RESEARCH ARTICLE

Effects of stress or infection on rat behavior show robust reversals due to environmental disturbance [version 1; referees: 1 approved, 1 approved with reservations]

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v1 **First published:** 06 Dec 2017, **6**:2097 (doi: 10.12688/f1000research.13171.1)
Latest published: 16 Jan 2018, **6**:2097 (doi: 10.12688/f1000research.13171.2)

Abstract

Background: The behavior of animals is intricately linked to the environment; a relationship that is often studied in laboratory conditions by using environmental perturbations to study biological mechanisms underlying the behavioral change.

Methods: This study pertains to two such well-studied and well-replicated perturbations, i.e., stress-induced anxiogenesis and Toxoplasma-induced loss of innate fear. Here, we demonstrate that behavioral outcomes of these experimental manipulations are contingent upon the ambient quality of the wider environment where animal facilities are situated.

Results: During late 2014 and early 2015, a building construction project started adjacent to our animal facility. During this phase, we observed that maternal separation stress caused anxiolysis, rather than historically observed anxiogenesis, in laboratory rats. We also found that Toxoplasma infection caused an increase, rather than historically observed decrease, in innate aversion to predator odors in rats.

Conclusion: These observations suggest that effects of stress and Toxoplasma are dependent on variables in the environment that often go unreported in the published literature.

Open Peer Review

Referee Status: ✔ ?

	Invited Referees 1	2
REVISED version 2 published 16 Jan 2018		
version 1 published 06 Dec 2017	✔ report	? report

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Competing interests: No competing interests were disclosed.

How to cite this article: Abdulai-Saiku S, Hegde A, Vyas A and Mitra R. **Effects of stress or infection on rat behavior show robust reversals due to environmental disturbance [version 1; referees: 1 approved, 1 approved with reservations]** *F1000Research* 2017, **6**:2097 (doi: 10.12688/f1000research.13171.1)

Grant information: This work was financially supported by Ministry of Education, Singapore (grant RG136/15 and RG 46/12).
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

First published: 06 Dec 2017, **6**:2097 (doi: 10.12688/f1000research.13171.1)

Introduction

Multiple laboratories have reported that stress causes anxiogenesis in rats[1–4]. Similarly, well-replicated studies indicate that infection of rats with protozoan *Toxoplasma gondii* reduces innate aversion to predator odor[5–11]. This report describes our serendipitous observations that the direction for both behavioral changes is intricately dependent on the broader environment where animal facilities are situated.

The primary aim of our experiments was to study proximate mechanisms of anxiogenesis and innate aversion in rats. We used routine paradigms of maternal separation and *Toxoplasma gondii* infection that cause anxiogenesis and loss of innate aversion, respectively. However, construction of a building was initiated during the experiment adjacent to the animal holding facility. Results from this quasi-experimental change provided us with an unplanned opportunity to study the effects of change in environment on rat anxiety and defensive behaviors.

Methods

Animals

Adult male and female Wistar Han rats (7 to 8 weeks at the start of the experiments) were procured from InVivos, Singapore. Rats were housed in groups of two per cage (males and females were housed separately) with ad libitum access to food and water (24–26°C; 60–70% relative humidity; 12h light-dark cycle with lights on at 0700h). For all tests, animals were allocated to groups in a random manner. Experiments were conducted by SA-S and AHN who were blind to group allocations. Analysis was done by AV who was also blind to group allocations. All procedures were approved by the Institutional Animal Care and Use Committee of the Nanyang Technology University. All efforts were made to ameliorate any suffering of animals. None of our procedure involved induction of sustained pain requiring pharmacological interventions. Animals were observed daily to confirm lack of sickness related behaviors and weighed weekly. The behavior tests do not involve any use of shock or other painful stimuli. The dose of parasites used in this study does not result in weight loss or sickness behavior in this strain of rats.

At the end of all experiments, animals used in the Toxoplasma infection paradigm were sacrificed by decapitation and their brains were removed and flash frozen. In the case of the stress paradigm, animals were sacrificed by cardiac perfusion using cold phosphate buffered saline (PBS) followed by cold 4% paraformaldehyde.

