 partial η^2=0.064
Time × Diet	F(2.92,87.68)=0.653 p=0.579 partial η^2=0.021
Sex × Diet	F(1,30)=0.000 p=0.990 partial η^2=0.000
Time × Sex × Diet	F(2.92,87.68)=2.404 p=0.074 partial η^2=0.074

Table 1B

Experiment 2	**No Shock – Context Fear Training**
Sex	F(1,30)=0.516 p=0.478 partial η^2=0.017
Diet	F(1,30)=0.012 p=0.915 partial η^2=0.000
Time	F(3.05,91.62)=6.466 **p<0.001** partial η^2=**0.177**
Time × Sex	F(3.05,91.62)=1.258 p=0.294 partial η^2=0.040
Time × Diet	F(3.05,91.62)=1.032 p=0.383 partial η^2=0.033
Sex × Diet	F(1,30)=3.135 p=0.087 partial η^2=0.095
Time × Sex × Diet	F(3.05,91.62)=0.710 p=0.551 partial η^2=0.023

Table 1C

Experiment 3	**No Shock – Context Fear Training**
Sex	F(1,27)=11.10 p=0.003 partial η^2=0.291
Diet	F(1,27)=0.245 p=0.625 partial η^2=0.009
Time	F(2.44,65.88)=3.016 p=0.046 partial η^2=0.100
Time × Sex	F(2.44,65.88)=3.091 **p=0.042** partial η^2=**0.103**
Time × Diet	F(2.44,65.88)=0.668 p=0.545 partial η^2=0.024
Sex × Diet	F(1,27)=2.753 p=0.109 partial η^2=0.093
Time × Sex × Diet	F(2.44,65.88)=1.590 p=0.207 partial η^2=0.056

Table 2. Three-way ANOVAs on context fear testing of control no shock mice across Experiments.

Context Fear Expression	**No Shock Groups Across Experiments**		
Sex	F(1,87)=2.852	p=0.095	partial η^2=0.032
Diet	F(1,87)=0.497	p=0.483	partial η^2=0.006
Experiment	F(2,87)=3.187	p=0.046	partial η^2=0.068
Sex × Diet	F(1,87)=0.748	p=0.390	partial η^2=0.009
Sex × Experiment	F(2,87)=6.137	**p=0.003**	partial η^2=**0.124**
Diet × Experiment	F(2,87)=0.839	p=0.436	partial η^2=0.019
Sex × Diet × Experiment	F(2,87)=0.447	p=0.641	partial η^2=0.010

Figure 2

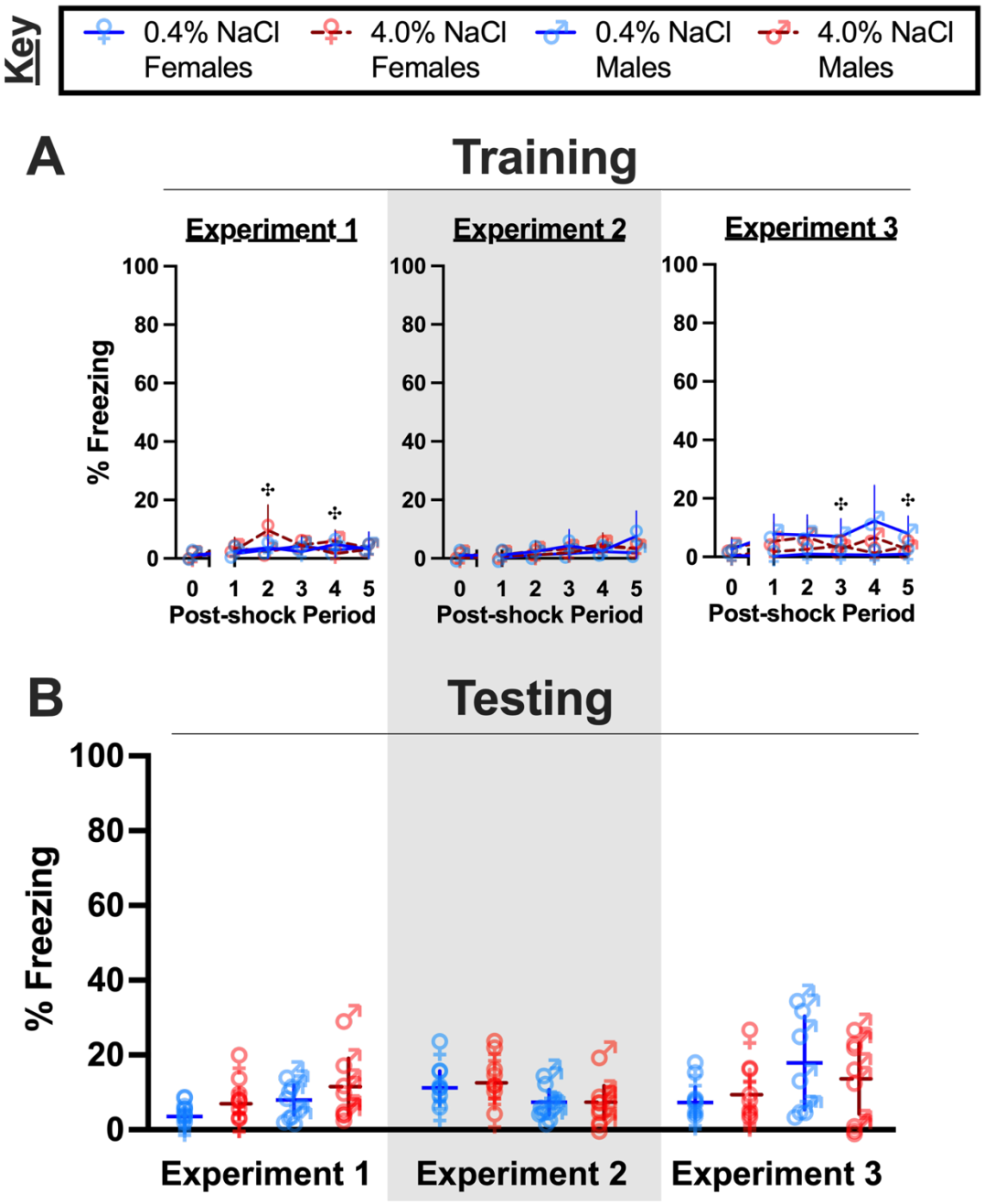

Figure 2. ***Fear behavior in no shock control mice across Experiments***.

Females represented by venus symbols, males by mars symbols; 0.4% NaCl represented by blue symbols and solid lines, 4.0% NaCl represented by red symbols and dashed lines. A) Percent freezing during 'training' of control mice exposed to the Training Context, but receiving

no foot shocks for the duration of the 6 min ‘training’ session. B) Percent freezing during testing of control no shock mice exposed to the Training Context for a 10 min testing session. Experiment 1: 0.4% NaCl females, n=9; 4.0% NaCl females, n=9; 0.4% NaCl males, n=8; 4.0% NaCl males, n=8. Experiment 2 (grey shading): 0.4% NaCl females, n=8; 4.0% NaCl females, n=8; 0.4% NaCl males, n=9; 4.0% NaCl males, n=9. Experiment 3: 0.4% NaCl females, n=8; 4.0% NaCl females, n=8; 0.4% NaCl males, n=7; 4.0% NaCl males, n=8. Data are graphed as mean ± 95% confidence interval. $^{+}$indicates $p<0.05$ difference between males consuming 0.4% and 4.0% NaCl diets at indicated time points.

