Investigating the behavioural and physiological responses associated with observational fear memory

Leonardo Piovanelli

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Supervisor: Prof. Estelle Barbier

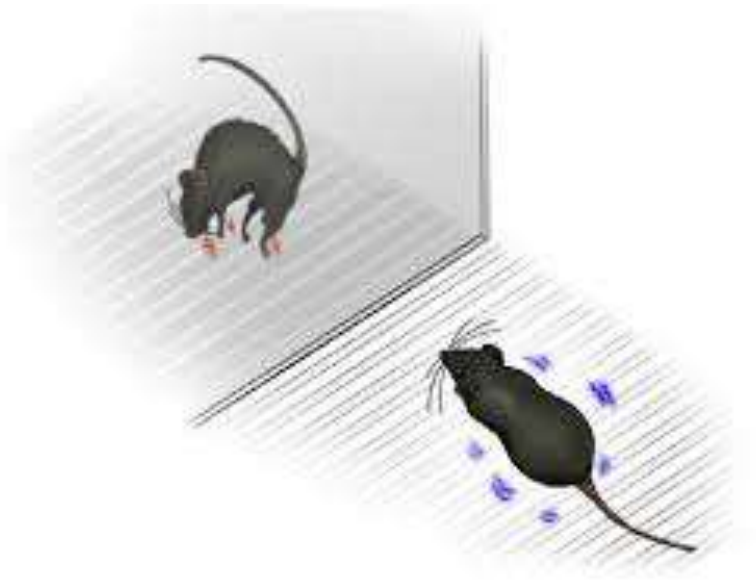
Examiner: Prof. Per Jensen

Centre for Social and Affective Neuroscience (CSAN), Linköping University

Background – Observational fear conditioning

The act of acquiring fear through vicarious stimuli

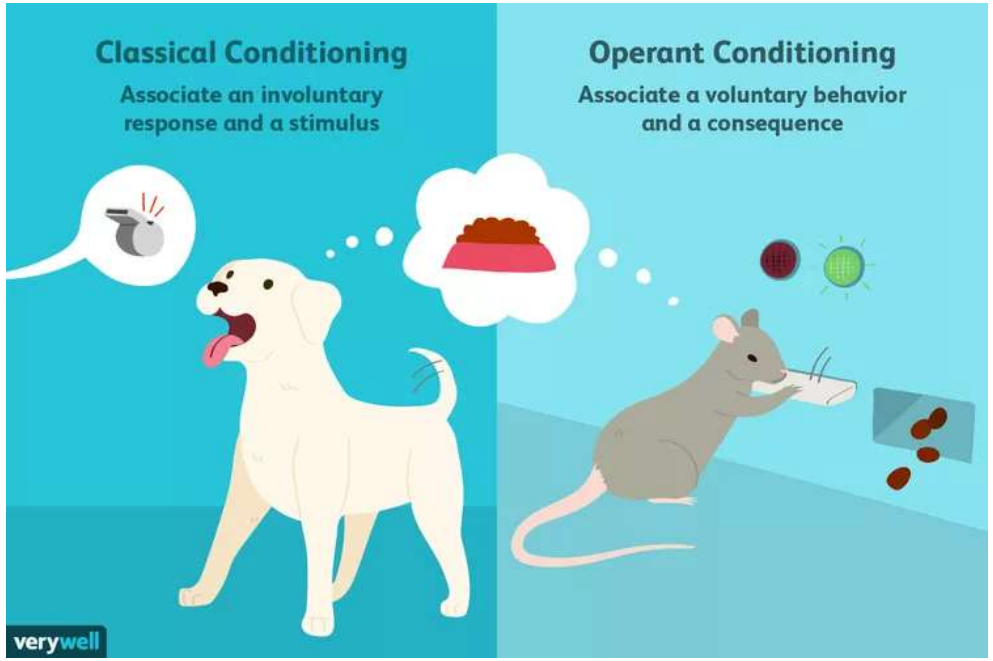
- Follows the same principles as regular conditioning (CS, US, UR, CR) but the conditioning is indirect.
- Many factors can influence the conditioning (level of familiarity, vocalizations, empathy…).
- Fear is quantified through behavioural reaction.
  - Freezing behaviour



Background – Operant fear conditioning

Acquisition of a fear stimulus through repeated operant training

- Operant training: conditioned behaviour performed by the subject to gain a reward.
- In fear conditioning, aversive stimulus (shock) is presented to the animal as a counterbalance for the positive reinforcement (reward).
- Fear is measured through change in the operant performance.
  - Suppression ratio: $SR = \frac{LP(Tone) - LP(Baseline)}{LP(Tone) + LP(Baseline)}$