Toxoplasma gondii infection and quantification of aversion to cat odor

Female rats were either injected with tachyzoites of type 2 Prugniaud strain of *Toxoplasma gondii* (5×10^6 tachyzoites in 500 µl phosphate buffered saline, *i.p.*;) or mock injected with the buffer alone between 2pm and 4pm. Parasites needed for the infection were maintained *in vitro* in human foreskin fibroblast cultures and were harvested using syringe lysis. Behavioral experiments were conducted seven weeks post-infection; a time-window consistent with chronic phase of the infection.

Aversion to cat odor was quantified in two different manners. For each run of the experiment, there was one control group and one *Toxoplasma*-infected group. Fifteen (15) animals were used in total for experiment 1 (8 control, 7 infected) and 19 animals were used in total for experiment 2 (10 control, 9 infected).

Aversion was first quantified in a rectangular arena with two opposite and identical arms (76 × 9 cm each), separated by a central part (9 × 9 cm in size; white Perspex). Animals were habituated to the arena for three consecutive days for 20 minutes each day. On the subsequent day, cat odors were presented in one bisect of the maze (1 ml each; bobcat urine from Maine Outdoor Solutions, USA). Animals were placed in the center of the maze and exploration time in both bisects of the arena was measured for 20 minutes. Trials were video recorded with offline analysis conducted using AnyMaze (Stoelting, USA). In this batch of animals, each received 500 µl of buffered saline intraperitoneally thirty minutes before the behavioral test.

Aversion to cat odor was also quantified in a circular arena that was arbitrally divided into four quadrants. Animals were habituated to the arena for three consecutive days for 20 minutes each day. On the subsequent day, cat odor, vanilla essence, water and the bedding from the animal's home cage were presented in each quadrant of the maze. Animals were placed in the center of the maze and exploration time in all quadrants of the arena was measured for 20 minutes. Trials were video recorded with offline analysis conducted using AnyMaze (Stoelting, USA).

Stress paradigm and quantification of anxiety

Eight week old breeders obtained from InVivos were allowed to acclimatize for at least 5 days before setting up breeding pairs (one male and one female per cage). Breeding cages were changed once a week as per normal, but with gentle handling of female, in case of pregnancy. Once pregnancy was certain (approx. 2 weeks), male was removed. 19 days after breeding pairs were set up (or if visually heavily pregnant), cages were checked daily for litters. Day of birth is assigned P0.

Maternal separation was used as the stress model (P2-P14, daily). 16 animals were used in total; 8 stressed, 8 unstressed. On each of these days, the dam was removed from the cage and placed in a new cage with unsoiled bedding. Pups were then retrieved into another cage with unsoiled bedding, transported to a separate room and put on a heating pad for three hours every morning. At the end of the separation period, pups and then dam were sequentially returned to the original soiled cage. Also, soiled bedding was changed on postnatal day 2, 9 and 14; by returning pups to a clean cage that had been supplemented with a scoop of soiled bedding and nesting material from the original cage. This practice was repeated on postnatal day 18 if the bedding was considered significantly soiled in case of large litter sizes. Pups were weaned on postnatal day 21. Anxiety was quantified when the male pups reached adulthood (7–8 weeks of age). Anxiety was measured using home cage emergence assay (adapted from 12) and elevated plus-maze[13].

In the home cage emergence assay, a rat placed in its home cage was transported to a well-lit room and habituated for five minutes. The cage was then left open by removal of the lid. The rat was offered a possibility of emerging from the home cage through a wire grid placed within the cage. The latency of emergence was recorded. Emergence was defined when all four limbs of the rat were placed on the grid. Trials were terminated at the emergence or at five minutes, whichever occurred earlier. Trials were video recorded and scored manually.

The elevated plus-maze consisted of a plus-shaped arena with two open (75 × 11cm, 1cm wall, 3–4 lux illumination) and two enclosed arms (75 × 11 cm, 26 cm wall). The arena was elevated to a height of 60 cm above the ground. The animal was placed at the center at the start of the trial. Exploration in open and enclosed arms was quantified for five minutes each.

All experiments for the stress paradigm were done using two groups of mice: stressed and unstressed.

Statistical analysis

The probability of type 1 error was calculated using unpaired two-tailed Student's t-test. The standardized effect size was calculated using Cohen's d[14]; with values above the magnitude of 0.8 interpreted as being of robust scale. Negative d values correspond to the comparisons where mean of experimental treatment was greater than that of respective controls. Mean inter-group difference was also calculated with 95% confidence intervals. Data is graphically presented as mean and standard error of the mean (SEM), along with individual values for each animal for each endpoint. Number of animals in each experimental group is noted in the figures. All statistical analysis was conducted using Graphpad Prism.