Acquisition of contextual fear resulted in no significant three- nor two-way interactions in any of the three Experiments within either sex (Tables 3-5). In fact, the only significant main effect was that of time, detected across all three Experiments (Tables 3-5). This illustrates all mice that underwent contextual fear conditioning with foot shocks present during training exhibited increasing levels of freezing (i.e., fear behavior) over the course of the training session (Figure 3). Pairwise comparisons indicated that, in Experiment 2, male mice consuming high salt and to be tested in the Neutral Context exhibited elevated freezing levels following the second and third of the five training foot shocks, relative to male mice consuming control diet and to be tested in the Neutral Context (Figure 3B, right panel). All other acquisition curves lacked significant differences across diets and future testing contexts (Figure 3), indicating that a high salt diet does not impact acquisition of contextual fear after two (Experiments 1, 3) or six (Experiment 2) weeks of consumption.

Table 3. Three-way repeated measures ANOVAs on context fear training for context fear conditioned mice of both sexes in Experiment 1.

Table 3A

Females	**Experiment 1 – Context Fear Training**
Diet	F(1,29)=0.145 p=0.706 partial η^2=0.005
Context	F(1,29)=0.009 p=0.927 partial η^2=0.000
Time	F(4.00,115.9)=111.3 **p<0.001** partial η^2=**0.793**
Time × Diet	F(4.00,115.9)=0.377 p=0.824 partial η^2=0.013
Time × Context	F(4.00,115.9)=0.844 p=0.500 partial η^2=0.028
Diet × Context	F(1,29)=0.024 p=0.879 partial η^2=0.001
Time × Diet × Context	F(4.00,115.9)=0.849 p=0.497 partial η^2=0.028

Table 3B

Males	**Experiment 1 – Context Fear Training**
Diet	F(1,28)=0.538 p=0.470 partial η^2=0.019
Context	F(1,28)=0.033 p=0.857 partial η^2=0.001
Time	F(3.56,99.68)=59.79 **p<0.001** partial η^2=**0.681**
Time × Diet	F(3.56,99.68)=0.167 p=0.941 partial η^2=0.006
Time × Context	F(3.56,99.68)=0.271 p=0.877 partial η^2=0.010
Diet × Context	F(1,28)=0.013 p=0.909 partial η^2=0.000
Time × Diet × Context	F(3.56,99.68)=0.266 p=0.880 partial η^2=0.009

Table 4. Three-way repeated measures ANOVAs on context fear training for context fear conditioned mice of both sexes in Experiment 2.

Table 4A

Females	**Experiment 2 – Context Fear Training**
Diet	F(1,29)=0.083 p=0.775 partial η^2=0.003
Context	F(1,29)=0.097 p=0.758 partial η^2=0.003
Time	F(4.11,119.1)=121.2 **p<0.001** partial η^2=**0.807**
Time × Diet	F(4.11,119.1)=0.354 p=0.845 partial η^2=0.012
Time × Context	F(4.11,119.1)=0.259 p=0.908 partial η^2=0.009
Diet × Context	F(1,29)=0.104 p=0.750 partial η^2=0.004
Time × Diet × Context	F(4.11,119.1)=0.537 p=0.714 partial η^2=0.018

Table 4B

Males	**Experiment 2 – Context Fear Training**
Diet	F(1,31)=2.080 p=0.159 partial η^2=0.063
Context	F(1,31)=0.111 p=0.741 partial η^2=0.004
Time	F(3.26,101.0)=103.1 **p<0.001** partial η^2=**0.769**
Time × Diet	F(3.26,101.0)=2.166 p=0.091 partial η^2=0.065
Time × Context	F(3.26,101.0)=0.402 p=0.768 partial η^2=0.013
Diet × Context	F(1,31)=1.332 p=0.257 partial η^2=0.041
Time × Diet × Context	F(3.26,101.0)=1.512 p=0.213 partial η^2=0.047

Table 5. Three-way repeated measures ANOVAs on context fear training for context fear conditioned mice of both sexes in Experiment 3.

Table 5A

Females	**Experiment 3 – Context Fear Training**
Diet	F(1,30)=0.777 p=0.385 partial η^2=0.025
Context	F(1,30)=0.514 p=0.479 partial η^2=0.017
Time	F(3.82,114.7)=100.9 **p<0.001** partial η^2=**0.771**
Time × Diet	F(3.82,114.7)=2.287 p=0.067 partial η^2=0.071
Time × Context	F(3.82,114.7)=1.008 p=0.404 partial η^2=0.033
Diet × Context	F(1,30)=0.250 p=0.621 partial η^2=0.008
Time × Diet × Context	F(3.82,114.7)=0.185 p=0.940 partial η^2=0.006

Table 5B

Males	**Experiment 3 – Context Fear Training**
Diet	F(1,27)=1.070 p=0.310 partial η^2=0.038
Context	F(1,27)=0.241 p=0.628 partial η^2=0.009
Time	F(2.84,76.71)=79.15 **p<0.001** partial η^2=**0.746**
Time × Diet	F(2.84,76.71)=0.332 p=0.792 partial η^2=0.012
Time × Context	F(2.84,76.71)=0.146 p=0.924 partial η^2=0.005
Diet × Context	F(1,27)=0.533 p=0.472 partial η^2=0.019
Time × Diet × Context	F(2.84,76.71)=0.413 p=0.733 partial η^2=0.015

