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# Automated phenotyping of social behaviour in mice : applications to autism spectrum disorders

Oscar Bauer

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# Université de Paris

École doctorale Frontières de l'Innovation en Recherche et Éducation (ED n°474)

*Laboratoire de génétique humaine et fonctions cognitives de l'Institut Pasteur*

## Automated phenotyping of social behaviour in mice

*Applications to autism spectrum disorders*

Oscar Bauer

Thèse de doctorat de neurosciences et troubles neuronaux

Dirigée par Elodie Ey

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Devant un jury composé de :

Sylvie Granon, PR, Université Paris-Saclay, rapporteur

Raphaël Marée, PhD, Université de Liège, rapporteur

Barbara König, PR, University of Zurich, examinatrice

Xavier Montagutelli, DR, Université Paris-Saclay, examinateur

Gilles Gheusi, MCF, Université Sorbonne Paris Nord, examinateur

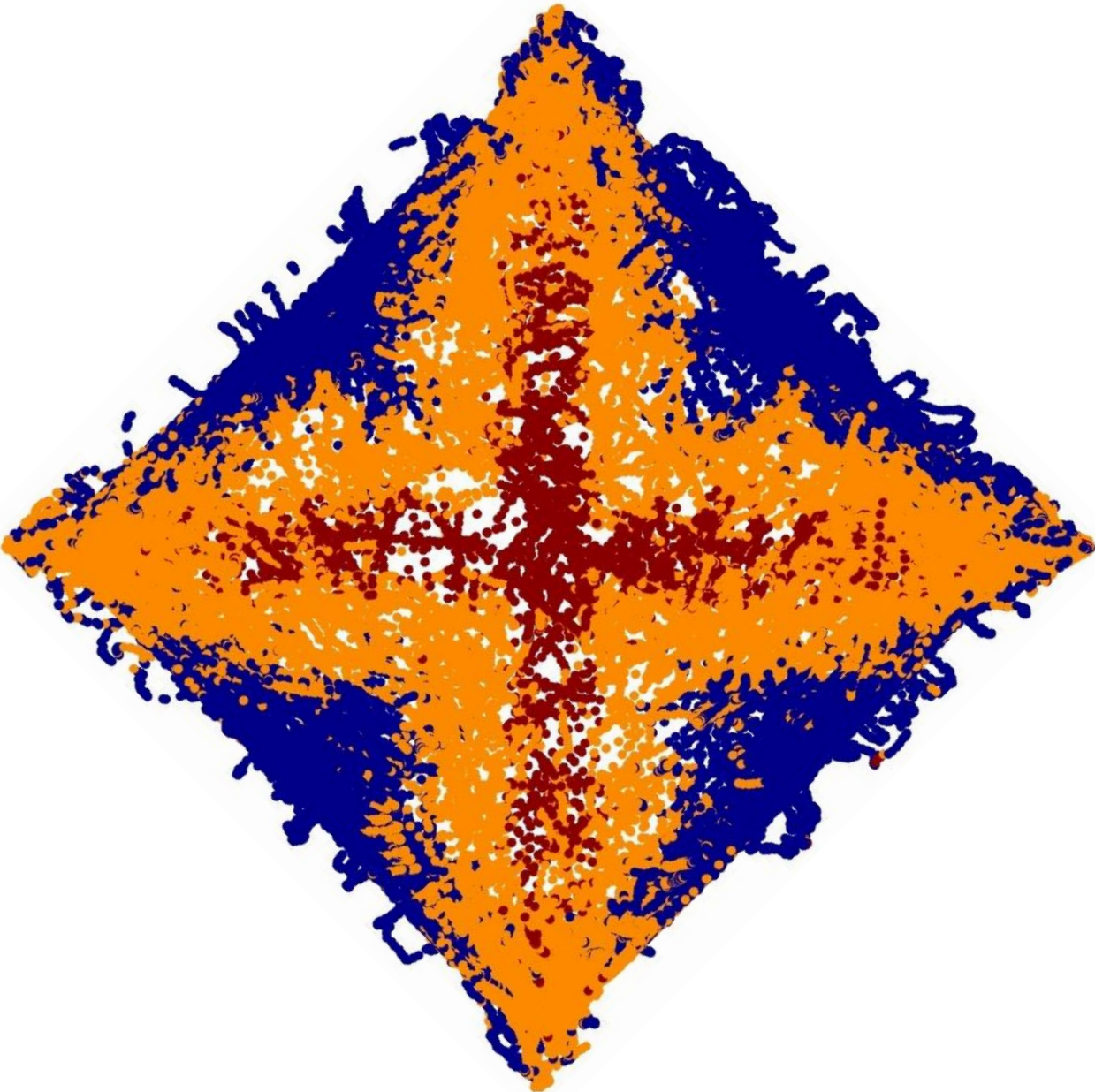
Thomas Bourgeron, PR, Université de Paris, président du jury

Élodie Ey, CR, Université de Strasbourg, directrice de thèse

Fabrice de Chaumont, PhD, Université de Paris, membre invité



A mon grand-père, Marc le Breton.







# “All science is either physics or stamp collecting.”

- Ernest Rutherford

Each step of this reasoning is made of discoveries, and the automation process used in this work should not hide the human reflection involved. I strived to let every data manipulation be led automatically, in order that I do not manually interfere with the logic I intellectually conceived. I justify this choice by both rigour and laziness, which are, in my opinion, the two breasts with which parsimony nourishes scientific discovery. If I first cited Rutherford allegation, my goal is not here to diminish biology. Instead, I want to show that the physical description of a complex subject can end in a kind of stamp collection. If we are dwarfs on giant's shoulders, examples with some well-known giants could help the reader to understand the way I personally conceive this work.

In a first time I designed SUCCUBE algorithm to understand how behaviour links different physical measurements acquired on animals. The discovery of such rules by SUCCUBE, in particular at the independent component analysis stage, is here the equivalent of the discovery of Newtonian laws of physics. Every further parts of the present work will still rely on this first discovery.

In a second time SUCCUBE applies the laws combining physical measurements, and observes the different states that the behaviour can take. The behavioural repertoire clustered on a Gaussian mixture model basis is here my Mendeleev periodic table of elements. This classification will be used in all behavioural analysis led afterward.

In a third time the application of SUCCUBE allows to describe the behaviour of 13 different strains. These results therefore take the form of a kind of extensive catalogue, which could be seen as the Linnaeus's *Systema Naturæ*. Each of those results are here obviously not necessary to answer the final question of this work. However, the extracted classification acquires its wideness from the diversity of subjects. Although each result can seem contingent, all of them are compiled in the catalogue to allow each reader to select the one of its interest.

This process, from physics to stamp collection, was necessary for me to, in a fourth time, achieve my final objective. Finding the behavioural affections related to *SHANK3* haploinsufficiency is my personal dwarf step. What follows is the story of how I did it.

## Abstract

Autism spectrum disorders are neurodevelopmental disorders affecting more than 1% of the global population. Among that, more than 1% of people with autism and intellectual disabilities carry a mutation in the gene coding for the postsynaptic scaffolding protein Shank3. Mouse models of such disorders have been studied through knock-out mutation on the homologous gene. Behavioural affections were found in homozygous knocked-out animals, but few effects have been reported on the heterozygous individuals, in widely used laboratory mouse strains such as C57BL/6J. Still, understanding the impact of *Shank3* haploinsufficiency is crucial, because human patients are heterozygous, and it will open the way to pharmaceutical strategies targeting an increase in *Shank3* expression.

In this optic, we first analysed the behaviour of 12 mouse strains from the Collaborative Cross, a panel of a hundred inbred strains emerging from the controlled breeding of eight founder strains. This first step was used to grab some of the behavioural diversity present in mice and to select genetic backgrounds of interest for their social abilities or activity levels.

In a second time, we generated *Shank3* heterozygous mutant mice on those mixed genetic backgrounds by crossing C57BL/6J.*Shank3*<sup>+/-</sup> mice with the selected CC strains. We analysed their behaviour as compared to their wild-type littermates. We used Live Mouse Tracker, a system to track mice in social group, to record and investigate the solitary and social behaviour of those mice.

We developed a new unsupervised behavioural classification algorithm called SUCCUBE, in order to discover new behavioural event categories and extend our investigation of potential behavioural affections. Based on independent component analysis followed by Gaussian mixture model based clustering, this unsupervised classifier is notably the first one to allow co-occurrences of different behavioural event on a same time point.

We here first compile wide descriptions of Collaborative Cross strain behaviour, in term of locomotor activity, exploration, stereotyped behaviour, thigmotaxis, neophobia and social interest. Such description can be beneficial to further development of other mouse models. We finally report the finding of three newly discovered behaviours, affected in their proportions of production, by *Shank3* haploinsufficiency, in a mixed background between C57BL/6J males and CC002 females. Those heterozygous mutants spent significantly less time rotating in acceleration, facing a conspecific or oriented in the same direction than a conspecific.

Such findings open the way to pharmaceutical trials aiming to restore the affected behaviour of such mutant models closer to the levels of wild-type ones. This also makes the demonstration of the potential of unsupervised behavioural classification to investigate the behaviour of animal models of neurological troubles.

# Résumé

Les troubles du spectre autistique sont des troubles du développement neurologique affectant plus de 1 % de la population mondiale. Ces troubles se manifestent par une communication sociale atypique, des comportements stéréotypés ou répétitifs ainsi que des intérêts restreints. Ils sont souvent aussi accompagnés d'autres troubles que l'on appelle de ce fait comorbidités, tels que l'hyperactivité, la déficience intellectuelle, la bipolarité, et l'épilepsie. Si les comorbidités peuvent souvent être traités, des traitements autres que la thérapie comportementale pour les symptômes clefs de l'autisme font pour le moment toujours l'objet de recherches. La majeure partie des causes de ces troubles sont génétiques. Cependant, les causes les plus pénétrantes sont des mutations de novo, qui ne sont donc pas héritées. Plus de 1 % des personnes avec autisme et retard mental portent une mutation du gène codant pour la protéine d'échafaudage postsynaptique Shank3. La mutation *Shank3* concerne une seule copie du gène. Les personnes portant ces mutations sont donc dites hétérozygotes ou haploinsuffisantes pour *Shank3*. Le fait que cette mutation ne touche qu'une copie laisse entrevoir une potentielle voie de restauration au travers d'une surexpression de la copie fonctionnelle restante.

Des modèles murins de ces troubles ont été étudiés via une mutation par inactivation (KO) du gène homologue. Les modèles les plus étudiés sont les mutants KO homozygotes. Ils ont démontré un comportement social affecté, des comportements stéréotypés ainsi qu'une hypoactivité. Les modèles mutants hétérozygotes ont quant à eux une meilleure validité structurelle. De la même façon que les patients humains, ils disposent toujours d'une copie fonctionnelle du gène *Shank3*, ce qui laisse entrevoir une potentielle voie de restauration du comportement au travers de la surexpression de cette copie. Cependant les modèles hétérozygotes ont été le sujet d'un plus petit nombre d'études, et celles-ci rapportent des effets plus subtils et moins reproductibles. Il est donc essentiel de comprendre l'impact de l'haploinsuffisance de *Shank3*, car les patients sont hétérozygotes et la compréhension de ces mécanismes ouvrira la voie à des stratégies pharmaceutiques visant une augmentation de l'expression de *Shank3*.

La majorité des études portant sur ces modèles ont été menés sur des fonds génétiques communs tels que C57BL/6J. Nous supputons néanmoins l'existence de gènes modulateurs qui pourraient permettre de mieux percevoir les différences de comportement induites par la mutation sur d'autres fonds génétiques.

Les difficultés à percevoir l'impact de l'haploinsuffisance de *Shank3* chez la souris peut aussi dépendre de notre capacité à distinguer des différences de comportement. Cette dernière est limitée par de nombreux facteurs. Les a priori humains restreints ou encore incorrects, comme dans le cas de l'anthropomorphisme, ainsi que les limites de nos capacités de perception ou encore d'attention, introduisent des biais et des imprécisions et sont autant de faiblesses qui peuvent réduire la puissance discriminative d'une analyse du comportement.

L'analyse computationnelle du comportement permet de dépasser les limites des méthodes traditionnelles. Nous nous sommes particulièrement intéressés à la classification comportementale qui permet d'associer des séquences d'observation d'animaux à des catégories de comportement. Les classifications comportementales non supervisées ont l'avantage de ne dépendre d'aucun a priori pour définir les répertoires comportementaux dans lesquels elle classeront les différentes séquences. Cela présente l'avantage majeur de permettre la découverte de nouvelles catégories de comportements. Ces méthodes définissent les catégories uniquement en fonction des données. Elles permettent donc aussi de se prémunir contre tous les biais liés à l'observation humaine. Cependant, toutes les méthodes non supervisées développées par le passé reposaient sur un partitionnement unique qui entraîne une seule segmentation temporelle des observations, dont toutes les catégories sont alors exclusives entre elles. D'autres méthodes de classification comportementale automatique, telles que celles basées sur des règles ou celles basées sur des exemples, permettent quant à elle de classer une même séquence dans plusieurs catégories.

Nous avons donc développé une méthode de classification de comportement non supervisé qui permet de gérer des catégories non-exclusives, i.e., les comportements co-occurent. Ceci est obtenu grâce à un partitionnement multiple. Celui-ci est basé sur la projection dans des sous-espaces orthogonaux. Une fois le comportement des souris enregistré par un système de suivi, les données de trajectoire permettent de calculer des mesures géométriques. Ce jeu de mesures basées sur les trajectoires comporte un nombre de particules en mouvements et 226 mesures de distances et d'angles. Elles peuvent être calculées en un seul point de temps si elles sont instantanées, ou comparer les informations de points de temps successifs pour donner des mesures dynamiques.

Ce jeu de mesures est par la suite traité par une analyse en composantes indépendantes. Celle-ci permet d'obtenir des dimensions orthogonales, qui serviront chacune de sous-espace pour accueillir un partitionnement différent. La fonction de séparation aveugle des sources permet d'obtenir un grand nombre de dimensions qui offrent chacune une mesure différente du comportement.

Chacune de ses composantes indépendantes sert enfin d'espace pour un partitionnement basé sur un mélange de gaussiennes. La distribution non-gaussienne des composantes indépendantes nous permet justement d'attendre un modèle comportant plusieurs gaussiennes, qui permet donc la ségrégation de plusieurs catégories de comportements par axe.

De nouvelles méthodes ont aussi été développées afin de définir le nombre de composantes indépendantes à extraire lors de l'analyse en composantes indépendantes et le nombre de distributions gaussiennes à mélanger lors du partitionnement basé sur un mélange de gaussiennes. Ainsi, la méthode ne nécessite aucune intervention humaine entre la collecte des données et les analyses statistiques qui seront finalement opérées à partir de la classification du comportement.

Nous avons développé ce nouvel algorithme de classification de comportements non supervisé afin de découvrir de nouvelles catégories d'événements comportementaux et d'étendre notre investigation des affections comportementales potentielles. Nous l'avons nommé SUCCUBE, pour System for Unsupervised Classification of Co-occurrent Underlying Behavioural Events. Cette méthode est la première classification comportementale non supervisée à permettre la co-occurrence d'événements comportementaux différents sur un même point de temps.



Nous avons par la suite utilisé Live Mouse Tracker, un système de suivi de souris en groupes sociaux, et SUCCUBE, pour enregistrer et étudier le comportement solitaire et social de multiples sujets murins. Ceci a été réalisé à partir de deux protocoles expérimentaux. Le premier, l'exploration solitaire d'un objet, consiste à suivre un individu isolé dans un environnement de 50 x 50 cm pendant une heure. Au milieu, au bout de 30 minutes, une maison rouge translucide est ajoutée à l'environnement pour y servir de nouvel objet. Le second protocole, le suivi d'un groupe sur le long terme, consiste à suivre un groupe préexistant de 4 individus de même sexe dans ce même environnement pendant 72 heures. Ces 72 heures sont marquées par un rythme jour nuit. Les animaux ont accès à la maison rouge translucide en guise d'abri, à de l'eau, de la nourriture, et du coton pour faire un nid.

Nous avons tout d'abord analysé le comportement de 12 souches de souris issues du Collaborative Cross, un panel d'une centaine de souches consanguines issues du croisement contrôlé de huit souches fondatrices. Cette première étape a permis de saisir une partie de la diversité comportementale présente chez les souris et de sélectionner des fonds génétiques d'intérêt pour leurs capacités sociales ou leurs niveaux d'activité.

Dans un deuxième temps, nous avons généré des souris mutantes hétérozygotes en *Shank3* sur des fonds génétiques mixtes en croisant des souris C57BL/6J.*Shank3*<sup>+/-</sup> avec les souches du Collaborative Cross sélectionnées pour leur comportement différent de celui des souris C57BL/6J. Nous avons analysé leur comportement par rapport à celui de leurs congénères non-mutants.

SUCCUBE a permis la découverte d'un répertoire comportemental de 116 catégories d'événements comportementaux à partir des données du protocole d'exploration solitaire d'un objet et d'un autre de 112 catégories à partir des données du protocole de suivi d'un groupe sur le long terme. Nous détaillons tout d'abord ces répertoires dont nous avons interprété et nommé chaque catégorie.

Nous compilons ensuite de larges descriptions du comportement des souches du Collaborative Cross, en termes d'activité locomotrice, d'exploration, de comportements stéréotypés, de thigmotaxie, de néophobie et d'intérêt social. Ces profils comportementaux peuvent être bénéfiques pour le développement ultérieur d'autres modèles murins. Ils peuvent aussi être les premières pierres à l'édifice d'une étude plus globale de la génétique des traits comportementaux. L'obtention d'un plus grand nombre de profils de souches du Collaborative Cross permettrait de conduire des analyses de Locus de traits quantitatifs. Il serait alors possible de trouver des gènes impliqués dans les différents traits comportementaux que notre classification permet de mesurer.

Nous rapportons enfin la découverte de trois nouveaux comportements, affectés dans leurs proportions de production, par l'haploinsuffisance en *Shank3*, dans un fond mixte entre mâles C57BL/6J et femelles CC002. Ces mutants hétérozygotes ont passé significativement moins de temps à tourner en accélération, à faire face à un congénère ou à être orienté dans la même direction qu'un congénère.

L'affection du comportement de rotation s'avère particulièrement intéressante dans la mesure où ces comportements de rotation ont été identifiés depuis longtemps (1973) comme étant des indices sensibles de la stimulation des récepteurs dopaminergiques. Sachant qu'un déséquilibre au niveau des récepteurs dopaminergiques D1 et D2 a déjà été observé chez des mutants pour le gène *Shank3*, nous pouvons émettre l'hypothèse que la réduction de la production du comportement de rotation en soit un signe extérieur.

Les comportements d'orientation sociale permettent quant à eux de tracer un lien entre une différence de comportement solitaire de rotation et une affection du comportement social. De tels comportements peuvent être impliqués dans les comportements de contacts nez-à-nez ou de reniflements ano-génitaux qui ont été observés en moindre fréquence chez certains modèles mutants homozygotes.

Il faut noter que succube a permis la découverte des catégories de comportements sur lesquels nous avons pu observer les effets de l'haploinsuffisance en *Shank3*. Ceux-ci ne consistaient pas en des comportements d'intérêt a priori. Nous pouvons même remarquer que le comportement des souris est généralement abordé sous l'angle des changements de localisation et des distances entre individus. Nous pouvons donc dire que la découverte de ces catégories d'événements comportementaux a conditionné l'obtention des résultats finaux de ces recherches.

De telles découvertes ouvrent la voie à des essais pharmaceutiques visant à rétablir le comportement affecté de ces modèles mutants plus près des niveaux de ceux des non-mutants. Cela permet également de démontrer le potentiel de la classification comportementale non supervisée pour étudier le comportement de modèles animaux de troubles neurologiques.

Les catégories d'événements comportementaux extraites par SUCCUBE pouvant co-occurrencer, celles-ci sont typiquement des éléments modulaires. Il sera donc par la suite particulièrement intéressant d'analyser les patterns d'associations simultanées et successives de ces catégories, afin de comprendre la structure temporelle de plus grande échelle du comportement et de pouvoir l'utiliser afin de trouver des différences de comportements toujours plus fines.

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# Abbreviations

A: Arena-oriented features

ADHD: Attention-Deficit with Hyperactivity Disorder

ADI-R: Autism Diagnostic Interview, Revised

ANK ankyrin repeat domain

ASD: Autism Spectrum Disorder

B: Body-oriented features

BD: Behavioural Descriptor

BEC: Behavioural Event Category

CARS: Childhood Autism Rating Scale

CATSS: Child and Adolescent Twin Study in Sweden

CC: Collaborative Cross

CCC: Children's Communication Checklist

CNVs: Copy Number Variants

DSM-5: Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition

E: Emitter

H: Head point

HAC: Hierarchical Agglomerative Clustering

GD: Gaussian Distribution

GMM: Gaussian Mixture Model

IC: Independent Component

ICA: Independent Component Analysis

IQ: Intellectual Quotient

KO: Knock-Out

M: Centre of mass point

NPR: national patient register

O: Object-oriented features

P: Particule-oriented features

PCA: Principle Component Analysis

PDZ: PSD-95/Discs large/ZO-1 domain

Pro: proline-rich region

PSD: postsynaptic density

R: Receiver

RBS: Repetitive Behaviour Scale

RFID: Radio Frequency Identification

RIX: Recombinant Inbred intercross

S: Socially-oriented features

SAM: sterile alpha motif domain

SHANK: SH3 and multiple Ankyrin repeat domains

SH3 Src homology 3 domain

SNVs: Single Nucleotide Variants

SRS: Social Responsiveness Scale

SUCCUBE: System for Unsupervised Classification of Co-occurrent Underlying Behavioural Events

T: Tail basis point

22q13: locus q13 of chromosome 22

Δ: deletion



# **Introduction**





# Autism spectrum disorder

## Initial description

The first mention of Autistic spectrum disorder (ASD) in the literature is the 1943 publication of Kanner “Autistic Disturbance of Affective Contact” [1]. This first study relates observations on 11 children (8 boys, 3 girls) from 1938. The observed subjects are described as displaying “a powerful desire for aloneness” and “an obsessive insistence on persistent sameness”. Leo Kanner considered such cases as grouped in a new mental condition, and named this condition “early infantile autism”.

In parallel Hans Asperger studied 4 children with similar characteristics, but without intellectual disability or language deficit. In his publication of 1944 he described his patients as clumsy, lacking empathy and having highly specific centres of interest [2].

## Clinical criteria

ASD is a neurodevelopmental condition. First symptoms therefore are observed in the early childhood. Patients can be consistently diagnosed at three years of age, although it may become clear only later in life [3], [4].

ASD is characterized by impairments in two areas (table 1):

- the social-communication domain
- the behavioural domain, which includes repetitive behaviours and restricted interests.

ASD is diagnosed on a behavioural basis, following guidelines of the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) [5]. The DSM-5 also introduced the notion of spectrum in order to account for the important diversity of symptoms among the patients.

Table 1: Criteria for the diagnosis of autism spectrum disorder from the *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5)*, in 2013. (APA 2013) [5]

- A. Persistent deficits in social communication and social interaction across multiple contexts, as manifested by the following, currently or by history (examples are illustrative, not exhaustive, see text):
1. Deficits in social-emotional reciprocity, ranging, for example, from abnormal social approach and failure of normal back-and-forth conversation; to reduced sharing of interests, emotions, or affect; to failure to initiate or respond to social interactions.
  2. Deficits in non-verbal communicative behaviours used for social interaction, ranging, for example, from poorly integrated verbal and non-verbal communication; to abnormalities in eye contact and body language or deficits in understanding and use of gestures; to a total lack of facial expressions and non-verbal communication.
  3. Deficits in developing, maintaining, and understanding relationships, ranging, for example, from difficulties adjusting behaviour to suit various social contexts; to difficulties in sharing imaginative play or in making friends; to absence of interest in peers.
- B. Restricted, repetitive patterns of behaviour, interests, or activities, as manifested by at least two of the following, currently or by history (examples are illustrative, not exhaustive; see text):
1. Stereotyped or repetitive motor movements, use of objects, or speech (e.g., simple motor stereotypies, lining up toys or flipping objects, echolalia, idiosyncratic phrases).
  2. Insistence on sameness, inflexible adherence to routines, or ritualized patterns or verbal non-verbal behaviour (e.g., extreme distress at small changes, difficulties with transitions, rigid thinking patterns, greeting rituals, need to take same route or eat food every day).
  3. Highly restricted, fixated interests that are abnormal in intensity or focus (e.g., strong attachment to or preoccupation with unusual objects, excessively circumscribed or perseverative interest).
  4. Hyper- or hyporeactivity to sensory input or unusual interests in sensory aspects of the environment (e.g., apparent indifference to pain/temperature, adverse response to specific sounds or textures, excessive smelling or touching of objects, visual fascination with lights or movement).
- C. Symptoms must be present in the early developmental period (but may not become fully manifest until social demands exceed limited capacities, or may be masked by learned strategies in later life).
- D. Symptoms cause clinically significant impairment in social, occupational, or other important areas of current functioning.
- E. These disturbances are not better explained by intellectual disability (intellectual developmental disorder) or global developmental delay. Intellectual disability and autism spectrum disorder frequently co-occur; to make comorbid diagnoses of autism spectrum disorder and intellectual disability, social communication should be below that expected for general developmental level.

These clinical and psychological criteria, considered on diagnosis, are evaluated through standardized procedures or tools. The most used ones are qualitative assessments:

- The *Autism Diagnostic Interview, Revised* (ADI-R) [6], an interview with the patient's parents, allowing to evaluate the evolution and potential deficits.
- The *Autism Diagnostic Observation Schedule* (ADOS) [7], an interaction with the patient structured with short activities and games, allowing the direct and standardised evaluation of deficits in the social communication and behaviour domains.

ADI-R and ADOS are the international standard evaluations. However, tools for the quantitative evaluation of the patient have also been developed. They allow to precise the deficits in particular domains, in complement of the ADOS :

- The *Childhood Autism Rating Scale* (CARS) [8], a scale evaluating the global deficit, of the patient, which can be associated with ASD core symptoms.
- The *Children's Communication Checklist* (CCC) [9], a scale evaluating the social communication ability of the patient.
- The *Social Responsiveness Scale* (SRS) [10], a scale evaluating specifically the social behaviours of the patient.
- The *Repetitive Behaviour Scale* (RBS) [11], a scale quantifying the repetitive and the stereotyped behaviours of the patient.

ASD can also be associated with comorbidities (figure 1). 75% of patients are displaying intellectual disabilities at different severity levels [12]–[14]. Patients with ASD and an Intellectual Quotient (IQ) of more than 70 are considered without intellectual disabilities and display what we call high-functioning autism.

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**Figure 1: Core symptoms and frequent comorbidities observed in individuals with ASD, from Huguet et al. in 2013 [15].** *“Arrows define clinical characteristics of the individuals, and the red line defines the level of severity (low at the centre and high at the periphery). Colours represent the combination of clinical features.”*

Anxiety disorders, which denote increased anxiety and fear, are present in 11 to 84.1% of patients according to the type of measure [16], [17], against 3 to 30% in the neurotypical population [18]. People with ASD may also present Attention-Deficit with Hyperactivity Disorder (ADHD) in 28.2 to 87 % of the cases [17], against 3.4% in neurotypical population [19]. They are also more subjected to mood disorder as bipolar disorders and depression, 2 to 57% of cases [17], against 3.8% in the neurotypical population [20]. Patients with ASD have also a greater risk to develop epilepsy, in 5 to 38% of cases the patients display seizure [21], when the overall lifetime prevalence of epilepsy is 0.76% [22].

## Epidemiology

The current prevalence of ASD is estimated between 1 and 2% of the global population. The sex ratio of ASD patients is unbalanced, male patients outnumbering females four to one [23]. The prevalence is particularly well documented in the United States and was estimated at 18.5 per 1000 children aged eight years, in 2016 [23]. However this prevalence is heterogeneous over the United States, varying from 13.1 to 31.4 per 1000 children, between states. Most of this variance can be explained by the numbers of health centres, psychiatrists and paediatricians in each state [24]. This prevalence has been constantly increasing since the 1960's and 1970's when it was comprised between 0.4 and 2 per 1000 children [25] until the most recent measurements from the United States Centers for Disease Control and Prevention (figure 2).

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Figure 2: Estimated prevalence of autism spectrum disorder in the United States of America from 2004 to 2020 based on Centers for Disease Control and Prevention reports, from Autism Speaks. *“Analysis of 2016 medical and/or school records of 8-year-olds from 11 monitoring sites across the United States.”*

Environmental factors could explain a part of this increase as we know that risk factors for ASD exist [26]. However, most of this increase is likely to be due to the change in diagnostic criteria and the inclusion of outpatient contacts [27]. For example, in Sweden, the real prevalence revealed to be stable between 1993 and 2002, but the diagnosis of ASD increased in this period (figure 3) [28].

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Figure 3: Annual prevalence of autism spectrum disorder in Child and Adolescent Twin Study in Sweden (CATSS), national patient register (NPR), and NPR diagnoses in Swedish twins, from Lundström et al. in 2015 [28]. *“\*Prevalence calculated on 19993 people responding in twin study born 1993-2002. †Prevalence calculated on all twins, irrespective of response in CATTS (n=26953). Diagnosis in NPR was ascribed before the children’s 10th birthday. ‡Prevalence calculated on all births in Sweden 1993-2002 (n=1078975). Diagnosis in NPR was ascribed before the children’s 10th birthday. Regression lines are depicted within 95% confidence intervals.”*



“The current treatment options for the core symptoms of autism are limited to psychosocial therapies, such as applied behaviour analysis” [29]. Such approaches have shown to be effective in improving social skills and communication. Pharmaceutical treatments are more used to treat comorbidities, such as risperidone and aripiprazole for irritability. Risperidone has also shown to be beneficial by reducing stereotyped behaviours. However those two drugs have limitations because they lead to increased appetite, weight gain and somnolence, what causes discontinuations in treatments [30]. The difficulties to discover appropriate treatments for ASD symptoms also depend on the patients heterogeneity, which itself depends on the diversity of ASD causes. In order to better target therapeutic trials and identify new potential therapeutic strategies, it is necessary to better understand the causes of ASD.

## **Genetic causes**

Twin studies have displayed a major genetic contribution to ASD. With concordances ranging from 82 to 92% in monozygotic twins compared with 10% in dizygotic twins, ASD appeared as the most genetic of neuropsychiatric disorders [31], [32]. The heritability of ASD is estimated around 50%. Most of this heritability relies on common genetic variants. However, our understanding of the genetics of ASD concerns almost exclusively highly penetrant de novo mutations, which are thus not inherited [33]. These de novo mutations targeting clinically relevant loci for ASD are carried by 10% of patients, particularly those with intellectual disabilities. Such mutations can be Copy Number Variants (CNVs), variations in the number of copies of a particular gene, or Single Nucleotide Variants (SNVs), variations in a single nucleotide (figure 4). In ASD-associated genes, 61% are expressed in the brain [15].

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**Figure 4: Relative contributions of genetics and environment in ASD, from Huguet and Bourgeron in 2016 [33].** *“Based on twin and familial studies, it is estimated that the genetic and environmental contributions to ASD are approximately 50=50%. Most of the inheritable part seems to be due to common variants observed in the general population, with a small contribution from rare variants. Importantly, de novo mutations are genetic causes of ASD, but they do not contribute to heritability because they are present only in the patient. These de novo events are therefore considered environmental causes of ASD, but act on the deoxyribonucleic acid molecule.”*

These many genes are involved in different cellular functions, such as chromatin remodelling, mRNA translation, metabolism, and 14% in synaptic function (Figure 5) [15]. However, the consequences of these mutations could converge towards an alteration of neuronal and synaptic homeostasis [34], [35].

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Figure 5: Biological pathways of proteins mutated in ASD or other disorders, from Huguet et al. In 2013 (Huguet, et al. 2013)<sup>15</sup>.

*“These proteins are involved in many functions, including the organization of the postsynaptic density, cytoskeleton dynamics, cellular signalling cascades, epigenetic regulation of transcription, and release of neurotransmitters. Proteins associated with ASD are in red, those associated with other psychiatric disorders are in purple, and those associated with intellectual disability are in blue.*”

Panel a illustrates the pre- and post-synapse; panel b illustrates the synapse and gene transcription regulation in the nucleus.”

## **SHANK3 gene**

In 2007, Durand and colleagues discovered that a mutation of a single copy of *SHANK3* gene on the locus q13 of chromosome 22 (22q13) can result in severe cognitive deficits, including language and speech disorder and ASD [36]. Mutations in *SHANK3* is present in 0.69% of patients with ASD, and in 2.12% of the patients with co-occurrent ASD and moderate to profound intellectual disability [37]. This makes *SHANK3* haploinsufficiency one of the more penetrant monogenic causes of ASD [38]. *SHANK3* is also associated to Phelan-McDermid syndrome, which is caused by the deletion of the 22q13 locus, intellectual disability, schizophrenia and bipolar disorder [39], [40]. People with *SHANK3* mutations can display severe speech deficits, some individuals are non-verbal, other may use single words or phrase speech. Some regression may be observed. Such mutations generally drive people to be hypoactive, they may display hypotonia and motor skill deficits. brain abnormalities and mild dysmorphic features can be present. Patients may also develop seizures as well as feeding and gastrointestinal problems [41].

## **Proteins**

*SHANK3*, also known as *ProSAP2*, is part of the SH3 and multiple Ankyrin repeat domains (*SHANKs*) gene family, which also includes *SHANK1* and *SHANK2* also known as *ProSAP1*. They encode for SHANKs which are scaffolding proteins of the postsynaptic density (PSD) of glutamatergic synapses [39]. Shank3 have five conserved protein domains (figure 6) [42]:

- an ankyrin repeat domain (ANK);
- a Src homology 3 domain (SH3);
- a PSD-95/Discs large/ZO-1 domain (PDZ);
- a proline-rich region containing homer-and cortactin-binding sites (Pro)
- a sterile alpha motif domain (SAM).

Figure 6: Some *SHANK3* mutations causing autism-related phenotypes, represented on the chromosome and in the protein domain by Jiang and Ehlers in 2013 [42]. “*SHANK3* is part of a large gene cluster associated with deletions in chromosome 22q13.3 deletion syndrome, or Phelan-McDermid syndrome, associated with autistic behaviors and intellectual disability (select genes are shown). The deletion sizes vary from 17 kb within *SHANK3* to 10 Mb in 22q13.3. *SHANK3* gene structure, mutations, and protein domains are shown. Human *SHANK3* has 23 exons as deduced from cDNA AB569469 deposited in GenBank. Exon 11a is a newly identified exon. The positions of six identified promoters are indicated as black arrows. The exons in red are alternatively spliced. The positions of point mutations are indicated as blue arrows and the nature of point mutations are as described above the arrow. Protein domains are shown and aligned to corresponding exons (ANK, ankyrin repeat domain; SH3, Src homology 3 domain; PDZ, PSD-95/Discs large/ZO-1 domain; Pro, a proline-rich region containing homer- and cortactin-binding sites; SAM, sterile alpha motif domain). CpG islands are sites of differential methylation and are indicated by green bars.”

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**Figure 7: SHANK3 Protein Interactions and Isoform-Specific Domain Structure, from Jiang and Ehlers in 2013 [42].**

*“(A) Schematic of the partial Shank protein interactome at the PSD with Shank3 as a model. Protein domains in Shank family members are similar. Many interacting proteins interact with all three Shank family proteins (Shank1, Shank2, and Shank3) in in vitro assays. The proteins in red font are altered in Shank3 mutant mice. (B) Diagram of SHANK3 protein isoforms SHANK3a-f. Protein domain structure was deduced from confirmed mRNAs expressed from different promoters in human and mouse brains. Polypeptides have not been validated due to the lack of isoform-specific antibodies. Pro, proline-rich region.”*

*SHANK* genes have intragenic promoters that condition a complex transcriptional regulation with multiple and extensive alternately spliced exons. *SHANK3* gene encompasses 22 exons and six promoters, that lead to the transcription of at least six mRNA and their translation in six main isoforms of *SHANK3* protein (figure 7 B) [42], [43]. This diversity of *SHANK3* isoforms is likely to play a role in synaptic signalling and on the protein composition of the PSD. *SHANK3* transcript structure being complex, point mutations, intragenic deletions and translocations of *SHANK3* carried by patients with ASD are isoform-specific. Mutations may have a different impact on the different *SHANK3* isoforms proportions, and this mix of translated isoforms will be specific to the mutation.

## Functions

*SHANK3* “may function as a master scaffold forming large sheets that may represent the platform for the construction of the PSD complex” (figure 7 A) [44], [45]. This PSD complex is formed by the binding of *SHANK3*, with many synaptic proteins it interacts with, as neuroligins and neuexins, at glutamatergic synapses. Such binding interactions regulates the structural organization of dendritic spines [36]. This structure of the glutamatergic dendritic spines would influence the synapse function and homeostasis.

*SHANK3* is also known to “have a positive effect on the induction and maturation of dendritic spines” [37]. This could explain its role in the formation of synapses [39]. *SHANK3* thus have an impact on neuronal development, what allows to understand its implication on neurodevelopmental disorders.



## ***Shank3* knockout mouse models**

Genetic studies proved the implication of *Shank3* and other genes mutations in patients with ASD. However the mechanisms by which the mutation impacts the physiology and behaviour remain hard to study on human patients. Animal models carrying such mutations therefore may allow to fill this gap of understanding and to test innovative candidate ways of treatment.

Mouse models are the most used ones because:

- they have a satisfying phylogenetic proximity to human, our lineage diverged between 70 and 80 millions years ago [46], and thus carry 99 % of genes homologous to human ones, including orthologous 80% of genes, as *Shank3* [47];
- they are easy to manipulate genetically to mimic the human mutations [48];
- they share physiological similarity with humans;
- they have behavioural traits that can be paralleled with some human ones, they can display social behaviours and stereotyped behaviours, that we can measure [49];
- they have an accelerated lifespan, their entire life cycle can be observed in three years;
- they are cost-effective and efficient, they are small and can be observed in apparatus fitting in animal facilities, they reproduce quickly, they are easy to handle and transport;
- they have been used as biomedical research models since 1678, so we understand this species particularly well;
- they can be inbred in order to obtain genetically identical strains. Such genetical uniformity increases accuracy and reproducibility of experiments, which reduces variance and increases statistical power. However, inbreeding may also be a limit of mouse models, as the strain used can become too specific and thus lose a part of its ability to mimic the effects observed in human. A potential way to avoid this limit is to study the impact of a mutation on different genetic backgrounds, in order to benefit from the different sets of modifier genes.

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Figure 8: Heat-map representation of the clustering of mouse models for ASD carrying mutations in synaptic proteins and their phenotypic traits, from Ferhat et al. 2017 [50]. Traits are “as indicated: social interest/motivation, social recognition, social communication, stereotypes, synaptic physiology and protein composition.” The colour of the cells indicates if each study found: *No significant difference between wild-type and mutant mice*; *Significantly higher or lower trait in mutant than in wild-type mice*; *Mixed evidence* or if the trait was *Not investigated*.

Researchers thus generated and explored mouse models to accumulate knowledge about the biology of autism [51]. We will here focus on the impact of a *Shank3* mutation on mouse behaviour, *Shank3* being the most studied genetic cause of ASD. If a *Shank3* mutation affects the behaviour of an animal in an ASD-like way, we can consider to some extent that it models the disorders. Once a model is defined, it could also allow to run pharmaceutical trials, which would again produce knowledge about ASD mechanisms. Finally, if a drug allows to restore the affected phenotype closer to the one of non-mutant individual, it would be a step toward human pharmaceutical rescue strategies.

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**Figure 9: Targeted Mutations in *Shank3* Genes in Mice, from Jiang and Ehlers in 2013 [42].**

*“Schematic of the mouse Shank3 gene structure deduced from cDNA AB230103 deposited in GenBank. The promoters are shown by arrows and alternatively spliced exons are indicated in red. The exact transcription starting site of promoter 3 remains to be determined. The positions of targeted mutations in five different lines of Shank3 mutant mice are shown. The transcripts from promoters upstream of deleted exons are predicted to be truncated or disrupted (red arrows) and the transcripts from promoter downstream of deleted exons are predicted to be intact in each mutant line of mice (black arrows). Bottom panels depict predicted isoform-specific expression of Shank3 mRNA and proteins in Shank3 mutant mice. The “-” indicates that the isoform is disrupted and “+” indicates the isoform remains intact. The full complement of Shank3 mRNA and protein isoforms that derive from combinations of alternative promoters and mRNA splicing remains unknown. Therefore, the pattern of isoform-specific expression and disruption by specific mutations is likely more complex than indicated.”*

### **Shank3 mutant phenotype**

Most studies observed the behaviour of homozygous mutant mice for *Shank3*, a fewer proportion evaluated heterozygous mutants (figure 8). Such mutations are not always targeting the same exon, and as in human, *Shank3* mutations carried by mouse are isoform-specific. That means that different mouse models, with different mutations, are not lacking the same Shank3 isoforms. [42]. For example (Figure 9):

- mice with deletions  $\Delta\text{ex4-7}$  [52] and  $\Delta\text{ex4-7}$  [53], [54] are only lacking Shank3 isoforms a and b;
- mice with deletion  $\Delta\text{ex11}$  [55] are lacking Shank3 isoforms a, b and c;
- mice with deletion  $\Delta\text{ex13-16}$  [52] are lacking Shank3 isoforms a to d.

**Table 2: decrease of activity between different studies and Shank3 mouse models.**

| Mutant models         | publications                | Effect in homozygous | Effect in heterozygous |
|-----------------------|-----------------------------|----------------------|------------------------|
|                       |                             | mutant               | mutant                 |
| Shank3 $\Delta$ 4-9B  | M. Yang et al., 2012[56]    | reduced              | not significant        |
|                       | Drapeau et al., 2014[57]    | not significant      | reduced                |
| Shank3 $\Delta$ 4-7   | Peça et al., 2011[52]       | reduced              | not investigated       |
| Shank3 $\Delta$ 4-9P  | Jaramillo et al., 2016[58]  | not significant      | not significant        |
| Shank3 $\Delta$ 4-9J  | Kabitzke et al., 2018[59]   | not significant      | not investigated       |
| Shank3 $\Delta$ 4-22J | X. Wang et al., 2016[60]    | reduced              | not significant        |
|                       | Bey et al., 2018[61]        | reduced              | not investigated       |
| Shank3 $\Delta$ 4-22B | Drapeau et al., 2018[62]    | reduced              | reduced                |
| Shank3 $\Delta$ 9     | Lee et al., 2015[63]        | not significant      | not investigated       |
| Shank3 $\Delta$ 11    | Vicidomini et al., 2017[64] | not significant      | not investigated       |
| Shank3 $\Delta$ 13-16 | Kabitzke et al., 2018[59]   | reduced              | not investigated       |
| Shank3 $\Delta$ 21P   | Duffney et al., 2015[65]    | not investigated     | not significant        |
|                       | kouser et al., 2013[66]     | reduced              | not investigated       |
| Shank3InsG3680        | Zhou et al., 2016[67]       | reduced              | reduced                |
| Shank3R1117X          |                             | reduced              | not significant        |
| Shank3InsGex21        | Speed et al., 2015[68]      | reduced              | not significant        |
| Shank3cKI             | Mei et al., 2016[69]        | reduced              | not investigated       |

Over 21 publications from the literature, the behaviour of 15 mouse models with different *Shank3* mutation was measured, for a total of 24 independent behavioural characterizations of *Shank3* mutant mice. In 17 of the 24 behavioural characterizations of shank3 mutants, researchers have evaluated the locomotor activity of mice. On the 16 testing homozygous mutants, 11 concluded in a reduction of the activity associated to the homozygous mutation. On the 9 testing heterozygous mutants, 3 concluded in a reduction of the activity associated to the heterozygous mutation (Table 2).

**Table 3: decrease of social interest between different studies and Shank3 mouse models.**

| Mutant models         | publications                | Effect in homozygous | Effect in heterozygous |
|-----------------------|-----------------------------|----------------------|------------------------|
|                       |                             | mutant               | mutant                 |
| Shank3 $\Delta$ 4-9B  | M. Yang et al., 2012[56]    | reduced              | reduced                |
|                       | Bozdagi et al., 2010[53]    | not investigated     | reduced                |
|                       | Drapeau et al., 2014[57]    | not significant      | not significant        |
| Shank3 $\Delta$ 4-7   | Peça et al., 2011[52]       | reduced              | not investigated       |
| Shank3 $\Delta$ 4-9P  | Jaramillo et al., 2016[58]  | reduced              | not significant        |
| Shank3 $\Delta$ 4-9J  | X. Wang et al., 2011[54]    | reduced              | not investigated       |
|                       | Kabitzke et al., 2018[59]   | not significant      | not investigated       |
| Shank3 $\Delta$ 4-22J | Bey et al., 2018[61]        | reduced              | not investigated       |
| Shank3 $\Delta$ 4-22B | Drapeau et al., 2018[62]    | reduced              | not significant        |
| Shank3 $\Delta$ 9     | Lee et al., 2015[63]        | not significant      | not investigated       |
| Shank3 $\Delta$ 11    | Vicidomini et al., 2017[64] | reduced              | not investigated       |
| Shank3 $\Delta$ 13    | Jaramillo et al., 2017[70]  | reduced              | reduced                |
|                       | Peça et al., 2011[52]       | not significant      | not investigated       |
|                       | J. Luo et al., 2017[71]     | reduced              | not investigated       |
| Shank3 $\Delta$ 13-16 | Kabitzke et al., 2018[59]   | reduced              | not investigated       |
|                       | Duffney et al., 2015[65]    | not investigated     | reduced                |
|                       | Qin et al., 2018[72]        | not investigated     | reduced                |
| Shank3 $\Delta$ 21P   | kouser et al., 2013[66]     | not significant      | not investigated       |
|                       | Bidinosti et al., 2016[73]  | reduced              | not investigated       |
| Shank3InsG3680        | Zhou et al., 2016[67]       | reduced              | not significant        |
| Shank3R1117X          |                             | reduced              | reduced                |
| Shank3InsGex21        | Speed et al., 2015[68]      | not significant      | not significant        |

In 22 of the 24 behavioural characterizations of *Shank3* mutants, researchers have evaluated the social interest of mice. On the 19 testing homozygous mutants, 13 concluded in a reduction of the social interest associated to the homozygous mutation. On the 11 testing heterozygous mutants, 6 concluded in a reduction of the social interest associated to the heterozygous mutation (Table 3).

**Table 4: reduction of interest in social novelty between different studies and Shank3 mouse models.**

| Mutant models         | publications                | Effect in homozygous | Effect in heterozygous |
|-----------------------|-----------------------------|----------------------|------------------------|
|                       |                             | mutant               | mutant                 |
| Shank3 $\Delta$ 4-9B  | M. Yang et al., 2012[56]    | not significant      | not significant        |
|                       | Drapeau et al., 2014[57]    | reduced              | reduced                |
| Shank3 $\Delta$ 4-7   | Peça et al., 2011[52]       | reduced              | not investigated       |
| Shank3 $\Delta$ 4-9J  | Kabitzke et al., 2018[59]   | not significant      | not investigated       |
| Shank3 $\Delta$ 9     | Lee et al., 2015[63]        | not significant      | not investigated       |
| Shank3 $\Delta$ 11    | Vicidomini et al., 2017[64] | reduced              | not investigated       |
| Shank3 $\Delta$ 13    | Jaramillo et al., 2017[70]  | reduced              | reduced                |
|                       | Peça et al., 2011[52]       | reduced              | not investigated       |
| Shank3 $\Delta$ 13-16 | J. Luo et al., 2017[71]     | reduced              | not investigated       |
|                       | Kabitzke et al., 2018[59]   | not significant      | not investigated       |
|                       | Duffney et al., 2015[65]    | not investigated     | not significant        |
| Shank3 $\Delta$ 21P   | kouser et al., 2013[66]     | not significant      | not investigated       |
|                       | Bidinosti et al., 2016[73]  | not significant      | not investigated       |
| Shank3InsG3680        | Zhou et al., 2016[67]       | reduced              | not significant        |
| Shank3R1117X          |                             | reduced              | reduced                |
| Shank3InsGex21        | Speed et al., 2015[68]      | not significant      | not significant        |

In 16 of the 24 behavioural characterizations of *Shank3* mutants, researchers have evaluated the interest in social novelty of mice. On the 15 testing homozygous mutants, 8 concluded in a reduction of the interest in social novelty associated to the homozygous mutation. On the 7 testing heterozygous mutants, 3 concluded in a reduction of the interest in social novelty associated to the heterozygous mutation (Table 4).

Table 5: decrease in ultrasonic vocalisation emissions between different studies and *Shank3* mouse models.

| Mutant models | publications              | Effect in homozygous mutant | Effect in heterozygous mutant |
|---------------|---------------------------|-----------------------------|-------------------------------|
| Shank3Δ4-9B   | Bozdagi et al., 2010[53]  | not investigated            | reduced                       |
| Shank3Δ4-9J   | X. Wang et al., 2011[54]  | reduced                     | not investigated              |
|               | Kabitzke et al., 2018[59] | reduced                     | not investigated              |
| Shank3Δ4-22J  | X. Wang et al., 2016[60]  | reduced                     | not significant               |
|               | Bey et al., 2018[61]      | reduced                     | not investigated              |
| Shank3Δ9      | Lee et al., 2015[63]      | not significant             | not investigated              |
| Shank3Δ13-16  | Kabitzke et al., 2018[59] | reduced                     | not investigated              |
| Shank3Δ21P    | kouser et al., 2013[66]   | not significant             | not investigated              |

In 8 of the 24 behavioural characterizations of *Shank3* mutants, researchers have evaluated the ultrasonic vocalisation emissions of mice. On the 7 testing homozygous mutants, 5 concluded in a reduction of the ultrasonic vocalisation emissions associated to the homozygous mutation. On the 2 testing heterozygous mutants, 1 concluded in a reduction of the ultrasonic vocalisation emissions associated to the heterozygous mutation (Table 5).



**Table 6: increase of stereotyped behaviours between different studies and *Shank3* mouse models.**

| Mutant models         | publications                | Effect in homozygous mutant | Effect in heterozygous mutant |
|-----------------------|-----------------------------|-----------------------------|-------------------------------|
| Shank3 $\Delta$ 4-9B  | M. Yang et al., 2012[56]    | increased                   | increased                     |
|                       | Drapeau et al., 2014[57]    | not significant             | not significant               |
| Shank3 $\Delta$ 4-7   | Peça et al., 2011[52]       | increased                   | not investigated              |
| Shank3 $\Delta$ 4-9P  | Jaramillo et al., 2016[58]  | increased                   | not significant               |
| Shank3 $\Delta$ 4-9J  | X. Wang et al., 2011[54]    | increased                   | not investigated              |
|                       | Kabitzke et al., 2018[59]   | not significant             | not investigated              |
| Shank3 $\Delta$ 4-22J | X. Wang et al., 2016[60]    | increased                   | not significant               |
|                       | Bey et al., 2018[61]        | increased                   | not investigated              |
| Shank3 $\Delta$ 4-22B | Drapeau et al., 2018[62]    | increased                   | not significant               |
| Shank3 $\Delta$ 9     | Lee et al., 2015[63]        | not significant             | not investigated              |
| Shank3 $\Delta$ 11    | Vicidomini et al., 2017[64] | increased                   | not investigated              |
| Shank3 $\Delta$ 13    | Jaramillo et al., 2017[70]  | increased                   | increased                     |
| Shank3 $\Delta$ 13-16 | Kabitzke et al., 2018[59]   | increased                   | not investigated              |
|                       | Duffney et al., 2015[65]    | not investigated            | increased                     |
| Shank3 $\Delta$ 21P   | Qin et al., 2018[72]        | not investigated            | increased                     |
|                       | kouser et al., 2013[66]     | increased                   | not investigated              |
|                       | Bidinosti et al., 2016[73]  | increased                   | not investigated              |
| Shank3InsG3680        | Zhou et al., 2016[67]       | increased                   | not significant               |
| Shank3R1117X          |                             | not significant             | not significant               |
| Shank3InsGex21        | Speed et al., 2015[68]      | increased                   | not significant               |
| Shank3cKI             | Mei et al., 2016[69]        | increased                   | not investigated              |

In 21 of the 24 behavioural characterizations of *Shank3* mutants, researchers have evaluated the stereotyped behaviours of mice. On the 19 testing homozygous mutants, 15 concluded in an increase of the stereotyped behaviours associated to the homozygous mutation. On the 11 testing heterozygous mutants, 4 concluded in an increase of the stereotyped behaviours associated to the heterozygous mutation (Table 6).

We can notice an important variability between studies, that may be imputable to differences in isoforms expressed, in number of individuals taken into account or to laboratory specific effects. Despite this, most findings about *Shank3* mutant mice can be considered as reminiscent to the observations in patients, or ASD-like.

Over the 25 behavioural characterizations of *Shank3* mutant we considered, homozygous mutant were more studied than heterozygous mutants. We took 5 categories of behavioural trait into account: activity; social interest; interest in social novelty, ultrasonic vocalisation emission and stereotyped behaviour. For each of those categories, the majority of the behavioural characterizations, evaluating this trait on homozygous mutants, concluded in a significant effect of the mutation. In contrast, the rate of significant findings on the impact of the heterozygous mutation, never exceeded 50%, for any of the trait.

In 2016, Mei and colleagues led a study on a novel mouse model with *Shank3* conditional knock-in mutation. Their mice, which were not expressing *Shank3*, displayed a social interaction deficit and repetitive grooming behaviours. But they showed that, re-expression of the *Shank3* gene, once mice were grown adult, could rescue certain behavioural abnormalities including social deficit and stereotyped grooming behaviour. Such results reveal the potential of post-developmental activation of *Shank3* to restore neural functions and behaviour [69].

## The potential of *Shank3* heterozygous mutant models

The majority of the mutations implied in ASD, including *Shank3*, affect a single allele. This suggests “a key role for gene dosage in susceptibility to ASD” [35]. Most patients affected by *Shank3* mutation are thus heterozygous, as the mutation disrupts only one copy of the gene. In 2007, when the implication of *Shank3* in ASD was discovered, Durand and her collaborators stated that this gene highlighted the importance of a “gene dosage–sensitive synaptic pathway” in ASD. [36] If the behaviour of mouse models can be partially restored through *Shank3* activation after development [69], the same could be possible for human patients, then increasing the transcription of their remaining *Shank3* allele would be a promising potential treatment pathway.

However, the restoration of behaviour through the increased translation of non mutated allele is not possible in homozygous mutant. Such therapeutic option can only be studied on a heterozygous *Shank3* mutant model. We noticed the imbalanced ratio of studies studying heterozygous mutant compared to the ones describing homozygous models. In the 2018 Drapeau et al. study, homozygous mutant mice were displaying more grooming than wildtype. However, no significant effects were found on heterozygous mice. This shows a major reason for the minor use of heterozygous models for *Shank3*. The deficits are typically more pronounced in homozygotes, and heterozygotes often display an intermediate phenotype [62].

In another publication of Drapeau and her collaborators, from 2014, an interesting way to valorise heterozygous model study is attempted: the use of new genetic backgrounds. It is frequent that the phenotypic impact of a single locus mutation can be modulated by other loci [57]. Such modulation should thus be maximal between two entirely different genetic backgrounds. In laboratory mice each strain corresponds to a distinct genetic background. A mutation impact on the phenotype is therefore often different across strains. Changing the strain in which we study a mutation is thus a way to identify modifier loci. In this particular study, strains C57BL/6, 129SVE and FVB/Ntac have been used to investigate potential genetic background modulations on the phenotypes of *Shank3* heterozygous and homozygous models and wild-type mice. However, the authors conclude in the absence of strong strain effects in the large battery of behavioural analyses led on these *Shank3 mutants*.

In this work we will pursue in this way of background investigation to obtain a clear model of behavioural affection caused by *Shank3* haploinsufficiency. However, in order to increase our ability to detect behavioural consequences of *Shank3* heterozygous mutations, we will also rethink our way to measure and evaluate the behaviour.

## Traditional behavioural analysis

The field of biology tackling animal behaviour, or ethology, preceded computer sciences. The methodologies previously developed in this field were therefore not dependent of any heavy computational resolution. In this thesis, we will use the term “traditional behavioural analysis” [74] to refer to these practices that are not based on computer processing.

Collected data have always been analysed from a mathematical point of view. However, without the help of modern technology, the collection of behavioural data meets natural limitations. Indeed, performing calculations while observing a behaviour is made difficult by the high rate of animal behaviour occurrence. For example, mice have been estimated to perform on average three blocks of different posture behaviours per second when they are active [75].

To face this difficulty, researchers have traditionally been constrained to choose rather to lead measurements which did not imply any computation or to rely on mental complex analytic resolution. This limitation therefore separates the two only possible ways left for traditional behavioural protocols [74]:

- Capturing simple aspects of the behaviour through a particular assay specifically designed to collect this precise kind of data (*i.e.* tube test [76], light dark box test [77], three chamber test [78]...)
- Leading wide observations of the behaviour, reported through manual descriptions by the human observer, which relies on their individual and natural understanding and conceptualisation of the behaviour. These two methods will allow us to introduce the traditional limitations found in pre-computational behavioural analysis.

## Behavioural assay limits

Simplicity and rapidity of implementation made behavioural assays efficient and well standardized. The principal limitation of such methods comes from their extremely low dimensionality. They indeed capture few measurements, often only one [76], which can be linked to several behavioural traits. As a result, behavioural assays may miss aspects of the behaviour and even the ones of interest.

For example, as pointed by Egnor and Branson [74], the tube test was first described and used in 1961 by Lindzey and colleagues to evaluate mouse dominance behaviour [76]. But in 1966, these same researchers tried to use this tube test to predict two other dominance measurements, success in fights and priority in access to food, which are more directly linked to fitness [79]. They counter-intuitively discovered an anti-correlation between such food and fight related dominance traits and results in the tube test. These different tests measure indeed different aspects of mouse aggression.

The three chamber test presented by Nadler et al. In 2004 [78] can also be discussed in such way. This test includes two steps. The first one evaluates social interest by comparing the time spent by the tested animal in a chamber containing another individual with the time spent in a chamber with no other animal. The second one evaluates the preference for social novelty by comparing the time spent by the tested animal in the chamber containing still the first other individual with the time spent in the other chamber where a second conspecific has been introduced. C57BL6/J mice significantly preferred the social chamber in first step and the novel individual chamber in second step.

However in 2010 Pearson et al. replicated the experiment with a change in step two [80]. The novel individual being introduced in the chamber were the first conspecific was installed during phase 1, this primarily encountered animal being then installed in the chamber which was empty of any conspecific during phase one. This experiment failed to demonstrate any preference for social novelty in C57BL6/J mice, proving that this second step of the experiment, was not only testing the preference for a novel individual. The interest of the test mouse could be simply driven by the changes in the environment between the two steps. In this case, if both chamber changes, and that both contain a conspecific, no preference would be expected.

Behavioural assays are highly limited in term of output diversity. Due to behavioural mechanistic complexity, or high dimensionality, such output may inform us on a completely different trait than expected *a priori*. Such example also shows how easily *a priori* opinions and language can shape our interpretation of a behavioural measurement.

This low dimensionality comes with a second burden, the impossibility to discover effects which were not precisely hypothesized by researchers. Assays are thus limited by the *a priori* of researchers on which aspects of behaviour should be affected by the conditions they study.

## **Manual annotation limits**

The manual annotation of behaviours, giving a wider description, seems to overcome such limitations. But while these defaults are still present in less sensible proportions, such methodology brings new limitations.

## **Observer subjectivity**

Subjectivity is a major burden of manual annotation. The observations and their report are biased depending on the observer. The classifications used by two observers can differ leading to significant variations in data annotated [81], [82]. Even the classification produced by one unique observer has been reported to be variable or not consistent along time [83]. A part of this problem can be imputed to the fact that manual annotations are limited by human language. “The identification or classification of actions and activities by human observers cannot always be captured by formal verbal definitions” [84]. The imprecision in subsequent descriptions makes criteria of human classifications hard to be shared and reproducibility between laboratories hard to be reached.

## **Human capacities**

Sensitive and cognitive capacities of the observers also limit deeply human observations. For example, the precision offered by the human visual system limits the perceptual abilities of an observer. Therefore, the visual description of an animal posture cannot benefit from the information present in too small spatial scales. Recognition of rapid movement patterns is also more and more difficult as we get close to human limits in short time scales. Attentional capacities also play a major role in human behavioural classification. For example, at a same time point, it is harder to recognise a social behaviour happening between two individuals which are spread in space. This shows that big spatial scales also constitute complex contexts for human observation. More generally, recording several behaviours of different types at the same time undermines the attentional abilities of an observer. “The number of different behaviours that can be measured concurrently is relatively small” [82], and subsequent annotations offer also a low dimensionality.



Attentional capacities also evolve along time and limit the length of an annotation of stable quality [81]. This means that behavioural sequences implicating a long temporal scale are harder to study with precision. Finally, from a statistical point of view, the shorter the observation durations, the less reliable the rates of production of behaviours ultimately computed.

### **A priori knowledge**

On another side, the necessary presence of a priori knowledge when using manual annotation is heavily bounding its outcomes. An observer can only annotate behaviours which are previously considered as being of interest and which are expected. Researchers are thus considering behaviours annotated by others previously, or ones they noticed themselves. In this way all behaviours used were first described by a human. But as we expressed earlier, human capacities to detect behaviours are limited. As we will see afterward, humans are less good at discovering new behaviours than computers can be [82]. Above this, as for behavioural assays, the choice of the behaviours to annotate in the context of the study of some particular conditions can be unjustified or suboptimal. Some demanding work may be done to annotate behaviours which does not respond to the studied variable when another one would have. If a researcher does not have such *a priori* information, which tells which are the behaviours of interest, he needs to acquire them through trials. The intuitions driving such choices can thus be some false *a priori* knowledge, as language can carry for example. Whatever if the *a priori* opinions turn out to be true or false, such information would not be necessary if the data collection produced results of higher dimensions, and that the researchers did not need to select the behaviours to be measured. We can also argue that Human *a priori*, in the case of animal behaviour studies, can be anthropomorphic and therefore consider the structure of the behaviour in a suboptimal way.