Background – Research Aim

Characterising observational fear in rats, to have a tool for molecular analysis

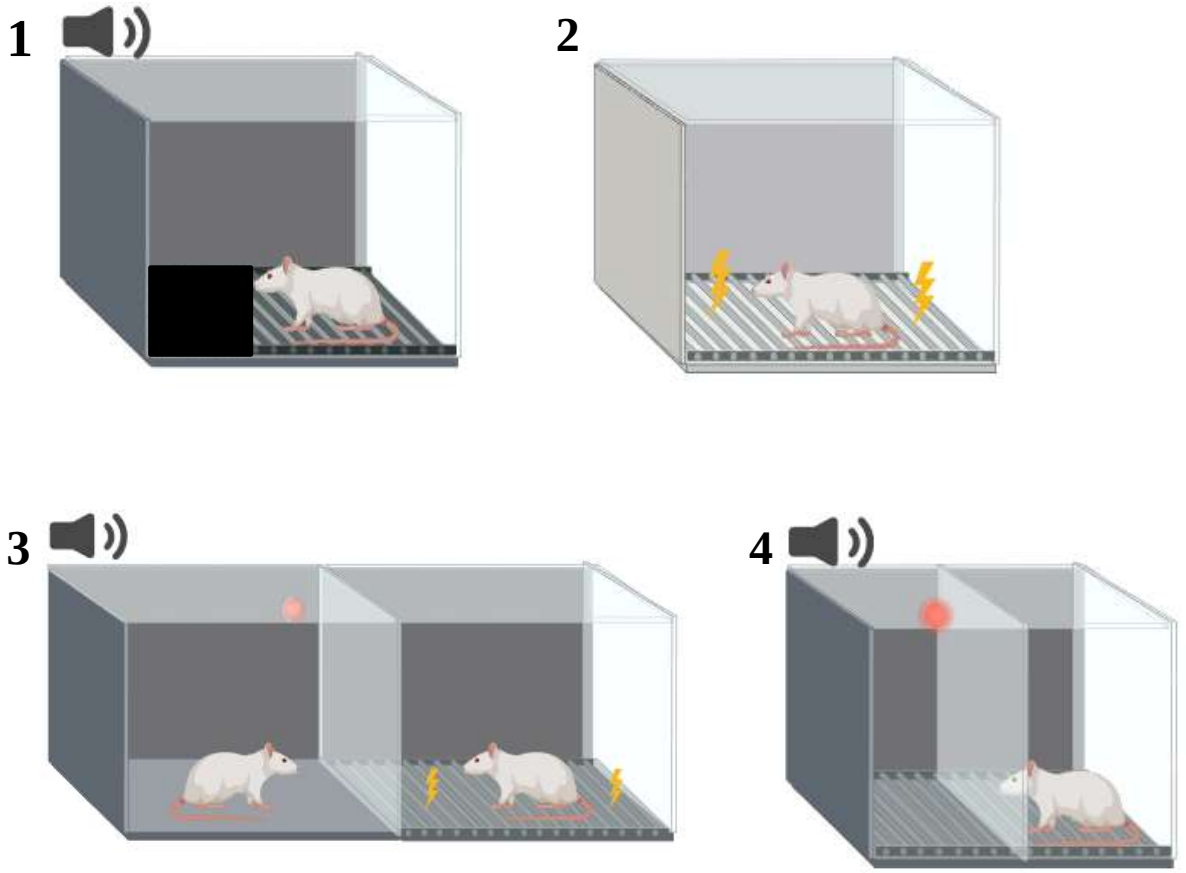
- Two ways: increase in freezing behaviour (observational) and decrease in suppression ratio (operant).

- Analyse freezing behaviour in relationship to the conditioned stimulus (during and after).
- Testing the possibility to include additional behaviours for fear measure.
- Look into the physiological reaction by measuring corticosterone (blood samples).

- Use of diazepam (anxiolytic) in the operant model for validation.

Methods – Observational fear experiment

- 56 male wistar rats (live animals) employed for the experiment
- 2 groups: demonstrators and observers
- Phase 1: **tone habituation** with 5 acoustic tones and a floor over half the grid
- Phase 2: **priming** of the observers (exposure to 6 shocks) in a different context
- Phase 3: **fear acquisition** (24h after phase 2). Demonstrator receives the shock, observer observe. 6 tones of two minutes are played, with a 2 second foot shock following
- Phase 4: **fear testing** (1 month after phase 3). Rats are exposed individually to the acoustic tone without the shock (2 tones of 30 seconds)



Methods – Corticosterone immunoassay analysis

- Blood samples collected at 4 timepoints:
  - **T0** Baseline (before any manipulation), **T1** After fear acquisition, **T2** 24h after fear acquisition , **T3** After fear testing
- Corticosterone is isolated and extracted with a speed vacuum
- Samples containing corticosterone are subsequently analysed with the Enzyme immunoassay kit
- Concentrations (pg/ml) are estimated using Softmax pro software with a plate reader

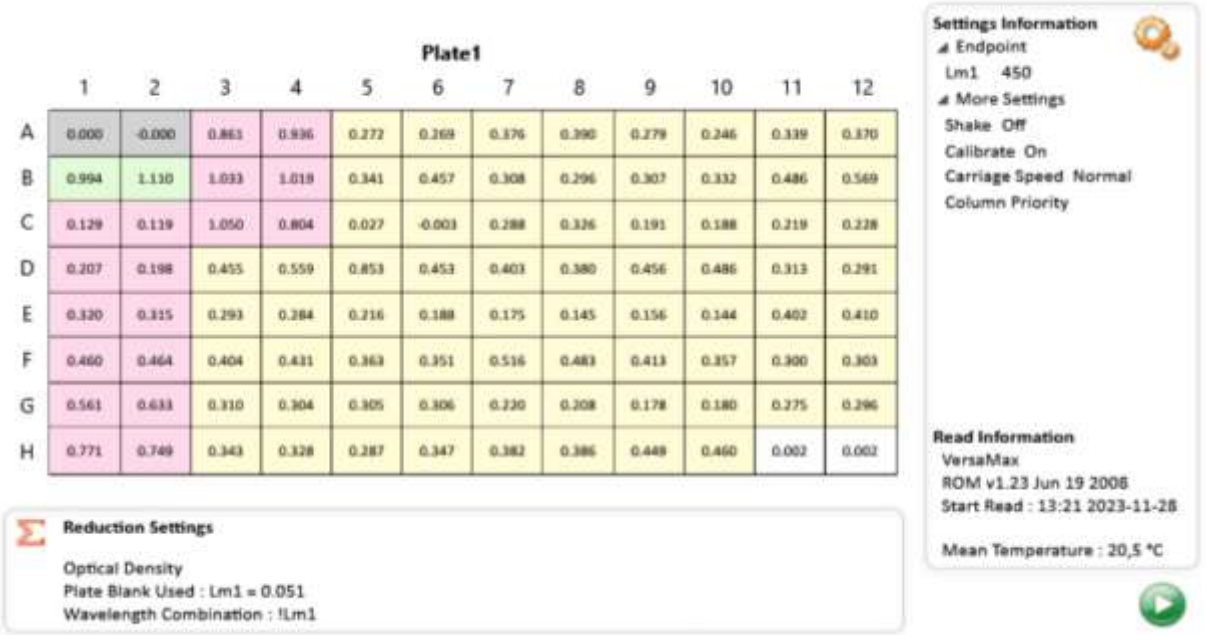


Plate1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.000	-0.000	0.863	0.936	0.272	0.269	0.376	0.390	0.279	0.246	0.339	0.370
B	0.994	1.110	1.033	1.019	0.341	0.457	0.308	0.296	0.307	0.332	0.486	0.569
C	0.129	0.119	1.050	0.804	0.027	-0.003	0.288	0.326	0.191	0.188	0.219	0.228
D	0.207	0.198	0.455	0.559	0.853	0.453	0.403	0.380	0.456	0.486	0.313	0.291
E	0.320	0.315	0.293	0.284	0.216	0.188	0.175	0.145	0.156	0.144	0.402	0.410
F	0.460	0.464	0.404	0.431	0.363	0.351	0.516	0.483	0.413	0.357	0.300	0.303
G	0.561	0.633	0.310	0.304	0.305	0.306	0.220	0.208	0.178	0.180	0.275	0.296
H	0.771	0.749	0.343	0.328	0.287	0.347	0.382	0.386	0.449	0.460	0.002	0.002

