3
Study 1

Genetic selection of two new rat lines displaying different levels of conditioned freezing behavior

3.1
Objectives

The main objective of the present work was to develop a bidirectional selective breeding program using Wistar rats, employing the conditioned freezing behavior in response to contextual cues previously associated with footshocks as selection criterion. Also, we aimed to introduce a third group of randomly mated rats (RND) in the selective breeding program, which could serve as a control for the selected lines.

3.2
Subjects

Albino Wistar rats were employed as subjects. The initial stock of these animals was obtained in 1995 from a local producer (Oswaldo Cruz Foundation), and since then they have been maintained in the colony room of the PUC-Rio Psychology Department with controlled room temperature (24 ± 1°C) and in a 12 h/12 h light/dark cycle (07:00-19:00 h). The selective breeding procedure described in this work began in March of 2006. Experiments occurred always during the light phase of the cycle. Six to eight days after birth, animals were marked by amputation of one toe from each foot and a small cut in one of the ears. Upon weaning at 21 days of age, animals were separated by sex and housed in groups of five to seven, according to their respective lines, in polycarbonate cages measuring 18×31×38 cm, with food and water always provided *ad libitum*. The animals were between 75 and 85 days of age at the beginning of the experiment. For five days before to the experiment, the animals were handled once daily for a period of 2 min. All experimental protocols employed in this work were approved by the PUC-Rio Psychology Department ethics committee and conformed to the Brazilian Society of Neuroscience and Behavior Guidelines for Care and Use of

Laboratory Animals (SBNeC), which are based on the US National Institutes of Health Guide for Care and Use of Laboratory Animals (revised in 1996).

3.3
Equipments

Contextual fear conditioning occurred in four observation chambers of Plexiglas (25 × 20 × 20 cm), each one placed inside a sound-attenuating box. A red light bulb (25 W) was placed inside the box, and a digital video camera was mounted in the back of the observation chamber so the animal's behavior could be observed on a monitor outside the experimental chamber. An observation program (GeoVision GV800, PCI Systems) was used to record all procedures (Figure 4). A ventilation fan attached to the box supplied background noise of 78 dB (A scale). The floor of the observation chamber consisted of 15 stainless steel rods (4 mm diameter) spaced 1.5 cm apart (center-to-center), which were wired to a shock generator and scrambler (Insight, São Paulo, Brazil). An interface with eight channels (Insight, São Paulo, Brazil) connected the shock generator to a computer, which allowed the analyst to apply an electric footshock. A digital multimeter (MD-1400 - ICEL, Manaus) was used to calibrate shock intensities before each experiment. An ammonium hydroxide solution (5%) was used to clean the chamber before and after each subject.

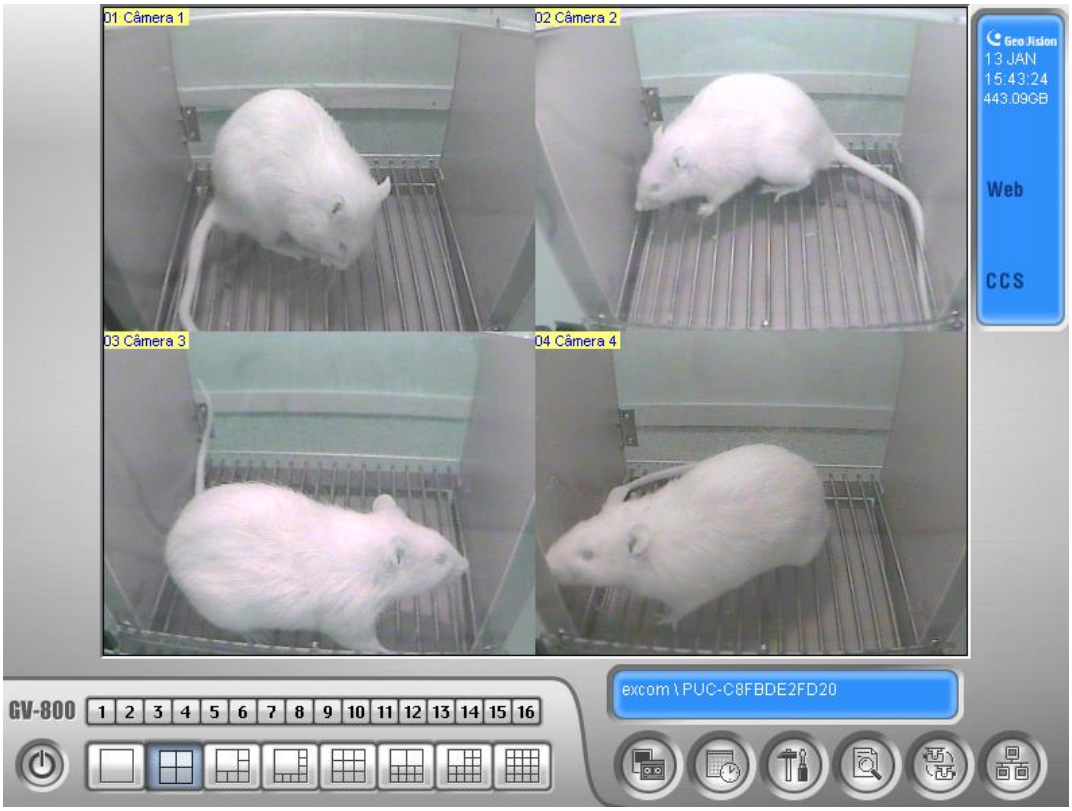

Figure 4 - Video recording program used for behavioral register.

3.4
Procedures

In order to develop a line of rats with a high rate of conditioned freezing, termed Carioca[3] High-Freezing (CHF), and another line of rats with a low rate of conditioned freezing, named Carioca Low-Freezing (CLF), 120 animals (60 males and 60 females) randomly bred in our colony room were used. These animals constituted the initial generation (S_0). The contextual fear conditioning protocol involved an acquisition session and a testing session. During acquisition, each animal was placed in the observation chamber for 8 min. At the end of this period, three unsignaled electrical footshocks were delivered, with each shock lasting 1 s and with an intershock interval of 20 s. The initial shock strength employed in the first five generations of selective breeding was 1.0 mA. In order to avoid ceiling effects, this footshock intensity was reduced in our breeding program in the 6th generation to 0.7 mA and in the 8th generation on forward to 0.6 mA. The animal was then returned to its home cage 3 min

[3] Carioca is the name given to those born in Rio de Janeiro.

after the last shock. The testing session occurred approximately 24 h after training. This test consisted of placing the animal for 8 min in the same chamber in which the three footshocks had been administered in the previous day. No footshock or other stimulation occurred during this period. A time-sampling procedure was employed to evaluate fear conditioning to contextual cues. Every 2 s the animal was observed and a well-trained observer (VCG), blind to the experimental conditions, recorded episodes of freezing, defined as the total absence of movement of the body or vibrissae except for movements required for respiration. In the first four generations (S_1-S_4), each rat was observed one at a time. In the S_5-S_8 generations, rats were observed in pairs; finally, in the subsequent generations (S_9-S_{14}), rats were observed in groups of four. Moreover, in S_{14} the freezing was manually scored by a different observer (CEB), in order to investigate the impact of an independent observation. Figure 5 shows the contextual fear conditioning paradigm.

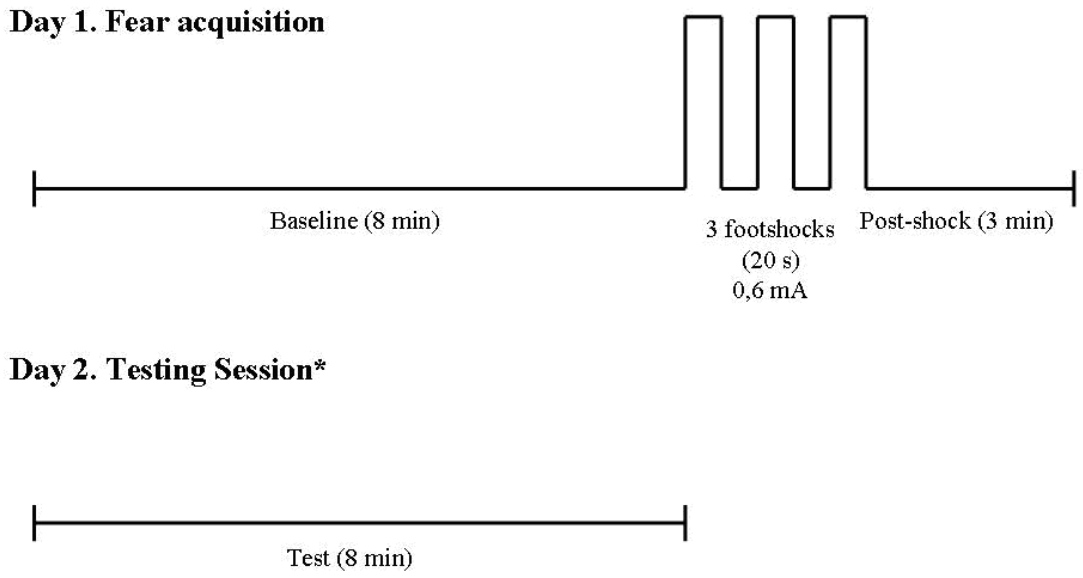

Figure 5 - Contextual fear conditioning procedure used for phenotyping. *Freezing registered in the testing session was employed as mating criteria.

The agreement between observers with respect to the scoring of freezing episodes in our laboratory is higher than 0.95. At the acquisition session, freezing was scored during the 8 min baseline period prior to the occurrence of the first footshock as well as during the 3 min post-shock period immediately after the occurrence of the third footshock. Freezing was also scored during the 8 min test session. The total amount of freezing behavior observed during the test session was used as the criterion for animal

mating. The 10 male and 10 female rats with the highest conditioned freezing scores, as well as the 10 male and 10 female rats with the lowest conditioned freezing rates, were selected to breed the CHF and CLF lines, respectively. From the 10 CHF families, 76 animals were born, while the 10 CLF families gave rise to 71 animals. These animals were the first-generation offspring of our breeding procedure (S_1). The same procedure was used for the production of new generations of selected animals (S_2 to S_{14}). Mating always occurred within each line. One exception occurred in S_2, when one female from the CLF line with the highest score of conditioned freezing was bred with a male from the CHF line that also had the highest score of conditioned freezing. The high- and low-family breeders were chosen after all animals from a given generation had been phenotyped. However, the number of breeding pairs varied through generations. Also, due to fertility problems, an additional crossing in the fifth generation, and two additional crossings in the seventh generation were made, and the resulting offspring was then incorporated to its respective generation. Also, an additional cross was made in the generation S_{12}, but the offspring was only employed in the third study of the present thesis.

From the fourth generation onwards, a third group of males and females were introduced in our breeding program. These animals underwent the same procedures employed to produce the high and low freezing lines. The only difference is that this third group, termed “Randomly Mated” (RND), did not receive any selection pressure after phenotyping, i.e. the mating was randomized among animals. The objective was to create a parallel control group for the selected lines, with an intermediate conditioned freezing response. The initial population of RND rats was produced from 16 breeding pairs of Wistar rats randomly bred in our colony. This stock had the same origin of the High and Low selected rats of our laboratory. Brother–sister breeding pairs were always avoided in all groups to reduce inbreeding, thus reducing genetic variability, which could lead a reduction in the animal's fertility and random changes in the development of the selected lines due to genetic drift (Falconer and MacKay, 1996). Table 2 shows the number of breeding pairs in each generation of selective breeding, as well the rate of females who gave birth.

Generation	High Freezing		Randomly Selected		Low Freezing	
	Breeding pairs	% Fertilized females	Breeding pairs	% Fertilized females	Breeding pairs	% Fertilized females
S_0	10	100%	-	-	10	100%
S_1	11	100%	-	-	11	91%
S_2	10	80%	-	-	10	80%
S_3	14	86%	-	-	14	100%
S_4	16	63%	16	69%	16	63%
S_5	15	60%	10	90%	15	47%
S_5/F_1	10*	80%	-	-	10*	50%
S_6	15	100%	9	100%	15	87%
S_7	15	40%	15	87%	15	46%
S_7/F_1	9*	33%	-	-	6*	22%
S_7/F_2	4*	80%	-	-	5*	50%
S_8	15	86%	10	80%	15	80%
S_9	15	66%	14	64%	15	73%
S_{10}	15	86%	14	71%	15	93%
S_{11}	15	100%	15	80%	15	73%
S_{12}	15	93%	15	93%	15	93%
S_{13}	15	93%	15	93%	15	93%
S_{14}	15	93%	15	93%	15	93%

Table 2 - Fertility rate throughout generations. *Same Breeders.

Despite the limited space of our facilities, the number of individuals of each population of the CHF and CLF selected lines was kept unaltered in all generations, except in cases of natural deaths. For the RND rats, however, no more than 70 rats were maintained in the first five generations (S_5-S_{10}), and after that the population was kept unchanged.