## Human effort

Finally, we can discuss some other aspects of manual annotation which do not lead to theoretical limits in data collection but to practical ones. Direct observation of animals is capital for researchers to have a spontaneous and intuitive understanding of the behaviour of an animal. Although, human observation-based measurements of behaviour are extremely time consuming. Even assisted by computer, annotation takes typically three times the length of the video [82]. This issue limits throughput of such method for financial and delay reasons. Less or shorter experiments including fewer sampled individuals lead to less statistical power and result reliability [85]. Such method has also been described as dull [82]. The boring aspect of manual annotation is not only a matter of comfort. The mental discomfort comes with an increase of error-rates diminishing the quality of acquired data, and inattention which limits the discovery of key behaviours of interest. It is obvious that if nowadays technology can complete such tasks, the working time saved this way for researchers allows way more creative work and more efficient use of human brain doing what it is skilled for.

## Computational analysis of behaviour or computational ethology

In a lot of fields of modern biology, science is shifting toward an interdisciplinary activity thanks to the beneficial interaction with mathematics, computer science and engineering. This is driving changes in the way we measure and model biological objects. The complexity of the analysis of freely moving animals makes this problematic particularly prone to such methodological revolution [82]. We can now expect to overcome a lot of the past limits in our understanding of behaviour by the use of modern tools, in both recording and analysing behavioural data.

### New tools to record the behaviour

In the study of animal behaviour, the first research activity we can revolutionize through technological advances is the process of data collection. New techniques of measurements include coils [86], touch screens [87], infrared sensors [88], laser Doppler vibrometry [89], accelerometers [90], frustrated Total Internal Reflection [91], microphone arrays [92]. The more commonly used method stays behavioural recording through video data, as it is relatively inexpensive, non-invasive and capturing a pretty general representation of what happens in an experiment. Transforming such videos into behaviourally relevant data require the use of computer-vision techniques [74]. In the present work, we used Live Mouse Tracker [93] a tracking computer-vision method which combines detection with infrared depth-sensing, Radio Frequency Identification of each animal and estimation of animal orientation by machine learning. In the case multiple mice are tracked simultaneously, it also monitors the quality of tracking through radio-frequency identification of each individual.

## Tracking as a data acquisition tool

Researchers use tracking systems to study the behaviour of animals on the basis of their postures and positions [74]. Tracking allows to follow points corresponding to animals or more precisely to some of their body-parts. We call trajectory data the list of coordinates of such points over time. On the one hand, tracking can be considered as an evolution from the manual annotation method, in the sense that it also allows the study of freely behaving animals. The behaviour of animals which are not restricted to a specific task permits to evaluate more different traits, it can then be said high-dimensional. Behavioural assays are way more reductionist and low-dimensional. But on the other hand, one of the major advantages of assays is that they offer precise measurements. An advantage that it shares with tracking procedures. Indeed, coordinates of trajectory data allow all kind of geometric measurements, from basic distances and angles to speed and acceleration by derivatives, and way more. By combining the best of those two traditional techniques, tracking data offers a wide range of possibilities to deepen.

First tracking processes have been led manually from video data [94]–[96]. This highly demanding work has the advantage not to suffer from the subjectivity of definitions of behavioural categories used in human annotation. The use of tracked points allows precise further geometric measurements, frame by frame. Such measurements avoid an important part of human perceptual and attentional limits, bringing more precision than direct observation of a video.

This considerable advantage is balanced by the fact that trajectory reliability can depend on the observer. Indeed, manual tracking produced by two different human observers can differ and the precision of the tracking can also suffer from attentional decreases. Finally, the choice of which points to track consists in an *a priori*, but any measurements from those points stay possible. This makes that such trajectory data can serve in processes allowing the discovery of behavioural traits of interest.

Through the technological evolutions of computer-vision techniques, tracking procedures have been progressively automated. Semi-automatic tools, as Mice Profiler [97], which still required human corrections on parts of the timeline, were a first increase in the reliability and efficiency. Finally, fully-automatic tracking systems arose [93], [98]–[105], freeing the process from any correction or control by a human observer. For example, Live Mouse Tracker [93] does not need any human intervention, from the disposition of animals in the tracking arena, to the production of the output database of trajectories.

Such automation conserves all the advantages brought by manual tracking. Trajectories extracted from automated tracking are no more depending on any human agent, what makes it way more reliable [98] and fully reproducible between different laboratories. Automatic methods also allow to benefit as much as possible from the precision of the video data, when human actions could still introduce imprecisions. Finally, such automation increases drastically the throughput of tracking, which can allow more experiments, bigger sample sizes and higher subsequent statistical power [82].

## Live Mouse Tracker specificities

The particular tracking system that we used in this work, Live Mouse Tracker [93], adds particular advantages compared to other automated tracking methods. The depth sensing which replaces basic video recording offers a tridimensional capture of what happens in the environment. It is more than obvious to tell that tracking points in three dimensions rather than two is increasing the dimensionality of acquired data. More than that, the animal-background segmentation of individuals is based on an altitude threshold, rather than a colour contrast one as used in most tracking algorithms [98]. This particularity opens the possibility of environmental enrichment. On one side food, bedding, nest building material and shelter allow sufficiently comfortable conditions for long experiments, here 72 hours, without impacting (negatively) the animal well-being.

Long experiments make possible more reliable behavioural traits measurements what increases ultimately the statistical power. On the other side such enrichment permits to the animals to express wider range of their behaviour, what also adds some richness to the data collected in such context.

The pose estimation through machine learning allows us to record the head and basis of the tail in supplement to the mass centre. This last one being the only point considered by most tracking systems [98]. Three points to track one individual thus also consist in an increase in data dimensionality.

This tracking is designed for social contexts and tracks accurately four mice simultaneously. Their identities are constantly verified by the RFID chip detections. Tracking three points in three dimensions for each of four mice at 30 time points by second for 72 hours in an enriched environment represent an unusually rich data collection for one behavioural experiment.

### **From trajectories to quantitative measurement**

Tracking and more even automated one allows to reduce drastically the limitations encountered on data collection. The trajectory data it produces are rich. Nevertheless, they are generally not statistically exploitable in this form, to evaluate any trait or its response to any condition [74]. No statistical test can compare two trajectories in their raw form of point succession, and such comparison would lack of sense. The common procedure to obtain a statistically relevant material is to treat trajectory data through behavioural classification. Such classified material is then understandable in the same way than the output of the traditional manual annotation.

## The behavioural classification approach

Behavioural classification is the process affecting parts of the experimental timeline to some categories of behaviour. Such categories can describe any action of one or several animal(s). We refer to the complete set of categories by the name of behavioural repertoire. The distribution of such categories over the timeline is then easy to be compared between animal groups or conditions. For example, the most basic information we can measure is the part of the timeline affected to a particular behavioural category. This informs us on the proportion of time during which an individual produces the particular action described by the category.

The traditional manual annotation of behaviour is integrating a manual behavioural classification. Behavioural classification thus can suffer from all limitation listed earlier. Our data collection system, Live Mouse Tracker [93], allows us to avoid an important part of such limitations. This gives the opportunity to continue avoiding such limitations while completing the analysis. As for tracking methods, new behavioural classification methods have progressively been developed to be more automatic, and avoid some limitations.

In order to make the better use of Live Mouse Tracker data, we thus aim at using a behavioural classification method which is:

- Free from observer subjectivity, to be more reliable and reproducible.
- Free from human capacities, to be more precise, reliable and to take into account more dimensions.
- Free from *a priori* knowledge, to take into account more dimensions and be able to discover new behavioural categories through a data-oriented process.
- Free from human effort, to be more feasible in term of human, financial and time resources.



Methods to automatize the behavioural classification can be of three types, depending on the way their behavioural repertoire and the criteria separating their behavioural categories are defined [74] :

- Rule-based classifiers, designed through the definition of rules describing the behavioural structure.
- Example-based Classifiers, trained through supervised machine learning on examples annotated by a human observer.
- Unsupervised classifiers, discovering the behavioural structure through unsupervised machine learning.

We will now describe these three ways to automatize behavioural classification in order to understand their impact on the limitations of classification, and which method would thus be the most appropriate to classify Live Mouse Tracker data.

### **Rule-based classifiers**

The simplest automatic system to classify behaviours are the rule-based classifiers [74]. In such systems, the behavioural repertoire is defined on the basis of thresholds set manually by humans [93], [97], [101], [106]–[109]. These algorithms divide the timeline between behavioural event categories by applying these thresholds on measurements extracted from the outputs of the tracking or other data collection system (Figure 10).

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**Figure 10: Example of some rule-based behavioural event category definitions, given by Martin in 2004 [106].** *“A: Schematic drawing of the arena used to quantify the spontaneous locomotor activity of a single fly. The square arena of 4 cm and 3.5 mm high is covered by a glass plate to reduce the fly vertical movement. The central “virtual” zone of 2 cm diameter is depicted (dotted line) and serves to quantify the spatial distribution of walking. The path trajectory shown represents the first minute of recording. We easily note that the fly follows mainly the border of the arena. B: Graphic representation of the parameters used to determine a fly’s movement. To smooth the jerky movement and removed the wobbling of the fly that occurs at the recording resolution (5 Hz), we established that a movement will be determined when the fly has moved a minimum of 4 mm/s. Conversely, to define a stop, we established that the walking speed is inferior to 2 mm/s. The range between 4 and 2 mm represents the buffer zone.”*

Once a rule-based classifier is developed, this last one does not need any human input to classify the behaviours. This method being fully automated it is obvious that it allows to avoid any human effort from researchers using it. As rules designed for this classification applies always in the same way, such methods are free from observer subjectivity. Their results are thus more reliable and reproducible between laboratories than manual ones. Nevertheless, rule-based classifiers depend on the subjectivity of the rule designer. This means that two classifiers can define with different rules a behaviour with the same name.

The applications of rules also do not depend on human senses, categories defined by thresholds on recorded measurement are thus more precise and reliable. However, even if no observer needs to annotate any behaviour along the timeline, the definition of the rules describing the behavioural repertoire generally results from human observation of the behaviour. It is therefore unlikely that a human would encode a behaviour when its perceptual or attentional capacities do not allow to discriminate it. Such limitations upcoming from the design of rule-based classifiers shows that this method is restricted by *a priori*. This method is thus unable to discover and annotate behavioural event categories which would not have been known previously. This makes rule-based classifiers able to manage fewer dimensions than if they could discover new categories.

We also can add to the advantages of rule-based classifiers that they allow to describe behavioural events occurring simultaneously. Two rules can be independent from each other, what means that the respect of one rule does not imply the respect of another one. In such case, each rule leads to an independent clustering, and the behaviour is thus described through multiple clusterings. An animal could then be described as performing two behaviours, each one defined by one rule, at the same time point. This is the case in the classifiers implemented in Mice Profiler [97] and Live Mouse Tracker [93]. The behavioural co-occurrences diversify the states in which animals can be described at each time point. We thus can say that this particularity gives a potential to manage dimensions.

### **Example-based classifiers**

More complex systems can be developed in order to let computers determine more optimal segmentations than humans would set manually [74]. A researcher may have a hypothesis about the behavioural categories of interest, without knowing which measurement could allow to discriminate it or the exact threshold to use. In this case, supervised machine learning can be used, rather than setting parameters of the classification manually [100], [107], [110]–[117]. In such method, the researcher only needs to manually annotate a restricted set of frames for the behaviours of interest. The supervised machine learning is then fed with this set of examples, in order to extract the parameters of the classification function. The parameters resulting from this machine learning finally permits to extend the manual classification led on examples by replicating automatically annotations on the entire dataset.

Example-based classifiers need annotation examples, as a human input, to classify the behaviours. Such methods therefore require a certain human effort from researchers training it. The amount of manually labelled frames is generally correlated with the quality of the subsequent automated classifier. However, once trained, the classifier can extend the annotations without any human effort. As the parameters of this classification are extracted from manual annotations, such methods are limited by the training observer subjectivity. Results stay more reliable than fully manual annotations, because the parameters extracted are rigorously defined, stable and directly applied on recorded measurement. They are also more reproducible between laboratories in the case the same training examples are used.

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Figure 11: Example of a supervised learning pipeline, from Kabra et al. in 2013 - JAABA overview [114].

*“Input trajectory (top left): the x, y position of a fly over 1,000 s of video is plotted in black (cyan portion corresponds to other plots). This video and trajectory are input to JAABA. JAABA interface (bottom): the users interact with the JAABA interface to encode their definition of the behaviour (for example, touching). Users are shown video of the animal (image) overlaid with its tracked position (bold triangle) and its future and past trajectory (bold line). They encode their definition by labelling frames (top timeline) in which they are certain the animal is (red) or is not (blue) performing the behaviour. Unlabelled frames are indicated in black. These labels are passed to the JAABA machinery, which creates a new behaviour detector. Visualizations of this behaviour detector are returned in the middle and bottom timelines, which show the detector's predictions (colour) and confidence (saturation). JAABA machinery (grey shading): the underlying JAABA machinery inputs the animals' trajectories. 'Per-frame' feature time series are computed from the input trajectories (examples in first row). Each plot corresponds to an example per-frame feature. The pink line indicates the frame shown. 'Window'-feature time series are computed from the inter-animal distance per-frame feature (second row). This window feature-based representation and the manual behaviour labels are input into the learning algorithm (green), which finds the automatic behaviour detector (orange) that inputs the window features and best predicts the input labels.”*

However, an observer needs to annotate some behaviours along part of the timeline. It is thus likely that a human would only annotate behaviours that its perceptual and attentional capacities allow to discriminate. Such limitations upcoming from the use of supervised machine learning in example-based classifiers shows that this method is restricted by *a priori*. As for the rule-based one, this method is unable to discover and annotate behavioural event categories which would not have been known previously. This makes example-based classifiers able to manage fewer dimensions than if they could discover new categories.

Example-based classifiers also present the advantage to allow the description of behavioural events occurring simultaneously. An observer can annotate a part of the timeline with two labels and other parts independently with one or the other, what means that belonging to one category does not imply belonging to another one. In such case each category of annotation leads to the training of an independent clustering, and the behaviour is thus described through multiple clusterings. An animal could then be described as performing two behaviours, each one defined by the parameters extracted by learning on different example annotations, at the same time point. This is the case in the classifiers implemented in JAABA [114] (Figure 11). The fact that such algorithm considers animals producing multiple behaviours simultaneously increases the diversity of states they can describe at each time point. Such ability of example-based classifiers therefore offers a higher dimensionality.

## Unsupervised classifiers

Previously mentioned methods of behavioural classification assume that the behavioural categories of interest are known *a priori*. As explained earlier, such necessity of *a priori* prevents the discovery of unpredicted category and therefore limits the number of behaviours which can be considered. In the case of animal studies, we can also question the legitimacy of a human observer to define the categories clustering the behaviour of an animal; anthropomorphic biases can limit our understanding. The behaviour of a human is also potentially conceived differently between its producer and an exterior human observer. We are not even sure that the category we conceive consciously are an appropriate description of the neuronal activity encoding our own behaviour.

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**Figure 12. Example of an unsupervised learning pipeline, from Berman et al. in 2014 - Overview of the data analysis pipeline [118].** *“Raw images of the *D. melanogaster* are segmented from the background, rescaled to a reference size and then aligned, creating a stack of images in the co-moving and co-rotating frame of the fly. These images are then decomposed via PCA into a relatively low-dimensional set of time series. A Morlet wavelet transform is subsequently applied to these time series, creating a spectrogram for each postural mode separately. After normalization, each point in time is mapped into a two-dimensional plane via t-SNE (Maaten and Hinton et al., 2008). Lastly, a watershed transform is applied to a Gaussian-smoothed density over these points, isolating individual peaks from one another.”*



*“How do we know that our definitions of behaviour correspond to behavioural distinctions that are meaningful to the animal or relevant for neurobiological control?”<sup>1</sup>*

The alternative consists in avoiding the use of any a priori knowledge by using unsupervised machine learning approaches on acquired data. Such method mines the inherent structure of behaviour by clustering parts of the timeline into behavioural categories depending on their similarity or differences [75], [118], [120]–[129] (figure 12).

Unsupervised classifiers do not need any human observation to train the machine learning, and do not need that any human set rules delimiting the categories. Such method therefore avoids all human effort to define the behavioural repertoire, from both user and developer. As humans do not interfere between the acquired measurements and the classification extracted, unsupervised classifiers are free from observer subjectivity. Their results are therefore more reliable and reproducible between laboratories than ones affected by observer biases. Nevertheless, unsupervised classifier categories are dependent on the data set that they cluster to extract it. This means that two classifiers, which have learned from different datasets, can define with different parameters a behaviour with the same name.

Such classifiers do not depend on human senses, the categories defined by thresholds on recorded measurement are thus more precise and reliable. Moreover, even the choice of the categories composing the behavioural repertoire do not result from human observation of the behaviour. It is therefore possible for an unsupervised classifier to segregate a behavioural category that human perceptual or attentional capacities would have not allow to discriminate it. Such ability of unsupervised classifiers shows that such method is not restricted by a priori. They are thus able to discover and annotate behavioural event categories which would not have been known previously. This makes unsupervised classifiers able to manage more dimensions than previously described methods. "Unsupervised learning software has a major benefit over supervised learning: it allows for the discovery of novel movements and recognition of deviations from usual movements that are not visible to human eye. It provides an unbiased reflection of the behaving animal" [130].

Several unsupervised behavioural classifiers have been developed since 2001 [75], [116]–[118], [120]–[126]. All of them were based on a single global clustering taking all features in account. This allows that the classification benefit from all dimensions collected to map the diversity of behaviour. However, the drawback is that such classification does not allow the description of behavioural events occurring simultaneously. One clustering divides the timeline with a certain number of labels, that are all mutually exclusive, what means that belonging to one category implies not to belong to another one. Such methods therefore describe animals performing only one behaviour at a time. This can be seen as a limitation on the diversity of states in which an animal can be described at each time point. Such limitation of those unsupervised algorithms therefore restrains the dimensionality they can manage.

## **SUCCUBE - the targeted behavioural classifier**

The unsupervised classifiers allow to avoid all limitations cited previously, but all past algorithms were based on single clusterings and thus did not allow behavioural categories to co-occur. Even if multiple clustering has always been used in supervised classifiers as rule-based and example-based ones, no technical limitation prevents its use in an unsupervised algorithm. In order to combine all advantages, we designed the first unsupervised behavioural classifier based on multiple clustering. SUCCUBE stands for System for Unsupervised Classification of Co-occurrent Underlying Behavioural Events.

### **The problem of event co-occurrence - the need for multiple clustering**

Animals can perform several independent actions simultaneously. For instance, they can be walking or stopped and at the same time they can be sniffing or not. Assuming that only one behavioural event category is produced at a given time is not adapted to the complexity of behaviour. Only multiple clustering approaches can manage such event co-occurrences.

**Figure 13.** Example of a behavioural event category co-occurrence, from Datta et al. in 2019 – **Challenges in Computational Ethology [131]**. *“A key challenge facing any segmentation of continuous behaviour into components is illustrated. When the mouse is sniffing and walking at the same time, is that a compositional behaviour whose basis set includes “walk” and “sniff,” or is “walking+sniffing” a fundamentally new behaviour?” Such question can not be assessed if the first observation that the animal is “sniffing and walking at the same time” is not available.*

## **Approaches of multiple clustering**

Our strategy requires the development of a multiple clustering displaying more than two non-redundant clusterings from one global high-dimensional tracking database. The method being unsupervised, we do not possess any given clustering to start with and the number of clusterings to be detected is not specified by the user. We target a simultaneous computation of multiple clustering solutions, in order to avoid any a priori distinction in term of importance of the different behavioural event categories. For example, we do not want “walking” category to take the advantage on the “sniffing” one in the clustering process (figure 13-14). We thus investigated the kind of procedures generally applied in such multiple clustering contexts [132].

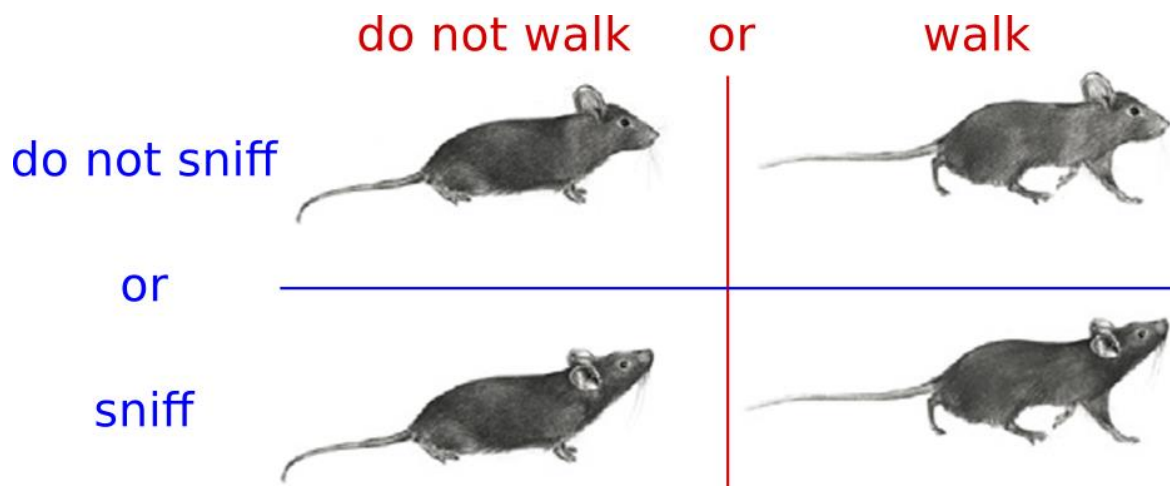


Figure 14. Example of a multiple clustering which accounts for behavioural event category co-occurrences. Here the behaviour is clustered in two different ways, *to walk or not to walk* in red for the first clustering and *to sniff or not to sniff* in blue for the second one. The situation in which the animal is “sniffing and walking at the same time” is classified in walking as when the mouse is only walking and in sniffing as when the mouse is only sniffing. A fourth situation thus need to be considered, in which the animal is neither sniffing nor walking. This last is classified in both not walking as when the mouse is only sniffing and not sniffing as when the mouse is only walking.

One way to obtain multiple clustering solutions from one space is the orthogonal subspace projections [133] (figure 15). After a first clustering of data in the complete space, the dimensions segregating the clusters are identified. Then data are projected in a subspace orthogonal to these dimensions and a new clustering is ran in this new subspace. This process continues iteratively displaying a new clustering at each iteration. As orthogonal dimensions are decorrelated, we expect the segregations of clusters in orthogonal spaces to be different. It is said that “Orthogonal concepts share no or only few common attributes”. Such method is particularly adapted to high dimensional datasets, because the more successive subspaces are used, the higher number of different clusterings we obtain. However the iterative process of such method is not desirable as we do not hypothesize any hierarchy between clusterings.

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**Figure 15.** Examples of orthogonal subspace projections, from Müller et al. [132]. *Two 3D graphs representing data points clustered in three groups (blue, green and red points) in a first subspace represented in **black** (A: axis; B: surface). The orthogonal subspace in which data can then be projected for next clustering are represented in **orange** (A: surface; B: axis).*

Some orthogonal subspace projection algorithms use Principle Component Analysis (PCA) to select the strongest principle components of cluster means [132]. This dimension reduction technique allows to determine a set of dimensions or subspaces which is the most implied in the segregation of the previous clustering. The afterward projection of data in the orthogonal subspace can then easily be done by selecting all principal components that explain few of the variance between the means of previous clusters. This notably helps with losing less dimensionality at each projection iteration. We can mention that PCA is particularly adapted to the research of orthogonal subspaces because of its orthogonality criterion.

Another method for multiple clustering is multiple spectral clustering which enforces different subspaces [134]. Here clustering does not precede the selection of subspaces, but cluster quality and subspace difference are both optimized through a simultaneous objective function [135].

The easiest way to measure the redundancy between two variables through their correlation coefficient. However, this aspect of redundancies is known to be limited to the capture of linear dependencies among random variables. More complex approaches are based on mutual information. In 2005, Gretton et al. proposed the Hilbert-Schmidt Independence Criterion [136] to measure the statistical dependence between subspaces. This allows to search for independent subspaces, in each of which the algorithm can then proceed to a clustering. The authors also showed that this criterion also allows to realize an Independent Component Analysis (ICA).

## **Machine learning pipelines in animal behaviour studies**

In behavioural biology, a number of technological breakthroughs allowed to collect progressively larger and more detailed datasets. At some point, the new diversity, complexity and high dimensionality of such datasets outstripped the analytical capacities of researchers. Some of those researchers made use of machine learning methodologies in order fill the gap and extract knowledge in this new context.

In their review “Applications of machine learning in animal behaviour studies”, Valletta et al. described how such methods are applied in the field [137]. In the case of unsupervised learnings, which are the methods we can use in our case, they present the two processes, dimensionality reduction and clustering.

Dimensionality reduction techniques are used to perform feature extraction. They allow to reduce the number of dimensions considered while focusing on a smaller number of more informative ones. Such process permits to unravel the structure linking the different variables measured. PCA is the most used method of this kind as it allows to reorganize dimensions in a set of orthogonal axes ordered from the more informative to the less informative one. ICA is another dimensionality reduction technique which can display a set of orthogonal dimensions in a potentially fewer number than in the original set.

Clustering is used to unravel the structure linking the different data points on the basis of the variables which have been measured for each of them. The K-means is the most used algorithm of this kind as its simplicity makes it easy to understand and to compute. The Hierarchical Agglomerative Clustering (HAC) is also widely used because it presents the advantage not to need any a priori number of cluster and to display the exhaustive list of points grouping. However this last method is particularly computationally expensive on large data-sets. Finally “Gaussian mixture model is a simple but powerful model that perform clustering via density estimation”. In this algorithm the distribution of data is modelled as the sum of several Gaussian distributions. Each data point is then affected to a cluster depending on which of the Gaussian distributions give the highest probability at its coordinates.

These two unsupervised machine learning approaches can be used together. In this case, dimensionality reduction is generally used before clustering, which will then be based on the previously reduced set of dimensions. It is the case in the application of unsupervised machine learning developed in Valletta et al. [137] (Figure 16).



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Figure 16. Example of an unsupervised learning pipeline from Valletta et al. in 2016 - Pheasant eggs case study analysis pipeline [137]. *In this case study the authors first used PCA as a dimension reduction technique in order to perform feature extraction. They followed with some k-means and hierarchical clusterings on the previously extracted feature set.*

## SUCCUBE pipeline

Live Mouse Tracker [93] provides us with rich trajectory data, which can be turned into a large set of trajectory-based features [74] through simple geometric measurements at each frame or between consecutive ones, as in JAABA algorithm [114]. Machine learning then allows to both reduce dimension and finally cluster frames [137]. The dimension reduction used to extract a new feature set could ideally allow to select a set of subspaces, in order to cluster frames independently for each subspace and reach multiple clustering. PCA and ICA both rely on an orthogonality criterion which is suited for orthogonal subspace projections [132].

The PCA would aim at the maximal dimensionality reduction. This is not our objective; we here consider high dimensionality as a richness. The more subspaces we select, the more behaviours can be discriminated at a single frame. We can also add that the distribution of the mixed components would likely be more normal as the sum of random distributions converge toward a Gaussian one [138]. Such normal distributions are not particularly prone to segregate in multiple clusters.

In the opposite, the second ICA criterion, non-Gaussianity, ensure to obtain components which are the least normally distributed [139]. Also the consideration of mutual information as in the Hilbert-Schmidt Independence Criterion [136] provides dimensions sharing less information which are supposed to lead to more different clusterings. In order to maximise the number of co-occurrent behavioural category at a single frame, the complete data space needs to be projected into a maximal number of orthogonal subspaces. On the basis of ICA, this can be achieved by projecting data into each Independent Component (IC) separately for a unidimensional clustering.

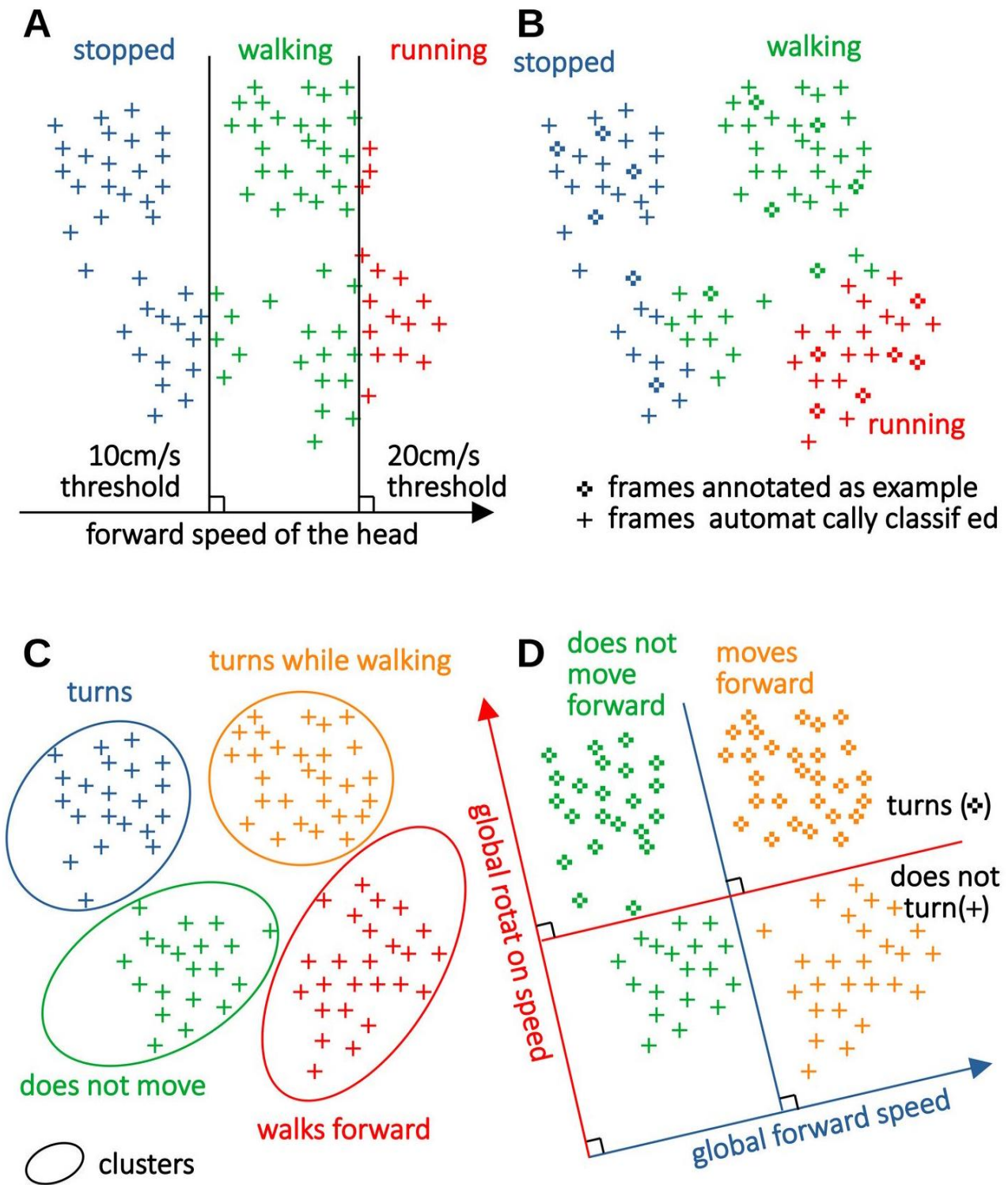


Figure 17: Examples of the different methods of behavioural classification of a same dataset of time-points.

A: Rule based classifier defining « **walking** » events as ones where the speed of the head of the individual is superior to an arbitrary threshold of 1 centimetre per second. Under this threshold the animal is considered as « **stopped** ».

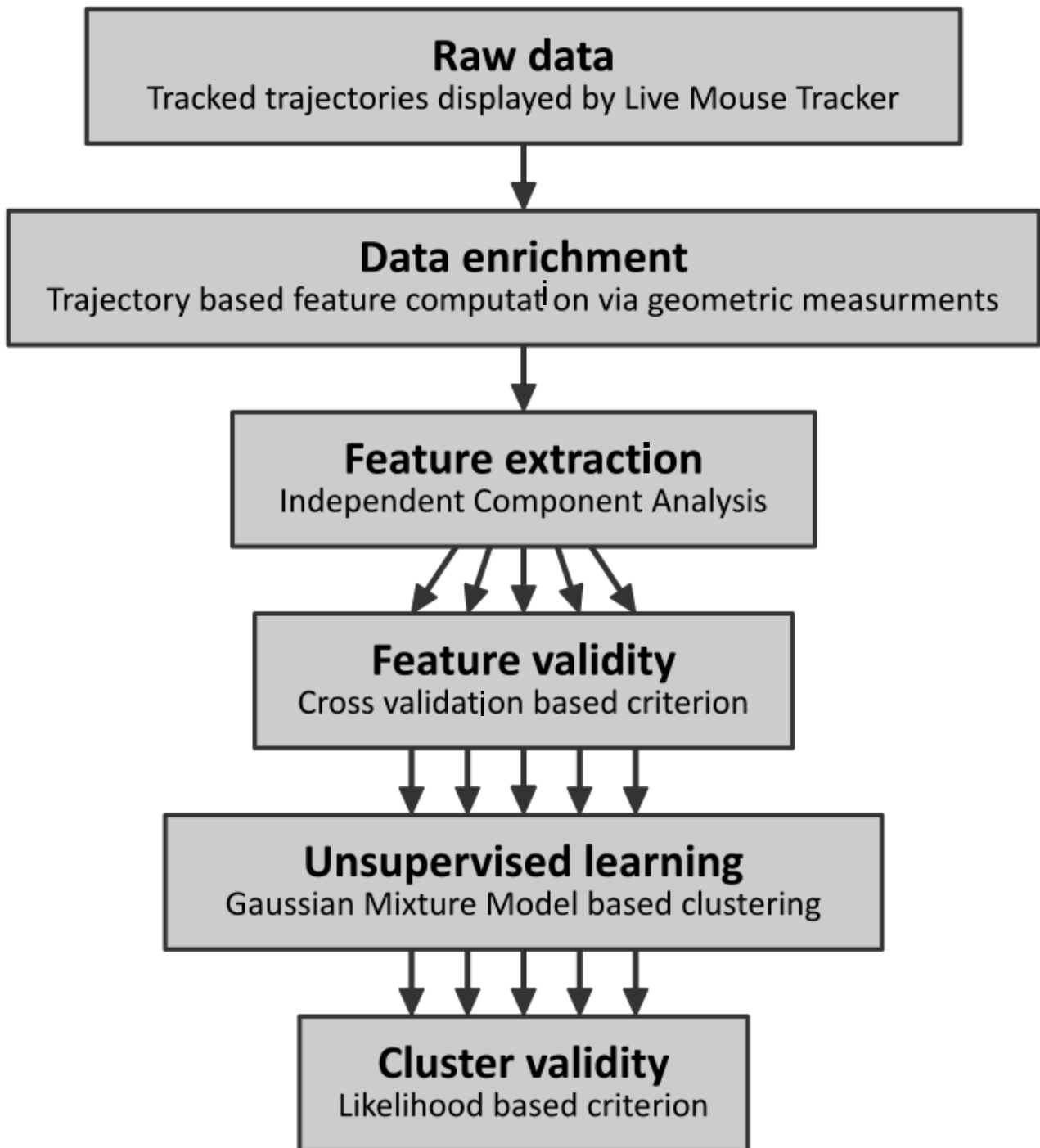
B: Example based classifier trained for the three behavioural event categories « **stopped** », « **walking** » and « **running** ». The supervised learning is here trained on the basis of six example frames manually annotated by an expert for each category. This training set is represented by the special points ( ) and the classification is automatically extended to the other time-points ( ).

C: Unsupervised classifier discovering four clusters of behavioural events, named afterwards « **does not move** », « **walks forward** », « **turns** » and « **turns while walking** ». Clustering techniques could be for instance: K means, K medoids, hierarchical clusterings, DBSCAN, OPTICS or expectation maximisation techniques. Such techniques need the number of clusters, here four, to be specified.

D: SUCCUBE classifier discovering two independent dimensions named afterwards « **global forward speed** » and « **global rotation speed** ». Each dimension led to the extraction of one threshold segregating two behavioural event categories. The « **global forward speed** » allows to segregate the « **moves forward** » and « **does not move forward** » categories. The « **global rotation speed** » allows to segregate the « **turns** » ( ) and « **does not turn** » ( ) categories. The number of dimensions and clusters used is optimally selected by SUCCUBE algorithm, without any user specification.

Such unidimensional clustering also ensures the subsequent segregation to be expressed in the simplest way, one unidimensional threshold between each cluster. Gaussian mixture model based clustering is particularly suited to produce such clustering on one dimension distributed in a non-Gaussian way. One non-Gaussian distribution requires very probably a mixture of at least two Gaussian distributions in order to be satisfyingly modelled. The use of GMM based clustering thus ensures that most of the ICs will lead to a clustering segregating at least two behavioural event categories.

This complete process, called SUCCUBE, therefore allows to extract multiple clustering of behavioural events from tracking data (figure 18). The behavioural event categories extracted from different clustering are independent, in the sense that they can happen at the same frame or not (figure 17). In order that this process can be considered and used without any human supervision, all parts of the analysis need to be parametrized depending on data. An important part of the present work therefore resides in the design of the criteria for the number of ICs extracted through ICA and for the number of Gaussian distributions used in GMM based clustering. These criteria are developed in the material and method chapter. As it is acquired without supervision, the richness of the obtained behavioural repertoire is dependent on the diversity of tracking data used as input.



*Figure 18. The unsupervised learning pipeline of SUCCUBE algorithm. We first used ICA as a dimension reduction technique in order to perform feature extraction. We followed with some GMM based clusterings on each previously extracted feature set. The division of arrows represent the fact that each IC obtained from the ICA is then treated separately in a particular GMM based clustering in order to achieve multiple clustering.*

## A step toward Behavioural building blocks

The discipline of behavioural classification aims to segment a more and more precise behavioural repertoire. Such tendency to optimise the training of behavioural classifiers thus made the link with another fundamental question of behavioural biology, the research of fundamental units of behaviour. All complex behaviours would then be interpretable as a construction made from the association of those behavioural building blocks [74]. More than providing an efficient and compact description of behaviour, such building blocks would allow to better understand the higher structure of behaviour. The more we decompose the behaviour toward those elementary blocks, the more we can unravel the structure of the behaviour they compose (figure 19).

Fentress and Stilwell first mentioned behavioural components in 1973 in their article “Grammar of a Movement Sequence in Inbred Mice” [140]. They discovered “hierarchical principles of organization by describing in detail the sequences of face grooming components in mice”. They introduced this novel approach of the behaviour being built by association of simpler components, with the idea that such building blocks are associated successively.

In Gris et al. 2017 [130], the behaviour is hierarchically broken into parts. The complete potential of behaviour, the ethome, is first restricted to a sub-ethome depending on the environment. This sub ethome is then dissected into easily interpretable units of behaviour, the ethograms. Such complete actions, that we can observe in a reproducible way, are then understood as a sequence of different movements. Those movements are themselves made of a succession of poses, which can finally be sampled as instantaneous snapshots.

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**Figure 19. Structure of behaviour from Gris et al. In 2017 [130].** *“Ethome of an animal consists of numerous sub-ethomes (a complete set of ethograms in a specified environment), which consist of ethograms (sets of repeatable, predefined, trainable, or innate movements), which consist of movements (the smallest complete motion). Movements are built from poses (postural snapshots in time). Examples of mouse behaviour are presented in the pyramid.”*

This highly practical vision allows to match the behaviour with our photographic way of acquiring data. But if we can see a movement as a succession of postures, we could see postures emerging from an assemblage of elementary movements. In the end, the behaviour is made of muscular activity, orchestrated by the brain to coordinate it. The concept of motor primitives inherited from microstimulation field seems to account for a different conception of building block association. “Complex movements are often described as the summation of simpler motor primitives” [141]. The summation operator as the concept of “muscle synergies” both seem to represent an idea of



simultaneous interaction of multiple motor primitives. This functional approach of how the behaviour is generated demonstrates that some behavioural components can be produced simultaneously in a coordinated way and thus co-occur. We could thus say that the behaviour deserves to be dissected in behavioural building blocks that can be associated both successively and simultaneously. Such units can take two forms, postures for the ones characterized through the position of the animal and its parts and movemes [142] for the ones characterized through the motion of the animal and its parts.

SUCCUBE presents four advantages in the research of such behavioural building blocks:

- First this automated method allows to treat tracking data acquired at a fast frame rate, 30 per second for Live Mouse Tracker[93]. This reduces the time scale of elements, multiplies the number of successive postures observed and divides motions in shorter movemes.
- The ability to classify co-occurrent behaviours allows their simultaneous association.
- The unsupervised character of the method allows to free the classification from human perceptual limits. From their high dimensionality to their small scales in both space and time, behavioural elements can lack sense in the usual interpretation we are used to conceive behaviour through. A human observer characterizes the behaviour depending on its understanding. SUCCUBE does not know such limitations and treats all dimensions available at the smaller scales sampled.
- Building the behavioural repertoire on the basis of a big data-set makes classified elements more reproducible and statistically relevant. Gomez\_Martin and colleagues argued that “the biggest service of big behavioural data would be to promote the development of new unifying frameworks for animal behaviour. This may even lead to the identification of fundamental behavioural units (let us call them ethons).” [143]

## The need for genetic background diversity

SUCCUBE is a machine learning algorithm, and thus needs to learn from a tracking dataset in order to propose a classification of behavioural categories. The quality of the classification depends on the training dataset. It depends on its size. The long experiments offering numerous frames and the numerous replications of the experiments on a large population of individuals are major factors of the resulting classification quality. But it also depends on its diversity. The long experiments offering multiple phases of behavioural activity, the enrichment of the environment and the presence of both sexes in the sample panel all contribute to the collection of a more diverse training dataset. A last diversity can be taken in account, the diversity of the genetic background. The use of multiple mouse strains will participate to make our classification more complete and accurate.

The use of several strains presents also another advantage, more specific to our *Shank3* haploinsufficiency question. In human, *SHANK3* haploinsufficiency is a highly penetrant cause of neurodevelopmental disorder [38]. In C57BL/6J mice, *Shank3* homozygous knock-out causes remarkable behavioural alterations when behavioural differences caused by *Shank3* heterozygous knock-out are scarce [50]. The severity of *Shank3* haploinsufficiency could be modulated by other genes from the genetic background [57]. Such background effects have been found on other models as, for example, heterozygous deletion of the 16p11.2 BP4-BP5 locus. Deleted C57BL/6N mice did not display any social interaction alterations, when some can be found on a F1 C57BL/6N × C3B hybrid genetic background [144]. The study of *Shank3* haploinsufficiency effect on the behaviour of animals with a different genetic background than C57BL/6J could potentially unravel such effects. Analysing the behaviour of multiple mouse strains thus can allow us to select candidate backgrounds to study *Shank3* haploinsufficiency.

## The Collaborative Cross

In order to base our behavioural classification on diverse genetic backgrounds and to test the impact of *Shank3* haploinsufficiency on other backgrounds than C57BL/6J we thus need to use a panel of strains. We chose to use strains from the Collaborative Cross.

### What is the Collaborative Cross?

The Collaborative Cross (CC) is a large panel of recombinant inbred lines. Such strains being inbred, they allow to produce unlimited number of genetically identical individuals [145]. As in most widely used strains, C57BL/6J for example, a long inbreeding leads to a high phenotypic homogeneity. Such similarity of individuals is an important factor of the high statistical power of laboratory animal studies. CC are not simply inbred, but recombinant inbred lines. Recombinant strains are obtained by crossing several parental inbred strains. Their chromosomes incorporate a permanent set of recombination events, between chromosomes inherited from the parental strains. In the case of CC strains, they are derived from eight parental inbred strains: A/J, C57BL/6J, 129S1/SvImJ, NOD/LtJ, NZO/H1LtJ, PWK/PhJ, WSB/EiJ and CAST/EiJ.

Genetic analysis showed that this diverse set of founder strains allow to capture almost 90% of the genetic variation known in the *Mus musculus* derived laboratory mouse genome. Such genetic diversity is significantly superior to what any other resource captures. The distribution of this diversity is also more uniform and has no blind spot. [146] With over 300 pre-CC strains [147] and around a hundred, today 84, of fixed fully inbred CC strains, derived from eight different founder strains, the CC is nowadays both the largest panel of recombinant inbred lines and the one which covers the largest genetic diversity. The CC thus serves as a genetic reference population.

In order to lead genetic analysis, the scientific community needs to genotype the CC strains. The fact that each strain needs to be genotyped only once is advantageously cheap in relation to the extensive number of studies destined to use it [145]. As each of their segment belongs to one of the eight founder strains, which are all already genotyped, the CC genome reconstructions are easy. Crossing genomic and phenomic information is the base of quantitative trait locus analyses which permit to find loci implied in the traits of interest [148].

The CC has been “designed specifically for complex trait analysis”. The goal was to provide researchers with a mammalian model resource matching our actual computational, statistical and genomics resources. The vast potential phenotypic diversity hidden in the common inbred strains is expressed by mixing them in many different combinations. The high number of strains allows to compute reliable statistics that can support genetic effect and phenotypic correlations. The controlled randomization of genetic factors between strains allows causal inference and thus permits to use particular statistic methods as quantitative trait locus [145].

## The generation of the Collaborative Cross

The Collaborative Cross is a panel of eight-way recombinant inbred lines. The eight founder strains have been selected to capture a high level of genetic diversity, higher than any previous resource [146]. Five of them are classical laboratory inbred strains: A/J, C57BL/6J, 129S1/SvImJ, NOD/LtJ and NZO/H1LtJ. The three others are wild-derived inbred strains representing the three major *Mus musculus* subspecies: PWK/PhJ for *M. m. musculus*, WSB/EiJ for *M. m. domesticus* and CAST/EiJ for *M. m. castaneus* (figure 20) [149], [150].

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Figure 20: Eight mice belonging each to a different founder strain of the Collaborative Cross, from UNC computational Genetics.

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**Figure 21: Crossing scheme that led to the inbred lines of the CC, from Panthier and Montagutelli in 2012 [147].** *“The crosses realized in first generation determine the parental origin of chromosomes X and Y and of mitochondrial DNA in the produced line.”*

The panel of eight-way RI strains, is made of new strains, crossed independently from each other. In order to obtain strains derived from the eight progenitor strains, with balanced genomic contributions, a particular breeding scheme, the eight-way funnel, is needed (Figure 21).

The individuals of the founder strains are considered as part of the G0 generation. The hybrids progeny of their intercrosses constitutes the G1 generation. The eight G0 founder strains allow theoretically 56 different combinations in G1 generation. Then, G1 individuals which do not share any G0 parental strains are crossed in order to obtain the four-way G2 generation. In the same way than previously, G2 individuals which do not share any G0 parental strains are crossed in order to obtain the eight-way G2:F1 generation [151].

All genes of the future CC strain are present in this G2:F1 generation. The contribution of the eight founder strains to G2:F1 are theoretically balanced. The following steps consist only in sib-mating in order to complete inbreeding and increase the homozygosity [150]. We will refer to each successive generation by G2:Fn, n being the number of generations, starting at 1 on G2:F1 [151].

Eight-way RI strains are expected to achieve 99% inbreeding by generation 23 [145]. In practice we consider the strain fully inbred at approximately G2:F22 [152]. Each CC strain is generated by a different eight-way breeding funnel. Each resulting CC strain genome is thus made of a unique random and independent combination of the eight founder strains genetics [151].

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**Figure 22: Example of the genetic composition of a pre-CC line, from Panthier and Montagutelli in 2012 [147].** *“It capture in average 143 chromosomic segments originating each of one of the eight parental lines. Some segments are here still not fixed at the homozygous state.”*

This theoretical mating scheme hides an unanticipated challenge. On all independent eight-way funnels initiated to generate a CC strain, a majority became extinct [149]. The mating scheme is thus progressively optimized to minimize unpredictable genomic interactions between strains [145]. In order to avoid mixing strains known to have an unproductive or even infertile cross, “The current design avoids (NZO × CAST) and (NZO × PWK) hybrids, which are reproductively incompatible, and (PWK × 129) males, which are infertile” [151]. The CC, which was originally planned to include a thousand of strains [145], concretely struggle with accumulating more than a hundred of them.



## Insights on the Collaborative Cross

Genome-wide analysis of the incipient strains have shown the high genetic diversity between them [150]. Depending on the breeding scheme producing them, CC strains are theoretically supposed to capture an average of 135 unique recombination events [145]. It has been shown that, in practice, the genome of a CC line is made of an average of 143 chromosomal segments, each inherited of one of the eight concurring parental strains (Figure 22) [150]. And even if those recombination sites are dense, they are homogeneously distributed on the complete genome. The same study controlled that, as in theory, each of the parental line contribute in a balanced way, around 12.5 %, to the origin of such segments. The inheritance of founder haplotypes respects the expected frequencies [149]. A panel of numerous and genetically diverse recombinant inbred lines, with dense and homogeneously distributed recombination sites, delimiting segments with balanced allele frequencies, has all qualities required for a resource of system genetics. All of those characteristics are increasing the statistical power “to map causative loci and understand complex disease-related traits” [150].

## Our advantages in the use of the Collaborative Cross strains

In 2018, Molenhuis et al. used 63 lines of the CC in order to study the variation of autism related behaviors between those strains [153]. Their work gives a first idea of the locomotor activity, social interest, grooming frequency and tendency to display stereotyped behavior of all those CC lines. They even found a locus potentially implied in route tracing stereotypy on the basis of quantitative trait locus analysis. We will preferentially use CC lines that have been studied in this article in order to be able to compare our results with the one previously published by this team.

In 2005, Zou et al. described the new approach of Recombinant Inbred intercross (RIX) [154]. RIX are produced by crossing the different recombinant inbred line of a same panel together (figure 23). They are the diallel crosses from this panel. Threadgill and Churchill announced in 2012 that “future applications of the CC will exploit this strategy of recombinant inbred intercrosses”. Such approach adds a new generation of diversity, with the square of the original number of CC lines. But RIX crosses also theoretically present statistically lower phenotypic variances. This means that analysis led on RIX individuals will have more statistical power.

In this work we want to study *Shank3* haploinsufficiency on new background. But, in our facilities, we maintain the mutation on C57BL/6J background. The easiest way to obtain mutant mice on new backgrounds using the collaborative cross is thus to realize intercrosses between CC lines and mutant C57BL/6J mice. C57BL/6J being one of the parental strain of the CC, their genetic distance to CC strains is in average the same than the distance between CC strains. We can thus hypothesize that our C57BL/6J X CC crosses will have similar characteristics with RIX populations, including the lower phenotypic variances which offers better statistical power.

**Figure 23: scheme of the production of RIX hybrids, from Zou et al. In 2005 [154].** *“The relationship between the parental strains and the derivative RIs along with the relationships between RIXs is shown.”*

In 2012, Sun et al. led a study on mouse brain transcriptome with the idea that it “may play a critical role in behavioral phenotypes”. They evaluated the transcriptional differences between the whole brain and specific regions and between two strains, which count as part of the CC founders, C57BL/6J and NOD/ShiLtJ. They discovered that inter-strain transcriptional differences are greater in discrete brain regions than what is detected when the whole brain is compared [155]. We could hypothesize that the behavioral phenotype, which depends on those different transcriptional rates, follow the same tendency, and that the separation of the behavioural phenotype in more sub-parts allow to discover even more variations between strains.

## Objectives and hypothesis

We investigate mouse behaviour in order to understand the impact of mutation on genetic models of neuropsychiatric disorders. Here the mutation of *Shank3* gene provides us with a mouse model of autism spectrum disorder. Technological breakthroughs allow behavioural analysis progresses through new data collection methods. Such methods gather more data of greater precision with less biases and effort. In our case we investigate mouse behaviour with Live Mouse Tracker[93].

Our main and theoretical goal is to empower our behavioural analysis capacities by identifying new behavioural categories which would have not be seen by a human. In order to do so, we chose to develop a new behavioural classifier. If the way of collecting behavioural data has evolved, the way to measure and understand the behaviour needs to match the high quantity of data and absence of bias. We thus target the development of a classifier both unsupervised and able to classify each time-point in several behavioural event categories. We hypothesize that such classifier, SUCCUBE, would allow to discriminate categories which would have not been considered without this one. But the algorithm needs to be confronted to real mouse data in order to proof its capacity to bring a new point of view on behaviour and shed light on new behaviours of interest.

We then applied SUCCUBE algorithm to two practical goals:

- Identifying genetic backgrounds of interest for the study of autism spectrum disorder models.
- Finding a new heterozygous model for *Shank3* haploinsufficiency.

In a first time, in order to study the genetic background, we chose to train SUCCUBE algorithm on a large dataset including 13 different mouse strains. This would allow us to gather behavioural profiles of 12 Collaborative Cross strains compared to C57BL/6J. We hypothesis that some of those strains would differ from the reference C57BL/6J. Such differences will allow us to select strains to cross with C57BL/6J heterozygous for *Shank3* in order to study this haploinsufficiency on mixed genetic background. This behavioural profile and the differences it displays will also be usable for future studies or model development requiring to select some Collaborative Cross strains for their behaviour.

In a second time, in order to study *Shank3* haploinsufficiency effect, we chose to apply the previously trained SUCCUBE algorithm on the crosses determined on the bases of first results. This would allow us to gather behavioural profiles of *Shank3* heterozygous mice from mixed genetic backgrounds compared to their wild-type littermates. We hypothesise that, in some of those crosses, heterozygous mice would differ from wild-type ones in term of solitary and social behaviour. Such differences will allow us to select one or several genetic background(s) which can be used as models for behavioural differences caused by *Shank3* haploinsufficiency. This behavioural profile and the differences it displays will be usable for future studies on *Shank3* haploinsufficiency effects and potential treatments targeting behavioural restoration.

# **Material and methods**



## mouse cohorts

### First cohort - Collaborative Cross strains

We experimented with mice from C57BL/6J strain, and 12 Collaborative Cross strains. Those strains were CC001, CC002, CC012, CC018, CC024, CC037, CC040, CC041, CC042, CC051, CC059 and CC061. We used 3 groups of 4 females and 3 groups of 4 males for C57BL/6J strain, and 2 groups of 4 females and 2 groups of 4 males for each Collaborative Cross strain. This cohort was thus made of 54 groups of 4 animals, i.e. 208 individuals. We received mice at age between one and two months old. We formed groups of 4 individuals on their arrival, if they did not arrive already grouped. From their arrival to the end of the experimental protocols, we kept those mice in a room at temperature between 20 and 23 °C. This room was enlightened on a 13/11 day/night rhythm, with light from 7h to 20h and no light from 20h to 7h. Mice had access to food and water *ad libitum*, and to some cylindrical compressed cottons to build nests.

### Second cohort - F1 crosses between the Collaborative Cross and *Shank3*<sup>+/-</sup>:

We crossed mice from 3 CC strains with *Shank3*<sup>+/-</sup> mice on C57BL/6J background [55]. These crosses differed in the strain of the CC parent and in the sex of each parent. It resulted in five different types of crosses (trios with one male and two females):

- A CC002 male with two *Shank3*<sup>+/-</sup> females.
- A *Shank3*<sup>+/-</sup> male with two CC002 females.
- A CC012 male with two *Shank3*<sup>+/-</sup> females.
- A *Shank3*<sup>+/-</sup> male with two CC012 females.
- A *Shank3*<sup>+/-</sup> male with two CC059 females.



## Weaning

We performed weaning between 28 and 42 days of age. After weaning, we genotyped mice, to know if they are wild-type (*Shank3*<sup>+/+</sup>) or heterozygous for *Shank3* (*Shank3*<sup>+/-</sup>). We identified each mouse through ear punches. We used the ear punch to genotype them. Once we got genotyping results, we formed groups of 4 individuals, 2 CCXXX.*Shank3*<sup>+/+</sup> and 2 CCXXX.*Shank3*<sup>+/-</sup>, of the same sex and each individual coming from a different litter.

From reproduction to the end of the experimental protocols, we kept these mice in a room at temperature between 20 and 23 °C. This room was enlightened on a 13/11 day/night rhythm, with light from 7h to 20h and no light from 20h to 7h. Mice had access to food and water *ad libitum*, and to some cylindrical compressed cottons to build nests. We provided a cardboard shelter in each cage.

## The experimental protocol

### RFID tagging

After a minimum of one week of habituation to the experimental facility, each individual was equipped with a Radio Frequency Identification (RFID) transponder (134 kHz glass probe ISO 11784/11785 2 × 12 mm). We performed the surgery under gas anaesthesia with isoflurane and subcutaneous local analgesia with lidocaine. After another week of recovery and when the animals were at least two months old, we performed first the single object exploration test and later on the long-term group monitoring.

## **Experimental setup**

We used as environment a squared plexiglass arena with 50-centimetre-long sides and 40 cm high walls, which is part of Live Mouse Tracker set-up. Between each experiment, we cleaned the cage with soap water and dried it using paper towels. Then we added approximately 1 cm layer of fresh wooden bedding on the floor of the arena. We then placed this arena on a RFID antenna floor and under a Kinect to complete the Live Mouse Tracker set-up [93]. We kept room temperature between 20 and 23 °C.

## **Single object exploration**

Each individual was first submitted to a solitary recording. The goal of this recording was to characterise the behaviour of the tested individual in a context of spatial exploration of a novel environment and in a context of exploration of a novel object which can also serve as a shelter.

## **Setting**

We performed all single object exploration experiments between 10h and 18h. The tracking environment was exposed to a light between 60 and 90 lux.

We first transported the subject's home-cage in the experimental room, at least 30 minutes prior starting the recording, to allow acclimation to the observation conditions. Then, the experimenter introduced one animal into the Tracking environment, placed in the centre, launched the recording with Live Mouse Tracker [93] on ICY platform [156], and left the room with the shortest delay possible. The experiment was then constituted of two 30 minutes long successive tracking phases.

## Phase 1

We tracked the mouse for a first period of 30 minutes in this new environment. The behaviour observed in this phase is expected to be similar to the one traditionally observed in open-field test. As in this widely used protocol, we aimed at measuring locomotor activity, exploratory behaviour and emotionality including anxiety [157].

This first recording phase was longer than the traditional open-field test, which often last between 2 and 10 minutes [157]. This extended time period should allow to observe more diverse facets of behaviour. Early activity can measure exploratory behaviour and response to novelty and anxiety. We should then obtain a more accurate proxy for baseline or spontaneous activity later on.

The light level, 60 to 90 lux, was also 2 to 3 times higher than the 30 lux used in most open-field tests [157]. This setting should increase thigmotaxis and decrease locomotion, and would thus give more information about the animal anxiety. The large-size environment we used is believed to provide more accurate data on other behavioural aspects than locomotor activity. As it allows a wider range of distance to the walls, this environment makes thigmotaxis easier to discriminate. The addition of bedding prevented us to observe fecal boli or urine, which could have informed us on the animal's anxiety level. But it allowed to observe digging, which diversified the exploration and could also be implied in stereotyped behaviours.

## Phase 2

Then, after the 30 minutes of the first phase, the experimenter added a red translucent house to the tracking environment, which marked the start of the second 30 minutes long phase. This house was rectangular with 5 cm high walls and two opposed 9 cm long open sides, and two 7.5 cm long close ones. The house was made of a tainted red plexiglass, which allowed human vision through red light and kinect depth sensing through infrared light, but which was opaque to the wavelengths visible by mice. Thanks to this colour, the house was a darker place for mice than the rest of the environment, while we could continue tracking under it. In order not to mix the particularities of corners or of the centre of the arena with the impact of the house, we disposed it at equal distance between a corner and the centre.

The mouse was tracked for a second period of 30 minutes in the environment which then contained the house. The behaviour observed in this phase was expected to contain some similar aspects to the one in the popular novel object exploration test [158]. It mostly differed from this last by the fact that the object was alone, presented only once, but for a longer period of observation of the animal in presence of the object what could allow a habituation. We thus expected that this recording could inform us on the reaction of individuals to novelty, neophobia. But we did not plan any evaluation of memory through this phase.

The object being a house, its physical particularities had to be taken into account. It has been proven that mice tend to explore longer object they can climb on than object they can only touch [158]. In the case of the object we use, mice could touch it, climb on it, and even get under it. As the range of interaction was again higher, we could expect *a priori* that mice would explore it even longer.

We can add that as this object is opaque to mouse vision, they were in a shadier environment when they sheltered under the house. The animal could reduce its exposition to light by going under the house. This particularity of the object introduced gave to this experiment a similar aspect to the light/dark box test[77]. We could thus gather information relative to mouse anxiety through their tendency to escape open spaces by sheltering under the house.

## **The long term group monitoring experiment**

Each group of 4 individuals was then submitted to a long term group recording. The goal of this recording was to characterise the behaviour of the tested individuals in a context of reciprocal social interaction with multiple conspecifics on an extended period and in an enriched environment.

### **Setting**

We used the same squared plexiglass arena with 50 cm long sides and 40 cm high walls as in the solitary test, washed it the same way with soap water and covered the floor with 1 cm layer of the same wooden bedding. We disposed a translucent red house, opaque to wavelength visible for mice but clear to infrared light of the kinect, between a corner and the centre of the arena. We let a handful of rodent food pellets between the opposite corner and the centre, and six cylindrical compressed cottons between a third corner and the centre. We then placed this arena on a RFID antenna floor and under a Kinect to complete the Live Mouse Tracker set-up. In the last corner, we disposed a water bottle, the end of the nipple passing through the wall of the arena by an adjusted hole. We kept room temperature between 20 and 23 °C all along the experiment. We exposed the environment to light on 13/11 day/night rhythm, of 75 lux from 7h to 20h and 0 lux from 20h to 7h. All long-term group monitorings were initiated between 18h and 19h and each lasted at least 70 hours.

