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Investigating the behavioural and physiological responses associated with observational fear memory.

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1. ABSTRATC

Observational fear studies are an important tool for the understanding of fear transmission in both humans and animals. By analysing the behavioural responses of an animal, namely freezing behaviour, it possible to quantify their fear response. An alternative method implies operant training behaviour, where the animal manifest fear by reduction of the mentioned behaviour. In this study, we looked at both methods of analysing fear in rats, by including additional possible fear-related behaviours in the scoring count, checking the physiological reactions by means of corticosterone concentrations and through use of diazepam for the validation of the operant training method. Results obtained indicated an increasing production of corticosterone after important phases of the experiment. The rats did manifest freezing behaviour, however the highest percentage was recorded not in relation to the acoustic stimulus but in the aftermath of it, showing an anticipatory response to the aversive stimulus. Vigilant behaviours would grow in percentage when the animal was exposed to the acoustic tone, with corresponding decrease in exploratory behaviours. The use of diazepam had an effect over the suppression of operant behaviour, with higher dosages keeping a lower suppression. The inclusion of additional behaviours is beneficial to the understanding of the dynamical shifts in behaviours during observational fear procedure, helping to understand the occurrence of fear at behavioural level. With the incoming development of a facial recognition AI software, behavioural analysis would be sped up, leaving more time to perform molecular analysis of fear transmission.

Keywords: Observational fear, Operant training, Corticosterone, Behaviours, Ethograms, Freezing, Operant suppression.

2. INTRODUCTION

2.1 Science of fear

Fear is a strongly unpleasant emotion, felt towards a known or perceived threat, or danger (Dunsmoor & Paz, 2015; Vlaeyen et al. 2016). Fear acts in an anticipatory way, eliciting physical and psychological responses from an individual in preparation of a plausible painful stimulus (Adolphs, 2013). As an innate reaction, theoretically every individual can experience it, although the intensity varies depending on multiple factors. One of them being the rationality of the fear itself: a phobia is defined by an intense irrational fear emotion towards a stimulus that is known to be rationally harmless (De Jong et al. 1997; LeDoux, 2014), such as the trypophobia or fear of "small clusters of organised holes" (Martínez-Aguayo et al. 2018).

Fear intensity refers to the array of reactions recorded for an individual during the fear stimulus exposure (Dymond et al. 2015). These are generally visible and quantifiable through factors such as heartbeat rate, facial distortion, and production of sweat. In summary, symptoms of stress can be used to evaluate the intensity of fear display (Mobbs et al, 2015). The similarity between the stress response and the emotion of fear has led many to assuming a correlation between them, at the very least.

Unfortunately, links between anxiety traits, development of PTSD and the influence of fear are still unknown, and it is not the only instance of misconception and lack of consensus over fear in science (VanElzakker et al. 2014; Shansky, 2015). The concept of fear itself was considered in some instances a psychological artificial construct, and not something that is regulated by the brain, as more recent studies suggest (Onat & Büchel, 2015). Specifically, fear studies have moved from stimulation of behavioural responses to identifying the molecular processes and functions in the brain that underly fear responses and fear memory (Méndez-Bertolo et al. 2016; Asok et al. 2019).

The notion of fear memory highlights the cognitive process of acquiring behavioural responses for appropriate fear stimuli, both at conscious (image memory, emotion memory) and unconscious (behavioural responses, physical reactions) (Albrecht et al. 2012; Izquierdo et al. 2016). The amygdala and nucleus accumbens play a significant role in administration of the fear memory process, which shares neural circuits with other functions such as pain responses and modulation of social behaviours (Méndez-Bertolo et al. 2016). With the amygdala already found responsible for regulating the reward threshold in alcohol-withdrawal and addiction studies, a possible correlation with the fear circuits has yet to be found (Asok et al. 2019).

However, multiple studies are currently underlying the functions of the neuronal channels of the amygdala, with new discoveries at a high rate.

2.2 Fear in animals

As previously mentioned, there are aspects of fear studies that are still awaiting consensus from scientists. Some suggest that the term fear is not suitable for animals as it is for humans. The reason being the lack of a possibility for the animals to directly communicate their sense of fear (Adolphs, 2013). The validity of such claim remains uncertain.

Recent discoveries made on rodents' brain showed highly specialised neural circuits for fear in contrast to the human circuits, which involves the regulation of multiple factors including fear (Ganella & Kim 2014). The behavioural response of an animal to a stressful stimulus can be interpreted following a specific model: the triggered responses fall under the concept of "fight or flight response" (Kozlowska et al. 2015). First studied by W.B. Cannon (1915), it highlights a stress response from the sympathetic nervous system for either fighting or fleeing a potential threat (Kohl et al. 2018). It has been used historically to help the classification of species as predators or prey, underlying their behavioural tendencies for fleeing or fighting (Gaynor et al. 2019).

Another aspect considered is the social structure of the species (Keysers et al, 2022), with gregarious animals generally found to flee more if alone but fighting more if together (Toth & Neumann, 2013; Monfils & Agee, 2018). Another variable is the familiarity of the fear stimulus. Known as novel object paradigm, it is used to assess the memory of a subject and their reactions toward a previously unknown object (Goode & Maren, 2014). This usually predicts a higher fear intensity towards the object during the first encounter, with memory retention and subsequent drop in intensity during the following encounters. The novel object paradigm is not, however, used directly to assess fear (Takola et al. 2021).

2.3 Animal models

Scientific discoveries, outside of specific contexts, have outlined the similarities between humans and other mammal species, first at biological level with similarities in terms of relative organ's size, production of substances and relative brain size and functions. Secondly at a behavioural level, with almost identical behavioural patterns concerning emotional manifestation and responses, learning and assimilation (Debiec & Olsson, 2017).

The first studies using animal models involved non-human primates' species, showing evidence for the acquisition of fear and fear memory (Mineka et al, 1984). However, a major issue with

using primates was the increasing difficulty in obtaining ethical permits and technical issues in obtaining the primate's brain for additional analysis. Currently, rodents are the main animal model used (Laxmi et al. 2003). The perks of using rodents in experiments are their availability and their short lifespan making it possible to monitor their growth constantly.

Specifically, mice (*Mus musculus*) were the first rodents to be consistently used in fear experiments, since they were prone for freezing in behavioural studies and were easy to house (Kondrakiewicz et al. 2019). Mice have been used in contexts of operant fear conditioning and observational fear. Although their solitary nature, they provided good insight when placed in an observational context. Analysis on their brain put a basis for studying the neural channels of fear and their connections with the amygdala (Kim et al. 2021).

Rats (*Rattus norvegicus*) are more gregarious than mice, for which they can be kept in pairs or groups of three. Rats are part of a different behavioural niche, having a more predatory lifestyle, giving out a different spectrum of behaviours (Luyten et al. 2011; Jones et al. 2018). The similarities in neural circuits for observational fear in rats, makes them perfect pre-clinical models for fear studies (Kiyokawa et al. 2019). Studies to develop new plans for inclusion of more animal models are in progress (de Rivera et al. 2016).

2.4 Observational fear

Almost 60 years ago (Askew & Field, 2008), Rachman (1968) and Bandura (1969) discussed the possibility of fear transmission on individual level, through direct observation of one's fear experience. Baptised as "Observational Fear learning", it has furthermore been studied by Rachman, in 1977, who put the observational fear learning as one of three possible ways of acquiring fear: the other two were conditioning, both classical and operant, and through direct information share.

The first suggestion made by Rachman was purely related to humans and regarded mainly the possibilities for phobias to be transmitted vicariously (Dou et al. 2023). However, he noted that for observational fear, the possibility to acquire fear was not related to the presence of an aversive stimulus, instead the idea of it is sufficient, mixed with the perception from who experienced it. On top of these presented claims, successive studies looked at the transmission of fear between a dyad of parent-children (Marin et al. 2020; Szczepanik et al. 2020) and developed new paradigms for confrontation (Skversky-Blocq et al. 2021; 2022).

In a study from Bilodeau-Houle et al. from 2023, it was hypothesized that a stronger association and fear transmission in children looking at their parents compared to strangers. The parents

were subjected to a twofold situation in an operant setting: one action causing a shock and one with no effect. The same setup was used by individuals unknown to the children. The younger subjects were then exposed to the videos of both their parents and the strangers, with consequential introduction to the same setup without shock responses. The fear responses from the children were congruent with the fear acquisition from both parents and strangers with the shock cue, in correlation with the blank cue. Additionally, comparing the fear learning in response to the parents and the strangers showed a correlation between the children and the parents, supporting the initial hypothesis that children exhibit a stronger fear transmission when observing their parents. In another similar case, the quality of the relationship parents-children was used as diversification factor for observational fear. Results showed a stronger fear response from children with less secure relationship with their parents, putting an opening for anxiety factors to influence observational fear transmission (Haaker et al, 2017; 2021).

Observational fear studies have focused on animals as well, with evidence of fear transmission between individuals (Pisansky et al. 2017; Shi et al. 2022). The uncertainty of fear transmission in animals solely relay on the absurd number of factors to be taken in consideration. These factors are often subject of studies using the observational fear method and looking at the importance of the factor itself (Terranova et al. 2023). Such factors can include familiarity with the environment or the individuals (Yusufishaq & Rosenkranz, 2013), role of vocalizations (Lidhar et al. 2017; Fendt et al. 2021), role of empathy (Atsak et al. 2011; Kim et al. 2018; Keum & Shin, 2019) etc…For this reason, consensus on observational fear results is yet to be found, and the possibility for unexpected outputs is high. Animal subjects used are mainly non-human primates (before) and rodents (nowadays).

2.4.1 Corticosterone and fear

As a measure of regulation of the stress level of an individual, glucocorticoid hormones are produced. These hormones not only help with stress regulation, but also energy regulation and immune reactions. In humans, the main glucocorticoid is cortisol, while in animals the main one is corticosterone. An interesting aspect of corticosterone has been found in correlation to fear studies. Animal subjected to fear recall through exposure to cue exhibited an increase in the level of corticosterone in their blood (Carcea & Froemke, 2019).

However, the increase of the corticosterone level coincides with a strengthened fear retention in the individuals subjected to the cue. Moreover, the administration of corticosterone in fear-reinstated subjects helped decrease the stress levels, showing a secondary opposite effect of

corticosterone on the mechanisms of fear retention. Both effects have shown a vast temporal span of action (Wang et al 2014).

2.4.2 Behaviour scoring

Expression of fear in animals is generally measured differently than in humans. Scientists have associated a defensive mechanism known as freezing to the physiological expression of fear. The animal, when freezing, undergo a state of tonic immobility only interrupted by light trembles. Tactile sensors such as whiskers are tensed and in an upright position, alerted. The animal tends to position itself in a in a cornered position, giving the back to the wall to cover its dead points. Head is generally upright and looking straight ahead.

