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Research Report

Natural compulsive-like behaviour in the deer mouse *(Peromyscus maniculatus bairdii)* is associated with altered gut microbiota composition

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Abstract

Obsessive-compulsive disorder (OCD) is a psychiatric illness that significantly impacts affected patients, and available treatments yield suboptimal therapeutic response. Recently, the role of the gut-brain axis (GBA) in psychiatric illness has emerged as a potential target for therapeutic exploration. However, studies concerning the role of the GBA in OCD are limited. To investigate whether a naturally occurring obsessive-compulsive-like phenotype in a rodent model, i.e. large nest-building in deer mice, is associated with perturbations in the gut microbiome, we investigated and characterized the gut microbiota in specific pathogen free bred and housed large (LNB) and normal (NNB) nest building deer mice of both sexes (n = 11 per group, including 3 males and 8 females). Following baseline characterisation of nest building behaviour, a single faecal sample was collected from each animal and the gut microbiota analysed. Our results reveal the overall microbial composition of LNB animals to be distinctly different compared to controls (PERMANOVA $p < 0.05$). While no genera were found to be significantly differentially abundant after correcting for multiple comparisons, the normal phenotype showed a higher loading of *Prevotella* and *Anaeroplasma*, while the OC-phenotype demonstrated a higher loading of *Desulfovermiculus, Aestuariispira, Peptococcus* and *Holdemanella* (cut-off threshold for loading at 0.2 in either the first or second component of the PCA). These findings not only provide proof-of-concept for continued investigation of the GBA in OCD, but also highlight a potential underlying etiological association between alterations in the gut microbiota and the natural development of obsessive-compulsive-like behaviours.

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1 Introduction

Obsessive compulsive disorder (OCD) is a multidimensional psychiatric disorder that is characterised by intrusive and persistent thoughts or ideas, i.e. obsessions and/or ritualistic behaviours (compulsions) that are often expressed in an attempt to mitigate the level of distress caused by the obsessive thoughts (APA, 2013; Abramowitz & Jacoby, 2015; Wu & Lewin, 2017). The condition is phenotypically heterogeneous and symptoms usually cluster along the basis of specific themes, e.g. fears of contamination and cleaning rituals, fears of harm and checking compulsions, a need for symmetry and just-right feelings associated with ordering compulsions, as well as covert inappropriate thoughts relating to, among others sexual misconduct, religion, and violence (Williams *et al.*, 2013; Abramovitch & Cooperman, 2015).

Chronic high-dose selective serotonin reuptake inhibitors (SSRIs) are currently recommended as first line pharmacotherapy for OCD (Albert *et al.*, 2018), while increasing the dose of the SSRI used or SSRI-antipsychotic augmentation strategies are all clinically employed in the treatment of SSRI-refractory OCD (Dold *et al.*, 2015). Nevertheless, a significant number of patients respond poorly to these options and other available pharmacotherapeutic interventions (Albert *et al.*, 2018). Therefore, a better understanding of the etiology and pathophysiological processes underlying OCD is needed to develop more effective treatment options.

During the past decade, the gut-brain axis (GBA) and its involvement in psychiatric disease has gained significant interest (Mayer *et al.*, 2014; Dinan & Cryan, 2017; Bastiaanssen *et al.*, 2018). Communication between the gut and the brain takes place on a number of functional levels, including via neural, neuroendocrine and immunological signalling (Foster & Neufeld, 2013; Cussotto *et al.*, 2018). Indeed, the gut microbiota is regarded as one of the major immunomodulatory influences in the human body (Zhao & Elson, 2018). While the exact etiological associations between psychiatric illness and the gut microbiota are still not fully understood, altered immune responses may be instrumental in the pathogenesis of brain disorders, including OCD (da Rocha *et al.*, 2008; Turna *et al.*, 2016). In light of this, several investigations into microbiota manipulation in animal models of psychiatric illness have been conducted, including models of depression (Kelly *et al.*, 2016a), anxiety (Crumeyrolle-Arias *et al.*, 2014), and OCD (Kantak *et al.*, 2014). However, the exact translational value of these findings remains to be established (Kelly *et al.*, 2016b).