Generation	High Freezing		Randomly Selected		Low Freezing	
	Males	Females	Males	Females	Males	Females
S_1 (n=147)	37	39	-	-	34	37
S_2 (n=143)	37	35	-	-	37	34
S_3 (n=158)	34	45	-	-	42	37
S_4 (n=151)	46	31	-	-	33	41
S_5 (n=217)	29	37	40	16	49	46
S_6 (n=175)	29	23	26	24	39	34
S_7 (n=308)	64	61	17	25	73	68
S_8 (n=184)	28	35	30	34	32	25
S_9 (n=279)	70	56	22	38	51	42
S_{10} (n=250)	44	37	42	40	37	50
S_{11} (n=353)	60	56	60	46	62	69
S_{12} (n=345)	67	55	54	70	49	50
S_{13} (n=338)	59	42	57	51	67	62
S_{14} (n=425)	56	69	86	73	69	72
Total (n=3473)	**660**	**621**	**434**	**417**	**674**	**667**

Table 3 - Distribution of the number of male and female rats behaviorally characterized among high conditioned freezing, randomly selected and low conditioned freezing animals along fourteen selected generations. *Note:* For S_0, N=120 (60 males and 60 females).

Body weight. To assess possible lines differences in growth rates, litters were weighed from S_9 to S_{13}, at 7 days of age, and individual animals were weighed on postnatal days (PND) 21 and 42, and also on the training day. Due to practical reasons, animals did not go to the conditioning training in the same day. In this case, weight on the conditioning day was considered in the range of 75-85 day old animals.

3.5
Statistics

Selective Breeding

Behavioral data from the S_0 population were compared by a Student's t-test only regarding sex effects, because the High and Low conditioned freezing lines did not exist yet. Since the number of selected groups varied during the selective breeding process, as well the intensity of footshocks, starting in S_1 behavioral data were analyzed separately for each generation. For the first four generations of selective breeding, a two-way ANOVA was used, either for baseline, post-shock or conditioned freezing: the first factor, with 2 levels, was breeding line (CHF and CLF), and the second factor, with 2 levels, was the animal's sex (male and female). For the other selected generations (S_5-S_{14}), a two-way ANOVA for each dependent variable was also conducted. But, in this case, the first factor, breeding line, had 3 levels (CHF, CLF and RND), and the second factor, with 2 levels, was related to the animal's sex (male and female). Additionally, an ANCOVA was also conducted, with the post-shock freezing as a covariant factor, to evaluate whether the breeding line effect on conditioned freezing during the test session was attributable to possible post-shock differences that these animals presented during the training session. The level of significance employed was of 0.05. Fisher's Least Significant Differences (LSD) test was used for post-hoc comparisons.

3.6
Results

Selective Breeding

The S_0 Population

Data of baseline and post-shock freezing were not registered in the S_0 generation. The results of the Student's t test regarding conditioned freezing in the testing session showed no significant differences among males and females (t_{118}= 0.018; p>0.05).

S_1 generation

In the first-generation offspring of selective breeding, the results of baseline freezing showed the absence of two-way interaction ($F_{1,142}$=0.077; p=0.781), and no main effects for line ($F_{1,142}$=0.043; p=0.835) and sex ($F_{1,142}$=0.114; p=0.735). For post-shock freezing, the analysis showed no two-way interaction ($F_{1,142}$=0.107; p=0.743), and no main effects for line ($F_{1,142}$=0.588; p=0.444) or sex ($F_{1,142}$=0.218; p=0.641). For conditioned freezing, the ANOVA showed a lack of two-way interaction ($F_{1,142}$=0.032; p=0.857), as well as a lack of main effects for line ($F_{1,142}$=0.283; p=0.595); however main effects for sex were observed ($F_{1,142}$=5.197; p<0.05).

The ANCOVA results showed no significant two-way interaction ($F_{1,141}$=0.3; p=0.864), and no main effects for line ($F_{1,141}$=0.263; p=0.609), and main effects for sex ($F_{1,141}$=5.2; p<0.0.5)

S_2 generation

Results of the baseline freezing in the second generation of selective breeding showed the absence of two-way interaction ($F_{1,139}$=0.9; p=0.344), and no main effects for line ($F_{1,139}$=0.109; p=0.741) or sex ($F_{1,139}$=0.043; p=0.835). For post-shock freezing, the analysis showed the absence of two-way interaction ($F_{1,139}$=1.66; p=0.199), and no main effects for line ($F_{1,139}$=1.418; p=0.235) or sex ($F_{1,139}$=0.602; p=0.439).

For conditioned freezing, results showed the absence of two-way interaction ($F_{1,139}$=0.274; p=0.6), and no main effects for line ($F_{1,139}$=3.298; p=0.071); however, results showed main effects for sex ($F_{1,139}$=4.044; p=0.046).

For the ANCOVA: absence of two-way interaction ($F_{1,138}$=0.273; p=0.602), and no main effects for line ($F_{1,138}$=3.232; p=0.74), but main effects for sex ($F_{1,138}$=3.991; p<0.05).

S_3 generation

In the third generation, results from baseline freezing showed a lack of two-way interaction ($F_{1,155}$=1.613; p=0.205), and the absence of main effects for line ($F_{1,155}$=1.463; p=0.228) and sex ($F_{1,155}$=3.104; p=0.08). The analysis of post-shock freezing showed the absence of two-way interaction ($F_{1,155}$=0.52; p=0.471), and no main effects for line ($F_{1,155}$=0.157; p=0.692) or sex ($F_{1,155}$=1.2; p=0.275).

The analysis of conditioned freezing found the absence of two-way interaction ($F_{1,155}$=0.207; p=0.65). However, main effects for line ($F_{1,155}$=43.758; p<0.001) and sex ($F_{1,155}$=4.072; p<0.05) were observed. Post-hoc comparisons found significant differences between CHF and CFL animals, for both males and females (all p <0.001).

For the ANCOVA, an absence of two-way interaction ($F_{1,154}$=0.217; p=0.642), and main effects for line ($F_{1,154}$=43.541; p<0.001) and sex ($F_{1,154}$=4.09; p<0.05) were found.

S_4 generation

Results of the baseline freezing in the fourth generation of selective breeding showed the absence of two-way interaction ($F_{1,147}$=0.762; p=0.383), and no main effects for line ($F_{1,147}$=0.179; p=0.672) or sex ($F_{1,147}$=0.388; p=0.534). For post-shock freezing, the absence of two-way interaction ($F_{1,147}$=0.066; p=0.797), and main effects for line ($F_{1,147}$=6.2; p<0.05), but not for sex ($F_{1,147}$=3.516; p=0.063) were observed.

For conditioned freezing, the analysis showed the absence of two-way interaction ($F_{1,147}$=0.093; p=0.76); however, main effects for line ($F_{1,147}$=60.160; p<0.001) and sex ($F_{1,147}$=10.393; p<0.001) were observed. Pairwise post-hoc comparisons showed that CHF differed from CLF animals for both males and females (all p <0.001).

For the ANCOVA, the absence of two-way interaction ($F_{1,146}$=0.55; p=0.815) and main effects for line ($F_{1,146}$=51.426; p<0.001) and sex ($F_{1,146}$=15.913; p<0.001) were observed.

S_5 generation

The analysis of baseline freezing in the fifth generation showed the absence of two-way interaction ($F_{2,211}$=0.38249; p=0.682) and no main effects for line ($F_{2,211}$=0.48182; p=0.618) or sex (F1,211=0.505; p=0.477). For post-shock freezing, the absence of two-way interaction ($F_{2,11}$=1.824; p=0.163) was observed; and results also showed a main effect for line ($F_{2,211}$=13.101; p<0.001), but not for sex ($F_{2,211}$=1.24; p=0.266). Fisher LSD post-hoc comparisons showed that male CHF animals presented significantly more post-shock freezing responses than males CLF and RND animals, and that female CHF rats presented more post-shock freezing responses than female RND animals (all p <0.001).

For conditioned freezing, ANOVA showed the absence of two-way interaction ($F_{2,11}$= 0.821; p=0.441), and main effects for line ($F_{2,211}$=26.400; p<0.001) or sex ($F_{1,211}$=28.259; p<0.001). Fisher LSD post-hoc comparisons showed that male CHF animals presented significantly more conditioned freezing than male CLF and RND animals (all p <0.001).

For the ANCOVA, the absence of two-way interaction ($F_{2,210}$=1.252; p=0.288), and main effects for line ($F_{1,210}$=17.771; p<0.001) and sex ($F_{1,210}$=32.418; p<0.001) were observed.

S_6 generation

In the sixth generation, ANOVA results for baseline freezing showed the absence of two-way interaction ($F_{2,169}$= 0.898; p=0.409), and a lack of main effects for line ($F_{2,169}$=1.042; p=0.354) and sex ($F_{1,169}$=0.033; p=0.854). The same patterns of results was found for post-shock freezing, with results showing no two-way interaction ($F_{2,169}$=0.898; p=0.409), and no main effects for line ($F_{2,169}$=1.042; p=0.354) or sex ($F_{1,169}$=0.033; p=0.854).

The analysis of conditioned freezing behavior showed no two-way interaction ($F_{2,169}$=0.153; p=0.858), but results showed main effects for line ($F_{2,169}$=7.422; p<0.001) and sex ($F_{1,169}$=14,321; p<0.001). Fisher LSD post-hoc comparisons showed that male CHF animals only differed from male RND animals, but not from male CLF animals. The same pattern of results was found for females (all p <0.001).

The ANCOVA showed a non-significant two-way interaction ($F_{2,168}$=173.703; p=0.71). Main effects for line ($F_{2,168}$=5.882; p<0.001) and sex ($F_{1,168}$=13,165; p<0.001) were also observed.

S_7 generation

In the seventh generation of selective breeding, the results for baseline freezing demonstrated the absence of two-way interaction ($F_{2,302}$=1.025; p=0.359), and the absence of main effects for line ($F_{2,302}$=1.307; p=0.272) and sex ($F_{1,302}$=0.048; p=0,826). The analysis of post-shock freezing also showed the absence of two-way interaction ($F_{2,302}$=0.522; p=0.593) and main effects for line ($F_{2,302}$=12.123; p<0.001) and sex ($F_{1,302}$=4.508; p<0.05). Post-hoc comparisons showed that, for males, CLF differed from CHF and RND animals. The same results were found for females (all p <0.001).

The results for conditioned freezing showed no two-way interaction ($F_{2,302}$=2.850; p=0.594); however main effects were observed for line ($F_{2,302}$=14.152; p<0.001) and sex ($F_{1,302}$=14.233; p<0.001). Fisher LSD post-hoc comparisons showed that CHF animals significantly differ from CLF and RND animals for males. For females, CHF differed only from CLF animals (all p <0.001).

The ANCOVA performed with the post-shock freezing as a covariant factor showed the absence of a two-way interaction ($F_{2,301}$=2.409; p=0.92). Main effects were also found for line ($F_{2,301}$=10.632; p<0.001) and sex ($F_{1,301}$=10.526; p<0.001).

S_8 generation

For baseline freezing, the results showed neither a two-way interaction ($F_{2,178}$=2.007; p=0.127) nor main effects for line ($F_{2,178}$=0.613; p=0.542) and sex ($F_{1,178}$=1.159; p=0.283). For post-shock freezing, the absence of two-way interaction was verified ($F_{2,178}$=0.997; p=0.371); also, main effects for line ($F_{2,178}$=4.581; p=0.011),

but not for sex ($F_{1,178}$=0.416; p=0.519) were found. Post-hoc pairwise comparisons showed differences for females, with CLF animals being significantly different from CHF and RND animals (all p <0.05).

For context freezing, ANOVA showed no two-way interaction ($F_{2,178}$=0.620; p=0.538), but main effects for line ($F_{2,178}$=3.371; p<0.05) and sex ($F_{1,178}$=8.003; p<0.05) were found. Post-hoc pairwise comparisons showed differences only between male CLF and male RND animals (p<0.05).

The ANCOVA showed a non-significant two-way interaction ($F_{2,177}$=0.617; p=0.541), and main effects for sex ($F_{1,177}$=7.643; p<0.05), but not for line ($F_{2,177}$=1.362,; p=0.259).

S_9 generation

The analysis for baseline freezing showed neither a two-way interaction ($F_{2,273}$=0.265; p=0.766) nor main effects for line ($F_{2,273}$=0.243; p=0.784) or sex ($F_{1,273}$=1.316; p=0.252). For post-shock freezing, the absence of a two-way interaction ($F_{2,273}$=1.956; p=0.143) and main effects for line (F2,273=5.550; p<0.05) but not for sex ($F_{1,273}$=0.920; p=0.338) were noted. Pairwise post-hoc comparisons showed that CHF animals differed from CLF animals, which differed from RND animals. For females, CHF differed from CLF animals (all p <0.05).