The amount of time spent freezing varies from individual to individual (Roelofs, 2017). When analysing fear expression, it is important to evaluate the side-behaviours that manifest in the absence of freezing or that can be related to it. The other behaviours are divided in macro-categories, putting together behaviours that relate to a similar state. Closer to freezing, while considered not as strong, are vigilant behaviours such as head-scanning, where the subject moves systematically its head from one direction to another, and the less intense free are sniffing, where the animal try to locate a stimulus in an unusual position above itself.

Other behaviours tend to manifest in neutral situations and manifest a less stress state of the subject. Between those behaviours, exploration for cues in the environment through sniffing and rearing, which is the bipedal position assumed for exploration of higher locations, is common. To a similar context are associated self-proactive behaviours like grooming, or more rarely playing (Whishaw & Kolb, 2020). The extent, complexity and categorisation of the aforementioned behaviours is usually left to the discretion of scientists themselves, while a general outline is present. The interactions between the behaviours are used to establish the relations with fear response, looking at possible associations of the subjects with experimental cues.

2.5 Operant conditioning

Similar in concept to Pavlov's (1928) classical conditioning but different in mechanisms and requirements, operant conditioning is another valid concept to be used in fear studies, developed by B. F. Skinner in 1937. In classical conditioning, a conditioned stimulus (CS) is paired with an unconditioned stimulus (US) or reinforcer to generate a conditioned response (CR) (Akpan, 2020). Operant fear conditioning expands the same concept by using a behavioural action of

the subject that is not automatic, on the contrary it acts deliberately on the environment (Laing & Harrison, 2021).

Operant conditioning works as a trigger for repeated actions that produces a favourable outcome (Bunzli et al. 2011). At the same time, the learning process suppress actions that produces non-desirable outcomes (Murphy & Lupfer, 2014). A reinforcer is introduced for increasing the repeatability of the action (Adamczyk et al. 2019). In general, the reinforcer falls between the parameters of primary or secondary reinforcer: primary being part of an individual's basic needs such as food or water rewards, secondary being an auxiliary need generating pleasure, such as a toy or similar (Akpan 2020).

Reinforcers can be positive or negative, depending on the experimental requirements (Zambetti et al. 2022). An example can be the suppression model, where a negative reinforcer (such as an electric shock) is used to reduce the conditioned behaviour or action. The regulation of the association CS-US is given to the amygdala, which works as a mediator for learning, while at the same time influencing cortical plasticity with changes in the connection of synapses (Davis, 1986; Mobbs & Kim, 2015).

2.5.1 Diazepam

Diazepam is a benzodiazepine medication used as an anxiolytic to treat anxiety symptoms and disorders, with hypothetical use for treating psychiatric disorders. Due to its stress-reducing nature, diazepam can be used for suppression model validation in operant fear conditioning experiments.

2.6 Aims of the study

The study aimed at characterising observational fear in rats, to have a tool for molecular analysis, by the means of two ways of evaluating fear: increase in freezing behaviour and suppression ratio.

For the first model, I wanted to analyse the array of behaviours displayed by rats during the observational fear experiment. Specifically, the instance of freezing in relation to the acoustic tone. Additionally, I wanted to test the possibility for freezing behaviour to extend over the duration of the CS. Moreover, by analysing additional behaviours, I wanted to account if other behaviours could be used to measure fear.

Forbye, I wanted to see if corticosterone collected through blood samples matched the fear expression shown by freezing behaviour, since corticosterone does not work as an indicator and cannot therefore predict freezing behaviour.

For the suppression model, I used the operant conditioning experimental setup and validated it through diazepam injections.

3. METHODS

3.1 General description of the experiments

The study was divided into two macro experiments: the observational fear procedure that included behavioural observations and corticosterone analysis, and the operant fear conditioning procedure with diazepam injections. Specimens used for one experiment could not be used for the other.

For the observational fear experiment, the animals were divided in two groups: observers and demonstrators. The procedure focused on preparing the animals for fear acquisition, with different requisites depending on their group. During fear acquisition two rats would be placed together in the experimental cage: an observer and a demonstrator. The demonstrator received a foot shock paired with an acoustic tone while the observer watches. After 30 days without further procedures, the rats were exposed to the acoustic tone again, individually. The procedure is called fear test.

Behaviours were recorded for both the fear acquisition and the fear test of each specimen through camera recordings, and behaviours were scored manually for each individual. Blood samples were taken from the rats at four timepoints and subsequently worked and analysed in the chemical lab. In summary, each rat produced four blood samples collected in different moments of the experiment.

For the operant fear experiment, the rats were individually trained for multiple weeks for operant task; pressing a lever to obtain a reward. Firstly, at fixed ratio of one (one press one reward) then at fixed ratio two (two presses one reward). The animals were then divided into two groups and exposed to different intensities of electric foot shock (0.4 mA and 0.8 mA) paired with an acoustic tone. One week after, the specimens undergo fear test, exposure to the acoustic tone alone. The suppression of operant behaviour was used for measuring the fear response.

3.2 Housing and facility

The totality of the experiments involving animals were conducted in the CBR-2 facility, part of the Linköping Hospital affiliated with Linköping University. The CBR staff was taking care of the animals for all non-experimental needs.

Wistar outbred rats were used for all the experiments. The animals were bought from an external supplier (Charles River). Animals involved in fear experiments would usually arrive at the facility at approximately two months of age, spending one week in the holding room with no contact with the experimenters. Subsequently, they were housed in rooms with reversed light cycle (red-white light). Temperature and humidity were constantly regulated, approx. 23C° and 45% humidity. Food and water were available ad libitum.

Rats were housed in pairs into individually ventilated cages (IVC) of 800cm². Cages were kept in racks, for a total of 35 cages per rack. The housing room had a designated space for each rack, for six total racks per room. Each cage was equipped with a card summarizing the essential information of the rats: ID number, line, date of birth, person in charge of the experiment, etc… Cards were colour-coded depending on the animal status.

Each housing room had a pre-room where routine procedures were executed, with occasional experimental procedures. The facility had several laboratories supplied with specific experimental equipment. Fear experiments were conducted mainly in the Laboratory S. The room was equipped with 16 operant boxes (MedAssociates) with modifiable designs. Other equipment included two desktop pc, 16 shockers (1.8–5V) and one control board (MedAssociates).

3.3 Observational fear experiment

A total of 64 rats were used for the experiment, all males. Due to a mistake during one session of fear test, eight rats had to be excluded from the results, leaving a total to 56 animals.

As initial step, the rats were divided into two groups: demonstrators and observers. The division criterion was limited to the marked number of each individual (even for observers & uneven for demonstrators). Each housing cage hosted an observer and a demonstrator.

The animals were treated 32 at the time in numerical order for practicality, with a 24 hours-difference between set of 32 to avoid major stressing. The rats were exposed to the operant chamber with the addition of a floor placed over half of the grid for 20 minutes. Two sessions over two days. During habituation, five acoustic tones (29 kHz, 65 Db) of two seconds were played with three-minute intervals between each tone (**fig. 1**).

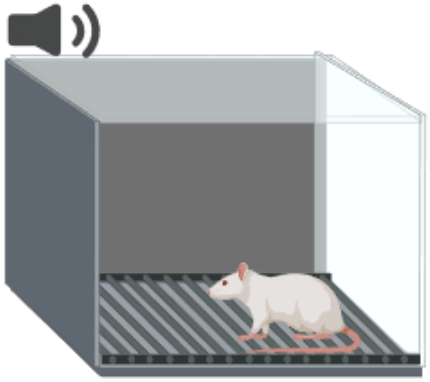

Figure 1: Representation of the Tone habituation session done with Biorender.

The next step was conducted only on the observers group. The subjects underwent one session of priming in a different operant cage. This was done to avoid associative learning with an already familiar environment. The observers received six non-signalled foot shocks (0.8 mA) of two seconds, with a random inter-shock interval (**fig .2**).

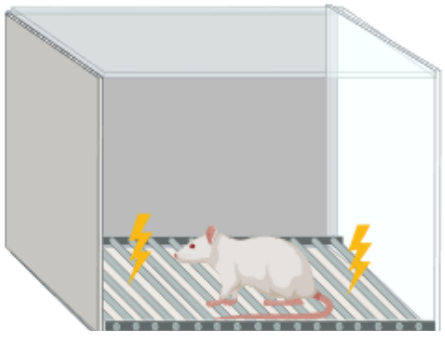

Figure 2: Representation of the Priming session for observers done with Biorender.

24 hours after priming, both groups of rats went through fear acquisition. The operant cages were prepared in two different contexts (**fig. 3**); with a separator in the middle for the rats to perceive each other's and to interact, and a floor on the internal side of the cage. Observers were placed on the floored side of the cage, while the demonstrators were on the grid side. During fear acquisition, an acoustic tone was played for 30 seconds, paired in the final two seconds with an electric foot shock (0.8 mA) received by the demonstrator while the observer watches. The tone + shock was delivered a total of six times throughout the session, with a three-minute interval. Each fear acquisition session lasted 20 minutes. Four sessions with four boxes used each time, for a total of eight sessions for two days. Bedding was changed after every session (**fig. 4**).

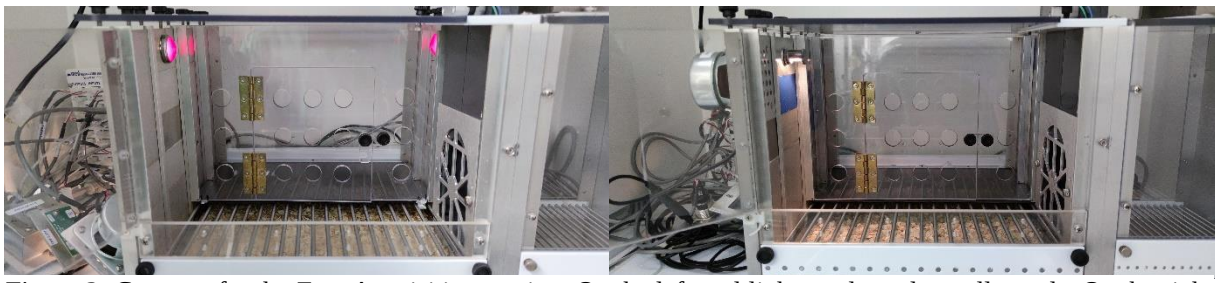

Figure 3: Contexts for the Fear Acquisition session. On the left, red lights and regular wall panels. On the right side, house light and blue wall panels with air grid.