Reduction Settings

Optical Density
Plate Blank Used : Lm1 = 0.051
Wavelength Combination : !Lm1

Settings Information
Endpoint
Lm1 450
More Settings
Shake Off
Calibrate On
Carriage Speed Normal
Column Priority

Read Information
VersaMax
ROM v1.23 Jun 19 2006
Start Read : 13:21 2023-11-28

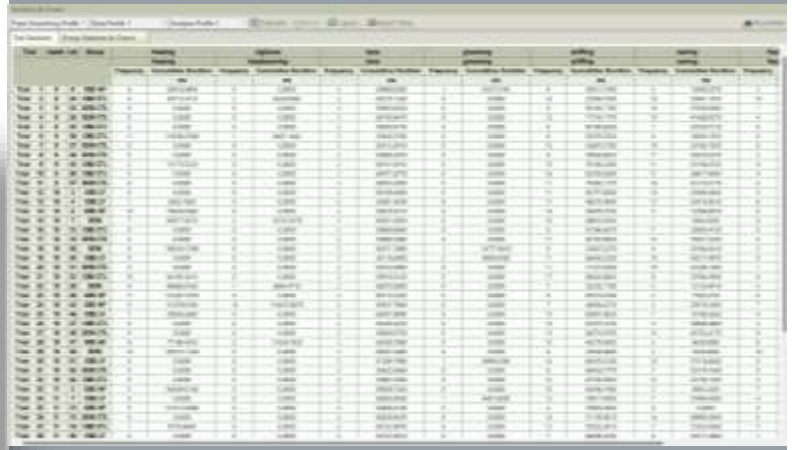
Mean Temperature : 20,5 °C

Methods: Behavioural scoring

- Additional 50 wistar rats used (video animals), 5 groups
- Recordings from acquisition and testing sessions
- In acquisition, 1st tone not scored
- Freezing behaviour scored during the acoustic tones and 1 minute after

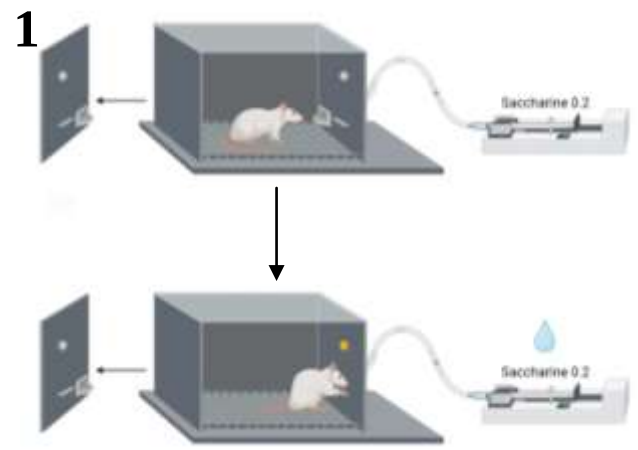
- Ethograms scored for:
  - Baseline (2 minutes before the tones)
  - During the tones
  - After the 1st tone (only testing)
- List of behaviours:
  - Grooming
  - Sniffing
  - Rearing
  - Free-air whisking
  - Head-scanning
  - Jumping (only acquisition)
  - Social interaction (only acquisition)
- Ethograms for video animals only

Program used: Ethovision XT

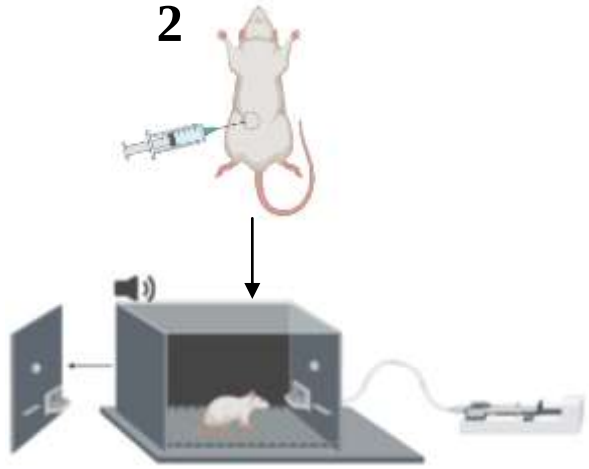


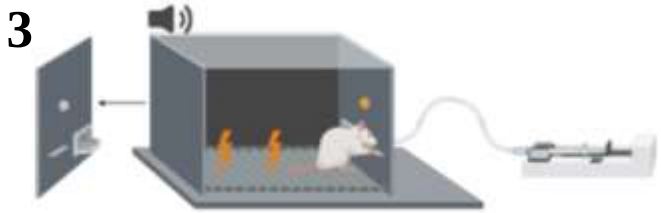
Methods – Operant fear experiment

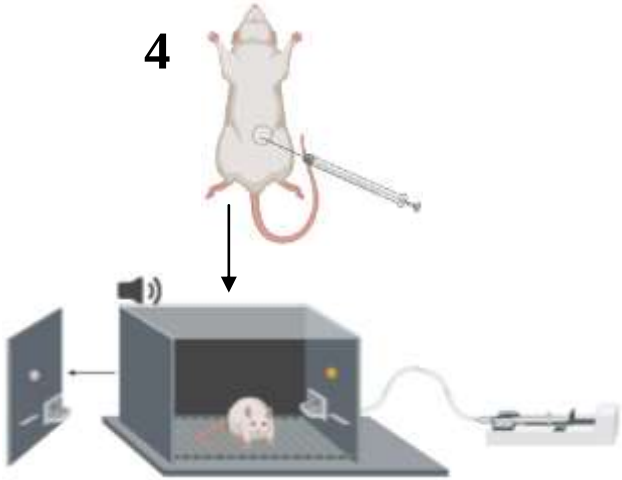
- 40 males and 51 female wistar rats employed for the experiment
- Phase 1: **operant training** on fixed ratio 1, then fixed ratio 2 (0.2% saccharine reward)
- Phase 2: **Injection and tone habituation**. Rats are injected with saline solution and habituated to the acoustic tones (2 of 30 seconds)
- Phase 3: **fear acquisition**. 2 groups (0.4 and 0.8 mA), 3 tones of 30 seconds with shock of 2 seconds
- Phase 4: **diazepam injections and fear testing**. 3 subgroups (control, 0.3 and 1 mg/kg), 2 tones of 2 minutes each