Results

Toxoplasma gondii infection increased aversion to cat odor

In the first set of animals, aversion to cat odor was quantified as percentage time in bisect containing cat odor relative to total trial duration. Rabbit odor was placed in the opposing bisect as a novel non-predator odor. Inter-group differences did not reach pre-determined threshold for statistical significance (Figure 1A; $t_{13} = 1.78$, $p = 0.098$). Despite the lack of sufficiently low type 1 error, the effect on mean was of robust magnitude (Cohen's $d = 0.949$; $\Delta = -11.61\%$ with 95% confidence intervals -25.68 to 2.46%). The maximum of animals from the infected group was below the median of the control animals. The robust effect size and the observation that infected mean was lower than controls in contrast to the multitude of published studies, led us to plan a further experiment to increase the statistical power.

In this second set of animals, aversion to cat odor was quantified in a circular arena congruent to the initial design of reported infection effects. One quadrant contained soiled bedding from home cage of the animal, serving as the home base for exploratory sorties. Cat odor and a novel vanilla odor were placed in two adjoining quadrants. The ratio of time spent in cat quadrant relative to sum time spent in both cat and novel odor quadrants was calculated (chance = 50%). Toxoplasma infection, in contrast to earlier observations in the similar design, reduced percentage time spent near cat urine (Figure 1B; $t_{17} = 2.70$, $p = 0.015$). The effect of infection on innate aversion was of robust magnitude (Cohen's $d = 1.239$; $\Delta = -16.46\%$ with 95% confidence intervals -29.31 to -3.61%). The maximum of animals from the infected group was again observed to be below the median of the control animals.

Serological examination confirmed that all animals in the infected groups sustained chronic infection with *Toxoplasma gondii*.

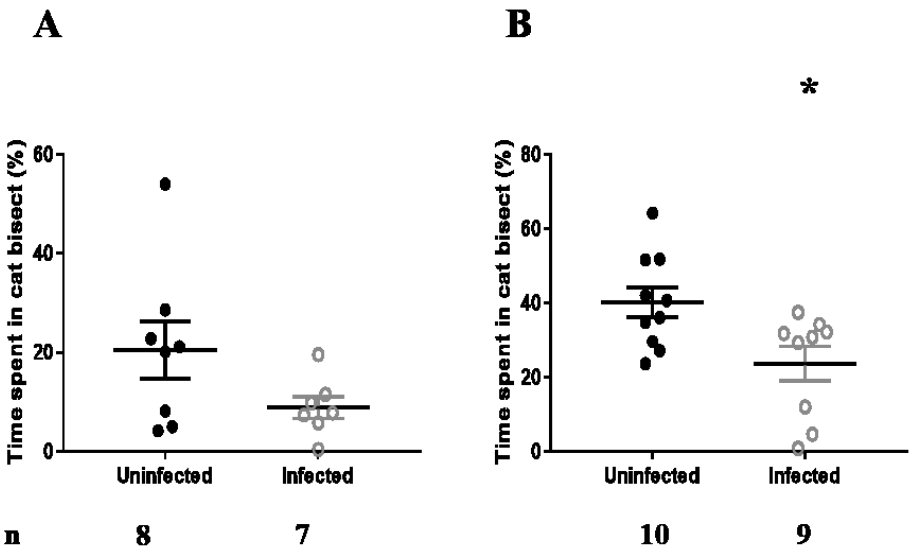

Figure 1. *Toxoplasma gondii*-infected female rats showed increased aversion to bobcat odor in two sequential experiments (**A** and **B**). Ordinate depicts time spent by female rats chronically infected with *Toxoplasma gondii* near bobcat odor. Line graphs depict mean and standard error of the mean for control (black) and infected (gray) female rats. *, $p < 0.05$; unpaired two-tailed Student's t-test.

Early life maternal separation stress resulted in anxiolytic behavior in male rats

Animals subjected to early life maternal separation stress were tested in the elevated plus maze and home cage emergence test to determine the effect of maternal separation on anxiety behavior during adulthood.