For conditioned freezing, the analysis showed the absence of a two-way interaction ($F_{2,273}$=0.597; p=0.551), and main effects for line ($F_{2,273}$=28.206; p<0.001) and sex ($F_{1,273}$=6.463; p<0.05). Post-hoc pairwise comparisons showed that CHF animals differed from CLF and RND animals, for both males and females (all p <0.001).

The ANCOVA analysis showed the absence of a two-way interaction ($F_{2,272}$=0.437; p=0.646) and main effects for line ($F_{2,272}$=26.042; p<0.001) and sex ($F_{1,272}$=9.152; p<0.001).

S_{10} generation

For baseline freezing, results showed the absence of a two-way interaction ($F_{2,244}$=1.796; p=0.168), and no main effects for line ($F_{2,244}$=0.542; p=0.582) or sex

($F_{1,244}$=0.000; p=1.000). For post-shock freezing, the analysis showed the absence of a two-way interaction ($F_{2,244}$=0.391; p=0.676), and no main effects for sex ($F_{1,244}$=3.197; p=0.075); however, main effects for line ($F_{2,244}$=13.927; p<0.001) were found. Post-hoc comparisons showed that CHF animals differed from CLF and RND animals, for both males and females.

For conditioned freezing, the results showed a two-way interaction ($F_{2,244}$=5.552; p<0.05), and main effects for line ($F_{2,244}$=18.902; p<0.001) and sex ($F_{1,244}$=13.516; p<0.001). Post-hoc comparisons found differences between CHF and CLF, RND animals, for both males e females (all p <0.001).

The ANCOVA performed with post-shock freezing as a covariant factor showed the presence of a significant two-way interaction ($F_{2,243}$=6.301; p<0.05), and also main effects for line ($F_{2,243}$=10.88; p<0.001) and sex ($F_{1,243}$=10.856; p<0.001).

S_{11} generation

For baseline freezing, ANOVA showed an absence of two-way interaction ($F_{2,347}$=0.462; p=0.629), and no main effects for line ($F_{2,347}$=2.727; p=0.066) or sex ($F_{1,347}$=2.498; p=0.114). For post-shock freezing, results showed the presence of a two-way interaction ($F_{2,347}$=6.763; p<0.001), and main effects for line ($F_{2,347}$=10.399; p<0.001) and sex ($F_{1,347}$=5.383; p<0.05). Post-hoc comparisons showed significant differences only for females, with RND animals differing from CHF and CLF animals (all p <0.001).

Results for conditioned freezing showed the absence of a two-way interaction ($F_{2,347}$=0.474; p=0.622) and main effects for line ($F_{2,347}$=23.121; p<0.001) and sex ($F_{1,347}$=33.379; p<0.001) were noted. Post-hoc comparisons showed that CHF animals differed from RND and CLF animals, and that RND differed from CLF animals, for males; for females, CHF animals significantly differed from RND and CLF animals (all p <0.05).

The ANCOVA performed with post-shock freezing as a covariant factor showed the absence of a two-way interaction ($F_{2,346}$=1.724; p=0.18), and main effects for line ($F_{2,346}$=15.761; p<0.001) and sex ($F_{1,346}$=27.801; p<0.001).

S_{12} generation

The results for baseline freezing showed the absence of a two-way interaction ($F_{2,339}$=2.875; p=0.057), and no main effects for line ($F_{2,339}$=2621; p=0.074) or sex ($F_{1,339}$=0.075; p=0.783). For post-shock freezing, the results showed the absence of a two-way interaction ($F_{2,339}$=0.43; p=0.65), but main effects for line ($F_{2,339}$=20.269; p<0.001) and sex ($F_{1,339}$=14.023; p<0.001) were observed. For males, post-hoc comparisons showed that CHF animals differed from CLF, but not from RND animals. Moreover, CLF differed from RND animals. The same pattern of results was found for females (all p <0.001).

For conditioned freezing, the results showed the absence of a two-way interaction ($F_{2,339}$=0.071; p=0.931), but main effects for line ($F_{2,339}$=18.128; p<0.001) and sex ($F_{1,339}$=41.856; p<0.001) were observed. For males, post-hoc comparisons showed that CHF animals differed from CLF, but not from RND animals. Furthermore CLF differed from RND animals. For females, CHF differed from CLF and RND animals. Finally, CLF differed from RND animals (all p <0.001).

The ANCOVA performed with post-shock freezing as a covariant factor showed a non-significant two-way interaction ($F_{2,338}$=0.195; p=0.823) and main effects for line ($F_{2,338}$=10.297; p<0.001) and sex ($F_{1,338}$=31.055; p<0.001) were also verified.

S_{13} generation

For baseline freezing, the results showed the absence of a two-way interaction ($F_{2,332}$=0.279; p=0.756), and no main effects for line ($F_{2,332}$=0.393; p=0.531) or sex ($F_{2,332}$=1.309; p=0.271) were found. For post-shock freezing, the absence of a two way interaction ($F_{2,332}$=0.322; p=0.724), and main effects for line ($F_{2,332}$=5.257; p<0.05), but not for sex ($F_{2,332}$=0.525; p=0.469) were noted. Post-hoc comparisons showed significant differences only for males, with CHF animals differing from CLF animals (p<0.05).

For conditioned freezing, a two-way interaction ($F_{2,332}$=3.207; p<0.05), and main effects for line ($F_{2,332}$=36.372; p<0.001) and sex ($F_{2,332}$=20.8; p=0.001) were observed. Pairwise post-hoc comparisons showed that, for males, CHF differed from CLF and

RND animals; for females, CHF differed from CLF and RND animals. Finally CLF differed from RND animals (all p <0.001).

The ANCOVA performed with post-shock freezing as a covariant factor showed a non-significant two-way interaction ($F_{2,331}$=0.324; p<0.723), and main effects for line ($F_{2,331}$=5.154; p<0.05), but not for sex ($F_{1,331}$=0.514; p=474).

S_{14} generation

Baseline results from S_{14} showed the absence of a two-way interaction ($F_{2,419}$=1.99; p=0.14), and main effects for line ($F_{2,419}$=17.76; p<0.001), but not for sex ($F_{1,419}$=1.53; p=0.21). Post-hoc comparisons showed that CLF differed from CHF and RND rats, for males (all p <0.001). For females, CHF differed from RND and CLF rats (all p <0.001). The analysis of post-shock freezing showed a non-significant two-way interaction ($F_{2,419}$=2.84; p=0.06), and main effects for line ($F_{2,419}$=17.12; p<0.001), but not for sex ($F_{1,419}$=0.28; p<0.6). Post-hoc comparisons indicate that, for males, CLF differed from CHF and RND animals (all p <0.05). The same pattern of results was found for females.

The analysis of conditioned freezing showed the presence of a significant two-way interaction ($F_{2,419}$=3.994; p<0.05), and main effects for line ($F_{1,419}$=67.23; p<0.001) and sex ($F_{1,419}$=25,65; p<0.001). Post-hoc comparisons showed that CHF, CLF and RND rats differed significantly among themselves (all p <0.05). The same pattern of results was found for females.

The ANCOVA results showed the absence of a significant two-way interaction ($F_{2,418}$=2.577; p=0.77), and main effects for line ($F_{2,418}$=54.065; p<0.001) and sex ($F_{1,418}$=30.06; p<0.001).

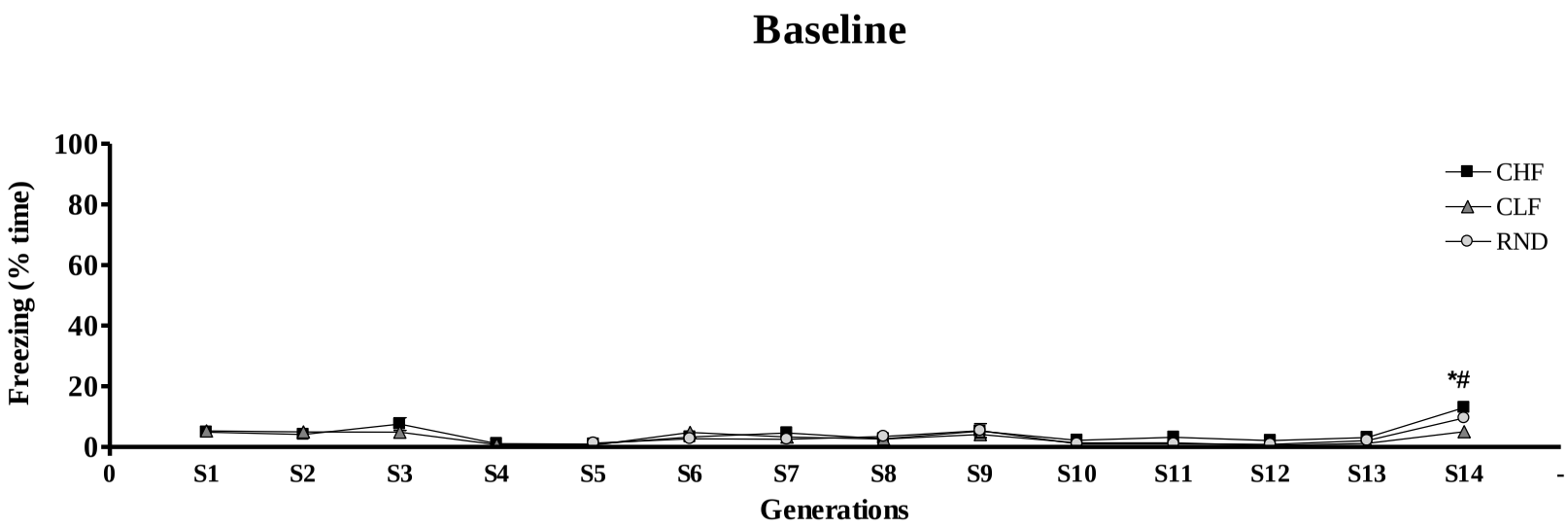

Figure 6 - Mean (± SEM) percentage of time spent freezing during the baseline acquisition session period of fourteen generations (S_1-S_{14}) of male and female rats selected for high (CHF) and low (CLF) levels of conditioned freezing, as well for randomly selected (RND) rats; * indicates significant differences between CHF and CLF rats; # indicates significant differences between CHF and RND rats (all $p < 0.05$).

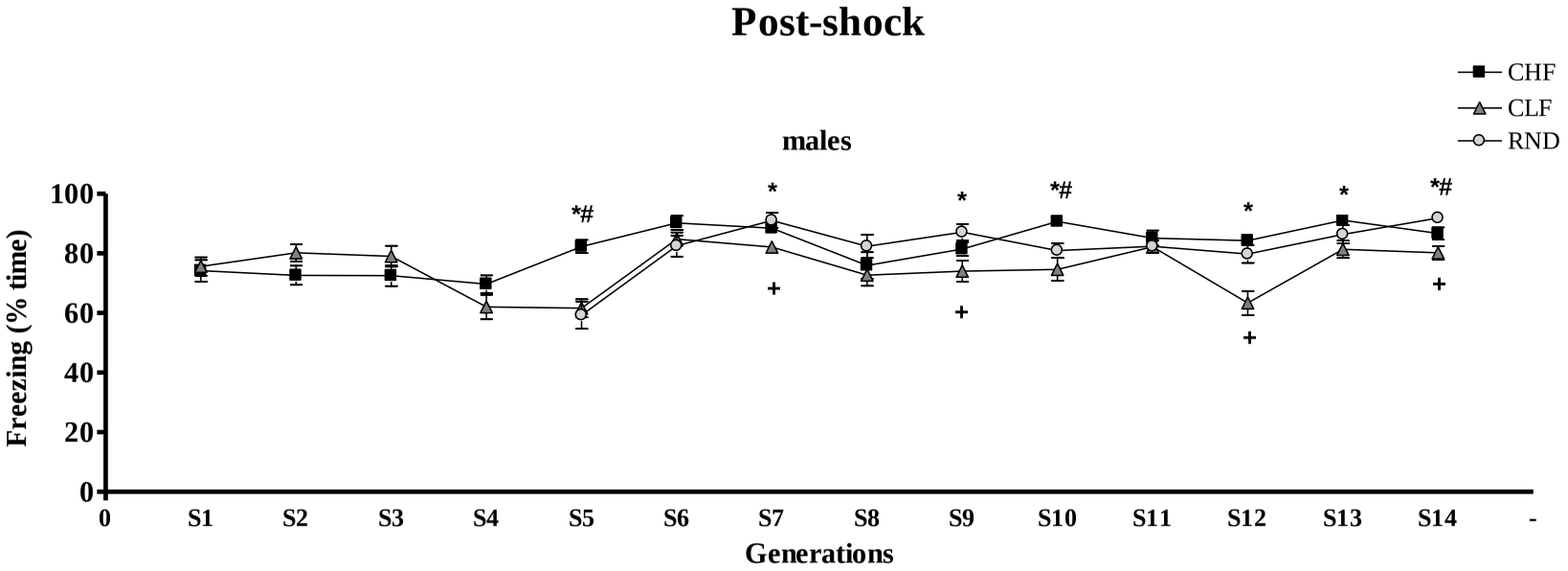

Figure 7 - Mean (± SEM) percentage of time spent freezing during the post shock acquisition session period for males of fourteen generations (S_1-S_{14}) of rats selected for high (CHF) and low (CLF) levels of conditioned freezing, as well for randomly selected (RND) rats; * indicates significant differences between CHF and CLF rats; # indicates significant differences between CHF and RND rats; + indicates significant differences between CLF and RND rats (all $p < 0.05$).