FR1 (2 weeks) → FR2 (2 weeks)





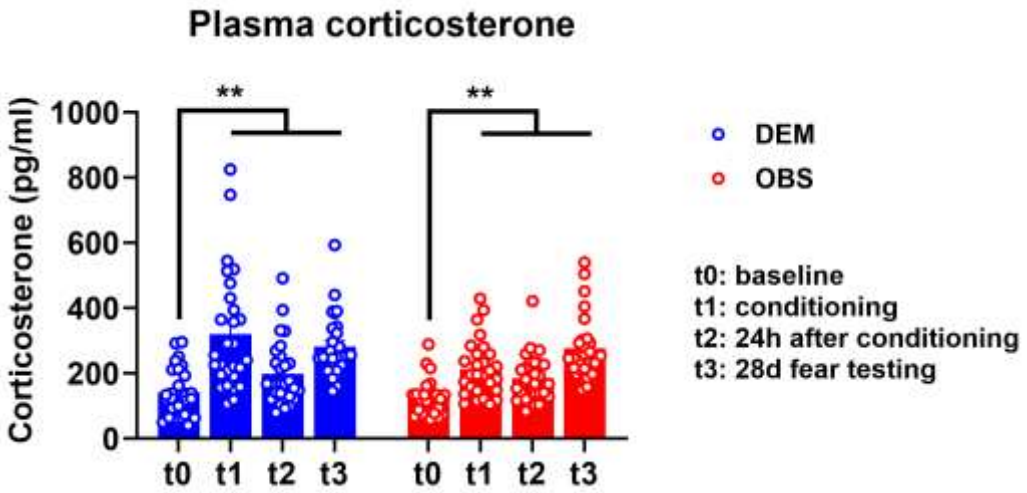


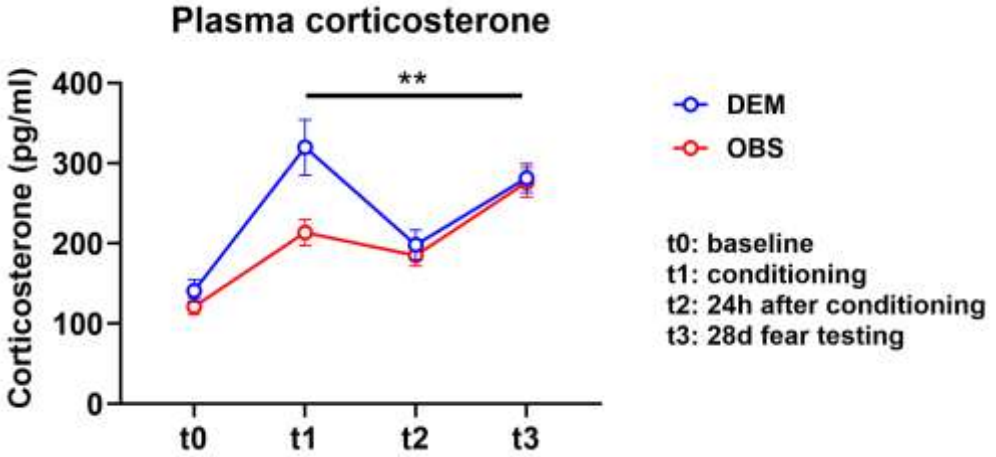
Results – Corticosterone

Two-way RM ANOVA:

Time x group: $F(3, 162) = 4.921$, $p = 0.0027$
Time: $F(2.090, 112.8) = 42.44$, $p < 0.0001$

Concentration of corticosterone was higher on t1-t3 compared to the concentration at t0



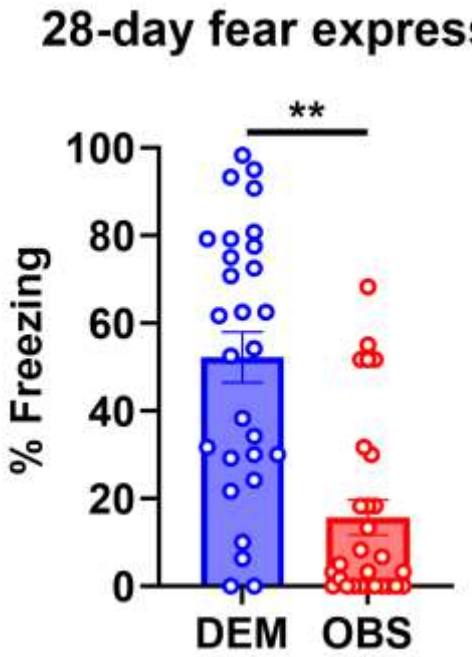


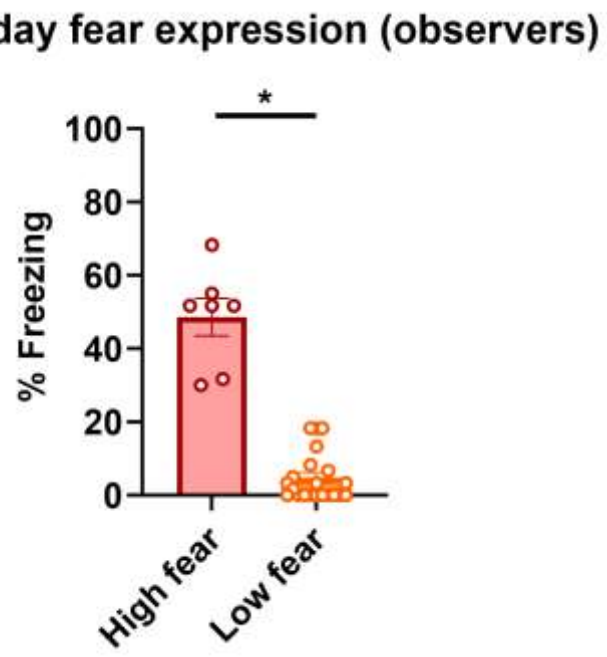
Results – Freezing scores (Live animals)