Stressed animals, in contrast to earlier observations in a similar design, exhibited significantly less anxiety compared to unstressed controls. This was evident as increased percentage entries into anxiogenic open arms of elevated plus-maze (Figure 2A; t_{14} = 4.21, p = 0.0009). Stress-induced anxiolysis was of robust magnitude (Cohen's d = -2.104; Δ = 27.77% with 95% confidence intervals 13.62 to 41.92%). The minimum of animals from the stressed group was higher than all but one animal from the unstressed group. Experimental treatment did not cause significant differences in number of entries made into non-anxiogenic enclosed arms of the maze (t_{14} = 1.98, p = 0.07). To preclude effects of entries in enclosed arms on open arm exploration, we further conducted a univariate analysis of variance for percentage open arm entries while employing number of enclosed entries as a covariate. This analysis revealed a significant increase in open arm exploration due to the stress independent of inter-group differences in enclosed arm entries ($F_{1,13}$ = 14.898, p = 0.002). This is congruent with significant increase in number of head dips made during the trial by stressed animals (t_{14} = 3.41, p = 0.0042; Δ = 13.25 with 95% confidence intervals 4.94 to 21.56).

Stress-induced anxiolysis was also confirmed by home cage emergence test. In this assay, anxiolysis manifests as reduced latency to emerge into a novel environment from home cage. Stress significantly decreased the latency of home cage emergence (Figure 2B; t_{14} = 3.14, p = 0.0072). Stress-induced anxiolysis was also of robust magnitude in this assay (Cohen's d = -1.57; Δ = -121.4s with 95% confidence intervals -204.2 to -38.5s). The maximum latency of animals from the stressed group was lower than median latency from the unstressed group.

Dataset 1. Cat odour avoidance assay

http://dx.doi.org/10.5256/f1000research.13171.d186327

Percentage time spent exploring the cat odour stimulus by control and *Toxoplasma*-infected rats in both experiment 1 and 2.

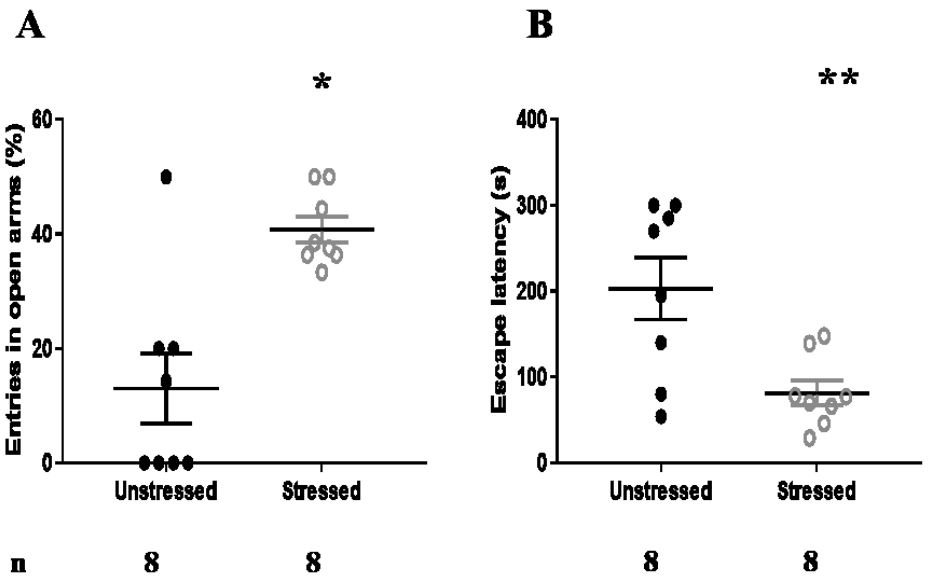

Figure 2. Early life maternal separation stress resulted in increased anxiolytic behavior in male rats. Ordinate depicts number of entries into the open arm relative to total entries in open and enclosed arms of the elevated plus maze (**A**) and latency to emerge from the home cage into a novel environment (**B**). Line graphs depict mean and standard error of the mean for unstressed (black) and stressed (gray) male rats. *, p < 0.05; **, p < 0.01; unpaired two-tailed Student's t-test.