Figure 3

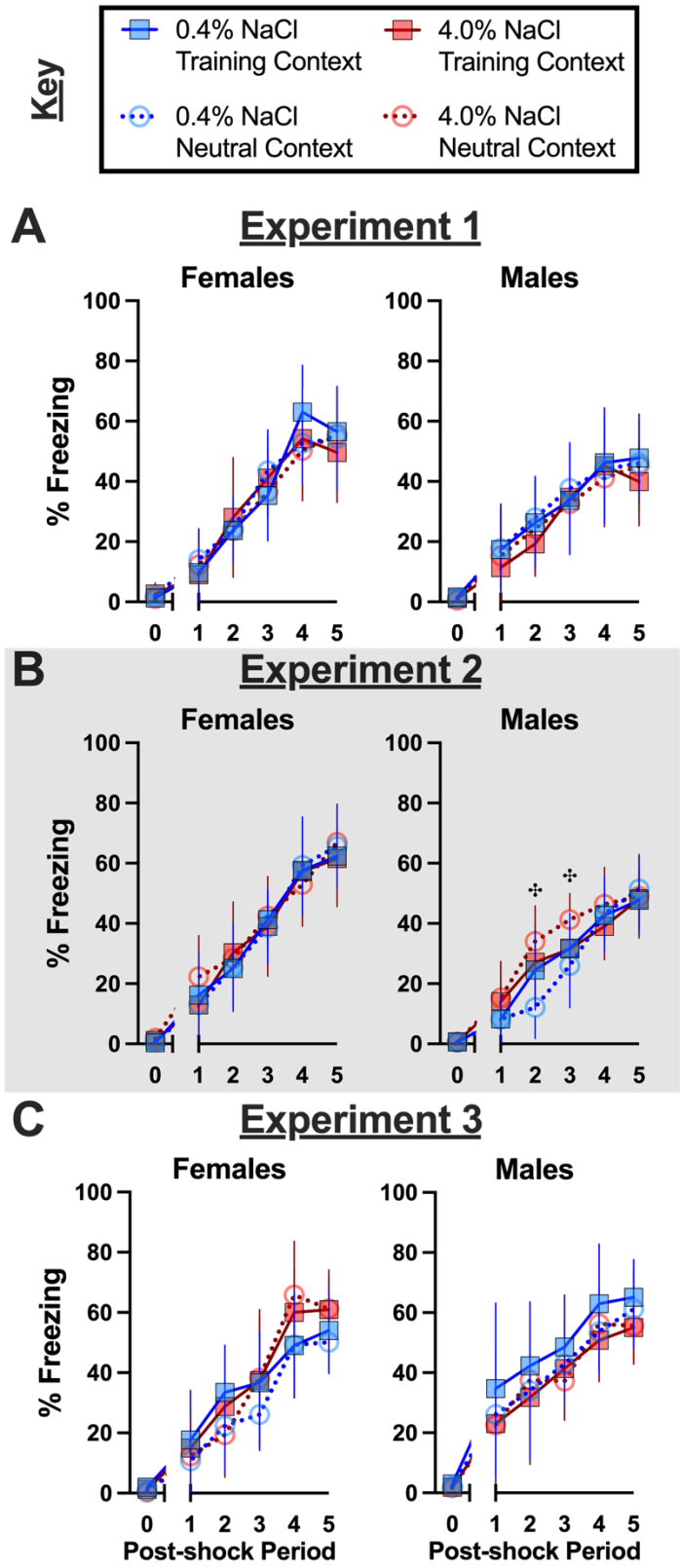
Key
0.4% NaCl Training Context
4.0% NaCl Training Context
0.4% NaCl Neutral Context
4.0% NaCl Neutral Context
A
Experiment 1
Females
Males
% Freezing
B
Experiment 2
Females
Males
% Freezing
C
Experiment 3
Females
Males
% Freezing
Post-shock Period
Post-shock Period

Figure 3. ***Context fear training across Experiments***.

Mice assigned to 0.4% NaCl represented by blue symbols, mice assigned to 4.0% NaCl represented by red symbols; mice to be tested in Training Context represented by squares and solid lines, mice to be tested in Neutral Context represented by circles and dotted lines. All mice were trained in the Training Context with five, pseudorandomized mild (1 s, 0.8 mA) foot shocks during a 6 min training session. Percent freezing during training of mice in A) Experiment 1, B) Experiment 2 (grey shading), and C) Experiment 3. Baseline percent freezing averaged across the first two minutes of the training session and plotted along x-axis as 0 time point. Average percent freezing for each 30 sec period following each of the five mild foot shocks are plotted along x-axis as Post-shock Periods 1-5. Experiment 1: 0.4% NaCl females Training Context, n=7; 0.4% NaCl females Neutral Context, n=9; 4.0% NaCl females Training Context, n=8; 4.0% NaCl females Neutral Context, n=9; 0.4% NaCl males Training Context, 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. $^{+}$indicates $p<0.05$ 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 sex × diet × context interactions, nor were there any significant diet × context interactions (Table 6). However, two-way sex × context interactions were significant for Experiments 2 and 3, and sex × diet was also significant for Experiment 3 (Table 6). Further, Experiment 2 had a significant main effect of diet, and Experiment 1 had significant main effects of sex and context (Table 6). 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 clarify the effects of high salt consumption on contextual fear expression and generalization across the sexes for the three different Experiment timelines employed. Experiment 1 involved training mice after two weeks of diet manipulation, and testing 48 h after training. The expected low (average ~20% or less) freezing levels in the Neutral Context, with higher (average ~40% or more) freezing levels in the Training Context, were observed across nearly all the sex and diet combinations (Figure 4A). A non-significant trend was noted for contextual fear expression in the Training Context between females and males consuming the high salt diet ($p=0.0542$; Figure 4A). Excepting this, Experiment 1 suggests that contextual fear expression after two weeks of high salt diet consumption is unaffected across contexts and sexes.

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 (Figure 4B); 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 Figure 2B 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 (Figure 4C). 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 (Figure 4C). On the contrary, males tested in the Neutral Context after consuming high salt exhibited significantly lower contextual fear than females tested in the Neutral Context after consuming high salt (Figure 4C). This indicates a bidirectional, sex-selective effect of high salt consumption on contextual fear generalization.

Table 6. Three-way ANOVAs on context fear expression for context fear conditioned mice across Experiment.

	Context Trained Shock Groups		
Context Fear Expression	**Experiment 1**	**Experiment 2**	**Experiment 3**
Sex	$F(1,57)=7.538$ **$p=0.008$** partial η^2=**0.117**	$F(1,60)=22.29$ $p<0.001$ partial η^2=0.271	$F(1,57)=15.19$ $p<0.001$ partial η^2=0.210
Diet	$F(1,57)=2.107$ $p=0.152$ partial η^2=0.036	$F(1,60)=6.665$ **$p=0.012$** partial η^2=**0.100**	$F(1,57)=0.002$ $p=0.962$ partial η^2=0.000
Context	$F(1,57)=114.1$ **$p<0.001$** partial η^2=**0.667**	$F(1,60)=171.5$ $p<0.001$ partial η^2=0.741	$F(1,57)=84.47$ $p<0.001$ partial η^2=0.597
Sex × Diet	$F(1,57)=0.191$ $p=0.663$ partial η^2=0.003	$F(1,60)=0.099$ $p=0.754$ partial η^2=0.002	$F(1,57)=5.380$ **$p=0.024$** partial η^2=**0.086**
Sex × Context	$F(1,57)=1.297$ $p=0.259$ partial η^2=0.022	$F(1,60)=4.938$ **$p=0.030$** partial η^2=**0.076**	$F(1,57)=9.414$ **$p=0.003$** partial η^2=**0.142**
Diet × Context	$F(1,57)=0.486$ $p=0.489$ partial η^2=0.008	$F(1,60)=0.316$ $p=0.576$ partial η^2=0.005	$F(1,57)=0.014$ $p=0.905$ partial η^2=0.000
Sex × Diet × Context	$F(1,57)=3.235$ $p=0.077$ partial η^2=0.054	$F(1,60)=1.682$ $p=0.200$ partial η^2=0.027	$F(1,57)=2.642$ $p=0.110$ partial η^2=0.044