We first transported the subject's home-cage in the experimental room, at least 30 minutes prior start of the recording, to allow acclimation to the observation conditions. Then, the experimenter introduced the four animals into the Tracking environment, placed in the centre, launched the Live Mouse Tracker [93] recording on the ICY platform [156], and left the room with the shortest delay possible. The experiment was then constituted of 1 to 2 hours of daylight, followed by three night phases of 11 hours, separated by two day phases of 13 hours and concluded on a final shorter day phase.

### **Recording**

We tracked the group of 4 mice for 70 hours, including 3 full night phases of 11 hours. We can see this test as an extension of social reciprocal interaction test to a longer period. Therefore, we also conducted the test in an enriched environment, which was expected to enhance the behavioural expression diversity of the subjects [159].

As on social testing individuals already completed the solitary test, they were not naive any-more to the red house. As a consequence, we could consider that this structure lost its status of novel object, and stayed mostly enrichment, which could serve as a hiding place and for climbing.

## SUCCUBE, the proposed classifier

The System for Unsupervised Clustering of Co-occurrent Underlying Behavioural Events (SUCCUBE) algorithm is an unsupervised behavioural classifier. A classifier aims to separate the behaviour of animals between several categories. We developed an unsupervised one, meaning that it segregates categories without a priori. The goal of this tool is to cluster frames of the experiment depending on the behaviour of animals. The threshold to separate those frames clusters are thus only based on the data. Extracted clusters group frames which are similar on some particular behavioural aspects. Inside one cluster, all frames contain thus a similar behavioural event. We thus called those clusters Behavioural Event Category (BEC). The algorithm defines those categories at the rate of one frame i.e. 1/30 of a second. Such part of the behaviour is shorter in time than most actions defined as behaviours. Those categories thus refer to more elementary components of the behaviour. Those elements can be associated successively along time to create more complex patterns. Most classifiers classify the behaviour at each frame, therefore a frame correspond to one global behaviour [93], [97], [114]. The main advantage of this algorithm is to allow the co-occurrence of BEC. For instance, others classifiers would label “mouse is rearing on the house” or “mouse is rearing on the field” whereas SUCCUBE would create 3 categories that can co-occur: “rearing”, “on the house”, “in the field” providing all possible combination. The BEC can thus also be associated simultaneously. This means it classifies each frames in several BEC, that is possible because it relies on multiple clusterings. This method is unsupervised, and it only builds categories depending on the data. It can thus permit to unravel categories which would not be known *a priori*.

## Algorithm

### Tracking data

This classifier relies on the tracking outputs which are trajectories. A trajectory is the location of a point that we follow at each time-point. Those locations are quantitative data and the classification will result on qualitative information. We aim to know if an animal is or is not performing a behaviour at each time-point.

Live Mouse Tracker[93] provides tracks with their identities. It provides the animal orientation and the positions  $(x,y,z)$  of three points per mouse, the head (H), the centre of mass (M) and the tail basis (T). We follow those points at a constant frame rate of 30 time-points per seconds.

### Trajectory based features extraction

The obtention of categories depends on variables permitting to distinguish between each. We do not want only to classify the different locations where an animal can be. We thus need to compute other variables than locations, allowing to segregate categories. Those intermediate variables are low-level representations of behaviour. They can be measurements relative to the shape or dynamic of the animals, or on its position relative to other points in the environment. They are "features extracted from the tracked positions of the animals and/or their parts over time" [74]. We thus called them trajectory-based features.



The computation of those features is an enrichment of the trajectory dataset. The goal is here to extract a maximum of information. We do not care for the partial redundancy in it. The JAABA algorithm [114] inspired us for the choice of some trajectory-based features. These features are geometric measurements relying on the tracked points locations. We extract them from locations of the three points H, M and T detected on the mice. Each of those can be expressed as a distance or an angle.

SUCCUBE extracts static features from point positions at a given frame. These ones describe the posture of the animal. In contrast, dynamic features rely on comparisons with the previous and successive frames. These ones describe the movement of the animal. No feature exceeds this three frames time-window. Behaviours exceeding this time scale will be described by the successive addition of those short units in the follow-up of this work (see discussion).

The tracking needs to recognize an individual to affect it with an identity. It also needs to find the animal orientation to provide the positions  $(x,y,z)$  of the basis of the tail, centre of mass and head. SUCCUBE thus compute features for frames  $f$ , for which frames  $f-1$ ,  $f$  and  $f+1$  fulfil those two conditions of identification and orientation, for each animal recorded. We do not extract information from other frames. If an animal is not identified or oriented, the frame contains a fewer amount of information.

This partial information could also carry biases: the tracker can miss animals, mix identities, fuse detection in a common shape, as explained in the validation section of Live Mouse Tracker[93]. Therefore, part of the information processed to create the feature could be wrong if an individual is not identified. This is why SUCCUBE only considers frames at which all individuals are identified. Individual features take into account only one animal considered as the Emitter (E). Social features consider two individuals in a dyadic oriented interaction. We called the other individual implied than the emitter the Receiver (R). More information is provided on the emitter than on the receiver. We did this in order to describe the behaviour of the emitter rather than the one of the receiver, while allowing descriptions of their interactions. The receiver of a dyad is the emitter of the dyad of opposed orientation and *vice versa*.

The complete feature list can be found in (Table annexe 1). We classified those features depending on the objects they consider:

- Body features (B), which describe the relative positions and movements of the emitter's body parts.
- Arena features (A), which describe the positions and movements of the emitter's body parts in relation to the arena walls, corners and centre.
- Object features (O), which describe the positions and movements of the emitter's body parts in relation to the house walls, open sides and corners. Those features were only used for the second phase of the single object exploration experiments. We could not consider the position of the house to be stable during the long-term group monitoring experiment. In consequence we did not use these features for the analysis of those recordings.
- Social features (S), which consider dyadic oriented interactions. They describe the positions and movements of the emitter's body parts in relation to the receiver's body parts positions or movements. SUCCUBE compute these features for each oriented pair of emitting and receiving individuals. These features were only used for the long-term group monitoring experiments.

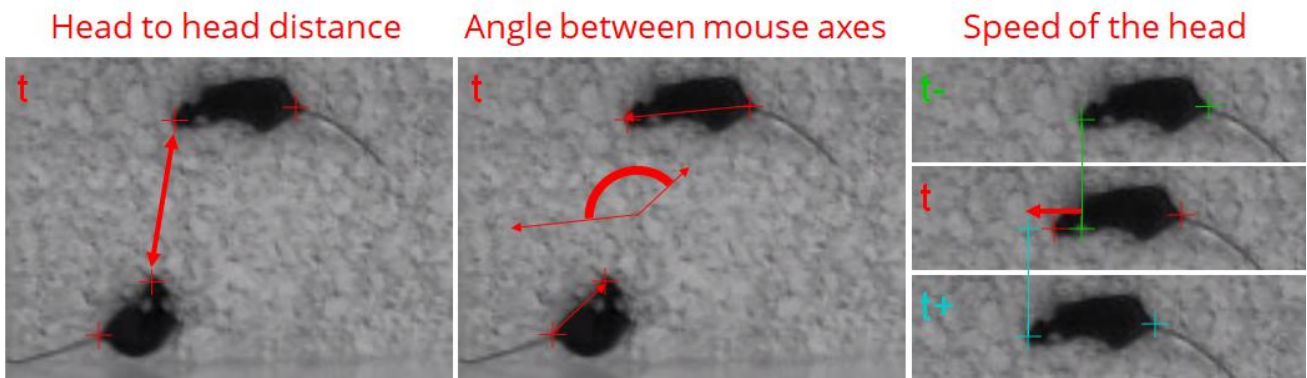


Figure 24: Example of three trajectory based features measurements on a filmed frame. A: Representation of the segment giving the distances between the heads of two animals (Table annex 1: N°159 distance HHr). B: Representation of the segments head-tail of two animals and of the angle between these segments. This angle gives the angle between the two mouse axes (Table annex 1: N°179 angle HT HTr). C: Representation of the head positions in previous and successive frames which gives the instant speed of the head (Table annex 1: N°25 speed H).

### Particles number feature

Live Mouse Tracker tracks any object moving in the cage. Then, if a moving object is not recognized as a mouse in a two seconds delay (60 frames), the track is interrupted. Live Mouse Tracker does not provide all the tracks of non-mouse movement in the cage, but it provides for each frame the number of object(s) detected as non-mouse. This number typically corresponds to the number of sawdust particles moving in the cage. It reflects the action of digging, fighting, or running which create different signature of appearance of sawdust. SUCCUBE takes this particle feature (P) into account and adds it with the trajectory based ones.

## Sub-sampling

By computing all features considered for all experiments, we obtain a large dataset which would be too heavy for computation. SUCCUBE thus proceeds to some subsampling in order to conceive several datasets which do not exceed computational capacities. The second goal of this subsampling is to divide data in order to run the following part of the analysis multiple times. Such multiple parallel runs will permit to target the more robust results. We built 100 of those subsamples.

We do not want the classification to be more specific to some individuals or some phases of the experiments. This could be the case if the subsamples contained more data-point from some parts of some experiment than from others. In order to avoid such bias, SUCCUBE proceeds to this sampling in a balanced way in consideration of several aspects.

For the single object exploration experiments, we sampled the same number of frames for each individual. We also considered as many frames before and after the addition of the house. For one experiment, we extracted 200 frames per phase per subsample. It means we used more than 11 min for each phase, more than 22 min in total, for each experiment, over all subsamples.

For the long-term group monitoring experiments, we did the same for each oriented animal dyads. We sampled equal numbers of frames in each night of the experiments. For one experiment, we extracted 300 frames per oriented dyad per night per subsample. It means we used 16 min 40 sec for each night, 50 min in total, per long-term group monitoring, over all subsamples.

We chose frame numbers to sample depending on the usable frame amount in the experiment phases. Most recordings presented more frames in which all features are computable than necessary. For these databases, we sampled at random without replacements over all subsamples. In a minor part of experiment phases, frames were insufficient. We still use these databases if they contained at least a fifth of the number of frames to sample. In this case, we proceeded to a random subsampling with replacements. This replacement has a risk to make the analysis of different subsamples more similar than it would be without. This means it could favour over-fitting and it is why we limited it.

More generally, frames which are not usable can depend on the behaviour of individuals. For example, when mice are grouped in close contact for a long time, LMT generally cannot segment them correctly. It means that the more mice exhibit behaviours which prevent us to use frames, the smaller is the part of their behaviour that we consider. This bias could make us think that rare events happened more frequently.

### **Standardization**

SUCCUBE scales features to be able to compare them with the ICA. We apply a global scaling of all data treated in this further analysis. We also want to balance this process between experiments, subjects and phases. For this reason, we standardize each feature over the whole set of subsamples. We first centre them by subtracting their global mean over all subsamples. Then we scale them by dividing them by the global standard deviation over all subsamples. We keep those balanced means and standard deviations, to be able to extend this scaling to other data points. As this we can afterward take any frame even not sampled one and apply the same standardization.

## Feature sets

Behavioural event categories considering more elements of the environment may hide the one which would consider less elements. For example, moving toward different locations of the area could hide the simpler category of moving forward. In order not to miss the simpler categories we divided further parts of the algorithm in several features set as in JAABA algorithm [114]. It also allows to express categories with a parsimonious amount of features.

These feature sets depend on the classification of feature and are the following:

- BP set is composed of body and particle features. It thus only considers one mouse and the bedding.
- BAP set is composed of body, arena and particle features. It thus considers one mouse in a recording arena with bedding.
- BAOP set is composed of body, arena, object and particle features. It thus considers one mouse in a recording arena with bedding, including a red house. We only analysed this set for the second phase of the single object exploration.
- BASP set is composed of body, arena, social and particle features. It considers an emitting mouse in a recording arena with bedding, including a receiving mouse. We only analysed this set in the long-term group monitoring.

## **Independent component analysis**

SUCCUBE then treats each subsample for each feature set in a separated way. It treats them through ICA. This process returns a new subspace made of orthogonal dimensions, which are the least normally distributed possible. In this way, these axes should each allow a different classification. These classifications will thus permit categories discriminated on different axes to co-occur.

We repeated those ICA for all numbers of extracted ICs from 2 to 60 or the maximum possible if lower. We cannot extract more IC than features in the feature set used. This will allow a further decision on the number of axis to extract (see hereafter “Independent component numbers criterion”). The ICA were conducted through the FastICA algorithm [160] with icafast function from ica package in R software [161].

## **Independent component numbers criterion**

The ICA needs a number of ICs to extract to be executed. We cannot keep independent components extracted with different numbers of ICs because they would not be independent between them. SUCCUBE thus need to choose a number of ICs to extract.

We here present the criterion we used to define an optimal number of IC to extract. For each number of IC to extract, we run the ICA on several subsamples (here 100) of the dataset. Each ICA returns an unmixing matrix. We can separate each unmixing matrix in contribution vectors. One exists for each IC extracted. Each contains the coefficients to apply to each feature in the linear mix to compute an IC.

For one number of ICs to extract, we get one unmixing matrix per subsample. SUCCUBE clusters all the contribution vectors extracted from all subsamples. It groups those vectors via an HAC. This clustering is based on average absolute Pearson's correlations. We keep grouping them while we do not group two vectors extracted from the same subsample. To select robust outputs, SUCCUBE keeps only clusters constituted by vectors extracted in at least half of subsamples. We call them level 1 clusters.

While we increase the number of ICs extracted, we aim at axes which fulfil three conditions:

- We do not limit to a minimum the number of axes in order to understand the biggest part of the behaviour possible.
- We conserve as much as we can the information present in axes extracted in fewer numbers. These ones would appear as the most evident axes to consider.
- We avoid to extract too many axes. The quality of the ICA would decrease if the axis follows less the ICA criteria. Axes could be more correlated between them or even more normally distributed.

SUCCUBE thus computes iteratively a score for each number  $N$  of extracted ICs. At each step, we take into account the contribution vectors of all numbers of ICs extracted from 2 to  $N$ . We agglomerate the level 1 clusters of contribution vectors with a second level of the same HAC. In the same way as before, the clustering goes on until it would merge two level 1 clusters from the same number of ICs. This allows to avoid grouping any pair of vectors generated from the same subsample. We call them level 2 clusters.



The score relies on a value that we will name the retention. We consider a level 2 cluster as a conserved cluster if it contains a level 1 cluster extracted when asking N ICs. The retention for N extracted ICs is the ratio of the level 1 clusters, which are part of conserved level 2 clusters. The score is the product of the number of level 1 clusters extracted when asking N ICs multiplied by the retention for N extracted ICs.

SUCCUBE stops scores computation if three conditions are fulfilled:

- The score is inferior to the one computed with fewer ICs. This is because we want to keep the number of ICs maximizing the score.
  - The increase of N has divided some level 2 clusters grouped when there were fewer axes. This shows that the new axes contain some ICs too correlated. This division of cluster would also lose the information on axis origins from fewer ICs.
  - No higher number of ICs offers a complete retention of 1. Complete retention would be a sign of quality of extracted ICs. We want to verify that complete retention will not come back in higher number of ICs. That is why we extracted ICs until at least 60 or the maximum possible if lower.
- Once SUCCUBE computed all scores until the stop, it keeps the number of ICs to extract giving the highest score.

### **Behavioural descriptors**

Our goal is to get axes describing the behaviour over all subsamples. We need to define common dimensions on which we can project all data. We thus extract a single axis of each level 1 cluster obtained when asking for the kept number of IC. For this purpose, SUCCUBE selects the centroids of each of those clusters. It chooses the axis with the highest average correlation to other axes from the same cluster. We named those axes behavioural descriptors.

### multi-set duplicates exclusion

For each experiment, we ran the ICA, on different feature-sets. For one experiment, all sets are imbricated. A bigger set contains at least all features of a smaller one. This allows to find behavioural descriptors that the presence of all features would hide.

At the same time, the addition of features does not hide all axes extracted from smaller features sets. We found similar behavioural descriptors in several feature-sets. In those cases, we did not analyse both behavioural descriptors.

To identify these duplicates, we applied a selection on behavioural descriptors. We compared the axes extracted with different features-set two by two using Pearson's absolute correlation. We computed the distance between behavioural descriptors over all subsamples.

We did not analyse further the axes extracted from more features if it correlated too much with an axis extracted from a simpler set. This choice allows us to be more parsimonious in two aspects. We get rid of kind of useless duplicates, and we keep the one constituted of the fewer number of features. The threshold of Pearson's correlation we chose here was an absolute coefficient of more than 75%. This selection does not change the conserved or the discarded behavioural descriptors. Discarded data remain available if needed later.

## Gaussian mixture model based clustering

To describe qualitative behavioural expressions at each frame, SUCCUBE clusters time points based on the Behavioural Descriptors (BD). We treat each BD independently, to get one independent classification per axis. In this way, categories extracted from two different BD will be able to co-occur at a same time point.

For one BD SUCCUBE gathers its values at all frames, over all subsamples. Then, on the basis of this distribution it segregates frames into classes. For this purpose, it uses a Gaussian Mixture Model (GMM) based clustering, that allows the definition of thresholds. These ones distribute all frames in several Behavioural Event Categories (BEC) along a BD.

In order to model a distribution through a GMM, we need to choose a number of Gaussian Distributions (GD) to mix (see hereafter “Gaussian distributions numbers criterion”). Thanks to ICA which produced them, BD should be the least normally distributed possible. One unique Gaussian distribution cannot provide a good model for such distribution. SUCCUBE uses at least two GD to model each behavioural descriptor. This choice gives good chances that most BD allow the segregation of at least two BEC.

We fit the GMM maximizing the likelihood

$$\mathcal{L} = \prod_{i=1}^T \sum_{j=1}^N p_j \mathcal{N}(x_i, \mu_j, \sigma_j^2)$$

Here,  $T$  is the number of data-points,  $N$  is the number of GD and  $p_j \mathcal{N}(x_i, \mu_j, \sigma_j^2)$  the weighted probability density of  $x_i$  in the  $j$ -th GD. SUCCUBE performs this computation through expectation maximization algorithm. The GMM based clustering were conducted with Mclust function from mclust package [162] in R software [161].

## Gaussian distributions numbers criterion

By computing those models, we here aim at getting a reliable clustering. We need to restrain the number of GDs mixed in the model for several reasons:

- In order to prevent over-fitting to the data-set. The more GD we add to a model, the more parameters we use and the more likely they can be specific to sampled frames against non-sampled ones. The goal of this classification is to be informative for frames that we did not sample, or even for further recordings.
- In order to limit GD overlapping, which statistically increases with the number of mixed GD, and makes the clustering less robust. Frames measured at values for which two GD overlap more would be classified with less confidence.
- In order to separate a fewer amount of BEC along each axis. The more BEC are described along an axis, the more complex is the interpretation of categories which end closer to each other. Limiting BEC number allows to reduce nuances to use while naming and interpreting the categories. Those nuances could make the interpreted data less informative. This approach needs to be parsimonious.

We select the number of GD which fits the best to the data while the mixed GD overlap the least possible. The criterion SUCCUBE uses to select GD number is the same than in Bauer et al., 2017 [163]:

“Several GMM are fitted with an increasing number of GDs, beginning with 2. We select the processed model by maximizing the function  $J_k$ , where  $k(i)$  denotes the number

$$\prod_{i=1}^T p_{k(i)} \mathcal{N}(x_i, \mu_{k(j)}, \sigma_{k(j)}^2)$$
 of the GD which gives the highest density probability for the frame  $x_i$ . This penalizes the models with respect to the overlapping of these GDs, and constrains

the number of GDs. For each BD, the GMM producing the first local maximum of this criterion will be used.”

SUCCUBE defines equality points of the probability of two GD as thresholds to segment frames along a BD. If some thresholds separate two clusters of at least 2.5% of sampled frames each, we only keep those thresholds. We do this to focus on the most representative categories of the axis, and for the number of categories to stay parsimonious. If all thresholds separate only extreme clusters of less than 2.5% of sampled frames, the BD is segregating rarer events. We here keep only the first threshold on each side, starting from the mean. These thresholds allow the classification of BEC.

### **Behavioural event categories**

For each BD, we cluster all time points in a certain number of BEC. An animal will thus produce as many BEC at each time point as the selected number of BD. As this clustering classified frames, the time scale of those categories is 1/30 of a second. We thus cannot expect those BEC to correspond to behaviours made of a sequence. Those BEC are closer to some behavioural building blocks [74]. Nevertheless, we could create sequences of behavioural building blocks by successions and potential co-occurrences of BEC. We separated two types of BEC. Posture events inform us on the configuration of the mouse's body and/or its body parts. Movemes [142] events inform us on the movements of the mouse and/or its body parts. Those BEC make sense at the scale of the individual or describe its interaction with the environment, including the arena, the house or a conspecific.

The first result we get from SUCCUBE is a behavioural repertoire. It is a list of behavioural event categories, which can append or that animals can produce. Those categories are exclusive between them when we classified them from the same BD. They can co-occur if they are extracted from different BD. And thus one BEC is active for each BD at each time point. Some BEC are thus general or common and just play the opposite role of more particular and rarer BEC from the same BD.

## Behavioural event categories interpretation

Once the classifier separated all BEC, we named them in a humanly readable way. As we got these categories without supervision, we did not know what they represented *a priori*. We provided names for each BEC using the following information:

- The contributions of features to the BD which segregate these BEC. We represent relative contributions exceeding 1, the mean relative contribution, on a bar-plot. This first graph allows us to interpret the direction of differences along the BD, in the features space (figure 25A).
- The distribution over all subsamples of the BD which segregate those BEC. We represent it on a histogram, on which we superpose the probability densities of the GMM and of each of its GD. We then represent the thresholds of the clustering. This second graph allows to locate BEC along BD and to see their relative proportions (figure 25B).
- The spatial repartition of the BEC. On an x,y spatial map of the environment, we represent each frame of an example experiments by a dot of colour. The colour of each dot depends on the active BEC at this frame. This third graph allows to examine if BEC appears more in some parts of the environment. It also shows the length of trajectories in each BEC, and the frequency of switches between those (figure 25C).

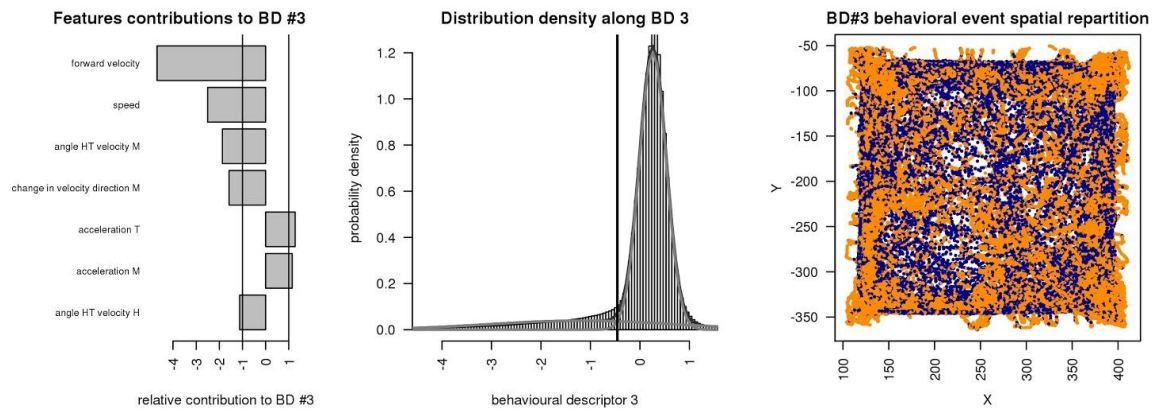


Figure 25: Interpretation figures for the BEC segregated on the 3rd BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “moves forward”
- the BEC on the right of B and orange in C: “does not move forward”

### Cluster use for performance

This algorithm has been adapted to the Institut Pasteur’s cluster in order to fasten the computation and enhance the live memory. The computation has been parallelized at several steps:

- On trajectory based features extraction, parallelized between each experiment.
- On subsample building, computation of the mean and standard deviation and on ICA, parallelized between each subsample.
- On GMM based clustering, parallelized between each BD.

## Comparative statistics

### Behavioural event exhibition time

Behavioural classification is a tool to turn trajectories into quantifiable behavioural categories. The quantification of the segmented behaviour is a measurement of a behavioural trait. On the basis of the classified frames, the easiest measurement is to count the number of frames in each category.

We cannot classify all frames because features are not extractable at each of those. The number of frames at which we can compute features differs between experiments. We do not want to compare tracking quality between experiments, but animal behaviour. We thus need to base this enumeration on the same frame number for each animal. The simpler behavioural trait to compute is then the timeshare proportions of each category.

We need an exploitable frame sample of equal length for each individual or dyad. The subsamples we created earlier for SUCCUBE algorithm fit exactly this purpose. They are balanced between individuals or dyads, and between each phase of experiments. We thus chose to use all frames over all subsamples to compute those proportions. We could then give the proportion depending on the emitter, on the phase, and on the receiver if one is present.

The information which interests us is those proportions. But time proportions do not distribute normally while real durations do. Parametric statistical tests deal better with normally distributed measurements. We thus avoided to divide those numbers of frames, in which an animal produces a BEC, by the complete duration. This means that we compared animals on the basis of the times spent exhibiting BEC, from an equal total time, in the goal to compare their proportions of BEC exhibition. This is exactly equivalent to say that we transformed all proportion data by multiplying all by the number of frames sampled by individual.



## Screening specific statistical considerations

The tracking automation provided by Live Mouse Tracker allowed sufficient efficiency in data collection to proceed to the screening of the behaviour of several strains in different contexts. The behavioural segmentation provided by SUCCUBE allowed the screening of a large number of behaviours for each strain and context. The goal of such screenings is to identify some strains presenting some amount of behavioural differences from C57BL/6J mice. This permitted the selection of strains of interest to cross with C57BL/6J *Shank3*<sup>+/-</sup>. This goal can be seen as a global question in which no particular strain or behavioural category is necessary or interesting *a priori*. This explorative screening procedure is used to discover differences when we do not know which variable they would affect.

This screening procedure thus makes the global null hypothesis, telling that: “all behaviours of all strains do not differ significantly from C57BL/6J behaviours”, of capital importance. We could not conclude that a particular strain differs significantly from C57BL/6J in the expression of a particular category if overall screening results suggest that no differences exist and that this particular one could have been found by chance. This means that we need to be cautious with the inflation of the family-wise error rate caused by multiple testing. If this problem is not considered, the extension of the screening to more behavioural categories or strains could be a way to artificially increase the probability to observe some behavioural differences.

We thus needed to apply corrections on the statistical test P values we obtained. Those corrections depend on the number of comparisons tested between independent variables. We thus needed to reduce the number of comparisons by avoiding useless ones between highly correlated variables or which would not serve to identify a strain of interest.

The number of collaborative cross strains being a particular richness of this study we compared each strain to C57BL/6J. But as our goal was to find interesting strains to use as models instead of C57BL/6J, we did not lead any comparison between collaborative cross strains.

The different experiments and their different phases were all *a priori* interesting. We thus did not exclude any from comparisons. But the information given by a particular behavioural event category proportion can be redundant and thus dependent between different phases. This is why we selected phases in which we compared measured proportions. Inter-phase difference tests were avoided.

Finally, behavioural event categories are independent from each other at the frame time scale. But their production proportions over the time of an experimental phase can correlate. This can be due to their potential association in patterns of succession. We can also see those correlations as the expression of more general behavioural syndromes [164]. Such syndromes would drive over-expression or under-expression of groups of behavioural event categories. In order to avoid useless statistics on highly correlated signals we needed to select the behavioural event category measurements to compare.

We thus proceeded to the comparison of the twelve Collaborative Cross strains to C57BL/6J. Each Collaborative Cross strain comparison was led on the same set of phase-specific behavioural event category measurements. The selection of this set of measurements needed to be based on the correlations between all measurements over all strains. These comparisons resulted in the conceptions of behavioural profiles which are homologous between each Collaborative Cross strains as they consider the same measurement set.

## Variable selection

SUCCUBE algorithm extracts a lot of different BEC. We measured exhibition time for each BEC in each experimental phase. This created a lot of measurements or behavioural traits. In order to avoid useless statistics on highly correlated signals, we needed to reduce the number of variables to study. A PCA could allow an interesting reduction of the number of dimensions. But this method has here two major defaults:

- The principal components condense too much the information. They could mix traits which would appear independent. This would reduce the amount of information we can extract about differences between individuals.
- The linear mixes of proportions of BEC would end up on new signals. These new signals, and the traits they define, would rely on different BEC expressed in different phases. This means that we would need a new level of interpretation to give a name to the principal components. This interpretation would be difficult as such signals are not used in the literature. And the resulting axes would be hard to link with understandable traits.

We thus preferred to use a clustering on the different BEC proportions measured in all phases. The goal was then to select one measurement per cluster as being representative of the cluster. It allowed us to work with variables easier to interpret, but in fewer number.

We thus clustered the BEC proportions depending on the exhibition times measured for each individual or dyad. We used a HAC based on the average absolute Pearson's correlations. We selected the number of clusters to segregate which gave the maximum product of number of category times the gap of correlation to the next cluster division.

We chose the measurements representative of clusters depending on three factors:

- We selected preferably measurements which aggregate early, in the sense that, along HAC, they group with other categories before some others.
- We selected measurements of behavioural event categories which seemed linked to the other categories of the cluster.
- We selected measurements lead on the same period (with/without object or night 1, 2 or 3) of the experiments as the majority of other measurements of the cluster.

## **Strains profile**

We used the selected time proportion measurements in order to build a behavioural profile of each CC strain recorded. We did this profile in comparison to the C57BL/6J strain which served as a reference. The important use of this strain in neuroscience and behavioural biology makes it a relevant point of comparison. Such profiles allowed afterward selection of strains of interest to study specific behaviours.

## Matching goals and test procedures

For the first cohort, when comparing collaborative cross strains to the C57BL/6J reference strain, our goal was to evaluate which strains differ more from the widely used reference strain in terms of behaviour. With four groups of four individuals per strain and five for the C57BL/6J for each sex, we could expect a decent discriminative power. At this step we were thus not lacking discriminative power. This is why the important Bonferroni correction applied for the product of the number of strains compared to C57BL/6J by the number of traits compared between these last is adequate. In the same way, as finding significant effects should be easy, we could do it separately on males and females even if it reduces our sample size.

We could afford to proceed to more comparisons with less animals. Finally, as a lot of trait measurements risked to be non-Gaussian or non-homoscedastic between strains, we directly applied non-parametric tests to all traits in order to compare each trait in the same way. The reduced p-values resulting from such tests were here not a concern.

For the second cohort, when comparing heterozygous and wild-type mice on mixed backgrounds, our goal was to find at least one background for which some behavioural traits differ significantly depending on *Shank3* haploinsufficiency. With 2 to 6 groups of four individuals per background and sex, including each two wild-type and two heterozygous animals, we could predict lower discriminative powers than for the first cohort.

At the same time, finding behavioural differences between individuals differing only in one copy of a gene was more challenging than the strain comparison of the first cohort. This is why we only applied Bonferroni correction for the number of trait compared between genotypes. In this way, we avoided the family-wise type 1 error-rate inflation with the extension of trait screening. We did not correct over all crosses in order to avoid inflation of the type 2 error-rate, more than it would be when testing only one genetic background. This result thus can be compared to the one that would be obtained if we already selected one genetic background to study *Shank3* mutation.

In the same way, as finding significant effects is here challenging we could not do it separately on males and females because it would reduce our sample size. We could not afford to proceed to more comparisons with less animals. This is why we compared all means by performing ANOVA with the genotype of the emitter, of the receiver in the case of social behaviour and the sex as factors and considering interactions between these factors. As such test is parametric, its discriminative power is better. And if some significant differences were obtained on measurements violating the Gaussianity or homoscedasticity hypothesis on the basis of Shapiro's and Levene's tests on residuals, we investigated further to prove such significance without such bias.



# **Results**





# Proof of concept

## ISBI: IEEE International Symposium on Biomedical Imaging, 2017

In this first article [163], we presented the basis of SUCCUBE. We used tracking data obtained through Mice Profiler [97] to compute trajectory-based features. We ran an ICA on these last and Gaussian mixture model based clusterings on the subsequent ICs. We showed, by cross-validation, that the behavioural event categories, obtained this way, correspond to the ones extracted by Mice Profiler [97]. We finally used the newly classified behavioural event categories to find back markers of hyperactivity and lack of social interest in *Shank2*<sup>-/-</sup> mice, another mouse model of autism spectrum disorder.

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## ISBI: IEEE International Symposium on Biomedical Imaging, 2018

In this second article [165], we adapted the embryo of SUCCUBE algorithm to the tracking data extracted by the newly developed Live Mouse Tracker [93]. In order to benefit from the richer trajectory data of this new tracking method, we increased drastically the number of trajectory-based features computed. We also started to extract behavioural descriptors by selecting ICs conserved between several ICA ran on different subsamples of data. Such innovations allowed us to deepen the understanding of the link between hyperactivity and lack off social interest in *Shank2*<sup>-/-</sup> mice.

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# Application to the Collaborative Cross

## Behavioural event categories extracted

The results are organized in chronological order. The data gathered by tracking the first cohort, black 6 and CC strains, have first been used as a basis for SUCCUBE to discover a wide behavioural repertoire. This one will first be presented here in tables 7 to 12.

We generated a behavioural repertoire separately for the single object exploration and for the long-term group monitoring experiments. Some categories are close between the two protocols, in this case we gave the same name to both categories. But we need to describe them separately for each protocol. The changes in the experimental protocol affect the characteristics of those categories. The same name does not refer to the exact same behaviour in different contexts. The mix of features contributing to the BD, as the threshold segregating each BEC on this one, can differ.

## The single object exploration

The single object exploration protocol's data led to the clustering of 116 BEC.

### The BP feature set

In the single object exploration data, on the set grouping body and particle features, SUCCUBE extracted 24 BD. Those 24 BD allowed to segregate 63 BEC.

Table 7: Experimenter given names for the BEC segregated on BD extracted from the BP features-set in the single object exploration experiment.

|       | BEC 1                                    |                             | BEC 2                                     |             |
|-------|--|-----------------------------|---|-------------|
| BD    | BEC 1                                    | BEC 2                       |   | BEC 3       |
|       | BEC 1                                    | BEC 2                       | BEC 3                                     | BEC 4       |
| BD 1  | does not rotate in deceleration          |                             | rotates decelerating                      |             |
| BD 2  | does not rotate in acceleration          |                             | rotates accelerating                      |             |
| BD 3  | moves forward                            |                             | does not move forward                     |             |
| BD 4  | contracts                                | stable extension            |   | extends     |
| BD 5  | is short on x-y axis                     | normal extension            |   | is extended |
| BD 6  | does not move backward                   |                             | moves backward                            |             |
| BD 7  | does not move the anterior part sideways |                             | moves the anterior part sideways          |             |
| BD 8  | is posterior deviating                   |                             | is not posterior deviating                |             |
| BD 9  | decelerates                              | stable speed                |   | accelerates |
| BD 10 | moves the posterior part sideways        |                             | does not move the posterior part sideways |             |
| BD 11 | is reared                                |                             | is not reared                             |             |
| BD 12 | moves without<br>agitating the bedding   | does not agitate<br>bedding | agitates bedding                          | digs        |
| BD 13 | unbends                                  | stable bending              |   | bends       |

|       |   |  |   |
|-------|---|--|---|
| BD 14 | is not bent   | is bent  |   |
| BD 15 | rears down  | stable altitude  | rears up  |
| BD 16 | centre of mass pushed forward                                   | centre of mass synchronized with other body parts                            | centre of mass pushed back                                      |
| BD 17 | tail base pushed back   | tail base synchronized with other body parts                                 | tail base pushed forward  |
| BD 18 | head pushed forward   | head synchronized with other body parts                                      | head pushed back  |
| BD 19 | orients the movements of the head forward                       | does not orient the movements of any body part forward                       | orients the movements of the tail forward                       |
| BD 20 | orients the movements of the head away from forward orientation | does not orient the movements of any body part away from forward orientation | orients the movements of the tail away from forward orientation |
| BD 21 | head-tail inversion on rearing                                  | no head-tail inversion on rearing  |   |
| BD 22 | lowers the head   | stable head altitude   | raises the head   |
| BD 23 | anterior acceleration   | anterior stable speed  | anterior deceleration   |
| BD 24 | lowers the tail   | stable tail altitude   | raises the tail   |

*BD 1 to BD 24 refer to the behavioural descriptors axes and BEC 1, BEC 2, BEC 3 and BEC 4 refer to the behavioural event categories segregated on those axes.*



The BEC “head-tail inversion on rearing” extracted from the 21st BD is describing events at which the animal has the head down and the basis of the tail high. This behaviour has not been observed, but this category is activated when features describe such inverted standing posture. This happens mostly when an animal retracts from a rearing on the wall. At those frames the high backward speed fools the tracking which inverses head and tail basis points. This results in an unusual tail basis altitude superior to the one of the head. BEC extracted from the 21st BD thus describe an event mixing a behaviour of an individual and an error of the tracking system.

### The BAP feature set

In the single object exploration data, on the set grouping body, arena and particle features, SUCCUBE extracted 31 BD. We kept 9 of those which did not correlate at more than 75% with BD extracted from BP feature set. Those 9 BD allowed to segregate 23 BEC.

**Table 8: Experimenter given names for the BEC segregated on BD extracted from the BAP features-set in the single object exploration experiment.**

| BD   | BEC 1                                   | BEC 2                               | BEC 3                     |
|------|---|-------------------------------------|---------------------------|
| BD 1 | is not oriented toward the closest wall | is oriented toward the closest wall |                           |
| BD 2 | is in a corner                          | is not in a corner                  |                           |
| BD 3 | orients away from the centre            | stable arena related orientation    | orients toward the centre |

|       |  |  |                                    |
|-------|--|--|------------------------------------|
| BD 6  | goes toward the wall                               | stable distance to the wall                        | goes away from the wall            |
| BD 7  | goes forward toward the corner                     | does not progress with the corner in front         | goes backward away from the corner |
| BD 8  | goes backward toward the corner                    | does not progress with the corner in the back      | goes forward away from the corner  |
| BD 11 | is in the near wall area                           | is in the corner or intermediate area              | is in the middle diagonal cross    |
| BD 15 | orients toward the corner                          | stable wall-corner orientation                     | orients toward the wall            |
| BD 17 | is oriented toward the wall rather than the corner | is oriented toward the corner rather than the wall |                                    |

*BD 1 to BD 17 refer to the behavioural descriptors axes and BEC 1, BEC 2 and BEC 3 refer to the behavioural event categories segregated on those axes.*

### **The BAOP feature set**

In the single object exploration data, on the set grouping body, arena, object and particle features, SUCCUBE extracted 14 BD. We kept 11 of those which did not correlate at more than 75% with BD extracted from BP and BAP feature set. These 11 BD allowed to segregate 30 BEC.

Table 9: Experimenter given names for the BEC segregated on BD extracted from the BAOP features-set in the single object exploration experiment.

| BD    | BEC 1                               |  | BEC 2                            |                                   |
|-------|-------------------------------------|--|----------------------------------|-----------------------------------|
|       | BEC 1                               | BEC 2                                    | BEC 2                            | BEC 3                             |
| BD 1  | is close to or in the house         |  | is far from the house            |                                   |
| BD 2  | get away from the house             | stable distance from the house           |                                  | goes toward the house             |
| BD 3  | is oriented toward the house        | no special house related orientation     |                                  | is oriented away from the house   |
| BD 4  | does not orient away from the house |  | orients away from the house      |                                   |
| BD 5  | orients toward the house            |  | does not orient toward the house |                                   |
| BD 7  | goes backward toward the house      | does not progress with house in the back |                                  | goes forward away from the house  |
| BD 8  | goes forward toward the house       | does not progress with house in front    |                                  | goes backward away from the house |
| BD 9  | angular decelerates                 | stable angular speed                     |                                  | angular accelerates               |
| BD 10 | orients toward the house            | stable house related orientation         |                                  | orients away from the house       |
| BD 11 | is not in the far from              | is in intermediate house related area    |                                  | is in the far from                |

|       |   |  |  |
|-------|---|--|--|
|       | house area  |  | house area   |
| BD 14 | goes in direction from<br>a house wall to an<br>open side | stable house open side to wall closeness | goes in direction from<br>a house open side to a<br>wall |

*BD 1 to BD 14 refer to the behavioural descriptors axes and BEC 1, BEC 2 and BEC 3 refer to the behavioural event categories segregated on those axes.*

### The long-term group monitoring

The long-term group monitoring protocol's data led to the clustering of 112 BEC.

#### The BP feature set

In the long term group monitoring data, on the set grouping body and particle features, SUCCUBE extracted 25 BD. We kept 24 of those because the GGM based clustering did not allow for any BEC segregation on the 21th BD. These 24 BD allowed to segregate 63 BEC.

Table 10: Experimenter given names for the BEC segregated on BD extracted from the BP features-set in the long term group monitoring experiment.

| BD    | BEC 1   |                                   | BEC 2  |                                |
|-------|---|-----------------------------------|--|--------------------------------|
|       | BEC 1   | BEC 2                             | BEC 2  | BEC 3                          |
| BD 1  | the movements of all body parts is oriented forward |                                   | the movements of some body parts is not oriented forward |                                |
| BD 2  | turns decelerating                                  |                                   | does not turn in deceleration                            |                                |
| BD 3  | turns accelerating                                  |                                   | does not turn in acceleration                            |                                |
| BD 4  | is extended   | normal extension                  |  | is short on x-y axis           |
| BD 5  | contracts   | stable extension                  |  | extends                        |
| BD 6  | moves forward                                       |                                   | does not move forward                                    |                                |
| BD 7  | accelerates   | stable speed                      |  | decelerates                    |
| BD 8  | is not deviating                                    |                                   | is deviating   |                                |
| BD 9  | does not move backward                              |                                   | moves backward   |                                |
| BD 10 | rears up  | stable altitude                   |  | rears down                     |
| BD 11 | is reared   | is not reared                     |  | has the head down              |
| BD 12 | has the tail down                                   | no head-tail inversion on rearing |  | head-tail inversion on rearing |

|       |   |  |  |
|-------|---|--|--|
| BD 13 | lowers the tail   | stable tail altitude   | raises the tail  |
| BD 14 | the movements of the tail is not oriented forward but the one of the head is                        | head and tail movements are each as oriented forward than the other                                  | the movements of the head is not oriented forward but the one of the tail is |
| BD 15 | bends   | stable bending   | unbends  |
| BD 16 | no bedding movements  | bedding movements  | some individuals dig   |
| BD 17 | lowers the head   | stable head altitude   | raises the head  |
| BD 18 | is bent   | is not bent  |  |
| BD 19 | head pushed forward   | head synchronized with other body parts  | head pushed back   |
| BD 20 | is arched in a U shape  | has the back straight  | has the back rounded   |
| BD 22 | tail base pushed back   | tail base synchronized with other body parts   | tail base pushed forward   |
| BD 23 | head deviation  | no partial deviation   | centre of mass deviation   |
| BD 24 | the movement of the centre of mass is not oriented forward but the one of the head and the tail are | the movements of the head and the tail are not oriented forward but the one of the centre of mass is |  |
| BD 25 | centre of mass synchronized with other body parts   | centre of mass pushed back   |  |

*BD 1 to BD 24 refer to the behavioural descriptors axes and BEC 1, BEC 2, BEC 3 and BEC 4 refer to the behavioural event categories segregated on those axes.*

The particle feature is the principal contributor to the 16th BD. It is not specific to one individual but global for the all environment. We thus cannot specify which animals take part to the BEC extracted from this axis.

The BEC “head-tail inversion on rearing” extracted from the 12st BD is similar to the one extracted from the 21st BD of the BP features-set from the single object exploration experiment (table 10). It thus also describes a mix behaviour of both an individual and the tracking system.

### The BAP feature set

In the long term group monitoring data, on the set grouping body, arena and particle features, SUCCUBE extracted 25 BD. We kept 7 of those which did not correlate at more than 75% with BD extracted from BP feature set. Those 7 BD allowed to segregate 17 BEC.

Table 11: Experimenter given names for the BEC segregated on BD extracted from the BAP features-set in the long term group monitoring experiment.

| BD   | BEC 1                               | BEC 2   | BEC 3                           |
|------|-------------------------------------|---|---------------------------------|
| BD 1 | is in a corner                      | is not in a corner                            |                                 |
| BD 2 | is oriented toward the closest wall | is not oriented toward the closest wall       |                                 |
| BD 3 | does not turn toward the centre     | turns toward the centre                       |                                 |
| BD 7 | goes forward away from the centre   | does not progress with the centre in the back | goes backward toward the centre |



|       |  |  |  |
|-------|--|--|--|
| BD 8  | goes forward toward the centre                     | does not progress with the centre in front         | goes backward away from the centre             |
| BD 15 | orients toward the wall rather than the corner     | stable wall-corner orientation                     | orients toward the corner rather than the wall |
| BD 16 | is oriented toward the wall rather than the corner | is oriented toward the corner rather than the wall |  |

*BD 1 to BD 17 refer to the behavioural descriptors axes and BEC 1, BEC 2 and BEC 3 refer to the behavioural event categories segregated on those axes.*

### The BASP feature set

In the long term group monitoring data, on the set grouping body, arena, social and particle features, SUCCUBE extracted 27 BD. We kept 13 of those which did not correlate at more than 75% with BD extracted from BP and BAP feature set. Those 13 BD allowed to segregate 32 BEC.

Table 12: Experimenter given names for the BEC segregated on BD extracted from the BASP features-set in the long term group monitoring experiment.

| BD    | BEC 1  |                                     | BEC 2   |  |
|-------|--|-------------------------------------|---|--|
|       | BEC 1  | BEC 2                               | BEC 2   | BEC 3                                    |
| BD 2  | same orientation as the receiver   |                                     | opposed orientation with the receiver                                       |  |
| BD 3  | is in contact with the receiver  |                                     | is at distance from the receiver  |  |
| BD 6  | emitter gets closer to the receiver  | neither getting closer or escaped   |   | receiver gets further from the emitter   |
| BD 7  | emitter gets further from the receiver                                     | neither getting further nor joined  |   | receiver gets closer to the emitter      |
| BD 11 | is back to the receiver  | no particular social orientation    |   | faces the receiver                       |
| BD 13 | has a small view angle on the receiver                                     |                                     | has a big view angle on the receiver  |  |
| BD 16 | the receiver is low  | the receiver is at average altitude |   | the receiver is high                     |
| BD 17 | the animals' anterior parts go in opposite directions                      |                                     | the animals' anterior parts go in same direction                            |  |
| BD 19 | the view angle on the receiver increases                                   | stable view angle on the receiver   |   | the view angle on the receiver decreases |
| BD 20 | emitter anterior part and receiver posterior part go in the same direction |                                     | emitter anterior part and receiver posterior part go in opposite directions |  |
| BD 21 | emitter posterior part and receiver anterior                               |                                     | emitter posterior part and receiver anterior                                |  |

|       |   |  |                          |
|-------|---|--|--------------------------|
|       | part go in opposite directions                        | part go in the same direction                          |                          |
| BD 22 | the animals' posterior parts go in the same direction | the animals' posterior parts go in opposite directions |                          |
| BD 24 | goes ahead the receiver                               | goes neither ahead nor behind the receiver             | goes behind the receiver |

*BD 1 to BD 14 refer to the behavioural descriptors axes and BEC 1, BEC 2 and BEC 3 refer to the behavioural event categories segregated on those axes.*

Those BEC describe also the behaviour of the receiver. This is particularly true for the BEC extracted on the 16th BD which only describes the behaviour of the receiver. Such categories can thus be redundant with other categories describing the behaviour of the emitter when emitter and receiver are switched.

## Behavioural event categories of interest

### Behavioural event categories of interest in single object exploration

The variable selection aim at reducing the subsequent number of statistical comparisons. The variations between the time proportion during which each individual display each BEC have then be used to cluster groups of BEC which are related in their expression. This will be presented in three dendrograms, figures 26 to 28. Such clustering allows to select one BEC to represent each cluster, in order to obtain a synthetic set of behaviors that summarizes the measured variations. This analysis has been led on the first cohort data, on which the full repertoire has been discovered.

Selection

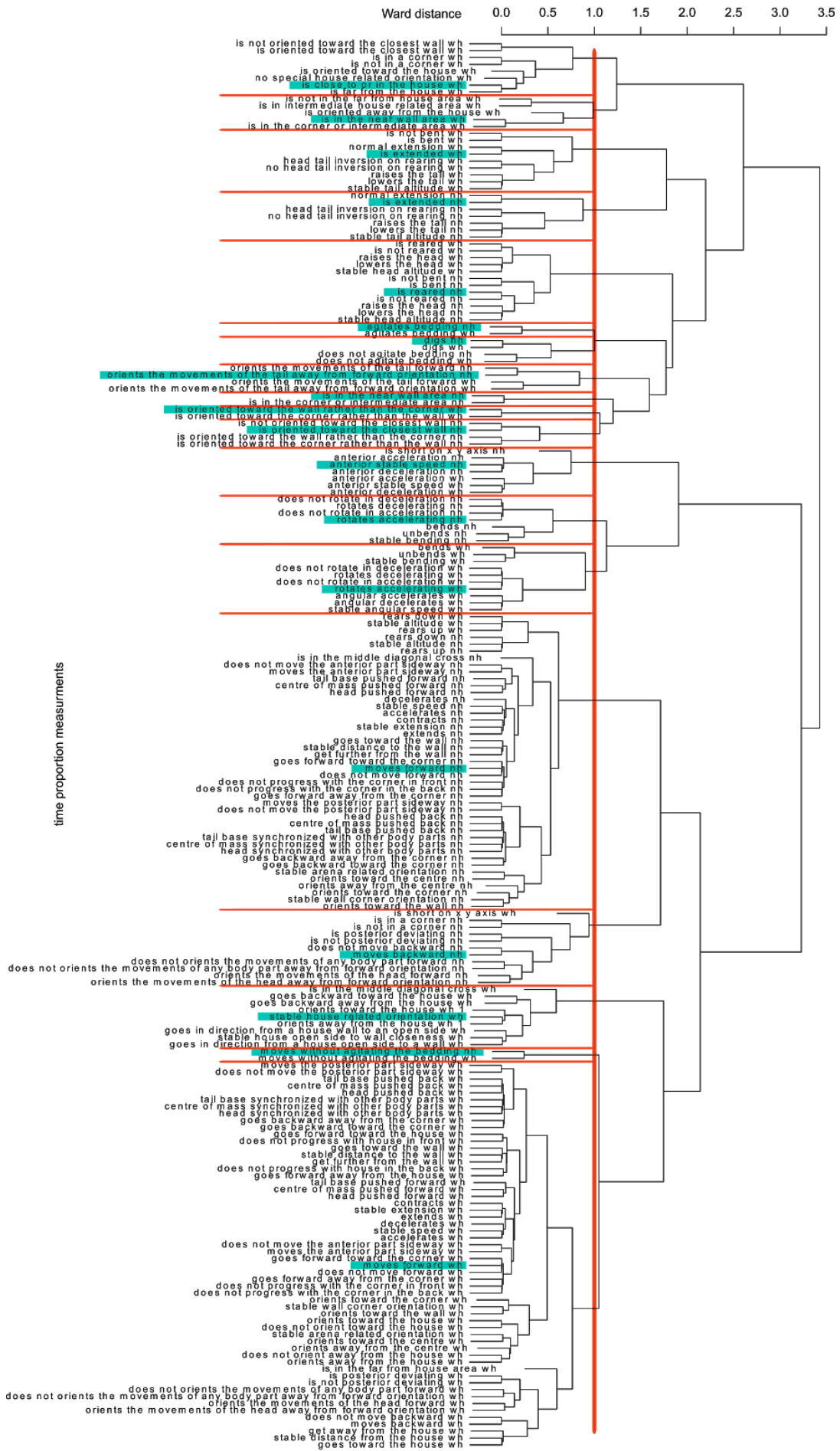


Figure 26: Dendrogram of the HAC of BEC measurements, depending on their proportions of production by individuals, in each phase of the single object exploration experiment. *The vertical red line marks the Ward distance threshold of 1 chosen for clustering. Horizontal red lines separate the BEC clusters. Blue rectangles highlight the BEC measurement chosen as representative of each cluster. Signs after the BEC names show in which phase of experiment we measured emission proportions. “nh” stands for “no house” or the first phase, before the addition of the house. “wh” stands for “with house” or the second phase, after the addition of the house.*

On the basis of the HAC we clustered BEC time proportions in the single object exploration experiment in 19 clusters. We selected in each one a category to represent the cluster (figure 26 in blue). We thus described the behaviour of mice in this experiment by a profile of 19 measurements.

## Interpretation

We consider six aspects of the solitary behaviour in the single object exploration experiment. We interpret BEC time proportions in terms of: locomotor activity; exploration; stereotyped behaviours; thigmotaxis; neophobia and interest for the house.

The behaviours of moving forward and of rotation in acceleration can be interpreted as locomotor activities, and exploratory behaviours. After the addition of the house we can also interpret a reduction of the production of such behaviours as a sign of neophobia induced by the novel object.

The fact of moving backward and to orient the movements of the tail away from forward orientation are not straight forward to interpret. Such motions could depend on locomotor activity.

The behavioural events consisting in having the anterior part at a stable speed are likely to happen mostly when the animal is immobile. As a matter of fact, motion sequences are often made of successive acceleration and deceleration events, what is even clearer for mice in a limited arena. This behavioural event is thus likely to be more displayed by animals with lower locomotor activity.

The posture of extension is part of the exploratory behaviours, as it allows mice to investigate their environment through olfactory and tactile clues. Such behaviour should be less present in animals afflicted with high stress or pain which generally adopt a balled posture. This behaviour can be compared to the stretched attend posture described in de Chaumont et. al.[93]. We can thus see it as a precautious exploratory behaviour, in particular after the addition of the house, this could be a sign of interest toward this novel object. The rearing behaviour can also be considered as an exploratory behaviour as it allows the scanning of the environment above them.

The behaviour consisting in moving without agitating the bedding necessitates a slow movement which is likely to be linked with exploration and curiosity. On the other hand, the fact to agitate the bedding is generally caused by fast movements which are more likely to reveal locomotor activity. The digging which is characterized by the highest amount of bedding movement can be an exploratory behaviour because it allows the mouse to discover underground environmental elements. Digging can also be a stereotypic behaviour, what can be interesting in the context of autism spectrum disorder model [166].

The fact to be in the near wall area or to be oriented toward the closest wall could be interpreted as thigmotaxis, which can itself be a response to stress or anxiety in mice. After the addition of the house such thigmotaxic behaviours can result from the neophobia stimulated by the novel object

The events when mice are close or in the added house show their curiosity and lack of neophobia. The house can also be a way to avoid some light and hide, a behaviour which could be produced as a response to stress.

Finally, the fact to be in a stable orientation relatively to the added house is likely to be due to the fact that the animal is oriented toward it to investigate it. This can be interpreted as a sign of curiosity.

## **Solitary behavioural event categories of interest in long term group monitoring**

### **Selection**





Figure 27: Dendrogram of the HAC of solitary BEC measurements, depending on their proportions of production by individuals, in each phase of the long term group monitoring experiment. *The vertical red line marks the Ward distance threshold of 1 chosen for clustering. Horizontal red lines separate the BEC clusters. Blue rectangles highlight the BEC measurement chosen as representative of each cluster. Signs after the BEC names show in which phase of experiment we measured emission proportions. "n1", "n2" and "n3" stands respectively for first, second and third nights.*

On the basis of the HAC we clustered BEC time proportions in the long-term group monitoring experiment in 18 clusters. We selected in each one a category to represent the cluster (figure 27 in blue). We thus described the behaviour of mice in this experiment by a profile of 18 measurements.

## Interpretation

We consider four aspects of the solitary behaviour in the long term group monitoring experiment. We interpret BEC time proportions in terms of: locomotor activity; exploration; stereotyped behaviours and thigmotaxis.

The behaviours of moving forward, backward and of deviation can be interpreted as locomotor activities, and exploratory behaviours. If studied on successive night we can say that the first night gives a better idea of the animal tendency to explore when the third night is more informative on the spontaneous locomotor activity.

The fact of moving backward and that the movements of the head is not oriented forward but that the one of the tail is, are not straight forward to interpret. Such motions could depend on locomotor activity.

The stable bending event is likely to happen mostly when the animal is immobile. This behavioural event is thus likely to be more express by animals with lower locomotor activity.

The posture of being arched in a U shape can make think about lordosis. We could hypothesis that, if this behaviour appears as an event category in the long-term group monitoring, it could consist of a socially relevant posture. If studied on successive night we can say that such trait evolution could reflect the evolution of the social organisation inside the group.

As in the single object exploration, the extension and rearing postures produced, during the long term group monitoring, are considered as exploratory behaviours.

Events of stability of body altitude and of tail altitude are not straight forward to interpret. Such motion could depend on locomotor activity and exploration.

The event when the bedding moves are generally caused by fast movements which are more likely to reveal locomotor activity. The digging which is characterized by the highest amount of bedding movement can be an exploratory behaviour because it allows the mouse to discover underground environmental elements. Digging can also be a stereotypic behaviour, what can be interesting in the context of autism spectrum disorder model.

The fact to be in a corner or to be oriented toward the closest wall could be interpreted as thigmotaxis, which can itself be a response to stress or anxiety in mice. Finally, events when the animal is oriented toward the wall rather than the corner are not straight forward to interpret. Such posture could depend on thigmotaxis.

## **Social behavioural event categories of interest in long term group monitoring**

### **Selection**

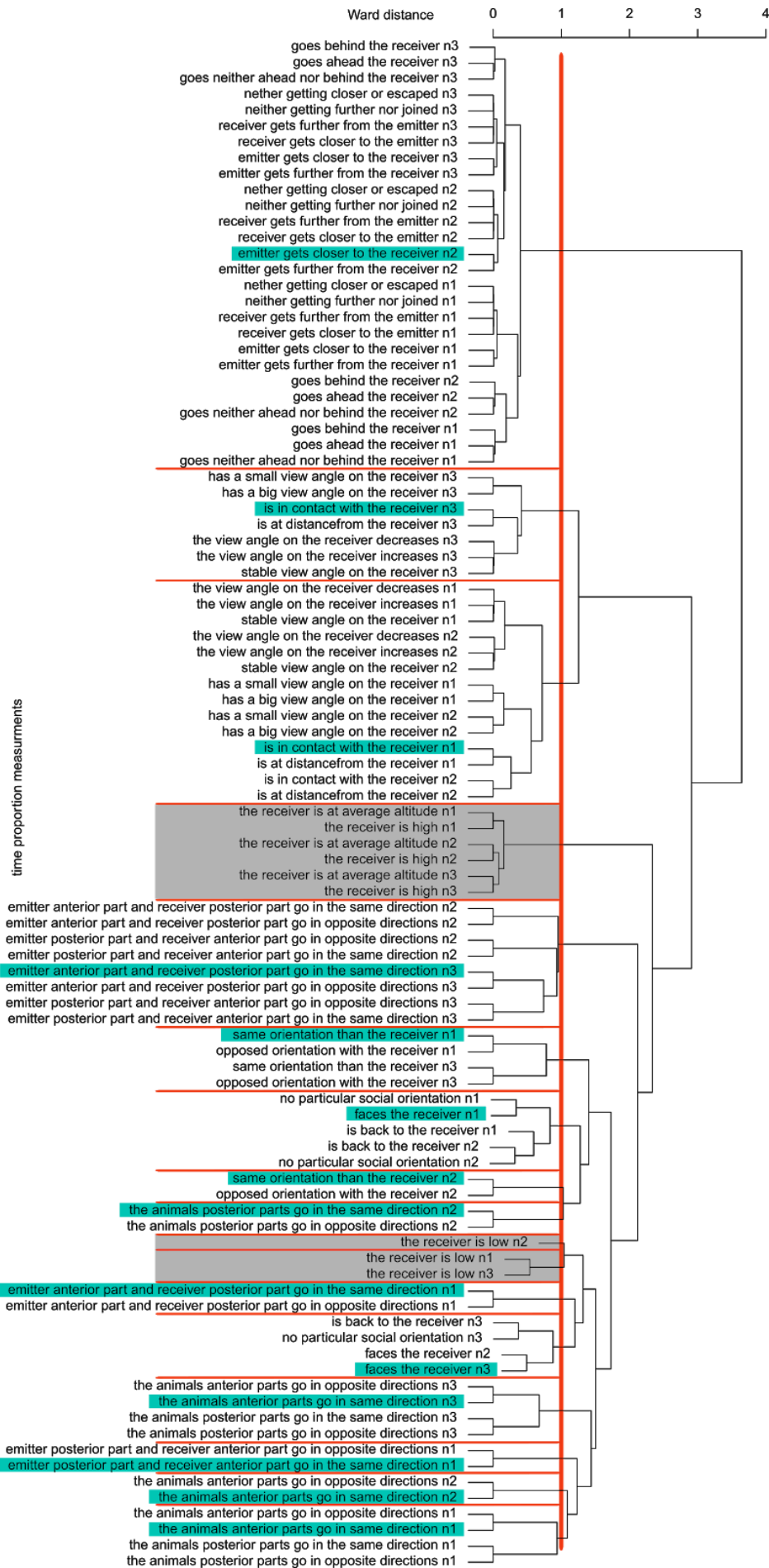


Figure 28: Dendrogram of the HAC of social BEC measurements, depending on their proportions of production by oriented dyads of mice, in each phase of the long term group monitoring experiment. *The vertical red line marks the Ward distance threshold of 1 chosen for clustering. Horizontal red lines separate the BEC clusters. Blue rectangles highlight the BEC measurement chosen as representative of each cluster. Grey rectangles highlight the clusters for which we didn't choose any representative measurements. Signs after the BEC names show in which phase of experiment we measured emission proportions. "n1", "n2" and "n3" stands respectively for first, second and third nights.*

On the basis of the HAC we clustered BEC time proportions in the long-term group monitoring experiment in 17 clusters. We selected, in 14 of them, one category to represent the cluster (figure 28 in blue). In the 3 other ones (figure 28 in grey), all BEC only described the behaviour of the receiver and we did not include such categories in the profile. We thus described the behaviour of oriented dyads in this experiment by a profile of 14 measurements.