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Regarded collectively, natural compulsive-like behavioural phenotypes expressed by deer mice *(Peromyscus maniculatus bairdii)*, i.e. large nest building (LNB) and high stereotypy, provide a well-validated naturalistic pre-clinical framework in which to study the etiopathology of OCD (Scheepers *et al.*, 2018). Approximately 30% of laboratory housed deer mice of both sexes express LNB (Wolmarans *et al.*, 2016). LNB manifests by the age of 8 weeks and is persistent and repetitive over the course of several trials. From a teleonomic perspective (Thornhill, 1996), LNB can be regarded as a maladaptation in a specific component of the normal behavioural repertoire of deer mice. Indeed, considering that mice build nests for the purposes of safety and protection, temperature regulation and to provide adequate nurseries for offspring (Jirkof, 2014), excessively large nests expressed in the laboratory serve no unique purpose. From an evolutionary perspective, nesting size and quality play a major role in mate choice and reproductive success in some species, e.g. birds (Holveck & Riebel, 2009), fish (Jamieson, 1995), and frogs (Felton *et al.*, 2006). However, this is not true for mice, which generally exercise mate choice by random or on the basis of amongst others, dominance, probability of genetic success, overall health, or patterns of ultrasonic vocalization (for a detailed review, see Latham and Mason (2004)). That both male and female deer mice engage in LNB, also excludes the likelihood of sex-related differences in nesting phenotype. Therefore, it is likely that excessive nest-building is expressed at the cost of other functions for which effort, time and energy is required, and may thus be regarded as a naturalistic maladaptation (Crespi, 2000).

We have previously shown that LNB is responsive to chronic high-dose (50 mg/kg/day) oral treatment with the SSRI, escitalopram (Wolmarans *et al.*, 2016). This is in line with the therapeutic, albeit varying, effect of SSRI intervention observed in the majority of patients with OCD (Fineberg *et al.*, 2006). Moreover, normal nest building (NNB) and LNB can be separated based on the underlying involvement of serotonin in its expression, as NNB remains wholly unaltered following such intervention, with nests neither decreasing or increasing in size (Wolmarans *et al.*, 2016). Such difference in treatment response also imply that it is not the behavioural act of nesting per se, but rather the aberrant cognitive processes that underlie such behaviour, that are modified by serotonergic intervention.

Considering the literature reviewed here and the fact that LNB spontaneously manifest in a sub-population of deer mice only, the present work aimed to investigate whether an association between such behaviour and natural modifications in the gut microbiota may exist. In fact,

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bearing in mind that theories pertaining to the involvement of the gut-brain axis in psychiatric illness point to developmental relationships (Borre *et al.*, 2014), the study of parallel, but equally intrinsic and non-induced changes in both behaviour and the gut microbiome may provide valuable insight into our understanding of neurodevelopmental psychiatric conditions.

2 Materials and Methods

2.1 Animals

Deer mice of both sexes were obtained from the deer mouse colony of the North-West University (NWU), Potchefstroom, South Africa (ethical approval number: NWU-00284-17-S5; AnimCare Research Ethics Committee, National Health Research Ethics Committees Registration Number: AREC-130913-015). The original breeding pairs were established using animals obtained from the *Peromyscus* Genetic Stock Centre at the University of South Carolina, USA. Since only 30% of deer mice express LNB behaviour (Wolmarans *et al.*, 2016), 40 deer mice (age 12 weeks) were screened to identify 11 (3 male and 8 female) deer mice of each nesting cohort, i.e. NNB and LNB. In line with the minimum recommendations set by the ARRIVE-guidelines (Kilkenny *et al.*, 2010), and considering an extensive review of recent literature (Cani *et al.*, 2008; Chang *et al.*, 2015; Desbonnet *et al.*, 2015; Luczynski *et al.*, 2016; Sampson *et al.*, 2016), group sizes of 3 – 8 animals are deemed sufficiently powerful in pre-clinical microbiome investigations.