Unpaired *t*-test:

DEM vs OBS: $t(54) = 5.20$, $p < 0.001$
OBS clusters: $t(26) = 11.5$, $p < 0.001$

Observer rats acquired fear with individual variance (high fear and low fear)



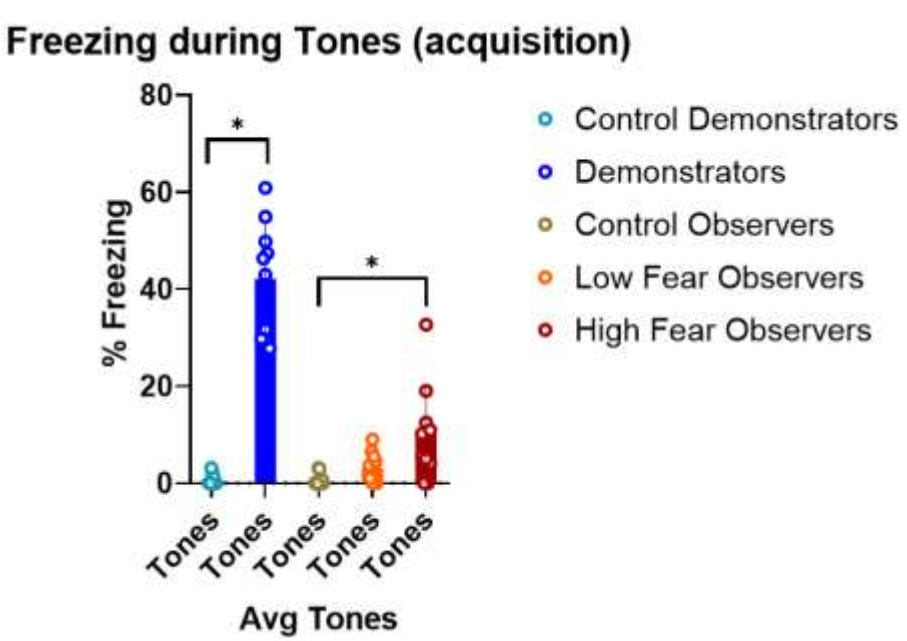


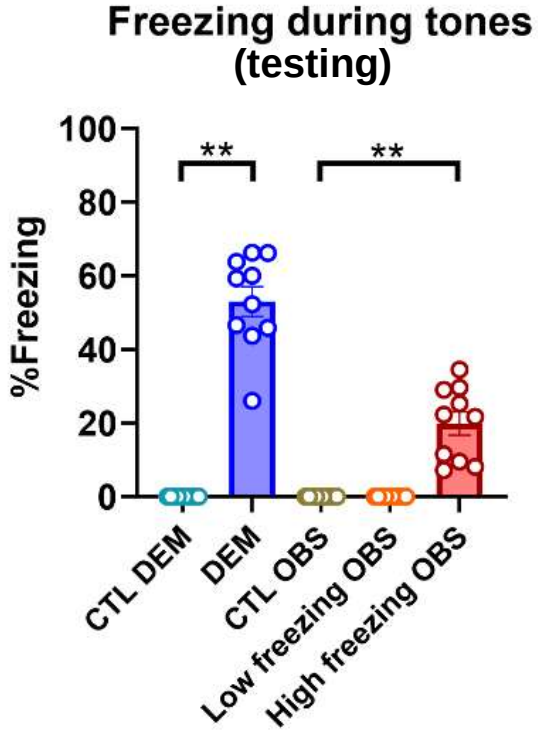
Results – Freezing scores (Video animals)

Kruskal-Wallis test:

Acquisition: *KW* statistic = 36.74 $p < 0,05$
Testing: *KW* statistic = 47.66, $p < 0,001$

Observers freeze during the tones, with a degree of variance (high fear and low fear observers)





Results – Freezing scores (Video animals)

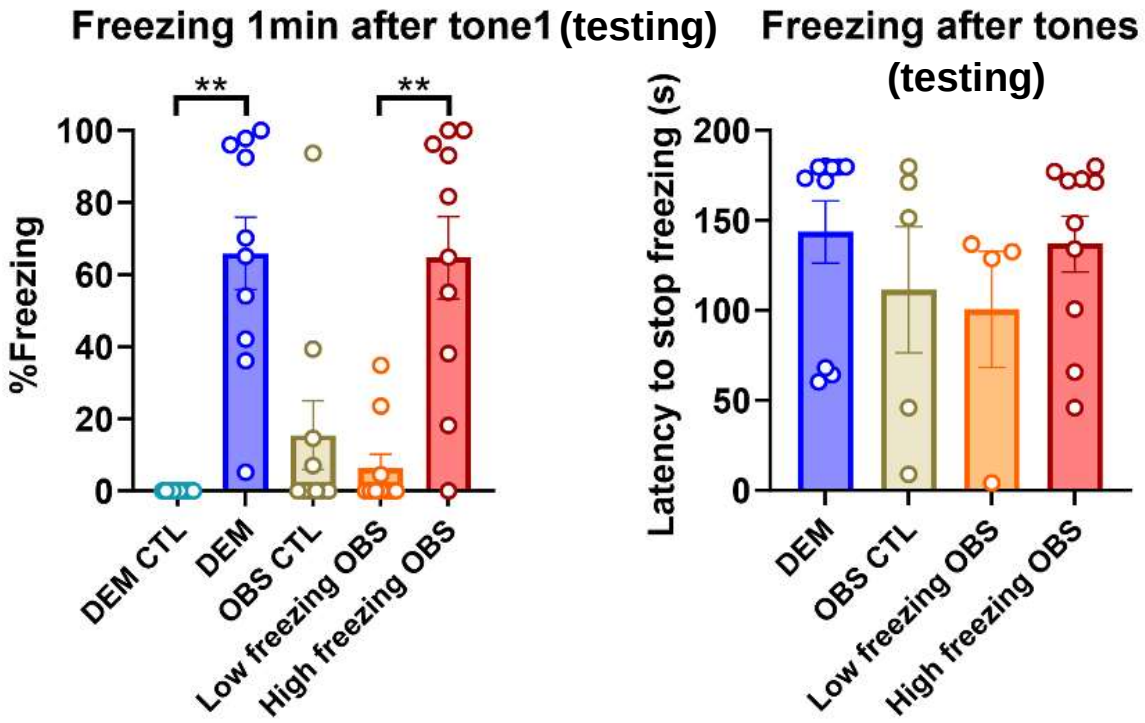
Kruskal-Wallis test:

Acquisition: *KW* statistic = 35.42, $p < 0.001$

Testing: *KW* statistic = 31.35 , $p < 0{,}001$
Latency: *KW* statistic = 4.181 , $p < 0{,}001$

Rats prolonged the freezing behaviour one minute after the tone.