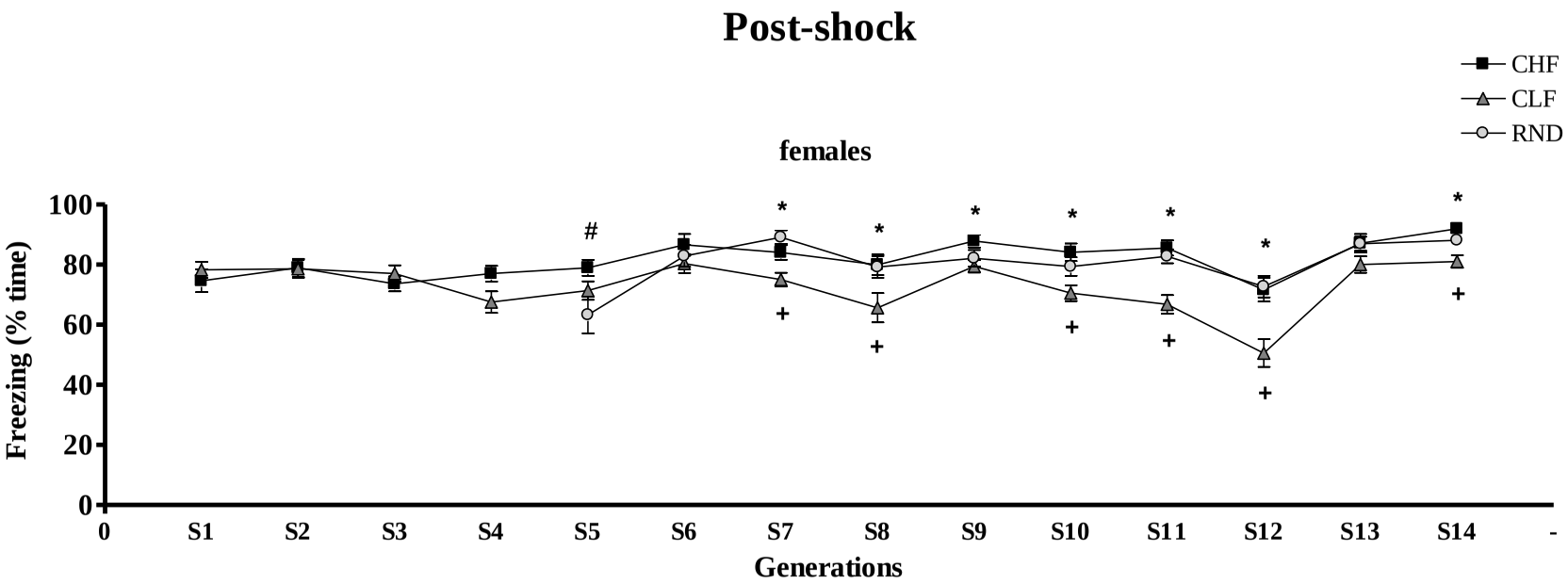

Figure 8 - Mean (± SEM) percentage of time spent freezing during the post shock acquisition session period for females of fourteen generations (S_1-S_{14}) of rats selected for high (CHF) and low (CLF) levels of conditioned freezing, as well for randomly selected (RND) rats; * indicates significant differences between CHF and CLF rats; # indicates significant differences between CHF and RND rats; + indicates significant differences between CLF and RND rats (all $p < 0.05$).

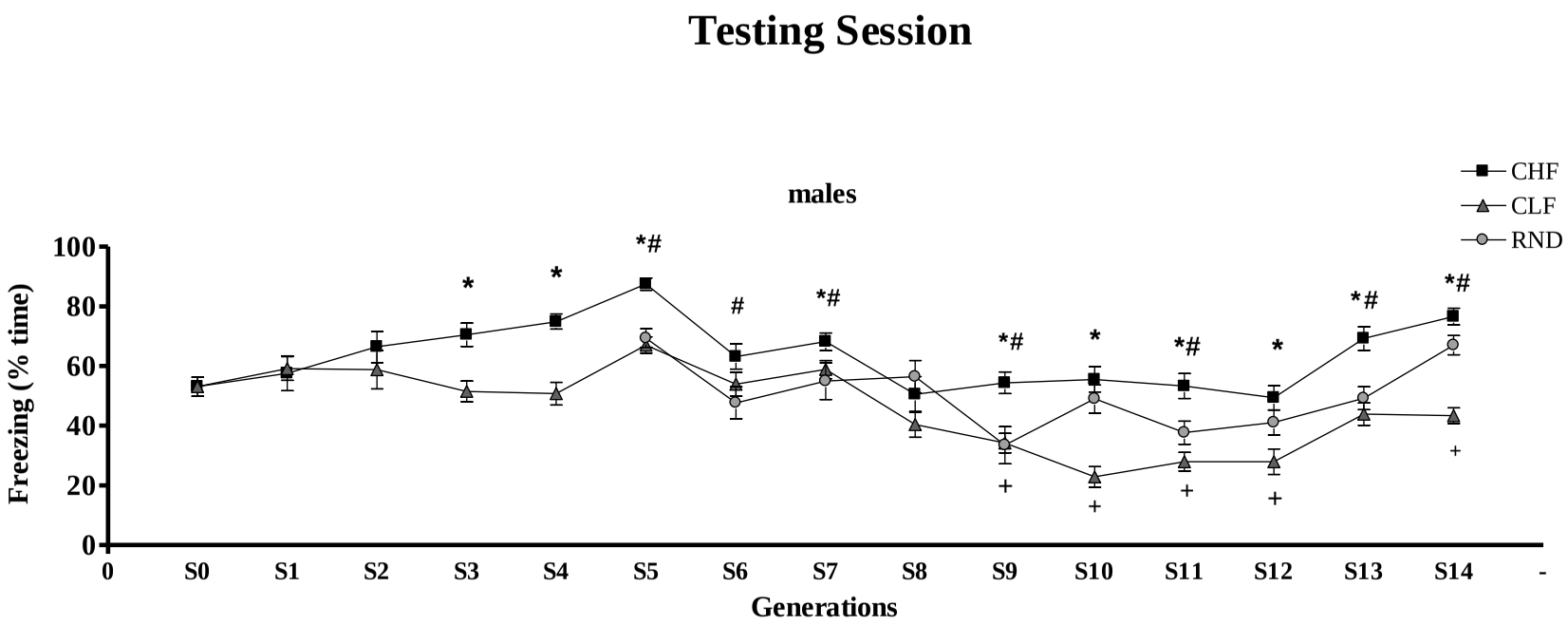

Figure 9 - Mean (± SEM) percentage of conditioned freezing during the testing session period for males rats selected for high (CHF) and low (CLF) levels of conditioned freezing, as well for randomly selected (RND) rats, in relation to the S_0 generation and the next fourteen generations (S_1-S_{14}); * indicates significant differences between CHF and CLF rats; # indicates significant differences between CHF and RND rats; + indicates significant differences between CLF and RND rats (all $p < 0.05$).

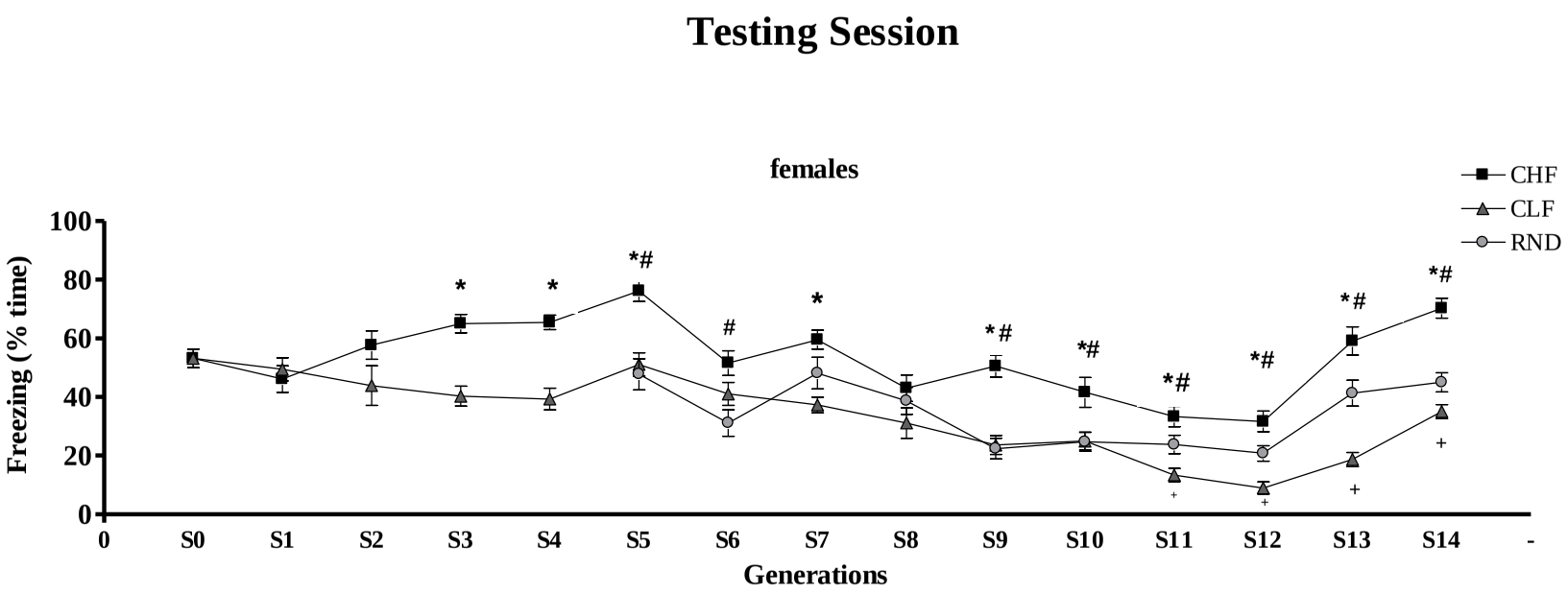

Figure 10- Mean (± SEM) percentage of conditioned freezing during the testing session period for males rats selected for high (CHF) and low (CLF) levels of conditioned freezing, as well for randomly selected (RND) rats, in relation to the S_0 generation and the next fourteen generations (S_1-S_{14}); * indicates significant differences between CHF and CLF rats; # indicates significant differences between CHF and RND rats; + indicates significant differences between CLF and RND rats; (all $p < 0.05$).

Different footshock intensities

The impact of different footshock intensities in CHF and CLF animals was also evaluated. The lines of each generation were clustered according to their respective footshock intensity (S_1-S_5: 1.0 mA; S_6-S_7: 0.7 mA; S_8-S_{14}: 0.6 mA). An initial three-way ANOVA for shock intensity (0.6; 0.7 and 1.0 mA), selected line (CHF and CLF) and for sex (male and female) was performed. It was found a significant two-way interaction for shock X line ($F_{2,2628}$=10.7; $p<0.001$), a significant two-way interaction for line X sex ($F_{1,2628}$=4.9; $p<0.05$), and main effects for line ($F_{1,2628}$=218.8; $p<0.001$) and sex ($F_{1,2628}$=101.6; $p<0.001$). However, it was found an absence of a significant three-way interaction regarding shock X line X sex ($F_{2,2628}$=0.53; $p>0.05$).