Animals were bred according to a standard out-bred protocol (Bateson, 1983) and housed and maintained in the specific-pathogen-free (SPF) area of the Vivarium at the North-West University, Potchefstroom, South Africa. The initial breeding pairs—used to breed the 40 mice screened in this investigation—were randomly allocated without prior knowledge of their nest building profiles. After weaning, offspring were housed in same-sex groups (4 – 6 animals per cage) in individually ventilated cages [(35cm (l) x 20cm (w) x 13cm (h); Techniplast® S.P.A., Varese, Italy], until one week prior to the onset of the first nest building analysis. From this point onwards, each animal was allocated to its own cage, while all experimental analyses were conducted in these same cages throughout the investigation. Animals were kept on a 12-hour light/dark cycle (06h00/18h00), at a temperature of 22 ± 1°C and relative humidity of 55 ± 5%. Food and water were provided ad lib. All mice received food from the same batch of pelleted rodent chow throughout the study. Cages were cleaned and new bedding material, consistently taken from the same batch of ground corncob, added once a week on the same day.

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2.2 Nest building analysis

Nest building behaviour was quantified as described previously (Wolmarans *et al.*, 2016). In short, nesting behaviour was assessed in each animal for 7 consecutive 24-hour periods. An excess of pre-weighed, sterile, hospital-grade, non-scented cotton wool was introduced in the roof of each home cage every day between 15h00 and 16h00. As mice generally build their nests just before dawn (Jirkof, 2014), the remaining cotton wool was only removed and weighed between 13h00 and 14h00 on the following day. Each day, built nests were removed, discarded and additional pre-weighed cotton wool supplied. Animals did not have access to any other form of nesting material, and food and water were supplied as normal. Daily nesting scores were expressed in grams of cotton wool used with a cumulative nesting score, describing nesting size and not quality per se, determined after one week (Wolmarans *et al.*, 2016). As nest building is a natural behaviour expressed by all rodents (Smithers, 1983), only animals that consistently built large nests over the course of 7 days were included in the LNB cohort. This was determined by plotting the total nesting scores against the coefficients of variance with respect to daily nesting behaviour, where LNB was defined as nesting behaviour that clustered within, or as near to, the upper quarter of the nesting score distribution, while demonstrating the lowest degree of variance (**Figure 1**). Likewise, NNB animals were identified as those individuals that built the smallest nests consistently over the course of 7 days—for a full review of this methodology, please refer to Wolmarans *et al.* (2016). A clear separation of the LNB and NNB cohorts (**Figure 2i – 2iv**) is important as the question asked is whether the compulsive-like phenotype is associated with unique perturbations in the gut microbiota. As such, for a control, we selected a group of mice that not only built smaller nests than the LNB group, but also did so with the lowest possible degree of variability (**Figure 1**). That said, while not a single animal refrained from engaging in nesting behaviour (**Figure 1**), NNB animals expressed nest building behaviour in a more adaptable manner (as indicated by the higher degree of between-day variance compared to LNB animals; **Figure 1**). These characteristics in nesting phenotype, point to a clear separation between the two cohorts, as LNB animals not only express the highest nesting scores, they do so without significant between-day variation. In other words, the motivational drive to engage in LNB is not only observed during a few nights of the 7-day assessment period, but across most of the trials (**Figure 1**), pointing to a OCD-like phenotype akin to behavioural inflexibility (Gillan *et al.*, 2011)**.**

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2.3 DNA analyses

2.3.1 Sample collection and DNA extraction

Fresh faecal samples were collected during the first hour of the dark (wake) cycle with a sterilized tweezer, transferred to 1.5ml Eppendorf® Safe-Lock tubes and immediately flash frozen in liquid nitrogen (Hong *et al.*, 2010). Samples were kept frozen at -80°C until the extraction of DNA. The QIAamp® PowerFecal® DNA kit (QIAGEN, Valencia, CA, USA) was used to extract the microbial DNA from faecal samples (0.25g/sample). DNA extraction was performed as per the manufacturer's instructions to ensure maximal cell lysis of bacterial cell wall components. The Thermo-Scientific® NanoDrop One Microvolume UV-Vis Spectrophotometer was used to assess the quality and quantity of the extracted microbial DNA.