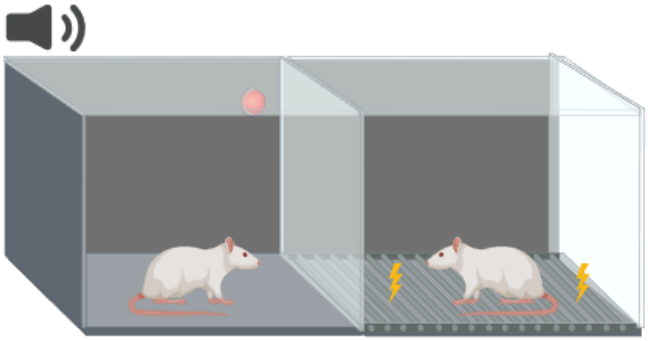

Figure 4: Representation of the Acquisition session done with Biorender. The operant cage is represented with two cages for clarification purposes (better visualisation) and for avoiding cramming.

Thereafter, no further procedure was conducted on the animals for four weeks, whith the exception of routinely weight sessions. The last step was the fear test. Eight operant boxes were prepared with two different contexts depending on the number of the box: context A **(fig. 5**) for odd numbers and context B (**fig. 6**) for even numbers. Contexts were:

- A: White panels, white divider, 2 red lights, EtOH sprayed and no social box grid

- B: Blue panels, striped divider, house light, DesiDos sprayed and social box grid

Rats were exposed to six acoustic tones of 30 seconds each with three-minute inter-tone intervals, in a 20-minute session. Eight sessions were performed over two days. Bedding was changed between each session (**fig. 7**).

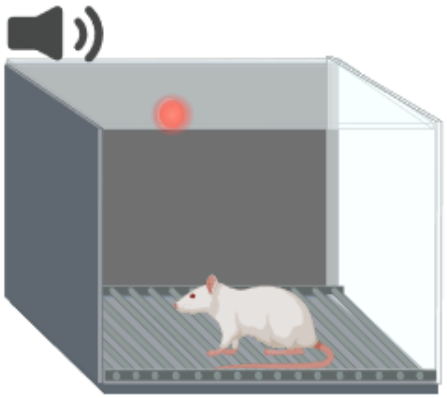

Figure 7: Representation of the Fear Testing session for observers done with Biorender.

3.4 Corticosterone enzyme immunoassay analysis

During the observational fear procedure, rats produced four blood samples collected at different timepoints:

- ❖ Baseline (t0): collected before any manipulation had happened on the animals.
- ❖ After fear acquisition (t1): collected approximately 10 minutes after each fear acquisition session.
- ❖ One day after fear acquisition (t2): collected the day after fear acquisition, approximately at the same time to respect the 24 hours interval.
- ❖ After fear test (t3): collected approximately 10 minutes after each fear test session.

Immediately after each extraction, artificial haemolysis took place by doing a centrifuge of the samples. The inorganic part (Plasma) was extracted. Samples were subsequently stored at -4C° temperature waiting for all timepoints and samples to be collected.

The next step was the extraction and isolation of corticosterone from the plasma, followed from a laboratory protocol. Samples were brought back to room temperature and shaken through vortex. From each sample, 10µl were taken. The serum was subsequently mixed with 100µl of ethyl acetate 100% pure (CH3COOC2H5) and placed in a thermos-shaker for two minutes, plus five minutes still. Afterwards, working in a hood, the composition of steroids and other inorganic substances was separated from the ethyl acetate and added to a previously prepared eppendorf with 100µl of MilliQ water. The samples were shaken again in the thermos-shaker for two minutes with additional 2 minutes still. The process from the addiction of ethyl acetate to the shaker after mixing with MilliQ had to be repeated twice for higher probability of isolating the corticosterone molecules.

After thermos-shaking for the second time, one final separation was performed: the solution containing the corticosterone was extracted and excluded from the milliQ water part of the sample. As a final step, samples were put into a speed vacuum, or speedvac, and ran for approximately 50 minutes at 35C°for the solution to dry and the corticosterone and other steroids to remain. The final samples were kept at -4C°. The whole process was repeated for all the samples produced.

The second part of the procedure was an enzyme immunoassay analysis performed with the DetectX Enzyme Immunoassay kit (Arbor Assays) (**fig. 8**). The kit contained all the equipment employed for the analysis of corticosterone. The samples previously extracted were re-suspended with Dissociation reagent, vortex and left to rest for five minutes.

Figure 8: Contents of the Enzyme Immunoassay kit from Arbor Assays. From left to right: Stop solution, DetectX corticosterone antibody, TMB substrate, DetectX corticosterone conjugate, Assay buffer, Corticosterone standard and Wash buffer.

The addition of Assay Buffer (1:5 dilution) brought the dilution of each sample to 1:100, standard dilution for blood samples coming from males. Afterwards, standard concentrations for corticosterone were prepared as indicator and margin of the analysis. Concentrations went from 10.000 pg/mL to 39.069 pg/mL subdivided in nine points. Plates were subsequently prepared, with samples used on duplicate wells to assess the difference in concentration for each sample. The distribution of wells was as such: one non-binder (75µl Assay buffer), one blank (50µl Assay buffer), nine standards and the remaining wells as samples (50µl). Samples were organised as to have all the timepoints of a single rat on the same plate.

Once all samples were added to the plate, 25µl of Corticosterone Conjugate (DetectX) were allocated to all wells and to all but the non-bindings, 25µl of Corticosterone Antibody were added. The plate was then covered and left on a shaker for one hour (700-900 rpm). After shaking, the plate was emptied and washed with Wash Buffer (1:20) for four times, drying on paper sheets in between every wash. Immediately after the washing, 100µl of TMB Substrate were administered into every well. Afterwards the plate was left to incubate for 30 minutes at room temperature. Lastly, 50µl of Stop Solution were added in each well.

The plate was subsequently placed in a plate reader connected to the SoftMax Pro software. This software was designed to produce a reading of the corticosterone concentration of each well in the plate. A layout of the plate with identical samples positions was required, with settings for optical density at 450 nm. The final readout would use the plate template to display the concentrations on each well, with a graphical representation of the standard concentrations for accuracy.

3.5 Behavioural Scoring

In addition to the animals used for the observational fear procedure and the corticosterone analysis, recordings of 50 additional male rats were used for behavioural scoring in both fear acquisition and fear testing. Said animals were divided in four groups: observers, demonstrators, and control groups for both. These animals were only used for behavioural scoring in an observational fear setup.

Fear acquisition and fear testing sessions were recorded with an infrared camera placed atop the operant chamber. Videos were then exported to be used for scoring behaviours.

The licensed software Ethovision XT (Noldus) was used to produce the behavioural data for the thesis. The software had a function for acquisition of video sources, transforming them into

sets of frames. The process allowed non-useful frames to be removed by setting the video-coordinates for a specific interval of time.

Said frames could be subsequently played in slideshow with an additional scoring function. Setting up a list of behaviour, it was possible to key-bind each behaviour to track it and score it over the slideshow. Each frame was also described with seconds and milliseconds, giving exact coordinates in the reference video for the scoring output.

The behaviours analysed in this experiment were:

- Freezing: can be defined as a state of tonic immobility, with occasional small movements, maintained by the animal for more than one second, usually positioned facing the centre of the cage.
- Head scanning: similar to freezing, it also implies immobility but with a rhythmic movement of the head from left to right.
- Grooming: action the animal does on itself, mainly with the front limbs or with the head.
- Sniffing: most common of the behaviours displayed, sniffing implies a movement and/or contact of the whiskers with a surface such as the wall or the grid of the cage. A separate scoring is done when the rat sniffs at no specific location, named free-air sniffing.
- Rearing: the action of standing on the back limbs from the animal. With sniffing, is considered as a macro-behaviour "exploration".
- Jumping: rare behaviour. The animal is in a position for which no limbs are touching the surface of the operant box.

The described behaviours were scored during all types of sessions. During fear acquisition, instances of physical interaction between the two subjects were also scored. Behaviours were scored in relation to the acoustic tones' onset and offset. Once scored, data was converted from seconds to percentages and averaged between the two tones. The average score is used for statistical analysis.

3.6 Operant fear experiment with diazepam

A total of 128 rats were used for this experiment, 64 males and 64 females. No sex difference was investigated, and the animals were not treated together at any point of the procedure. The protocol for males and females did not vary. Out of the 128 animals used, 24 males and 13 females were excluded as they did not meet the criterion for statistical analysis.

The first phase of the procedure revolved around training the animals for operant behaviour. The operant cages were set up with two levers and two receptacles, on the left side and on the right side, with one white light on the right. The left lever, when pressed, would administer around 100µl of saccharine solution, which was positioned in a syringe outside the operant box and connected to the left receptacle.

The rats underwent training session every day of the week for a 20-minute session. The operant behaviour consisted of the animal pressing the lever and obtaining a reward. Firstly, the administration of saccharine was on fixed ratio one (FR1). When FR1 is considered as learned, approximately after two weeks of training, the fixed ratio is increased to 2. Two lever presses per one saccharine reward. FR2 went on for an additional two weeks (**fig. 9**).

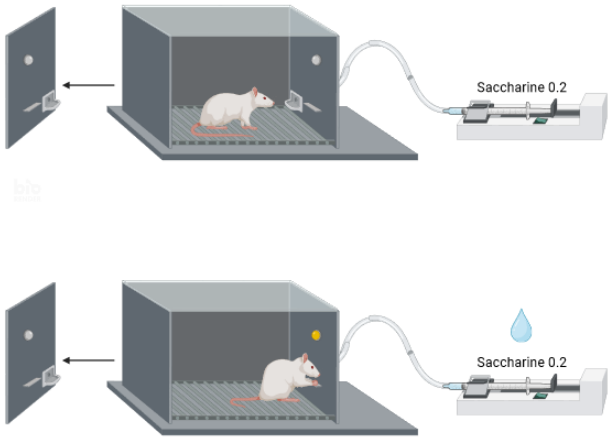

Figure 9: Representation of the Operant training phase, done with Biorender.

Rats were subsequently habituated to both being injected with saline solution before a training session, two-four times with increasing dosage, and to an acoustic tone (29 kHz, 65 Db) of two minutes repeated two times per session (**fig. 10**). Around six sessions of tone habituation were required. Afterwards the animals did additional FR2 sessions to re-establish a valid baseline of operant ratio.

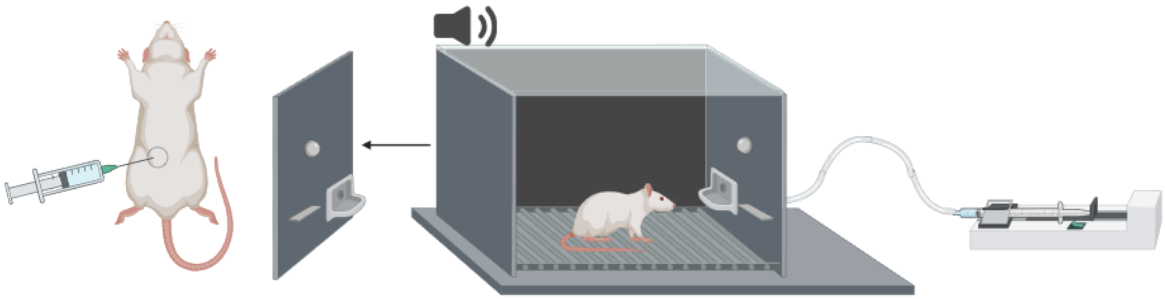

Figure 10: Representation of the saline solution injection training (left) and the acoustic tone habituation (right), done with Biorender.