Figure 4

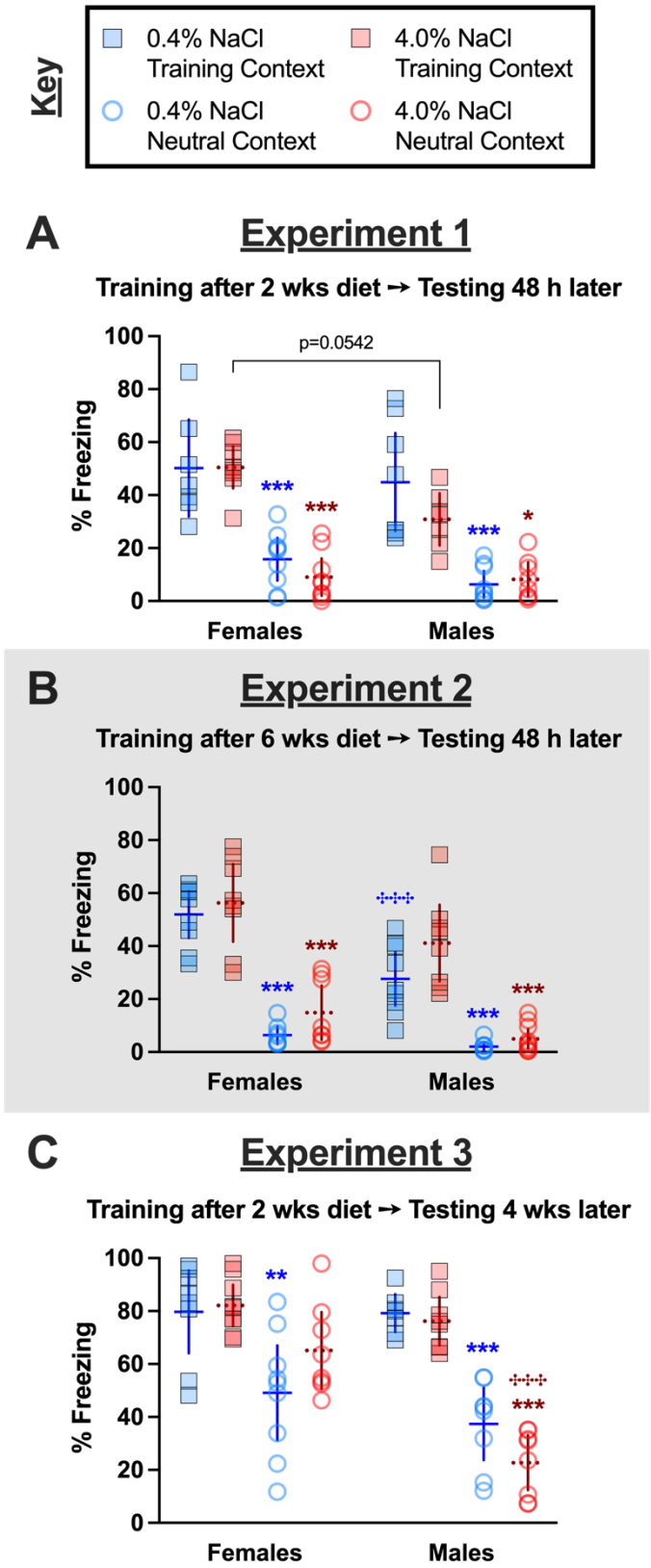
Key
0.4% NaCl Training Context
4.0% NaCl Training Context
0.4% NaCl Neutral Context
4.0% NaCl Neutral Context
A
Experiment 1
Training after 2 wks diet → Testing 48 h later
p=0.0542
% Freezing
100
80
60
40
20
0
***
***
***
*
Females
Males
B
Experiment 2
Training after 6 wks diet → Testing 48 h later
% 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

Figure 4. ***Context fear expression across contexts and Experiments***.

Mice assigned to 0.4% NaCl represented by blue symbols, mice assigned to 4.0% NaCl represented by red symbols; mice tested in Training Context represented by squares and solid lines, mice tested in Neutral Context represented by circles and dotted lines. Percent freezing during minutes two through six of the 10 min testing session are graphed for all mice. Testing occurred A) 48 h after training in Experiment 1, during which mice underwent two weeks of diet manipulation; B) 48 h after training in Experiment 2 (grey shading), during which mice underwent six weeks of diet manipulation; and C) four weeks after training in Experiment 3, during which mice underwent six total weeks of diet manipulation (training occurred after two weeks of diet manipulation). Experiment 1: 0.4% NaCl females Training Context, n=7; 0.4% NaCl females Neutral Context, n=9; 4.0% NaCl females Training Context, n=8; 4.0% NaCl females Neutral Context, n=9; 0.4% NaCl males Training Context, 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. *$p<0.05$, **$p<0.01$, ***$p<0.001$ 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.

Regardless of diet condition, all mice had *ad libitum* access to drinking water. Nonetheless, we evaluated if serum osmolality was affected across diet condition, sex, or testing Context in mice trained with foot shocks, as well as across diet, sex, and Experiment in control no shock mice. In control no shock mice, no three-way interaction of sex × diet × Experiment was observed, but a significant two-way interaction between sex × Experiment was detected (Table 7). No main effect of diet was observed. Despite the significant sex × Experiment interaction, Holm-Šídák's post-hoc testing revealed no significant differences in serum osmolality between sexes or diets in any Experiment, nor within any sexes or diets across Experiments (Figure 5).

Analyses of mice trained with foot shocks revealed zero three- or two-way interactions, and no significant main effects (Table 8). Likewise, Holm-Šídák's post-hocs indicated no significant differences across diets or sexes in any of the three Experiments (Figure 6), suggesting serum osmolality was unaltered in mice, and thus the behavioral changes observed were not the consequence of osmotic stress. For food, water, salt, and kcal consumption data, plus body weight records, readers are referred to the Supplemental Material.

Table 7. Three-way ANOVAs on serum osmolality in control no shock mice across Experiments.

Osmolality	**No Shock Groups Across Experiments**		
Sex	F(1,89)=0.424	p=0.517	partial η^2=0.005
Diet	F(1,89)=0.046	p=0.830	partial η^2=0.001
Experiment	F(1,89)=3.639	p=0.030	partial η^2=0.076
Sex × Diet	F(1,89)=0.510	p=0.477	partial η^2=0.006
Sex × Experiment	F(1,89)=4.287	**p=0.017**	partial η^2=**0.088**
Diet × Experiment	F(1,89)=1.729	p=0.183	partial η^2=0.037
Sex × Diet × Experiment	F(1,89)=1.331	p=0.270	partial η^2=0.029