2.3.2 DNA sequencing

Paired-end sequencing of the V3 to V4 hypervariable regions of the 16S rRNA was performed by Macrogen® Inc. (South-Korea) using the FWD 5'-CCTACGGGNGGCWGCAG-3' and REV 5'-GACTACHVGGGTATCTAATCC-3' primer pair on a MiSeq (Illumina) platform. The V3 to V4 hypervariable region was used as high inter-taxon variability of these regions can be used to distinguish between closely related bacteria. Library preparation was performed as per the 16S Metagenomic Sequencing Library, Preparation Part #15044223, Rev. B protocol, except when using the Hercules II Fusion DNA Polymerase (Agilent, Santa Clara, USA), the Nextera Index Kit V2 (Illumina, San Diego, USA) and the V3-V4 primers. Indexed adapter-ligated fragments were pooled and then gel purified and PCR amplified.

2.4 Statistical analysis

First, quality control of raw *fastq* sequencing files was performed using *fastqc* and *multiqc* programs respectively (Ewels *et al.*, 2016). Second, a Divisive Amplicon Denoising Algorithm (DADA) 2 (version 1.8) (Callahan *et al.*, 2016) in R studio (R version 3.4.3; R-studio version 1.1.456) (Gandrud, 2016) was used for the construction of an amplicon sequence variant (ASV) table (Callahan *et al.*, 2016). The DADA 2 workflow consisted of the following steps: inspecting the read quality profiles, filtering and trimming low-quality reads, identifying error rates, dereplication (eliminating redundant comparisons), sample inference, merging paired reads, constructing an amplicon sequence variant (ASV) table, removing chimera's and assigning taxonomy. Taxonomy was assigned using the Ribosomal Database Project (RDP) as a reference

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database (Cole *et al.*, 2005). The *vegan* package in R was used to evaluate β-diversities. For PCA, Aitchison distance was calculated using the *ALDEx2* library (Fernandes *et al.*, 2014; Gloor *et al.*, 2017). The Shannon, Simpson, Chao1, Observed and Fisher diversity indices were used to evaluate α-diversity. Filtering was performed to only include taxa that were observed more than once in at least 15% of the animals. Centred log-ratio transformed (clr) relative abundance for each ASV was also determined using *ALDEx2*. To test for statistically significant differences in the relative abundance of ASVs between the gut microbial composition of NNB and LNB animals, we used permutational multivariate analysis of variance (PERMANOVA; *vegan* package). The Mann-Whitney U test was used to compare alpha diversity metrics, a p-value of < 0.05 was deemed significant in all cases.

3 Results

To test for differences between the gut microbial composition of NNB and LNB animals (3 male and 8 female animals per group, respectively), a total of 22 faecal samples were analysed, each from the same time point in the sleep cycle. All samples passed quality control (QC) with a minimum read count threshold of 10 000 and median read depth of 34 686 reads per sample. From this, 86 genera were detected.

The Mann-Whitney U test revealed no differences in α-diversity between NNB and LNB animals, as measured by Chao 1, Shannon, Simpson, Observed and Fisher distance matrices, respectively. However, β- diversity using Aitchison distance at the genus level, revealed a clear clustering of NNB and LNB cohorts (**Figure 3**). PC1 and PC2 accounted for 13.77% and 10.91% of the variance observed respectively, and PERMANOVA revealed this distinction to be statistically significant ($p < 0.05$). In this regard, two clusters, having a 20% loading in either PC1 or PC2 (Camacho *et al.*, 2010) were observed that associated with the control (driven by the prevalence of *Prevotella* and *Anaeroplasma*), and the OC-phenotype (driven by the prevalence of *Desulfovermiculus, Aestuariispira, Peptococcus* and *Holdemanella*), respectively.

4 Discussion

The major findings of the present work are that 1) there is a significant difference in the overall gut microbiota composition of NNB and LNB animals, and 2) such difference is driven by the

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prevalence of *Prevotella* / *Anaeroplasma*, and *Desulfovermiculus / Aestuariispira / Peptococcus* / *Holdemanella,* respectively.

