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Adult bacterial exposure increases behavioral variation and drives higher repeatability in field crickets

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Abstract

Among-individual differences in behavior are now a widely studied research-focus within the field of behavioral ecology. Furthermore, elements of an animal's internal state, such as energy or fat reserves, and infection status can have large impacts on behaviors. Despite this, we still know little regarding how state may affect behavioral variation. Recent exposure to pathogens may have a particularly large impact on behavioral expression given that it likely activates costly immune pathways, potentially forcing organism to make behavioral tradeoffs. In this study we investigate how recent exposure to a common bacterial pathogen, *Serratia marcescens*, affects both the mean behavioral expression and the among-individual differences (i.e. variation) in boldness behavior in the field cricket, *Gryllus integer*. We find that recent pathogen exposure does not affect mean behavioral expression of the treatment groups, but instead affects behavioral variation and repeatability. Specifically, bacterial exposure drove large among-individual variation, resulting in high levels of repeatability in some aspects of boldness (willingness to emerge into a novel environment), but not others (latency to become active in novel environment), compared to non-infected crickets. Interestingly, sham injection resulted in a universal lack of among-individual differences. Our results highlight the sensitivity of among-individual variance and repeatability estimates to ecological and environmental factors that individuals face throughout their lives.

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INTRODUCTION

Although among-individual differences in behavior (aka animal personalities) are widely studied topic in the field of behavioral ecology, there is comparatively little research on how changes in the internal state of an organism affects individuality. The existence of among-individual differences in a population or group of individuals is quantified as significant among-individual variance in focal trait and is often expressed in its standardized form: among-individual variation relative to the total variation, i.e. repeatability (Dingemanse and Dochtermann 2013). Several studies have considered how factors such as body condition affect the expression of among-individual differences (David et al. 2012; DiRienzo and Montiglio 2016), yet few have considered how immune activation and recent pathogen exposure in adults influences among-individual differences in behavior. Given the far-reaching evolutionary and ecological importance of individual variation (Sih et al. 2012; Wolf and Weissing 2012), and that most organisms are exposed to pathogens throughout their life, it is critical to understand how factors such as pathogens can modify the expression of behavioral repeatability.

It has been proposed that the relative costs of both investing in immune function and expressing behavior could promote individual differences in behavior (Kortet et al. 2010). Such an outcome could occur through two different mechanisms. Tradeoffs between the two traits may drive individuals who invest in immune function to express less costly behaviors, and those who express costly behaviors (e.g. aggression, activity, antipredator behavior) to invest less in immune function (Rigby and Jokela 2000; Kortet et al. 2007). Alternatively, positive feedbacks between immune function and behavior may result in individuals who invest in immune function being able to better express potentially costly behaviors (e.g. activity, aggression) as they are in turn better able to cope with the increased pathogen encounter rates (i.e. costs) associated with the expression of these behaviors (McElreath et al. 2007; Kortet et al. 2010; Luttbeg and Sih 2010). Several studies have attempted to explore this relationship through a developmental approach (Butler et al. 2012; DiRienzo et al. 2015). These studies have shown that exposure to pathogens or immune challenges during the juvenile stage can impact both mean behavioral expression and repeatability later in adult stage. Yet, these studies have not emphasized how recent exposure after reaching sexual maturity affects repeatability estimates.

To date, only one study has experimentally manipulated adult infection status to assess its associated impact on behavioral variation. Kekäläinen et al. (2014) found that previously uninfected Eurasian minnows (*Phoxinus phoxinus*) that are subsequently infected with a brain-encysted trematode parasite, *Diplostomum phoxini*, show higher levels of behavioral repeatability. Interestingly, infection status did not influence the mean level of behavioral expression (Kekäläinen et al. 2014). Several other studies have looked at wild-caught organisms that varied in infection status upon capture. In amphipods (*Paracalliope fluviatilis*), individuals infected with with *Microphallus sp.* expressed lower behavioral repeatability (Coats et al. 2010), but did not show differences in their mean behavioral response relative to uninfected amphipods. Alternatively, in periwinkle snails (*Littorina littorea*), those infected by the trematode *Cryptocotyle lingua* showed changes in mean behavior but not behavioral repeatability (Seaman and Briffa 2015). Collectively, it is

unclear why infections by some pathogens/parasites drive changes in among-individual but not mean behavioral expression, or vice-versa. Studies mentioned above have all focused on multicellular parasites that affect the brain. Behavioral manipulation of a host by pathogens might differ qualitatively depending on whether pathogens are exploiting the host as part of an intermediate stage of the parasite's life cycle or instead are exploiting host resources.