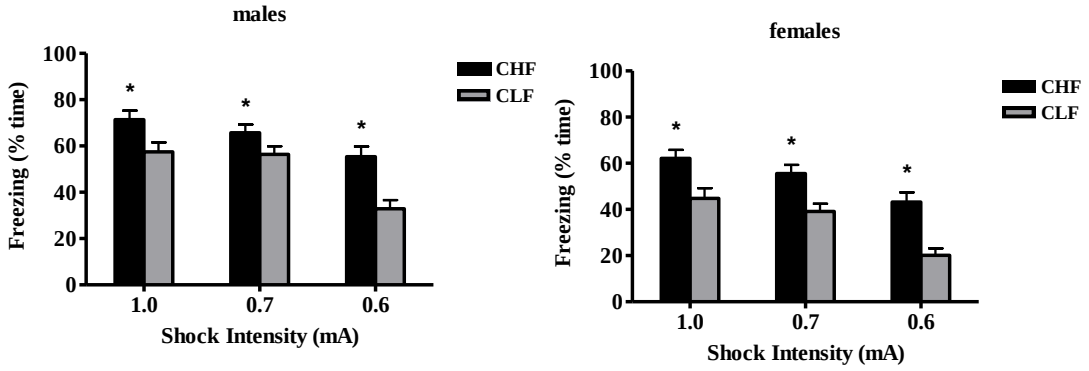

Figure 11: Mean (±SEM) percentage of conditioned freezing at different footshock intensities in CHF and CLF rats, among males and females; * denotes significant differences ($p<0.05$)

A subsequent two-way ANOVA only for shock intensity (0.6; 0.7 and 1.0 mA) and selected line (CHF and CLF) was then performed. Results showed a significant two-way interaction ($F_{2,2634}$=10.69; $p<0.001$), and main effects for shock intensity ($F_{2,2634}$=115.82; $p<0.001$) and line ($F_{1,2634}$=207.67; $p<0.001$). Significant differences between CHF and CLF were found in all shock levels (all $p<0.05$). Moreover, it was observed higher differences between CHF and CLF rats at the shock intensity of 0.6 mA (Figure 11). Indeed, this impression was

confirmed by a Delta comparison between CHF and CLF rats, among males and females, at every shock level (Figure 12).

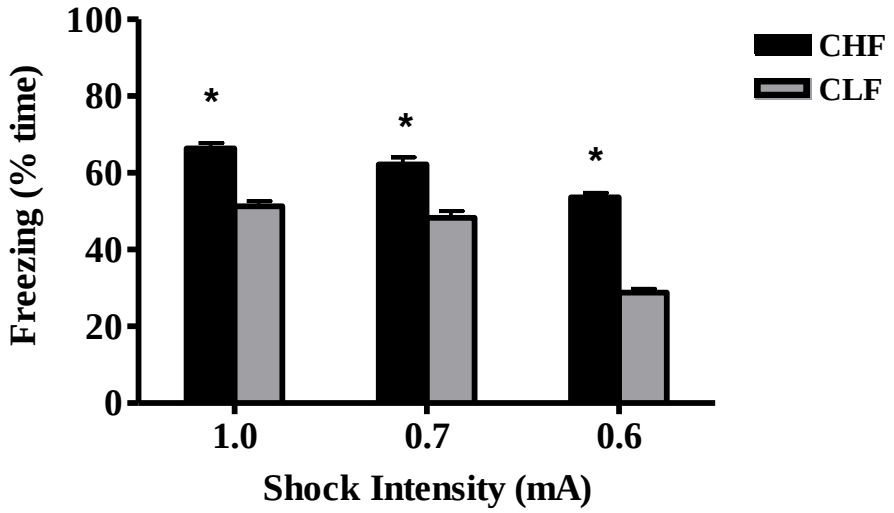

Figure 12: Mean (±SEM) percentage of conditioned freezing at different footshock intensities in CHF and CLF rats; * denotes significant differences ($p<0.05$)

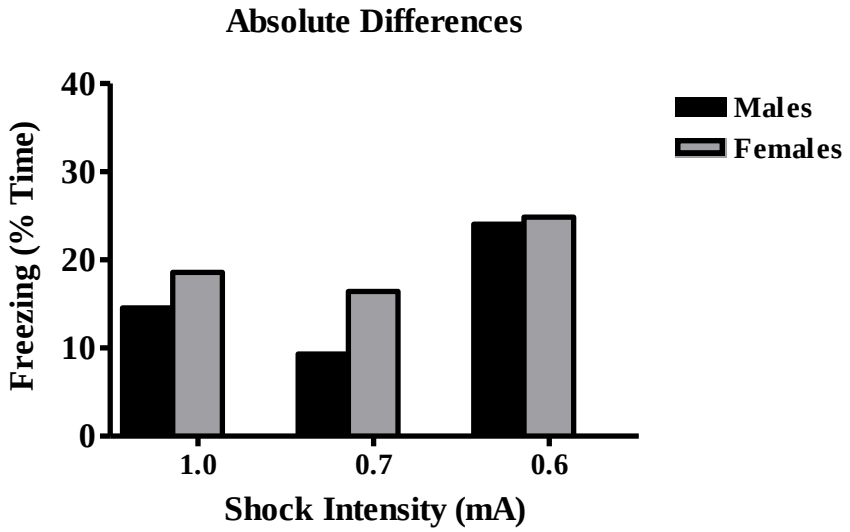

Figure 13: Absolute differences of conditioned freezing between CHF and CLF rats, among males and females, at different shock intensities.

Impact of a randomly selected (RND) control group

To asses the influence of RND animals in the selective breeding program since their introduction, conditioned freezing was analyzed through a two-way ANOVA, including only generations S_5 to S_{14}, but pooling together data from all these generations. The first factor, with 3 levels, was breeding line (CHF, CLF and RND); the second factor, with two levels, was related to the animal's sex (male and female). A significant two-way interaction ($F_{2,2868}$=4.87; $p<0.05$), and main effects for line ($F_{2,2868}$=152.173; $p<0.001$) and sex ($F_{1,2868}$=182.85; $p<0.001$) were observed. Post-hoc comparisons showed that CHF and CLF differed from each other and from RND animals, for both males and females (all $p <0.001$). Figure 13 shows the mean (+SEM) of conditioned freezing of CHF, CLF and RND rats, for males and females.

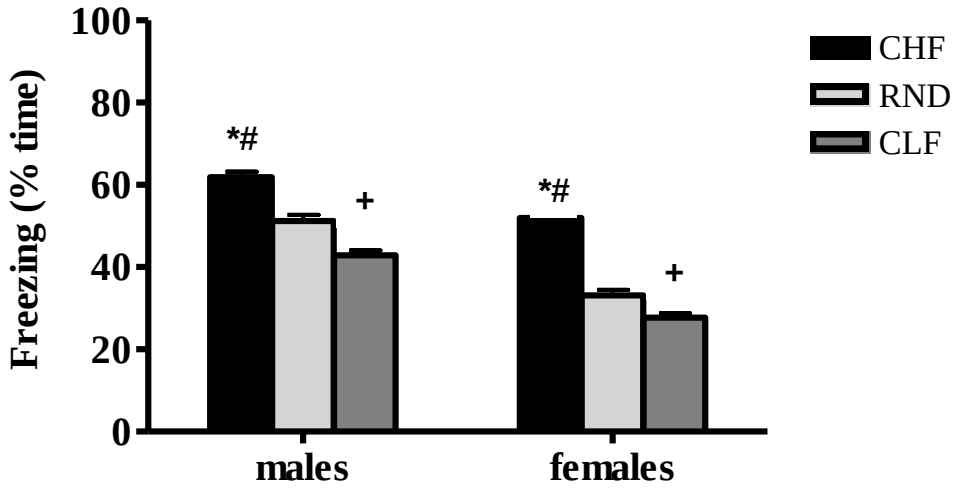

Figure 14: Mean (+SEM) percentage of conditioned freezing in generations S_5-S_{14}, all pooled together, of CHF, CLF and RND rats, for males and females. * indicates significant differences between CHF and CLF rats; # indicates significant differences between CHF and RND rats; + indicates significant differences between CLF and RND rats (all $p < 0.05$).

Sex Differences

Sex differences were evaluated for post-shock freezing and conditioned freezing. For post-shock freezing, males rats froze more in the post shock acquisition period (79.1% ± 0.49) than females (78.4% ± 0.52). A Student's t test analysis showed significant differences between the groups (t_{3471}=2.064; $p<0.05$). The same pattern of results was found for conditioned freezing registered in the training session. In general, male rats froze 53.38 (± 0.72), whereas females froze 39.78% (± 0.71) in testing sessions. Student's t test indicates a significant difference between the two groups (t_{3471}=13.0425; $p<0.001$).

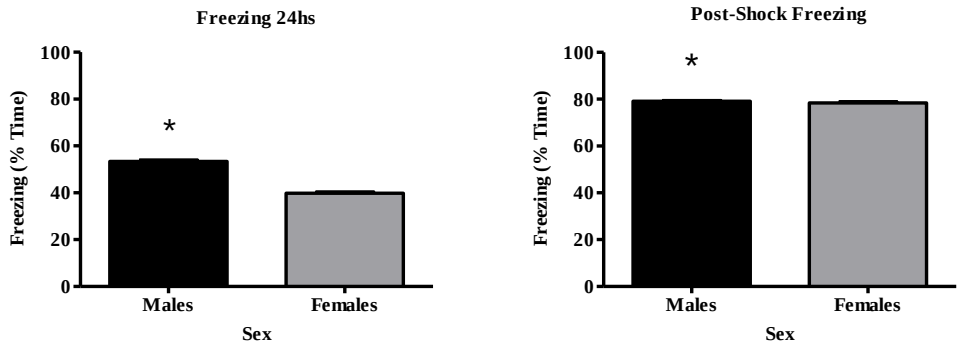

Figure 15: Mean (± SEM) of conditioned freezing registered 24hs after the training session (left) and of freezing registered during the post-shock acquisition period (right) among males (n=1768) and females (n=1705) rats; * indicates significant differences ($p<0.05$).

Absolute differences between CHF, CLF and RND animals through generations

In order to evaluate the strength of the selection procedure, absolute differences in conditioned freezing between CHF, CLF and RND animals, among males and females, was assessed through a Delta comparison of each generation of selective breeding (CHF x CLF; CHF x RND; CLF x RND).

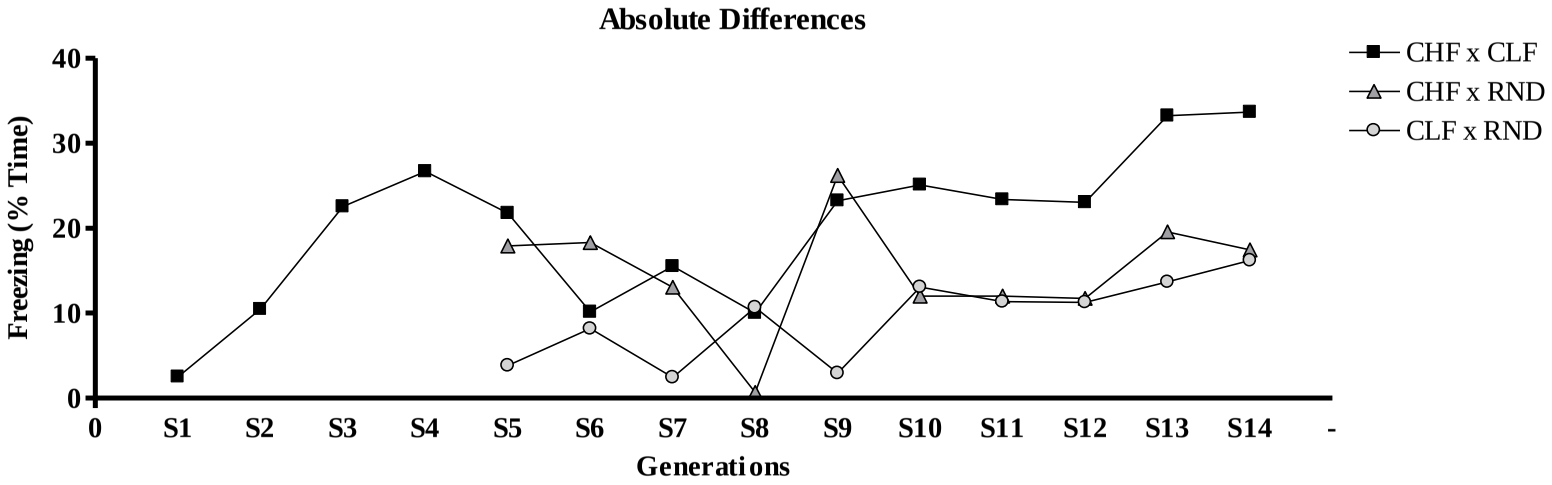

Figure 16: Absolute differences in conditioned freezing behavior between selected lines along thirteen generations of selective breeding.

To evaluate the absolute differences accumulated between the comparison groups during thirteen generations of selective breeding, we performed a One-Way ANOVA, with the Delta means for each comparison group as a factor. It was found a significant interaction ($F_{2,31}$=6.12; $p<0.05$), and post-hoc analysis showed that the comparison between CHF x CLF and between CHF x RND rats differed significantly from the CLF x RND comparison ($p<0.05$).