## Interpretation

All social behavioural events selected here should reflect the level of social interest. Such traits are thus of high interest in the context of autism spectrum disorder model. We will consider three different aspects of social interest:

- social proximity expressed by contact events;
- social orientation that we can interpret from facing and sharing orientation behaviours;
- social motion showed when the emitter gets closer to the receiver or event during which the two animals body parts move in the same direction.

If studied on successive nights, we can say that such trait evolution could reflect the evolution of the social organisation inside the group.

## **Collaborative Cross strain behavioural profiles**

The behavioral profiles of the collaborative cross strains are then obtained by comparing the proportions of time spent producing each previously selected BEC between this CC line and the C57BL/6J. These profiles are therefore based on the data from the first cohort only. They are represented by figures 29, 31 and 33 for males and 30, 32 and 34 for females.

## **Behavioural profile in single object exploration experiment**

We compared the behaviour in single object exploration of the 12 CC to C57BL/6J mice on the basis of 19 BEC measurements. We compared all means by performing Mann-Whitney non-parametric tests which were adapted to non-Gaussian and non-homoscedastic populations. In order to avoid the family-wise type 1 error-rate inflation with the extension of this screening we chose to correct all tests here led through Bonferroni method for 228 tests.



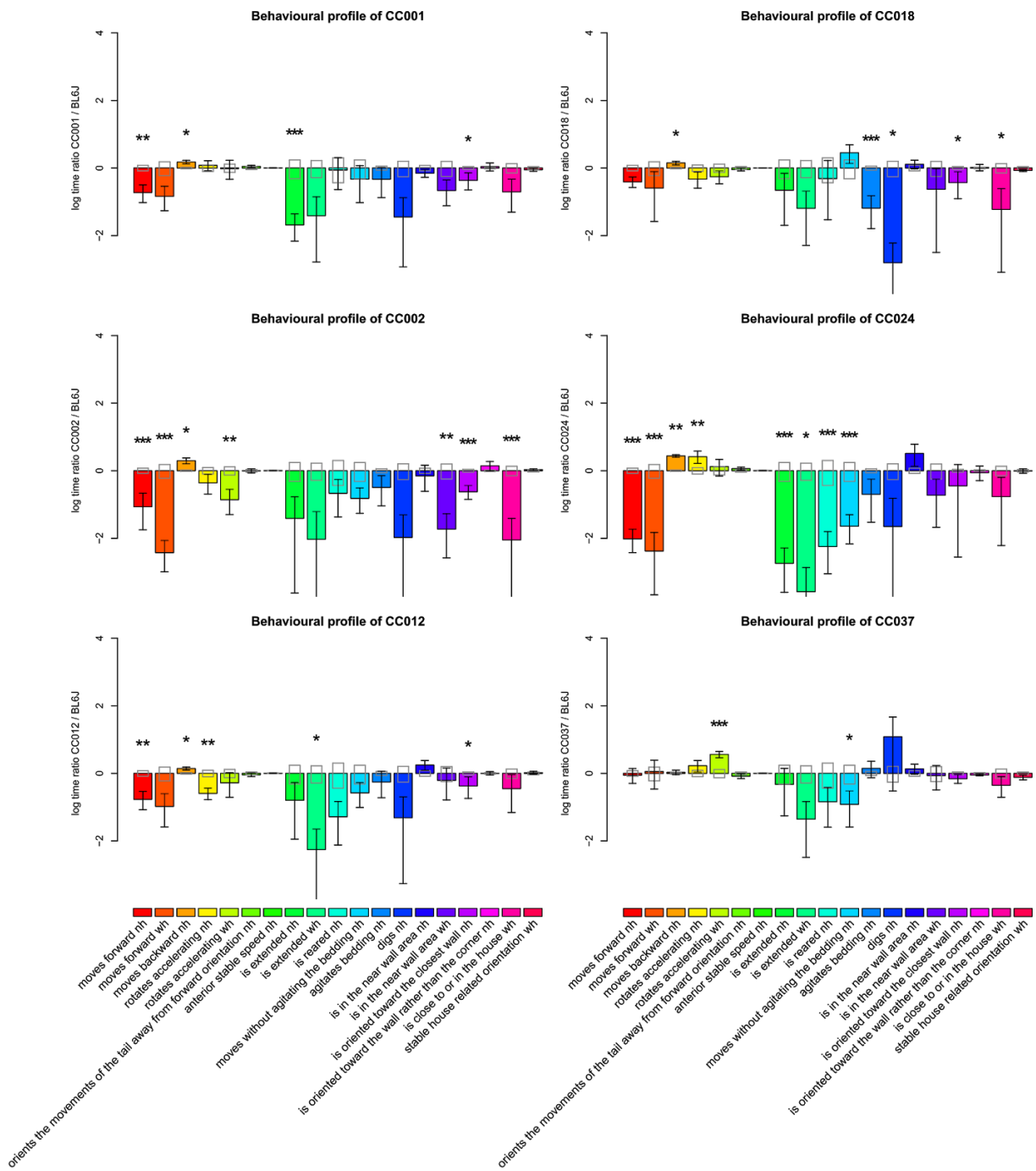
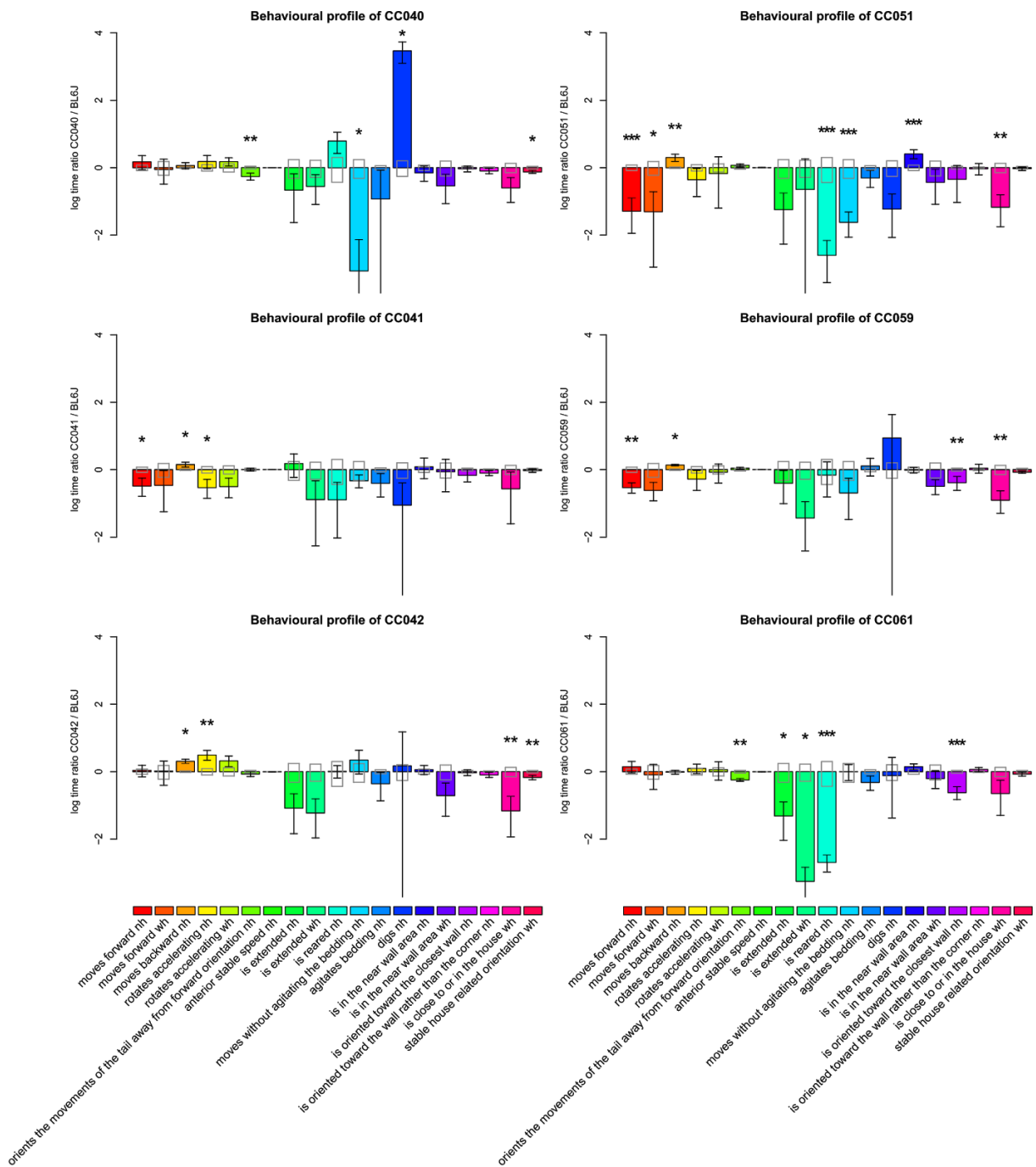


Figure 29: Behavioural profile of CC strain males in single object exploration, barplot of the logarithm of the ratio of time proportions of BEC production between CC and C57BL/6J male mice. Each bar gives the logarithm of the ratio (CC / C57BL/6J) in proportion of production of one BEC in one phase of an experiment. Black error bars display the confidence interval of CC strains means around the ratios.



Grey rectangles display the confidence interval of C57BL/6J means around the origin. Stars represent the significance of the Mann-Whitney tests comparing strains means on the basis of the P values (\*: <0.05 ; \*\*: <0.01 ; \*\*\*: <0.001), corrected for 228 tests through Bonferroni method. Signs after the BEC names show in which phases of the experiments we measured emission proportions. “nh” and “wh” stands respectively for first and second phases of the single object exploration.

During the single object exploration, before the addition of the house, CC001 males spent less time moving forward, being extended and being oriented toward the closest wall (respective P-values =  $2.04e-03$  ;  $5.84e-04$  ;  $2.8e-02$ ), and more time moving backward (P-value =  $1.96e-02$ ), than C57Bl/6J males.

This suggests that CC001 males demonstrate less locomotor activity, exploration and thigmotaxis than C57Bl/6J males.

Before the addition of the house, CC002 males spent less time moving forward and being oriented toward the closest wall (respective P-values =  $9.49e-04$  ;  $9.49e-04$ ), and more time moving backward (P-value =  $4.27e-02$ ), than C57Bl/6J males. Once we added the house, CC002 males spent less time moving forward, rotating in acceleration, being in the near wall area and being close to or in the house (respective P-values =  $9.49e-04$  ;  $1.9e-03$  ;  $1.9e-03$  ;  $9.49e-04$ ), than C57Bl/6J males.

This suggests that CC002 males demonstrate less locomotor activity, exploration and thigmotaxis and more neophobia than C57Bl/6J males.

Before the addition of the house, CC012 males spent less time moving forward, rotating in acceleration and being oriented toward the closest wall (respective P-values =  $2.04e-03$  ;  $1.17e-03$  ;  $2.8e-02$ ), and more time moving backward (P-value =  $1.96e-02$ ), than C57Bl/6J males. Once we added the house, CC012 males spent less time being extended (P-value =  $2.57e-02$ ), than C57Bl/6J males.

This suggests that CC012 males demonstrate less locomotor activity, exploration, thigmotaxis and interest toward the house than C57Bl/6J males.

Before the addition of the house, CC018 males spent less time agitating the bedding, digging and being oriented toward the closest wall (respective P-values =  $2.92e-04$ ;  $1.62e-02$  ;  $1.31e-02$ ), and more time moving backward (P-value =  $2.8e-02$ ), than C57BL/6J males. Once we added the house, CC018 males spent less time being close to or in the house (P-value =  $1.31e-02$ ), than C57BL/6J males. This suggests that CC018 males demonstrate less exploration, potentially stereotyped behaviours and thigmotaxis and more neophobia than C57BL/6J males.

Before the addition of the house, CC024 males spent less time moving forward, being extended, being reared and moving without agitating the bedding (respective P-values =  $2.92e-04$ ;  $2.92e-04$  ;  $5.84e-04$  ;  $2.92e-04$ ), and more time moving backward and rotating in acceleration (respective P-values =  $5.55e-03$  ;  $8.76e-03$ ), than C57BL/6J males. Once we added the house, CC024 males spent less time moving forward and being extended (respective P-values =  $2.92e-04$  ;  $1.61e-02$ ), than C57BL/6J males. This suggests that CC024 males demonstrate less locomotor activity, exploration, thigmotaxis and interest toward the house and more neophobia than C57BL/6J males.

Before the addition of the house, CC037 males spent less time moving without agitating the bedding (P-value =  $3.97e-02$ ), than C57BL/6J males. Once we added the house, CC037 males spent more time rotating in acceleration (P-value =  $5.84e-04$ ), than C57BL/6J males.

This suggests that CC037 males demonstrate less exploration and neophobia than C57BL/6J males.

Before the addition of the house, CC040 males spent less time putting the movement of their tail out of their axis and moving without agitating the bedding (respective P-values =  $3.5e-03$ ;  $1.61e-02$ ), and more time digging (P-value =  $1.62e-02$ ), than C57BL/6J males. Once we added the house, CC040 males spent less time having a stable house related orientation (P-value =  $3.97e-02$ ), than C57BL/6J males. This suggests that CC040 males demonstrate less exploration, potentially stereotyped behaviours and interest toward the house than C57BL/6J males.

Before the addition of the house, CC041 males spent less time moving forward and rotating in acceleration (respective P-values =  $3.97e-02$ ;  $2.8e-02$ ), and more time moving backward (P-value =  $1.96e-02$ ), than C57BL/6J males.

This suggests that CC041 males demonstrate less locomotor activity and exploration than C57BL/6J males.

Before the addition of the house, CC042 males spent more time moving backward and rotating in acceleration (respective P-values =  $1.96e-02$ ;  $2.04e-03$ ), than C57BL/6J males. Once we added the house, CC042 males spent less time being close to or in the house and having a stable house related orientation (respective P-values =  $1.17e-03$ ;  $8.76e-03$ ), than C57BL/6J males.

This suggests that CC042 males demonstrated more locomotor activity, exploration and neophobia and less interest toward the house than C57BL/6J males.

Before the addition of the house, CC051 males spent less time moving forward, being reared and moving without agitating the bedding (respective P-values =  $2.92e-04$  ;  $2.92e-04$  ;  $2.92e-04$ ), and more time moving backward and being in the near wall area (respective P-values =  $8.76e-03$  ;  $2.92e-04$ ), than C57BL/6J males. Once we added the house, CC051 males spent less time moving forward and being close to or in the house (respective P-values =  $1.96e-02$  ;  $1.17e-03$ ), than C57BL/6J males. This suggests that CC051 males demonstrate less locomotor activity and exploration and more thigmotaxis and neophobia than C57BL/6J males.

Before the addition of the house, CC059 males spent less time moving forward and being oriented toward the closest wall (respective P-values =  $8.76e-03$  ;  $8.76e-03$ ), and more time moving backward (P-value =  $1.96e-02$ ), than C57BL/6J males. Once we added the house, CC059 males spent less time being close to or in the house (P-value =  $1.17e-03$ ), than C57BL/6J males.

This suggests that CC059 males demonstrate less locomotor activity, exploration and thigmotaxis and more neophobia than C57BL/6J males.

Before the addition of the house, CC061 males spent less time putting the movement of their tail out of their axis, being extended, being reared and being oriented toward the closest wall (respective P-values =  $1.17e-03$  ;  $1.96e-02$  ;  $2.92e-04$  ;  $2.92e-04$ ), than C57BL/6J males. Once we added the house, CC061 males spent less time being extended (P-value =  $1.61e-02$ ), than C57BL/6J males.

This suggests that CC061 males demonstrate less exploration, thigmotaxis and interest toward the house than C57BL/6J males.

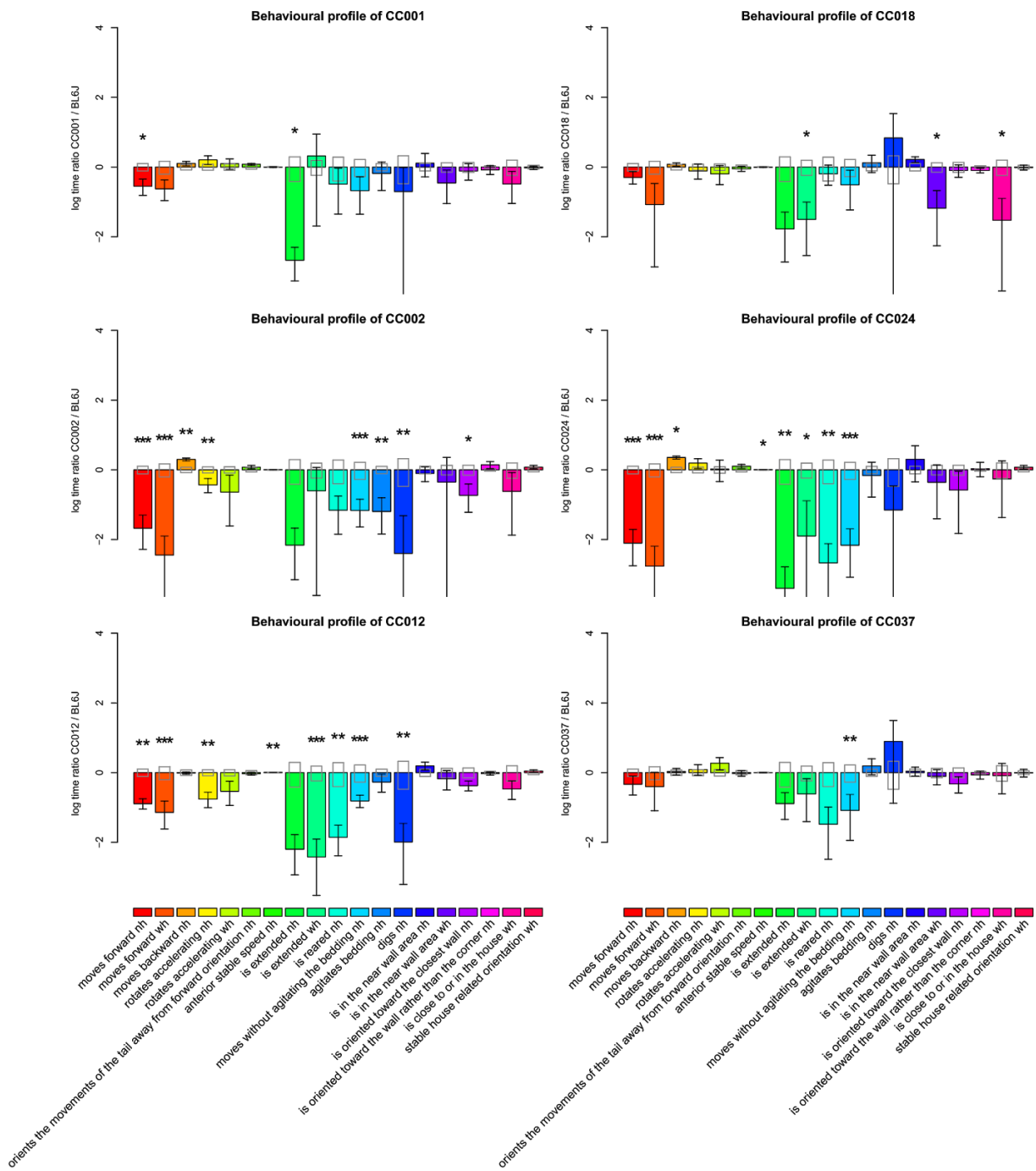
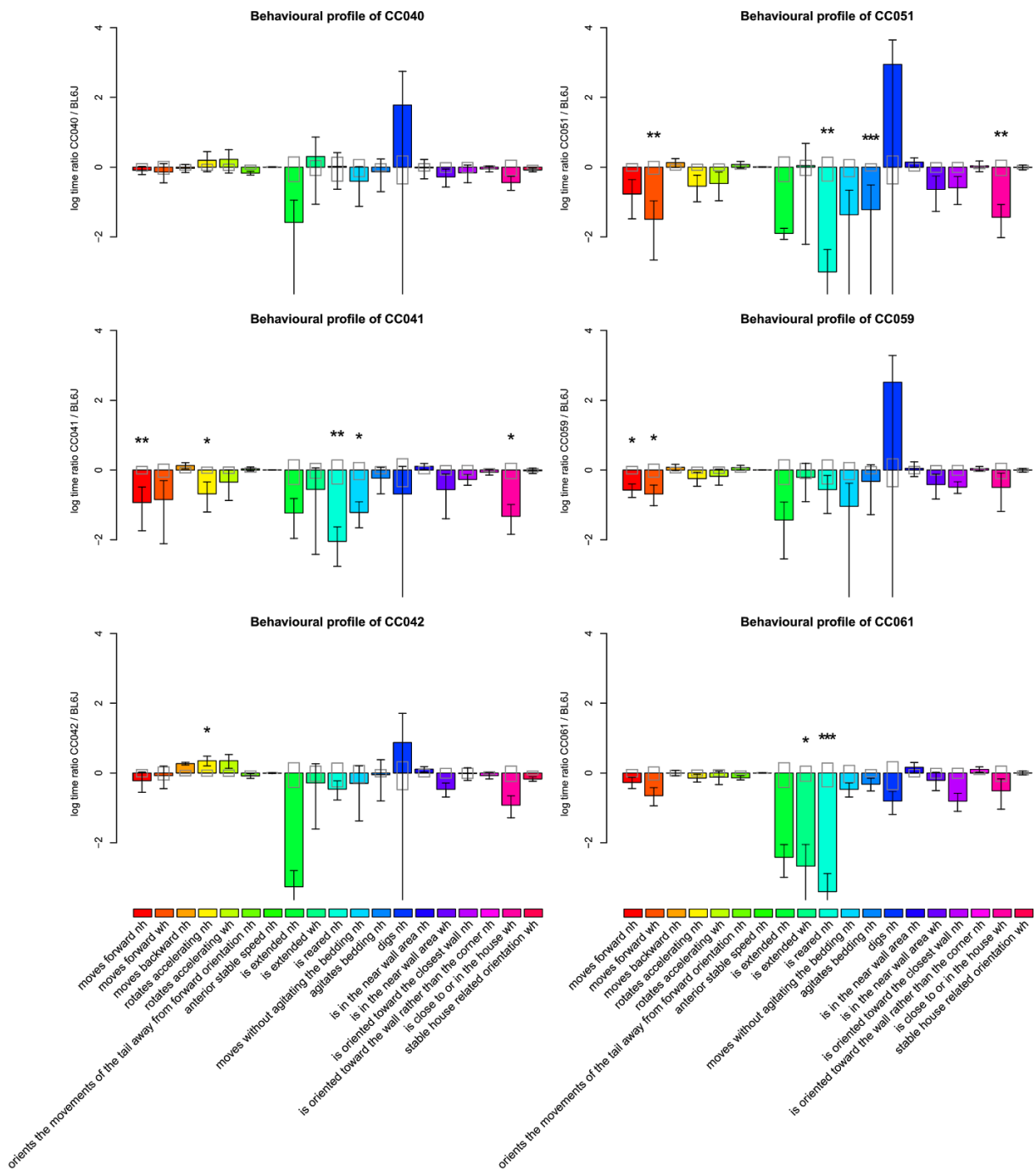


Figure 30: Behavioural profile of CC strain females in single object exploration, barplot of the logarithm of the ratio of time proportions of BEC production between CC and C57BL/6J female mice. Each bar gives the logarithm of the ratio (CC / C57BLI/6J) in proportion of production of one BEC in one phase of an experiment. Black error bars display the confidence interval of CC strains means around the ratios.



Grey rectangles display the confidence interval of C57BL/6J means around the origin. Stars represent the significance of the Mann-Whitney tests comparing strains means on the basis of the P values (\*:  $<0.05$  ; \*\*:  $<0.01$  ; \*\*\*:  $<0.001$ ), corrected for 228 tests through Bonferroni method. Signs after the BEC names show in which phases of the experiments we measured emission proportions. “nh” and “wh” stands respectively for first and second phases of the single object exploration.



During the single object exploration, before the addition of the house, CC001 females spent less time moving forward and being extended (respective P-values =  $4.15e-02$  ;  $1.18e-02$ ), than C57BL/6J females.

This suggests that CC001 females demonstrate less locomotor activity and exploration than C57BL/6J females.

Before the addition of the house, CC002 females spent less time moving forward, rotating in acceleration, moving without agitating the bedding, agitating the bedding, digging and being oriented toward the closest wall (respective P-values =  $3e-05$  ;  $2.92e-03$  ;  $1.8e-04$  ;  $1e-03$  ;  $7.33e-03$  ;  $1.78e-02$ ), and more time moving backward (P-value =  $4.08e-03$ ), than C57BL/6J females. Once we added the house, CC002 females spent less time moving forward (P-value =  $3e-05$ ), than C57BL/6J females. This suggests that CC002 females demonstrate less locomotor activity, exploration, potentially stereotyped behaviours and thigmotaxis and more neophobia than C57BL/6J females.

Before the addition of the house, CC012 females spent less time moving forward, rotating in acceleration, being reared, moving without agitating the bedding and digging (respective P-values =  $2.08e-03$  ;  $2.61e-03$  ;  $1.45e-03$  ;  $4.5e-04$  ;  $2.92e-03$ ), and more time having the anterior part at stable speed (P-value =  $5.4e-03$ ), than C57BL/6J females. Once we added the house, CC012 females spent less time moving forward and being extended (respective P-values =  $6.75e-04$  ;  $6e-05$ ), than C57BL/6J females.

This suggests that CC012 females demonstrate less locomotor activity, exploration, potentially stereotyped behaviours and interest toward the house and more neophobia than C57BL/6J females.

The behaviours of CC018 females were not significantly different from the behaviour of C57BL/6J females before we added the house. Once we added the house, CC018 females spent less time being extended, being in the near wall area and being close to or in the house (respective P-values =  $1.86e-02$  ;  $1.18e-02$  ;  $1.18e-02$ ), than C57BL/6J females.

This suggests that CC018 females demonstrate less interest for the house and more neophobia than C57BL/6J females.

Before the addition of the house, CC024 females spent less time moving forward, being extended, being reared and moving without agitating the bedding (respective P-values =  $6.2e-04$  ;  $2.48e-03$  ;  $2.48e-03$  ;  $6.2e-04$ ), and more time moving backward and having the anterior part at stable speed (respective P-values =  $4.15e-02$  ;  $2.29e-02$ ), than C57BL/6J females. Once we added the house, CC024 females spent less time moving forward and being extended (respective P-values =  $6.2e-04$  ;  $1.86e-02$ ), than C57BL/6J females.

This suggests that CC024 females demonstrate less locomotor activity, exploration and interest toward the house and more neophobia than C57BL/6J females.

Before the addition of the house, CC037 females spent less time moving without agitating the bedding (P-value =  $2.48e-03$ ), than C57BL/6J females. The behaviours of CC037 females were not significantly different from the behaviour of C57BL/6J females once we added the house.

This suggests that CC037 females demonstrate less exploration than C57BL/6J females.

During the single object exploration, CC040 females did not spend significantly different proportions of time producing any behavioural event category, compared to C57BL/6J females.

This suggests that CC040 females demonstrate behavioural traits close to the ones of C57BL/6J females.

Before the addition of the house, CC041 females spent less time moving forward, rotating in acceleration, being reared and moving without agitating the bedding (respective P-values =  $7.44e-03$  ;  $1.18e-02$  ;  $7.44e-03$  ;  $2.29e-02$ ), than C57BL/6J females. Once we added the house, CC041 females spent less time being close to or in the house (P-value =  $2.79e-02$ ), than C57BL/6J females.

This suggests that CC041 females demonstrate less locomotor activity and exploration and more neophobia than C57BL/6J females.

Before the addition of the house, CC042 females spent more time rotating in acceleration (P-value =  $1.3e-02$ ), than C57BL/6J females. The behaviours of CC042 females were not significantly different from the behaviour of C57BL/6J females once we added the house.

This suggests that CC042 females demonstrated more locomotor activity and exploration than C57BL/6J females.

Before the addition of the house, CC051 females spent less time being reared and agitating the bedding (respective P-values =  $1.24e-03$  ;  $6.2e-04$ ), than C57BL/6J females. Once we added the house, CC051 females spent less time moving forward and being close to or in the house (respective P-values =  $2.48e-03$  ;  $7.44e-03$ ), than C57BL/6J females.

This suggests that CC051 females demonstrate less locomotor activity and exploration and more neophobia than C57BL/6J females.

Before the addition of the house, CC059 females spent less time moving forward (P-value =  $4.15e-02$ ), than C57BL/6J females. Once we added the house, CC059 females spent less time moving forward (P-value =  $4.15e-02$ ), than C57BL/6J females.

This suggests that CC059 females demonstrate less locomotor activity, exploration and more neophobia than C57BL/6J females.

Before the addition of the house, CC061 females spent less time being reared (P-value =  $6.2e-04$ ), than C57BL/6J females. Once we added the house, CC061 females spent less time being extended (P-value =  $2.94e-02$ ), than C57BL/6J females.

This suggests that CC061 females demonstrate less exploration and interest toward the house than C57BL/6J females.

### **Solitary behavioural profile in long term group monitoring experiment**

We compared the solitary behaviour in long term group monitoring of the 12 CC strains to C57BL/6J mice on the basis of 18 BEC measurements. We compared all means by performing Mann-Whitney non-parametric tests. These tests were adapted to non-gaussian and non-homoscedastic populations. In order to avoid the family-wise type 1 error-rate inflation with the extension of this screening we chose to correct all tests here led through Bonferroni method for 216 tests.

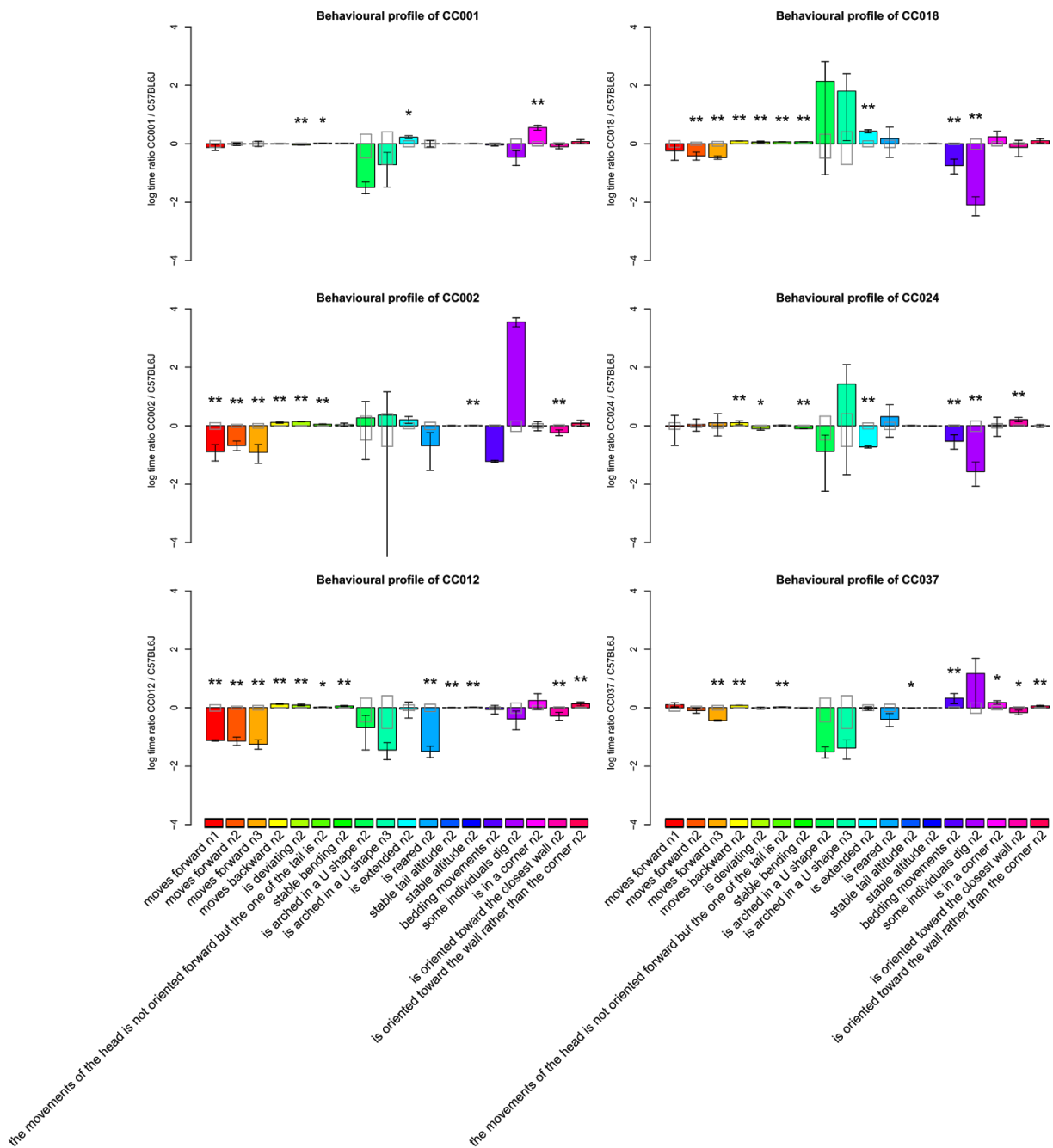
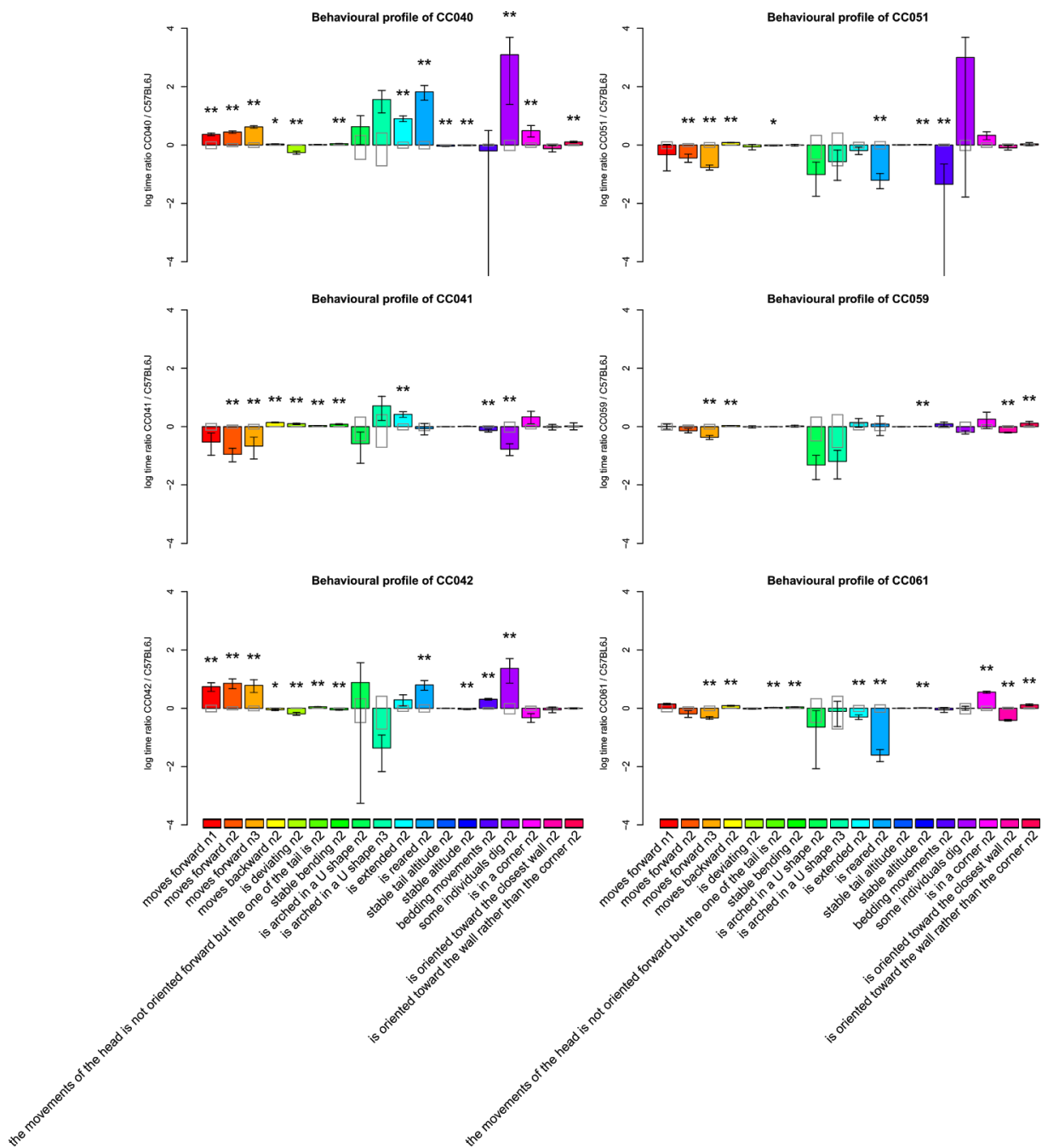


Figure 31: Solitary behavioural profile of CC strain males in long term group monitoring, barplot of the logarithm of the ratio of time proportions of BEC production between CC and C57BL/6J male mice. Each bar gives the logarithm of the ratio (CC / C57BL/6J) in proportion of production of one BEC in one phase of an experiment. Black error bars display the confidence interval of CC strains means around the ratios.



Grey rectangles display the confidence interval of C57BL/6J means around the origin. Stars represent the significance of the Mann-Whitney tests comparing strains means on the basis of the P values (\*: <0.05 ; \*\*: <0.01 ; \*\*\*: <0.001), corrected for 216 tests through Bonferroni method. Signs after the BEC names show in which phases of the experiments we measured emission proportions. “n1”, “n2” and “n3” stands respectively for first, second and third nights of the long term group monitoring.

During long term group monitoring, on the second night, CC001 males spent less time deviating from their past trajectories (P-value =  $3.43e-03$ ), and more time putting the movement of their head out of their axis, being extended and being in a corner (respective P-values =  $1.37e-02$  ;  $4.12e-02$  ;  $3.43e-03$ ), than C57BL/6J males.

This suggests that CC001 males in social context demonstrate more exploration and thigmotaxis than C57BL/6J males.

During long term group monitoring, on the first night, CC002 males spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J males. On the second night, CC002 males spent less time moving forward and being oriented toward the closest wall (respective P-values =  $3.43e-03$  ;  $3.43e-03$ ), and more time moving backward, deviating from their past trajectories, putting the movement of their head out of their axis and being at a stable altitude (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), than C57BL/6J males. On the third night, CC002 males spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J males.

This suggests that CC002 males in social context demonstrate less locomotor activity, exploration and thigmotaxis than C57BL/6J males.

During long term group monitoring, on the first night, CC012 males spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J males. On the second night, CC012 males spent less time moving forward, being reared and being oriented toward the closest wall (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), and more time moving backward, deviating from their past trajectories, putting the movement of their head out of their axis, having a stable bending, having a stable tail altitude, being at a stable altitude and being oriented toward the wall rather than the corner (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $1.37e-02$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), than C57BL/6J males. On the third night, CC012 males spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J males.

This suggests that CC012 males in social context demonstrate less locomotor activity, exploration and thigmotaxis than C57BL/6J males.

During long term group monitoring, on the second night, CC018 males spent less time moving forward, agitating the bedding and digging (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), and more time moving backward, deviating from their past trajectories, putting the movement of their head out of their axis, having a stable bending and being extended (respective P-values =  $3.43e-03$  ;  $6.86e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), than C57BL/6J males. On the third night, CC018 males spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J males.

This suggests that CC018 males in social context demonstrate less locomotor activity, exploration and potentially stereotyped behaviours than C57BL/6J males.



During long term group monitoring, on the second night, CC024 males spent less time deviating from their past trajectories, having a stable bending, being extended, agitating the bedding and digging (respective P-values =  $2.4e-02$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), and more time moving backward and being oriented toward the closest wall (respective P-values =  $3.43e-03$  ;  $3.43e-03$ ), than C57BL/6J males.

This suggests that CC024 males in social context demonstrate less locomotor activity, exploration and potentially stereotyped behaviours and more thigmotaxis than C57BL/6J males.

During long term group monitoring, on the second night, CC037 males spent less time having a stable tail altitude and being oriented toward the closest wall (respective P-values =  $1.37e-02$  ;  $1.37e-02$ ), and more time moving backward, putting the movement of their head out of their axis, agitating the bedding, being in a corner and being oriented toward the wall rather than the corner (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $4.12e-02$  ;  $6.86e-03$ ), than C57BL/6J males. On the third night, CC037 males spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J males.

This suggests that CC037 males in social context demonstrate first more exploration and thigmotaxis and then less locomotor activity on the third day than C57BL/6J males.

During long term group monitoring, on the first night, CC040 males spent more time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J males. On the second night, CC040 males spent less time deviating from their past trajectories, having a stable tail altitude and being at a stable altitude (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), and more time moving forward, moving backward, having a stable bending, being extended, being reared, digging, being in a corner and being oriented toward the wall rather than the corner (respective P-values =  $3.43e-03$  ;  $2.4e-02$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), than C57BL/6J males. On the third night, CC040 males spent more time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J males.

This suggests that CC040 males in social context demonstrate more locomotor activity, exploration, potentially stereotyped behaviours and thigmotaxis than C57BL/6J males.

During long term group monitoring, on the second night, CC041 males spent less time moving forward, agitating the bedding and digging (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), and more time moving backward, deviating from their past trajectories, putting the movement of their head out of their axis, having a stable bending and being extended (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), than C57BL/6J males. On the third night, CC041 males spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J males.

This suggests that CC041 males in social context demonstrate less locomotor activity, exploration and potentially stereotyped behaviours than C57BL/6J males.

During long term group monitoring, on the first night, CC042 males spent more time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J males. On the second night, CC042 males spent less time moving backward, deviating from their past trajectories, having a stable bending and being at a stable altitude (respective P-values =  $4.12e-02$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), and more time moving forward, putting the movement of their head out of their axis, being reared, agitating the bedding and digging (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), than C57BL/6J males. On the third night, CC042 males spent more time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J males.

This suggests that CC042 males in social context demonstrate more locomotor activity, exploration and potentially stereotyped behaviours than C57BL/6J males.

During long term group monitoring, on the second night, CC051 males spent less time moving forward, putting the movement of their head out of their axis, being reared and agitating the bedding (respective P-values =  $3.43e-03$  ;  $1.37e-02$  ;  $3.43e-03$  ;  $3.43e-03$ ), and more time moving backward and being at a stable altitude (respective P-values =  $3.43e-03$  ;  $3.43e-03$ ), than C57BL/6J males. On the third night, CC051 males spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J males.

This suggests that CC051 males in social context demonstrate less locomotor activity and exploration than C57BL/6J males.

During long term group monitoring, on the second night, CC059 males spent less time being oriented toward the closest wall (P-value =  $3.43e-03$ ), and more time moving backward, being at a stable altitude and being oriented toward the wall rather than the corner (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), than C57BL/6J males. On the third night, CC059 males spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J males.

This suggests that CC059 males in social context demonstrate less locomotor activity, exploration and thigmotaxis than C57BL/6J males.

During long term group monitoring, on the second night, CC061 males spent less time being extended, being reared and being oriented toward the closest wall (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), and more time moving backward, putting the movement of their head out of their axis, having a stable bending, being at a stable altitude, being in a corner and being oriented toward the wall rather than the corner (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), than C57BL/6J males. On the third night, CC061 males spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J males.

This suggests that CC061 males in social context demonstrate less locomotor activity and exploration and more thigmotaxis than C57BL/6J males.

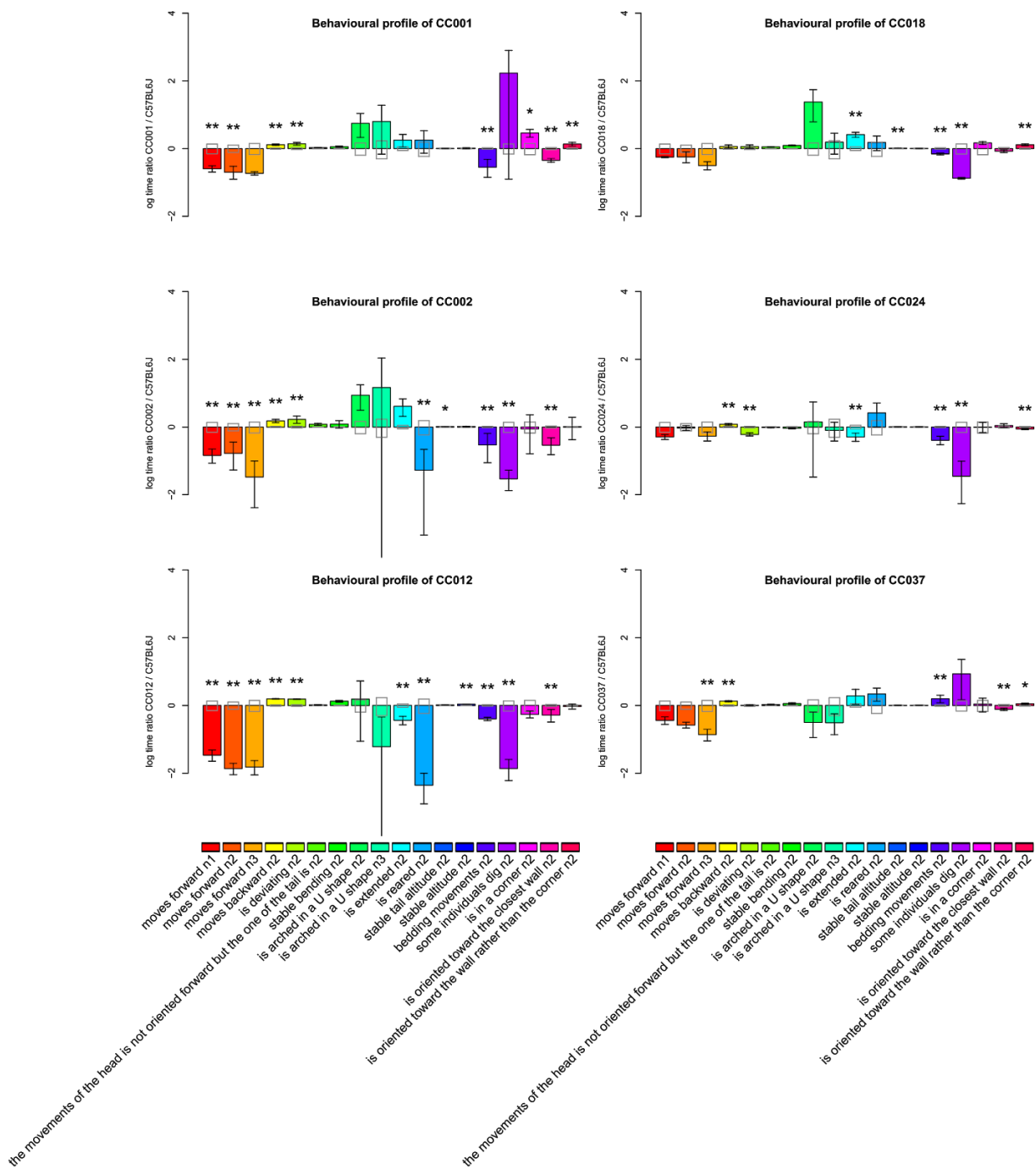
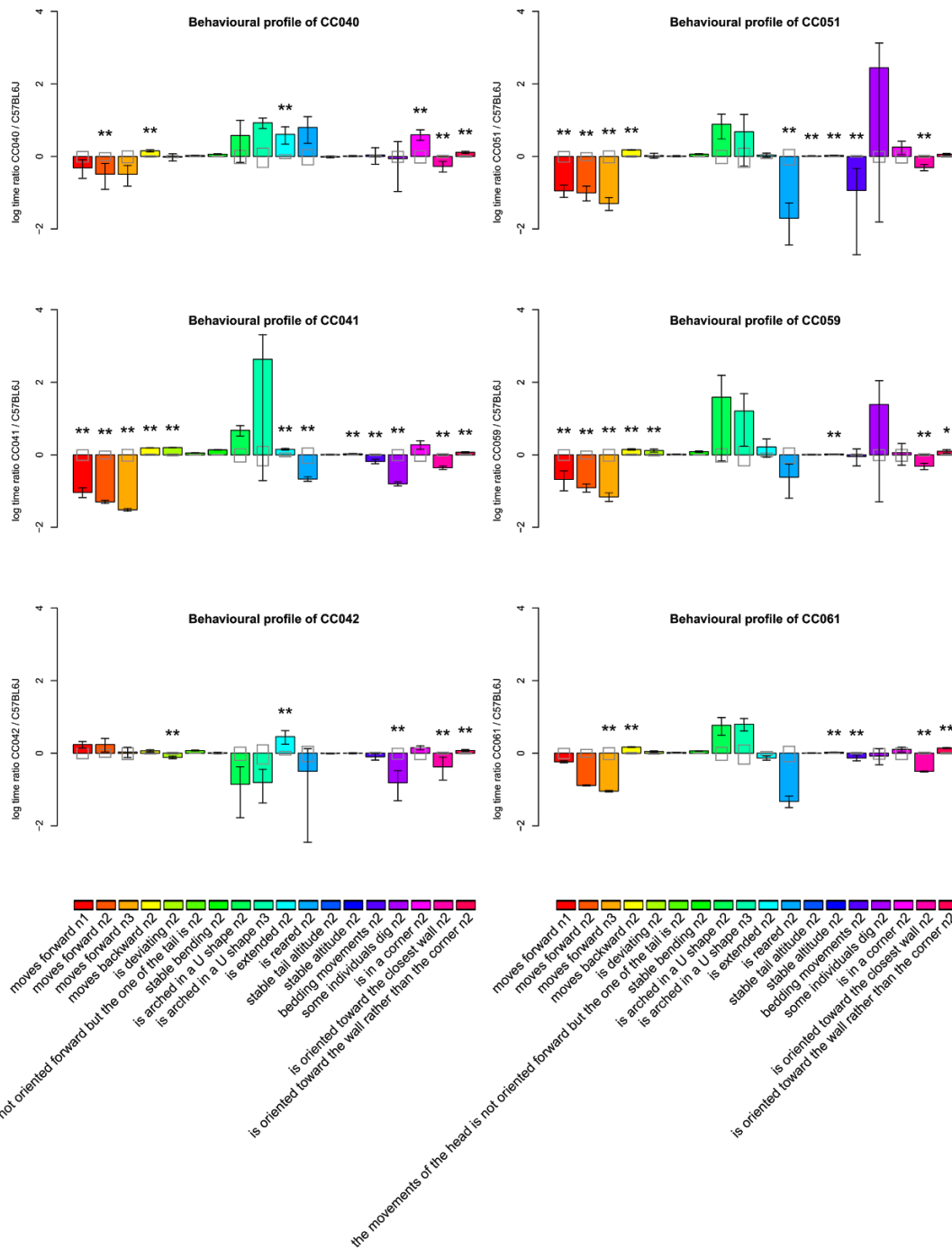


Figure 32: Solitary behavioural profile of CC strain females in long term group monitoring, barplot of the logarithm of the ratio of time proportions of BEC production between CC and C57BL/6J female mice. Each bar gives the logarithm of the ratio (CC / C57BL/6J) in proportion of production of one BEC in one phase of an experiment. Black error bars display the confidence interval of CC strains means around the ratios.



Grey rectangles display the confidence interval of C57BL/6J means around the origin. Stars represent the significance of the Mann-Whitney tests comparing strains means on the basis of the P values (\*: <0.05 ; \*\*: <0.01 ; \*\*\*: <0.001), corrected for 216 tests through Bonferroni method. Signs after the BEC names show in which phases of the experiments we measured emission proportions. “n1”, “n2” and “n3” stands respectively for first, second and third nights of the long term group monitoring.

During long term group monitoring, on the first night, CC001 females spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J females. On the second night, CC001 females spent less time moving forward, agitating the bedding and being oriented toward the closest wall (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), and more time moving backward, deviating from their past trajectories, being in a corner and being oriented toward the wall rather than the corner (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $1.37e-02$  ;  $3.43e-03$ ), than C57BL/6J females.

This suggests that CC001 females in social context demonstrate less locomotor activity and exploration and more thigmotaxis than C57BL/6J females.

During long term group monitoring, on the first night, CC002 females spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J females. On the second night, CC002 females spent less time moving forward, being reared, agitating the bedding, digging and being oriented toward the closest wall (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), and more time moving backward, deviating from their past trajectories and having a stable tail altitude (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $1.37e-02$ ), than C57BL/6J females. On the third night, CC002 females spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J females.

This suggests that CC002 females in social context demonstrate less locomotor activity, exploration, potentially stereotypic behaviours and thigmotaxis than C57BL/6J females.

During long term group monitoring, on the first night, CC012 females spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J females. On the second night, CC012 females spent less time moving forward, being extended, being reared, agitating the bedding, digging and being oriented toward the closest wall (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), and more time moving backward, deviating from their past trajectories and being at a stable altitude (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), than C57BL/6J females. On the third night, CC012 females spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J females.

This suggests that CC012 females in social context demonstrate less locomotor activity, exploration and thigmotaxis than C57BL/6J females.

During long term group monitoring, on the second night, CC018 females spent less time agitating the bedding and digging (respective P-values =  $3.43e-03$  ;  $3.43e-03$ ), and more time being extended, having a stable tail altitude and being oriented toward the wall rather than the corner (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), than C57BL/6J females.

This suggests that CC018 females in social context demonstrate less locomotor activity, exploration and potentially stereotyped behaviours than C57BL/6J females.

During long term group monitoring, on the second night, CC024 females spent less time deviating from their past trajectories, being extended, agitating the bedding, digging and being oriented toward the wall rather than the corner (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), and more time moving backward (P-value =  $3.43e-03$ ), than C57BL/6J females.

This suggests that CC024 females in social context demonstrate less locomotor activity, exploration and potentially stereotyped behaviours than C57BL/6J females.



During long term group monitoring, on the second night, CC037 females spent less time being oriented toward the closest wall (P-value =  $3.43e-03$ ), and more time moving backward, agitating the bedding and being oriented toward the wall rather than the corner (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $4.12e-02$ ), than C57BL/6J females. On the third night, CC037 females spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J females.

This suggests that CC037 females in social context demonstrate less thigmotaxis and less locomotor activity on the third day than C57BL/6J females.

During long term group monitoring, on the second night, CC040 females spent less time moving forward and being oriented toward the closest wall (respective P-values =  $3.43e-03$  ;  $3.43e-03$ ), and more time moving backward, being extended, being in a corner and being oriented toward the wall rather than the corner (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), than C57BL/6J females.

This suggests that CC040 females in social context demonstrate less locomotor activity, exploration and more thigmotaxis than C57BL/6J females.

During long term group monitoring, on the first night, CC041 females spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J females. On the second night, CC041 females spent less time moving forward, being reared, agitating the bedding, digging and being oriented toward the closest wall (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), and more time moving backward, deviating from their past trajectories, being extended, being at a stable altitude and being oriented toward the wall rather than the corner (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), than C57BL/6J females. On the third night, CC041 females spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J females.

This suggests that CC041 females in social context demonstrate less locomotor activity, exploration, potentially stereotyped behaviours and thigmotaxis than C57BL/6J females.

During long term group monitoring, on the second night, CC042 females spent less time deviating from their past trajectories, digging and being oriented toward the closest wall (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), and more time being extended and being oriented toward the wall rather than the corner (respective P-values =  $3.43e-03$  ;  $3.43e-03$ ), than C57BL/6J females.

This suggests that CC042 females in social context demonstrate less locomotor activity, exploration, potentially stereotyped behaviours and thigmotaxis than C57BL/6J females.

During long term group monitoring, on the first night, CC051 females spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J females. On the second night, CC051 females spent less time moving forward, being reared, agitating the bedding and being oriented toward the closest wall (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), and more time moving backward, having a stable tail altitude and being at a stable altitude (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), than C57BL/6J females. On the third night, CC051 females spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J females.

This suggests that CC051 females in social context demonstrate less locomotor activity, exploration and thigmotaxis than C57BL/6J females.

During long term group monitoring, on the first night, CC059 females spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J females. On the second night, CC059 females spent less time moving forward and being oriented toward the closest wall (respective P-values =  $3.43e-03$  ;  $3.43e-03$ ), and more time moving backward, deviating from their past trajectories, being at a stable altitude and being oriented toward the wall rather than the corner (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$  ;  $4.12e-02$ ), than C57BL/6J females. On the third night, CC059 females spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J females.

This suggests that CC059 females in social context demonstrate less locomotor activity, exploration and thigmotaxis than C57BL/6J females.

During long term group monitoring, on the second night, CC061 females spent less time agitating the bedding and being oriented toward the closest wall (respective P-values =  $3.43e-03$  ;  $3.43e-03$ ), and more time moving backward, being at a stable altitude and being oriented toward the wall rather than the corner (respective P-values =  $3.43e-03$  ;  $3.43e-03$  ;  $3.43e-03$ ), than C57BL/6J females. On the third night, CC061 females spent less time moving forward (P-value =  $3.43e-03$ ), than C57BL/6J females.

This suggests that CC061 males in social context demonstrate less locomotor activity, exploration and thigmotaxis than C57BL/6J males.

### **Social behavioural profile in long term group monitoring experiment**

We compared the social behaviour in long term group monitoring of the 12 CC strains to C57BL/6J mice on the basis of 14 BEC measurements. We compared all means by performing Mann-Whitney non-parametric tests which was adapted to some non-gaussian and non-homoscedastic populations. In order to avoid the family-wise type 1 error-rate inflation with the extension of this screening we chose to correct all tests here led through Bonferroni method for 168 tests.

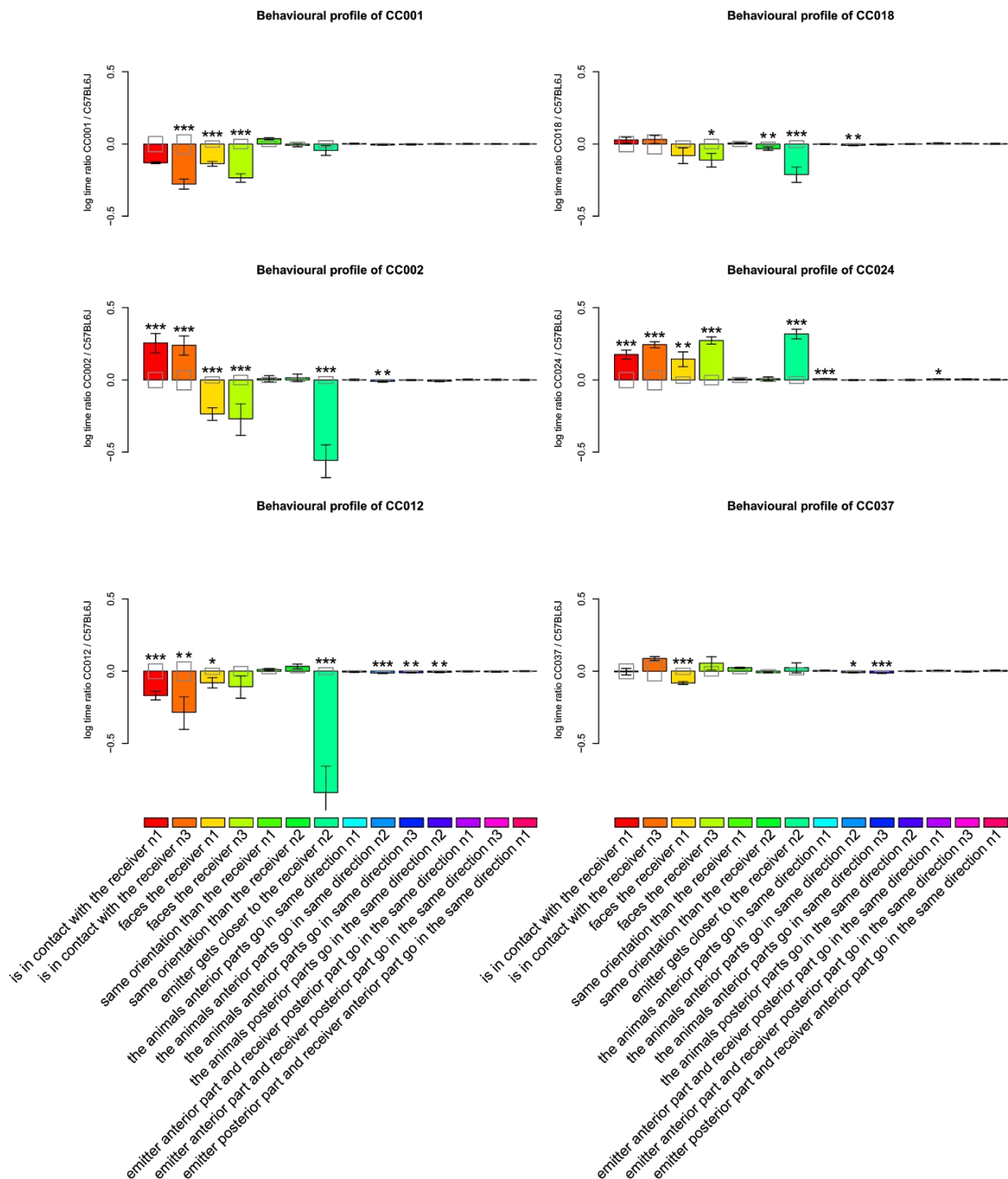
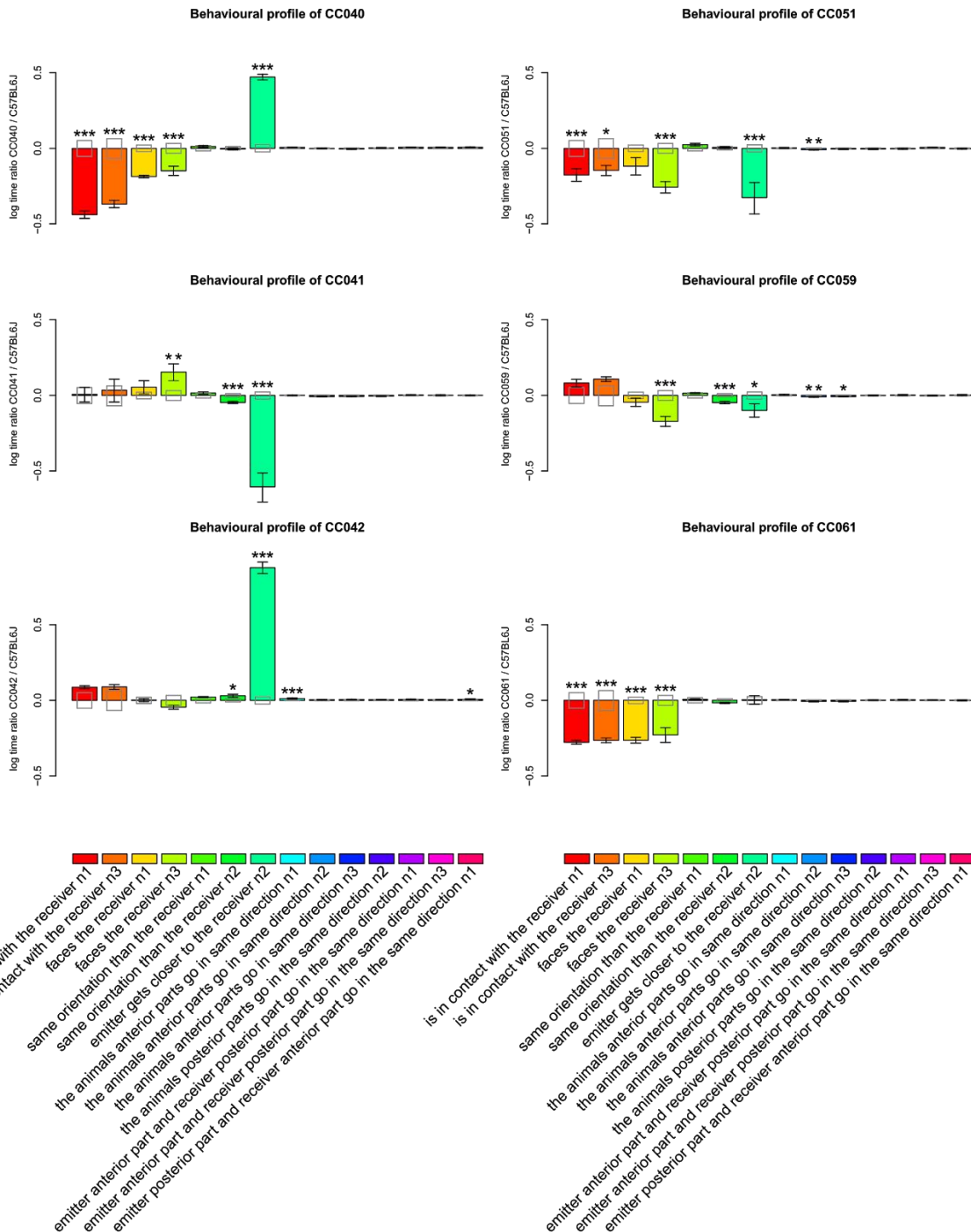


Figure 33: Social behavioural profile of CC strain males in long term group monitoring, barplot of the logarithm of the ratio of time proportions of BEC production between CC and C57BL/6J male mice. Each bar gives the logarithm of the ratio (CC / C57BL/6J) in proportion of production of one BEC in one phase of an experiment. Black error bars display the confidence interval of CC strains means around the ratios.