At this point, rats were divided in two groups depending on performance, representing two different shock intensities: 0.4 mA and 0.8 mA. The two groups went through fear acquisition with two different setups:

- Uneven-numbered boxes: white divider excludes the receptacle and lever.
- Even-numbered boxes: striped divider excludes the receptacle and lever.
- Exception for box 1 and 6 where no house light was present, replaced with red light.

Each session consisted of three acoustic tones of 30 seconds with a foot shock in the last two seconds, with a five-minute interval between the tones, for a total of 20 minutes. There were four sessions in total. Bedding is changed between each session (**fig. 11**).

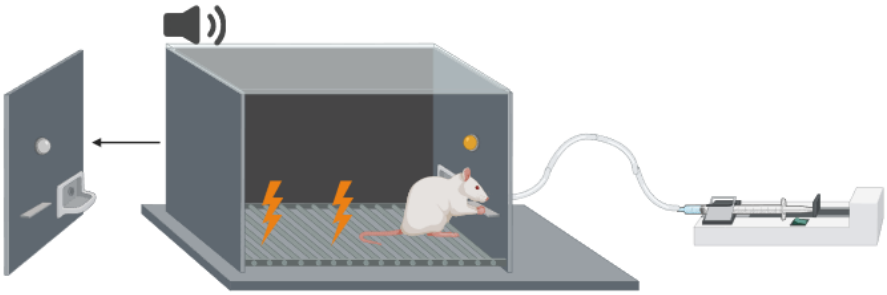

Figure 11: Representation of the Fear Acquisition session, done with Biorender.

After the fear acquisition, other sessions of recovery took place until the rats had a stable ratio again. Rats were re-divided into three additional groups for the injection of diazepam: control or vehicle (injected with saline solution), 0.3 mg/kg diazepam injection and 1 mg/kg diazepam injection.

Finally, fear test sessions were conducted approximately 10 minutes after each injection, by placing the rats in the operant context with access to the levers and the receptacles. Here, the animals were exposed to the acoustic tones again, without the foot shock. Again, bedding is

changed between each session (**fig. 12**) Transcripts of the number of lever presses were collected after each session (from FR1 to fear test) through the MedPC software.

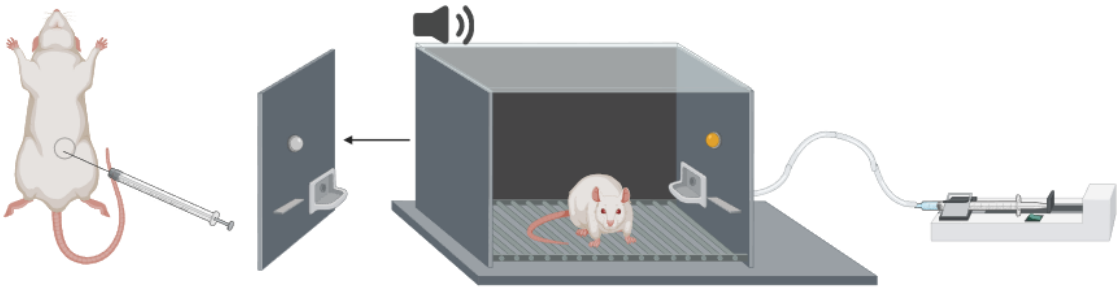

Figure 12: Representation of the Diazepam injection (left) and the Fear Testing session, done with Biorender.

3.7 Statistical Analysis

Data collected from Ethovision XT (Behaviour scoring), SoftMax pro (Corticosterone) and MedPC (lever presses) was convoluted in specific excel sheets, where the data would be polished for the statistical analysis. Graphical information and experiment's trends were produced in Graphpad Prism. Statistical analysis was performed with the software Statistica (Stat Soft). Significance statistical level was set up at 0.05. Statistical tests used were, in order of appearance: Unpaired t test, Two-way ANOVA, Mann-Whitney U test, Simple linear regression, Kruskal-Wallis test, One-way ANOVA, Repeated measures ANOVA and Newman-Keuls test.

In addition to the statistical tests, cluster analysis and factor analysis were performed. Cluster analysis was used to divide the observer groups (from both the 56 experimental rats and the 50 video rats) into high fear clusters (high freezing percentage) and low fear clusters (low freezing percentage). The clusters were made with Statistica.

Factor analyses were performed on the behavioural scoring data obtained from the 50 video rat subjects. The factor analysis pooled together all the behavioural values for each behaviour and determined several factors that influence the variance between said values. To estimate the number of factors required, I applied the Kaiser criterion (Kaiser, 1960), which takes in consideration the eigenvalues of each factor and excludes the ones below 1. The eigenvalues are coefficients that explains the percentage of variance between the variables. As a result, two factors were used for the analysis.

4. RESULTS

4.1 Observational fear experiment

In the observational fear experiment, results include corticosterone concentrations in pg mg^{-1} and behavioural scores in percentages, with focus on freezing. Additionally, the results of the factor analysis are shown in factor score (interval between 2 and -2).

4.1.1 Corticosterone concentrations

Concentration of corticosterone was higher in rats after fear acquisition and fear testing procedures compared to the concentration at baseline. Corticosterone concentration was analysed in relation to the blood collection timepoints (t0 to t3), showing a highly significant difference in concentration amount between t0 baseline and the other timepoints. This applied to both demonstrators and observers (**fig. 13**) (*Two-way ANOVA, distribution of the corticosterone measurement between timepoints, F = 4.921, p = 0.0027*). Variance in concentration from baseline (t0) was calculated for each timepoint. Statistical evidence showed a high difference between variances t1-t0 and t3-t0, at acquisition and test timepoints (**fig. 14**).

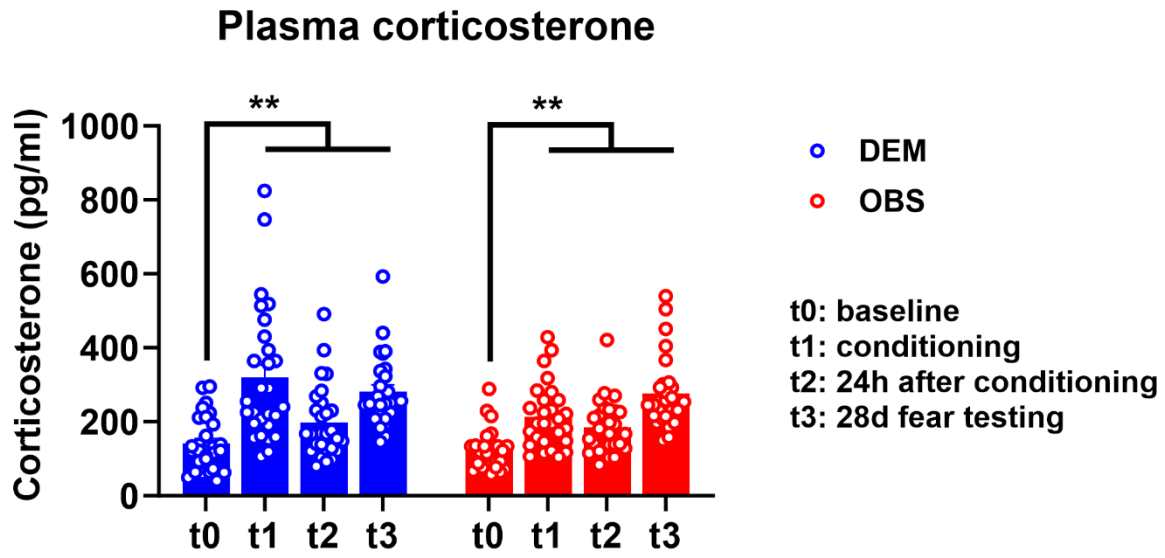

Figure 13: Corticosterone concentration (pg mg^{-1}) per individual plotted on all timepoints (t0 to t3) Groups showed in this comparison are demonstrators (blue) and observers (red). Coloured bars represent the total mean; dots represent individual values and whiskers the standard deviation. Significant difference is shown through black bars and asterisks (*= significant at 0.05, **= significant at 0.001).

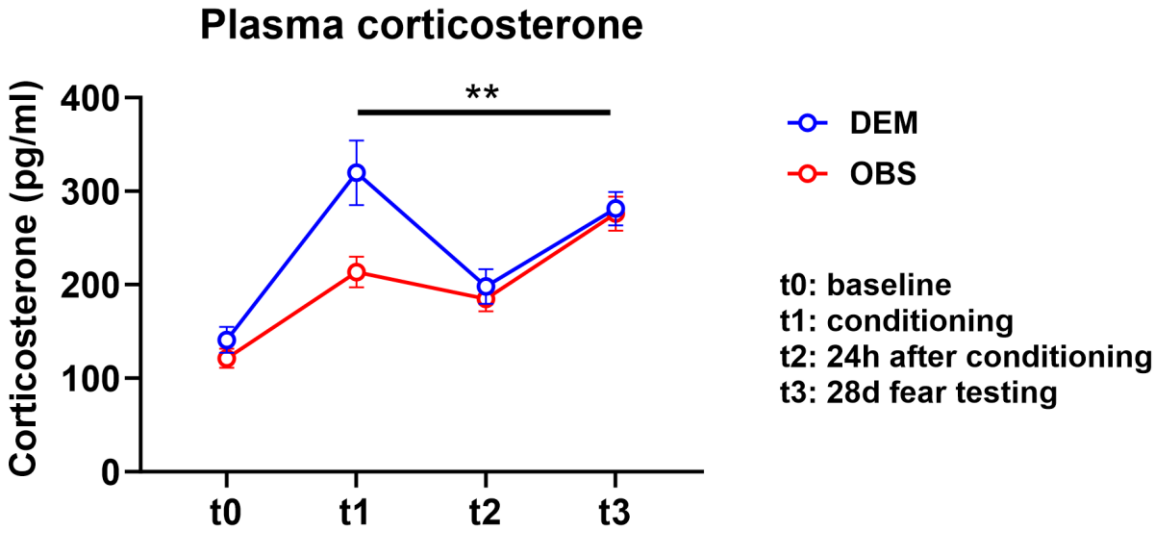

Figure 14: Corticosterone amount variance (pg mg^{-1}) per means (dots) on all timepoints (t0 to t3). Groups showed in this comparison are demonstrators (blue) and observers (red). Whiskers represent the standard deviation. Significant difference is shown through black bars and asterisks (*= significant at 0.05, **= significant at 0.001).