Dataset 2. Elevated plus maze anxiety test

http://dx.doi.org/10.5256/f1000research.13171.d186328

Escape latency and percentage open arm entries for stressed and unstressed animals.

Discussion

Experimental treatment in the present report caused robust effects, as evidenced by substantial effect size and clear departure of mean differences from the chance. The direction of these effects is in stark contrast to those observed in previous reports[1,6–8,15–17]. For example, multiple experiments in several laboratories indicate that chronic *Toxoplasma gondii* infection causes loss of innate fear to predator odor in male and female rats[6–8,11]. Data in the present report, however, argue for a significant increase in innate fear post-infection. Similarly, stress-induced anxiogenesis has been reported across several laboratories and several paradigms. The current dataset, in contrast, exhibits significant anxiolysis post-stress. The cause of this discrepancy cannot be ascertained with confidence. In fact, we have observed stress-induced anxiogenesis and the infection-induced loss of fear in the same animal facility and same animal strain before these experiments[1,5–7,18]. The only difference between the experimental circumstances has been a construction project that was ongoing during the present experiments. The construction started across the road from the animal facility after our preceding baselines were conducted and during the present period of the behavioral testing. In fact, we observed reversal to *Toxoplasma*-induced loss of fear in female rats in experiments conducted in the animal facility after the cessation of building construction. It remains unclear if the effects of construction related to the change in ambient vibrational environment or some hitherto unknown variable. Although the acoustic noise in frequency range audible to humans remained unchanged during the period, we are not confident that the construction did not change the acoustic environment in sub-audible frequencies. It is interesting that the effects observed here do not correspond to a simplistic notion of greater baseline stress during the period. Effects of stress on anxiety are often presented to have an inverse U kind of reaction norm, whereby increasing stress enhances its effects on the behavioral and health parameters[19–22]. We observed an anxiolysis by experimental stress rather than greater anxiogenesis due to the accumulative stress of the treatment and environmental change. Thus, the present observations reiterate the often complex interactions between environment and behavior that could impose significant bounds on the interpretation of laboratory experiments. Related to this, same transgenic mice are known to exhibit divergent behavioral phenotypes across three experimental locations despite careful alignment of experimental protocols[23].

Conclusions

Often, unforeseen changes in the environment near animal facilities can significantly alter the direction of experimental effects in rodent research. This highlights the crucial role of often unreported and unquantified environmental context in the interpretation and replicability of the behavioral data.

Data availability

Dataset 1: Cat odour avoidance assay. Percentage time spent exploring the cat odour stimulus by control and *Toxoplasma*-infected rats in both experiment 1 and 2. 10.5256/f1000research.13171.d186327[24]

Dataset 2: Elevated plus maze anxiety test. Escape latency and percentage open arm entries for stressed and unstressed animals. 10.5256/f1000research.13171.d186328[25]

Competing interests

No competing interests were disclosed.

Grant information

This work was financially supported by Ministry of Education, Singapore (grant RG136/15 and RG 46/12).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Data Source

25. Abdulai-Saiku S, Hegde A, Vyas A, *et al.*: **Dataset 2 in: Effects of stress or infection on rat behavior show robust reversals due to environmental disturbance.** *F1000Research.* 2017.
Data Source

Open Peer Review

Current Referee Status: ✔ ?

Version 1

Referee Report 19 December 2017

doi:10.5256/f1000research.14287.r28761

? **Terence Y. Pang**
Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, Vic, Australia

This is a well-written, clearly presented manuscript describing unexpected behavioural phenotypes of two well-established rodent models which routinely lead to rats having anxiolytic or anxiogenic behaviours. In the field of behavioural neuroscience where robustness of results and reproducibility is vital, the reporting of negative or contrary outcomes remains important. This report raises substantial concern for the rodent research occurring across that time period. It is crucial that universities and research institutes be educated on the impact that infrastructure development has on researchers, and the time and financial costs it imposes on research teams/projects.

In the Introduction, which is rather short, it would be useful to include one or two paragraphs referencing evidence that both Toxoplasma infection and maternal separation models are prone to environmental modification. One of the included references (Koe et al., Transl Psychiatry 2016) is an example of environmental modification of a robust maternal separation-induced adult phenotype. See also Sahafi E et al., Physiol Behav 2018 as another example of an external modifier of anxiety behaviour.