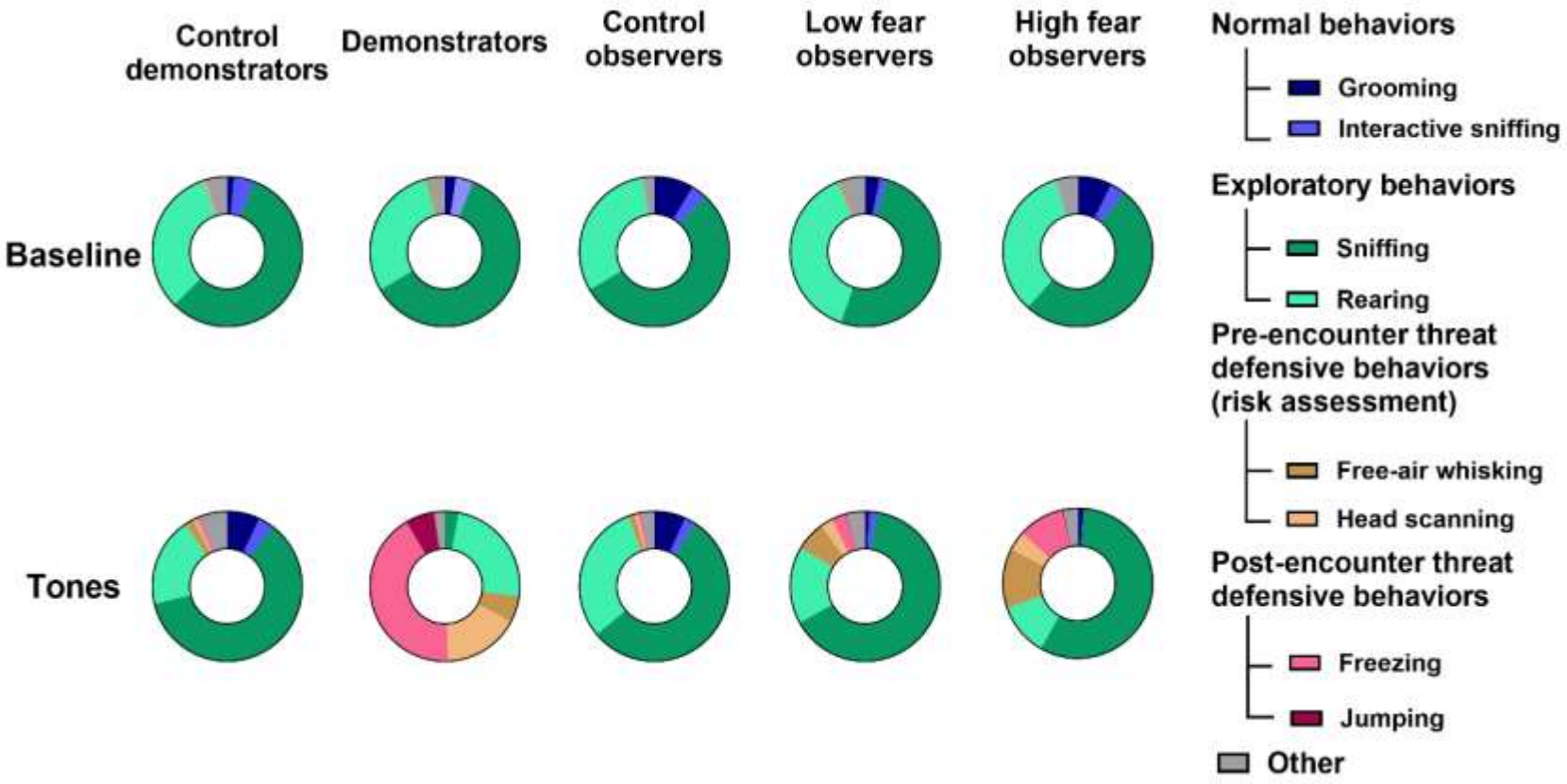
Latency for freezing behaviour extend up to almost three minutes in freezing test sessions