Grey rectangles display the confidence interval of C57BL/6J means around the origin. Stars represent the significance of the Mann-Whitney tests comparing strains means on the basis of the P values (\*: <0.05 ; \*\*: <0.01 ; \*\*\*: <0.001), corrected for 216 tests through Bonferroni method. Signs after the BEC names show in which phases of the experiments we measured emission proportions. "n1", "n2" and "n3" stands respectively for first, second and third nights of the long term group monitoring.

During long term group monitoring, on the first night, CC001 males spent less time facing a conspecific (P-value =  $4.14e-06$ ), than C57BL/6J males. On the third night, CC001 males spent less time being in contact with a conspecific and facing a conspecific (respective P-values =  $6.37e-12$  ;  $1.58e-07$ ), than C57BL/6J males.

This suggests that CC001 males demonstrate less social interest in term of proximity and motion than C57BL/6J males.

During long term group monitoring, on the first night, CC002 males spent less time facing a conspecific (P-value =  $1.74e-07$ ), and more time being in contact with a conspecific (P-value =  $4.48e-05$ ), than C57BL/6J males. On the second night, CC002 males spent less time getting closer to a conspecific and moving their anterior parts in the same direction than a conspecific anterior part (respective P-values =  $1.32e-08$  ;  $8.27e-03$ ), than C57BL/6J males. On the third night, CC002 males spent less time facing a conspecific (P-value =  $4.05e-04$ ), and more time being in contact with a conspecific (P-value =  $5.38e-05$ ), than C57BL/6J males.

This suggests that CC002 males demonstrate more social interest in term of proximity and motion but less in term of orientation than C57BL/6J males.

During long term group monitoring, on the first night, CC012 males spent less time being in contact with a conspecific and facing a conspecific (respective P-values =  $8.33e-04$  ;  $3.06e-02$ ), than C57BL/6J males. On the second night, CC012 males spent less time getting closer to a conspecific, moving their anterior parts in the same direction than a conspecific anterior part and moving their posterior parts in the same direction than a conspecific posterior part (respective P-values =  $2.77e-08$  ;  $1.72e-06$  ;  $6.38e-03$ ), than C57BL/6J males. On the third night, CC012 males spent less time being in contact with a conspecific and moving their anterior parts in the same direction than a conspecific anterior part (respective P-values =  $1.28e-03$  ;  $1.34e-03$ ), than C57BL/6J males.

This suggests that CC012 males demonstrate less social interest in term of proximity, orientation and motion than C57BL/6J males.

During long term group monitoring, on the second night, CC018 males spent less time having the same orientation than a conspecific, getting closer to a conspecific and moving their anterior parts in the same direction than a conspecific anterior part (respective P-values =  $6.17e-03$  ;  $2.15e-06$  ;  $1.38e-03$ ), than C57BL/6J males. On the third night, CC018 males spent less time facing a conspecific (P-value =  $3.16e-02$ ), than C57BL/6J males.

This suggests that CC018 males demonstrate less social interest in term of orientation and motion than C57BL/6J males.



During long term group monitoring, on the first night, CC024 males spent more time being in contact with a conspecific, facing a conspecific, moving their anterior parts in the same direction than a conspecific anterior part and moving their anterior parts in the same direction than a conspecific posterior part (respective P-values =  $9.56e-07$  ;  $2.86e-03$  ;  $3.62e-04$  ;  $4.51e-02$ ), than C57BL/6J males. On the second night, CC024 males spent more time getting closer to a conspecific (P-value =  $1.88e-06$ ), than C57BL/6J males. On the third night, CC024 males spent more time being in contact with a conspecific and facing a conspecific (respective P-values =  $1.38e-08$  ;  $2.28e-08$ ), than C57BL/6J males. This suggests that CC024 males demonstrate more social interest in term of proximity, orientation and motion than C57BL/6J males.

During long term group monitoring, on the first night, CC037 males spent less time facing a conspecific (P-value =  $1.46e-04$ ), than C57BL/6J males. On the second night, CC037 males spent less time moving their anterior parts in the same direction than a conspecific anterior part (P-value =  $1.25e-02$ ), than C57BL/6J males. On the third night, CC037 males spent less time moving their anterior parts in the same direction than a conspecific anterior part (P-value =  $4.31e-07$ ), than C57BL/6J males. This suggests that CC037 males demonstrate less social interest in term of orientation and motion than C57BL/6J males.

During long term group monitoring, on the first night, CC040 males spent less time being in contact with a conspecific and facing a conspecific (respective P-values =  $1.25e-08$  ;  $1.1e-06$ ), than C57BL/6J males. On the second night, CC040 males spent more time getting closer to a conspecific (P-value =  $1.25e-08$ ), than C57BL/6J males. On the third night, CC040 males spent less time being in contact with a conspecific and facing a conspecific (respective P-values =  $9.32e-15$  ;  $6.67e-05$ ), than C57BL/6J males.

This suggests that CC040 males demonstrate more social interest in term of motion but less in term of proximity and orientation than C57BL/6J males.

During long term group monitoring, on the second night, CC041 males spent less time having the same orientation than a conspecific and getting closer to a conspecific (respective P-values =  $2.2e-07$  ;  $1.25e-08$ ), than C57BL/6J males. On the third night, CC041 males spent more time facing a conspecific (P-value =  $4.15e-03$ ), than C57BL/6J males.

This suggests that CC041 males demonstrate less social interest in term of motion and orientation sharing and more in term of orientation toward the conspecific than C57BL/6J males.

During long term group monitoring, on the first night, CC042 males spent more time moving their anterior parts in the same direction than a conspecific anterior part and moving their posterior parts in the same direction than a conspecific anterior part (respective P-values =  $4.87e-04$  ;  $4.78e-02$ ), than C57BL/6J males. On the second night, CC042 males spent more time having the same orientation than a conspecific and getting closer to a conspecific (respective P-values =  $4.25e-02$  ;  $1.25e-08$ ), than C57BL/6J males.

This suggests that CC042 males demonstrate more social interest in term of orientation and motion than C57BL/6J males.

During long term group monitoring, on the first night, CC051 males spent less time being in contact with a conspecific (P-value =  $5.86e-04$ ), than C57BL/6J males. On the second night, CC051 males spent less time getting closer to a conspecific and moving their anterior parts in the same direction than a conspecific anterior part (respective P-values =  $2.31e-07$  ;  $1.01e-03$ ), than C57BL/6J males. On the third night, CC051 males spent less time being in contact with a conspecific and facing a conspecific (respective P-values =  $1.64e-02$  ;  $1.58e-07$ ), than C57BL/6J males.

This suggests that CC051 males demonstrate less social interest in term of proximity, orientation and motion than C57BL/6J males.

During long term group monitoring, on the second night, CC059 males spent less time having the same orientation than a conspecific, getting closer to a conspecific and moving their anterior parts in the same direction than a conspecific anterior part (respective P-values =  $7e-07$  ;  $2.88e-02$  ;  $1.83e-03$ ), than C57BL/6J males. On the third night, CC059 males spent less time facing a conspecific and moving their anterior parts in the same direction than a conspecific anterior part (respective P-values =  $1.42e-05$  ;  $4.38e-02$ ), than C57BL/6J males.

This suggests that CC059 males demonstrate less social interest in term of orientation and motion than C57BL/6J males.

During long term group monitoring, on the first night, CC061 males spent less time being in contact with a conspecific and facing a conspecific (respective P-values =  $9.32e-15$  ;  $2.78e-08$ ), than C57BL/6J males. On the third night, CC061 males spent less time being in contact with a conspecific and facing a conspecific (respective P-values =  $3.07e-08$  ;  $1.88e-06$ ), than C57BL/6J males.

This suggests that CC061 males demonstrate less social interest in term of proximity and orientation than C57BL/6J males.



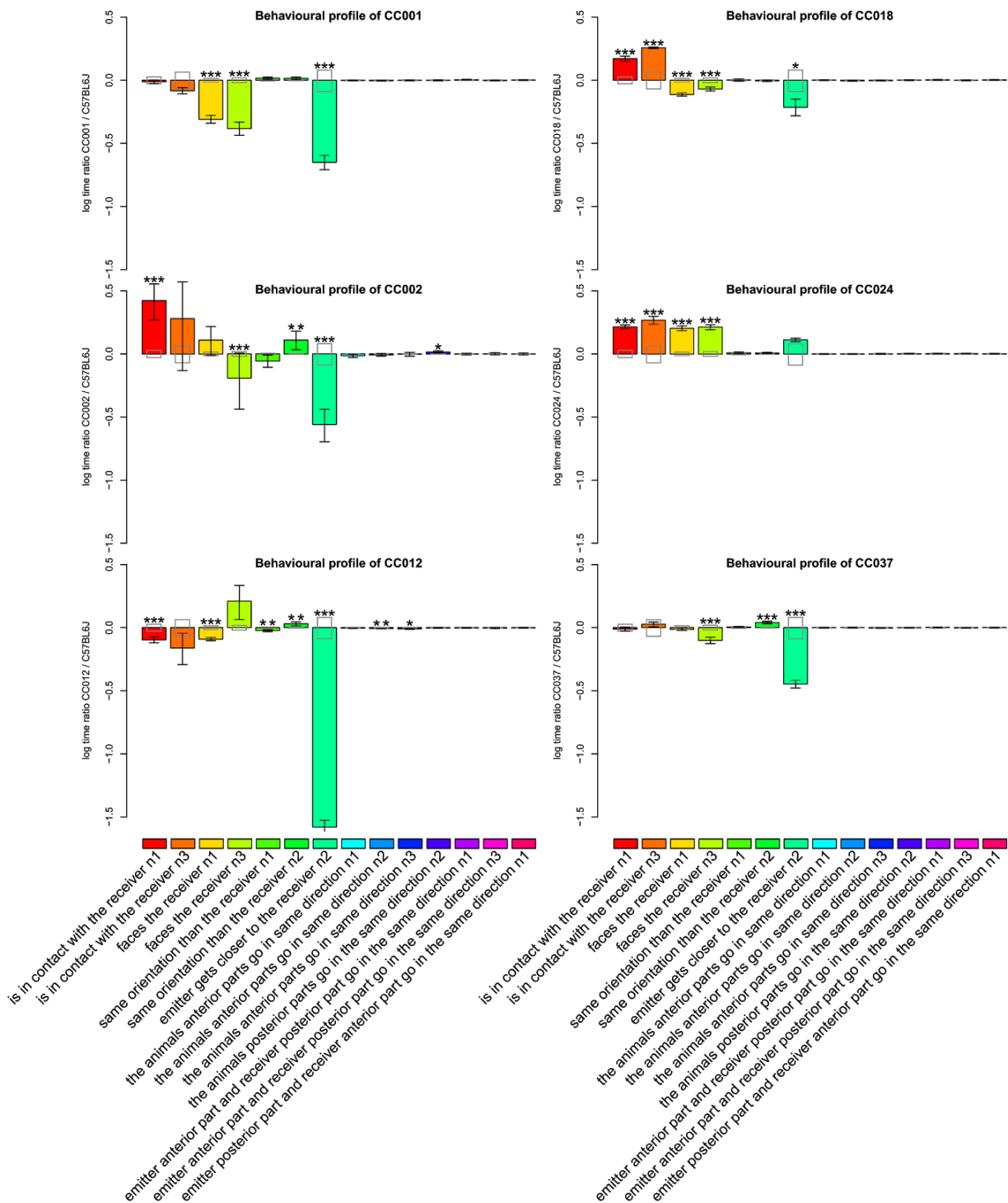
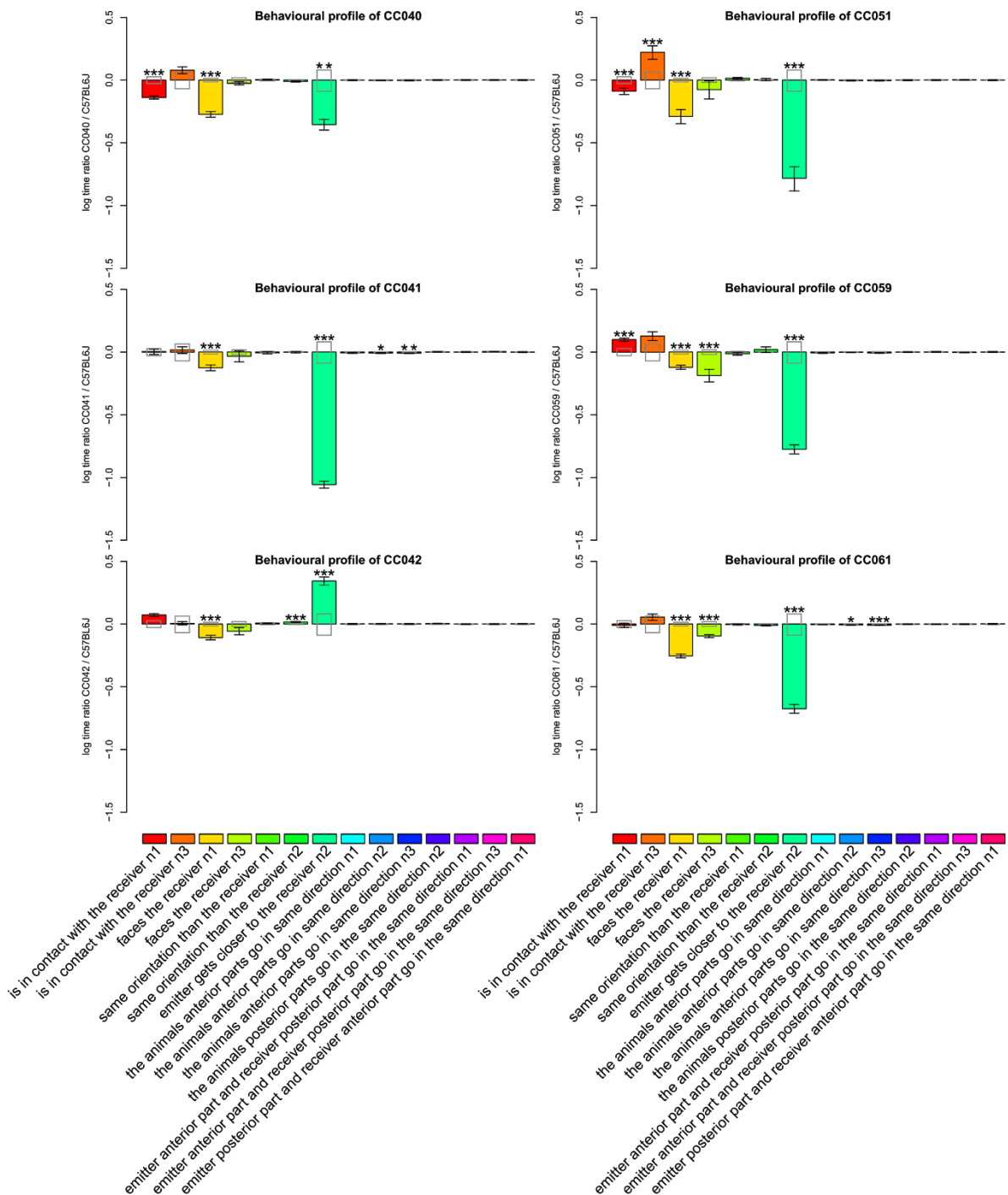


Figure 34: Social behavioural profile of CC strain females in long term group monitoring, barplot of the logarithm of the ratio of time proportions of BEC production between CC and C57BL/6J female mice. Each bar gives the logarithm of the ratio (CC / C57BL/6J) in proportion of production of one BEC in one phase of an experiment. Black error bars display the confidence interval of CC strains means around the ratios.



Grey rectangles display the confidence interval of C57BL/6J means around the origin. Stars represent the significance of the Mann-Whitney tests comparing strains means on the basis of the P values (\*: <0.05 ; \*\*: <0.01 ; \*\*\*: <0.001), corrected for 216 tests through Bonferroni method. Signs after the BEC names show in which phases of the experiments we measured emission proportions. "n1", "n2" and "n3" stands respectively for first, second and third nights of the long term group monitoring.

During long term group monitoring, on the first night, CC001 females spent less time facing a conspecific (P-value =  $9.32e-15$ ), than C57BL/6J females. On the second night, CC001 females spent less time getting closer to a conspecific (P-value =  $1.25e-08$ ), than C57BL/6J females. On the third night, CC001 females spent less time facing a conspecific (P-value =  $9.32e-15$ ), than C57BL/6J females. This suggests that CC001 females demonstrate less social interest in term of orientation and motion than C57BL/6J females.

During long term group monitoring, on the first night, CC002 females spent more time being in contact with a conspecific (P-value =  $3.64e-06$ ), than C57BL/6J females. On the second night, CC002 females spent less time getting closer to a conspecific (P-value =  $9.18e-07$ ), and more time having the same orientation than a conspecific and moving their posterior parts in the same direction than a conspecific posterior part (respective P-values =  $2.96e-03$  ;  $2.19e-02$ ), than C57BL/6J females. On the third night, CC002 females spent less time facing a conspecific (P-value =  $3.11e-04$ ), than C57BL/6J females.

This suggests that CC002 females demonstrate more social interest in term of proximity, orientation sharing and synchronized motion and less in term of orientation toward a conspecific and joining motion than C57BL/6J females.

During long term group monitoring, on the first night, CC012 females spent less time being in contact with a conspecific, facing a conspecific and having the same orientation than a conspecific (respective P-values =  $2.58e-04$  ;  $2.1e-07$  ;  $4.01e-03$ ), than C57BL/6J females. On the second night, CC012 females spent less time getting closer to a conspecific and moving their anterior parts in the same direction than a conspecific anterior part (respective P-values =  $1.25e-08$  ;  $5.97e-03$ ), and more time having the same orientation than a conspecific (P-value =  $7.5e-03$ ), than C57BL/6J females. On the third night, CC012 females spent less time moving their anterior parts in the same direction than a conspecific anterior part (P-value =  $1.77e-02$ ), than C57BL/6J females.

This suggests that CC012 females demonstrate less social interest in term of proximity, orientation and motion than C57BL/6J females.

During long term group monitoring, on the first night, CC018 females spent less time facing a conspecific (P-value =  $3.73e-08$ ), and more time being in contact with a conspecific (P-value =  $1.25e-08$ ), than C57BL/6J females. On the second night, CC018 females spent less time getting closer to a conspecific (P-value =  $1.25e-02$ ), than C57BL/6J females. On the third night, CC018 females spent less time facing a conspecific (P-value =  $1.07e-05$ ), and more time being in contact with a conspecific (P-value =  $9.32e-15$ ), than C57BL/6J females.

This suggests that CC0018 females demonstrate more social interest in term of proximity but less in term of orientation and motion than C57BL/6J females.



During long term group monitoring, on the first night, CC024 females spent more time being in contact with a conspecific and facing a conspecific (respective P-values =  $9.32e-15$  ;  $1.86e-14$ ), than C57BL/6J females. On the third night, CC024 females spent more time being in contact with a conspecific and facing a conspecific (respective P-values =  $3.3e-10$  ;  $9.32e-15$ ), than C57BL/6J females. This suggests that CC024 females demonstrate more social interest in term of proximity and orientation than C57BL/6J females.

During long term group monitoring, on the second night, CC037 females spent less time getting closer to a conspecific (P-value =  $1.25e-08$ ), and more time having the same orientation than a conspecific (P-value =  $6.07e-08$ ), than C57BL/6J females. On the third night, CC037 females spent less time facing a conspecific (P-value =  $1.61e-05$ ), than C57BL/6J females.

This suggests that CC037 females demonstrate less social interest in term of orientation toward a conspecific and motion and more in term of orientation sharing than C57BL/6J females.

During long term group monitoring, on the first night, CC040 females spent less time being in contact with a conspecific and facing a conspecific (respective P-values =  $3.73e-08$  ;  $9.32e-15$ ), than C57BL/6J females. On the second night, CC040 females spent less time getting closer to a conspecific (P-value =  $7.03e-03$ ), than C57BL/6J females.

This suggests that CC040 females demonstrate less social interest in term of proximity, orientation and motion than C57BL/6J females.

During long term group monitoring, on the first night, CC041 females spent less time facing a conspecific (P-value =  $4.54e-08$ ), than C57BL/6J females. On the second night, CC041 females spent less time getting closer to a conspecific and moving their anterior parts in the same direction than a conspecific anterior part (respective P-values =  $1.25e-08$  ;  $2.4e-02$ ), than C57BL/6J females. On the third night, CC041 females spent less time moving their anterior parts in the same direction than a conspecific anterior part (P-value =  $4.89e-03$ ), than C57BL/6J females.

This suggests that CC041 females demonstrate less social interest in term of orientation and motion than C57BL/6J females.

During long term group monitoring, on the first night, CC042 females spent less time facing a conspecific (P-value =  $8.53e-12$ ), than C57BL/6J females. On the second night, CC042 females spent more time having the same orientation than a conspecific and getting closer to a conspecific (respective P-values =  $3.11e-05$  ;  $8.96e-06$ ), than C57BL/6J females.

This suggests that CC042 females demonstrate less social interest in term of orientation toward a conspecific and more in term of orientation sharing than C57BL/6J females.

During long term group monitoring, on the first night, CC051 females spent less time being in contact with a conspecific and facing a conspecific (respective P-values =  $2.14e-04$  ;  $9.32e-15$ ), than C57BL/6J females. On the second night, CC051 females spent less time getting closer to a conspecific (P-value =  $1.25e-08$ ), than C57BL/6J females. On the third night, CC051 females spent more time being in contact with a conspecific (P-value =  $6.6e-05$ ), than C57BL/6J females.

This suggests that CC051 females demonstrate first less social interest in term of proximity, orientation and motion on the two first nights and then more in term of proximity on the third night than C57BL/6J females.

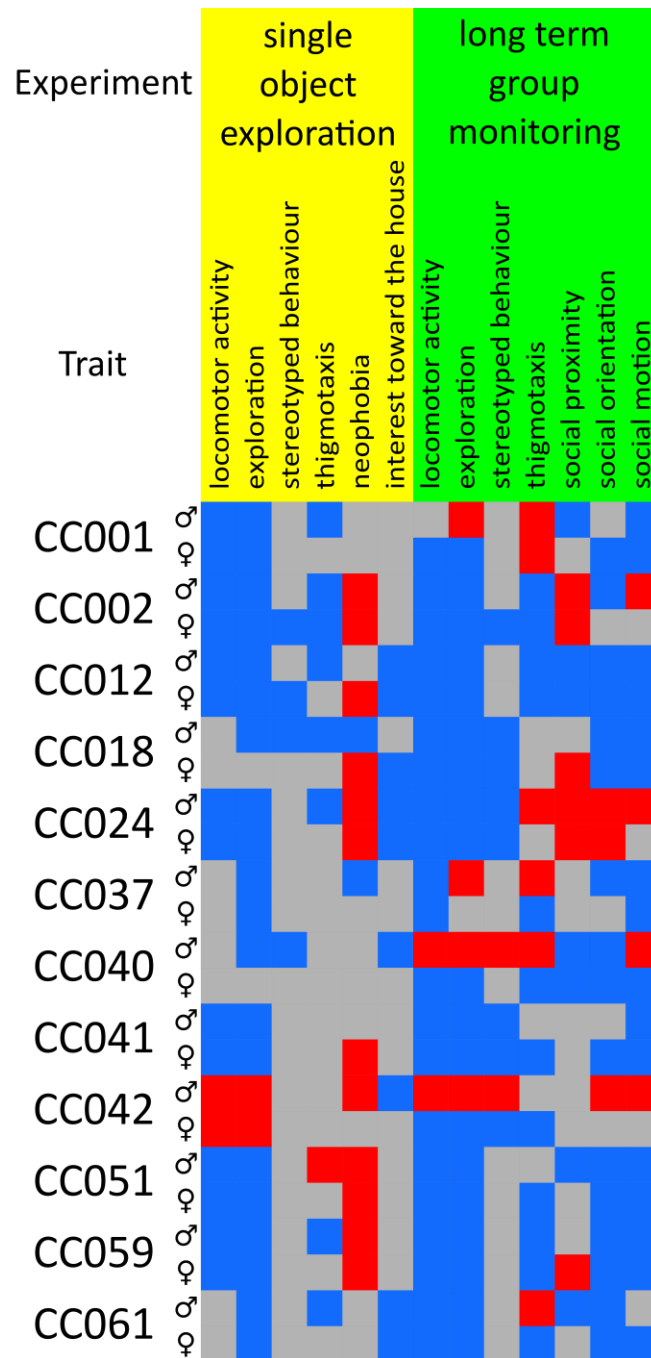
During long term group monitoring, on the first night, CC059 females spent less time facing a conspecific (P-value =  $6.37e-12$ ), and more time being in contact with a conspecific (P-value =  $2.65e-08$ ), than C57BL/6J females. On the second night, CC059 females spent less time getting closer to a conspecific (P-value =  $1.25e-08$ ), than C57BL/6J females. On the third night, CC059 females spent less time facing a conspecific (P-value =  $2.66e-10$ ), than C57BL/6J females.

This suggests that CC059 females demonstrate less social interest in term of orientation and motion and more in term of proximity than C57BL/6J females.

During long term group monitoring, on the first night, CC061 females spent less time facing a conspecific (P-value =  $1.25e-08$ ), than C57BL/6J females. On the second night, CC061 females spent less time getting closer to a conspecific and moving their anterior parts in the same direction than a conspecific anterior part (respective P-values =  $1.25e-08$  ;  $1.47e-02$ ), than C57BL/6J females. On the third night, CC061 females spent less time facing a conspecific and moving their anterior parts in the same direction than a conspecific anterior part (respective P-values =  $1.98e-09$  ;  $9.7e-04$ ), than C57BL/6J females.

This suggests that CC061 females demonstrate less social interest in term of orientation and motion than C57BL/6J females.

Table 13: Heat-map summary of interpreted trait differences between CC strains and C57BL/6J.



The blue squares represent traits interpreted as significantly lower than in C57BL/6J, the red squares represent traits interpreted as significantly higher than in C57BL/6J, the grey squares represent traits interpreted as not significantly different from C57BL/6J.

## F1 crosses selection

Table 14: Number of significant proportion differences between the behavioural profiles of CC strains and C57BL/6J.

| Strain: CC0...                                   | 01 | 02 | 12 | 18 | 24 | 37 | 40 | 41 | 42 | 51 | 59 | 61 |
|--|----|----|----|----|----|----|----|----|----|----|----|----|
| Males:   |    |    |    |    |    |    |    |    |    |    |    |    |
| Behaviour in single object exploration           | 4  | 7  | 5  | 5  | 8  | 2  | 4  | 3  | 4  | 7  | 4  | 3  |
| Solitary behaviour in long term group monitoring | 4  | 8  | 12 | 9  | 7  | 8  | 13 | 9  | 11 | 7  | 5  | 10 |
| Social behaviour in long term group monitoring   | 3  | 6  | 7  | 4  | 7  | 3  | 5  | 3  | 4  | 5  | 5  | 4  |
| Total in males                                   | 11 | 21 | 24 | 18 | 22 | 13 | 22 | 15 | 19 | 19 | 14 | 17 |
| Females:   |    |    |    |    |    |    |    |    |    |    |    |    |
| Behaviour in single object exploration           | 2  | 8  | 8  | 3  | 8  | 1  | 0  | 5  | 1  | 4  | 2  | 2  |
| Solitary behaviour in long term group monitoring | 8  | 10 | 11 | 5  | 6  | 5  | 6  | 12 | 5  | 9  | 8  | 6  |
| Social behaviour in long term group monitoring   | 3  | 5  | 7  | 5  | 4  | 3  | 3  | 4  | 3  | 4  | 4  | 5  |
| Total in females                                 | 13 | 23 | 26 | 13 | 18 | 9  | 9  | 21 | 9  | 17 | 14 | 13 |
| Total  | 24 | 44 | 50 | 31 | 40 | 22 | 31 | 36 | 28 | 36 | 28 | 30 |

As past studies found few significant effects of *Shank3* haploinsufficiency on C57BL/6J background, we chose to first cross it with strains exhibiting a lot of behavioural differences with it. We thus selected the strains CC012 and CC002, exhibiting respectively 44 and 50 significant behavioural differences, for those F1 crosses (table 14 in red).

In opposition, we then tried the mutation on a mixed background with a strain exhibiting fewer behavioural differences with C57BL/6J. We thus selected the strain CC059, exhibiting 28 significant behavioural differences, for this F1 cross. CC059 mice were noticed to be easier to manipulate than other strains and therefore potentially less stressed.

## **F1 crosses behavioural profiles**

The behavioural profiles of the F1 crosses of the second cohort are similar to those of the first cohort in the sense that they concern the same selected BEC. However, they differ in that here the haploinsufficient mutants of a genetic background are compared to their wild-type littermates of the same cross. Such profiles finally describe the behavioural differences caused by the mutation.

## **Behavioural profile in single object exploration experiment**

We compared the behaviour in single object exploration of the five F1 crosses mice between wild-types and heterozygous knockout for *Shank3* on the basis of 19 BEC measurements. We compared all means by performing ANOVA with the genotype of the emitter and its sex as factors and the interaction between these factors. In order to avoid the family-wise type 1 error-rate inflation with the extension of this screening we corrected all tests of each strain with the Bonferroni method for 19 tests.

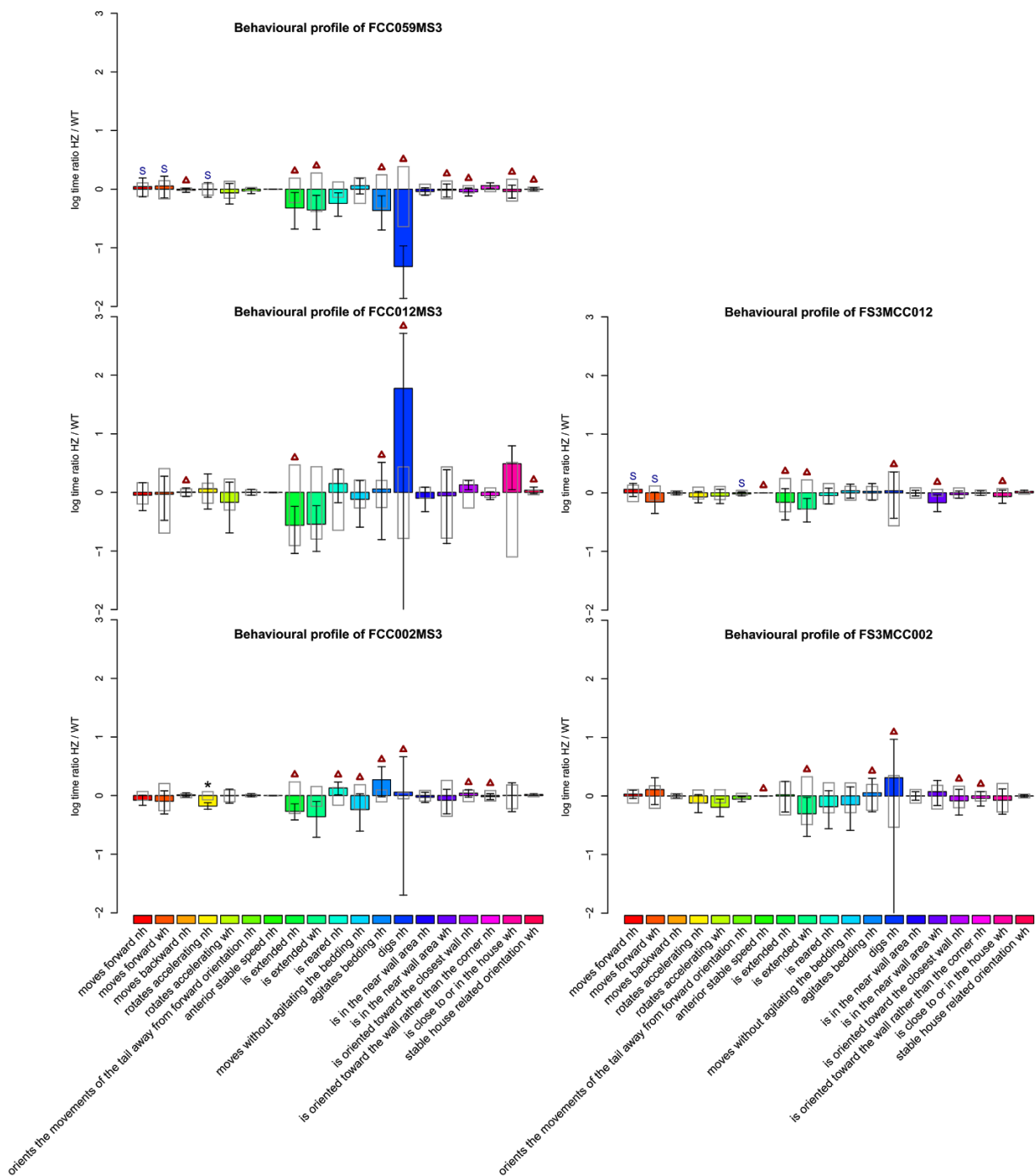


Figure 35: Behavioural profile of heterozygous *Shank3* knockout in F1 crosses in single object exploration, barplot of the logarithm of the ratio of time proportions of BEC production between heterozygous and wild-type mice. Each bar gives the logarithm of the ratio (HZ / WT) in proportion of production of one BEC in one phase of an experiment. Black error bars display the confidence interval of heterozygous mutant means around the ratios. Grey rectangles display the confidence interval of wild-type means around the origin. Red  $\Delta$  indicates the measurements for which the hypotheses of

ANOVA were not respected on the basis of Shapiro's or Levene's tests. Stars represent the significance given by the ANOVA, for genotype effect, on the basis of the P values (\*: <0.05 ; \*\*: <0.01 ; \*\*\*: <0.001), corrected for 19 tests through Bonferroni method. Blue S indicates the measurements for which the ANOVA reveals a significant sex effect on the basis of P values <0.05 after Bonferroni correction for 19 tests. Signs after the BEC names show in which phases of the experiments we measured emission proportions. "nh" and "wh" stands respectively for first and second phases of the single object exploration. "WT" and "HZ" stands respectively for wild-types and heterozygous knockout for *Shank3*.

For CC059xC57BL/6J.*Shank3*, CC012xC57BL/6J.*Shank3*, C57BL/6JxCC012.*Shank3* and C57BL/6JxCC002.*Shank3* crosses, no time of production of any behavioural event category differed significantly between genotypes, in the single object exploration.

In CC002xC57BL/6J.*Shank3* cross, the time spent rotating in acceleration before the addition of the house was significantly lower for heterozygous mice than for wild-type ones (P-value = 1.99e-02). The reduction of such movement BEC is coherent with the hypoactivity observed in homozygous *Shank3* knockout models and thus expected as potential effect of *Shank3* haploinsufficiency.





## Solitary behavioural profile in long term group monitoring experiment

We compared the solitary behaviour in long term group monitoring of the 5 F1 crosses mice between wild-types and heterozygous for *Shank3* on the basis of 18 BEC measurements. We compared all means by performing ANOVA with the genotype of the emitter, of the sex, of the experimental groups and interactions between those factors. It needs to be specified that, for some measurements, the gaussianity and homoscedasticity hypothesis were not respected on the basis of Shapiro's and Levene's tests on residuals. We still kept using ANOVA because the test is relatively robust to such conditions, and no significant genotype-related differences have been found in tests which did not respect these hypotheses. In order to avoid the family-wise type 1 error-rate inflation with the extension of this screening we chose to correct all tests of each strain Bonferroni method for 18 tests.

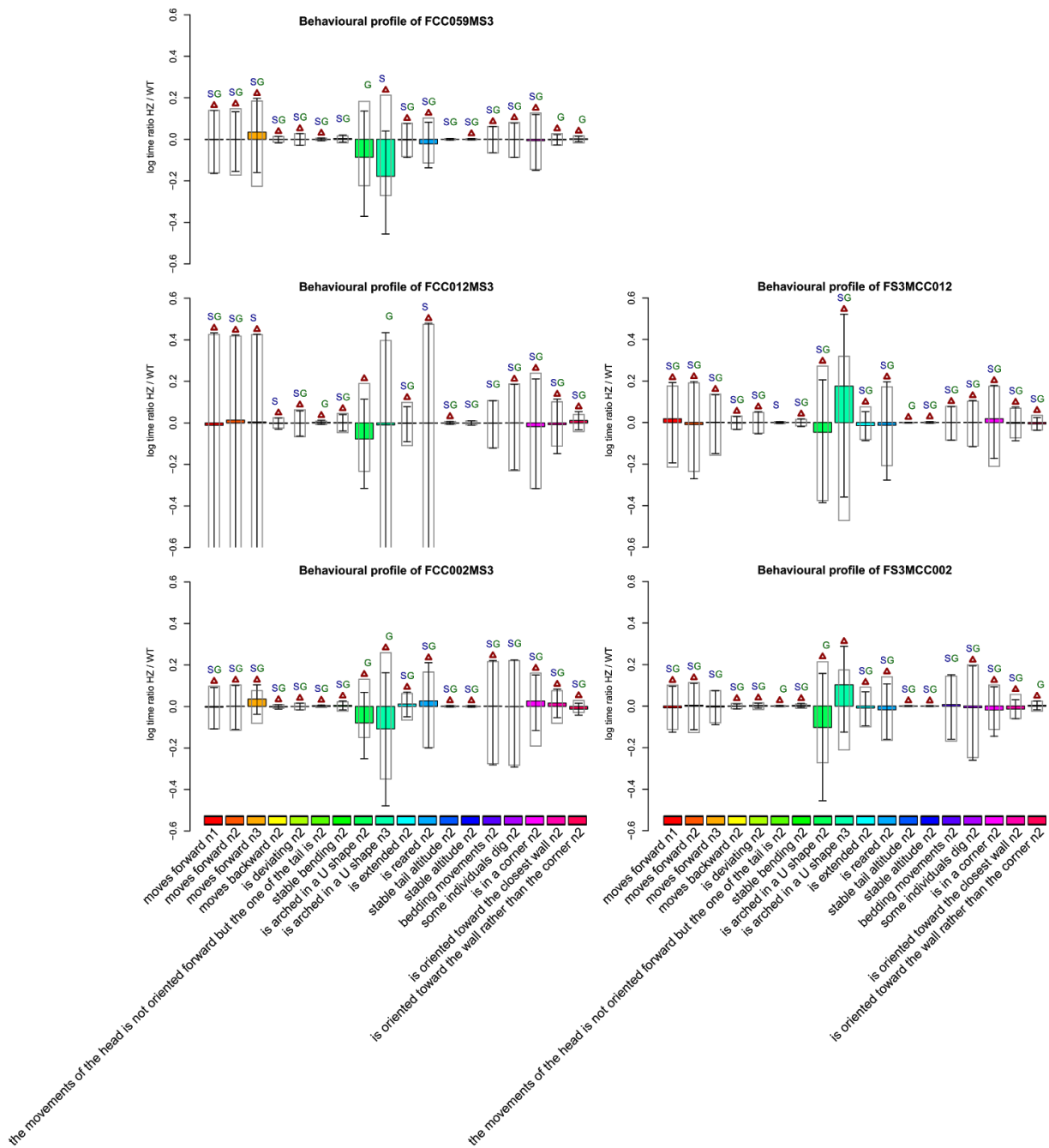


Figure 36: Solitary behavioural profile of heterozygous *Shank3* knockout in F1 crosses in long term group monitoring, barplot of the logarithm of the ratio of time proportions of BEC production between heterozygous and wild-type mice. Each bar gives the logarithm of the ratio (HZ / WT) in proportion of production of one BEC in one phase of an experiment. Black error bars display the confidence interval of heterozygous mutant means around the ratios. Grey rectangles display the confidence interval of wild-type means around the origin. Red  $\Delta$  indicates the measurements for which the hypotheses of ANOVA were not respected on the basis of Shapiro's or Levene's tests. Stars

represent the significance given by the ANOVA, for genotype effect, on the basis of the P values (\*: <0.05 ; \*\*: <0.01 ; \*\*\*: <0.001), corrected for 18 tests through Bonferroni method. Blue **S** indicates the measurements for which the ANOVA reveals a significant sex effect on the basis of P values <0.05 after Bonferroni correction for 18 tests. Green **G** indicates the measurements for which the ANOVA reveals a significant group effect on the basis of P values <0.05 after Bonferroni correction for 18 tests. Signs after the BEC names show in which phases of the experiments we measured emission proportions. "n1", "n2" and "n3" stands respectively for first, second and third nights of the long term group monitoring. Signs bellow bars show in which genotype we measured emission times. "WT" and "HZ" stands respectively for wild-types and heterozygous knockout for Shank3.

For all crosses, no time of production of any solitary behavioural event category differed significantly between genotypes, in any phase of the long term group monitoring. Significant group and sex effects have been found for a majority of the time production measurements of solitary behavioural event categories, in the long term group monitoring.



## Social behavioural profile in long term group monitoring experiment

We compared the social behaviour in long term group monitoring of the 5 F1 crosses between wild-types and *Shank3* heterozygous mice on the basis of 14 BEC measurements. We compared all means by performing ANOVA as a function of the genotype of the emitter, the genotype of the receiver, the sex of the animals, the experimental group and interactions between these factors. It needs to be specified that, for some measurements, the gaussianity and homoscedasticity hypotheses are not respected on the basis of Shapiro's and Levene's tests on residuals. We still kept using ANOVA because the test is relatively robust to such conditions. One significant genotype-related difference has been found in tests which did not respect these hypotheses. In this case, we checked that the significance did not depend on the sex factor, which separated groups with significantly different variances, following Levene's test. In order to avoid the family-wise type 1 error-rate inflation with the extension of this screening, we corrected all tests of each strain Bonferroni method for 14 tests.

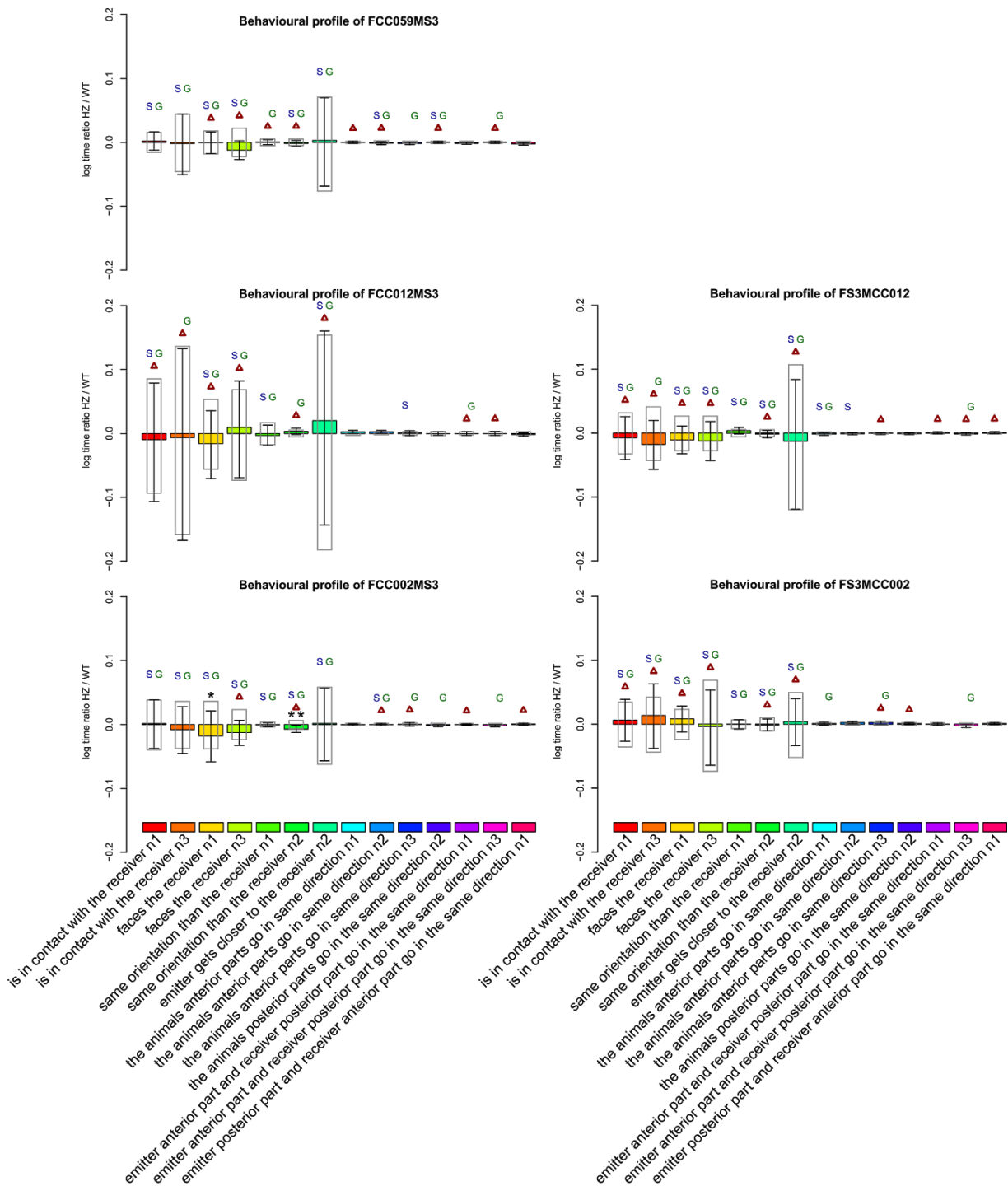


Figure 37: Social behavioural profile of heterozygous *Shank3* knockout in F1 crosses in long term group monitoring, barplot of the logarithm of the ratio of time proportions of BEC production between heterozygous and wild-type mice. Each bar gives the logarithm of the ratio (HZ / WT) in proportion of production of one BEC in one phase of an experiment. Black error bars display the confidence interval of heterozygous mutant means around the ratios. Grey rectangles display the confidence interval of

wild-type means around the origin. Red  $\Delta$  indicates the measurements for which the hypotheses of ANOVA were not respected on the basis of Shapiro's or Levene's tests. Stars represent the significance given by the ANOVA, for genotype effect, on the basis of the P values (\*: <0.05 ; \*\*: <0.01 ; \*\*\*: <0.001), corrected for 14 tests through Bonferroni method. Blue S indicates the measurements for which the ANOVA reveals a significant sex effect on the basis of P values <0.05 after Bonferroni correction for 14 tests. Green G indicates the measurements for which the ANOVA reveals a significant group effect on the basis of P values <0.05 after Bonferroni correction for 14 tests. Signs after the BEC names show in which phases of the experiments we measured emission proportions. "n1", "n2" and "n3" stands respectively for first, second and third nights of the long term group monitoring. Signs bellow bars show in which genotype we measured emission times. "WT" and "HZ" stands respectively for wild-types and heterozygous knockout for Shank3.

For CC059xC57BL/6J.*Shank3*, CC012xC57BL/6J.*Shank3*, C57BL/6JxCC012.*Shank3* and C57BL/6JxCC002.*Shank3* crosses, no time of production of any social behavioural event category differed significantly between genotypes of the emitter, in the long term group monitoring.

In CC002xC57BL/6J.*Shank3* cross, the time spent facing a conspecific on the first night and having the same orientation than a conspecific on the second one, were significantly lower for heterozygous mice than for wild-type ones (respective P-values = 1.01e-02 ; 6.80e-03). The second difference was first proven significant on the basis on an ANOVA which did not respect the homoscedasticity hypothesis. Levene's test here found that the sex factor separated groups with significantly different variances. We thus checked in a second time, that the significance did not depend on the sex factor by testing through ANOVA without this factor. The P-value for the genetic factor appearing unchanged (P-value = 6.80e-03), the confidence in this result is not affected.



The reduction of such socially-related orientation BEC is coherent with the lack of social interest observed in homozygous *Shank3* knockout models and thus expected as potential effect of *Shank3* haploinsufficiency.

No significant receiver's genotype effect has been found on the production time of any social behavioural event category of any cross, in the long term group monitoring. Significant sex and group effects have been found for a majority of the production time measurements of social behavioural event categories, in the long term group monitoring.

# **Discussion**



## Behavioural diversity in mice

In this work we found a new repertoire for mice behaviour and we phenotyped the behaviour of C57BL/6J and 12 CC strains over a representative set of categories from this repertoire. We could expect the behaviour to vary between CC strains. We need to remind that we did not lead statistical comparisons between CC strains, but only ones between CC strains and C57BL/6J, this last serving as a reference. Anyway, the differences in the patterns in which strains differ from this reference one inform us indirectly on the behavioural diversity between CC strains.

## C57BL/6J highly active and social behaviour

We chose C57BL/6J mice as a reference strain because it is widely used in behavioural biology. The fact that such mice are highly active and social makes this strain a good subject for numbers of behavioural studies. However, we could suggest that such an active and socially interested genetic background potentially could hide *Shank3* mutation effect. The hypoactivity and lack of social interest could have been compensated in a way by the particular tendencies of this strain.

The first information we can extract from the Collaborative Cross strain comparison confirms that the reference strain we used, C57BL/6J, does not have an average behaviour. We found significant differences in interpreted traits between C57BL/6J and each of the Collaborative Cross strain. And such differences occur generally in the same direction for each strain comparison. The clearest C57BL/6J tendencies are high locomotor activity and exploration. Compared to most CC strains, C57BL/6J expressed significantly more a lot of the movemes which make the animals change location, as moving forward, or orientation, as turning in acceleration, and this in both of our experiments. We can also notice the high social interest of C57BL/6J, particularly in term of orientation and motion.

We know the highly active behaviour of C57BL/6 since decades. In 1953 Thompson compared 15 mouse strains, including C57BL/6 [167]. In the open-field test, C57BL/6 showed the second highest exploratory activity. This score was also significantly higher than the one of 9 of those strains (Table 15). We can note that the three most active strains were then all C57 strains.

Table 15: Mean amount of exploratory activity shown by each of fifteen mouse strains, and the P values of differences between them, from Thompson in 1953 [167].

| No. | Strain   | Mean score | <i>p</i> value of differences* |          |
|-----|----------|------------|--------------------------------|----------|
|     |          |            | .05-.01                        | .01-.001 |
| 1   | C57BR/a  | 459        | > 3-4                          | 5-15     |
| 2   | C57BL/6  | 361        | > 7-9                          | 10-15    |
| 3   | C57BL/10 | 359        | > 7-9                          | 10-15    |
| 4   | DBA/1    | 334        | > 8-10                         | 11-15    |
| 5   | ND       | 308        | > 8-11                         | 12-15    |
| 6   | BDP      | 286        | > 10-12                        | 13-15    |
| 7   | DBA/2    | 253        | > 11-13                        | 14-15    |
| 8   | LP       | 194        | > 13-15                        |          |
| 9   | AKR      | 188        | > 13-15                        |          |
| 10  | C3H      | 177        | > 13-15                        |          |
| 11  | Ob.      | 149        | > 15                           |          |
| 12  | TC3H     | 117        |                                |          |
| 13  | BALB/c   | 74         |                                |          |
| 14  | AK/e     | 60         |                                |          |
| 15  | A/C1     | 20         |                                |          |

Thompson measured the exploratory activity score as the number of crossed lines, in 10 minutes, in a square arena separated in 36 smaller squares. “\*Read, for example, as follows: No. 1, Strain C57BR/a has a mean of 459 crosses., significantly greater than Strains Nos. 3 and 4 ( $p = .05-.01$ ) and Nos. 5 to 15 ( $p = .01-.001$ ).”

Table 16: Locomotor activity ranks of C57BL/6J between other strains on their in open-field test.

| publication                             | duration | sex | day | C57BL/6J rank / strain number | more active strain rate |
|---|----------|-----|-----|-------------------------------|-------------------------|
|   |          |     | 1   | 1 / 10                        | 0                       |
|   |          | ♀   | 2   | 1 / 10                        | 0                       |
| Bolivar et al., 2000[168]               | 5 min    |     | 3   | 3 / 10                        | 0.22                    |
|   |          |     | 1   | 1 / 10                        | 0                       |
|   |          | ♂   | 2   | 4 / 10                        | 0.33                    |
|   |          |     | 3   | 4 / 10                        | 0.33                    |
| Bolivar et al., 2009[169]               | 15 min   |     | 1   | 5 / 14                        | 0.3                     |
|   |          | ♂   | 2   | 6 / 14                        | 0.38                    |
|   |          |     | 3   | 6 / 14                        | 0.38                    |
| O'Leary et al., 2013[170]               | 5 min    | ♀   | 1   | 5 / 14                        | 0.3                     |
|   |          | ♂   | 1   | 6 / 14                        | 0.38                    |
| Chesler et al., 2013[171]               | 20 min   | ♀   | 1   | 5 / 9                         | 0.5                     |
|   |          | ♂   | 1   | 4 / 9                         | 0.37                    |
| Delprato et al., 2017[172]              | 20 min   | ♀   | 1   | 9 / 55                        | 0.14                    |
|   |          | ♂   | 1   | 9 / 55                        | 0.14                    |
| Liu and Gershenfeld, 2003[173]          | 5 min    | ♂   | 1   | 3 / 12                        | 0.18                    |
| GMC01                                   |          | ♀   | 1   | 2 / 8                         | 0.14                    |
| Kollmus et al., 2020[174]               | 20 min   | ♂   | 1   | 2 / 8                         | 0.14                    |
| Lipkind et al., 2004[175]               | 30 min   | ♂   | 1   | 2 / 8                         | 0.14                    |
| de Mooij-van Malsen et al., 2009[176]   | 60 min   | ♂   | 1   | 1 / 6                         | 0                       |
| Kliethermes et al., 2006[177]           | 30 min   | ♀   | 1   | 4 / 14                        | 0.23                    |
|   |          | ♂   | 1   | 4 / 14                        | 0.23                    |
| Takahashi et al., 2006[178]             | 10 min   | ♀   | 1   | 1 / 12                        | 0                       |
|   |          | ♂   | 1   | 2 / 12                        | 0.09                    |
| Geuther et al., 2019[179]               | 55 min   | ♀   | 1   | 24 / 58                       | 0.4                     |
|   |          | ♂   | 1   | 18 / 58                       | 0.29                    |
| Lad et al., 2010[180]                   | 5 min    | ♂   | 1   | 1 / 8                         | 0                       |
| Gubner et al., 2010[181]                | 15 min   | ♂   | 2   | 6 / 15                        | 0.35                    |
| Moy et al., 2008[182]                   | 10 min   | ♂   | 1   | 18 / 31                       | 0.56                    |
|   |          |     | 1   | 1 / 15                        | 0                       |
| Mhyre et al., 2005[183]                 | 45 min   | ♂   | 2   | 1 / 15                        | 0                       |
|   |          |     | 3   | 2 / 15                        | 0.07                    |
| Keum et al., 2016[184]                  | 5 min    | ♂   | 1   | 3 / 11                        | 0.2                     |
| Loos et al., 2009[185]                  | 10 min   | ♂   | 1   | 3 / 12                        | 0.18                    |
| Wiltshire et al., 2015[186]             | 30 min   | ♂   | 1   | 23 / 45                       | 0.5                     |
| Schoenrock et al., 2016[187]            | 10 min   | ♀   | 1   | 12 / 37                       | 0.3                     |
| Thompson et al., 1953[167]              | 10 min   | ♂♀  | 1   | 2 / 15                        | 0.07                    |
| Thomsen et Caine, 2011[188]             | 180 min  | ♀   | 1   | 2 / 15                        | 0.07                    |
|   |          | ♂   | 1   | 6 / 15                        | 0.35                    |
|   |          | ♀   | 1   | 7 / 15                        | 0.42                    |
| Thomsen et al., 2011[189]               | 180 min  | ♂   | 1   | 7 / 15                        | 0.42                    |
|   |          | ♀   | 1   | 6 / 15                        | 0.35                    |
|   |          | ♂   | 1   | 6 / 15                        | 0.35                    |
| Trullas et Skolnick, 1993[190]          | 5 min    | ♂   | 1   | 7 / 10                        | 0.66                    |
| Wahlsten et al., 2003[191]              | 5 min    | ♀   | 1   | 3 / 21                        | 0.1                     |
|   |          | ♂   | 1   | 7 / 21                        | 0.3                     |
| Wiltshire et al., 2010 unpublished[192] | 10 min   | ♀   | 1   | 15 / 37                       | 0.38                    |
|   |          | ♂   | 1   | 14 / 37                       | 0.36                    |

*All publications acquired a measure of locomotor activity in an open-field test or a closely similar protocol, estimated through travelled distance or number of lines crossed. The test duration is quite variable, from 5 to 180 minutes. For each publication we give the number of strain studied and the rank of C57BL/6J between the other strains. We also computed the ratio of other strains which exhibited more locomotor activity than C57BL/6J for each study, and the mean across all such ratio. We placed the results of Thompson's study in the table but marked this line in grey, because this study concerns C57BL/6 and not C57BL/6J, which was not considered apart in 1953.*

Later on, two distinct sub-strains of C57BL/6 mice emerged because of the separation of breeding colonies, giving C57BL/6J in the Jackson Laboratories and C57BL/6N at NIH [194]. C57BL/6J locomotor activity has been studied in a lot of publications which also concerned other strains. We found 27 studies in which this measure of locomotor activity has been acquired through open-field tests or equivalent protocols. Out of those 27 studies 5 of them found C57BL/6J to be the most active in at least one of their measurements. In order to better understand the relatively high locomotor activity of C57BL/6J among mouse strains, we got interested in the proportion of other strains which are evaluated as more active. For the measurements which give C57BL/6J in first rank this proportion is thus of 0. We can note that most of the proportions are between 0 and 0.5 with rare exceptions above 0.5 and a maximum of 0.66 or 2/3. We can thus say that C57BL/6J is, in most studies, part of the most active half of all strains used. If we compute a mean of all such proportions for each measurement across the 27 studies, the value obtained, although a bit abstract, is a proportion of 0.25. This indicates that we can generally expect 1/4 of other strains studied to be more active than C57BL/6J and 3/4 to exhibit less locomotor activity. The fact that, in the literature, a majority of other strains are less active than C57BL/6J is consistent with our results suggesting that C57BL/6J exhibit more locomotor activity than most of the CC strains we recorded.