Field crickets are an ideal model system for the study of both behavioral variation and immune function. Multiple studies have shown that field crickets show individual differences in boldness behavior, such that some individuals are more willing to take risks than others (Niemelä et al. 2012a; Niemelä et al. 2012b). Individual differences have also been shown in other behavioral traits related to risk taking such as flight initiation distance (Niemelä et al. 2015) and activity level (Fisher et al. 2015). Crickets, and other insects, have a number of independent aspects of immune function, including phenoloxidase enzymes, lysozomes, and an encapsulation responses (Rantala and Kortet 2003; Adamo 2004). Furthermore, in crickets aspects of immune function are known to be energetically costly. Encapsulation rate, for example, has been shown to be negatively related to adult body size and development time, suggesting a tradeoff between immune function and juvenile growth (Rantala and Roff 2005). Cricket immune function is also related a male's success in a fight, suggesting that immune function is related to individual differences in aggression (Rantala and Kortet 2004). Additionally, in populations with high parasite risk, shy behavioral types show a greater encapsulation response compared to bold types (Kortet et al. 2007).

In this study we investigated the effect of recent pathogen exposure on adult boldness behavior. Three experimental groups of adult field crickets (*Gryllus integer*) were subjected to either a low-dose bacterial injection of a common pathogen *Serratia marcescens*, a sham injection (control 1), or no injection (control 2). After experimental manipulation, boldness behavior was assessed twice, allowing decomposition of total phenotypic variation into its among-individual component and estimation of repeatabilities. We predicted that if all individuals have similar immunocompetence towards opportunistic pathogens, then there should be a reduction in mean boldness behavior but little change in among-individual variation. Yet, if individuals vary in their immune function such that bolder individuals have greater immunocompetence relative to less-bold individuals, then mean behavior will decrease, but among-individual variation will increase. Alternatively, if shy individuals have greater immunocompetence relative to bold individuals, then the mean behavior will decrease and among-individual variation will decrease.

MATERIAL AND METHODS

Study Animals

This study was conducted from November, 2011 through March, 2012 at the University of Oulu, Finland. We used the field cricket, *Gryllus integer,* from a laboratory population (approximately 8-9th generation) that was founded by individuals from a wild population (Davis, California, USA). The population periodically received additional wild-caught crickets in order to avoid potential inbreeding and increase genetic diversity. Crickets were maintained at the Experimental Unit of the University of Oulu. As rearing density can affect the development of cricket behaviors (Niemelä et al. 2012c), at the start of the experiment,

we sorted 238 nymphs (~ 1 week old) from the laboratory population into individual plastic containers (length 128 × width 98 × height 73 mm) and assigned each container with a unique identification number. Crickets were held at a 12:12h light:dark cycle at 27°C +/-1°C, and provided *ad libitum* food (reindeer pellets, Rehuraisio OY, poron herkku) and water. Nymphal body mass ranged from 0.0004 to 0.0010g (n = 10). Due to their small size, we did not weigh any experimental nymphs in order to avoid possible damage from handling.

Treatment groups

A total of three treatment groups were created: (1) bacterial injection, (2) sham injection, (3) no injection (control). Crickets from treatments groups 1 and 2 were injected with a Hamilton microsyringe between the 3rd and 4th segment of the abdomen five days after their adult molt. Group one received a 5μl injection of a 10^{-4} dilution of a 24-hour culture of the opportunistic bacterial pathogen *Serratia marcescens*. *S. marcescens* is a gram negative broad-spectrum opportunist pathogen that commonly infects plants, nematodes, insects, fish, and mammals (Grimont and Grimont 1978). Pilot data revealed that injections at stronger concentrations (10^{-3}) were lethal (see also Kortet et al. 2012). The sham injection group (control 1) received a 5μl injection of sterile nutrient broth. The control group (control 2) received no injection. A new bacteria culture was created for each day in which crickets were injected. Bacterial growth was evident each day given the change in color in the growth medium. Individual body mass in all treatment groups was also taken at this same time point. All crickets were checked if they experienced their adult molt three times a week on Monday, Wednesday, and Friday. A total of 204 adult crickets were used in the three treatment groups (bacterial injection n = 63, sham injection n = 68, control n = 73). The control treatment group was also used in an separate study that was conducted concurrently investigating the developmental effects of bacterial exposure (DiRienzo et al. 2015). All crickets were euthanized 48 hours after the behavioral trials were completed.