Results – Ethograms

Rats exhibit exploratory behaviours majorly during baseline

During the tones, increase in vigilant behaviour and freezing

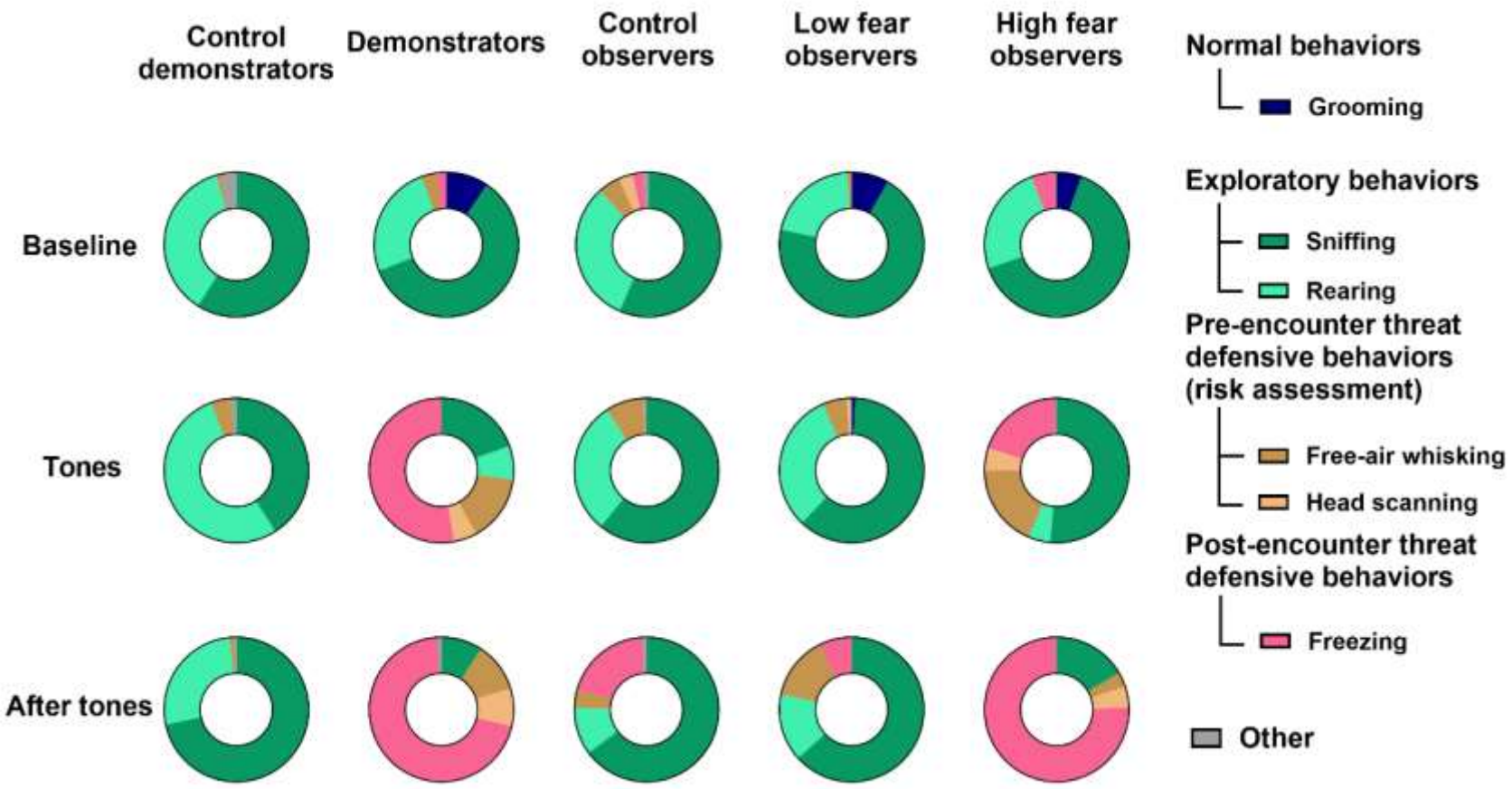


Results – Ethograms

Rats exhibit exploratory behaviours majorly

During the tones, increase in vigilant behaviour and freezing

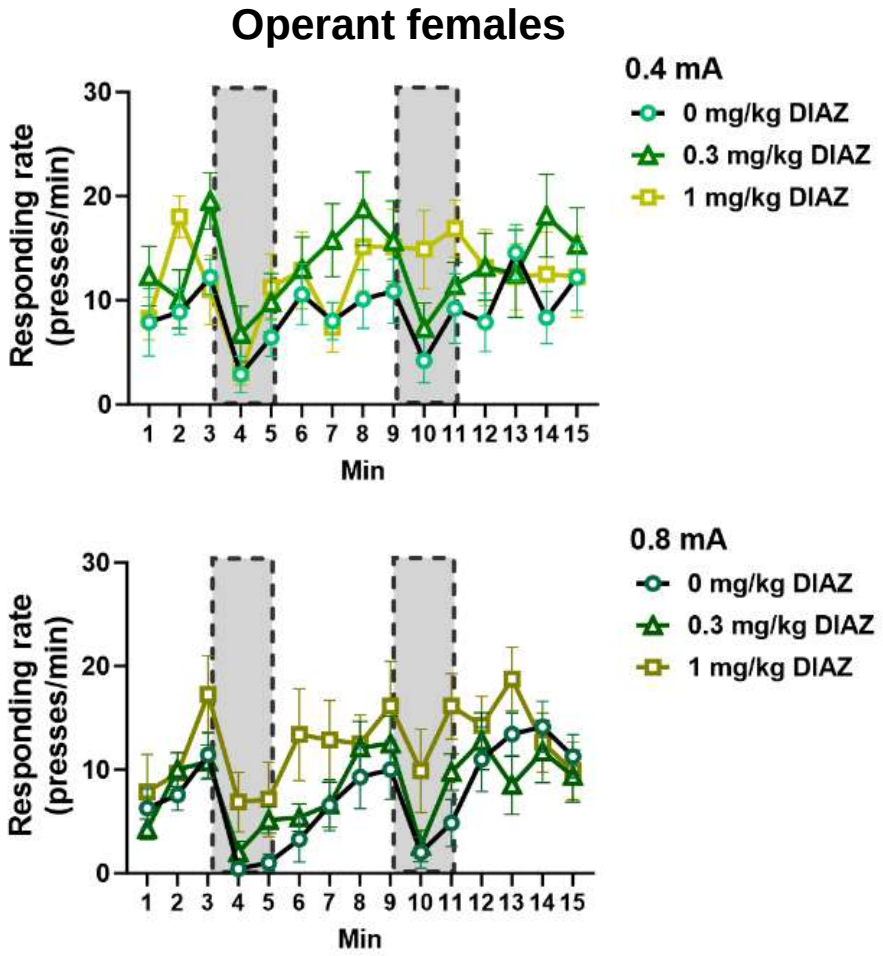
Freezing becomes predominant after the acoustic tones, with similar percentages between high fear observers and demonstrators



Results – Operant performance

Operant males: drop in operant behaviour during the acoustic tone in testing session affected by the shock intensity group

Operant females: more pronounced drop during the acoustic tone in testing session



Results – Suppression Ratio

Males

Two-way ANOVA:

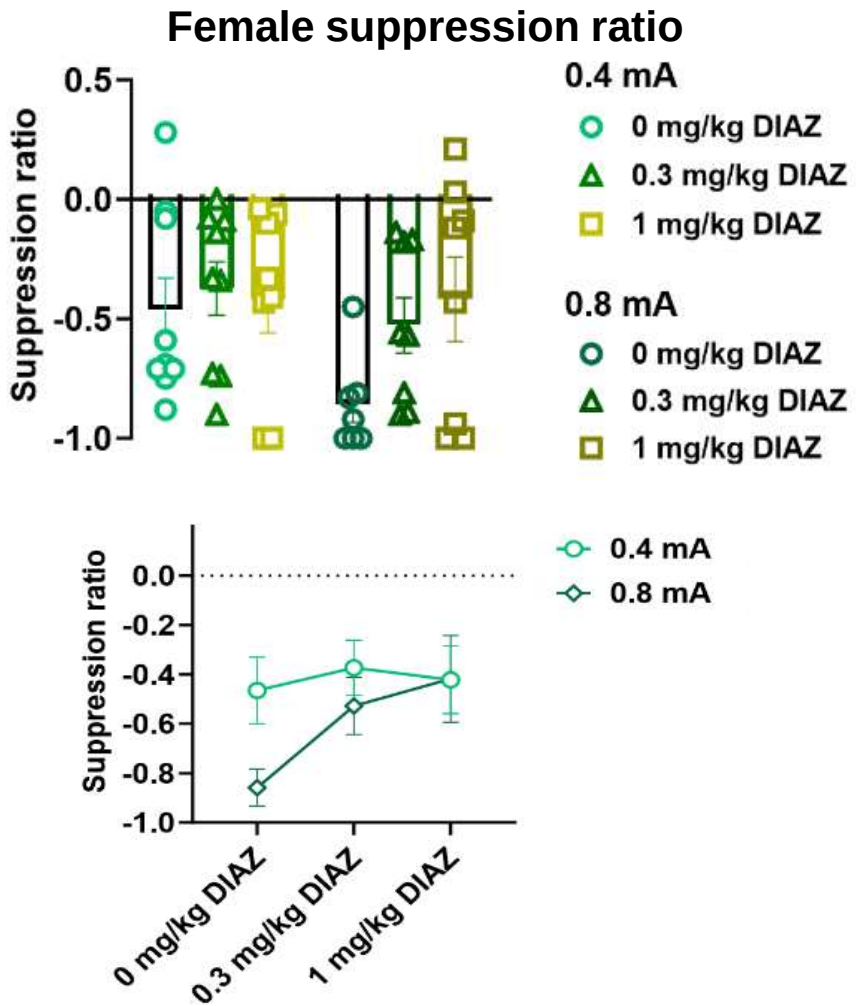
- Shock groups: $F(1) = 18.625$, $p < 0.001$
- Shock groups x dosage groups: $F(2) = 3.768$, $p = 0.033$

Females

Two-way ANOVA:

- Shock groups: $F(1) = 3.418$, $p = 0.071$
- Shock groups x dosage groups: $F(2) = 0.704$, $p = 0.5$

Suppression ratio was affected by the injection of different concentrations of diazepam, for both males and female. Only for males, there was significant difference.



Discussion – Corticosterone

- Corticosterone measurements indicates a growing stress in the animal for each timepoints, with peaks at fear acquisition (t1) and fear teasting (t3) and a drop 24 hours after acquisition (t2). Unfortunately, physiological stress response does not predict fearful behavioural response.

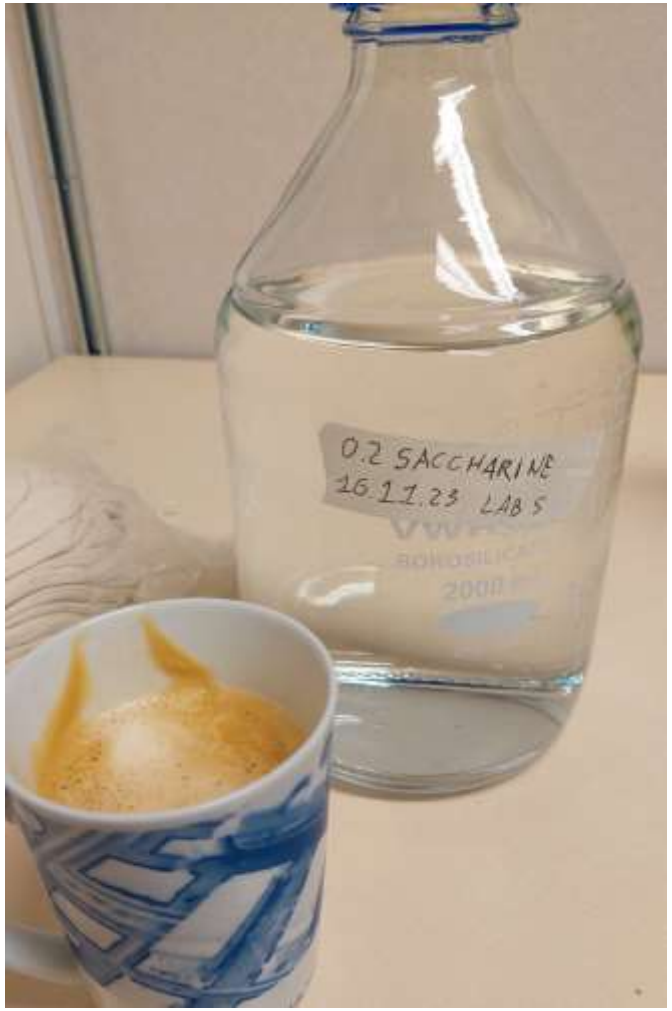
Discussion – Behaviours

- Rats exhibited freezing after one month from fear acquisition, in comparison to one week.
  - Observers showed different freezing scores at individual level
- Freezing behaviour increased in the minute after the acoustic tone, showing anticipatory fear for the shock
  - In fear testing, latency could go up to the full inter-tone time (3 minutes)
- Ethograms confirmed the increase of freezing during and after the tone, contextualising it with increases of vigilant behaviours and decrease of exploratory behaviours
  - Since vigilant behaviours anticipates freezing, they could be included as fear measurments

Discussion – Operant

- Implement of saccharine as a reward was successful, replacing the alcohol administration
  - Rats developed operant behaviour within the same time frame

- Injections of diazepam showed reduction in suppression ratio, depending on the different shock intensity recieved, showing an effect of all factors involved



For future studies

- Current work on development on AI software for facial recognition in rats (made with DeepLabCut), to fasten behavioural scoring.
- The behavioural scores conducted here will be used for the machine learning of said AI.
- The work sets the grounds for molecular and neurological work on observational fear.

Thank you for listening!

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