**Table 17: Social behaviour ranks of C57BL/6J males between other strains on different social tests.**

| publication          | duration | measured time                              | C57BL/6J rank |
|----------------------|----------|--|---------------|
| Bolivar et al., 2007 | 20 min   | social interaction with a novel mouse      | 4/5           |
|                      | 30 min   | social interaction with former novel mouse | 6/7           |
| Moy et al., 2008     | 10 min   | in chamber with a first novel mouse        | 6/17          |
|                      |          | sniffing cage with a first novel mouse     | 9/17          |
|                      | 10 min   | in chamber with former novel mouse         | 8/17          |
|                      |          | in chamber with a second novel mouse       | 11/17         |
|                      |          | sniffing cage with former novel mouse      | 9/17          |
|                      |          | sniffing cage with a second novel mouse    | 11/17         |
| Shin et al., 2016    | 10 min   | in chamber with a first novel mouse        | 3/11          |
|                      | 10 min   | in chamber with former novel mouse         | 4/11          |
|                      |          | in chamber with a second novel mouse       | 2/11          |

*In Bolivar et al., the time on social interaction is measured on two successive presentations, 20 then 30 minutes, of a novel mouse to a test mouse. The two other publications investigated social behaviour through the three-chambered test. In this protocol, a first novel mouse is presented to the test mouse in a first 10 minutes period, and two mice including the former one and a second novel one in a second 10 minutes period. We only kept the measured time spent in chambers containing a conspecific or sniffing it. For each measurement of each publication we give the rank of C57BL/6J between the other strains on the number of strains studied.*

We can see that the rank of C57BL/6J males on the different social behaviours measured in the literature seems to depend mostly on the study they come from. In Bolivar et al., C57BL/6J exhibited few social interactions what gave them low ranks from 6/7 to 4/5. In Moy et al., the social interest of C57BL/6J in the three-chambered test seems close to the average, giving ranks from 11/17 to 6/17 with a mean of 9/17, the middle rank. In Shin et al., the time spent by C57BL/6J test mice in the chambers occupied by a conspecific are high, with ranks from 4/11 to 2/11. Considering such different ranks of C57BL/6J males, in social behaviour studies which have screened multiple strains, it is hard to conclude in any tendency for low or high social interest. We need to keep in mind that such measure of social interest toward a novel individual are likely to be different than what we would be obtained on familiar individuals as in our long-term group monitoring protocol.

C57BL/6J mice have also been compared to other C57BL/6 sub-strains [194]. In open field test, C57BL/6J mice travelled a significantly longer distance than C57BL/6N and C57BL/6C mice. In social interaction test, C57BL/6J and C57BL/6C showed significantly more contacts than C57BL/6N. This last discovery is consistent with our observation of C57BL/6J relatively high social interest.

## Locomotor activity and exploration in the Collaborative Cross strains

This particular high locomotor activity and exploration of the reference strain also points out the even higher locomotor activity and exploration of CC042. Males and females CC042 spent significantly more time turning in acceleration than C57BL/6J during the single object exploration. Males CC042 spent significantly more time moving forward than C57BL/6J during each night of the long term group monitoring. And females CC042 spent more time moving forward than C57BL/6J during each night of this experiment, but this difference is not significant.

CC002 and CC012 are the two strains that we selected to cross with C57BL/6J because they expressed the highest amount of significant behavioural differences with this reference strain. Among those differences we can note the lower locomotor activity and exploration. This is noticeable at the fewer frames at which animals exhibited the move forward category in both experiment for each sex.

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**Figure 38: Locomotor activity obtained from 52 CC lines, from Molenhuis et al. in 2018 [153].** *“Plots are expressed as mean±SEM, with CC lines ordered along the x-axis by mean per phenotype.” The bottom strain names on the original figure are specific to their facilities of origin, we added the collaborative cross number of the strains we used in this work in order to make them easier to recognize and locate.*

In 2018, Molenhuis et al. lead an extensive behavioural screening of 53 different CC strains [153]. Our findings on CC042 corroborate the relatively long distance this team observed CC042 mice to travel, in their kind of open-field experiment. Depending on their measures, this strain is the 10<sup>th</sup> on 52 with the highest locomotor activity (figure 38). It is also the second one on the 12 we used on the basis of this same measurements.

This study also gave CC002 and CC012 the respective 17<sup>th</sup> and 37<sup>th</sup> position on 52 in term of locomotor activity, what corresponds to the 5<sup>th</sup> and 9<sup>th</sup> in the 12 CC strain we used (figure 38). The fact that those positions are below the one of CC042 is coherent with our own findings. We could say the same thing for the ten strains that both our study and Molenhuis' one found less active than CC042, what let think that our measurements of activity are globally concordant.

We can question the fact that this team observed even more activity in CC061, what did not happen in our experiments. The low sample size, 6 males per strain in average, of Molenhuis screening can be a cause of the fact that we cannot reproduce the same results. The strain used in this paper are not exactly the same as the one we used because they were note totally fixed at this time, this could also be a factor preventing the reproduction of the results. We finally need to keep in mind that even if the measured traits are expected to be the same, the two experimental contexts in which animal are observed and the measurements led on those last are different.

## Social behaviour in the Collaborative Cross strains

The high social interest of the reference strain also points out the even higher social interest of CC024. Males CC024 spent significantly more time in contact with their conspecific, facing them and getting closer to them, than C57BL/6J males did. Females CC024 spent significantly more time in contact with their conspecific and facing them, than C57BL/6J females did.

For the social behaviour, both CC002 and CC012 tend to differ from C57BL/6J, but each in a different direction. This is noticeable at the fewer frames at which animals exhibited the move forward category in both experiment for each sex. CC002 males and females spent significantly more time in contact with other individuals, than C57BL6/J mice did, what can be interpreted as a sign of higher social interest. CC012 mice spent significantly less time in contact with their conspecific, facing them and getting closer to them, than C57BL/6J mice did. We interpreted such traits as signs of lower social interest.

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**Figure 39: Social interaction from 52 CC lines, from Molenhuis et al. in 2018 [153].** *“Plots are expressed as mean±SEM, with CC lines ordered along the x-axis by mean per phenotype.” The bottom strain names on the original figure are specific to their facilities of origin, we added the collaborative cross number of the strains we used in this work in order to make them easier to recognize and locate.*

Our observation of a high social interest in CC024 mice seems completely opposed to Molenhuis findings [153]. Depending on their measures, this strain is the 9<sup>th</sup> on 52 with the least time spent in social interaction (figure 39). It is also the second shorter time on the 12 we used on the basis of this same measurements. In the same way, CC002 mice got the 15<sup>th</sup> shorter time in social interaction on 52, or 3<sup>rd</sup> on 12, when in our study we observed it to perform a higher time in contact than C57BL/6J which is already highly social. And on the opposite CC012 mice got the 5<sup>th</sup> longer time in social interaction on 52, or 3<sup>rd</sup> on 12, when in our study we observed it to be poorly socially interested (figure 39).

Here we can think that this precise measurement is not comparable to the different measurements we led to evaluate social traits. We could even think that our results on social interest in CC mice correlate negatively with Molenhuis' measure of social interaction. This can be due to differences in the protocol. In Molenhuis' social task, the time in social interaction is evaluated on a short period, on the first encounter of an unfamiliar individual. In our long term group monitoring, socially relevant behavioural categories are observed on three successive nights, spent in the group with which animals are living their everyday life. Our experiment does not include the aspect of social novelty that is present in Molenhuis' experiment.

It is interesting to note that differences in the protocol between Molenhuis' study and ours seemed to impact more the measurement of social interest than the measurement of locomotor activity. We can think that social behaviour being more complex than locomotor one, it is more sensible to the differences of experimental protocols, and its measurements are thus harder to replicate. Maybe that these same complexity and sensibility of social behaviour are conditioning the fact that social behaviour is affected by disorders which do not affect much other behavioural traits, as autism spectrum disorder.

## Behavioural impact of *Shank3* haploinsufficiency

We succeeded in observing some behavioural differences related to *Shank3* haploinsufficiency, in both experiments and thus both on solitary and social behaviours. This was our major goal. All of those differences have been observed in the CC002xC57BL/6J.*Shank3* cross. All impacts of *Shank3* mutation, described hereafter between wild-type mice and heterozygous knock-out, thus only concern the CC002xC57BL/6J.*Shank3* cross genetic background.

### Effects on solitary activity

The time spent rotating in acceleration before the addition of the house was significantly lower for heterozygous mice than for wild-type ones. This measurement is not comparable to the distance travelled in an open-field, which would certainly be more correlated with the time spent moving forward. But the shorter time spent turning can be described as an inhibition of a movement BEC and be interpreted as a sign of hypoactivity. This is coherent with the hypoactivity observed in homozygous *Shank3* knockout models [59].

In neuroscience, turning behaviour of mice and rats has been particularly studied in the context of research relative to Parkinson's disease. Unilateral injections of 6-hydroxydopamine into the striatum or substantia nigra are used to destroy the dopaminergic nerve terminals in it. Injected mice show an ipsilateral reduction in forebrain dopamine concentrations and exhibit more ipsilateral turning and less contralateral turning, they turn preferentially toward the injected side. Such animals serve as models of Parkinson's disease [195], [196].

Most psychomotor stimulants as amphetamine, amantadine and caffeine increase the ipsilateral turning. But we can induce contralateral turning with the dopamine precursor L-dopa [197], dopaminergic agonists as apomorphine or selective dopaminergic D<sub>1</sub> agonists as SKF 38393 and D<sub>2</sub> agonists as LY 171555 [198]. “Turning behavior as performed in these experiments is a simple but very sensitive index of central dopaminergic receptor stimulation.” [199]

This can be put in perspective with the findings from Ferhat and colleagues which studied gene expression in different parts of the brain of mice homozygous knocked out for *Shank3* (*Shank3<sup>Δ11/Δ11</sup>*). They found that the striatum is the structure showing the maximum of significantly differentially expressed genes between *Shank3<sup>Δ11/Δ11</sup>* and *Shank3<sup>+/+</sup>* mice. In *Shank3<sup>Δ11/Δ11</sup>* mice, under-expressed genes were enriched in genes expressing dopamine 1 receptor (D1-SPN) and over-expressed genes were enriched in genes expressing dopamine 2 receptor (D2-MSN) (unpublished).

In other respects, Peça et al. [52] recorded cortico-striatal synaptic circuitry in acute brain slices of 6–7-week-old animals. They found that *Shank3<sup>KO/KO</sup>* field population spikes were significantly reduced in comparison with control animals. As presynaptic function was not altered, this result suggests postsynaptic impairments in synaptic function and/or a reduction in the number of functional synapses in the striatum.

Altogether, we could hypothesize that an abnormal expression of D1 and D2 receptors relative genes in the striatum in *Shank3* mutants could lead to an abnormal postsynaptic impairments on dopaminergic neurones. Such reduced number of spikes due to the postsynaptic impairments could inhibit the turning behaviour mediated by those connections. This would allow to understand why *Shank3* haploinsufficiency reduces specifically turnings behaviour.



## Effects on social orientation

The time spent facing a conspecific on the first night and having the same orientation than a conspecific on the second one, were significantly lower for heterozygous mice than for wild-type ones. The fact to exhibit less behavioural event categories of socially relevant orientations can be linked to the fact that the animals spent less time turning. If the turning behaviour is reduced, the physical orientation of the animal's body is more rarely updated by a rotation. All animals in the group move, change location and orientation regularly. Turning behaviour thus-needs to be achieved for an animal to orient itself in relation to its conspecific and produce new behaviours of social orientation.

Even if the reduced proportions of socially relevant orientations can be caused by the fact that animals turn less, it can, at the same time, be interpreted as a lack of social interest. Such orientation has an impact on mouse capacities to perceive other individual visually and through olfaction. Such behaviour could lead to the fact that heterozygous mice collect less social cues than wild-type ones.

Socially-relevant orientations were not studied in this form in the past literature, but they are part of more complex behavioural sequences as sniffing and following. Many studies using the three-chambered test did not only evaluate time spent in each chamber but also the time spent sniffing the conspecific [200], [182]. This is a proof that, even when measures of social distances do not show an effect on social behaviour, we may find some by the addition of a criteria of social orientation.

Studies observing dyadic pairs in free interaction can also take into account behaviours involving social orientation. For example, Peça and colleagues have shown that pairs involving a wild-type mouse and one mutated homozygously for *Shank3* displayed a lower frequency of nose-to-nose interaction and anogenital sniffing, compared to pairs of two wild-type animals [52]. This last finding is coherent with what we find, by showing a lack of social interest through reduced socially-relevant orientations from *Shank3* homozygous mutant. Such reduced production of socially relevant orientations caused by *Shank3* haploinsufficiency could thus be expected, and it may be implied in less frequent sniffing behaviours.

### **Selection of a *shank3* haploinsufficiency model**

We showed by the present results that the mixed background, obtained from a CC002 mother and a C57BL/6J father, CC002xC57BL/6J, heterozygously knocked-out for *Shank3*, exhibit significant genotype dependent behavioural differences. In our animal facility, this particular cross offered an efficient reproduction, with female starting gestation quickly, generous litters of about ten pups, and a good survival rate until weaning. This genetic background is thus the one we would advise to use in order to study *Shank3* haploinsufficiency related behavioural affections.

Such model should thus be subjected to the usual test procedures for self-grooming, hypoactivity and dyadic social interaction. Such tests would allow to compare it with other pre-existing models of Autism spectrum disorder, in particular *Shank3* mutant ones. We could also introduce other *Shank3* mutations on this mixed genetic background. If more effects on the behaviour are found on this background, differences between different mutations could also be investigated. This would help to better understand the role of the different protein isoforms in the behavioural affection of *Shank3* mutations.

## Limits of the current *Shank3* haploinsufficiency study

This research is explorative, in the sense that it is data oriented and takes into account an important number of statistical tests. Due to those numerous tests, Bonferroni corrections for multiple testing lower the significance thresholds in high proportions. In this context, the best thing to do to counterbalance this loss of statistical power is to increase the sample size. Anyway, the increase in the cohort sizes is also an obvious way to increase the reliability of observed differences. It is clear that the F1 cohort used here, to discover the effects of *Shank3* heterozygous knock-out on the behaviour, is understaffed for this purpose. The number of recorded individuals should be increased. This also leads to ethical considerations, linked to the reduction of the number of animals used. An effective way to statistically compare bigger groups without using too many animals would be to select only one strain to study this mutation. After what we preliminary found we would suggest the use of CC002xC57BL/6J.*Shank3* F1 cross which both reproduced well and gave the biggest amount of differences in both solitary and social contexts.

We can think about a potential behavioural convergence effect on behaviour in mouse social groups. The behaviour of mice living the same group potentially influences the social behaviour of each group member. Therefore, control mice from one mixed-genotype group might not behave in the same way as in a group including only control mice. Knowing this we can suggest that the social protocol we chose to discriminate between heterozygous and wild-type behaviour is not optimal. Even if the mutation causes differences in the behaviour, if both types of animals are present in the same group and if groups tends to make the behaviour more uniform between individuals, then the original differences can be hidden. We thus propose further experiments grouping only individuals of the same genotype. This would allow to observe the mutant and wild-type behaviour separately, without any potential of convergence between them.

## Methodological considerations

### The richness of long experiments

Long experiments are a major specificity of our data collection protocols. Live Mouse Tracker allows a fully automated tracking with regular identity validations. It is thus not limited in tracking duration. Long recordings have three principal advantages for behavioural data. First, the accumulation of time points of features measurement increases the reliability of the behavioural classification. Bigger datasets increase both the quality of the blind source separation obtained through ICA and the precision of thresholds extracted by the GMM based clustering on BD distributions. In a second time, the more time points we use to compute rates of production of the different BEC, the more reliable are those rates. By comparing individuals on the basis of less artificially variant measurements, we thus increase our statistical discriminative potential. Finally, we can dissect long recordings in different phases in order to separate different effect on the behaviour. For example, with the decreasing novelty of the environment we can separate exploration from spontaneous mobility [201]. This can also help to understand the evolution of the social relationship with time, which plays a role in the definition of hierarchy in groups [202, p. 20]. We here measured BEC proportions at each night of the long term group monitoring experiments. Some BEC rates seem highly correlated between nights, others evolve between days. In some cases, we thus have an obvious justification for the use of the same BEC, to extract different pieces of information, depending on the phase in which we count their occurrences. For example, it is without surprise that the proportions of moving forward BEC are shown to be partly decorrelated between successive nights (figure 27-28). We are thus motivated to think that such variations allow us to evaluate different traits from this unique BEC, as we argued earlier: exploration and spontaneous mobility. This is why we compared this BEC proportions separately in each night, between strains and finally between genotypes.

## **The discovered repertoire**

This method allowed us to discover wider behavioural repertoires than what has been done in previous studies [97], [114], [75], [93]. This means that we are now able to discriminate and evaluate new behavioural categories which we could not work with earlier. In the same time, if we do not define behavioural event categories of interest, this rich repertoire can be hard to investigate because of the wide screening and the subsequent necessary corrections for multiple testing. The here discovered behavioural event categories can be interpreted as parts of more complex behavioural sequences. If another method than SUCCUBE allows researchers to select a set of behavioural sequences, it is then possible to evaluate the enrichment of this set in some specific behavioural event extracted from SUCCUBE. Behavioural event enriched in the sequences of interest can be selected as behavioural event of interest.

## **Limits of previous classifiers**

By the past, less behavioural categories were considered. Their selection has first been driven by human visual description of the behaviour, which is thus limited by Human perceptive capacities. Visual descriptions of solitary behaviour focused on movement leading to a change of location which were easier to perceive by the fact that our attention to motion is highly stimulated by those wide motions. Visual descriptions of social behaviour focused on contact, and socially relevant changes of location. Contact events were easier to recognize by the fact that mice were close to one another and therefore can be observed at the same time. And, as for solitary behaviour, the changes of location are widely extended in the environment and easy to notice (figure 40).

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Figure 40: Representation of behavioural events categories automatically labelled by Live Mouse Tracker from de Chaumont et al. in 2019 [93]. A list of extracted behaviours, classified into five major groups: individual, social dyadic, dyadic dynamic, configuration, and group-making and breaking events (dashes indicate that the animals are not moving).

Rule-based classifications label categories hard-coded by humans, on the basis of their *a priori* understanding of behaviour. Thus they naturally first focus on location changing movemes and social contact postures. We can see for example, that in the behavioural events already labelled by Live Mouse Tracker (figure 40), movemes are represented by:

- jumping behaviour, which is highly stimulating for human perception by the speed, the unusual altitude change, and the noise associated.
- Moving behaviour, which is based on location changes and thus stimulates easily the observer because of the spread of localisation in space.

And social behaviours are represented by:

- Contact and group postures, which are based on the co-localization, and thus easy to observe because all animals implied are visible in the same area.
- Contact making or breaking, which adds this co-localized posture to moving behaviour resulting in its appearance or disappearance and is thus profiting of a synergistic potential of visual stimulation on the observer.
- Trains and following behaviour, which focus on the shared direction of movement, two separated objects moving in similar patterns being easily recognized by human perception.

Unsupervised behavioural classification relying on a single clustering can also be biased in favour of those behaviours which impact location related measurements. Such measurements have a great variance and will participate in important proportions to the variance of datasets.

On the opposite, genotype-related alterations of behaviour that we here found are orientation changing movements and socially oriented postures. This capacity to cluster behaviours which are not the most evident for human senses or in term of variance of measurements is, in our opinion, the advantage of a method of unsupervised behavioural classification relying on multiple clustering as SUCCUBE. It here seems that this ability to discriminate between co-occurrent behaviours allowed to measure the traits altered by *Shank3* haploinsufficiency.





# **Conclusion**



## Main contributions

The main product of this work is the SUCCUBE algorithm. This new method allows, for the first time, the unsupervised discovery of a behavioural repertoire whose categories can co-occur. In addition to considerably reducing the inherent biases of supervised classification, this new method allows to consider more different aspects of the behaviour and to accurately describe what the animals are doing through the simultaneous association of categories.

The application of the SUCCUBE algorithm uncovered a large behavioural repertoire based on the diversity of behaviour among 216 individuals from the 13 strains of the first cohort. With 116 BEC for the single object exploration experiment and 112 BEC for the long-term group monitoring experiment, these repertoires are larger than anything previously described, especially when the combinatorial aspect of these behavioural categories is taken into account.

The profiles of the 12 collaborative cross strains we studied are also the most complete descriptions of the behaviour of these lines for now. It also provides insight into the relationship between genetic diversity and behavioural diversity. This information can be used in the future for the research of new mouse models.

Finally, the fact that we found a cross, CC002xC57BL/6J, that shows robust differences in behaviour due to a haploinsufficiency in *Shank3* is our final result. This strain can now be used as a model for preclinical testing.

## Perspectives

### Validation

The validation process would consist in the fact to encode, in Live Mouse Tracker behavioural repertoire, behavioural event categories inspired by the ones segregated through SUCCUBE. This validation would first permit to confirm the effects of *Shank3* haploinsufficiency on the behaviour. We would look for similar effects on simpler trait measurements, mixing less features and quantified with one threshold for each feature. Obtaining similar statistical results would corroborate findings made with SUCCUBE's categories. This should also help to confirm our interpretation of behavioural event categories. The construction of such simpler trait measurements would reflect our interpretation of SUCCUBE's categories. Such new measurements will finally be usable to further investigate *Shank3* haploinsufficiency effects on mouse behaviour and potential restoration trial.

### Methodological improvements

In further development SUCCUBE'S algorithm can be modified. New features can be added to complement the repertoire. The method can also be adapted to datasets acquired through other methods and recording setups, as for example accelerometry. Other dimension reduction techniques can be tried as auto-encoder which could allow non-linear feature mixes.

## **Temporal structure analysis**

SUCCUBE also enables further investigations on the nature of behaviour. SUCCUBE here allowed us to extract a rich and modular behavioural repertoire from tracking data and to classify time-points in its categories. The temporal structure linking the production of those behavioural events can thus now be studied. The co-occurrence rates of the different behavioural event categories can serve as new measurements to characterize mice. Succession patterns between the categories can be studied in similar ways by their proportions. The behavioural descriptors and classifications along time may also be analysed through autocorrelation in order to identify potential rhythms of behavioural mechanisms. In social context the temporal structure of behaviour can also help to understand the reaction of one animal to the behaviour of its relatives. For example, concerning the present work, a link could be drawn between the reductions of turning behaviour on one side and socially related orientations on the other. Temporal analysis of the behaviour could inform us on their potential co-occurrences or even to recurrent successions suggesting a causal link.

## **Further investigations**

### **Continuation with the Collaborative Cross**

After C57BL/6J, it would be interesting to profile the behaviour of the other founding strains of the Collaborative Cross. Two Collaborative Cross strains share an eighth of their genes, and it is the same between a founding strain and a collaborative strain, but the genetic distance between two founding strains is potentially higher.

An increase of the number of profiled CC strains could also be interesting as it may allow the use of quantitative trait locus analysis. For example Molenhuis found a locus which has great probabilities to be implied in the determination of the amount of route tracing stereotypy exhibited by mice, which could be relevant in the context of ASD [153]. This would permit to discover genes implied in different aspects of the behavioural variability. This could also help in the identification of modifier genes of *Shank3* mutant phenotype. Which in last instance would make easier the selection of better models for *Shank3* haploinsufficiency impact on the behaviour.

### **Pharmaceutical strategy trials**

Now that we identified a model in which the behaviour is affected by *Shank3* haploinsufficiency, both in their solitary and social phenotype, CC002xC57BL/6J.*Shank3* F1 cross, this one can be used for pharmacological trials. It is now possible to compare wild-types and non-treated heterozygotes to heterozygotes treated with drugs increasing *Shank3* transcription. Lithium could be an interesting candidate as it has proven its ability to increase *SHANK3* transcription in *in vitro* human neurons [203]. It was shown to rescue some symptoms in human *SHANK3* patients with ASD [204]. Lithium also rescued some repetitive grooming in *Shank3* knockout mice [205] If a drug allows some restoration of the phenotype closer to the wild-type one, it would be the first indices in favour of future human pharmacological treatment opportunity.

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# **Annexes**



Table annex 1: SUCCUBE features

| N° | Feature names                  | measurement | time scale | category |
|----|--------------------------------|-------------|------------|----------|
| 1  | particle number                | number      | dynamic    | particle |
| 2  | length HT                      | distance    | static     | body     |
| 3  | length MT                      | distance    | static     | body     |
| 4  | length HM                      | distance    | static     | body     |
| 5  | change in length HT            | distance    | dynamic    | body     |
| 6  | change in length MT            | distance    | dynamic    | body     |
| 7  | change in length HM            | distance    | dynamic    | body     |
| 8  | angle HMT                      | angle       | static     | body     |
| 9  | change in angle HMT            | angle       | dynamic    | body     |
| 10 | altitude H                     | distance    | static     | body     |
| 11 | altitude M                     | distance    | static     | body     |
| 12 | altitude T                     | distance    | static     | body     |
| 13 | change in altitude H           | distance    | dynamic    | body     |
| 14 | change in altitude M           | distance    | dynamic    | body     |
| 15 | change in altitude T           | distance    | dynamic    | body     |
| 16 | angular velocity HT            | angle       | dynamic    | body     |
| 17 | angular velocity MT            | angle       | dynamic    | body     |
| 18 | angular velocity HM            | angle       | dynamic    | body     |
| 19 | angular acceleration HT        | angle       | dynamic    | body     |
| 20 | angular acceleration MT        | angle       | dynamic    | body     |
| 21 | angular acceleration HM        | angle       | dynamic    | body     |
| 22 | change in velocity direction H | distance    | dynamic    | body     |
| 23 | change in velocity direction M | distance    | dynamic    | body     |
| 24 | change in velocity direction T | distance    | dynamic    | body     |
| 25 | speed H                        | distance    | dynamic    | body     |
| 26 | speed M                        | distance    | dynamic    | body     |
| 27 | speed T                        | distance    | dynamic    | body     |
| 28 | forward velocity H             | distance    | dynamic    | body     |
| 29 | forward velocity M             | distance    | dynamic    | body     |
| 30 | forward velocity T             | distance    | dynamic    | body     |
| 31 | sideway velocity H             | distance    | dynamic    | body     |
| 32 | sideway velocity M             | distance    | dynamic    | body     |
| 33 | sideway velocity T             | distance    | dynamic    | body     |
| 34 | acceleration H                 | distance    | dynamic    | body     |
| 35 | acceleration M                 | distance    | dynamic    | body     |
| 36 | acceleration T                 | distance    | dynamic    | body     |
| 37 | angle HT velocity H            | angle       | dynamic    | body     |
| 38 | angle HT velocity M            | angle       | dynamic    | body     |
| 39 | angle HT velocity T            | angle       | dynamic    | body     |
| 40 | distance to centre H           | distance    | static     | arena    |
| 41 | distance to centre M           | distance    | static     | arena    |
| 42 | distance to centre T           | distance    | static     | arena    |
| 43 | change in distance to centre H | distance    | dynamic    | arena    |
| 44 | change in distance to centre M | distance    | dynamic    | arena    |

|    |   |          |         |        |
|----|---|----------|---------|--------|
| 45 | change in distance to centre T                                      | distance | dynamic | arena  |
| 46 | angle HT centre H   | angle    | static  | arena  |
| 47 | angle HT centre M   | angle    | static  | arena  |
| 48 | angle HT centre T   | angle    | static  | arena  |
| 49 | change in angle HT centre H   | angle    | dynamic | arena  |
| 50 | change in angle HT centre M   | angle    | dynamic | arena  |
| 51 | change in angle HT centre T   | angle    | dynamic | arena  |
| 52 | distance to closest wall H  | distance | static  | arena  |
| 53 | distance to closest wall M  | distance | static  | arena  |
| 54 | distance to closest wall T  | distance | static  | arena  |
| 55 | change in distance to closest wall H                                | distance | dynamic | arena  |
| 56 | change in distance to closest wall M                                | distance | dynamic | arena  |
| 57 | change in distance to closest wall T                                | distance | dynamic | arena  |
| 58 | angle HT closest projection on a wall H                             | angle    | static  | arena  |
| 59 | angle HT closest projection on a wall M                             | angle    | static  | arena  |
| 60 | angle HT closest projection on a wall T                             | angle    | static  | arena  |
| 61 | change in angle HT closest projection on a wall H                   | angle    | dynamic | arena  |
| 62 | change in angle HT closest projection on a wall M                   | angle    | dynamic | arena  |
| 63 | change in angle HT closest projection on a wall T                   | angle    | dynamic | arena  |
| 64 | distance to closest corner H  | distance | static  | arena  |
| 65 | distance to closest corner M  | distance | static  | arena  |
| 66 | distance to closest corner T  | distance | static  | arena  |
| 67 | change in distance to closest corner H                              | distance | dynamic | arena  |
| 68 | change in distance to closest corner M                              | distance | dynamic | arena  |
| 69 | change in distance to closest corner T                              | distance | dynamic | arena  |
| 70 | angle HT closest corner H   | angle    | static  | arena  |
| 71 | angle HT closest corner M   | angle    | static  | arena  |
| 72 | angle HT closest corner T   | angle    | static  | arena  |
| 73 | change in angle HT closest corner H                                 | angle    | dynamic | arena  |
| 74 | change in angle HT closest corner M                                 | angle    | dynamic | arena  |
| 75 | change in angle HT closest corner T                                 | angle    | dynamic | arena  |
| 76 | distance to closest house wall H                                    | distance | static  | object |
| 77 | distance to closest house wall M                                    | distance | static  | object |
| 78 | distance to closest house wall T                                    | distance | static  | object |
| 79 | change in distance to closest house wall H                          | distance | dynamic | object |
| 80 | change in distance to closest house wall M                          | distance | dynamic | object |
| 81 | change in distance to closest house wall T                          | distance | dynamic | object |
| 82 | sum of distances to house walls H                                   | distance | static  | object |
| 83 | sum of distances to house walls M                                   | distance | static  | object |
| 84 | sum of distances to house walls T                                   | distance | static  | object |
| 85 | change in the sum of distances to house walls H                     | distance | dynamic | object |
| 86 | change in the sum of distances to house walls M                     | distance | dynamic | object |
| 87 | change in the sum of distances to house walls T                     | distance | dynamic | object |
| 88 | angle to closest projection on a wall in reference to animal axis H | angle    | static  | object |
| 89 | angle to closest projection on a wall in reference to animal axis M | angle    | static  | object |
| 90 | angle to closest projection on a wall in reference to               | angle    | static  | object |

|     |  |          |         |        |
|-----|--|----------|---------|--------|
|     | animal axis T  |          |         |        |
| 91  | change in the angle to closest projection on a wall<br>in reference to animal axis H | angle    | dynamic | object |
| 92  | change in the angle to closest projection on a wall<br>in reference to animal axis M | angle    | dynamic | object |
| 93  | change in the angle to closest projection on a wall<br>in reference to animal axis T | angle    | dynamic | object |
| 94  | sum of angles to projections on walls in reference to<br>animal axis H               | angle    | static  | object |
| 95  | sum of angles to projections on walls in reference to<br>animal axis M               | angle    | static  | object |
| 96  | sum of angles to projections on walls in reference to<br>animal axis T               | angle    | static  | object |
| 97  | change in the sum of angles to projections on walls<br>in reference to animal axis H | angle    | dynamic | object |
| 98  | change in the sum of angles to projections on walls<br>in reference to animal axis M | angle    | dynamic | object |
| 99  | change in the sum of angles to projections on walls<br>in reference to animal axis T | angle    | dynamic | object |
| 100 | distance to closest house gate H   | distance | static  | object |
| 101 | distance to closest house gate M   | distance | static  | object |
| 102 | distance to closest house gate T   | distance | static  | object |
| 103 | change in distance to closest house gate H   | distance | dynamic | object |
| 104 | change in distance to closest house gate M   | distance | dynamic | object |
| 105 | change in distance to closest house gate T   | distance | dynamic | object |
| 106 | sum of distances to house gates H  | distance | static  | object |
| 107 | sum of distances to house gates M  | distance | static  | object |
| 108 | sum of distances to house gates T  | distance | static  | object |
| 109 | change in the sum of distances to house gates H                                      | distance | dynamic | object |
| 110 | change in the sum of distances to house gates M                                      | distance | dynamic | object |
| 111 | change in the sum of distances to house gates T                                      | distance | dynamic | object |
| 112 | angle to closest projection on a gate in reference to<br>animal axis H               | angle    | static  | object |
| 113 | angle to closest projection on a gate in reference to<br>animal axis M               | angle    | static  | object |
| 114 | angle to closest projection on a gate in reference to<br>animal axis T               | angle    | static  | object |
| 115 | change in the angle to closest projection on a gate<br>in reference to animal axis H | angle    | dynamic | object |
| 116 | change in the angle to closest projection on a gate<br>in reference to animal axis M | angle    | dynamic | object |
| 117 | change in the angle to closest projection on a gate<br>in reference to animal axis T | angle    | dynamic | object |
| 118 | sum of angles to projections on gates in reference<br>to animal axis H               | angle    | static  | object |
| 119 | sum of angles to projections on gates in reference<br>to animal axis M               | angle    | static  | object |
| 120 | sum of angles to projections on gates in reference                                   | angle    | static  | object |



|     |  |          |         |        |
|-----|--|----------|---------|--------|
|     | to animal axis T   |          |         |        |
| 121 | change in the sum of angles to projections on gates<br>in reference to animal axis H | angle    | dynamic | object |
| 122 | change in the sum of angles to projections on gates<br>in reference to animal axis M | angle    | dynamic | object |
| 123 | change in the sum of angles to projections on gates<br>in reference to animal axis T | angle    | dynamic | object |
| 124 | distance to closest house corner H   | distance | static  | object |
| 125 | distance to closest house corner M   | distance | static  | object |
| 126 | distance to closest house corner T   | distance | static  | object |
| 127 | change in distance to closest house corner H   | distance | dynamic | object |
| 128 | change in distance to closest house corner M   | distance | dynamic | object |
| 129 | change in distance to closest house corner T   | distance | dynamic | object |
| 130 | sum of distances to house corners H  | distance | static  | object |
| 131 | sum of distances to house corners M  | distance | static  | object |
| 132 | sum of distances to house corners T  | distance | static  | object |
| 133 | change in the sum of distances to house corners H                                    | distance | dynamic | object |
| 134 | change in the sum of distances to house corners M                                    | distance | dynamic | object |
| 135 | change in the sum of distances to house corners T                                    | distance | dynamic | object |
| 136 | angle to closest corner in reference to animal axis H                                | angle    | static  | object |
| 137 | angle to closest corner in reference to animal axis M                                | angle    | static  | object |
| 138 | angle to closest corner in reference to animal axis T                                | angle    | static  | object |
| 139 | change in the angle to closest corner in reference to<br>animal axis H               | angle    | dynamic | object |
| 140 | change in the angle to closest corner in reference to<br>animal axis M               | angle    | dynamic | object |
| 141 | change in the angle to closest corner in reference to<br>animal axis T               | angle    | dynamic | object |
| 142 | sum of angles to corners in reference to animal axis<br>H                            | angle    | static  | object |
| 143 | sum of angles to corners in reference to animal axis<br>M                            | angle    | static  | object |
| 144 | sum of angles to corners in reference to animal axis<br>T                            | angle    | static  | object |
| 145 | change in the sum of angles to corners in reference<br>to animal axis H              | angle    | dynamic | object |
| 146 | change in the sum of angles to corners in reference<br>to animal axis M              | angle    | dynamic | object |
| 147 | change in the sum of angles to corners in reference<br>to animal axis T              | angle    | dynamic | object |
| 148 | biggest angle of view of a house wall  | angle    | static  | object |
| 149 | biggest angle of view of a house gate  | angle    | static  | object |
| 150 | angle of view of the house   | angle    | static  | object |
| 151 | change in the biggest angle of view of a house wall                                  | angle    | dynamic | object |
| 152 | change in the biggest angle of view of a house gate                                  | angle    | dynamic | object |
| 153 | change in the angle of view of the house   | angle    | dynamic | object |
| 154 | part of the animal recovered by the house  | distance | static  | object |
| 155 | change in the part of the animal recovered by the                                    | distance | dynamic | object |

|     | house                   |          |         |        |
|-----|-------------------------|----------|---------|--------|
| 156 | social altitude Hr      | distance | static  | social |
| 157 | social altitude Mr      | distance | static  | social |
| 158 | social altitude Tr      | distance | static  | social |
| 159 | distance HHr            | distance | static  | social |
| 160 | distance HMr            | distance | static  | social |
| 161 | distance HTr            | distance | static  | social |
| 162 | distance M Hr           | distance | static  | social |
| 163 | distance M Mr           | distance | static  | social |
| 164 | distance M Tr           | distance | static  | social |
| 165 | distance T Hr           | distance | static  | social |
| 166 | distance T Mr           | distance | static  | social |
| 167 | distance T Tr           | distance | static  | social |
| 168 | change in distance HHr  | distance | dynamic | social |
| 169 | change in distance HMr  | distance | dynamic | social |
| 170 | change in distance HTr  | distance | dynamic | social |
| 171 | change in distance M Hr | distance | dynamic | social |
| 172 | change in distance M Mr | distance | dynamic | social |
| 173 | change in distance M Tr | distance | dynamic | social |
| 174 | change in distance T Hr | distance | dynamic | social |
| 175 | change in distance T Mr | distance | dynamic | social |
| 176 | change in distance T Tr | distance | dynamic | social |
| 177 | angle HT HHr            | angle    | static  | social |
| 178 | angle HT HMr            | angle    | static  | social |
| 179 | angle HT HTr            | angle    | static  | social |
| 180 | angle HT M Hr           | angle    | static  | social |
| 181 | angle HT M Mr           | angle    | static  | social |
| 182 | angle HT M Tr           | angle    | static  | social |
| 183 | angle HT T Hr           | angle    | static  | social |
| 184 | angle HT T Mr           | angle    | static  | social |
| 185 | angle HT T Tr           | angle    | static  | social |
| 186 | angle HT HrTr           | angle    | static  | social |
| 187 | angle HT MrTr           | angle    | static  | social |
| 188 | angle HT HrMr           | angle    | static  | social |
| 189 | angle MT HrTr           | angle    | static  | social |
| 190 | angle MT MrTr           | angle    | static  | social |
| 191 | angle MT HrMr           | angle    | static  | social |
| 192 | angle HM HrTr           | angle    | static  | social |
| 193 | angle HM MrTr           | angle    | static  | social |
| 194 | angle HM HrMr           | angle    | static  | social |
| 195 | angle velocities H Hr   | angle    | dynamic | social |
| 196 | angle velocities H Mr   | angle    | dynamic | social |
| 197 | angle velocities H Tr   | angle    | dynamic | social |
| 198 | angle velocities M Hr   | angle    | dynamic | social |
| 199 | angle velocities M Mr   | angle    | dynamic | social |
| 200 | angle velocities M Tr   | angle    | dynamic | social |
| 201 | angle velocities T Hr   | angle    | dynamic | social |
| 202 | angle velocities T Mr   | angle    | dynamic | social |

|     |                            |          |         |        |
|-----|----------------------------|----------|---------|--------|
| 203 | angle velocities T Tr      | angle    | dynamic | social |
| 204 | angle HrHTr                | angle    | static  | social |
| 205 | angle HrHMr                | angle    | static  | social |
| 206 | angle MrHTr                | angle    | static  | social |
| 207 | change in angle HrHTr      | angle    | dynamic | social |
| 208 | change in angle HrHMr      | angle    | dynamic | social |
| 209 | change in angle MrHTr      | angle    | dynamic | social |
| 210 | difference velocities H Hr | distance | dynamic | social |
| 211 | difference velocities H Mr | distance | dynamic | social |
| 212 | difference velocities H Tr | distance | dynamic | social |
| 213 | difference velocities M Hr | distance | dynamic | social |
| 214 | difference velocities M Mr | distance | dynamic | social |
| 215 | difference velocities M Tr | distance | dynamic | social |
| 216 | difference velocities T Hr | distance | dynamic | social |
| 217 | difference velocities T Mr | distance | dynamic | social |
| 218 | difference velocities T Tr | distance | dynamic | social |
| 219 | speed H toward Hr          | distance | dynamic | social |
| 220 | speed H toward Mr          | distance | dynamic | social |
| 221 | speed H toward Tr          | distance | dynamic | social |
| 222 | speed M toward Hr          | distance | dynamic | social |
| 223 | speed M toward Mr          | distance | dynamic | social |
| 224 | speed M toward Tr          | distance | dynamic | social |
| 225 | speed T toward Hr          | distance | dynamic | social |
| 226 | speed T toward Mr          | distance | dynamic | social |
| 227 | speed T toward Tr          | distance | dynamic | social |

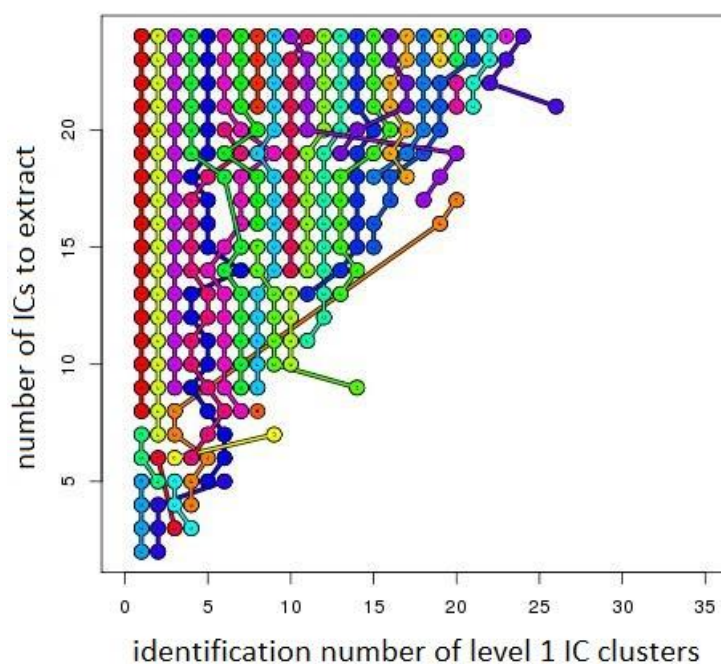


Figure annex 1: IC clusters over numbers of IC to extract for the BP feature set of the single object exploration dataset. Each dot represent a level 1 cluster of IC, each line of dot of unique colour represent a level 2 cluster of IC.

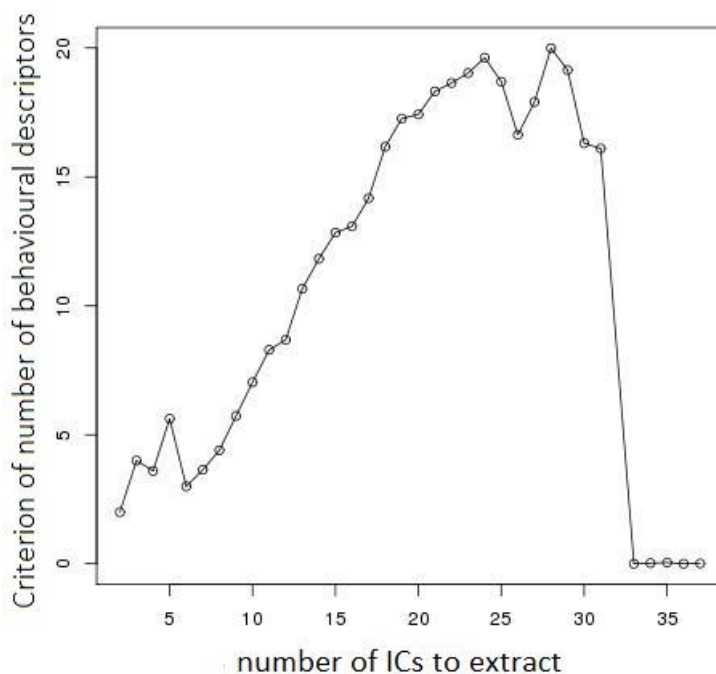


Figure annex 2: Criterion of number of behavioural descriptors in function of the number of IC for the BP feature set of the single object exploration dataset. 24 behavioural descriptors kept for the BP feature set of the single object exploration dataset.

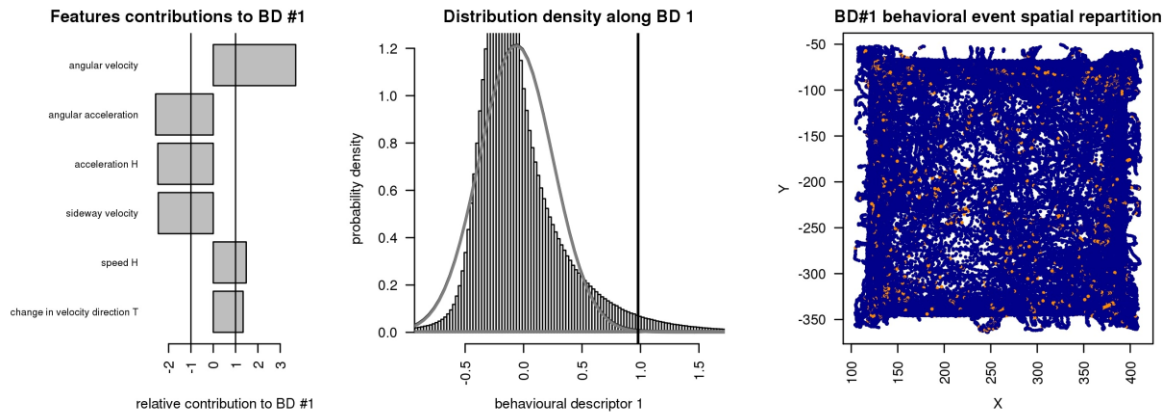


Figure annex 3: Interpretation figures for the BEC segregated on the 1st BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “does not rotate in deceleration”
- the BEC on the right of B and orange in C: “rotates decelerating”

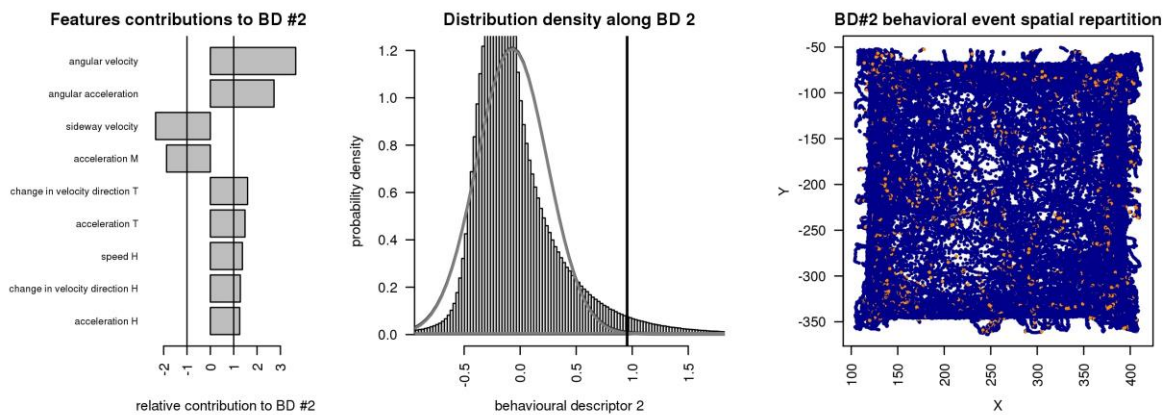


Figure annex 4: Interpretation figures for the BEC segregated on the 2nd BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “does not rotate in acceleration”
- the BEC on the right of B and orange in C: “rotates accelerating”

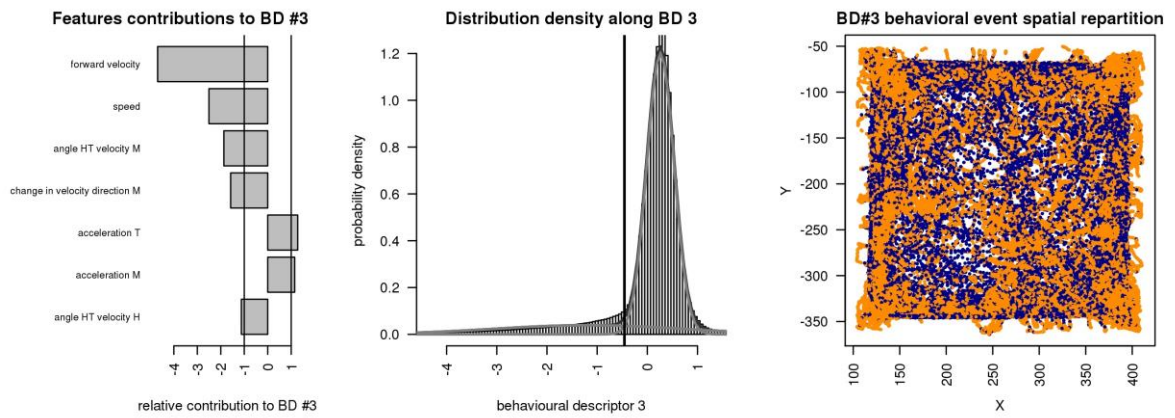


Figure annex 5: Interpretation figures for the BEC segregated on the 3rd BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “moves forward”
- the BEC on the right of B and orange in C: “does not move forward”

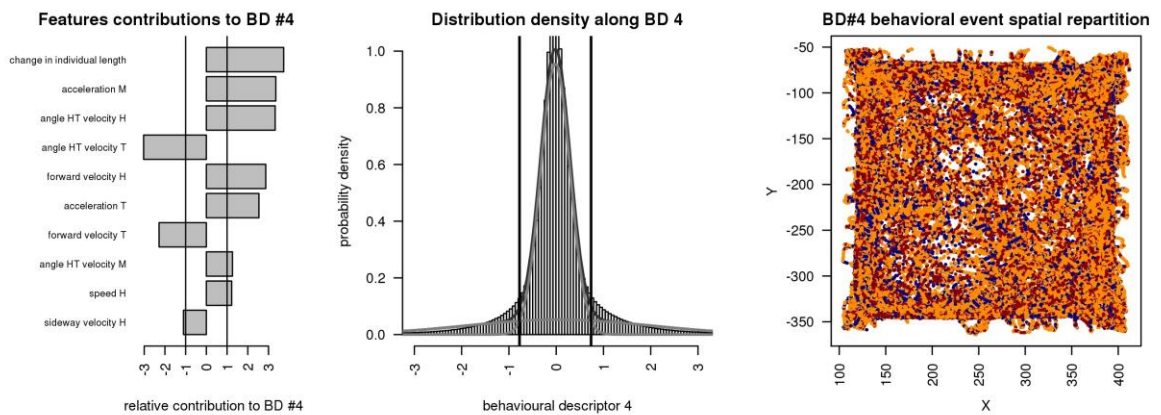


Figure annex 6: Interpretation figures for the BEC segregated on the 4th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “contracts”
- the BEC on the middle of B and orange in C: “stable extension”
- the BEC on the right of B and red in C: “extends”



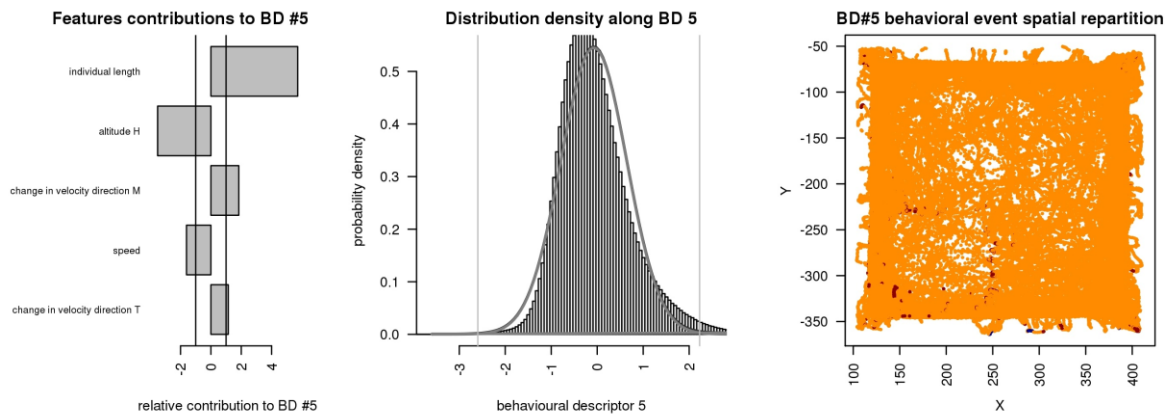


Figure annex 7: Interpretation figures for the BEC segregated on the 5th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“is short on x-y axis”*
- the BEC on the middle of B and orange in C: *“normal extension”*
- the BEC on the right of B and red in C: *“is extended”*

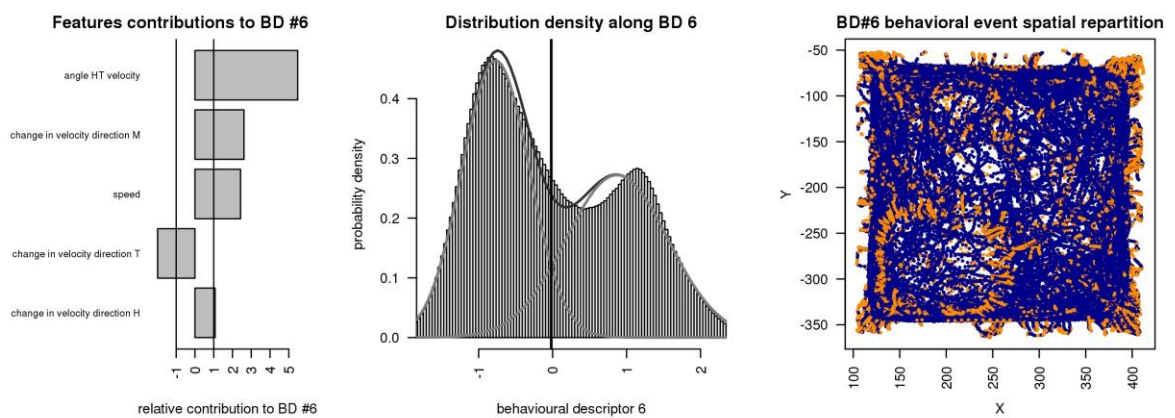


Figure annex 8: Interpretation figures for the BEC segregated on the 6th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“does not move backward”*
- the BEC on the right of B and orange in C: *“moves backward”*

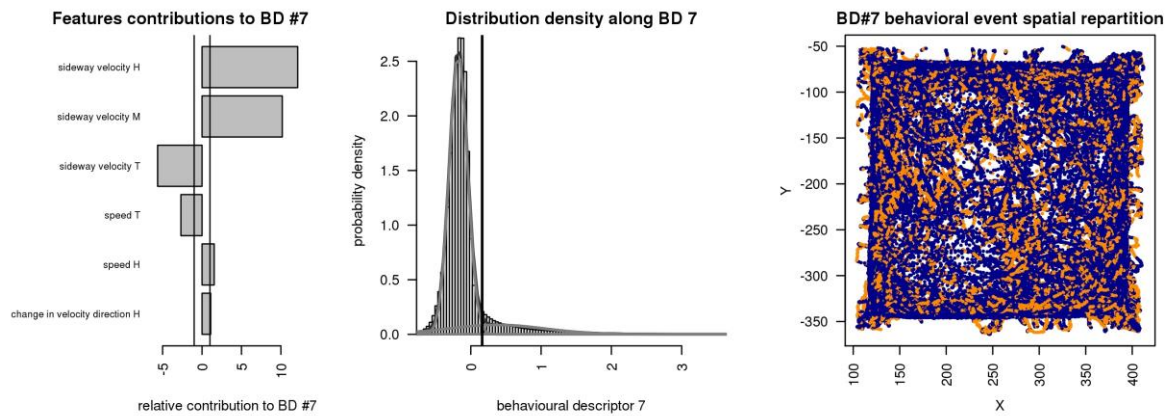


Figure annex 9: Interpretation figures for the BEC segregated on the 7th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“does not move the anterior part sideways”*
- the BEC on the right of B and orange in C: *“moves the anterior part sideways”*

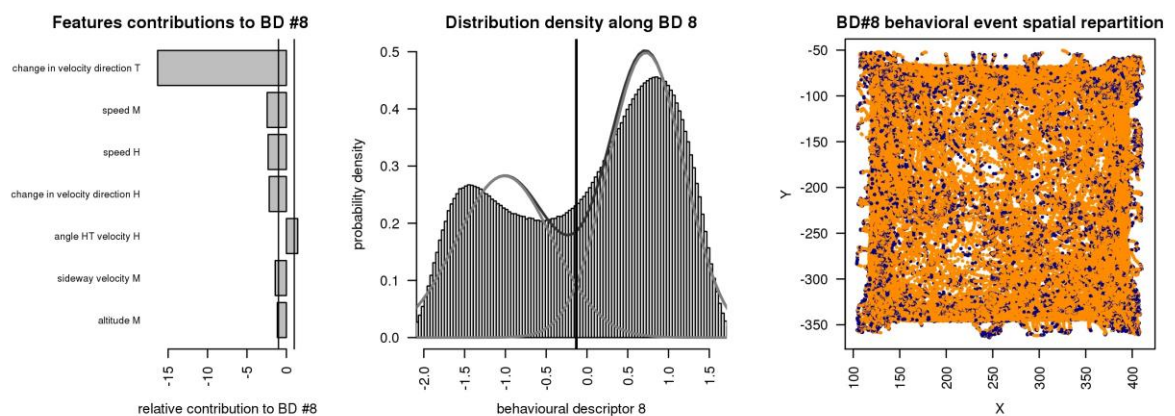


Figure annex 10: Interpretation figures for the BEC segregated on the 8th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“is posterior deviating”*
- the BEC on the right of B and orange in C: *“is not posterior deviating”*



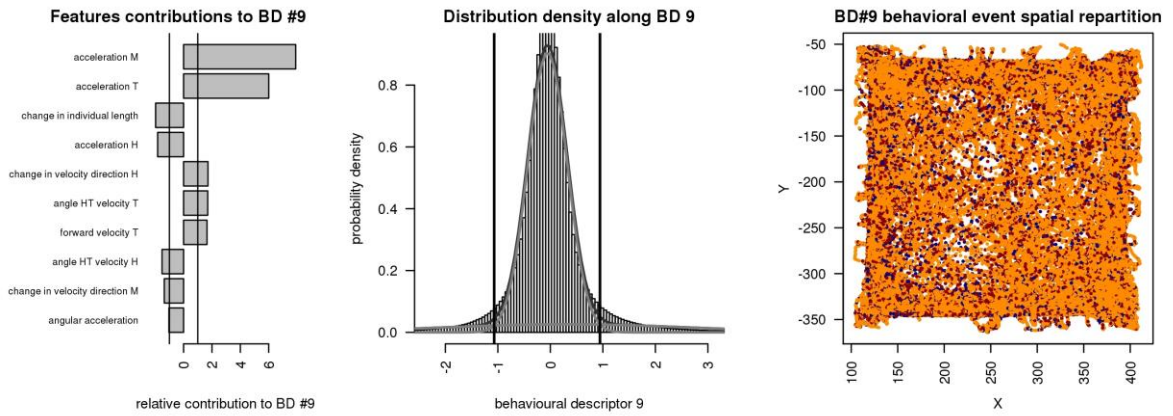


Figure annex 11: Interpretation figures for the BEC segregated on the 9th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“decelerates”*
- the BEC on the middle of B and orange in C: *“stable speed”*
- the BEC on the right of B and red in C: *“accelerates”*

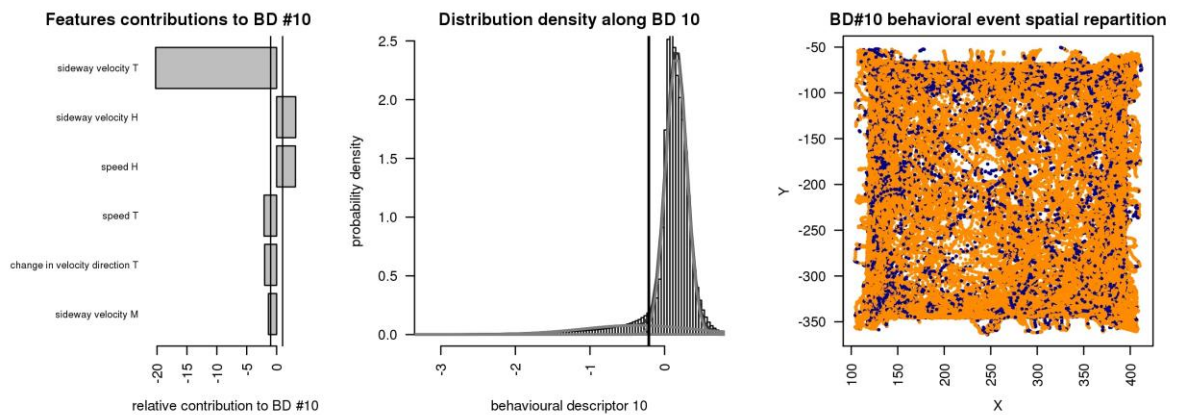


Figure annex 12: Interpretation figures for the BEC segregated on the 10th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“moves the posterior part sideways”*
- the BEC on the right of B and orange in C: *“does not move the posterior part sideways”*

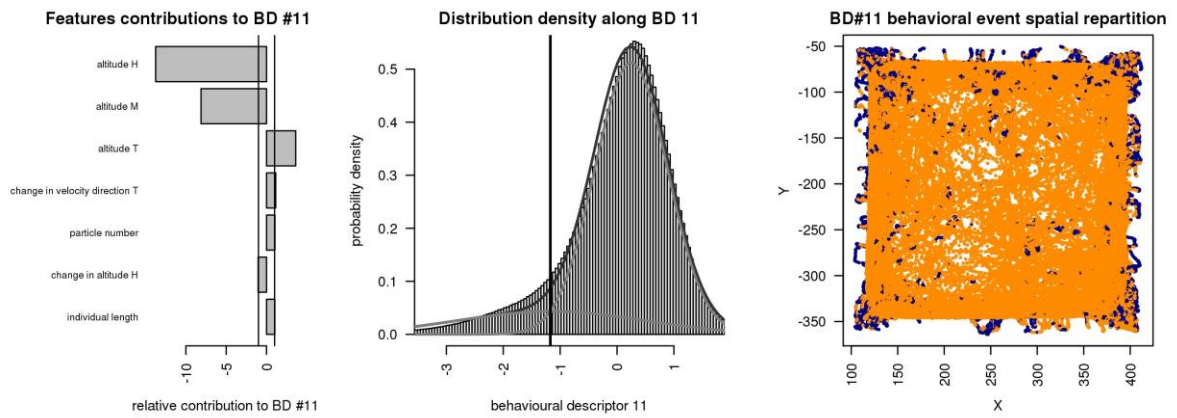


Figure annex 13: Interpretation figures for the BEC segregated on the 11th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“is reared”*
- the BEC on the right of B and orange in C: *“is not reared”*