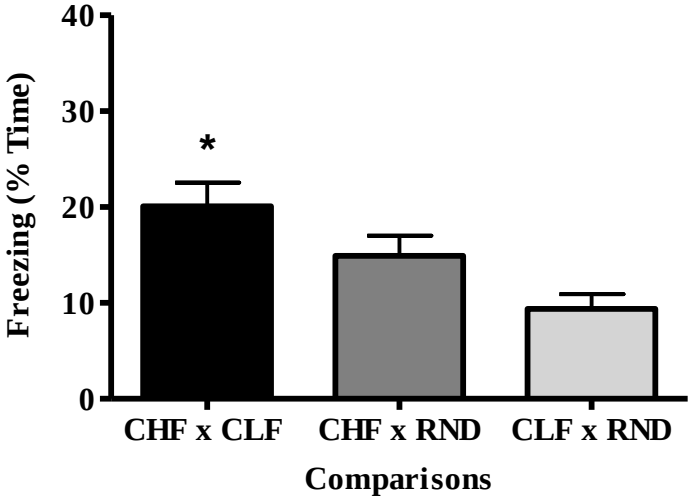

Figure 17: Mean (± SEM) of absolute differences of conditioned freezing between selected lines; * denotes significant difference between CHF x CLF and CLF x RND comparisons ($P<0.05$).

Heritability

The heritability ratio (h^2b), also called broad heritability, measures the degree of phenotypic variation (V_P) due to genetic factors for a single population under the limits of environmental variability during the study. In this sense, h^2b measures the variation observed in the phenotype, i.e., expresses the proportion of variance due to the genetic component. However, in selection experiments, researchers are more interested in the improvement of one specific trait (e.g. Freezing Behavior), which is regulated by the effects of additive genes. This more limited estimate has been called narrow heritability (h^2n). The basic formula to

calculate the narrow heritability is: $h^2n=R/S$, where "R" is the "Genetic Gain", obtained by subtracting the mean of one generation from the mean of the previous generation; and "S" is the selection differential, obtained by subtracting the mean of the selected individuals from the mean of its respective generation. Thus, to obtain the effects of additive genes, we use the formula $R=h^2n \times S$ (Klug & Cummings, 1991).

By applying this formula we evaluate the degree of heritability for freezing behavior, as well as the estimates of Genetic Gain in CHF and CLF rats, among males and females, during the selective breeding procedure. Due to variations in the shock intensity during the phenotyping process, only generations under the same protocol regarding shock intensity were compared. Table… shows the generations of CHF and CLF rats evaluated and their respective previous generation. Figure… shows the h^2n ratio of CHF and CLF during the selective breeding process and figure… shows the Genetic Gain observed in CHF and CLF lines across the selective breeding procedure.

Comparisons

Generation	Previous Generation	Shock Intensity
S_1	S_0	1.0mA
S_2	S_1	1.0mA
S_3	S_2	1.0mA
S_4	S_3	1.0mA
S_5	S_4	1.0mA
S_7	S_6	0.7mA
S_9	S_8	0.6mA
S_{10}	S_9	0.6mA
S_{11}	S_{10}	0.6mA
S_{12}	S_{11}	0.6mA
S_{13}	S_{12}	0.6mA
S_{14}	S_{13}	0.6mA

Table 4: Generations of CHF and CLF rats employed in the estimation of heritability and genetic gain.

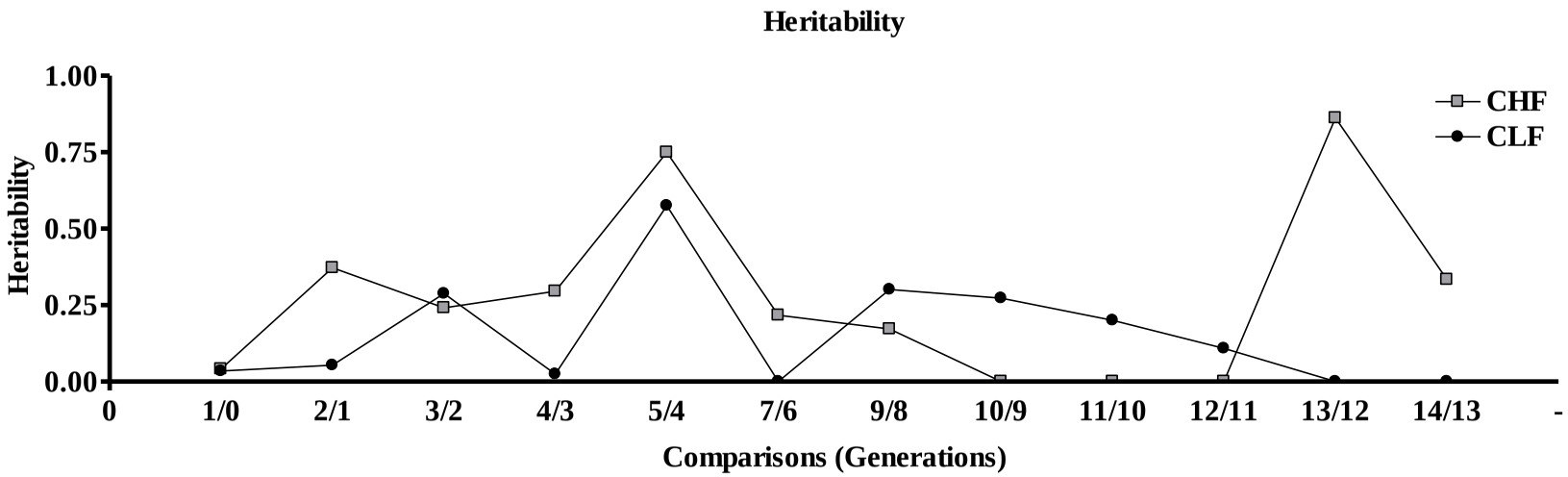

Figure 18: Estimates of narrow heritability (h^2) of freezing behavior for CHF and CLF rats along 14 generations of selective breeding.

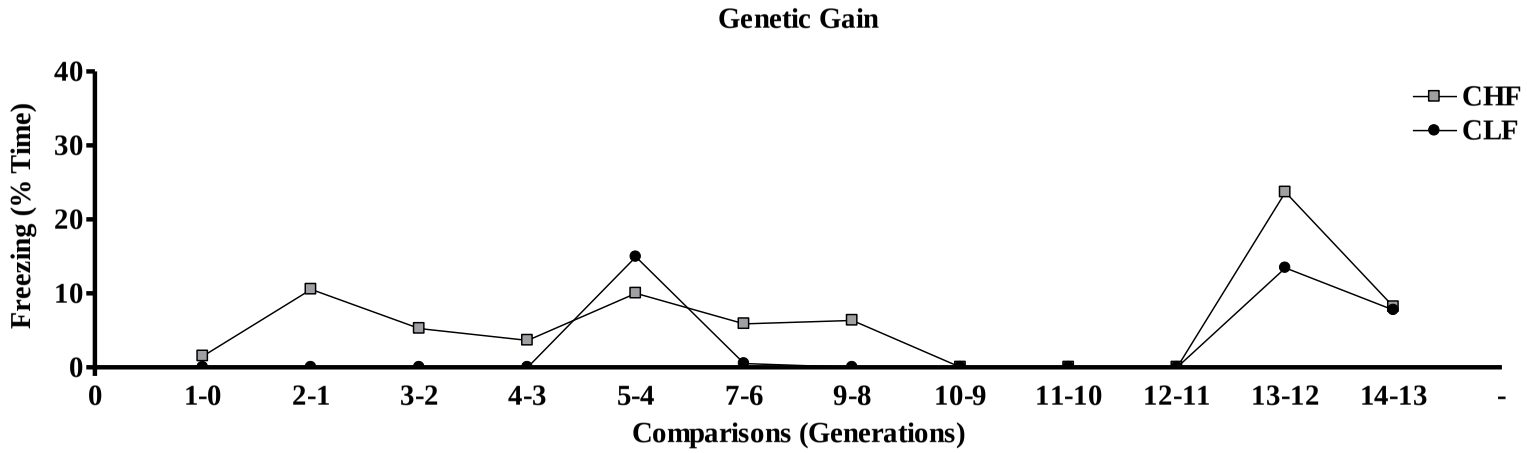

Figure 19: Estimates of Genetic Gain for CHF and CLF rats along 14 generations of selective breeding.

Body Weight

Figure 12 shows the mean (± SEM) of litter weight through 5 generations of selective breeding (S_9-S_{13}). Weight at PND 7 was analyzed using a one-way ANOVA for each generation. Non-significant differences between CHF, CLF and RND in all generations were observed (all $p < 0.05$).

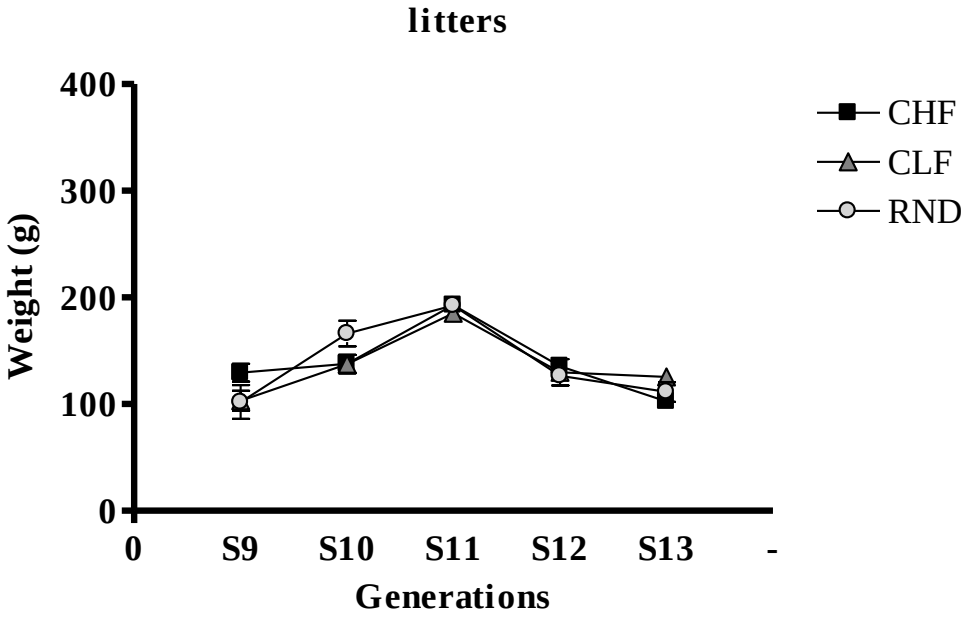

Figure 20 - Mean (± SEM) of litter weight from generation S9-S13 (all $p < 0.05$).

Rats are sexually dimorphic and body differences are expected. However, the pattern of change in body weight for males and females in the present study was similar. So, data for males and females was presented together.

Generation	Age (Days)	High Freezing		Randomly Selected		Low Freezing	
		Mean Weight (g)	(±) SEM	Mean Weight (g)	(±) SEM	Mean Weight (g)	(±) SEM
S_9	21	40,24	0,72	43,93	0,84	43,44	1,04
	42	134,06	1,57	133,09	1,82	129,59	2,27
	75-85	289,59	6,35	278,01	7,39	271,7	9,21
S_{10}	21	39,76	0,64	38,93	0,62	40,48	0,63
	42	128,37	9,2	139,64	8,88	125,07	9,15
	75-85	249,21	5,86	234,56	5,65	253	5,82
S_{11}	21	48,62	0,69	45,16	0,64	54,14	0,72
	42	135,35	2,04	132,06	1,91	149,54	2,13
	75-85	228,04	4,53	222,14	4,25	243,74	4,72
S_{12}	21	33,3	0,47	32,56	0,52	34,58	0,46
	42	117,59	1,43	118,95	1,58	111,9	1,42
	75-85	229,43	4,61	220	5,12	222,59	4,57
S_{13}	21	38,81	0,56	37,88	0,49	44,49	0,54
	42	176,8	7,09	161,25	6,27	166,53	6,86
	75-85	258,74	5,43	250,91	4,8	257,06	5,24

Table 5: Mean (± SEM) of body weight in S_9-S_{13} generations among CHF, RND and CLF rats.

To assess changes in body development among the lines, data from PND 21 and PND 42, as well as from the training day, were analyzed using a linear regression model correlating weight X age for each generation. Results showed a steady increase of body weight during development in all groups. Table 5 shows the linear model for each generation and line.

Generation	High Freezing		Randomly Selected		Low Freezing	
	Linear Regression Model	R^2	Linear Regression Model	R^2	Linear Regression Model	R^2
S_9	y = 124.68x - 94.72	0.98	y = 117.04x - 82.403	0.9814	y = 114.13x - 80.017	0.9804
S_{10}	y = 104.73x - 70.337	0.9922	y = 106.26x - 73.003	0.9863	y = 97.815x - 57.92	0.9997
S_{11}	y = 89.71x - 42.083	0.9996	y = 94.8x - 40.46	0.9999	y = 88.49x - 43.86	0.9999
S_{12}	y = 98.065x - 69.357	0.9935	y = 94.005x - 64.987	0.9896	y = 93.72x - 63.603	0.998
S_{13}	y = 109.97x - 61.813	0.9788	y = 106.29x - 56.543	0.9927	y = 106.52x - 63.017	0.9917

Table 6: Linear trend model for generation S_9-S_{13} in CHF, RND and CLF rats.