Boldness trials

Individual boldness was quantified using a novel environment test, which is an established method for assessing boldness in field crickets (Hedrick 2000; Hedrick and Kortet 2006; Kortet and Hedrick 2007; DiRienzo et al. 2013). Boldness in these crickets was previously shown to be repeatable (Niemelä et al. 2012b; DiRienzo et al. 2015). Seven days after their adult molt (two days after injection), individual crickets were placed inside a semi-opaque vial within an unfamiliar arena (19 × 19 × 11 cm). The vial was placed in a vertical position to prevent the cricket from exiting. After a 2 minute acclimation period the vial was gently placed in the horizontal position allowing the possibility for a cricket to exit. We recorded three measures of boldness: the latency for the cricket to become active after being placed in the horizontal position, the latency for the cricket's head to emerge, and the latency for the cricket to fully emerge from the vial. Low values of these measures indicate high levels of boldness (i.e. willingness to expose oneself to risk in a novel environment). We conducted the behavioral trials 48 hours after injection to avoid any potential handling effects from influencing our behavioral trials. All trials were conducted in dark conditions with only dim red light. Cricket vision is poor in red light (Briscoe and Chittka 2001); thus this minimized external and observer influences while mimicking nocturnal conditions. The boldness trial

was repeated on the following day using the same procedures. The vial was cleaned with 70% isopropyl alcohol and the sand in the arena was changed after each trial. We did not use blinded methods since treatment number was associated with individual ID. In order to account for any potential bias, observer ID (n = 3) was included in the statistical models (see Statistical Analysis). All boldness trials were conducted between 9:30 and 15:00. Trial duration was limited to 20 minutes for logistical reasons.

Statistical analysis

The influence of treatment on boldness behavior was assessed using generalized linear mixed models. In the models we included each of the measured behavioral traits separately as the response variable with individual ID as a random effect and treatment, weight, sex, and observer as fixed effects. The log-transformed latency to become active was modeled with Gaussian errors, while whether the individual's head and full body emerged were modeled with binomial errors and logit links. Cricket weight was standardized to a mean of zero and standard deviation of one prior to fitting in the model. Treatment, observer, and sex were coded as factors. All models were estimated with the lme4 package for R (Bates et al. 2013).

The presence of significant repeatability (i.e. existence of "personality") within each treatment group was defined as the presence of significant among-individual variation. Adjusted repeatability scores were calculated following the methods described by Nakagawa & Schielzeth (Nakagawa and Schielzeth 2010). In sum, we fitted mixed models with one of the three behavioral measurements as the response variable and then individual ID as a random effect and body weight, sex, and experimental observer as fixed effects. These models were fit individually to each of the treatment groups. Latency to become active was modeled with Gaussian errors while whether the individual's head and full body emerged from the vial were both modeled with binomial error structure and logit link function. We calculated 95% confidence intervals through bootstrapping methods (n = 1000 samples) (Bates et al. 2013). We tested for significance of among-individual variance for each treatment by using likelihood ratio tests (over one degree of freedom, (Pinheiro and Bates 2000) comparing the model with the individual random effect to a model without it, while keeping the fixed effect structure constant). The statistical software package R version 3.1.1 (R Core Team 2014) was used for all the analyses.

RESULTS

Influence of bacterial treatment on mean behavior

Treatment had no influence on any of the mean behavioral responses. The latency for crickets to become active was affected by cricket weight such that the heavier the cricket was, the more shy it was, as indicated by the longer latency to become active (β= -0.240, SE = 0.079, P = 0.002). Full model outputs are included as supplementary material (S1T1).