4.1.2 Behavioural scores - Freezing

The two following figures took in consideration only the 56 rats subjected to the observational fear procedure.

Rats showed defensive behaviours during the acoustic tones play. Demonstrator rats froze in higher percentage than observer rats during the two minute's acoustic tones of the fear testing session (**fig. 15**). There was a statistically high significant difference between demonstrators and observers in freezing percentages (*Unpaired t test, fear expression difference between two groups, t (54) = 5.20, p < 0.001).*

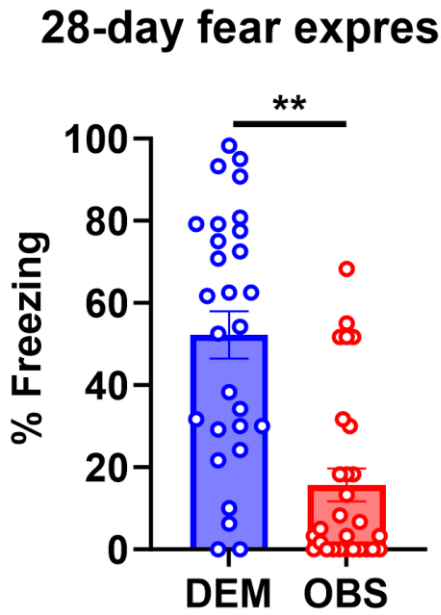

Figure 15: Fear expression by means (coloured bars) of freezing percentages (dots) during the fear test session (28 days after fear acquisition) of the groups demonstrators (blue) and observers (red). Whiskers represent the standard deviation. Significant difference is shown through black bars and asterisks (*=0.05, **=0.001).

Between the observers, there was a group of animals (seven individuals) that exhibited major defensive behaviour. Clusters for the observer freezing scores were made, with the high fear cluster's percentages higher than the low cluster's (**fig. 16**). There was a significant difference between the high fear cluster scores and the low fear cluster scores (*Unpaired t test, fear expression difference between two clusters, t (26) = 11.5, p < 0.001*).

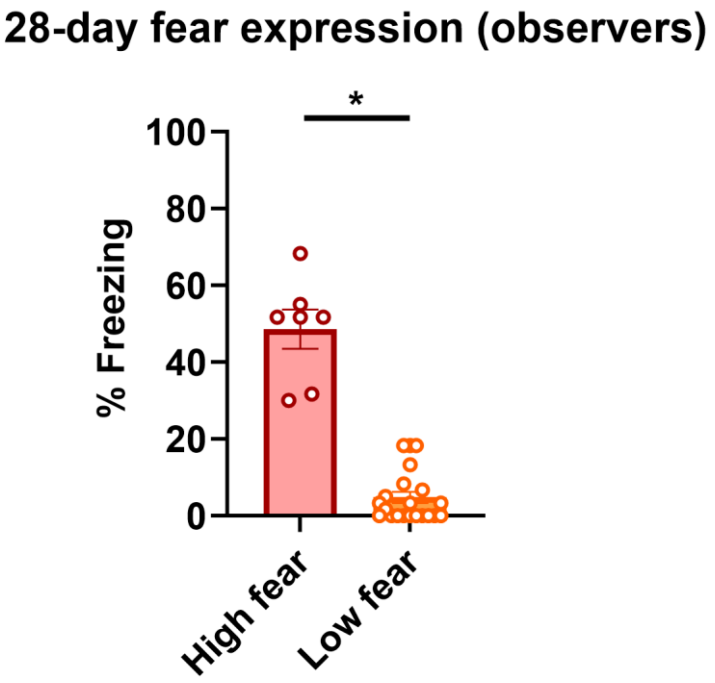

Figure 16: Fear expression by means (coloured bars) of freezing percentages (dots) during the fear test session (28 days after fear acquisition) of the observer clusters high fear (red) and low fear (orange). Whiskers represent the standard deviation. Significant difference is shown through black bars and asterisks (*=0.05, **=0.001).

The following figures considered the behavioural scores obtained from the 50 additional video-subject rats.

During fear acquisition, rats that were not the control groups exhibited freezing behaviour. Specifically, demonstrators and high fear observers had high percentages of defensive behaviours (**fig. 17**). There was a statistically significant difference between the freezing scores of demonstrators and control demonstrators, and between high fear observers and control observers (*Kruskal-Wallis test, variance between the averaged means, KW statistic: 32.83, $p < 0.0001$*)

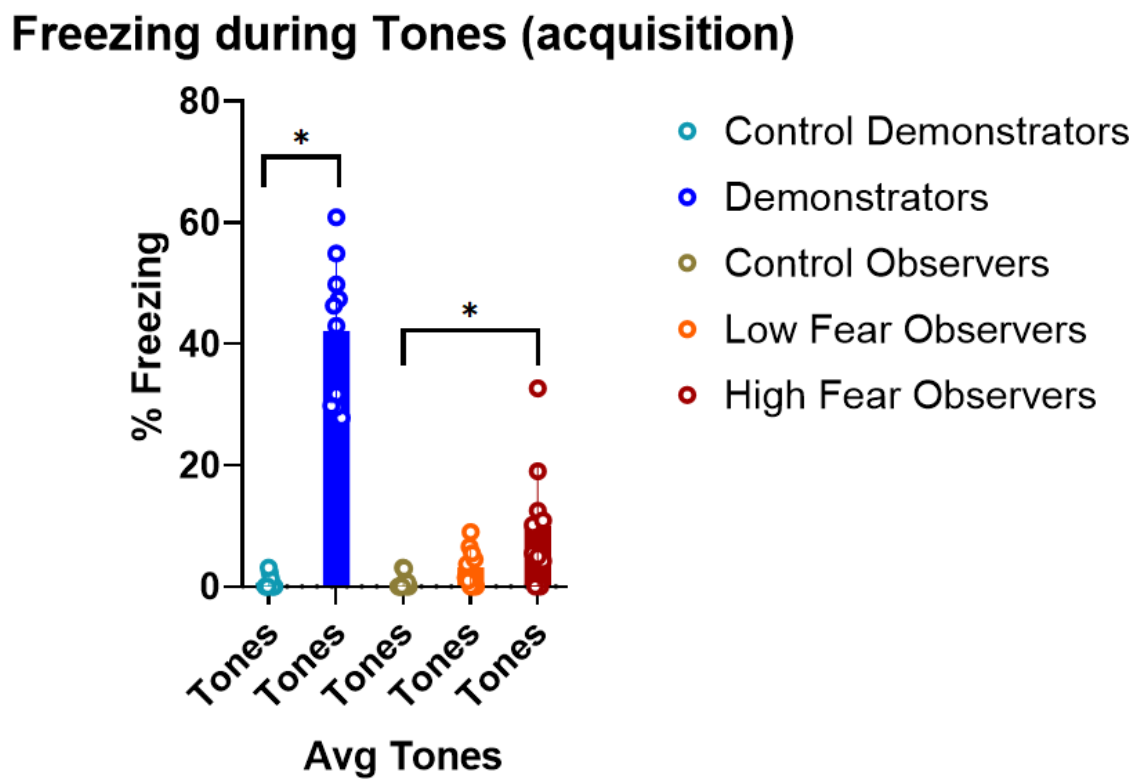

Figure 17: Freezing percentages per means (coloured bars) and individual values (dots) during fear acquisition. The values are calculated during the acoustic tones. Whiskers represent the standard deviation. Significant difference is shown through black bars and asterisks (*= significant at 0.05, **= significant at 0.001).

Additionally, animals from the demonstrator and both high and low observer groups prolonged the freezing behaviour in the average minute after receiving the shock. Specifically, low fear observers increased their defensive behaviours (**fig. 18**). The resulting statistic evidenced a highly significant difference in the distribution of the means between the two demonstrator groups and between the control observer group and the high fear observer group (*Kruskal-Wallis test, variance between the averaged means, KW statistic = 35.42, $p < 0.001$*).

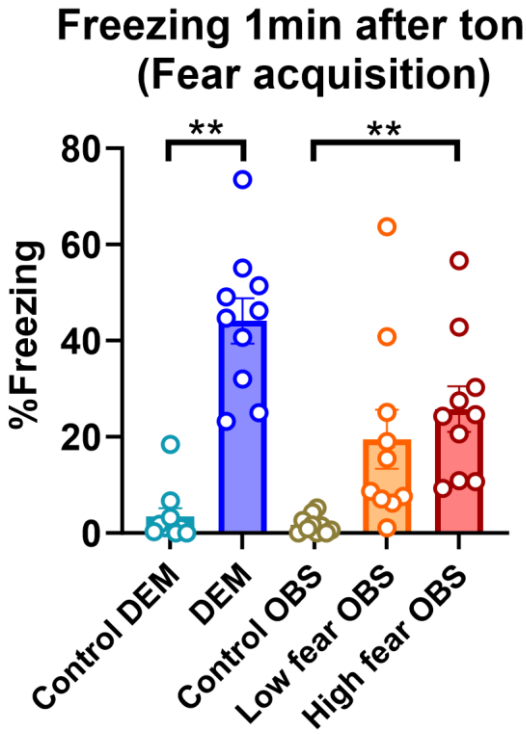

Figure 18: Freezing percentages per means (coloured bars) and individual values (dots) during fear acquisition. The values are calculated one minute after the acoustic tones. Whiskers represent the standard deviation. Significant difference is shown through black bars and asterisks (*= significant at 0.05, **= significant at 0.001).

Rats shows greater signs of freezing behaviour one or more minutes after the acoustic tone's offset. In the context of fear testing, results are similar to the fear acquisition. Demonstrator rats and high fear observer rats had the highest freezing percentages both during the tones and one minute after **(fig. 19A, B)**. Prolonging the freezing scoring to a total of 3 minutes (time inter-tone), four groups out of five shows important latency for freezing behaviour (**fig. 19C**).

Highly statistical significance was found between demonstrator groups and between control observers and high fear observers during the tones (*Kruskal-Wallis test, variance of means for average tones freezing scores between groups, KW statistic = 47.66, p < 0,001*). Additional highly statistical significance was found between demonstrator groups and between high and low fear observers groups at one minute after the tone's end (*Kruskal-Wallis test, variance of means for one minute after tone freezing scores between groups, KW statistic = 31.35 , p < 0,0001*), however the variance in latency of freezing was not significant (*Kruskal-Wallis test, variance of means for latency freezing scores between groups, KW statistic = 4.181 , p < 0,0001*).