This manuscript is limited in that there is no molecular data to be paired with the interesting behavioural phenotype. A comparison of monoamine-relevant genes ala Récamier-Carballo S et al., Behav Pharmacol 2017 would have been ideal. But this can be speculated upon in the Discussion. Could also mention the involvement of environment-induced epigenetic changes, see McCoy CR et al., Eur J Neurosci 2016 : DNA methylation changes in the hippocampus.

The major shortcoming of this manuscript is that I am unsure about how one would go about quantifying structural disturbances. Is it based solely on the unexpected behavioural phenotype observed? Or has the phenotypes consistently shifted during the stated period before returning to “normal”? Have there been anecdotal accounts of construction noise in the rodent facility? Is there any data about building vibrations? (Civil engineers would have the equipment to measure structural vibrations).

Assuming the significant external source of variability (as compared to a new experimenter who is a inexperienced at handling rodents and conducting the behavioural tests), it would be useful to include litter sizes and M/F sex ratios. Is there body weight data in the event that feeding behaviour was also altered?

It is unusual to only present EPM data as % entries in open arm. What about total time in open arms as a % of the test duration?

Is it possible to include schematics of the different test arenas for Figure 1? Do the authors have habituation data (total time spent moving, distance travelled) for odor aversion tests? If the infected rats are anxious, they could be observed to display non- or lesser habituation even at baseline in the absence of a predator odor. If this was the case, it would only serve to strengthen the interpretation.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: Rodent behaviour testing, anxiety, stress

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 27 Dec 2017

Rupshi Mitra, Nanyang Technological University, Singapore

We thank reviewer for suggestions and comments. This has helped us to improve this manuscript during the revision. We have now submitted version 2 of this manuscript to the F1000Research.

Introduction has been modified in version 2 to include prior work showing that effects of Toxoplasma infection and maternal separation are subject to environmental modification (references 12 through 16 in the bibliography).

We have also revised the discussion to include plausible proximate mechanisms including epigenetic changes and monoamines. Please see paragraphs immediately preceding the conclusions.

We now return to the reviewer's comment about ambivalent nature of quantifying structural disturbances. We have indeed observed return to stress-induced anxiogenesis and Toxoplasma-induced loss of fear once construction project abated. Toxoplasma effects were

eventually published (reference 7 in the revised bibliography; DOI: 10.1016/j.bbi.2017.04.005). Same set of experimenters conducted experiments before, during and after the construction project. Thus, congruent stress- and infection- effects before and after construction project suggest that the environmental modification brought about by the construction explains atypical effects in the interim.

Experimental groups were coded during the experiment. For example, in case of Toxoplasma infection, experimenter did not know infection status of the individual animals; and groups were merely identified with codes during the statistical analysis. Hence we did not notice the reversal until long after the experiment was over, data was analyzed and infection status was confirmed using serology. This precluded systematic investigation of the environmental variables during the period of experiment itself. Although the acoustic noise in frequency range audible to humans remained unchanged during the period, we are not confident that the construction did not change the acoustic environment in sub-audible frequencies. Similarly we did not have opportunity to measure structural vibrations as the project was finished while we were analyzing the data and confirming group assignments using serology.

Toxoplasma gondii infection did not cause significant change in body weight of animals (179.1 ± 4.708, n = 8 for control; 183.1 ± 2.706, n = 7 for infected; p = 0.5). This information is now included in methods section of the revised manuscript. We have revised the manuscript to include date for percentage open arm time ($p < 0.001$) for EPM in figure 2. We have also included schematics of test arena in revised Figure 1. Please note that this has changed panel number for figures in the results and legends.

Unfortunately, we did not record videos for habituation sessions. We have earlier shown that Toxoplasma infection does not affect locomotion or exploration in open field arena.

Competing Interests: No competing interests were disclosed.

Referee Report 11 December 2017

doi:10.5256/f1000research.14287.r28765

Jaroslav Flegr
Department of Biology, Charles University in Prague, Prague, Czech Republic

The authors present interesting data showing that, most likely, an unknown environmental factor or factors can qualitatively modify the behavioral responses of experimental animals on various standard stimuli (here the maternal separation stress and the *Toxoplasma* infection), which could result in unexpected results of standard experiments. The methods are clear and with sufficient details described, and the collected data are analyzed, presented and interpreted in a proper way. The authors suggested that the most probable factor that influenced the outputs of their experiments was (acoustical or mechanical) disturbance from a building construction project that had started adjacent to their animal facility during their experiments. This explanation seems to be reasonable, however, it would be a little bit difficult (and expensive) to test its validity.