Repeatability of boldness

Changes in among-individual variation drove different patterns of repeatability among the different treatments. Latency to become active within the vial was repeatable in the control

group (R (95% CI): 0.271 (0.049 - 0.473)) (Fig 1), and there was significant among-individual variation in the control group in if the cricket fully emerged from the vial (LRT = 4.463, p = 0.035), although the confidence interval of the repeatability estimate overlapped zero (0.340 (0.000 - 0.545)) (Table 1). Both measures of emergence (head and full body) were highly repeatable in the bacterial injection group (0.608 (0.110 - 0.854) & 0.614 (0.122 - 0.852)), but not in the sham injection group (0.037 (0.000 - 0.252) & 0.260 (0.000 - 0.467)) (Fig 1, Table 1). These patterns of emergence were driven by a large increase in among-individual variation for the bacterial injection treatment (head emerge Var_{ID} = 5.109; body emerge Var_{ID} = 5.234) relative to the control group (head emerge Var_{ID} = 0.679; body emerge Var_{ID} = 1.694) and sham injection group (head emerge Var_{ID} = 0.125; body emerge Var_{ID} = 1.156).

DISCUSSION

This study adds to a growing body of literature demonstrating that pathogen exposure may not necessarily affect the population mean behavioral phenotype, but instead influence the amount of among-individual variation (Coats et al. 2010; Kekäläinen et al. 2014; Seaman and Briffa 2015). Against our predictions, individuals did not, on average, down regulate their boldness. However, bacterial exposure dramatically increased individual differences: infected crickets displayed between 3.09 and 7.52 times greater among-individual variance relative to controls in both of the emergence measures causing increased repeatabilities in these behaviors. One potential explanation for this result is that individuals do indeed vary in their immunocompetence, and there is positive feedback between immunocompetence and boldness behavior. Individuals with greater immunocompetence towards pathogens could be easily maintaining, or even increasing the expression of behavior, while those with lower immunocompetence may have a more intense infection and down regulate their boldness (Poulin 2010). This would lead to fanning out of the individual variance, in turn increasing the repeatability estimates, without affecting the mean behavioral expression. Such a relationship is common in other contexts, such as sexual selection, where signal quality is typically positively correlated with individual condition (Kotiaho 2000; Holzer et al. 2003; Freeman-Gallant et al. 2010). In wolf spiders, for example, males who experience an increase in condition are able to produce higher courtship drumming rates relative to those whose condition decreased, and the courtship drumming is positively correlated with immune function (Mappes et al. 1996; Ahtiainen et al. 2006). Interestingly, we saw a lack of repeatability in the sham injected treatment. Such sham injections have been demonstrated to activate some aspects of the immune system in invertebrates (Márkus et al. 2005; Ardia et al. 2012). It is possible that bacterial injection vs. just puncturing the cuticle activates different components of the cricket immune system, in turn driving the different behavioral responses. Still, the mechanism that would create these differences in behavioral repeatability remains elusive. Importantly, it is known that certain pathogens can effect behaviors depending on their interactions with the host's immune system (e.g. Adamo et al. 2014).

Interestingly, we found that not all behaviors were affected equally by the treatments. Specifically, although exposure to bacteria appeared to drive significant among-individual differences in both emergence measures, the same treatment appeared to have an opposite

effect on the latency to become active. The lack of repeatability in this measure was driven by a combination of reduced among-individual variation (Var_{ID}) along with high levels of within-individual variation (Var_r). This suggests that overall, the individuals in a population became more similar in this behavioral measurement, yet individuals demonstrated more plasticity across the two trials. These results may be a byproduct of using terms such as boldness, which risk lumping fundamentally distinct behaviors into a one single general term (Réale et al. 2007; Briffa and Weiss 2010; Carter et al. 2013). In our case, the early boldness measure (latency to become active) and later measures (whether the cricket emerges) may represent fundamentally different behaviors, each of which is regulated by different physiological, neurological, or cognitive, processes, thus the difference in repeatability between the measures within the same treatment. Indeed, many studies in field crickets show differences in repeatability in different aspects of what is called boldness (Niemelä et al. 2012a; DiRienzo et al. 2015). Furthermore, many studies investigating the personality-immune relationship show similar differential effects of parasite infection on more fundamentally distinct behavioral categories (e.g. activity vs. boldness) (Kekäläinen et al. 2014; Horváth et al. 2016). Our results suggest that the immune-personality relationship is highly complex, and that a single experience (e.g. bacterial exposure) can have very different effects on what we lump into being functionally similar behaviors.