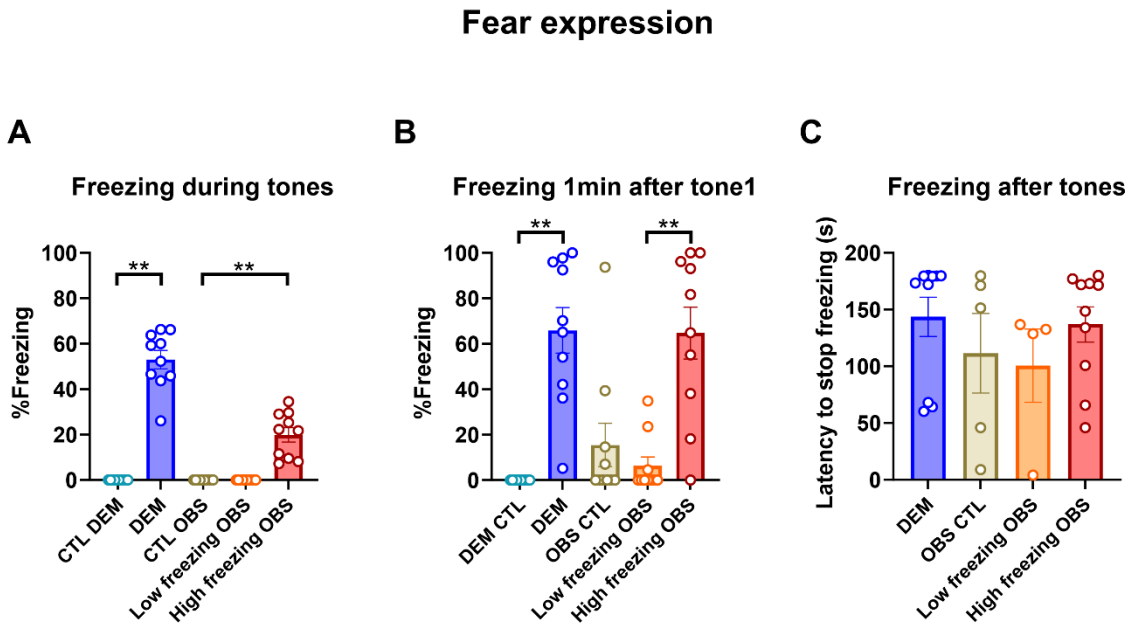

Figure 19: Freezing percentages per means (coloured bars) and individual values (dots) during fear testing. The values are calculated during the tones (**A**) and one minute after the tone's offset (**B**). Freezing latency (in seconds) is used for the inter-tone time (**C**). Whiskers represent the standard deviation. Significant difference is shown through black bars and asterisks (*= significant at 0.05, **= significant at 0.001).

4.1.3 Behavioural scores - Ethograms

Scores obtained for all behaviours were pooled in ethograms. Ethograms were done for scores done for the two minutes before the tones (baseline).

For the fear acquisition procedure, the animals exhibited exploratory behaviours during the baseline timepoint. Vigilant behaviours increased throughout the acoustic tones, apart for the control groups. Demonstrators exhibited a high percentage of freezing behaviour, with high and low fear observers showing a minor percentage (**fig. 20**).

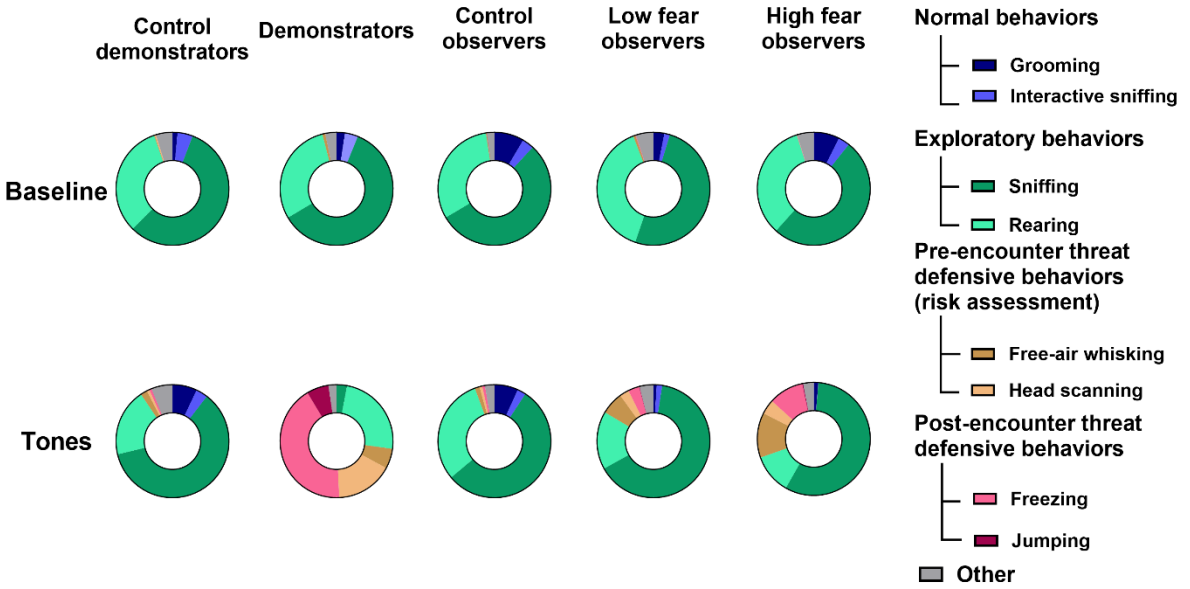

Figure 20: Ethograms divided by timepoints (vertical left) and groups (horizontal top), with a descriptive legend to the right describing the different behaviours scored and the different macro categories for each behaviour. The behaviour "other" refers to all non-accountable behaviours performed.

Factors 1 and 2 of the factor analysis explained 65% of the variation between behaviours, in each group of subjects. Relationships between behaviours evidenced changes in the dynamics related to, on one side, reduction in exploratory behaviours and increase in vigilance/defensive behaviours (factor 1). On the other side, increase in free-air whisking influenced decrease in rearing (factor 2) (**fig. 21A**). Factor scores also showed the behavioural diversity between experimental groups, with each factor analysed independently (**fig. 21B, C**).

Resulting factor analysis showed a highly significant difference for factor 1 between the demonstrator groups (*One-way ANOVA, influence of factor 1 for all groups, $F = 160.952$, $p < 0.001$*), and a highly significant difference for factor 2 between the control observer group and the two other observer groups (*One-way ANOVA, influence of factor 2 for all groups, $F = 8.422$, $p = 0.001$*).

A

Behaviors	Factor 1	Factor 2
Grooming	-0.33	-0.50
Interaction	-0.41	-0.53
Sniffing (all)	-0.95	0.10
Rearing	0.25	-0.77
Free-air whisking	0.06	0.78
Head scanning	0.60	0.32
Freezing	0.89	0.15
Jumping	0.96	0.01
Eigenvalue	3.48	1.72
% Total variance	43.51	21.49

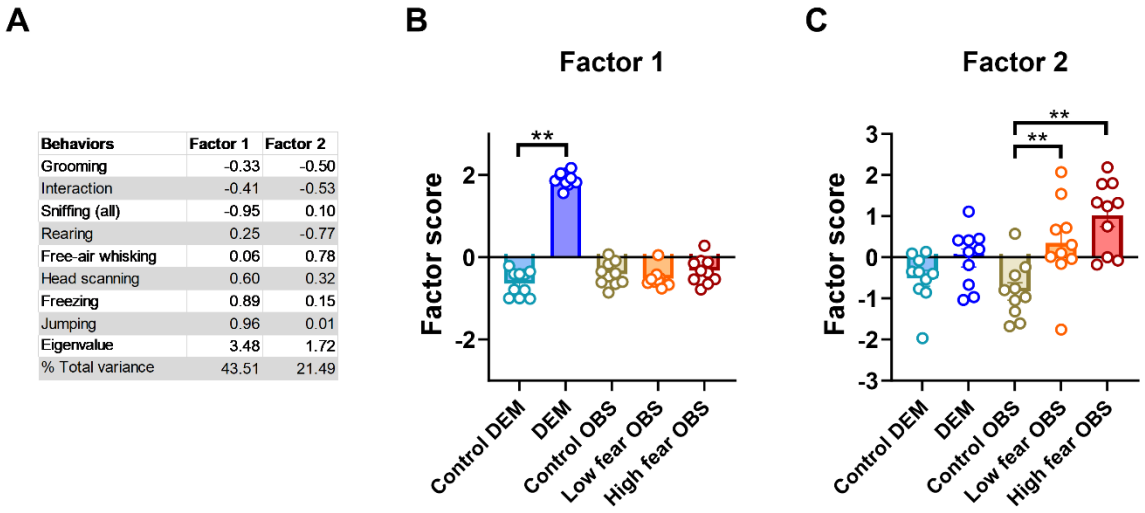

Figure 21: Table of factor values for each behaviour (**A**), graphical representation of the individual factor analysis between the groups (X-axis), during fear acquisition. Factor 1 (**B**) and factor 2 (**C**). Factor scores are expressed per means (coloured bars) and individual values (dots). Whiskers represent the standard deviation. Significant difference is shown through black bars and asterisks (*= significant at 0.05, **= significant at 0.001).

Ethograms produced from behavioural data obtained during fear testing sessions showed similar trends to the ones produced during fear acquisition: high percentages of exploratory behaviour during baseline and increased vigilance (and freezing for demonstrators and high fear observers) during the acoustic tones play. Additional ethograms were provided for the inter-tones time. In this last instance, freezing exponentially increased for high fear observers, matching the demonstrator's percentages. Other increased freezing percentages could be recorded in control observers and low fear observers (**fig. 22**).

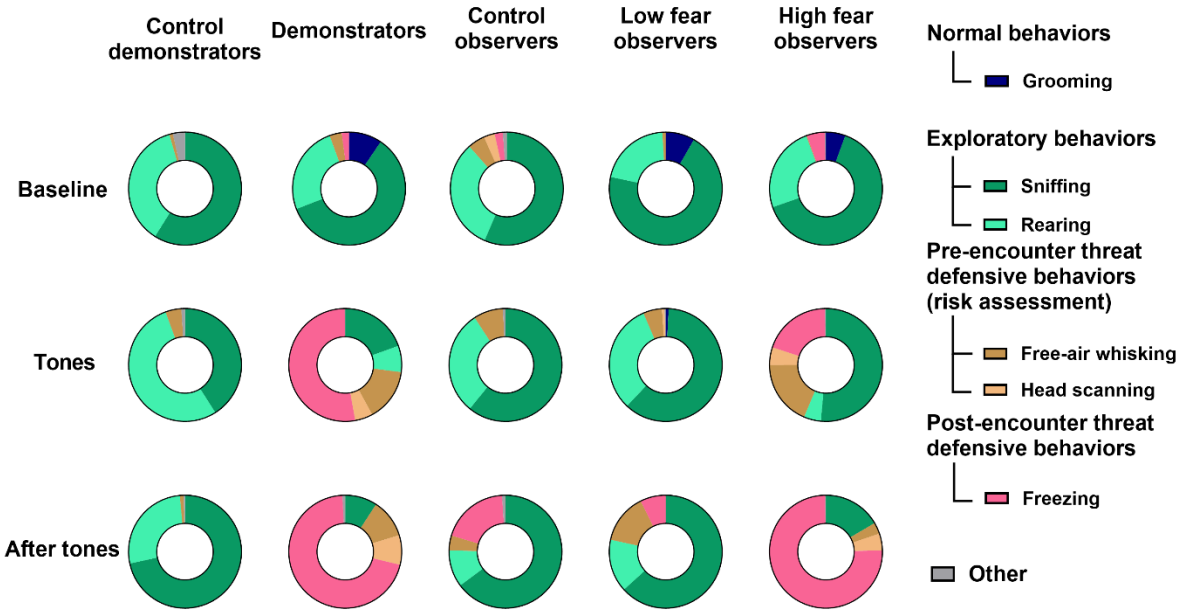

Figure 22: Ethograms divided by timepoints (vertical left) and groups (horizontal top), with a descriptive legend to the right describing the different behaviours scored and the different macro categories for each behaviour. The behaviour "other" refers to all non-accountable behaviours performed.