I consider the results (and conclusions) of this study to be not only very interesting, but also very important. It is highly probable that the same or similar phenomena are frequently seen by many

researchers, however, they are mostly considered to be just the results of some technical error – “This new student/technician is really terrible, he certainly confused the labels on the cages/test tubes!”. We can just hope that the publication of the present paper will have the “#MeToo effect” – that it will encourage other researchers to publish their own puzzling results.

I agree that the inverted-U shape (or U-shape) relations between many physiological variables is mostly responsible for frequently observed opposite reaction of a biological system on the same stimuli. Under one situation (e.g., when no building construction project is going on) the background level of stress is low and adding some stress factor, e.g., infecting animals with *Toxoplasma*, will shift the behavioral response toward the maximum of the inverted-U function. Under another situation, when the background level of stress is higher (when the building construction project is going on) additional stress (e.g., the infection with *Toxoplasma*) will shift the behavioral response behind the maximum of the inverted-U function, which will result in an opposite behavioral reaction on the infection. In the review article on the methodological problems of studying the effects of toxoplasmosis using *Toxoplasma*-human model (Flegr, 2013), I showed that on genetically polymorphic outbred animals, including humans, the same factor often influences some individuals in one way and another individuals in an opposite way, depending on their (unknown) genotype. Very often, we can see that population means of the output variable in the affected individual and in the controls remains the same; however, variance of the output variable in the population of affected individuals grows significantly. For example, comparison of Cattell’s 16PF personality profiles of women showed that infected women had higher intelligence and lower guilt proneness than *Toxoplasma*-free women. At the same time, they differed in the variance of four other personality factors, namely protension, surgency, shrewdness and self-sentiment integration (Flegr and Havlíček, 1999). It is therefore very important to study the effects of particular factors not only on population mean of the output variable but also on the variance of this variable. We should never forget that the F-test (or a permutation test performed on squared Z- scores) is not just a pesky technique for testing presumptions of parametric statistical tests, but often it can also be an important and powerful tool for detecting biologically relevant effects of the factor under study.

Exactly the same mechanism can explain why males and females so often react to the same factor in an opposite way. In most animal species, males and females are not same. Therefore, many physiological parameters of males and females differ in their (mean) position in relation to the maximum of the inverted-U function. Consequently, they will respond to the same factor by the opposite-direction shifts. For example, 10 of 16 Cattell’s personality factors are shifted in an opposite direction in men and women in reaction to the *Toxoplasma* infection (Flegr *et al.*, 2000; Flegr *et al.*, 1996). Similarly, *Toxoplasma* -infected men rate the smell of highly diluted cat urine as more attractive while infected women rate this smell as less attractive than their non-infected peers (Flegr *et al.*, 2011). It is worthwhile in the context of the present Abdyla-Saiku *et al.* article to mention that our recent study showed the very opposite pattern, namely higher attractiveness of the smell in the infected women and lower in the infected men, when undiluted cat urine was used as the stimulus (Flegr *et al.* 2017).

Back to the present article. It can be published in its present form. I would just suggest that the authors cite the old study (Vyas *et al.*, 2007) showing the inverted-U shaped response of infected rats on the smell of cat urine. When describing their experimental setup, the authors should better emphasize the fact that stressed mothers, not stressed pubs were used in all ethological tests. Authors should also double-check whether all Latin names of species and genera are printed in italic, both in the main text and in the References.

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: Evolutionary biology, evolutionary parasitology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 27 Dec 2017

Rupshi Mitra, Nanyang Technological University, Singapore

We thank reviewer for suggestions and comments. This has helped us to improve this manuscript during the revision. We have now submitted version 2 of this manuscript to the F1000Research.

In the version 2, we have included a discussion of non-monotonic response of Toxoplasma in the introduction. We would also like to clarify that we tested stressed pups not their mothers. Pups

were maternally deprived before weaning, allowed to reach adulthood and then tested.

This has now been made clear during the revision.

We have carefully checked and corrected all Latin names.

Competing Interests: No competing interests were disclosed.