The neurobiological and pathophysiological processes underlying OCD are not yet fully elucidated, with current treatment options also yielding suboptimal results (Atmaca, 2016). During the past decade, our understanding of the GBA has expanded significantly (Sherwin *et al.*, 2018), having been shown to play a role in the pathophysiology of a number of psychiatric illnesses, including anxiety and depression (Foster & Neufeld, 2013). In terms of OCD, very little research has been conducted to elucidate if and how the gut microbiota may be associated with the condition (Kantak *et al.*, 2014; Turna *et al.*, 2016). Further, although some clinical results have been reported that may be indicative of the potential therapeutic value of microbiotic modification in the treatment of central nervous system disorders (Messaoudi *et al.*, 2011), it remains difficult to translate these findings to clinical studies (Kelly *et al.*, 2016b).

In this investigation we interrogated possible associations between a *naturally* developing compulsive-like phenotype, i.e. LNB in the deer mouse (Wolmarans *et al.*, 2016), and alterations in the gut microbiota. Our finding that the community composition of the gut microbiota in LNB animals is significantly different from that in the NNB cohort is noteworthy. Taking into account that LNB transpires naturally over the course of development and given that animals included in this investigation have been randomly selected without litter bias and housed individually, the differences observed in microbial composition parallel the differences observed in behavioural expression; this association is therefore likely naturalistic. Our finding that a clustering of *Prevotella* and *Anaeroplasma* was driving the compositional ordination in NNB compared to LNB animals, is noteworthy. Interestingly, while the human gut microbiota demonstrates significant biogeographical stratification (Donaldson *et al.*, 2016), most organisms cluster within the phyla *Firmicutes* and *Bacteroidetes*, which includes the genus *Prevotella* (Albenberg & Wu, 2014; Marchesi *et al.*, 2016). Further, both *Prevotella* (Shenker *et al.*, 1991) and *Anaeroplasma* (Beller et al., 2019) have been associated with significant anti-inflammatory properties, while children diagnosed with autism, a condition also characterized by persistent behavioural phenotypes, have been shown to present with lower gut *Prevotella* abundance (Kang *et al.*, 2013).

In terms of the gut microbiota composition of LNB animals, members of the phylum Proteobacteria, of which *Aestuariispira* is an example, have been proposed as microbial signatures

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of among others, inflammatory conditions (Rizzatti *et al.*, 2017). Further, hydrogen sulphide releasing bacteria, of which *Desulfovermiculus* (Loubinoux *et al.*, 2002), and *Peptococcus* (Bourgault & Rosenblatt, 1979; Van Eldere et al., 1988) are examples, have been associated with gut mucosal injury and inflammatory pathology (Loubinoux *et al.*, 2002). Interestingly, *Peptococcus,* has also been implicated in other models of adult neurodevelopmental aberrancies following exposure to prenatal stress (Golubeva *et al.*, 2015). Therefore, considering that LNB develops spontaneously over time, and that this phenotype is associated with a lower loading of *Prevotella* and *Anaeroplasma*, it is possible that the composition of the microbiota in LNB animals reported here, can exert a gut-to-brain neuroimmune-associated etiological influence on the expression of compulsive-like nest building in the deer mouse (Heijtz *et al.*, 2011; Furtado & Katzman, 2015; Turna *et al.*, 2016). This possibility should be elaborated in future investigation.

5 Conclusion

The data presented here indicates for the first time in a pre-clinical model that *naturally* developing OC-like behaviour is associated with inherent differences in the gut microbial composition, compared to that in normal controls. Future investigations into a possible causal role of the gut microbiota in the etiology of compulsive phenotypes are warranted. Specifically, the relationship between obsessive-compulsive behaviour, stress, and immune alterations on the one hand, and adaptations in the microbiota of normal and compulsive-like animals on the other, needs further elucidation. Further, using gnotobiotic mice and other means of microbiota modification, e.g. antibiotic treatment, it would be of value to characterize the behavioural response in LNB deer mice under circumstances of microbiota alterations. Such studies will potentially contribute to a better understanding of the neurobiology underlying OCD and may ultimately lead to the development of better treatment.

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Conflict of Interest Statement

None declared.