Factors 1 and 2 of the factor analysis explained approximately 58% of the variation between behaviours, in each group of subjects. Relationships between behaviours evidenced changes in the dynamics related to, on one side, the increased performance of sniffing and other exploratory behaviours for the reduced performance of vigilant behaviours and freezing (factor 1). On the other side, a strong reduction of rearing and grooming behaviours meant a minor increase in the remaining behaviours (factor 2) (**fig. 23A**). Factor scores also showed the behavioural diversity between experimental groups, with each factor analysed independently (**fig. 23B, C**). For factor 1, highly statistical significance was found between the two demonstrator groups scores (*One-way ANOVA, variance of factor 1 scores between experimental groups, $F = 18.186$, $p < 0,001$*). For factor 2, highly statistical significance was found between the two demonstrator groups scores and statistical significance was found between control observer and high fear observer groups *(Kruskal-Wallis test, variance of factor 2 scores between experimental groups, KW statistic = 28.98824, $p < 0,05$*)

A

Behaviors	Factor1	Factor2
Grooming	0.03	-0.41
Sniffing	0.97	0.21
Rearing	0.14	-0.94
Free-air Whisking	-0.48	0.48
Head-Scanning	-0.40	0.30
Freezing	-0.71	0.45
Eigenvalue	2.37	1.11
% Total variance	39.44	18.47

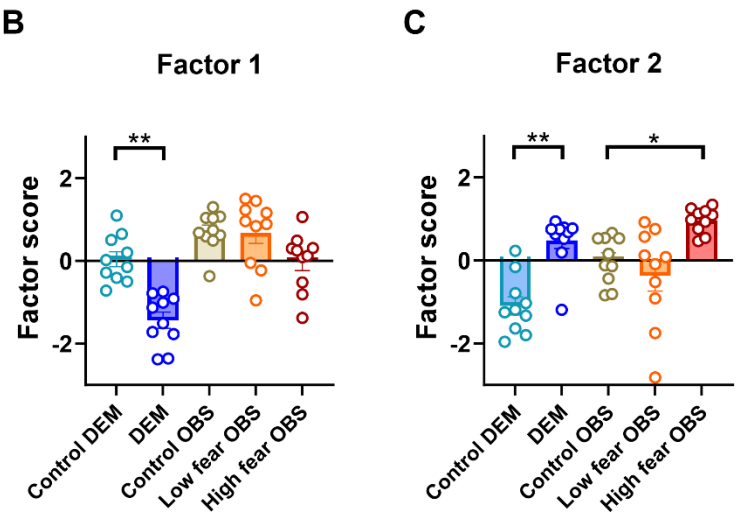

Figure 23: Table of factor values for each behaviour (**A**), graphical representation of the individual factor analysis between the groups (X-axis), during fear acquisition. Factor 1 (**B**) and factor 2 (**C**). Factor scores are expressed per means (coloured bars) and individual values (dots). Whiskers represent the standard deviation. Significant difference is shown through black bars and asterisks (*= significant at 0.05, **= significant at 0.001).

4.2 Operant fear experiment

Male rats experienced a drop in operant performances during the acoustic tones, of higher intensity when exposed to a higher shock voltage (**fig. 24A, B**). Suppression ratio was influenced by the shock intensity, with higher shock subgroups closer to full suppression. Diazepam in different concentrations also influenced the suppression ratio (**fig. 24C, D**).

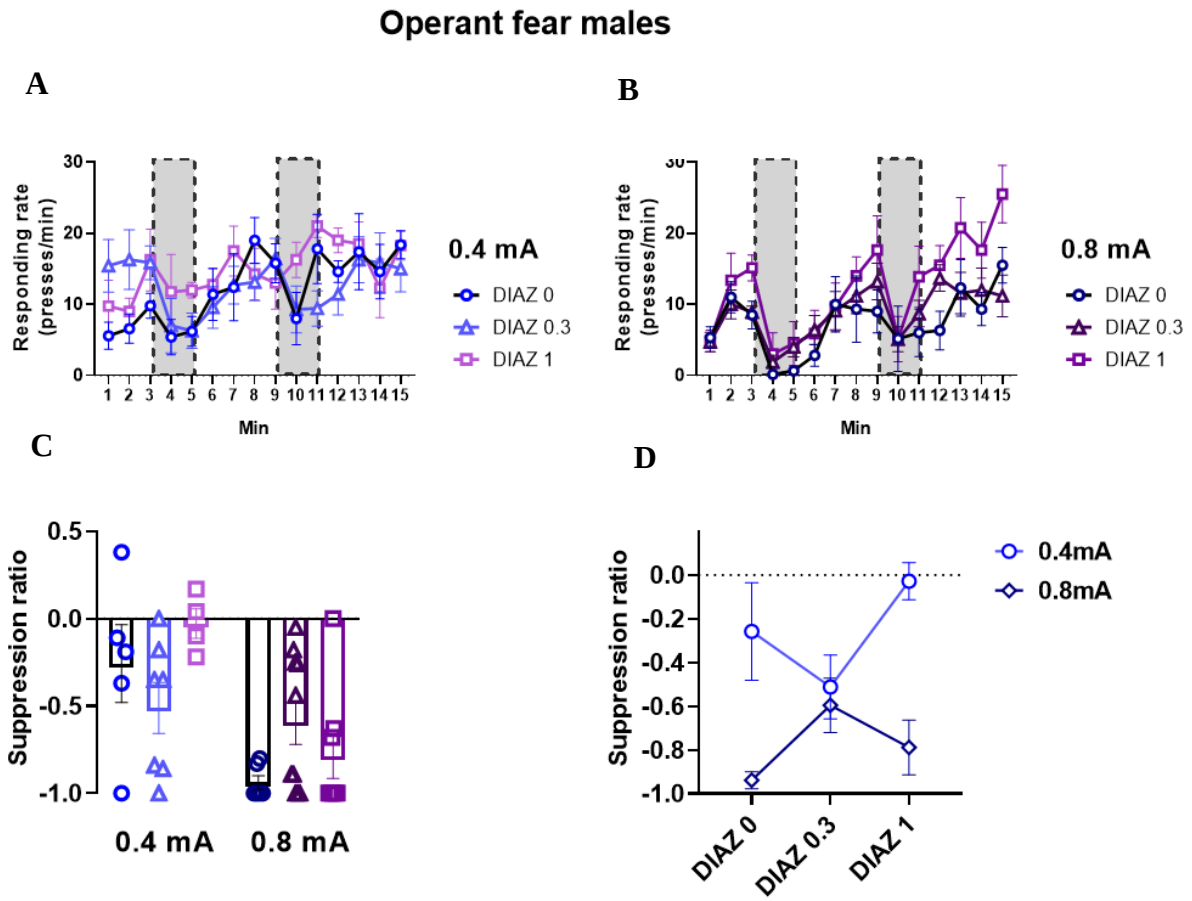

Figure 24: Summary table showing the responding rate during the fear expression session for the 0.4 mA group and diazepam subgroups (**A**), responding rate during the fear expression session for 0.8 mA group and diazepam subgroups (**B**). Additionally, graphical representation of suppression ratio at individual level for both groups and subgroups (**C**), and at group level as progression line (**D**). Whiskers represent the standard deviation. In **A** and **B**, squares, triangles and dots are mean values from the individuals.

At statistical level, ANOVA results showed highly significant difference in the tone variable (*Repeated measures ANOVA, Variance between dependent and independent variable, $F = 57.556$, $p < 0.001$*). Results also indicated a significant difference in intensity variable (*Repeated measures ANOVA, Variance between dependent and independent variable, $F = 5.795$, $p < 0.05$*), in the intersection of tone and intensity (*Repeated measures ANOVA, Variance between dependent and independent variable, $F = 7.192$, $p < 0.05$*) and in the intersection of tone, intensity, and dose (*Repeated measures ANOVA, Variance between*

dependent and independent variable, $F = 5.735$, $p < 0.05$). The variable of dose showed a strong trend (*Repeated measures ANOVA, Variance between dependent and independent variable, $F = 3.088$, $p = 0.059$*).

Post Hoc results showed a major variance in the 0.8 mA group compared to the 0.4 mA group. (*Newman-Keuls test, Probability for mistake between, within and pooled data, MSE = 101.45, df = 55.559*)

Finally, results from two-way ANOVA with suppression ratio and grouped values revealed a highly significant difference between intensity groups *(Two-way ANOVA, Variance in suppression ratio between groups, $F = 18.625$, $p < 0.001$*) and significant difference in the interception between intensity groups and dosage subgroups (*Two-way ANOVA, Variance in suppression ration in the interception of groups and subgroups, $F = 3.768$, $p < 0.05$*). By means of a Post Hoc test (*Newman-Keuls test, Probability for mistake between data, MSE = 12681, df = 34.000*), results evidenced a highly significant difference between the 1 mg kg^{-1} dose subgroups (*$p < 0.001$*) and a statistically significant difference between the 0 mg kg^{-1} dose subgroups (*$p < 0.05$*).

Female rats experienced a drop in operant performances during the acoustic tones, of similar intensity when exposed to a higher shock voltage (**fig. 25A, B**). Suppression ratio was influenced by the shock intensity, with only the higher shock subgroup closer to full suppression. Diazepam in different concentrations also influenced the suppression ratio (**fig. 25C, D**).