Terminal reproductive investment would be an alternative explanation for the detected results if different individuals adopted different strategies (i.e. only some exhibited terminal reproductive investment), and this would have potentially increased the among-individual variation in the population. One may also argue that immune exposure to pathogens and the associated altered immune function impacts behavior for reasons other than traditional trade-offs. For example, changes in behavior may be part of a host response to increase the chance of recovery (e.g. behavioral fever (Louis et al. 1986; Adamo 1998), and it may not be an energetic trade off, *per se*. The detected changes in 'bold' behavioral variation after immune challenge may be due to differences in anti-predator behavior that are potentially caused by oxidative stress (e.g. Janssens and Stoks 2014). Therefore, we feel that more studies are needed to fully understand possible causality pathways, as these mechanistic details remain largely unknown in invertebrates.

One fundamental ecological and evolutionary question is the temporal nature of the plastic responses of variance components and repeatability to environmental factors. Many behavioral traits have been shown to be plastic in response to experiences during development (Sinn et al. 2008; Butler et al. 2012; DiRienzo et al. 2012; Niemelä et al. 2012a; DiRienzo et al. 2015; Liedtke et al. 2015; DiRienzo and Montiglio 2016). Developmental plasticity is largely assumed to be an adaptive, and mostly irreversible, response where an organism experiences an environmental cue during a sensitive period and adjusts its traits accordingly for the future (West-Eberhard 2003; Groothuis and Trillmich 2011). If individuals differ in these permanent environmental effects, they may generate individual differences in behaviors that are irreversible, i.e. true personality. Yet, it is mostly unknown whether similar cues during adulthood can have equally lasting effects on behavioral variation. Given the short-term duration of this study, it is unknown if the observed changes in variance are permanent, or if the amount of among-individual variation will return back to their "original expression" once all individuals have recovered from the

potential infection. If the latter is the case, then our observations could be an example of "pseudo-personality," or personality that is induced by reversible environmental variation, and not true individual differences induced by genetic or permanent environmental effects (Martin and Réale 2008; Westneat et al. 2011; Niemelä et al. 2015). Thus, any fitness effects associated with a change in behavioral variation would be short term. Alternatively, if these effects are lasting even after the infection has cleared, then exposure to such pathogens could indirectly have long term effects to ecological and evolutionary processes within the host population (Sih et al. 2012; Wolf and Weissing 2012). Collectively, longer-term studies are needed to study both how pathogen exposure affects behavioral variation but also the long-term effects of such exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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SIGNIFICANCE STATEMENT