Figure 5

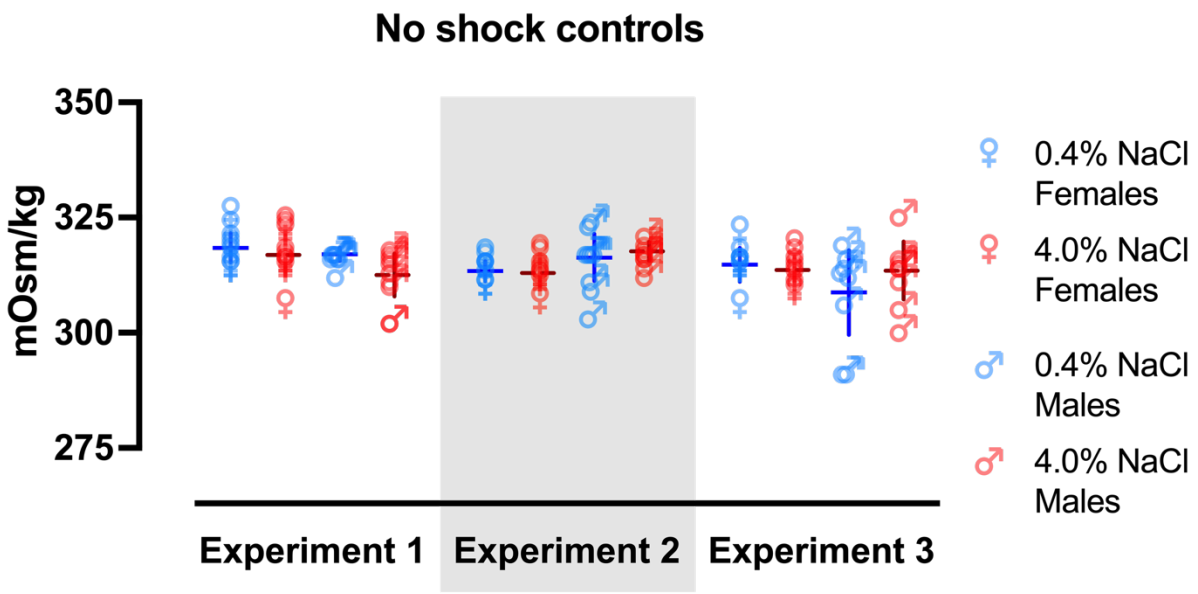

Figure 5. ***Serum osmolality in no shock control mice across Experiments***. Females represented by venus symbols, males by mars symbols; 0.4% NaCl represented by blue symbols, 4.0% NaCl represented by red symbols. Experiment 1: 0.4% NaCl females, n=9; 4.0% NaCl females, n=9; 0.4% NaCl males, n=8; 4.0% NaCl males, n=9. Experiment 2 (grey shading): 0.4% NaCl females, n=8; 4.0% NaCl females, n=8; 0.4% NaCl males, n=9; 4.0% NaCl males, n=9. Experiment 3: 0.4% NaCl females, n=8; 4.0% NaCl females, n=8; 0.4% NaCl males, n=8; 4.0% NaCl males, n=8. Data are graphed as mean ± 95% confidence interval.

Table 8. Three-way ANOVAs on serum osmolality in context fear conditioned mice across Experiments.

	Context Trained Shock Groups		
Osmolality	**Experiment 1**	**Experiment 2**	**Experiment 3**
Sex	$F(1,59)=1.662$ $p=0.202$ partial $\eta^2=0.027$	$F(1,62)=0.096$ $p=0.758$ partial $\eta^2=0.002$	$F(1,55)=0.086$ $p=0.771$ partial $\eta^2=0.002$
Diet	$F(1,59)=001$ $p=0.979$ partial $\eta^2=0.000$	$F(1,62)=0.673$ $p=0.415$ partial $\eta^2=0.011$	$F(1,55)=0.745$ $p=0.392$ partial $\eta^2=0.013$
Context	$F(1,59)=1.339$ $p=0.252$ partial $\eta^2=0.022$	$F(1,62)=0.005$ $p=0.941$ partial $\eta^2=0.000$	$F(1,55)=1.440$ $p=0.235$ partial $\eta^2=0.026$
Sex × Diet	$F(1,59)=0.012$ $p=0.912$ partial $\eta^2=0.000$	$F(1,62)=0.152$ $p=0.698$ partial $\eta^2=0.002$	$F(1,55)=0.213$ $p=0.646$ partial $\eta^2=0.004$
Sex × Context	$F(1,59)=0.482$ $p=0.490$ partial $\eta^2=0.008$	$F(1,62)=0.020$ $p=0.888$ partial $\eta^2=0.000$	$F(1,55)=0.154$ $p=0.696$ partial $\eta^2=0.003$
Diet × Context	$F(1,59)=1.903$ $p=0.173$ partial $\eta^2=0.031$	$F(1,62)=0.055$ $p=0.815$ partial $\eta^2=0.001$	$F(1,55)=0.781$ $p=0.381$ partial $\eta^2=0.014$
Sex × Diet × Context	$F(1,59)=0.003$ $p=0.959$ partial $\eta^2=0.000$	$F(1,62)=1.901$ $p=0.173$ partial $\eta^2=0.030$	$F(1,55)=0.856$ $p=0.359$ partial $\eta^2=0.015$

Figure 6

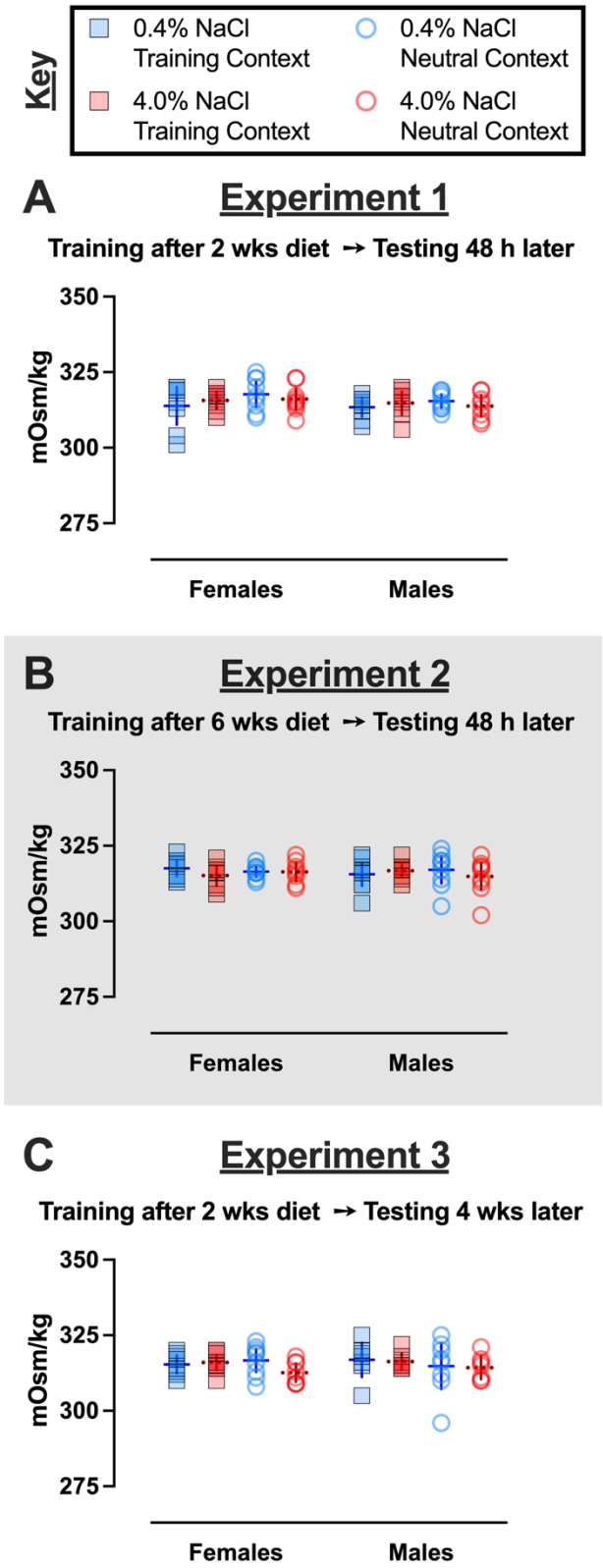
Key
0.4% NaCl Training Context
0.4% NaCl Neutral Context
4.0% NaCl Training Context
4.0% NaCl Neutral Context
A
Experiment 1
Training after 2 wks diet → Testing 48 h later
mOsm/kg
350
325
300
275
Females
Males
B
Experiment 2
Training after 6 wks diet → Testing 48 h later
mOsm/kg
350
325
300
275
Females
Males
C
Experiment 3
Training after 2 wks diet → Testing 4 wks later
mOsm/kg
350
325
300
275
Females
Males