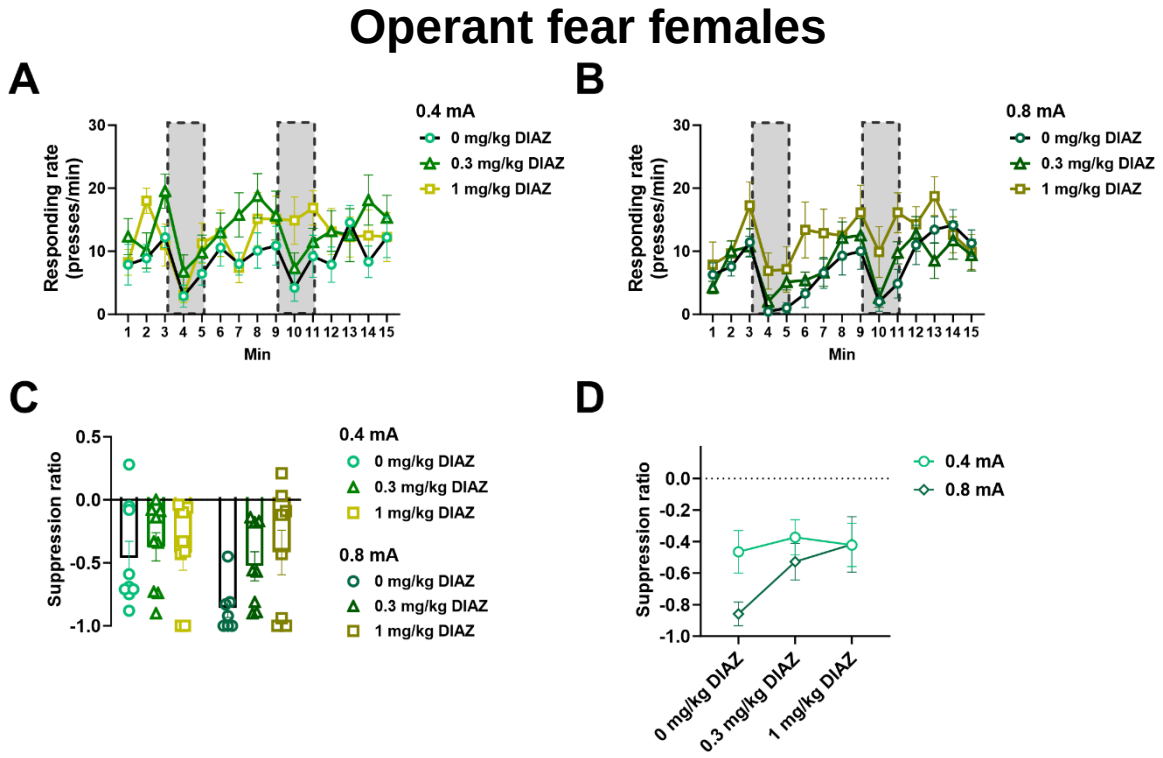

Figure 25: Summary table showing the responding rate during the fear expression session for the 0.4 mA group and diazepam subgroups (**A**), responding rate during the fear expression session for 0.8 mA group and diazepam subgroups (**B**). Additionally, graphical representation of suppression ratio at individual level for both groups and subgroups (**C**), and at group level as progression line (**D**). Whiskers represent the standard deviation. In **A** and **B**, squares, triangles and dots are mean values from the individuals.

At the statistical level, output of the ANOVA test uncovered a highly significant variance on the tone variable (*Repeated measure ANOVA, response rate as dependent variable against independent variables, $F = 66.048$, $p < 0.001$*) but otherwise no statistically significant variance in the other variables.

Subsequent Post Hoc test (*Newman-Keuls test, Probability for mistake between, within and pooled data, MSE = 129.20 , df = 71.773*) showed statistically significance for variation within subgroups: 0.3 and 1 mg kg^{-1} for group 0.4 mA ($p < 0.05$), 0 and 0.3 mg kg^{-1} for group 0.8 mA ($p < 0.05$).

Two -way ANOVA conducted with suppression ratio as a dependent variable produced no statistically significant variation between the datasets (*Two-Way ANOVA, variance in suppression ratio, F = 2.842; 2.004; 1.142 , p = 0.099; 0.147; 0.329*).

5. DISCUSSION

5.1 Summary of results

For the observational fear experiment, rats increased the production of corticosterone after the experimental procedures of fear acquisition and fear testing (t1 & t3), but within 24 hours from an experimental procedure, corticosterone measurements would drop (t2) to a similar level to the baseline (t0).

In terms of behavioural scoring, rats exhibited multiple behaviours during the different recorded phases. Both demonstrators and observers exhibited freezing behaviour during the acoustic tones play. In the observers, the response was more variable, and they got divided in two cluster for high fear and low fear. Freezing was exhibited in greater percentages by observers in the one minute after the tone's execution. In fear testing, the three-minute inter-tone time had continuous freezing instances by demonstrators and both fear clusters of observers.

When looking at all the analysed behaviours, rats exhibited mainly exploratory behaviours (sniffing/rearing) before (baseline) and during (tones) the fear acquisition phase. With the tone, vigilance behaviour increased for the observers and demonstrators, with freezing becoming the main behaviours for the demonstrators. In the fear testing situation, the output was similar in terms of expressed behaviours. Additionally, animals exhibited higher freezing after the end of the acoustic tone, including the control observers.

For the operant fear experiment, both male and female rats experienced a drop in operant performance during the acoustic tone play. Said drop was more important for rats that had received a higher voltage shock (0.8 mA). The injection of different amounts of diazepam had a visible effect on the suppression ratio in both female and male rats, of both shock intensity groups.

5.2 Discussion over observational fear

Overall, the animals subjected to the observational fear experiment exhibited freezing behaviours as a response to acquiring the fear stimulus vicariously, confirming the validity of the procedure. In comparison to previous datasets from the same laboratory, the time between fear acquisition and fear testing was delayed from one week to one month. This proved to be a

correct choice, as the freezing scores increased drastically, showing a longer period for fear learning to be acquired by the animal's brain.

Moreover, the inclusion of freezing scores in the time after the acoustic tone showed how the subjects might act in a predictive way towards the conditioned stimulus: the greater amount of freezing happening after the end of the tone might be indicative of the animal's anticipatory behaviour toward the electric shock, recognising the order of "tone then shock".

Alternatively, with the inclusion of vigilant behaviours, it is possible to visualise the change in behaviours of the animal. During the tone, the animal does not need to freeze since the danger (shock) comes afterwards. Instead, the subjects tend to stay in a vigilant state (free-air whisking/head-scanning), waiting for the shock at the end. This vigilant position could be interpreted as a factor for fear, while not as extreme as the freezing behaviour.

Suppression ratio in an operant fear conditioning setting proved to be another valid unit to measure fear expression, although the risk of excluding animals can be higher due to the training requirements and the strict exclusion criterion. However, the use of diazepam injections validates the procedure when compared with different shock intensities, as a way of quantifying the fear response through more intense fear stimuli. The results from the testing session followed a trend, detectable in other operant fear procedures done by the lab and by others.

5.3 Discussion over fear and freezing

The evaluation of fear solemnly on freezing scores must be modified for the inclusion of other factors. While being the predominant behaviour in a defensive situation, freezing alone does not describe the full instance of fear. Fear can be influenced by many factors such as stress, but the two unit of measure (corticosterone concentrations and freezing percentages) do not correlate with each other. This lack of a correlation suggests that the physiological stress response of the animal is not caused by or related to fear.

The inclusion of other behaviours in the scoring helped understanding the dynamic relationships between said behaviours and freezing. The knowledge about the animal's movements and actions helped categorizing such behaviours and highlighted the power dynamics of the same behaviours within groups. Contrary to the previous experiments, where only freezing was considered for the experiment. On the same level as the inclusion of behaviours, the scoring of freezing after the tone's offset proved to be critically important in understanding the process of conditioning to a cue in the animal, potentially shifting the

importance of the scores from the tone duration to the time afterwards. Both these factors should be considered when working on a new experiment in observational fear.

5.4 Discussion over behavioural scoring

When analysing fear through behavioural scores (freezing), researchers tend to limit the timeframe for analysis. The limitation usually refers to the fear testing phase, where the subjects are confronted with the conditioned stimulus (the tone) that elicit the conditioned response (freezing).

This limitation is usually due to the amount of time consumed over the experimental procedures, with the additional task of scoring behaviours prolonging the time needed to obtain valid behavioural scores. Since the behaviour scores are never performed individually, to avoid personal biases, training is usually required before starting to score. The training consumes additional time, reducing the amount of scoring obtainable. Scorers need to agree on a scoring method before performing the task, which sometimes can create confusion and can possible slow down the whole process.

The possibility of implementing a faster method for behavioural scoring will be analysed in the near future. An AI face-recognition programme built to specifically analyse rats is in development. By letting a programme score behaviours, time lost in all previously mentioned steps could be saved to create more data through additional analyses. The current behavioural scores are part of a dataset used for the development of the software, through machine-learning. The software will adapt to the parameters already in use, developing a faster way of behavioural recognition. With the possibility to fasten up the behavioural research, openings for more molecular and neurological studies on observational fear could be created

6. ETHICAL AND SOCIAL CONSIDERATIONS

All the experimental procedures were grouped under the ethical permit number 16869-2022 ID4498 approved on 2022-11-03 by the regional body for animal research. The experiment presented moderate hazardous threats for the animals: some of them included the exposure to electric foot shock, intraperitoneal injections, tail blood sampling. The exposure to such procedures classified the experiment for moderate suffering, which requested the termination of all animals after the end of the practical procedure.

Animals were euthanised following the standard procedure for chemical euthanasia: animals are placed in a housing cage with just bedding, and isoflurane is administered until the animals are asleep, then the flow switches to carbon dioxide instead. Once believed euthanised, the

animals are removed from the housing cage to inspect for any signs of life. Subsequently, their diaphragm is broken through perforation, to confirm then animal's death.

The study used rats as preclinical model animals for understanding of observational fear process and the subsequent physiological and behavioural reactions. Similar studies are generally base grounds for targeted studies at molecular and neural level, which also requires model animals. What motivates these studies is usually the search for knowledge and development. In this specific case, unlike other studies performed on animals, the possibility to replace the animal with an artificial model is not possible. This due to the relatively little knowledge on the molecular mechanisms of fear and the considerable amount of discussion over the quantification of fear in a behavioural and physiological unit.

However, the procedures are refined and rediscussed continuously for improvements in the animal health and welfare. One example comes from the operant fear conditioning procedure, where the administration of alcohol was replaced by 0.2% saccharine, which was shown to be a valid alternative solution. The administration of saccharine did not affect the learning of the operant fear behaviour and there was no risk for addiction in the rats.

In terms of social revenues, the experimentation on observational fear and the development of molecular studies on it, could bring benefits in the forms of targeted areas of the brain for administration of fear. The amygdala has already been found responsible for regulating neural circuits for stress and fear, but new discoveries could increase the knowledge over how animals firstly and humans secondly process fear.

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