3.8
Discussion

In any selection experiment aiming at the study of emotionality, the main objective is to produce groups of animals with opposite behavioral responses to the same threatening environmental stimuli, and then investigate their genetic underpinnings. Emotionality is a construct that mirrors the emotional reactivity of an animal to its environment. Therefore, the use of multiple measures of emotionally is crucial to dissect the appropriate overlay between the different dimensions of this construct. This was the case of several studies described in the previous section of this work, which employed innate (Broadhurst, 1957; Ramos et al., 2003; Fujita, 1975; Liebsch et al., 1998a; Brunelli et al., 1996) or learned fear responses (Bignami, 1965; Brush, 1966; Gomes & Landeira-Fernandez, 2008).

In this sense, the main purpose of the present study was to develop a bidirectional selective breeding program employing Wistar rats, using the conditioned freezing in response to contextual cues previously associated with footshocks as selection criterion. The hypothesis is that the phenotypical differences in learned fear may be associated with functionally different conditioned fear circuits. The preliminary results of this ongoing procedure in our laboratory represent the first successful attempt to select rats with reliable and selective differences in conditioning freezing, and extend the findings of our previous report (Gomes & Landeira-Fernandez, 2008). Also, we introduced a third group of randomly mated rats (RND) in the selective breeding program, which may serve as a control group for the selected lines. Results from this continuous selective breeding program in our laboratory indicate a progressive divergence of the conditioned freezing phenotype in both male and female rats. Differences between CHF and CLF lines became clear after three breeding generations. Reports from mouse studies have indicated that only one generation was sufficient to differentiate high- and low- conditioned freezing lines (Ponder et al., 2007; 2008; Radcliffe et al., 2000), whereas the present results detected a reliable difference after three generations. This result may suggest subtle differences between the two species. The present CHF and CLF lines are

particularly meaningful since most behavioral, pharmacological, and neuroanatomical experiments studying conditioned fear have been conducted using rats.

Very low levels of freezing behavior were observed among all groups during the baseline periods of the acquisition sessions (Figure 6). ANOVA results did not show significant differences between all groups. The only exception was in S_{14}, with CHF animals demonstrating more unconditioned freezing than the other groups. This analysis clearly demonstrates that differences in conditioning freezing observed between CHF and CLF animals in the testing session were not related with the initial levels of animal activity during the baseline period. It also reveals that handling for 5 days results in very low levels of unconditioned freezing responses prior to the occurrence of footshocks.

However, results of post-shock freezing registered in the acquisition sessions are still unclear. Although the ANOVA analysis showed sex effects only in the S_5, S_7, S_{11} and S_{12} generations, in general males rats froze more in the post shock acquisition period (79.1% $\pm$ 0.49) than females (78.4% $\pm$ 0.52). A Student's t test analysis showed significant differences between the groups (t_{3471}=2.064; $p<0.05$). Also, the ANOVA results showed differences in the amount of post-shock freezing between CHF and CLF animals. These differences were not observed in our original report that employed the first three generations of these two lines (Gomes & Landeira-Fernandez, 2008), but it was detected in a recent work (Gomes et al, 2011a). Furthermore, differences between these two lines and the RND group were also observed, for both males and females (see Figure 9 and 10).

Three possibilities may explain these discrepant results. One is that the footshock intensity used to phenotype animals until the present generation (0.6 mA) was much lower than the intensity used during the first five generations (1.0 mA). Therefore, the higher footshock intensity could lead to a ceiling effect so that differences in post-shock freezing behavior might not be observed. Indeed, the footshock intensity was reduced in our breeding program in the 6th generation to 0.7 mA and in the 8th generation to the present intensity (0.6 mA) to prevent possible ceiling effects produced by this relatively strong (1.0 mA) footshock intensity. A second possibility could be related to the fact that freezing observed

immediately after footshock reflects associative learning between contextual cues and the aversive footshock (Fanselow, 1980, 1990; Vianna et al., 2001b). For example, when the footshock is presented simultaneously with the rat's placement in the chamber, no contextual fear conditioning is observed (Landeira-Fernandez et al., 1995). Moreover, placing the animal in a different context from the one in which the footshock was delivered did not produce any freezing behavior (Fanselow, 1980). Therefore, differences in the post-shock freezing between CHF and CLF animals could be a consequence of the fact that CHF rats have a greater propensity for exhibiting higher conditioned freezing responses compared with CLF animals because of the continuous bidirectional selection over different generations. A third possible explanation for these incongruent results might be related to differences in pain sensitivity between these two lines. This is an important issue because freezing observed immediately after footshock is closely related to pain sensitivity and shock intensity (Cordero et al., 1998; Fanselow, 1984b). According to this possibility, selection for high and low conditioned freezing might independently lead to co-selection of other contributing factors that are not genetically linked but contribute to the phenotype that is being selected, such as differences in pain sensitivity to footshock. However, further studies are necessary to thoroughly test this possibility.

The major finding of the present study was the divergence between CHF and CLF rats in the conditioned freezing behavior in response to contextual cues previously associated with footshocks, along fourteen generations of selective breeding. Differences became significant after three generations and have stabilized (at least onefold) since then. These data reveal that conditioned fear is a highly heritable trait and can be rapidly, bidirectionally selected after a few generations.

We evaluate the degree of narrow heritability (h^2n) of freezing behavior in CHF and CLF rats, among males and females, during the selective breeding procedure (see Results). Figure 18 shows the h^2n ratio of CHF and CLF during the selective breeding process. In the first five generations of selective breeding, we observed a high degree of heritability of freezing behavior for CHF and CLF rats, but this rate decreased in the subsequent generations. However, for CHF rats, the degree of heritability increased in the S_{13} and S_{14} generations again, suggesting

that the genetic gain observed in these two generations (Figure 19) could be explained by the effects of the continuous selective breeding process, whereas this explanation maybe not suitable for CLF rats, since they showed low levels of heritability in generations S_{13} and S_{14}. In this sense, we believe that we need more generations of selective breeding in order to make the characteristics of this recently developed model more clearer and consistent.

Even with stable differences between CHF and CLF rats observed in the majority of generations, two exceptions occurred during the phenotyping process. Although an observed trend of divergent levels of freezing between CHF and CLF rats was noted, in S_6 and S_8, no statistically significant difference was observed between CHF and CLF, for both males and females. A possible explanation for this inconsistency might be related with the fertility problems found (most probably to inbreeding depression effects) in generations S_5 and S_7, which gave rise to S_6 and S_8, respectively. To circumvent this problem, in S_5 an additional cross was made, whereas in S_7 two additional crosses were made. The inclusion of offspring with different days of birth may be affected by subtle and involuntary alterations in the experimental context. Another, more plausible, hypothesis is related to the decreases occurred in the footshock intensities in our program, specifically in S_6 and S_8. As discussed above, the conditioned freezing response is particularly sensitive to shock strength (Fanselow and Bolles, 1979).

Indeed, a crucial variable of the present study is footshock intensity. As discussed above, the footshock intensity was reduced in our breeding program in the 6th generation to 0.7 mA and in the 8th generation to 0.6 mA, in order to prevent possible ceiling effects produced by the relatively strong intensity of 1 mA. Most importantly, strong footshock magnitudes could affect the selective breeding process as well, recruiting alternate pathways capable of mediating fear-related responses that are not directly associated with the conditioned freezing response (Ponnusamy et al, 2007). We evaluated the impact of different footshock intensities in CHF and CLF animals (see Results). The lines of each generation were clustered according to their respective footshock intensity (S_1-S_5: 1.0 mA; S_6-S_7: 0.7 mA; S_8-S_{14}: 0.6 mA). An initial three-way ANOVA for shock intensity (0.6; 0.7 and 1.0 mA), selected line (CHF and CLF) and for sex (male and female) was performed, but it was found an absence of a significant three-way interaction

regarding shock X line X sex. A subsequent two-way ANOVA only for shock intensity (0.6; 0.7 and 1.0 mA) and selected line (CHF and CLF) showed a significant two-way interaction, and main effects for shock intensity and line. Importantly, significant differences between CHF and CLF rats were found in all shock levels (all $p<0.05$). Moreover, it was observed higher differences between CHF and CLF rats at the shock intensity of 0.6 mA (Figure 13). A Delta comparison between CHF and CLF rats at every shock level confirmed this impression (Figure…). In this regard, results suggest that the footshock intensity of 0.6 mA could be the ideal for our phenotyping process.

Inter-chamber footshock reliability is essential for minimizing errors within experimental groups. Variations in footshock intensity (US) between chambers add additional variables that make interpreting the data difficult (Chang et al, 2009). For this reason, all conditioning chambers were calibrated with a multimeter before each experiment. However, a recurrent problem employing rodents in fear conditioned experiments is the accumulation of animal waste (i.e. urine, feces) in the stainless steel rods of conditioned chambers, as well in the scrambler circuit. This could affect the experimental procedure itself, reducing footshock intensities and leading to a reduction in freezing levels in all experimental groups. In this context, we employed new conditioned boxes in S_{13} and S_{14}, which probably led to an increase in the freezing levels observed in all groups. However, the behavioral divergence already observed between the selected lines in previous generations was maintained.

Another important feature of the present selective breeding program is the development of a randomly selected group of animals (RND), with intermediate levels of freezing behavior. This group of animals might serve as a control for the high- and low- conditioned freezing lines. The RND group was introduced when CHF and CLF rats were in the fifth generation of selective breeding (S_5). Based on data collected so far, RND animals in our selective breeding program showed promising results. A significant interaction was observed, with RND animals presenting intermediate freezing levels, in S_{10} and S_{14} for males and S_{12}, S_{13} and S_{14} for females.

We assessed the influence of RND animals in the selective breeding program since their introduction through a two-way ANOVA, including only

generations S_5 to S_{14}, but pooling together data from all these generations. It was found a significant two-way interaction, and main effects for line and sex. Importantly, post-hoc comparisons showed that CHF and CLF differed from each other and from RND animals, for both males and females (all $p < 0.001$). We evaluate absolute differences in conditioned freezing between CHF, CLF and RND animals, among males and females through a Delta comparison of each generation of selective breeding (Figure 16) and also absolute differences accumulated during thirteen generations of selective breeding. ANOVA results showed that the comparison between CHF x CLF and between CHF x RND rats differed significantly from the CLF x RND comparison ($p<0.05$).

These findings suggest that RND animals are presenting intermediate levels of freezing compared with the high and low lines. This is an important issue since, despite the importance of a randomly selected control group most of the selective programs reviewed in this work did not employ a group of random controls (Broadhurst, 1957, 1958; Ramos et al., 2003; Fujita, 1975, 1984; Liebsch et al., 1998a, b; Bignami, 1965). The only exception was the High and Low USV lines, widely divergent from each other and from the respective Random line, which has maintained N:NIH strain USV rates overall from generation to generation. However, in general, it was observed that High USV lines demonstrate more behavioral measures consistent with an "anxious" phenotype, whereas the Low line usually shows few behavioral differences from random control animals (Brunelli, 2005).

Due the fact that conditioned freezing response is a function of shock intensity, dependent on the association between conditioned and unconditioned stimuli, and is sensitive to a series of manipulations that interfere with its associative strength (Fanselow and Bolles, 1979; Landeira-Fernandez, 1996; Landeira-Fernandez et al., 1995), it is possible that differences in contextual fear conditioning between CHF and CLF animals might reflect differences in the pain sensitivity of these two groups. Previous studies indicate that post-shock freezing and freezing observed 24 h after contextual fear conditioning are mediated by associative learning (see Landeira-Fernandez, 1996 for a review). In this regard, an ANCOVA was performed, with post-shock freezing as a covariant factor, for each generation of selective breeding, in order to evaluate whether the breeding

line effect on conditioned freezing during the test session was attributable to post-shock differences that these animals presented during the training session. The ANCOVA results showed the absence of significant results in all generations (see Results). The only exception occurred in S_{10}, where a significant two-way interaction was observed ($F_{2,243}$=6.301; $p<0.05$). In this regard, an additional ANCOVA was performed, with post-shock freezing response as a covariant factor. But, in this case, the RND line was excluded from the analysis. The first factor, with 2 levels, was breeding line (CHF and CLF), and the second factor, with 2 levels, was the animal's sex (male and female). In this situation, results showed a non-significant two-way interaction ($F_{1,163}$=3.837; $p>0.05$), and main effects for line ($F_{1,163}$=21.042; $p<0.001$) but not for sex ($F_{1,163}$=1.023; $p=0.313$). Together, these findings weaken the possibility of differences in pain sensitivity being responsible for differences in conditioned fear, suggesting dissociation between the post-shock freezing response and the conditioned freezing response in the present study. Also, we propose that these two forms of freezing behavior might be mediated by a distinct set of genes that in turn regulates different neural mechanisms associated with each form of learning. In accordance to this view, it has been shown that freezing 24 h after conditioning, but not post-shock freezing, is mediated by N-methyl D-aspartate receptors (Kim et al., 1991; Kim et al., 1992). However, future studies are important to further evaluate whether CHF and CLF rats might present differences in pain sensitivity.