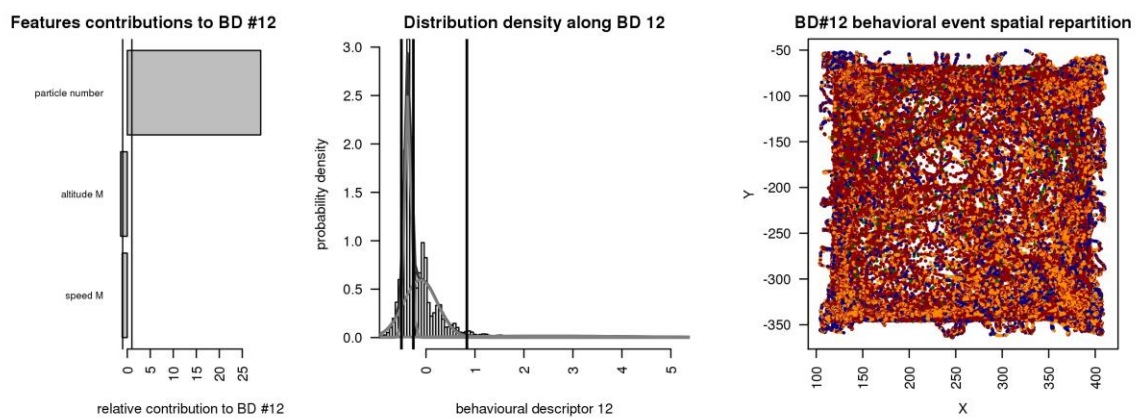


Figure annex 14: Interpretation figures for the BEC segregated on the 12th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“moves without agitating the bedding”*
- the BEC on the middle-left of B and orange in C: *“does not agitate bedding”*
- the BEC on the middle-right of B and red in C: *“agitates bedding”*
- the BEC on the right of B and red in C: *“digs”*

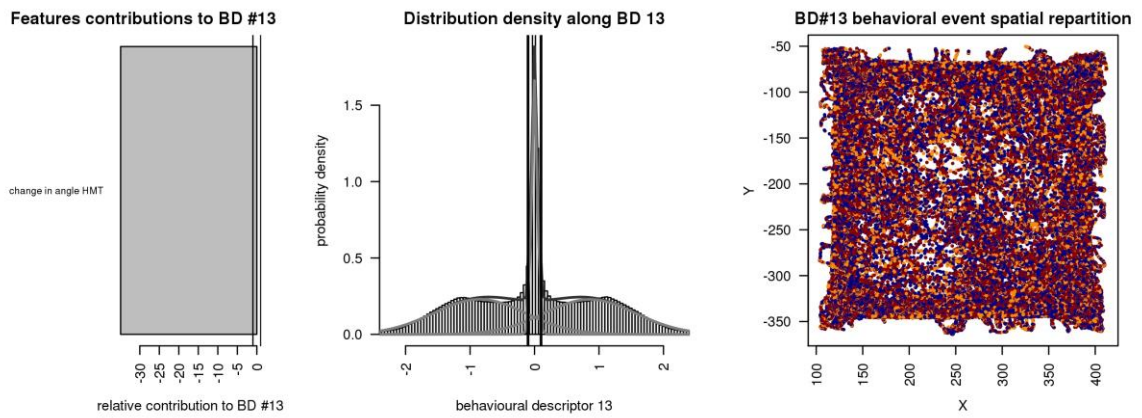


Figure annex 15: Interpretation figures for the BEC segregated on the 13th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “unbends”
- the BEC on the middle of B and orange in C: “stable bending”
- the BEC on the right of B and red in C: “bends”

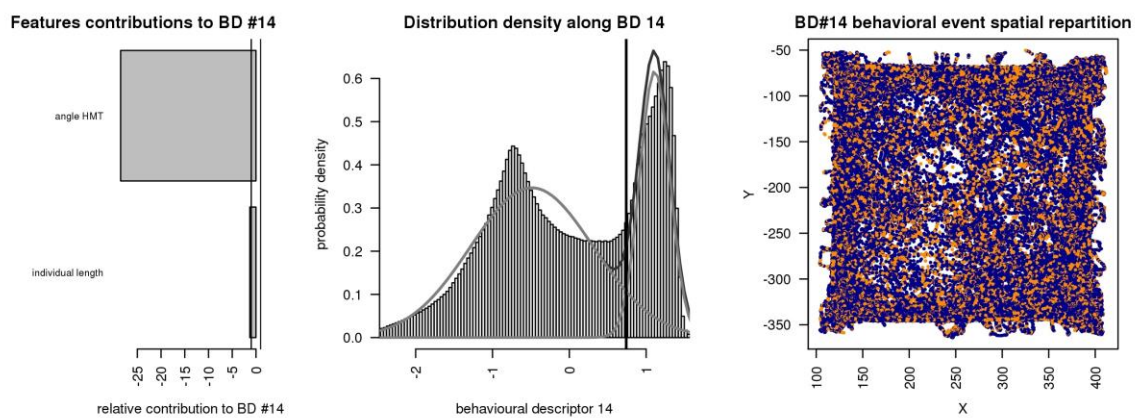


Figure annex 16: Interpretation figures for the BEC segregated on the 14th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “is not bent”
- the BEC on the right of B and orange in C: “is bent”

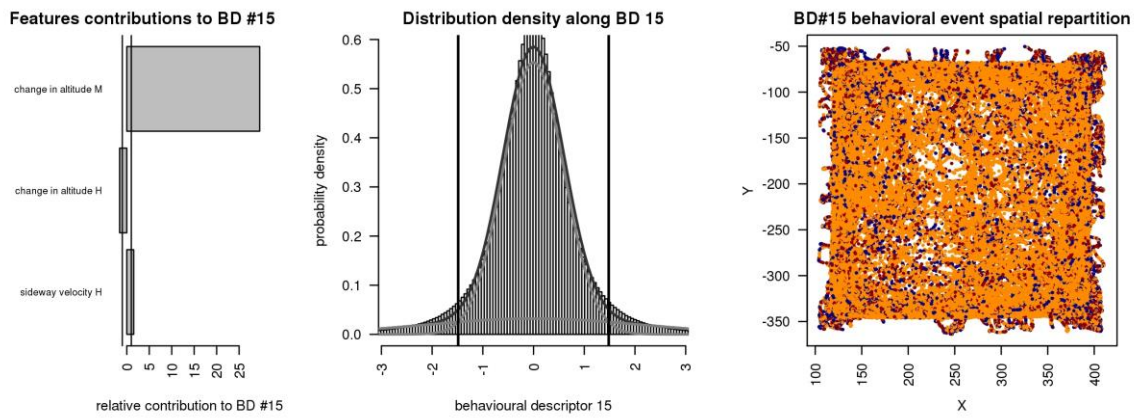


Figure annex 17: Interpretation figures for the BEC segregated on the 15th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“rears down”*
- the BEC on the middle of B and orange in C: *“stable altitude”*
- the BEC on the right of B and red in C: *“rears up”*

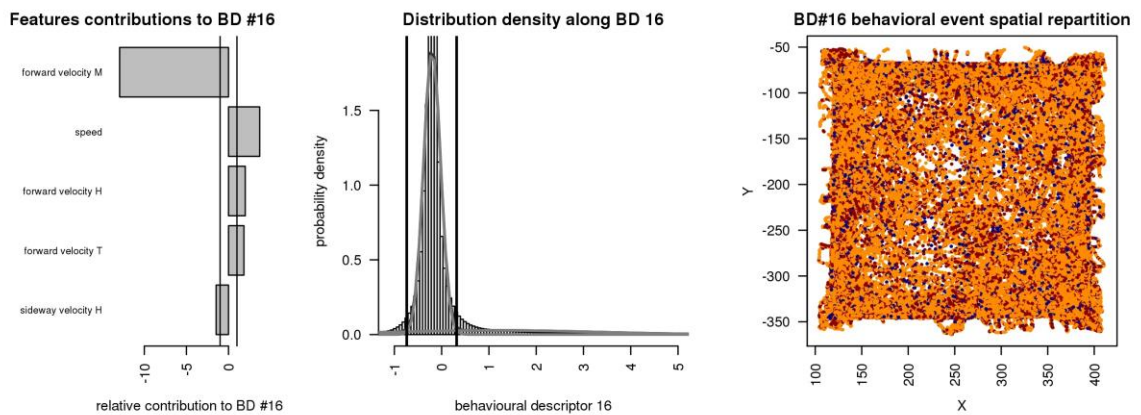


Figure annex 18: Interpretation figures for the BEC segregated on the 16th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“centre of mass pushed forward”*
- the BEC on the middle of B and orange in C: *“centre of mass synchronized with other body parts”*
- the BEC on the right of B and red in C: *“centre of mass pushed back”*



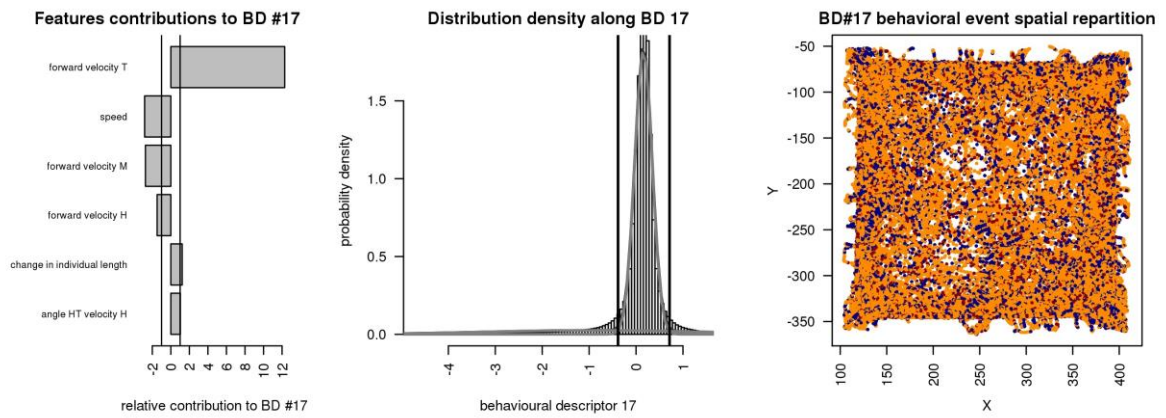


Figure annex 19: Interpretation figures for the BEC segregated on the 17th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: **“tail base pushed back”**
- the BEC on the middle of B and orange in C: **“tail base synchronized with other body parts”**
- the BEC on the right of B and red in C: **“tail base pushed forward”**

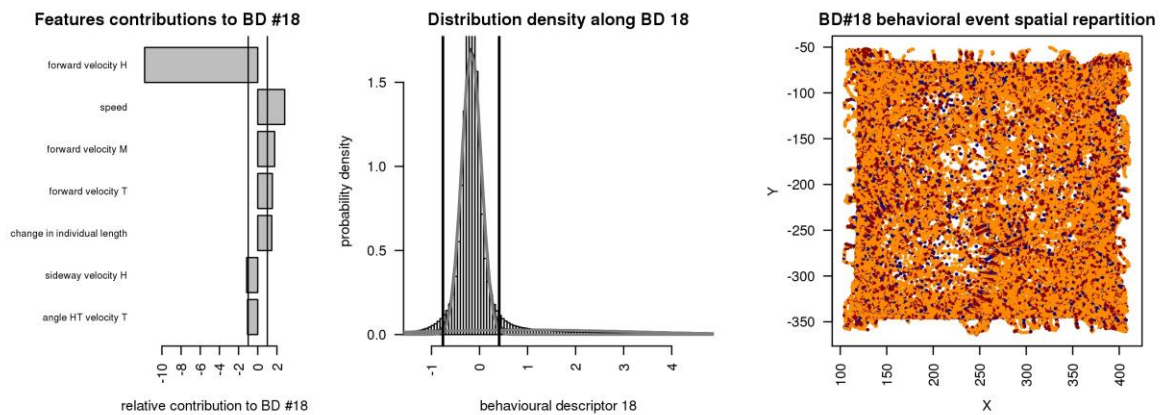


Figure annex 20: Interpretation figures for the BEC segregated on the 18th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: **“head pushed forward”**
- the BEC on the middle of B and orange in C: **“head synchronized with other body parts”**
- the BEC on the right of B and red in C: **“head pushed back”**

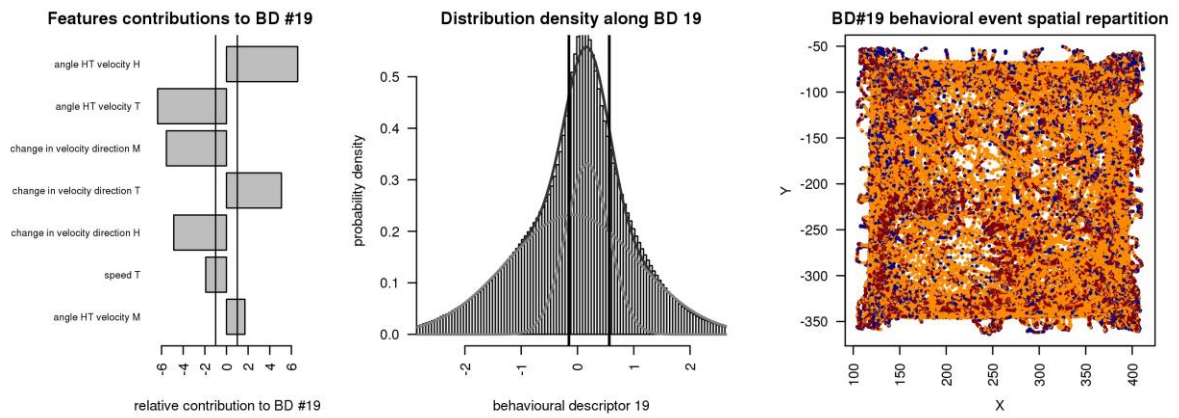


Figure annex 21: Interpretation figures for the BEC segregated on the 19th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“orients the movements of the head forward”*
- the BEC on the middle of B and orange in C: *“does not orient the movements of any body part forward”*
- the BEC on the right of B and red in C: *“orients the movements of the tail forward”*

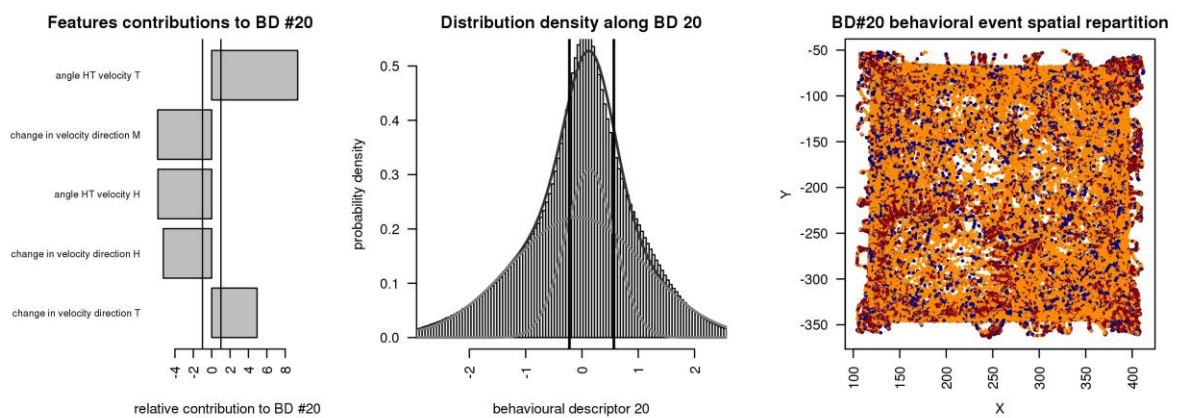


Figure annex 22: Interpretation figures for the BEC segregated on the 20th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“orients the movements of the head away from forward orientation”*
- the BEC on the middle of B and orange in C: *“does not orient the movements of any body part away from forward orientation”*
- the BEC on the right of B and red in C: *“orients the movements of the tail away from forward orientation”*

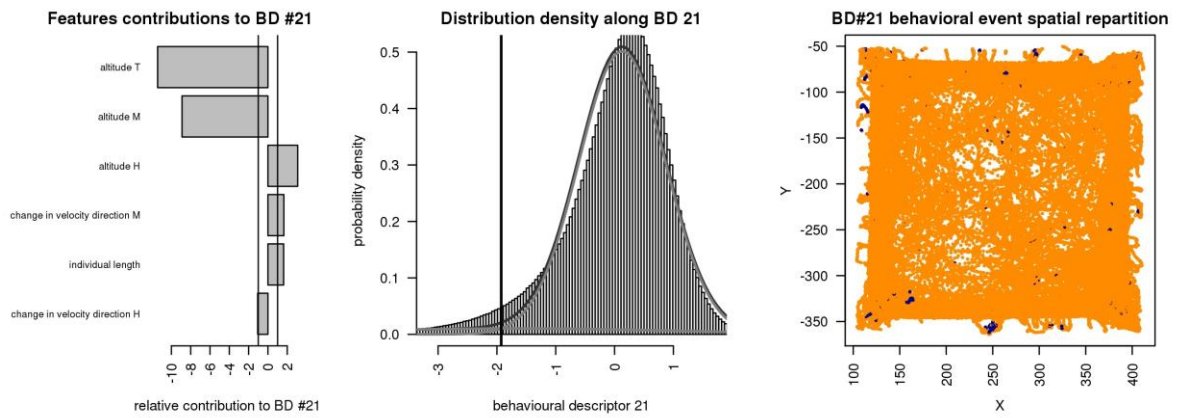


Figure annex 23: Interpretation figures for the BEC segregated on the 21th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “head-tail inversion on rearing”
- the BEC on the right of B and orange in C: “no head-tail inversion on rearing”

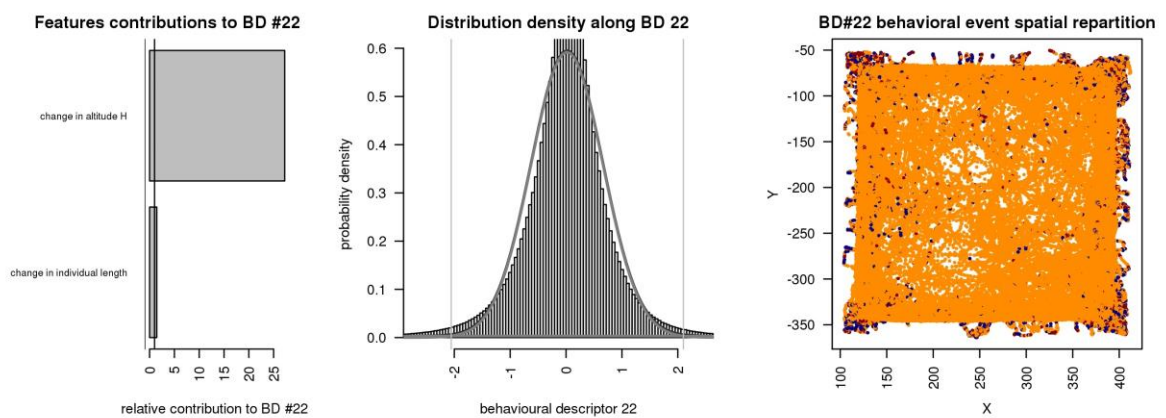


Figure annex 24: Interpretation figures for the BEC segregated on the 22th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “lowers the head”
- the BEC on the middle of B and orange in C: “stable head altitude”
- the BEC on the right of B and red in C: “raises the head”

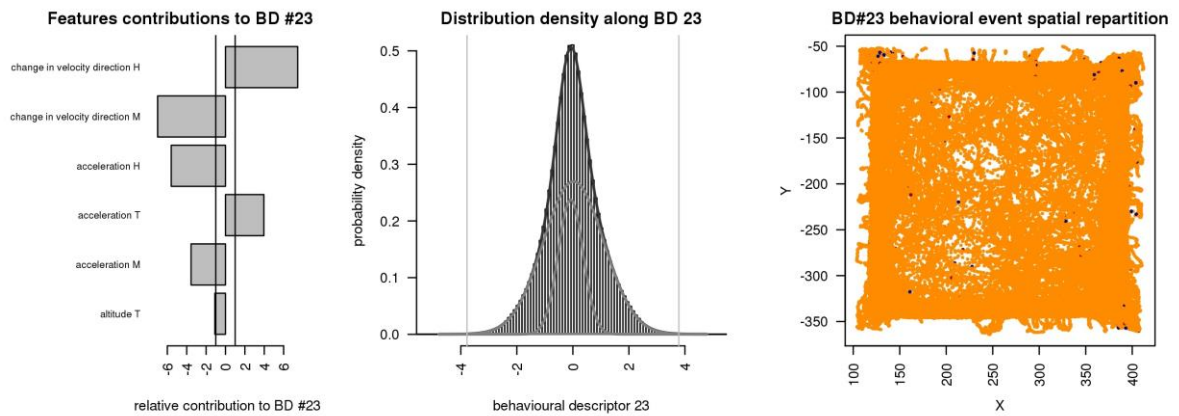


Figure annex 25: Interpretation figures for the BEC segregated on the 23th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “*anterior acceleration*”
- the BEC on the middle of B and orange in C: “*anterior stable speed*”
- the BEC on the right of B and red in C: “*anterior deceleration*”

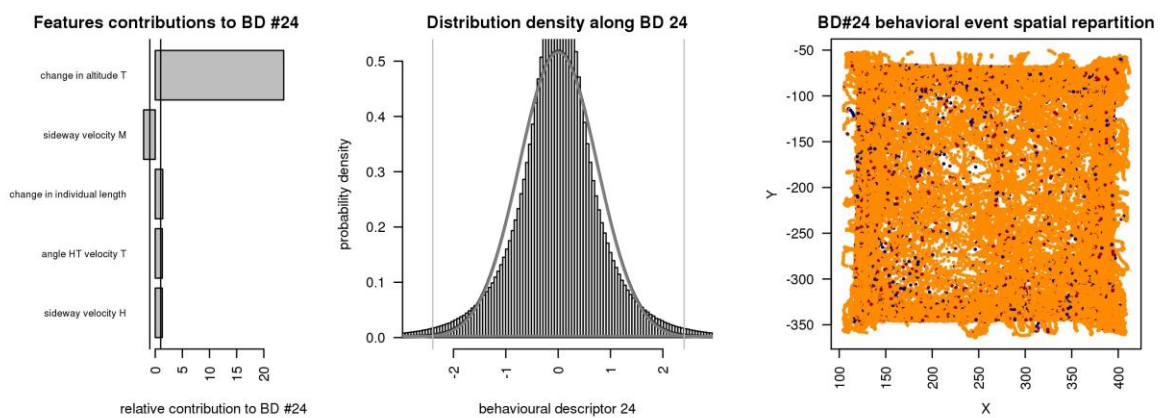


Figure annex 26: Interpretation figures for the BEC segregated on the 24th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “*lowers the tail*”
- the BEC on the middle of B and orange in C: “*stable tail altitude*”
- the BEC on the right of B and red in C: “*raises the tail*”



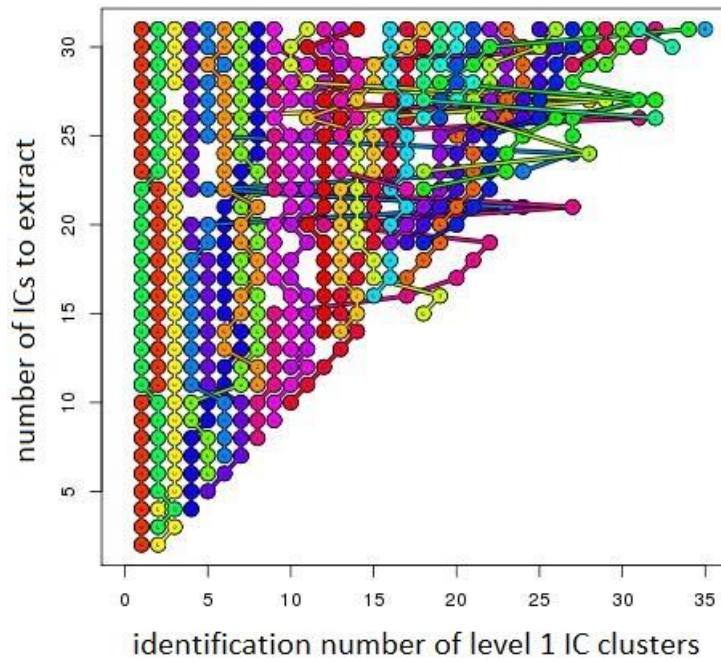


Figure annex 27: IC clusters over numbers of IC to extract for the BAP feature set of the single object exploration dataset. Each dot represent a level 1 cluster of IC, each line of dot of unique colour represent a level 2 cluster of IC.

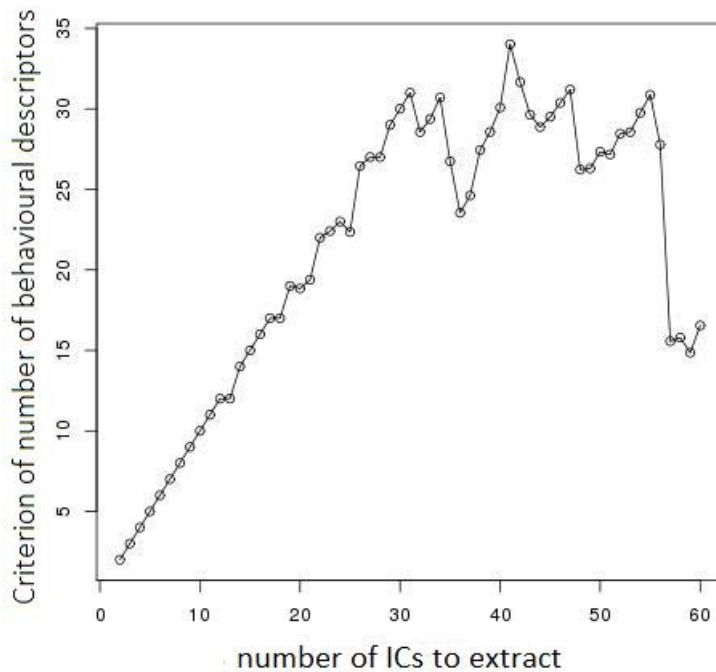


Figure annex 28: Criterion of number of behavioural descriptors in function of the number of IC for the BAP feature set of the single object exploration dataset. 31 behavioural descriptors kept for the BAP feature set of the single object exploration dataset.

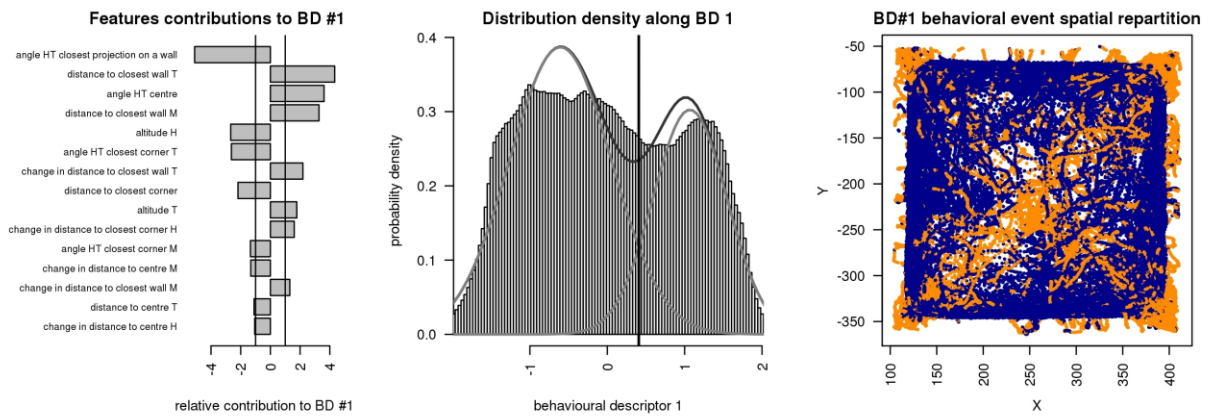


Figure annex 29: Interpretation figures for the BEC segregated on the 1st BD extracted from the BAP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“is not oriented toward the closest wall”*
- the BEC on the right of B and orange in C: *“is oriented toward the closest wall”*

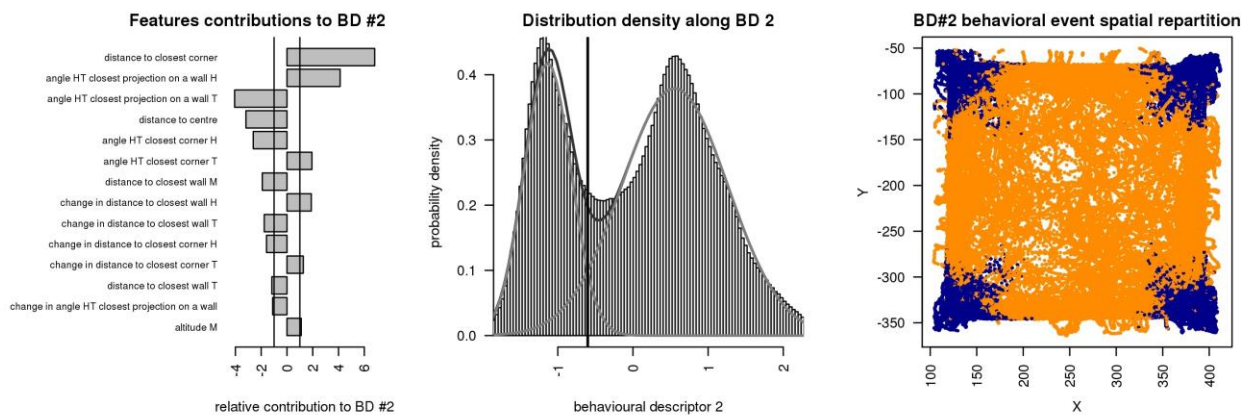


Figure annex 30: Interpretation figures for the BEC segregated on the 2nd BD extracted from the BAP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“is in a corner”*
- the BEC on the right of B and orange in C: *“is not in a corner”*

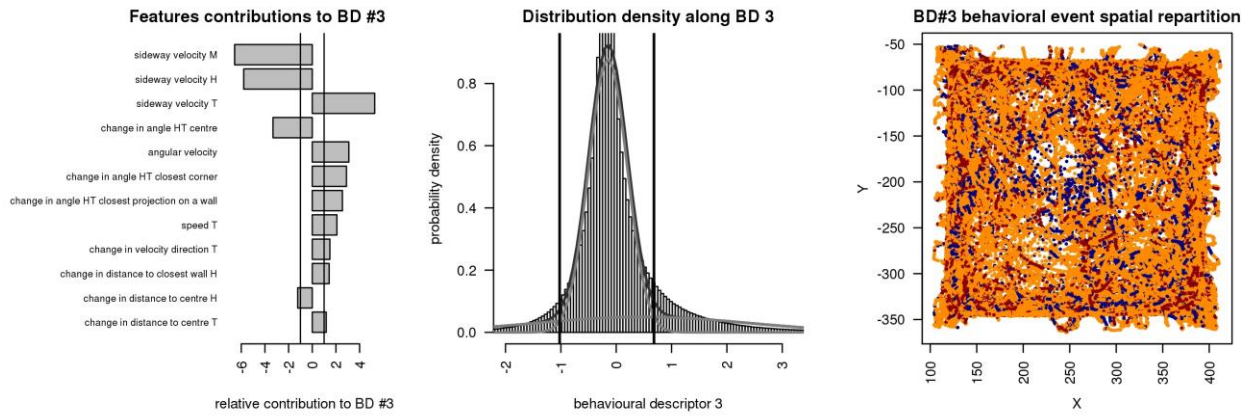


Figure annex 31: Interpretation figures for the BEC segregated on the 3rd BD extracted from the BAP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“orients away from the centre”*
- the BEC on the middle of B and orange in C: *“stable arena related orientation”*
- the BEC on the right of B and red in C: *“orients toward the centre”*

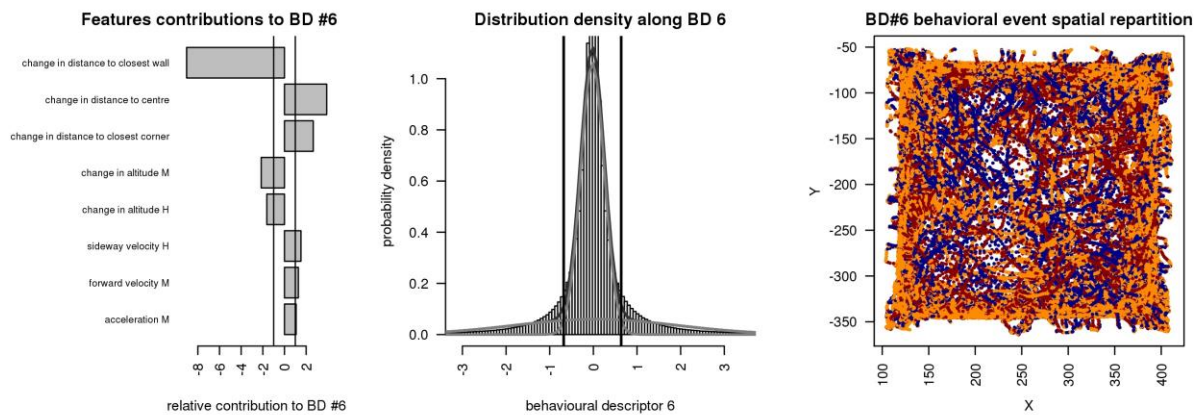


Figure annex 32: Interpretation figures for the BEC segregated on the 6th BD extracted from the BAP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“goes toward the wall”*
- the BEC on the middle of B and orange in C: *“stable distance to the wall”*
- the BEC on the right of B and red in C: *“goes away from the wall”*

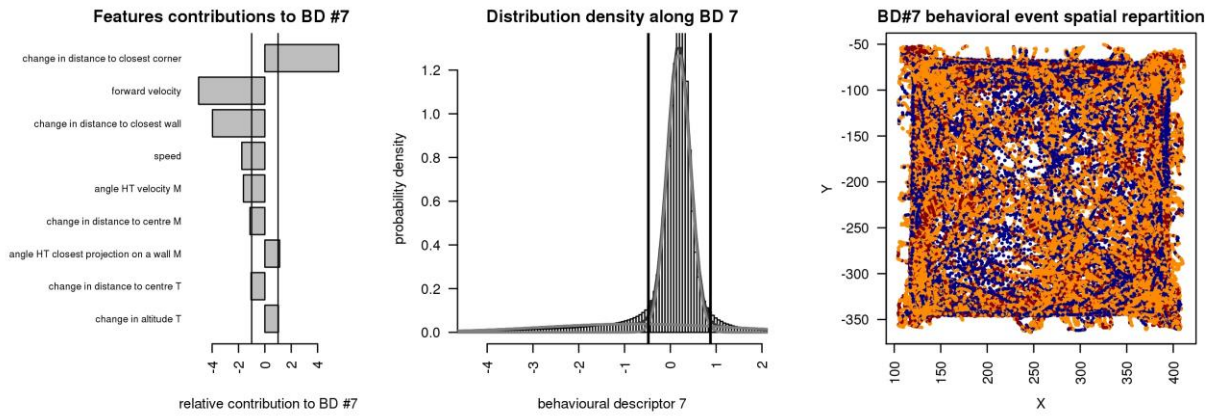


Figure annex 33: Interpretation figures for the BEC segregated on the 7th BD extracted from the BAP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “goes forward toward the corner”
- the BEC on the middle of B and orange in C: “does not progress with the corner in front”
- the BEC on the right of B and red in C: “goes backward away from the corner”

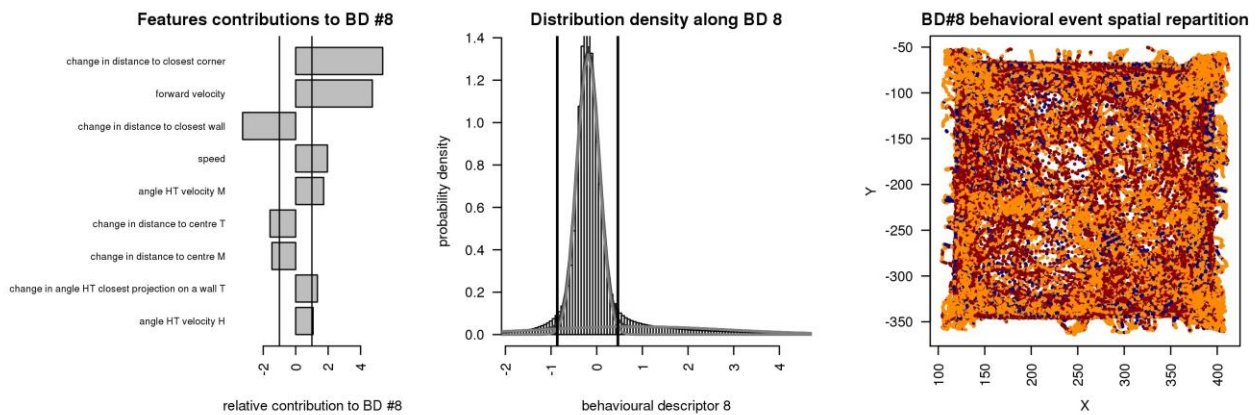


Figure annex 34: Interpretation figures for the BEC segregated on the 8th BD extracted from the BAP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “goes backward toward the corner”
- the BEC on the middle of B and orange in C: “does not progress with the corner in the back”
- the BEC on the right of B and red in C: “goes forward away from the corner”



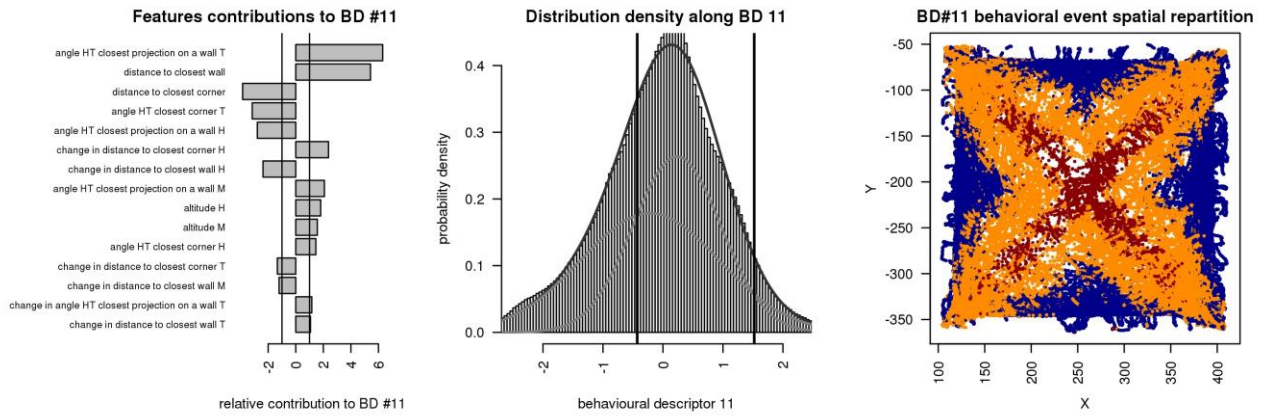


Figure annex 35: Interpretation figures for the BEC segregated on the 11th BD extracted from the BAP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“is in the near wall area”*
- the BEC on the middle of B and orange in C: *“is in the corner or intermediate area”*
- the BEC on the right of B and red in C: *“is in the middle diagonal cross”*

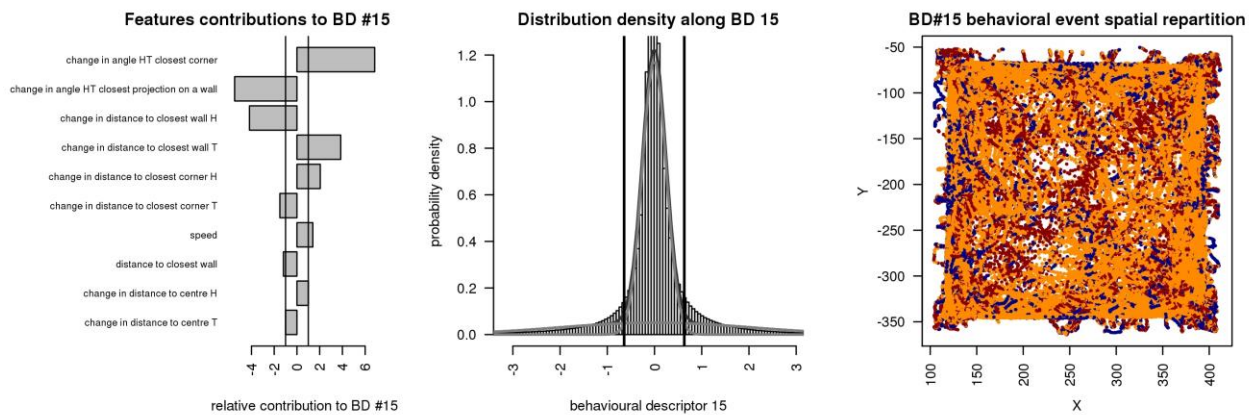


Figure annex 36: Interpretation figures for the BEC segregated on the 15th BD extracted from the BAP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“orients toward the corner”*
- the BEC on the middle of B and orange in C: *“stable wall-corner orientation”*
- the BEC on the right of B and red in C: *“orients toward the wall”*

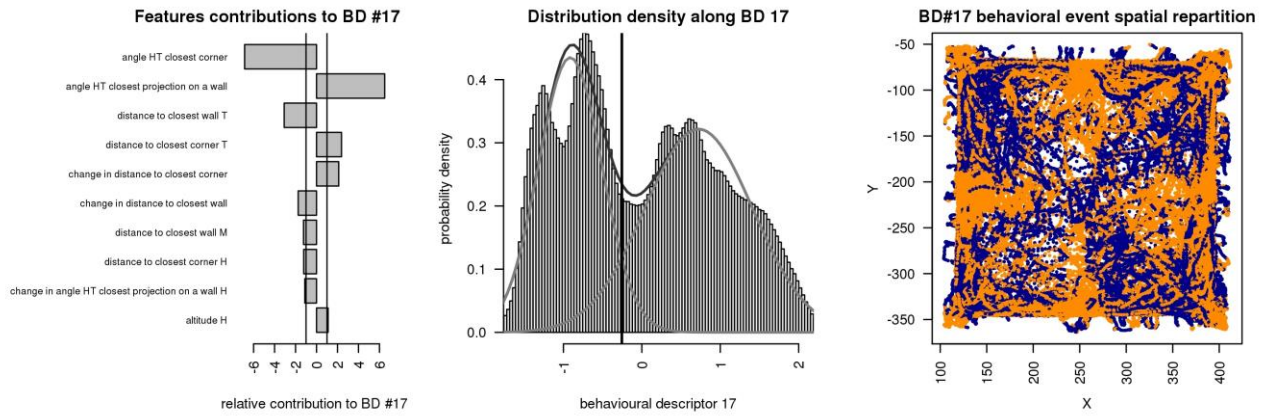


Figure annex 37: Interpretation figures for the BEC segregated on the 17th BD extracted from the BAP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“is oriented toward the wall rather than the corner”*
- the BEC on the right of B and orange in C: *“is oriented toward the corner rather than the wall”*

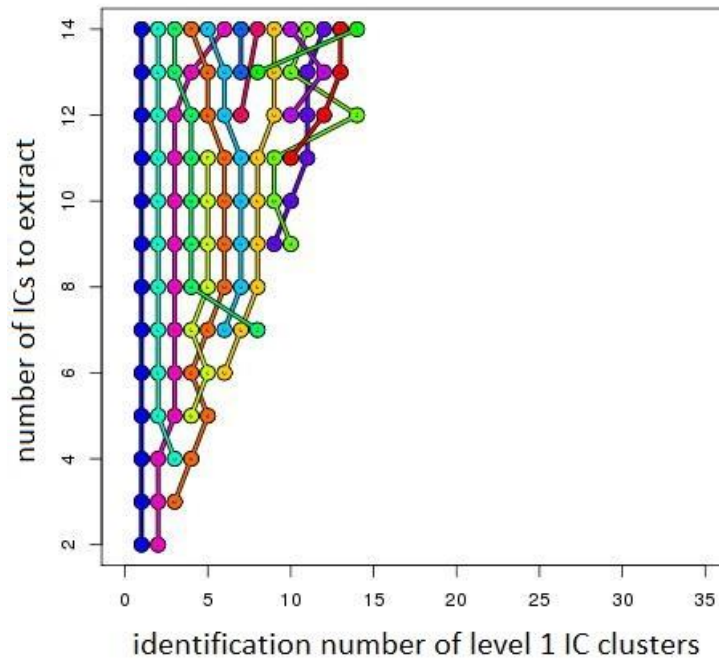


Figure annex 38: IC clusters over numbers of IC to extract for the BAOP feature set of the single object exploration dataset. Each dot represent a level 1 cluster of IC, each line of dot of unique colour represent a level 2 cluster of IC.

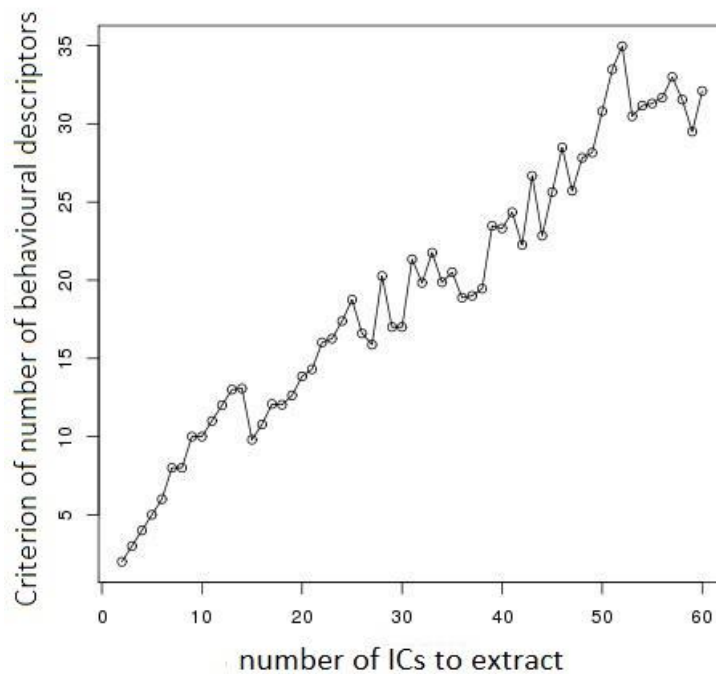


Figure annex 39: Criterion of number of behavioural descriptors in function of the number of IC for the BAOP feature set of the single object exploration dataset. 14 behavioural descriptors kept for the BAOP feature set of the single object exploration dataset.

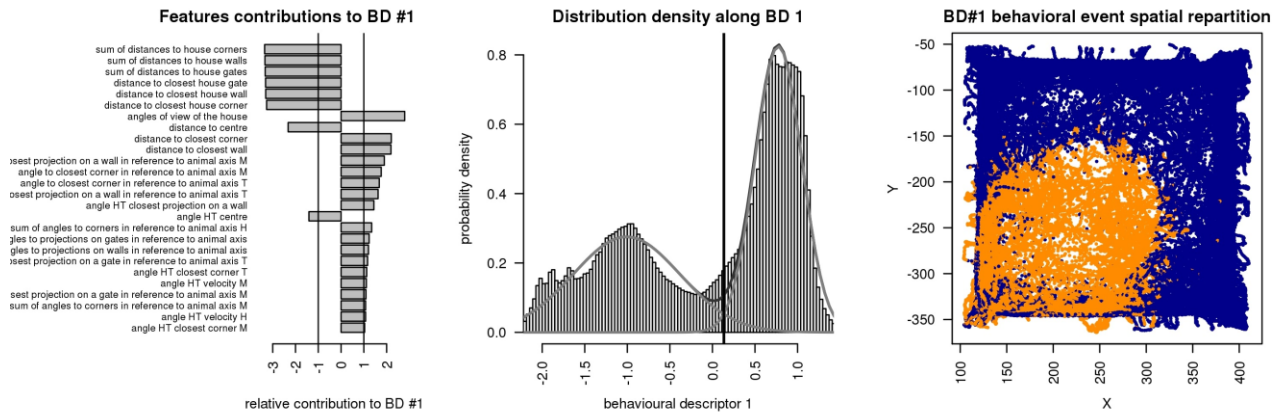


Figure annex 40: Interpretation figures for the BEC segregated on the 1st BD extracted from the BAOP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“is close to or in the house”*
- the BEC on the right of B and orange in C: *“is far from the house”*

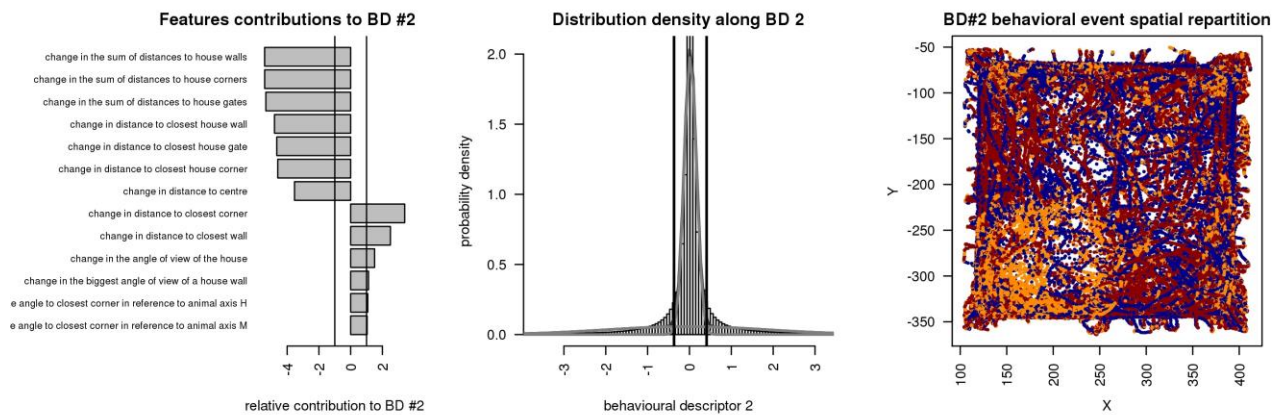


Figure annex 41: Interpretation figures for the BEC segregated on the 2nd BD extracted from the BAOP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“get away from the house”*
- the BEC on the middle of B and orange in C: *“stable distance from the house”*
- the BEC on the right of B and red in C: *“goes toward the house”*



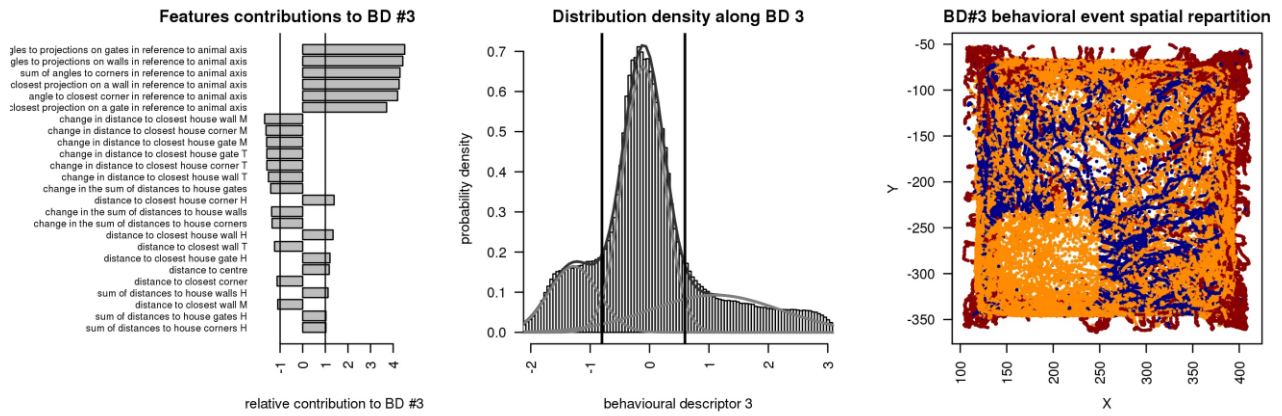


Figure annex 42: Interpretation figures for the BEC segregated on the 3rd BD extracted from the BAOP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“is oriented toward the house”*
- the BEC on the middle of B and orange in C: *“no special house related orientation”*
- the BEC on the right of B and red in C: *“is oriented away from the house”*

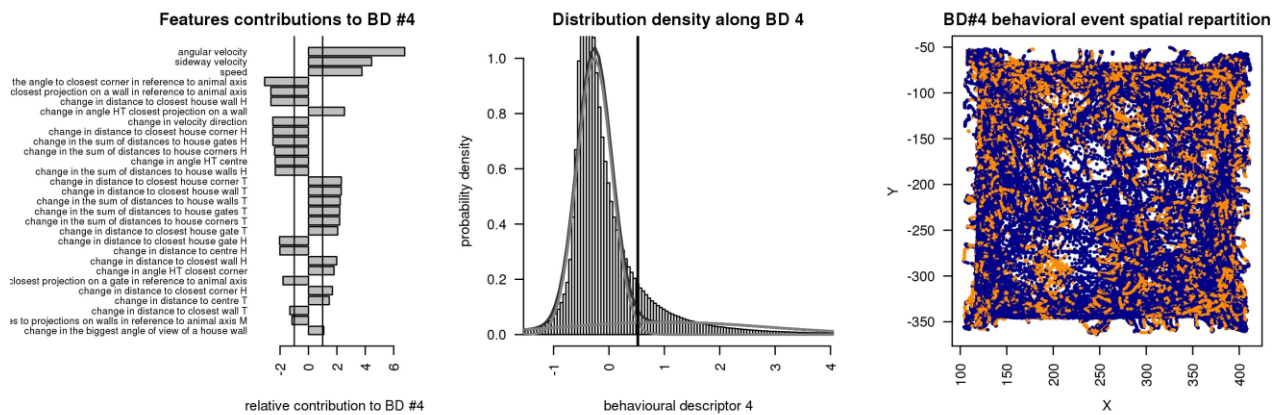


Figure annex 43: Interpretation figures for the BEC segregated on the 4th BD extracted from the BAOP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“does not orient away from the house”*
- the BEC on the right of B and orange in C: *“orients away from the house”*

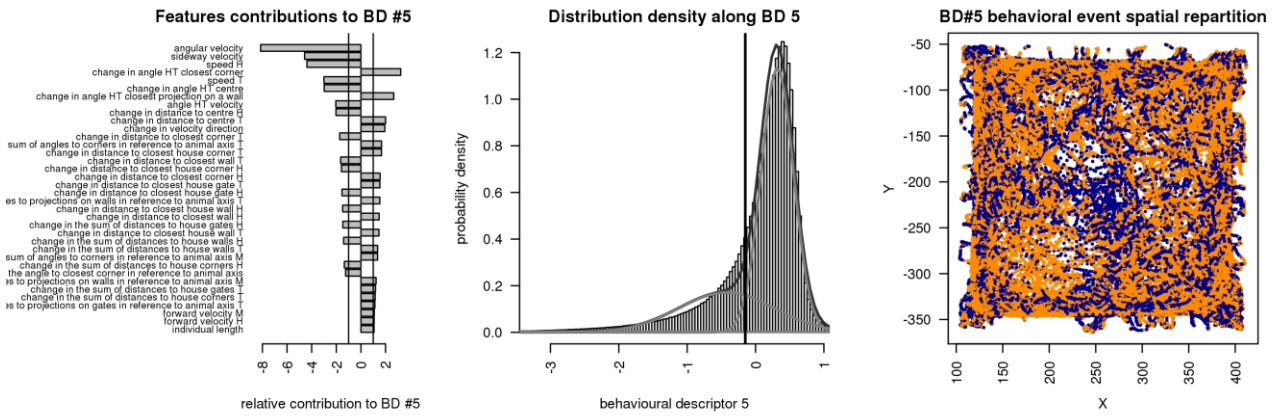


Figure annex 44: Interpretation figures for the BEC segregated on the 5th BD extracted from the BAOP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “*orients toward the house*”
- the BEC on the right of B and orange in C: “*does not orient toward the house*”

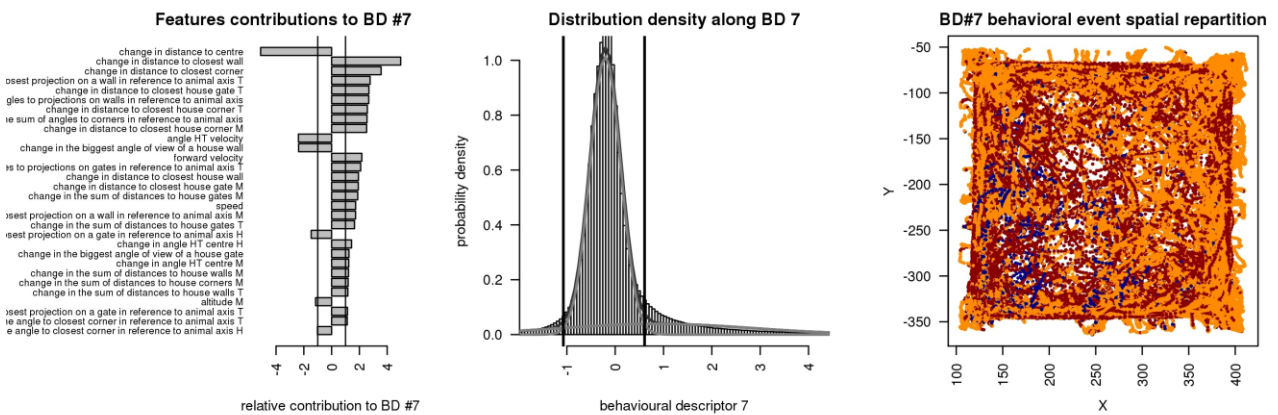


Figure annex 45: Interpretation figures for the BEC segregated on the 7th BD extracted from the BAOP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “*goes backward toward the house*”
- the BEC on the middle of B and orange in C: “*does not progress with house in the back*”
- the BEC on the right of B and red in C: “*goes forward away from the house*”

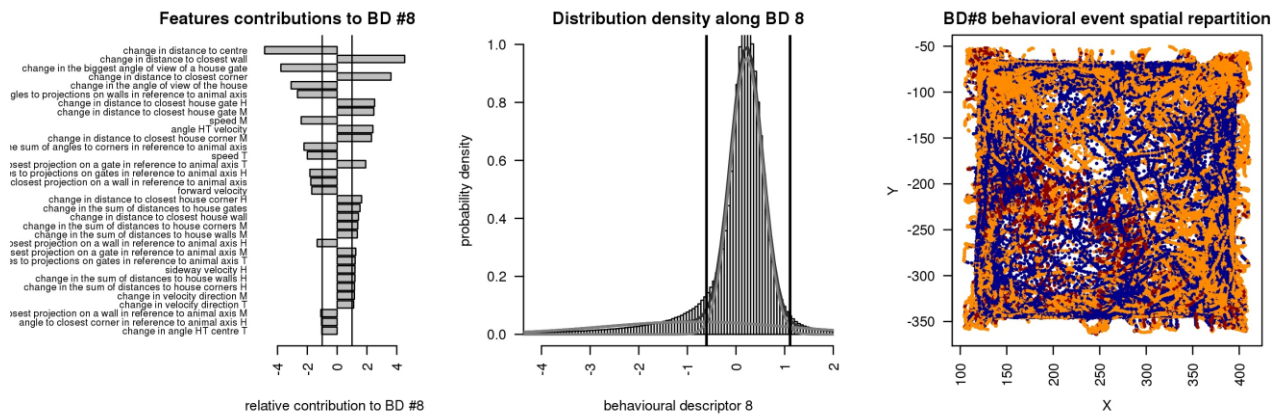


Figure annex 46: Interpretation figures for the BEC segregated on the 8th BD extracted from the BAOP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: **“goes forward toward the house”**
- the BEC on the middle of B and orange in C: **“does not progress with house in front”**
- the BEC on the right of B and red in C: **“goes backward away from the house”**

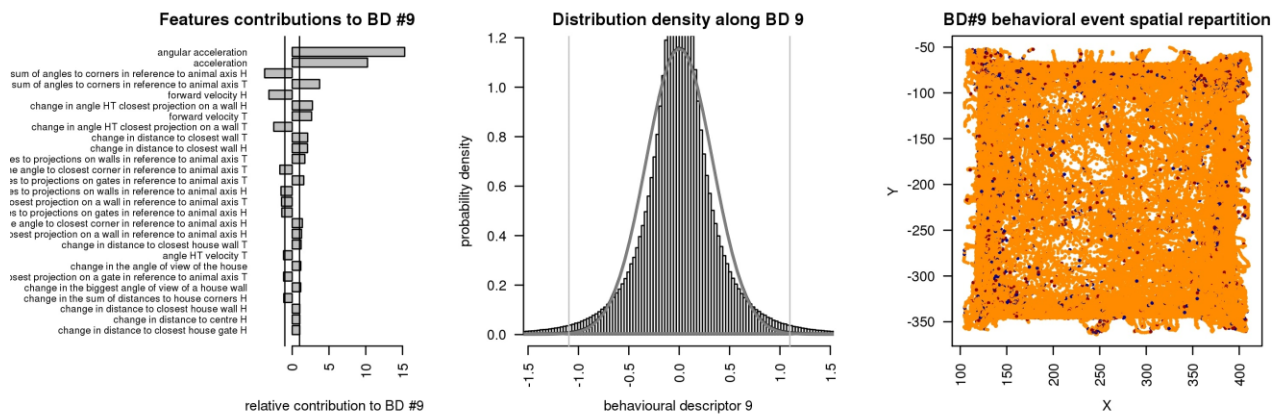


Figure annex 47: Interpretation figures for the BEC segregated on the 9th BD extracted from the BAOP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: **“angular decelerates”**
- the BEC on the middle of B and orange in C: **“stable angular speed”**
- the BEC on the right of B and red in C: **“angular accelerates”**



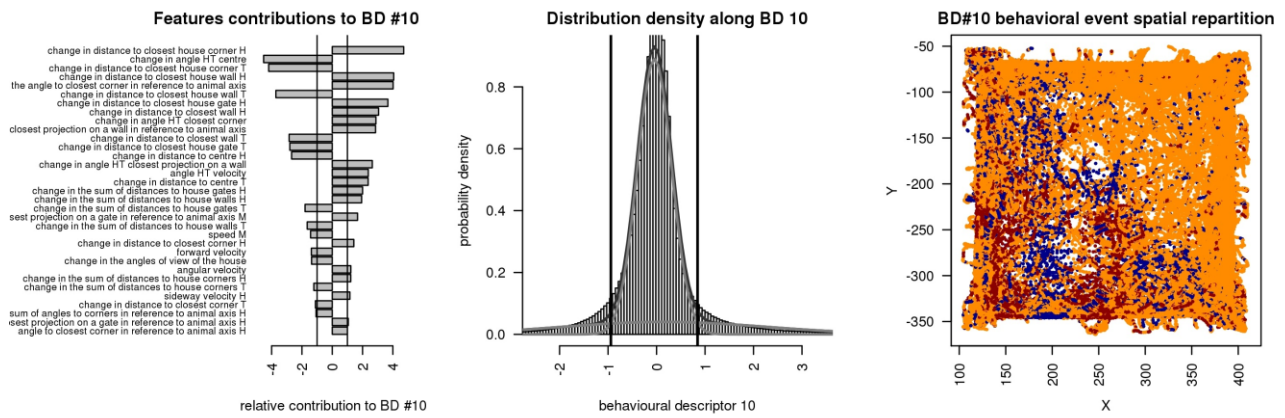


Figure annex 48: Interpretation figures for the BEC segregated on the 10th BD extracted from the BAOP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “*orients toward the house*”
- the BEC on the middle of B and orange in C: “*stable house related orientation*”
- the BEC on the right of B and red in C: “*orients away from the house*”

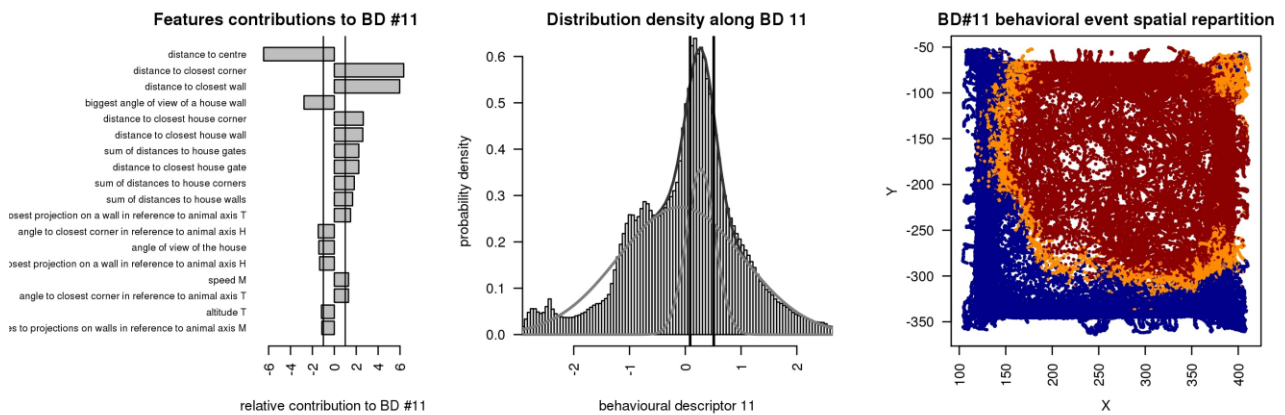


Figure annex 49: Interpretation figures for the BEC segregated on the 11th BD extracted from the BAOP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “*is not in the far from house area*”
- the BEC on the middle of B and orange in C: “*is in intermediate house related area*”
- the BEC on the right of B and red in C: “*is in the far from house area*”

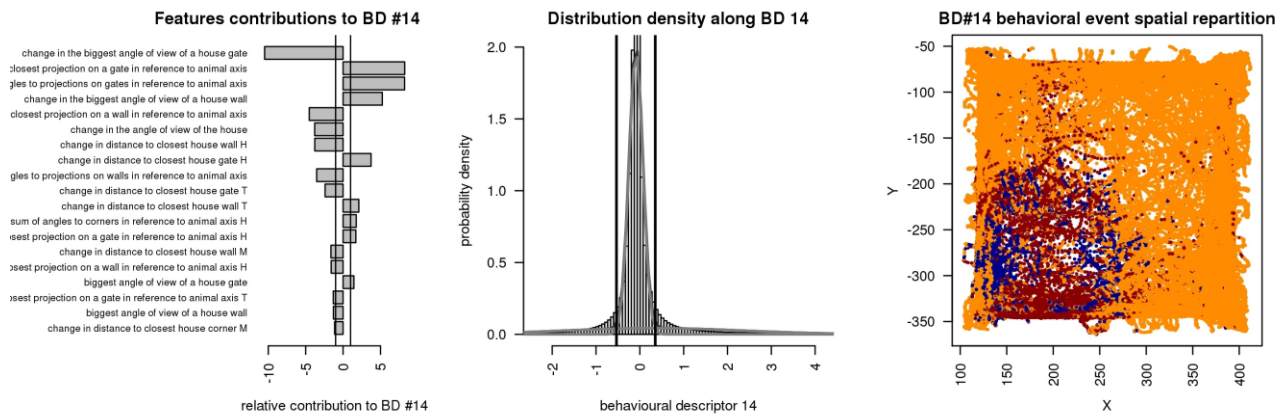


Figure annex 50: Interpretation figures for the BEC segregated on the 14th BD extracted from the BAOP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “goes in direction from a house wall to an open side”
- the BEC on the middle of B and orange in C: “stable house open side to wall closeness”
- the BEC on the right of B and red in C: “goes in direction from a house open side to a wall”

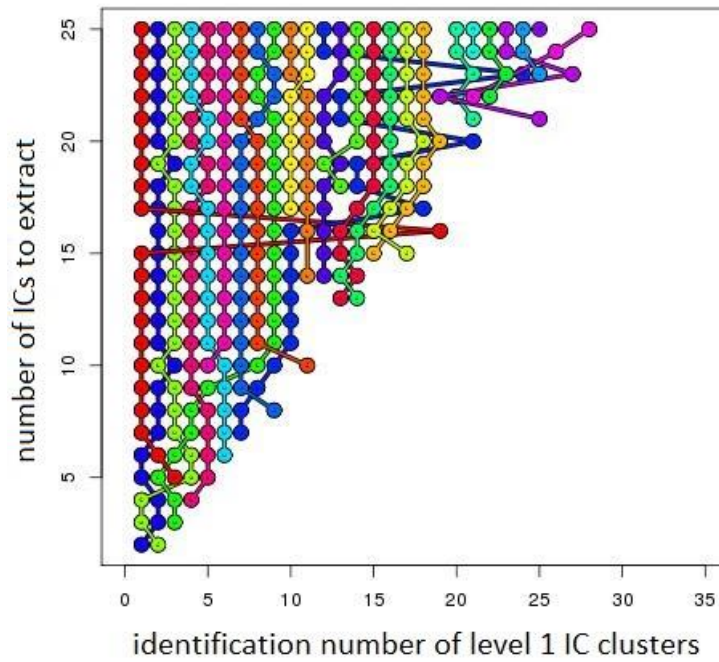


Figure annex 51: IC clusters over numbers of IC to extract for the BP feature set of the *long-term group monitoring*. Each dot represent a level 1 cluster of IC, each line of dot of unique colour represent a level 2 cluster of IC.