Animals are known to express consistent among-individual differences in behavior, also known as animal personalities, such that some individuals are always more bold, aggressive, or active relative to others. Yet, it is relatively unknown how factors such as energy reserves and exposure to pathogens impact these differences in behavior. Here we investigated how exposure to a pathogenic bacteria as adults affects both the mean behavioral expression as well as among-individual differences. Our results show that pathogen exposure results in large levels of among-individual differences in some aspects of boldness behavior (willingness to expose oneself to risk), but not others (latency to become active in a novel environment). These results highlight the sensitivity of behavioral differences to elements of state, such as infection status.
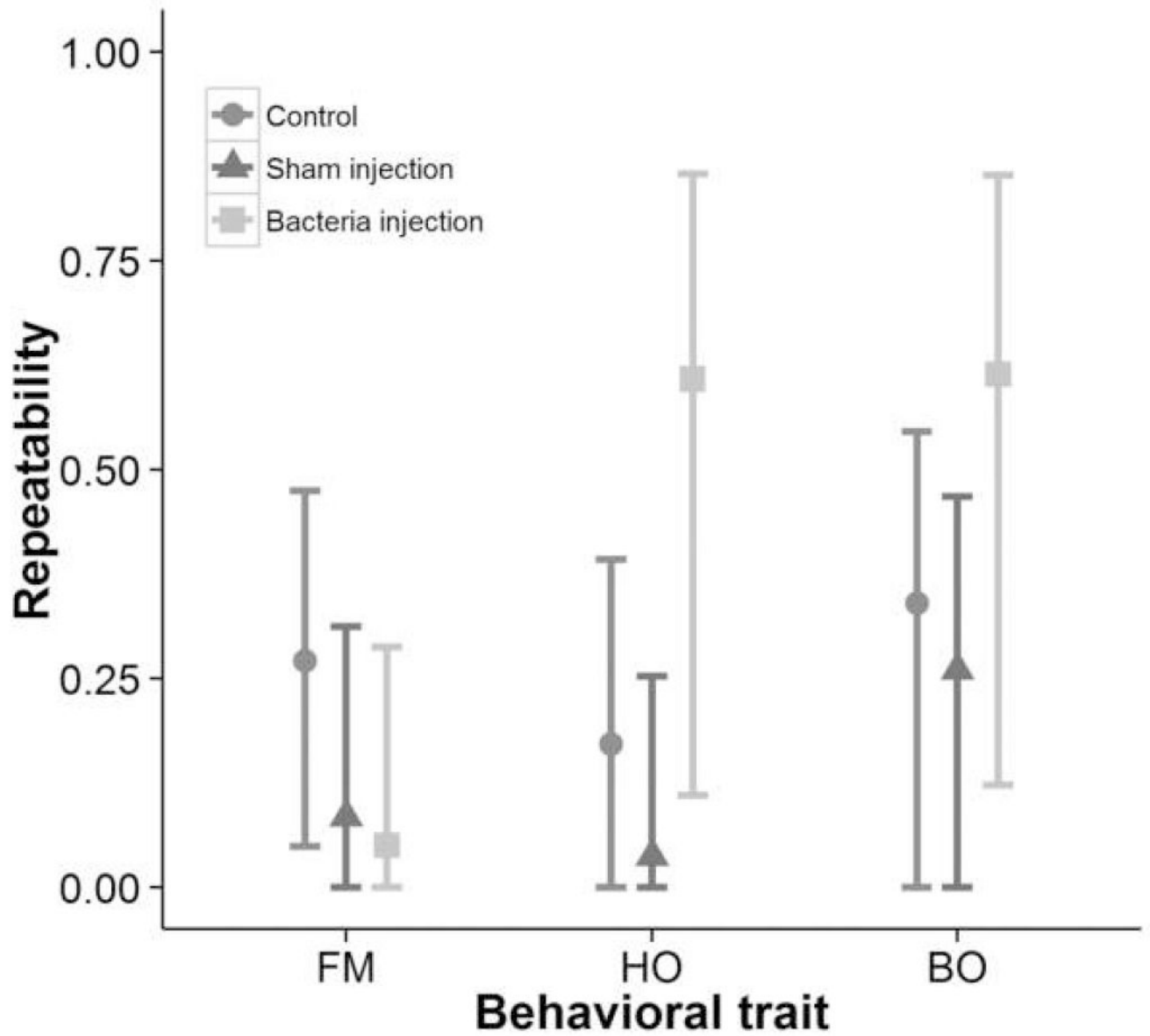
Fig 1.
Repeatability of the three treatment groups across the three behavioral measurements (FM: first movement, HO: head out, BO: body out). Error bars indicate 95% confidence intervals

Table 1

Individual and residual variance (Var_{ID} and Var_R), adjusted repeatabilities (R adj.), 95% confidence intervals (95% CI), and likelihood ratios (LRT) within the three treatments for all measured behavioral traits.

First Movement	Var_{ID}	Var_{ID} 95% CI	Var_R	Var_R 95% CI	R adj	95% CI	LRT	P
Control	0.477	0.077-0.932	1.284	0.900-1.711	0.271	0.049-0.473	13.717	< 0.001
Sham injection	0.182	0.000-0.741	1.983	1.395-2.589	0.084	0.000-0.312	-11.505	1
Bacteria injection	0.117	0.000-0.726	2.207	1.501-2.842	0.050	0.000-0.297	-4.884	1

Head Out	Var_{ID}	Var_{ID} 95% CI	Var_{Resid}	Var_{Resid} 95% CI	R adj	95% CI	LRT	P
Control	0.679	0.000-2.123	-	-	0.171	0.000-0.392	1.458	0.227
Sham injection	0.125	0.000-1.106	-	-	0.037	0.000-0.252	0.087	0.768
Bacteria injection	5.109	0.408-19.272	-	-	0.608	0.110-0.854	19.220	< 0.001

Body out	Var_{ID}	Var_{ID} 95% CI	Var_{Resid}	Var_{Resid} 95% CI	R adj	95% CI	LRT	P
Control	1.694	0.000-3.994	-	-	0.340	0.000-0.545	4.463	0.035
Sham injection	1.156	0.000-2.819	-	-	0.260	0.000-0.467	3.10	0.08
Bacteria injection	5.234	0.456-18.923	-	-	0.614	0.122-0.852	11.156	< 0.001