A possible criticism regarding the present selective breeding procedure is related to the accurate measurement of the conditioned freezing response itself. In the present study, freezing behavior was scored manually by an experienced observer (VCG), blind to the experimental conditions. However, freezing manual score is a long and tedious method that often requires multiple independent observations, being seriously susceptible to potential bias. Indeed, in order to ensure robust measures of freezing behavior, several studies have attempted to develop automated methods for the analysis of rodent motion during fear conditioning procedures (Contarino et al., 2002; Fitch et al., 2002; Marchand et al., 2003; Takahashi, 2004; Kopec et al, 2007; Anagnostaras et al, 2000), including our own (Gomes et al, 2009). Although some advantages were observed, these algorithms have some drawbacks. For example, several require

very sophisticated hardware that measure animal activity indirectly (e.g. photo-beam interruption of force-transduction). Others show poor time resolution, produce non-linear results, or only score motion and not freezing. Unfortunately, neither of these systems was suitable for our current experimental conditions. However, if more robust methods of automated freezing register were to be developed in the future, they would be incorporated in our selective breeding program in order to strengthen the phenotyping process.

Finally, another important aspect regarding the accurate measurement of conditioned freezing is the use of independent observations. Indeed, the data collected in S_{14} came from a different observer (CEB) from the other first thirteen generations. Results were very consistent, with the same behavioral pattern observed in the majority of generations.

Body Weight

Another interesting finding of the present study is related to body development among all rat groups. Body weight began to be measured in S_9. Litters were weighed at PND 7, and individual animals were weighed in PND 21, PND 42 and on the training day. Rats were sexually dimorphic and differences in body weight were as expected. Results showed a steady increase of body weight during development in all groups. This suggests that the continuous selective breeding process has not affected, so far, growth rates and body development measured as body weight. However, it is not unusual to observe differences in body weight in artificial selection experiments. For example, it was observed that Low line USV birth weights became significantly lower than both High and Random line weights since the 14^{th} generation of selective breeding. However, by the time of weaning Low line weights were not different from the High and Random lines for either sex, up to adulthood. The prenatal and/or genetic mechanisms underlying this long-term reduction in Low USV line birth weight are not yet known. The hypothesis is that the Low line fetuses may be genetically programmed for smaller sizes, or that the Low Line maternal uterine environment might be in some way unfavorable for fetus growth and development (Brunelli, 2005).

Sex Differences

Results from Study 1 also indicate that male rats consistently exhibit more conditioned freezing in the testing session than females during the development of the CHF and CLF lines, with the same being true for the RND control group. As a whole, male rats froze 53.38 (± 0.72), whereas females froze 39.78% (± 0.71) in testing sessions. Student's t test indicates a significant difference between the two groups (t_{3471}=13.0425; $p<0.001$). Significant differences between males and females were also found in post-shock freezing (see above). Sex differences favoring males have been observed in contextual fear conditioning (Maren et al., 1994; Markus & Zecevic, 1997) as well as in other spatial learning, such as in the 12-arm radial maze (Williams et al., 1990) and the Morris water maze (Roof, 1993). According to Steimer & Driscoll (2005), most of experimental studies involving anxiety and stress in rodents employed male animals. Basically, this preference is associated to the negative effects caused by physiological and behavioral variations that females present due the estrous cycle. Such variations are related to fluctuations of the hormones estrogen and progesterone. For example, it was observed that females from "Roman High Avoidance" rats are more active and less anxious in the proestrus in comparison to females in the diestrus. Besides, sex hormones might influence other tasks associated with learning and cognitive performance.

Sexual differences in anxiety tests employing rodents were firstly observed in the open field. In this model, males usually show less locomotor activity and higher defecation levels than females. Such results are traditionally interpreted as an indicative that males are more "fearful" or "anxious" than females. However, these differences could arise from other reasons, such as differences in metabolism levels, for example. Tests carried in other three anxiety models (Social Interaction, Elevated Plus Maze and Vogel Conflict Test) also indicate sexual differences. Nevertheless, the differences varied throughout the tests, with females demonstrating less anxiety in the Elevated Plus Maze, and being more anxious in the Vogel Conflict Test. Blanchard and colleagues (1991) showed that

females are more anxious than males in situations of potential danger, such as in the presence of a cat (for a review, see Palanza, 2001).

It has been suggested that these differences may be related to sexual dimorphisms observed in hippocampal anatomy and physiology. Indeed, electrophysiological studies have found that male rats that acquired contextual fear more rapidly than female rats also showed a higher magnitude of LTP induced at perforant path synapses in the dentate gyrus of the hippocampal formation (Maren et al., 1994; Maren, 1995). Therefore, it is possible that the marked sex differences observed in the present study are associated with a higher magnitude of male hippocampus LTP compared to female rats.

Conditioned fear and the interaction between two-way avoidance and freezing responses

Historically, the pavlovian fear conditioning was closely associated with one of the main causes of pathological anxiety (i.e. neurosis; Pavlov, 1927; Watson & Rayner, 1920). Surprisingly, the two-way avoidance response has been the main conditioned phenotype criterion employed for developing bidirectionally selected lines or strains based on learned aversive paradigms. That is the case for the Roman and Syracuse animals (discussed in the introduction section of this work) and other lines, such as Australian (Bammer, 1983), Koltushi (Ryzhova et al., 1983), and Hatano (Ohta et al., 1995) animals.

The use of the two-way avoidance response as the phenotype criterion for the development of so many genetic models of fear conditioning is curious because the learning mechanisms involved in the acquisition of this response are still unclear. In fact, two-way avoidance learning represents one of the oldest theoretical debates in behavioral sciences (for an elegant review of this debate, see Bolles, 1972). The two-factor theory (Mowrer and Lamoreaux, 1946) was one of the first attempts to address this issue. This theory posits that two different forms of learning are responsible for the acquisition of two-way avoidance. Initially, an animal undergoes classic aversive conditioning between the CS and US. Subsequently, the animal learns the instrumental response of crossing from one compartment to the other to terminate the CS and thus negatively reinforces the

response through a fear reduction process. According to this theory, the more "afraid" the animal is of the CS (respondent learning), the better the acquisition of the two-way avoidance response (instrumental learning). Therefore, an instrumental response is employed to measure the amount of fear triggered by the CS.

Although the two-factor theory has the appealing feature of integrating respondent and operant learning processes, several results have raised serious criticisms regarding this theoretical framework. As previously discussed, higher electrical footshocks are associated with lower two-way avoidance performance (Levine, 1966; McAllister et al., 1971). Moreover, manipulations that decrease contextual fear conditioning enhance the acquisition of the two-way avoidance response (Dieter, 1977). Finally, two-way avoidance can be bidirectionally modulated pharmacologically. Anxiolytic drugs enhance, whereas anxiogenic compounds impair, the acquisition of this response (Fernández-Teruel et al., 1991; Savić et al., 2005). However, these results contrast with the two-factor theory and indicate that the less emotionally reactive the animal is to the aversive situation, the better the animal will learn the shuttle box response. This fact imposes a certain problem with this animal model of learned fear, since the inability to learn the two-way avoidance response (i.e., a negative result) is employed as an index of the presence of a conditioned fear reaction. An alternative view of the two-way avoidance learning process is based on the fact that fear becomes classically conditioned, not only in response to a discrete CS that signals the occurrence of the footshock but also in response to contextual cues of each compartment where the US is presented (Landeira-Fernandez, 1996). Freezing in response to contextual cues and the CS might interfere with two-way avoidance acquisition. In fact, recent results indicate that animals tend to freeze when required to go to the compartment where they were previously shocked (Vicens-Costa et al., 2011).

On the other hand, the Carioca lines employed a much simpler procedure that involved contextual aversive conditioning, with freezing used as a direct and prominent measure of conditioned fear. Contextual fear conditioning is a useful paradigm for studying long-term memory in animals and has been widely shown to be a reliable behavioral index of associative fear (Fanselow, 1984). Moreover, contextual fear conditioning in rats is a highly heritable trait that can be rapidly

and bidirectionally selected (Gomes & Landeira-Fernandez, 2008). Anatomical and electrophysiological studies have described the neural circuitry involved in both CS and contextual fear conditioning, including the entire extent of sensory inputs to endocrine, autonomic, and behavioral outputs (Delgado et al., 2008; Fanselow, 1994; LeDoux, 2000). Long-term potentiation in the amygdala has also been shown to mediate the formation of fear conditioning (Goosens and Maren, 2004; Sigurdsson et al., 2007). Finally, isomorphism appears to exist between the freezing response to contextual stimuli paired with electrical shocks and generalized anxiety disorder (for a review, see Brandão et al., 2008). Therefore, the Carioca lines might represent an ideal system for studying the molecular and cellular bases of conditioned fear.

Genetics of Pavlovian fear phenotypes

Several studies employing phenotypic differences in conditioned fear are just beginning to identify how divergent conditioned fear memories responses may be related with different sets of genes. Clinical studies with human PTSD patients, for example, consistently indicate that they are more “conditionable”, have greater emotional valence for conditioned stimuli and take longer to extinguish fear once established than non-PTSD subjects (Peri et al., 2000; Orr et al., 2007; Blechert et al., 2005; Norrholm et al., 2011). Corroborating genetic studies of fear learning recurrently mention the elucidation of genes involved in human anxiety disorders as a goal (Moldin, 2000)

The individual variability in traits related with associative fear has been determined in rats and mice through the use of selected lines and inbred strains. The major advantage of employing inbred strains is that the differences in fear related behavior between strains provide direct evidence of variability. This was confirmed by several studies demonstrating that diverse inbred strains of mice differ in their performance in cued and contextual fear conditioning (Gerlai, 1998; Nguyen et al, 2000; Owen et al, 1997b; Paylor et al, 1994; Stiedl et al, 1999; Bolivar et al, 2001). Furthermore, a number of quantitative trait loci (QTL) have been identified as influencing fear conditioning in mice. For example, QTLs were located on chromosomes 1, 10 and 16 for freezing in response to cued stimulus

(Caldarone et al, 1997; Owen et al, 1997; Valentinuzzi et al, 1998; Wehner et al, 1997). Besides, selection studies with mice showed that significant differences in conditioned fear can be achieved using selective breeding, which also establishes their genetic underpinnings (Radcliffe et al, 2000; Ponder et al, 2007a; 2008).

Phenotypical differences in neural networks underlying fear responses are likely to contribute to phenotypical differences in conditioned fear behavior. Evidence indicates that differences in neuronal structure present in the fear circuitry are associated with differences in fear behaviors. An important study reported by Wellman et al (2007) used a 5-HTT transporter (5-HTT) knockout mouse as a model, and demonstrated a phenotype deficit in fear extinction recall. Moreover, associated structural changes in the medial prefrontal cortex (MPFC) and amygdala were also found, when neurons in these pathways showed increased dendrite length and increased spine density, respectively. The same authors (Izquierdo et al, 2006) also produced a reduced fear extinction phenotype using behavioral stress. In this case, a brief and uncontrollable stress led to dendrite retraction in neurons located in the mPFC, with an associated reduction in fear extinction.

However, most of the studies related to genetics of conditioned fear employed mice as subjects. It is our hypothesis that the recently developed CHF and CLF animals could be a suitable model in the search for the underlying genes related with conditioned fear response in rats. The combination of a variety of genetic approaches, such as selective breeding, with a large body of data on the cellular plasticity mechanisms and neural networks of conditioned fear shows great potential in attempting to explain why some individuals are capable to form longer lasting and stronger conditioned fear memories and to what extent this defensive outcome is related to other anxiety-related responses. The last 20 years have seen the precise identification of the input and output circuits of the amygdala, as well as emerging data regarding synaptic plasticity which encodes fear memories. Undoubtedly, the combination of the data on neural and cellular mechanism of conditioned fear and genetics of fear phenotypes is fundamental to our understanding of fear pathologies, in particular the Human Anxiety Disorder (Johnson et al, 2012).

In sum, the present study introduces two new lines of rats bidirectionally selected for their enhanced (CHF) or reduced (CLF) contextual fear conditioning, as measured by freezing behavior. A divergence between these two lines was observed after three generations, indicating a strong heritable component of this trait. A random (RND) line of randomly selected rats was also used as a control group for the CHF and CLF lines.