Figure 6. ***Serum osmolality in context fear conditioned mice across Experiments***. Mice assigned to 0.4% NaCl represented by blue symbols, mice assigned to 4.0% NaCl represented by red symbols; mice tested in Training Context represented by squares and solid lines, mice tested in Neutral Context represented by circles and dotted lines. Serum osmolality in context fear conditioned mice from A) Experiment 1, B) Experiment 2 (grey shading), and C) Experiment 3. Experiment 1: 0.4% NaCl females Training Context, n=8; 0.4% NaCl females Neutral Context, n=9; 4.0% NaCl females Training Context, n=8; 4.0% NaCl females Neutral Context, n=9; 0.4% NaCl males Training Context, n=8; 0.4% NaCl males Neutral Context, n=9; 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 sex × diet × Experiment interaction was detected in log-transformed serum corticosterone (cort) levels of no shock control mice, but a two-way sex × Experiment interaction was found (Table 9). Unlike with serum osmolality, Holm-Šídák's post-hoc tests revealed diet-specific differences in cort levels within sexes across Experiments, and across sexes within an Experiment (Figure 7). Specifically, females consuming control diet in Experiment 2 displayed lower cort levels relative to females on control diet in Experiment 1 ($p=0.0423$). Similarly, males consuming the high salt diet in Experiment 3 exhibited lower cort levels relative to males on high salt diet in Experiment 1 ($p=0.0109$). Within Experiment 3, females consuming high salt had significantly higher cort levels relative to high salt males in Experiment 3 ($p=0.0143$). 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 in Experiments 1 and 2 challenges this interpretation.

Mice that underwent context fear conditioning with foot shocks had no three- nor two-way significant interactions between contexts, diets, or sexes. Experiments 1 and 3 had significant main effects of sex, whereas Experiment 2 had a significant main effect of context. For Experiments 1 and 2, Holm-Šídák's post-hoc tests indicated no significant differences within sexes across contexts, nor across sexes within contexts (Figure 8A, 8B). In Experiment 3, males consuming control diet and tested in the Training Context exhibited significantly lower cort levels ($p=0.0183$) relative to females consuming control diet and tested in the Training Context (Figure 8C). Similar, but non-significant, trends were noted between females and males tested in the Neutral Context consuming control diet ($p=0.0549$) or consuming high salt ($p=0.0859$; Figure 8C). Generally, cort levels did not consistently vary as a result of sex, diet, testing context, nor their interaction, indicating that shifts in circulating corticosterone are not

responsible for the observed changes in contextual fear expression nor contextual fear generalization. Circulating cort levels were largely not correlated with contextual fear expression, except in females consuming high salt and tested in the Training Context in Experiment 3 ($p=0.039$; see Supplemental Figure S15).

Table 9. Three-way ANOVAs on log-transformed serum corticosterone levels in control no shock mice across Experiments.

Corticosterone	**No Shock Groups Across Experiments**		
Sex	$F(1,81)=12.77$	$p<0.001$	partial $\eta^2=0.136$
Diet	$F(1,81)=2.498$	$p=0.118$	partial $\eta^2=0.030$
Experiment	$F(2,81)=12.81$	$p<0.001$	partial $\eta^2=0.240$
Sex × Diet	$F(1,81)=1.654$	$p=0.202$	partial $\eta^2=0.020$
Sex × Experiment	$F(2,81)=3.498$	**$p=0.035$**	partial $\eta^2=$**0.080**
Diet × Experiment	$F(2,81)=0.527$	$p=0.592$	partial $\eta^2=0.013$
Sex × Diet × Experiment	$F(2,81)=1.512$	$p=0.227$	partial $\eta^2=0.036$

Figure 7

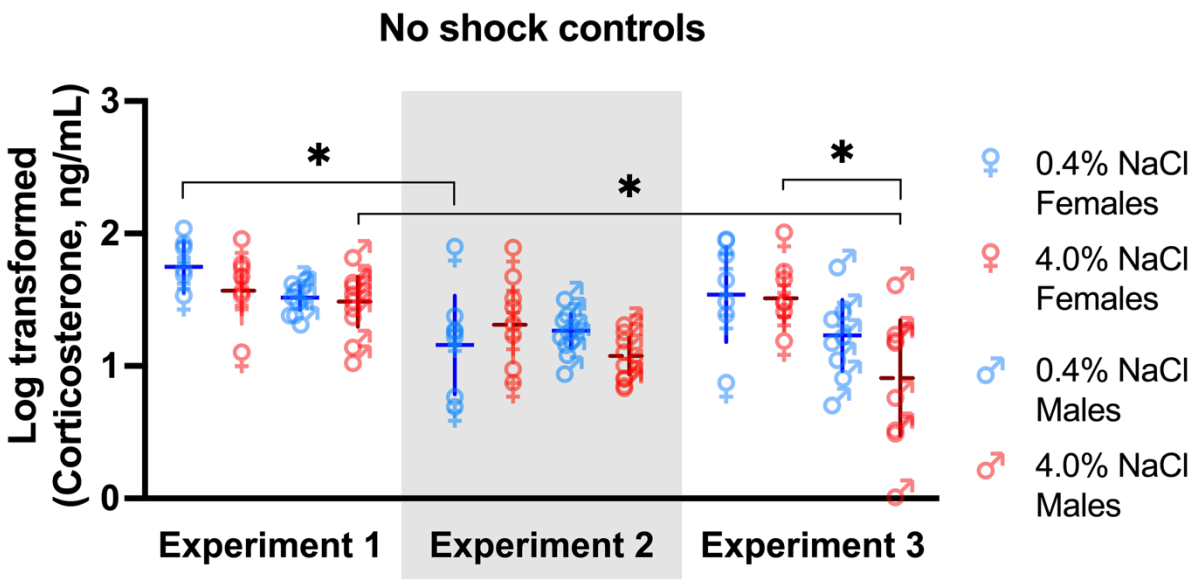

Figure 7. ***Log-transformed serum corticosterone levels of no shock control mice across Experiments***.