Author contributions

All authors contributed equally to this work.

Data Accessibility Statement

Data not presented in this manuscript (archived by DWW, SMM and TFB) will be made available on the basis of reasonable request.

Abbreviations

OCD: obsessive-compulsive disorder; GBA: gut-brain axis; LNB: large nest building; NNB: normal nest building; SSRI: selective serotonin reuptake inhibitor; NWU: North-West University, South Africa; SPF: specific pathogen-free area; DNA: deoxyribonucleic acid; DADA: divisive amplicon denoising algorithm; ASV: amplicon sequence variant; RDP: Ribosomal Database Project

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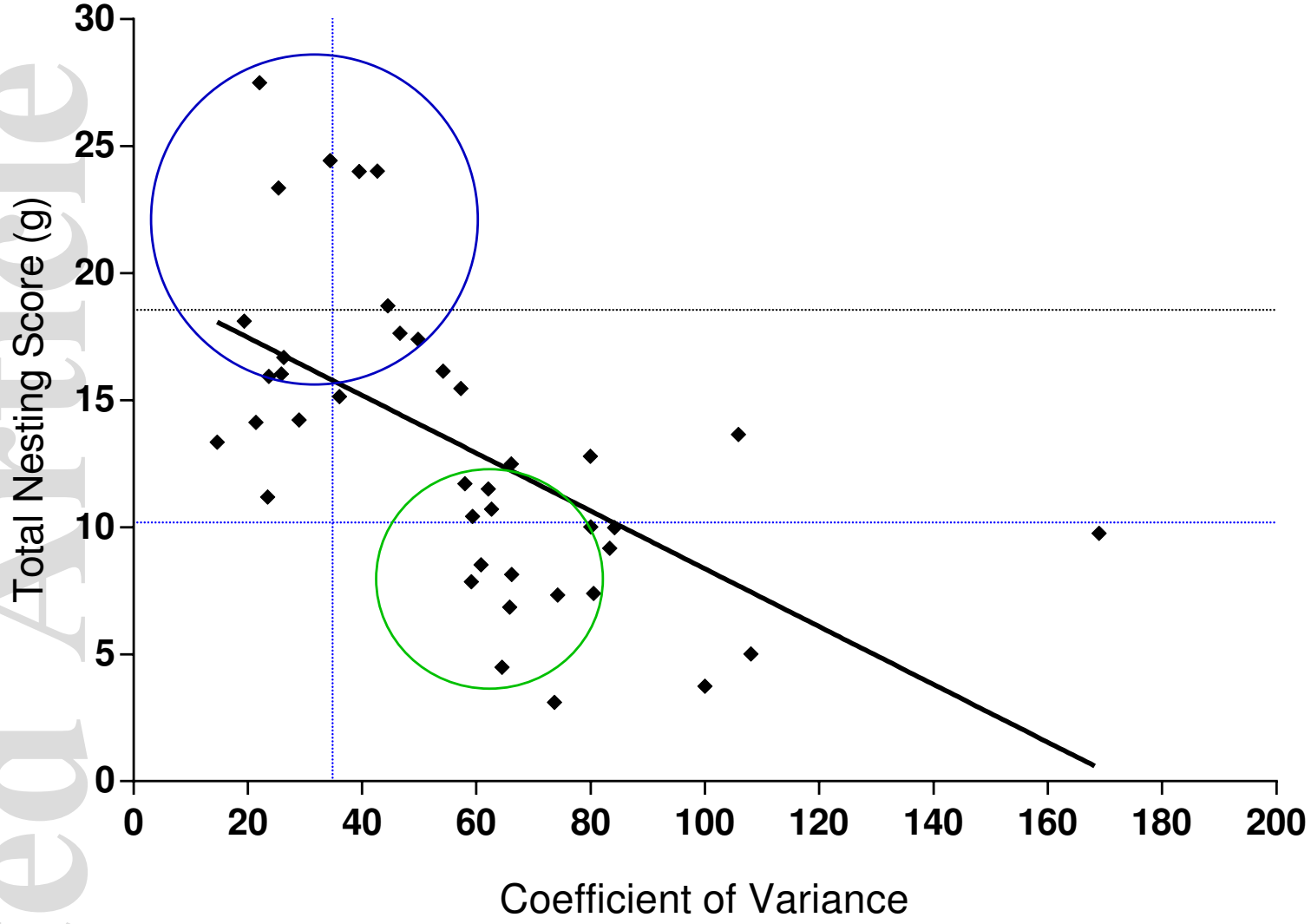
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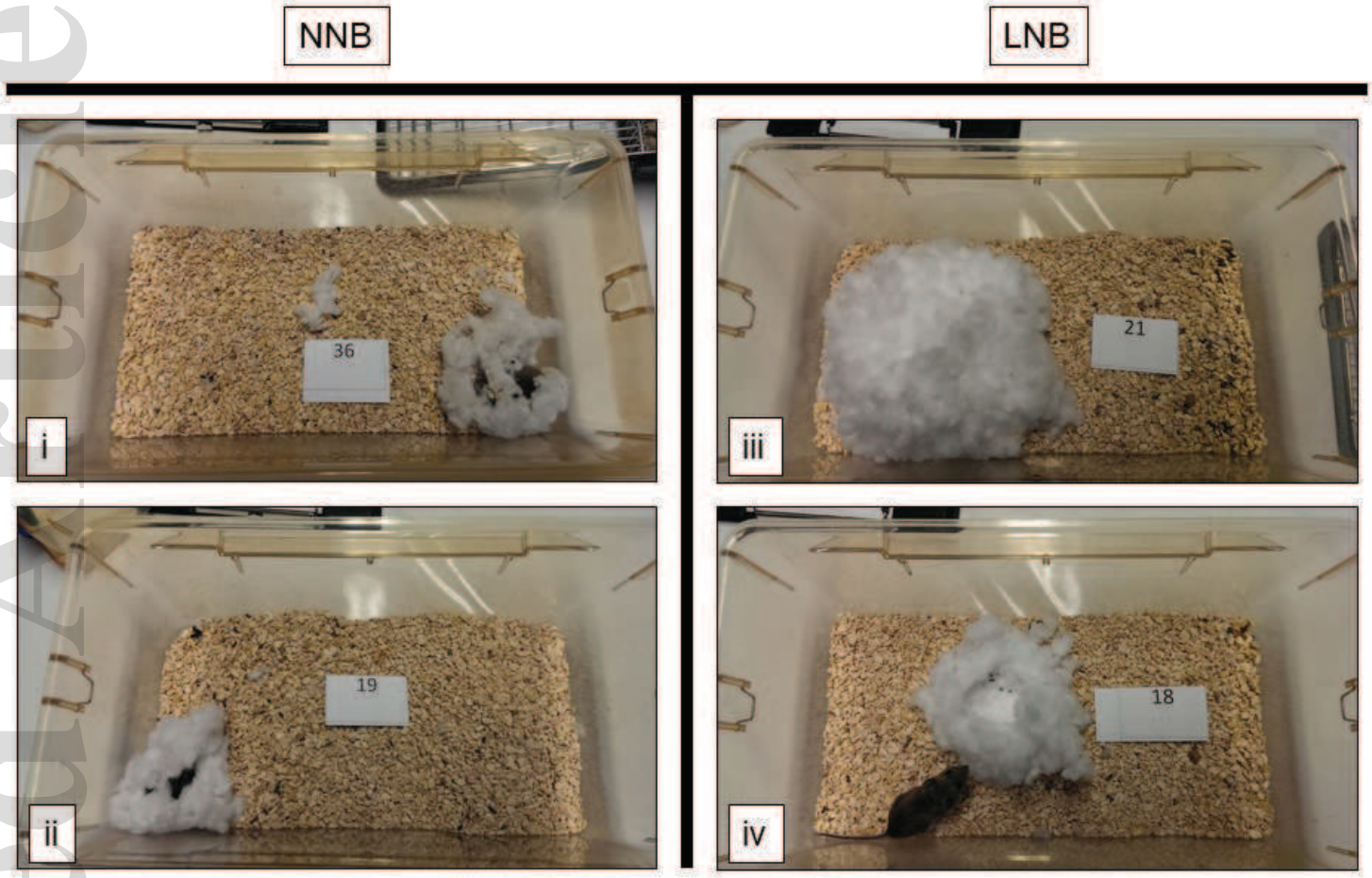
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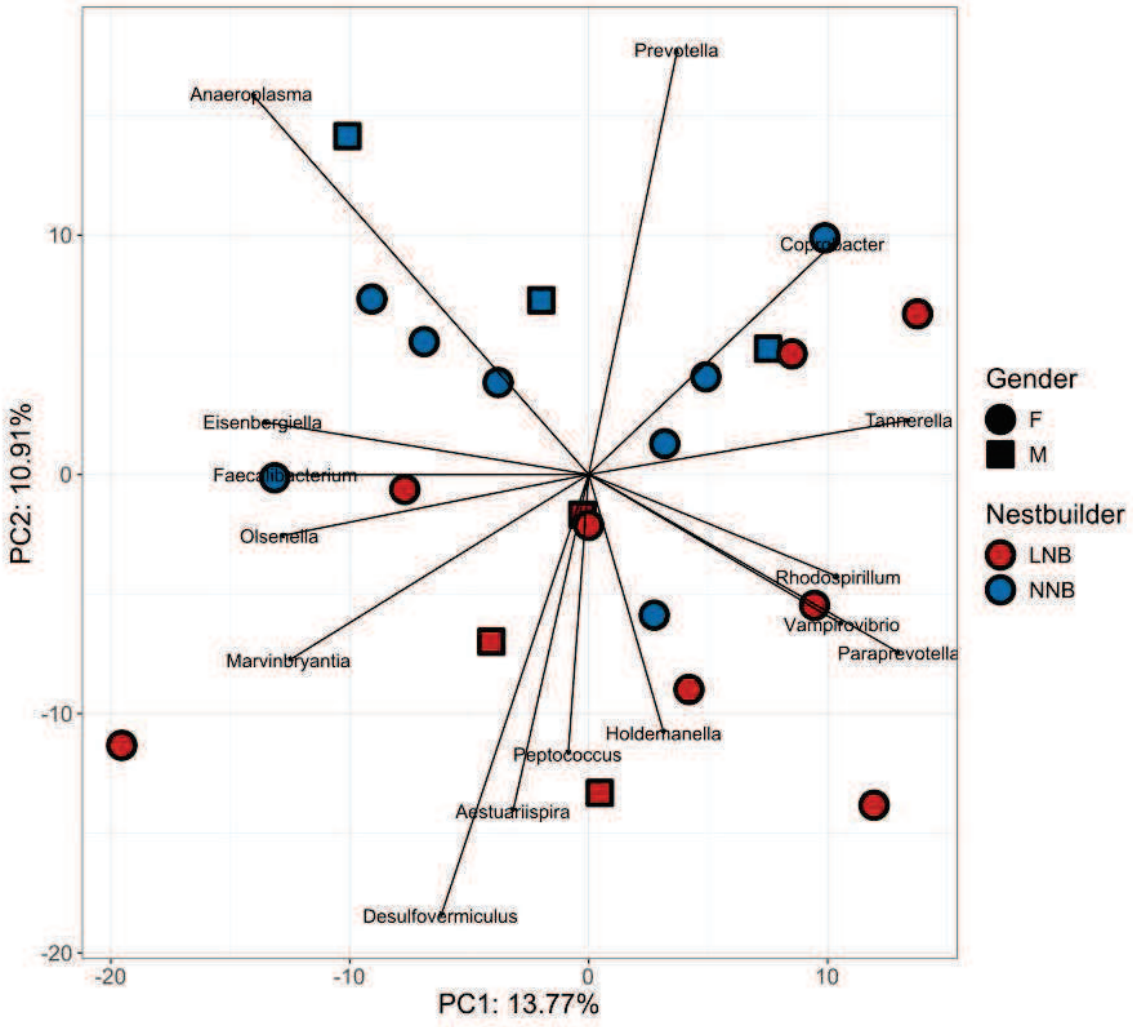


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