Females represented by venus symbols, males by mars symbols; 0.4% NaCl represented by blue symbols, 4.0% NaCl represented by red symbols. Experiment 1: 0.4% NaCl females, n=6; 4.0% NaCl females, n=7; 0.4% NaCl males, n=8; 4.0% NaCl males, n=9. Experiment 2 (grey shading): 0.4% NaCl females, n=7; 4.0% NaCl females, n=8; 0.4% NaCl males, n=9; 4.0% NaCl males, n=9. Experiment 3: 0.4% NaCl females, n=7; 4.0% NaCl females, n=7; 0.4% NaCl males, n=8; 4.0% NaCl males, n=8. Data are graphed as mean ± 95% confidence interval. *$p<0.05$ indicate difference between marked groups within same diet.

Table 10. Three-way ANOVAs on log-transformed serum corticosterone in context fear conditioned mice across Experiments.

	Context Trained Shock Groups		
Corticosterone	**Experiment 1**	**Experiment 2**	**Experiment 3**
Sex	F(1,52)=5.924 **p=0.018** partial η^2=**0.102**	F(1,59)=0.973 p=0.328 partial η^2=0.016	F(1,53)=38.87 **p<0.001** partial η^2=**0.423**
Diet	F(1,52)=1.107 p=0.298 partial η^2=0.021	F(1,59)=1.540 p=0.220 partial η^2=0.025	F(1,53)=1.170 p=0.284 partial η^2=0.022
Context	F(1,52)=0.325 p=0.571 partial η^2=0.006	F(1,59)=5.074 **p=0.028** partial η^2=**0.079**	F(1,53)=0.000 p=0.992 partial η^2=0.000
Sex × Diet	F(1,52)=2.815 p=0.099 partial η^2=0.051	F(1,59)=1.041 p=0.312 partial η^2=0.017	F(1,53)=0.186 p=0.668 partial η^2=0.003
Sex × Context	F(1,52)=2.355 p=0.131 partial η^2=0.043	F(1,59)=19.69 p=0.166 partial η^2=0.032	F(1,53)=0.012 p=0.913 partial η^2=0.000
Diet × Context	F(1,52)=0.033 p=0.856 partial η^2=0.001	F(1,59)=0.056 p=0.814 partial η^2=0.001	F(1,53)=0.966 p=0.330 partial η^2=0.018
Sex × Diet × Context	F(1,52)=0.164 p=0.687 partial η^2=0.003	F(1,59)=0.155 p=0.695 partial η^2=0.003	F(1,53)=0.204 p=0.654 partial η^2=0.004

Figure 8

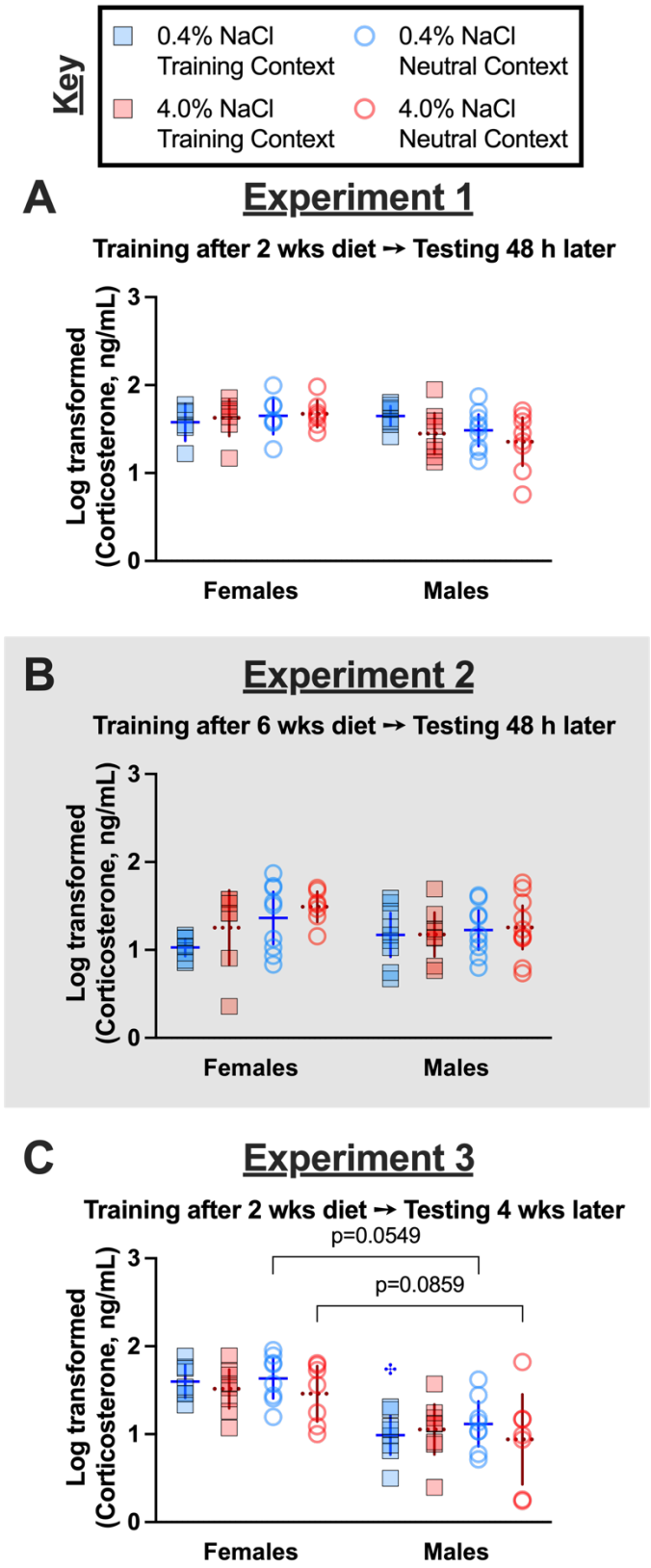
Key
0.4% NaCl Training Context
0.4% NaCl Neutral Context
4.0% NaCl Training Context
4.0% NaCl Neutral Context
A
Experiment 1
Training after 2 wks diet → Testing 48 h later
Log transformed (Corticosterone, ng/mL)
0
1
2
3
Females
Males
B
Experiment 2
Training after 6 wks diet → Testing 48 h later
Log transformed (Corticosterone, ng/mL)
0
1
2
3
Females
Males
C
Experiment 3
Training after 2 wks diet → Testing 4 wks later
p=0.0549
p=0.0859
Log transformed (Corticosterone, ng/mL)
0
1
2
3
Females
Males

Figure 8. ***Log-transformed serum corticosterone levels in fear conditioned mice across Experiments***.

Mice assigned to 0.4% NaCl represented by blue symbols, mice assigned to 4.0% NaCl represented by red symbols; mice tested in Training Context represented by squares and solid lines, mice tested in Neutral Context represented by circles and dotted lines. Log-transformed serum corticosterone levels in context fear conditioned mice in A) Experiment 1, B) Experiment 2 (grey shading), and C) Experiment 3. Experiment 1: 0.4% NaCl females Training Context, n=6; 0.4% NaCl females Neutral Context, n=7; 4.0% NaCl females Training Context, n=7; 4.0% NaCl females Neutral Context, n=7; 0.4% NaCl males Training Context, n=8; 0.4% NaCl males Neutral Context, n=9; 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=8; 0.4% NaCl females Neutral Context, n=9; 4.0% NaCl females Training Context, n=7; 4.0% NaCl females Neutral 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. $^{+}$indicates $p<0.05$ 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[43] or male mice[33]. These studies both used higher salt diets of 8.0% NaCl, compared to the 4.0% NaCl diet used here[33,43]. Both teams reported attenuated contextual fear expression, indexed by freezing behavior. This freezing behavior was measured during testing that occurred in the Training Context 24 h[33], or both 2 and 24 h[43], after training. Their observations align with the trend ($p=0.0542$) that we noted for male mice consuming the high salt diet towards reduced fear behavior in the Training Context relative to females on the same diet. Another 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[33]. However, this raises an important caveat regarding both investigations - rodents were trained using a cued fear paradigm with an auditory tone [33,43], rather than training the rodents with a traditional context fear conditioning protocol that lacks discrete cues. These different training procedures engage distinct brain regions[44,45], and likewise elicit distinguishable patterns of neuronal activation[46–48]. Thus, in the

present study we assessed contextual fear expression specifically following contextual fear conditioning, rather than cued fear conditioning.

Generalization of contextual fear is a hallmark symptom of many anxiety disorders, and is a conserved cross-species neurobehavioral phenomenon that is useful for understanding how neurophysiological changes influence behaviors[26,49]. The majority of contextual fear generalization investigations in rodents have been performed in males[27], though studies have recently begun to include females. Such sex-inclusive studies indicate that rodents of both sexes can and do exhibit generalized contextual fear, at timepoints both days[50] and weeks[51] after context fear training. Sex differences in the influence of various contextual modalities have been reported (see review[27]), and in our study contextual differences included tactile, olfactory, and visual modalities. We observed robust discrimination between contexts across the sexes when training was temporally proximal to testing, as well as convincing evidence of contextual fear generalization in both sexes consuming control diet when testing occurred four weeks following training. In females consuming high salt for 6 weeks prior to training and then testing 48 h later in the Neutral Context, we noted an unexpected bimodal distribution in freezing levels, with one sub-group averaging below 10% freezing and the other sub-group averaging near 30% freezing (Figure 4B). If this proves to be a reproducible occurrence in future experiments, this could provide intriguing evidence for individual differences in excessive salt consumption affecting females' abrupt (i.e., soon after training) contextual fear generalization behaviors. Longer term contextual fear generalization was apparent in mice of both sexes consuming control diet. In contrast to our hypothesis that high salt intake would enhance contextual fear generalization in males, we instead found an attenuation of this measure in males, but an enhancement in females. Given the near absence of investigations into how excess salt intake affects female behaviors (see review[52]), plus the relatively scant literature on contextual fear generalization in females (see review[27]), we did not have enough information to formulate an *a priori* hypothesis for females consuming high salt. Some researchers have suggested excess

salt intake exacerbates neuropsychiatric symptoms and conditions [23,24]. Our data support this relationship, at least in females. However, the relationship between high salt consumption and anxiety-relevant behaviors was not supported in our male mice, and in fact our data suggest an inverse relationship. Given frequent comorbidity between cardiovascular diseases and pathological anxiety[11–15] (see also reviews[16,17]), and the adverse impact of overconsumption of salt on cardiovascular disease risk[5,10], this finding in males was surprising. Nonetheless, it may be that in males, high salt-mediated cardiovascular pathology is necessary for emergence of increased anxiety-related behaviors, whereas in females excess salt is sufficient.

Along these lines, we evaluated if mice consuming excess salt exhibited peripheral indicators of osmotic (serum osmolality) or physiological (serum corticosterone) stress. Changes in these measures could suggest an overall disrupted state of homeostasis that might confound contextual fear learning and/or expression. For example, osmotic stress can reduce sensitivity to pain[53]. Though the shock level (0.8 mA) and duration (1 sec) that we use are designed to only elicit discomfort/distress, and not pain, it remains possible that osmotic stress could skew sensory perception of this brief aversive experience. Similarly, endogenous corticosterone levels can influence temporal development of contextual fear generalization as well as the persistence of contextual discrimination[54]. The absence of any sex-, diet-, or context-specific differences in serum osmolality indicates that osmotic stress was not present in our experimental animals. Though in Experiment 3 there were a couple of non-significant trends across the sexes of mice tested in the Neutral Context, no significant correlations between cort levels and fear expression in the Neutral Context were detected for either sex in Experiment 3. Indeed, of all the mice trained in the Training Context with foot shocks, only Experiment 3 females consuming high salt and tested in the Training Context displayed a significant (negative) correlation between context fear expression and cort levels (Supplemental Figure S15). The absence of any significant correlations between cort levels and any other

sex/diet/context combination across Experiments indicates that cort levels overall probably did not confound observed context fear expression and generalization.

Limitations of this study include singly housing of mice to allow for tracking of individual food/water/salt/kcal consumption and changes in body weight, meaning that mice were excluded from social stimulation. Diet manipulations were relatively short (two or six weeks), restricted to non-breeding adults, and contained no other accompanying unhealthy dietary components (e.g., low fiber, high fat). Experiments occurred during the light cycle, i.e., when mice would typically be resting or sleeping, and near the nadir of their natural circadian corticosterone pattern[55]. Only a single shock level and context fear conditioning paradigm was used, and mice were only tested in a single context. Thus, for future studies it will be important to evaluate behavior in group housed mice, assess longer diet manipulation periods and include additional unhealthy dietary components, and study how consumption of excess salt by younger mice affects their fear behaviors. Testing of behavior during the natural activity period of mice would be informative, as would assessing generalization and concurrent cort levels during different stages of the circadian cycle.

Our study provides foundational evidence of sex-selective consequences of excess salt intake on context fear expression and generalization. Additional studies will be essential to flesh out these initial findings and determine what brain cell populations are most impacted by excess salt intake, and how the effects of high salt might be mitigated. One logical starting point is evaluation of microglia, given converging evidence that both fear processing[56,57] and high salt intake[58–60] affect microglial activation. Moreover, oral administration of the brain-penetrant anti-inflammatory drug minocycline can mitigate microglial activation and behavioral changes in rodents consuming high salt[58], and the same drug attenuated fear measures in human participants[61]. Future studies should also assess how excess salt intake affects cued fear expression and discrimination, and in so doing begin identifying how specific limbic brain regions might differentially be affected in their functionality and/or connectivity following

extended periods of excess salt consumption. The evidence presented here, in conjunction with past literature focused on salt's effects on behaviors outside of the fear domain[52], indicates that salt is an underappreciated, non-caloric influence on conserved mammalian behaviors.

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