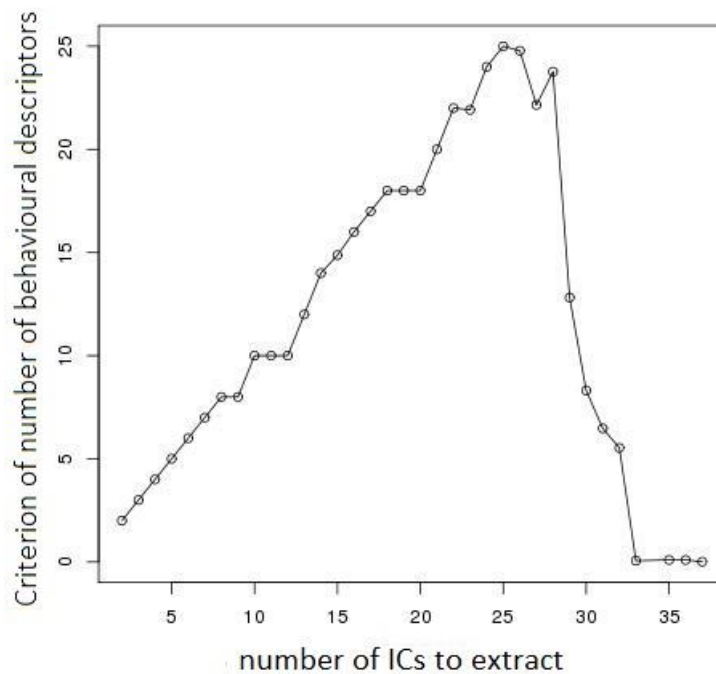


Figure annex 52: Criterion of number of behavioural descriptors in function of the number of IC for the BP feature set of the single object exploration dataset. 25 behavioural descriptors kept for the BP feature set of the *long-term group monitoring*.

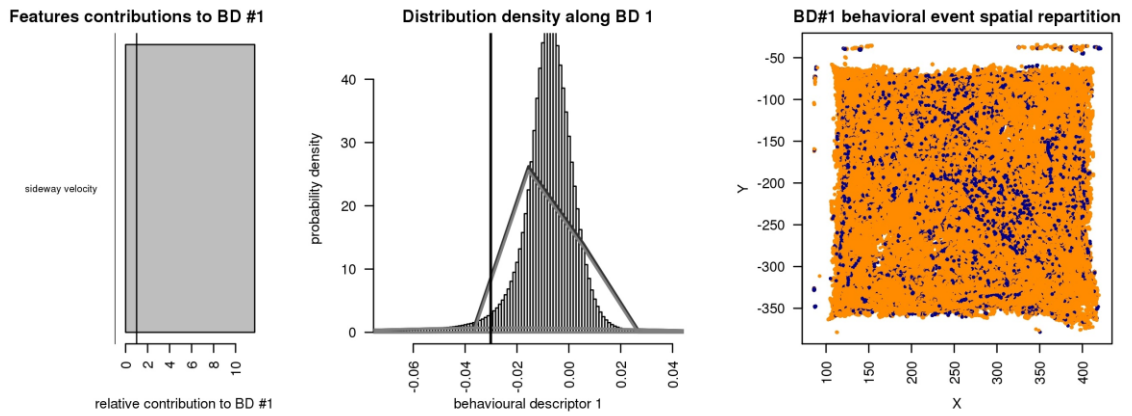


Figure annex 53: Interpretation figures for the BEC segregated on the 1st BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“the movements of all body parts is oriented forward”*
- the BEC on the right of B and orange in C: *“the movements of some body parts is not oriented forward”*

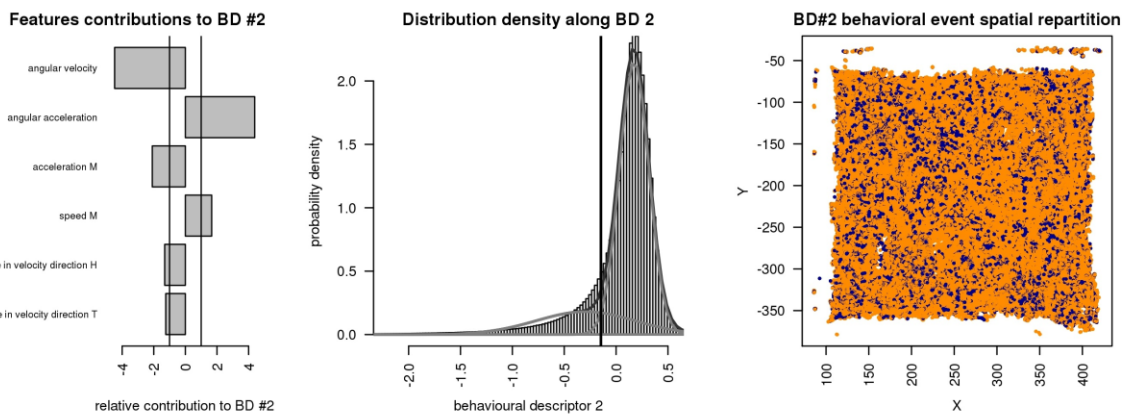


Figure annex 54: Interpretation figures for the BEC segregated on the 2nd BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“turns decelerating”*
- the BEC on the right of B and orange in C: *“does not turn in deceleration”*

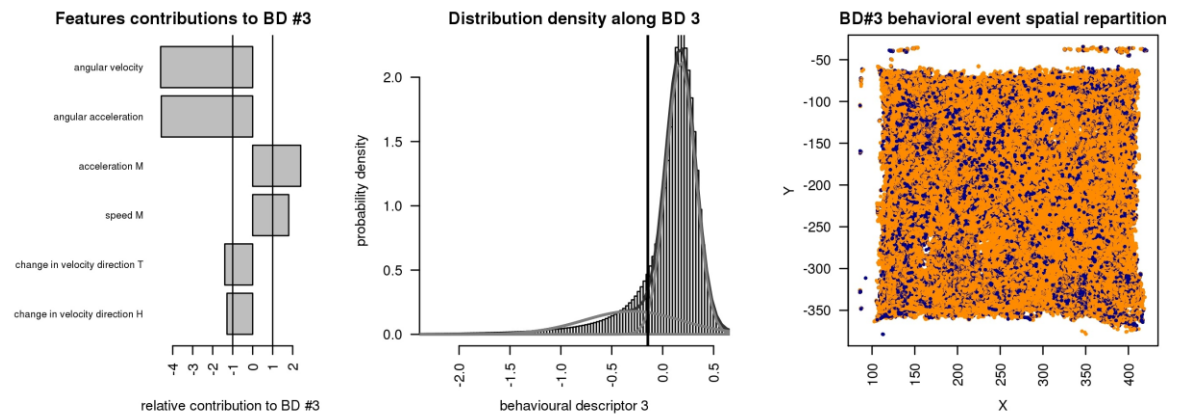


Figure annex 55: Interpretation figures for the BEC segregated on the 3rd BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“turns accelerating”*
- the BEC on the middle of B and orange in C: *“does not turn in acceleration”*

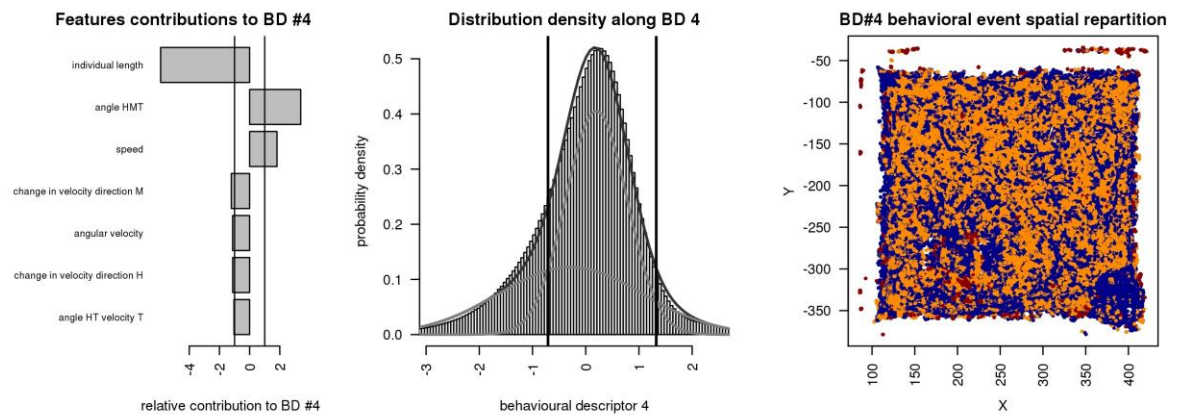


Figure annex 56: Interpretation figures for the BEC segregated on the 4th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“is extended”*
- the BEC on the middle of B and orange in C: *“normal extension”*
- the BEC on the right of B and red in C: *“is short on x-y axis”*



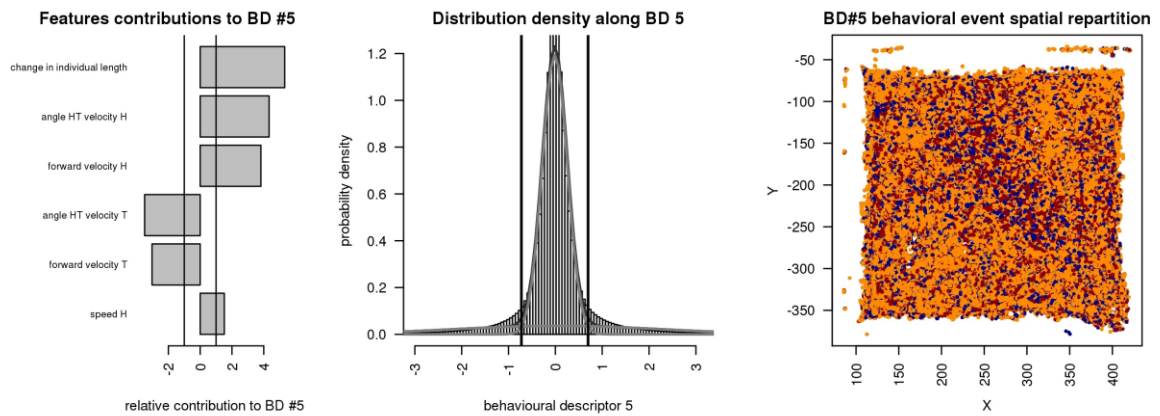


Figure annex 57: Interpretation figures for the BEC segregated on the 5th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “contracts”
- the BEC on the middle of B and orange in C: “stable extension”
- the BEC on the right of B and red in C: “extends”

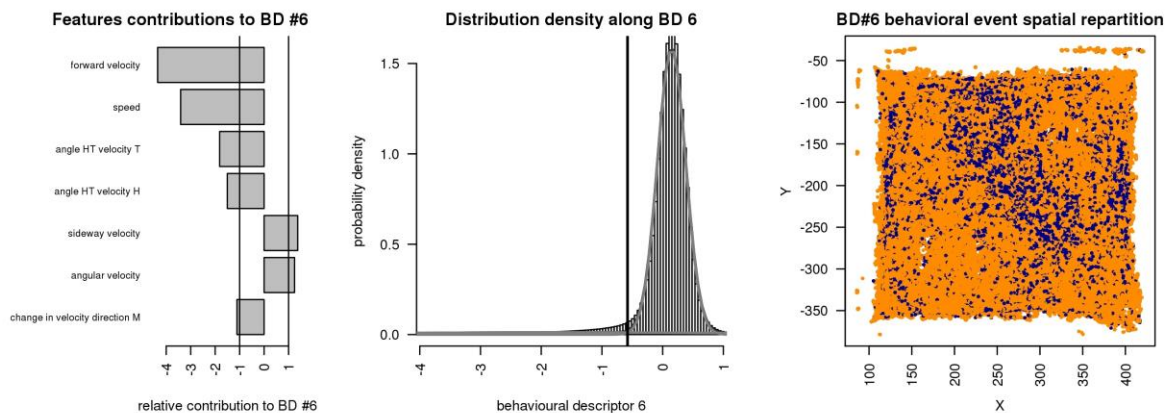


Figure annex 58: Interpretation figures for the BEC segregated on the 6th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “moves forward”
- the BEC on the right of B and orange in C: “does not move forward”

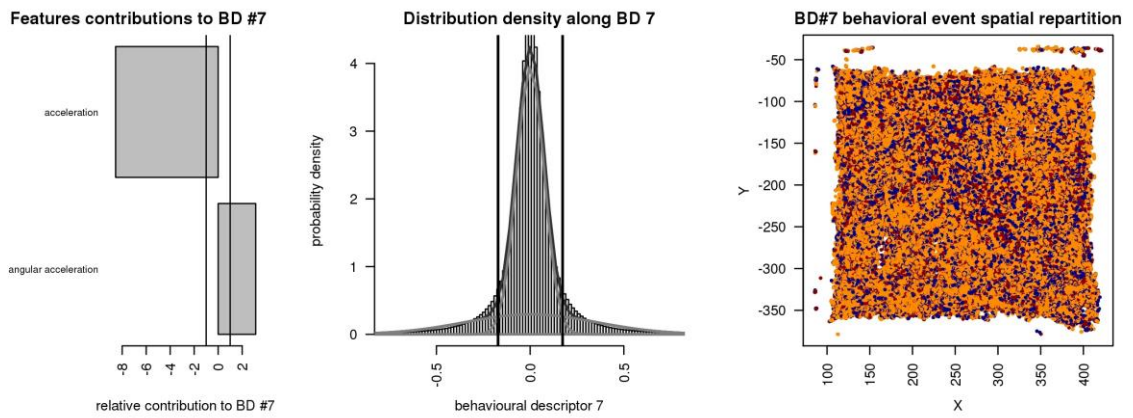


Figure annex 59: Interpretation figures for the BEC segregated on the 7th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “accelerates”
- the BEC on the middle of B and orange in C: “stable speed”
- the BEC on the right of B and red in C: “stable speed”

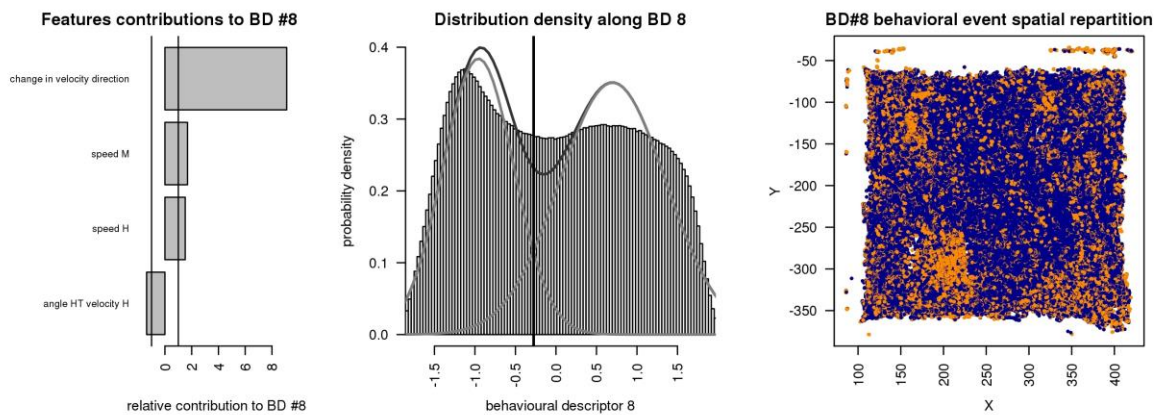


Figure annex 60: Interpretation figures for the BEC segregated on the 8th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “is not deviating”
- the BEC on the right of B and orange in C: “is deviating”

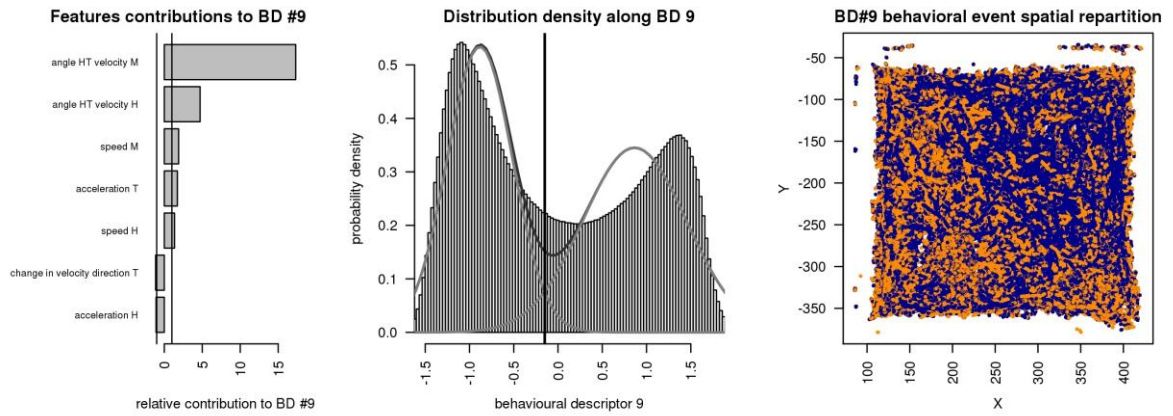


Figure annex 61: Interpretation figures for the BEC segregated on the 9th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“does not move backward”*
- the BEC on the right of B and orange in C: *“moves backward”*

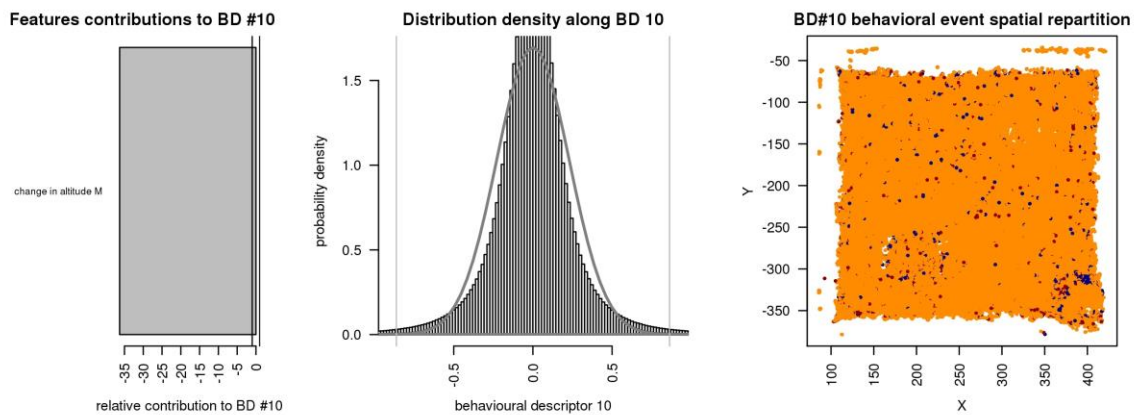


Figure annex 62: Interpretation figures for the BEC segregated on the 10th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“rears up”*
- the BEC on the middle of B and orange in C: *“stable altitude”*
- the BEC on the right of B and red in C: *“rears down”*

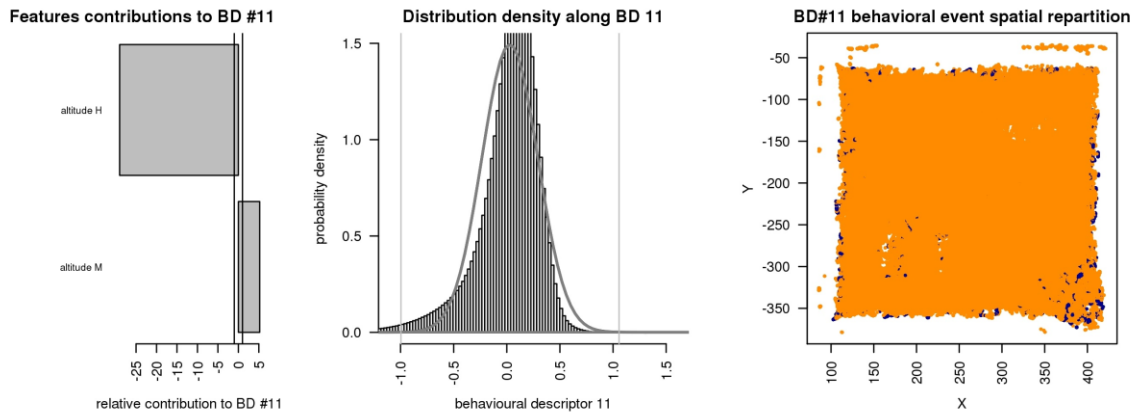


Figure annex 63: Interpretation figures for the BEC segregated on the 11th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“is reared”*
- the BEC on the middle of B and orange in C: *“is not reared”*
- the BEC on the right of B and red in C: *“has the head down”*

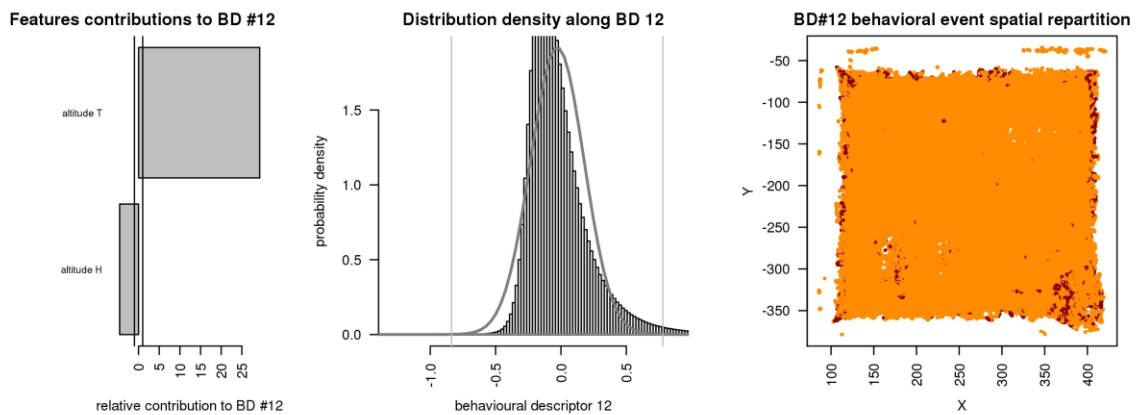


Figure annex 64: Interpretation figures for the BEC segregated on the 12th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“has the tail down”*
- the BEC on the middle of B and orange in C: *“no head-tail inversion on rearing”*
- the BEC on the right of B and red in C: *“head-tail inversion on rearing”*

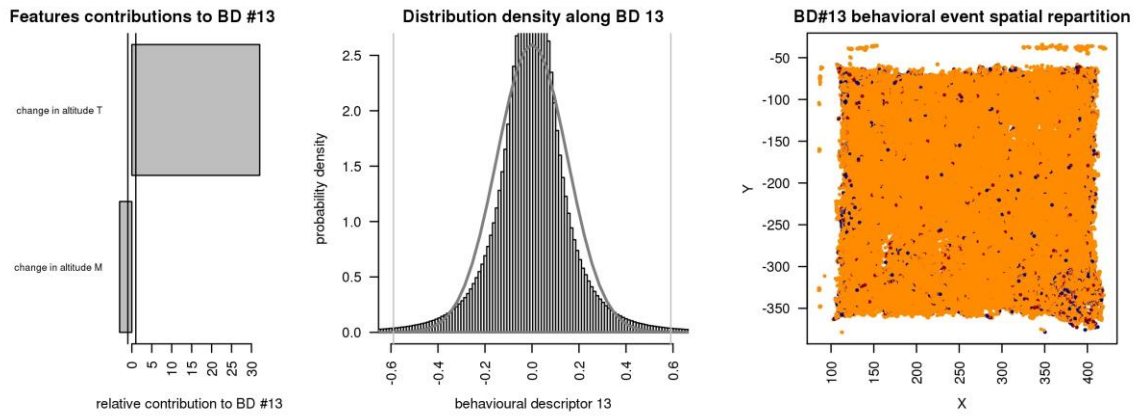


Figure annex 65: Interpretation figures for the BEC segregated on the 13th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“lowers the tail”*
- the BEC on the middle of B and orange in C: *“stable tail altitude”*
- the BEC on the right of B and red in C: *“raises the tail”*

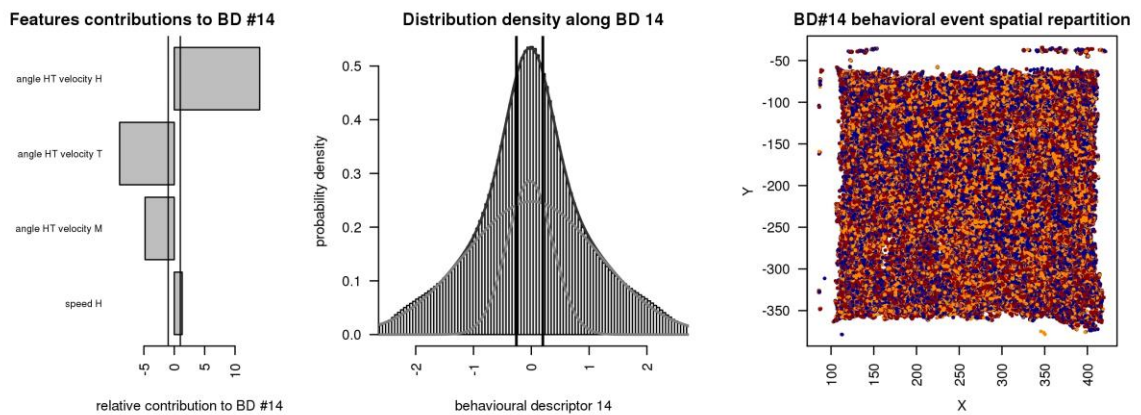


Figure annex 66: Interpretation figures for the BEC segregated on the 14th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“the movements of the tail is not oriented forward but the one of the head is”*
- the BEC on the middle of B and orange in C: *“head and tail movements are each as oriented forward than the other”*
- the BEC on the right of B and red in C: *“the movements of the head is not oriented forward but the one of the tail is”*



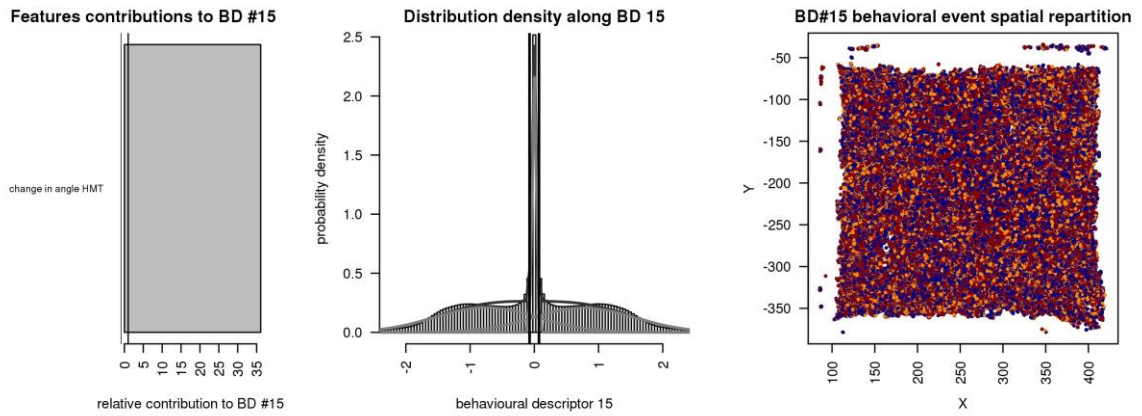


Figure annex 67: Interpretation figures for the BEC segregated on the 15th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “bends”
- the BEC on the middle of B and orange in C: “stable bending”
- the BEC on the right of B and red in C: “unbends”

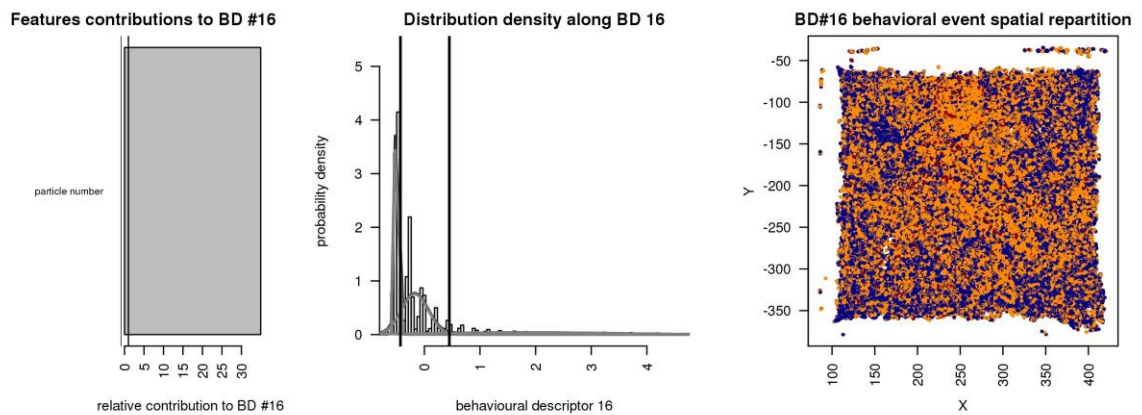


Figure annex 68: Interpretation figures for the BEC segregated on the 16th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “no bedding movements”
- the BEC on the middle of B and orange in C: “bedding movements”
- the BEC on the right of B and red in C: “some individuals dig”

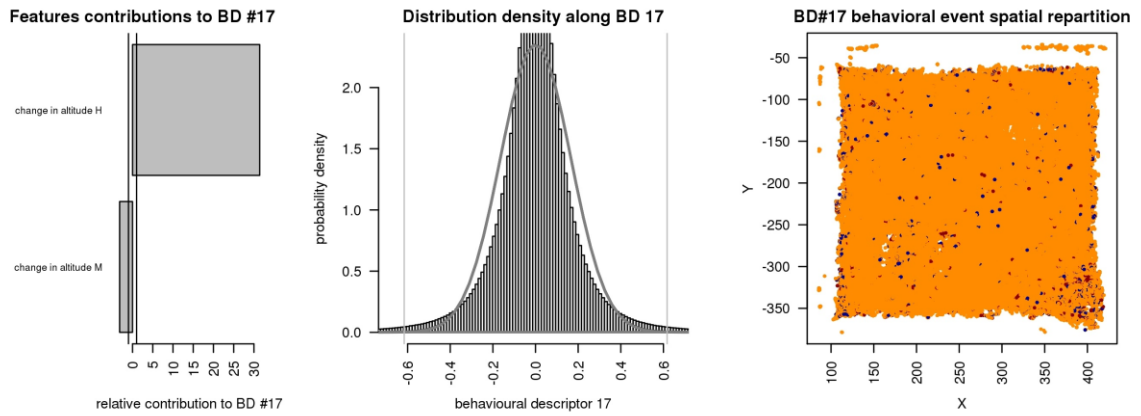


Figure annex 69: Interpretation figures for the BEC segregated on the 17th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “lowers the head”
- the BEC on the middle of B and orange in C: “stable head altitude”
- the BEC on the right of B and red in C: “raises the head”

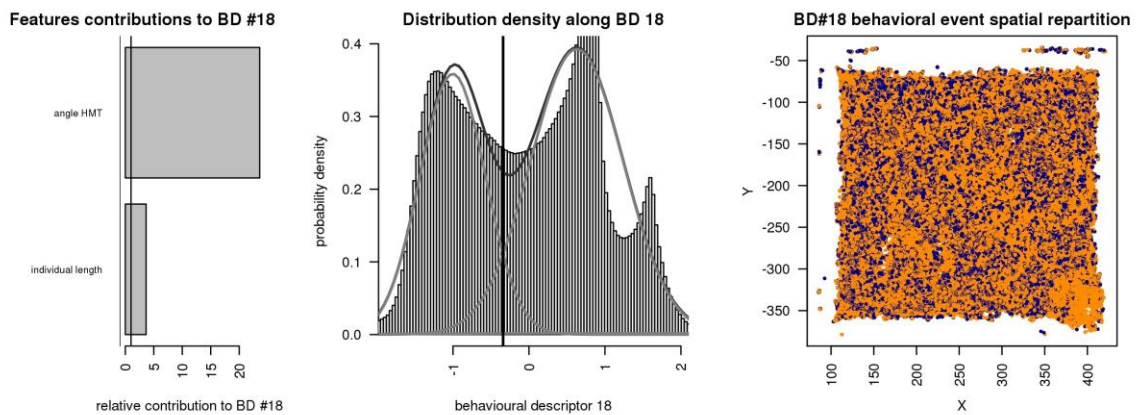


Figure annex 70: Interpretation figures for the BEC segregated on the 18th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “is bent”
- the BEC on the right of B and orange in C: “is not bent”

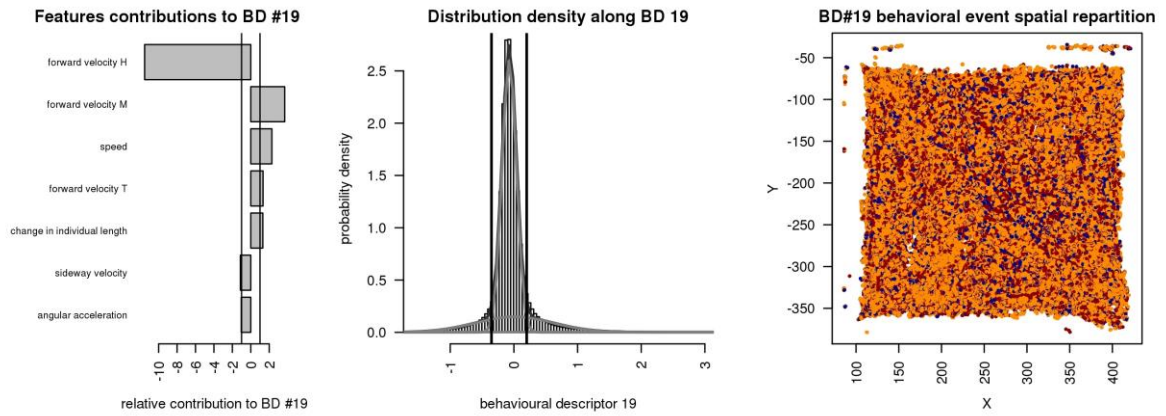


Figure annex 71: Interpretation figures for the BEC segregated on the 19th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: **“head pushed forward”**
- the BEC on the middle of B and orange in C: **“head synchronized with other body parts”**
- the BEC on the right of B and red in C: **“head pushed back”**

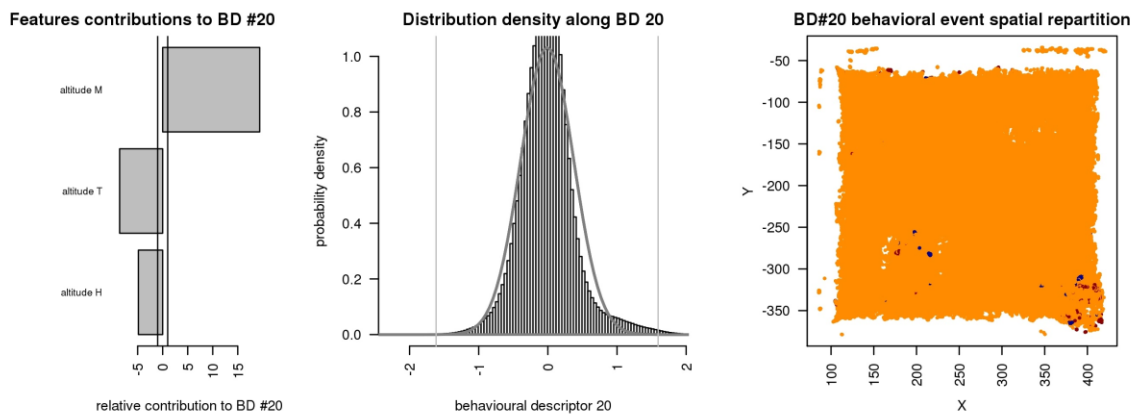


Figure annex 72: Interpretation figures for the BEC segregated on the 20th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: **“is arched in a U shape”**
- the BEC on the middle of B and orange in C: **“has the back straight”**
- the BEC on the right of B and red in C: **“has the back rounded”**



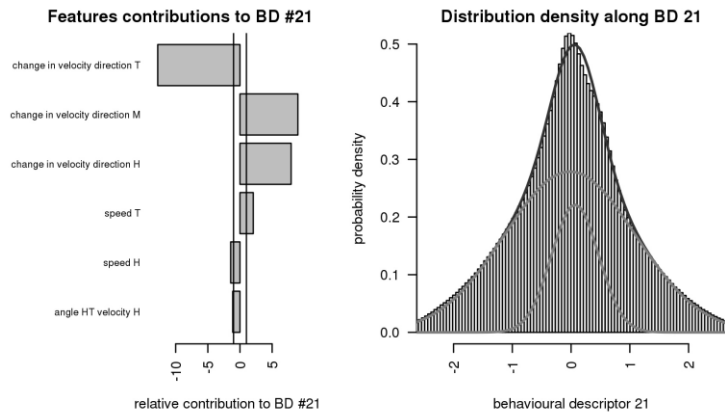


Figure annex 73: Interpretation figures for the BEC segregated on the 21th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM, no clusters are segregated.

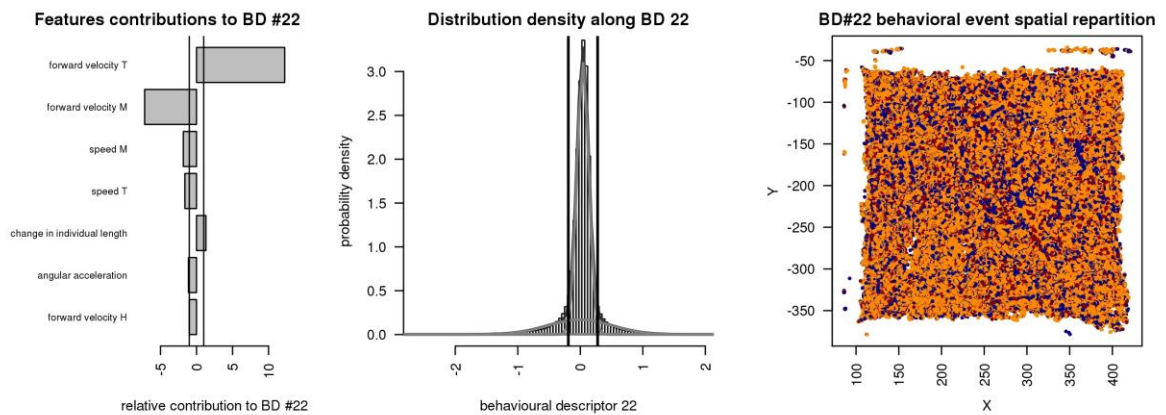


Figure annex 74: Interpretation figures for the BEC segregated on the 22th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: **“tail base pushed back”**
- the BEC on the middle of B and orange in C: **“tail base synchronized with other body parts”**
- the BEC on the right of B and red in C: **“tail base pushed forward”**

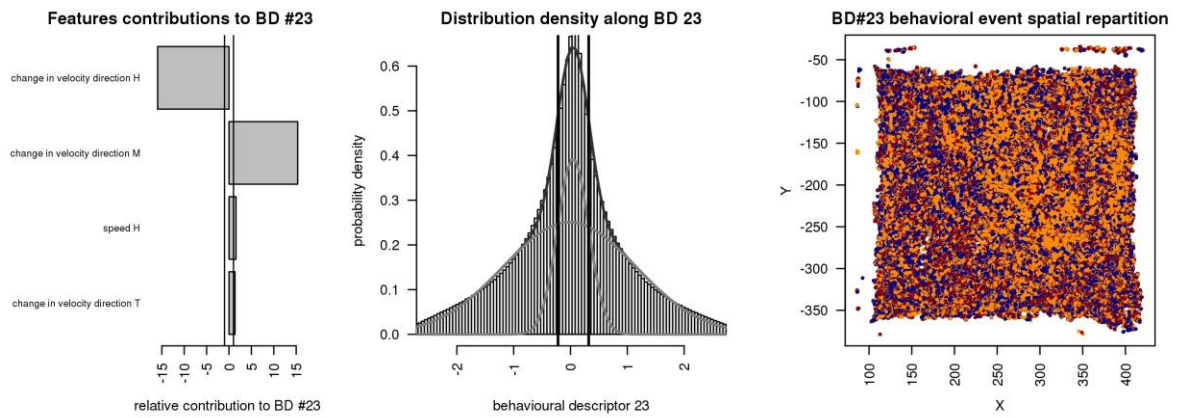


Figure annex 75: Interpretation figures for the BEC segregated on the 23th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: **“head deviation”**
- the BEC on the middle of B and orange in C: **“no partial deviation”**
- the BEC on the right of B and red in C: **“centre of mass deviation”**

#### anterior deceleration / anterior stable speed / anterior acceleration

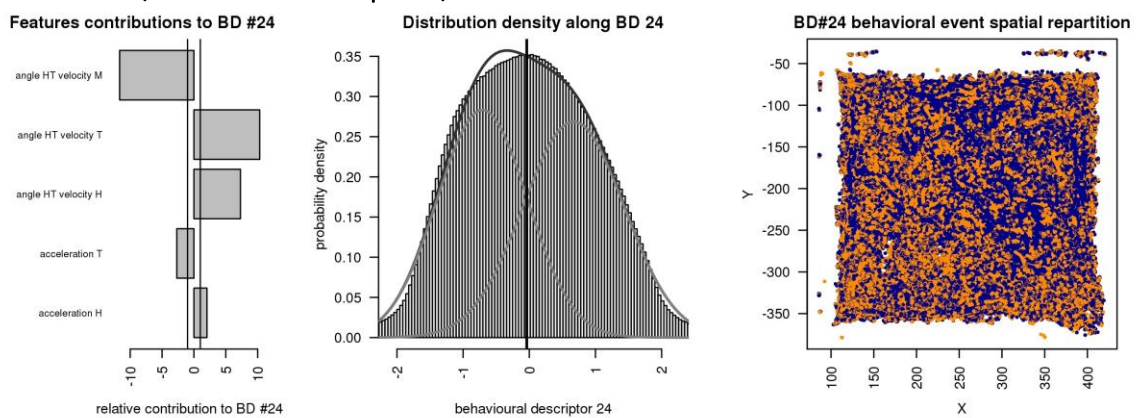


Figure annex 76: Interpretation figures for the BEC segregated on the 24th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: **“the movement of the centre of mass is not oriented forward but the one of the head and the tail are”**
- the BEC on the right of B and orange in C: **“the movements of the head and the tail are not oriented forward but the one of the centre of mass is”**

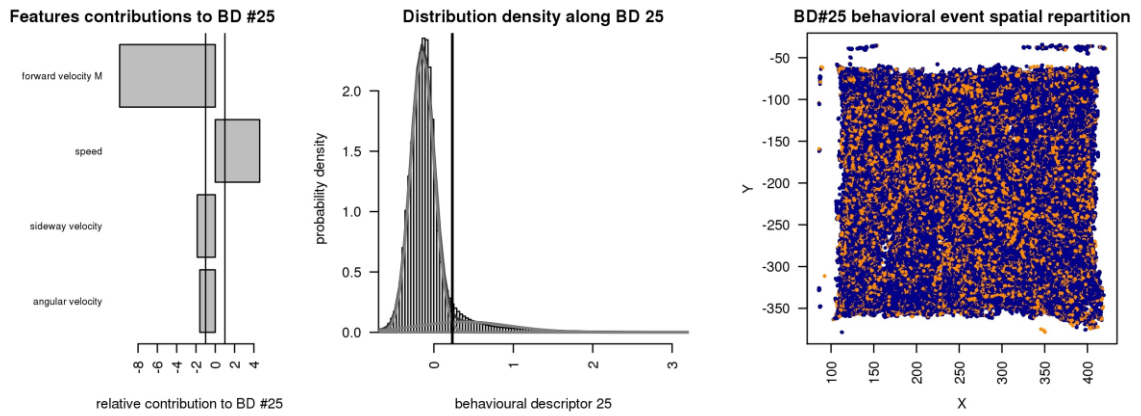


Figure annex 77: Interpretation figures for the BEC segregated on the 25th BD extracted from the BP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “centre of mass synchronized with other body parts”
- the BEC on the right of B and orange in C: “centre of mass pushed back”

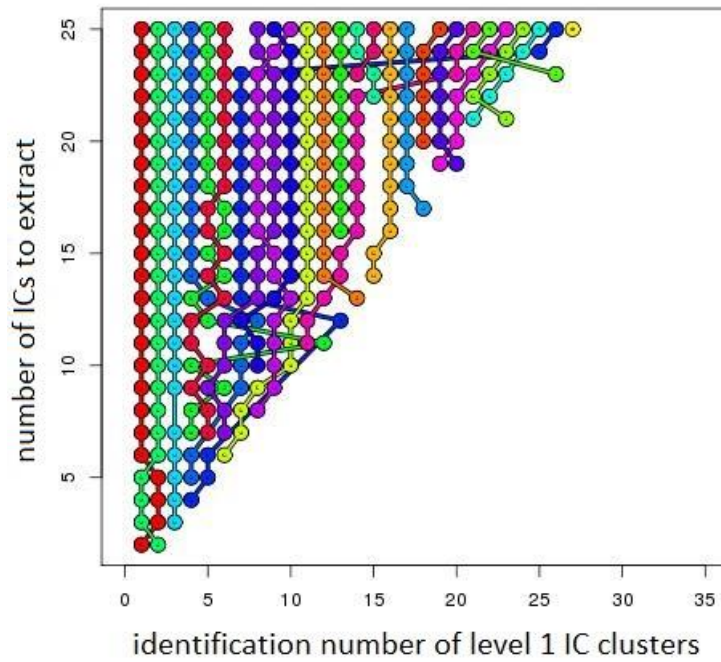


Figure annex 78: IC clusters over numbers of IC to extract for the BAP feature set of the *long-term group monitoring*. Each dot represent a level 1 cluster of IC, each line of dot of unique colour represent a level 2 cluster of IC.

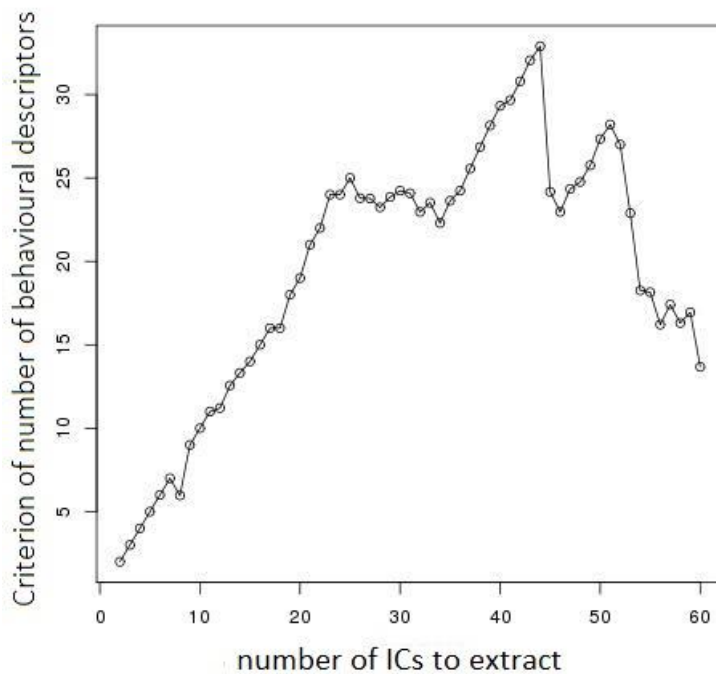


Figure annex 79: Criterion of number of behavioural descriptors in function of the number of IC for the BAP feature set of the single object exploration dataset. 25 behavioural descriptors kept for the BAP feature set of the long-term group monitoring.

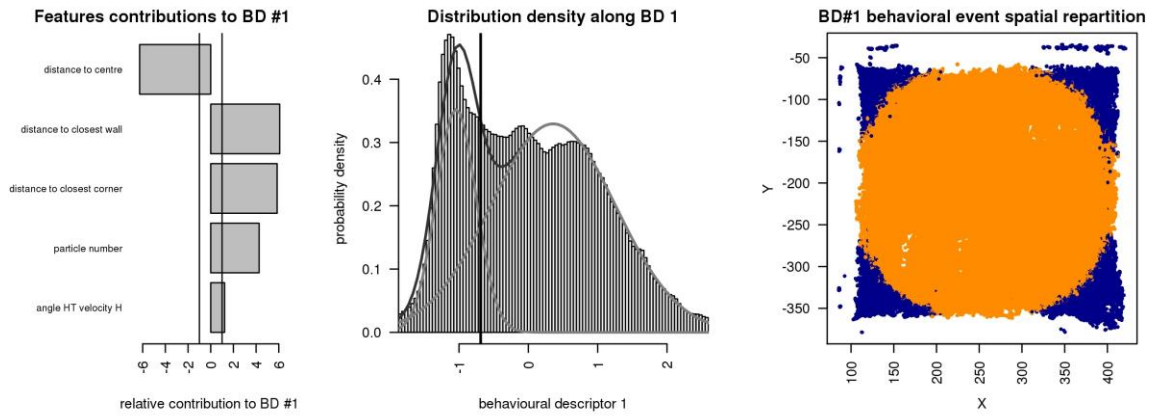


Figure annex 80: Interpretation figures for the BEC segregated on the 1st BD extracted from the BAP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“is in a corner”*
- the BEC on the right of B and orange in C: *“is not in a corner”*

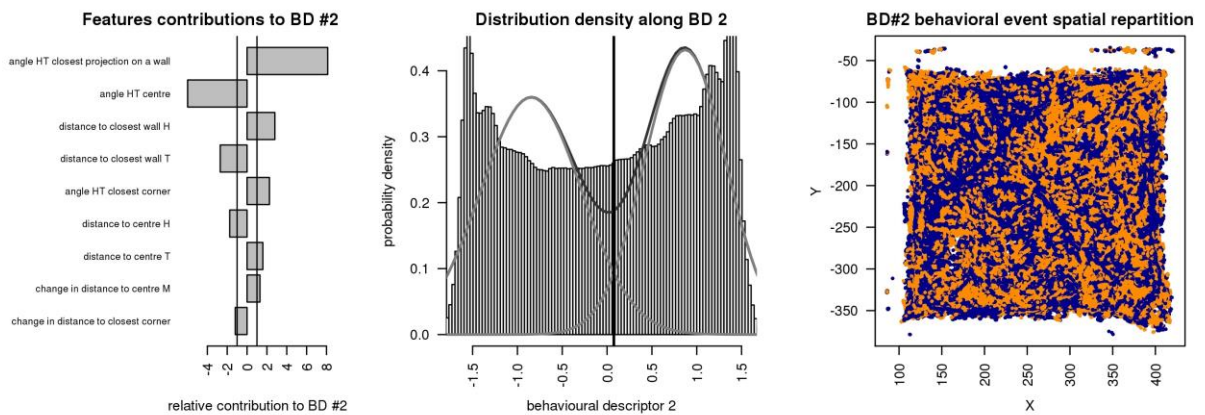


Figure annex 81: Interpretation figures for the BEC segregated on the 2nd BD extracted from the BAP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“is oriented toward the closest wall”*
- the BEC on the right of B and orange in C: *“centre of mass pushed back”*



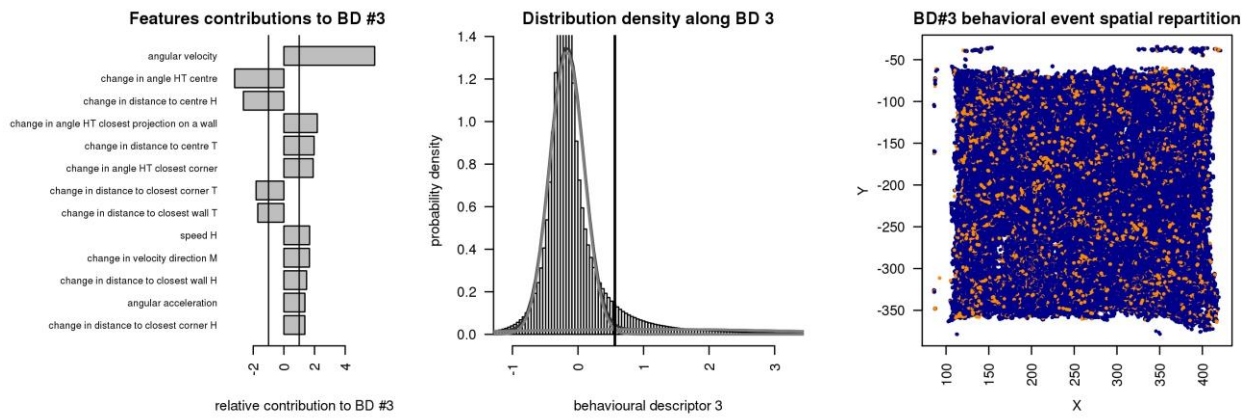


Figure annex 82: Interpretation figures for the BEC segregated on the 3rd BD extracted from the BAP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “does not turn toward the centre”
- the BEC on the right of B and orange in C: “turns toward the centre”

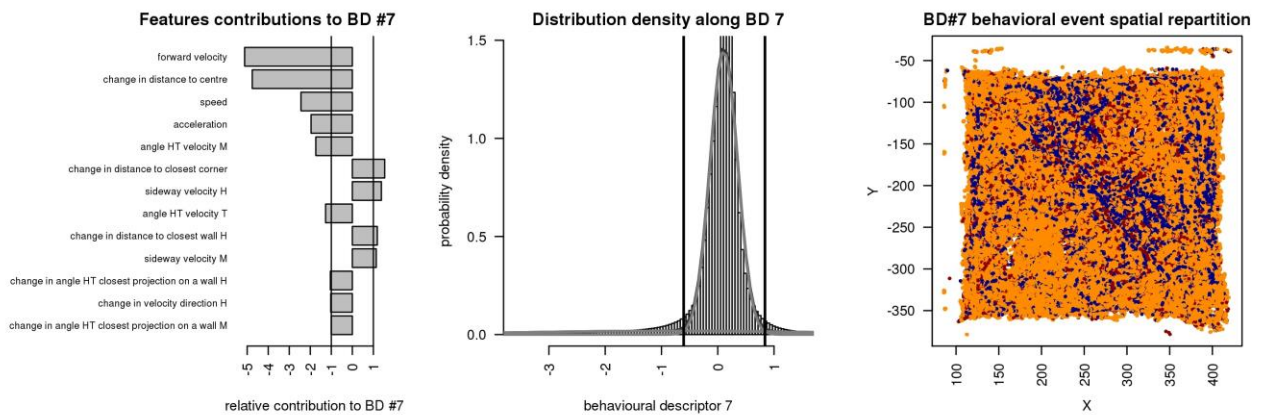


Figure annex 83: Interpretation figures for the BEC segregated on the 7th BD extracted from the BAP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “goes forward away from the centre”
- the BEC on the middle of B and orange in C: “does not progress with the centre in the back”
- the BEC on the right of B and red in C: “goes backward toward the centre”

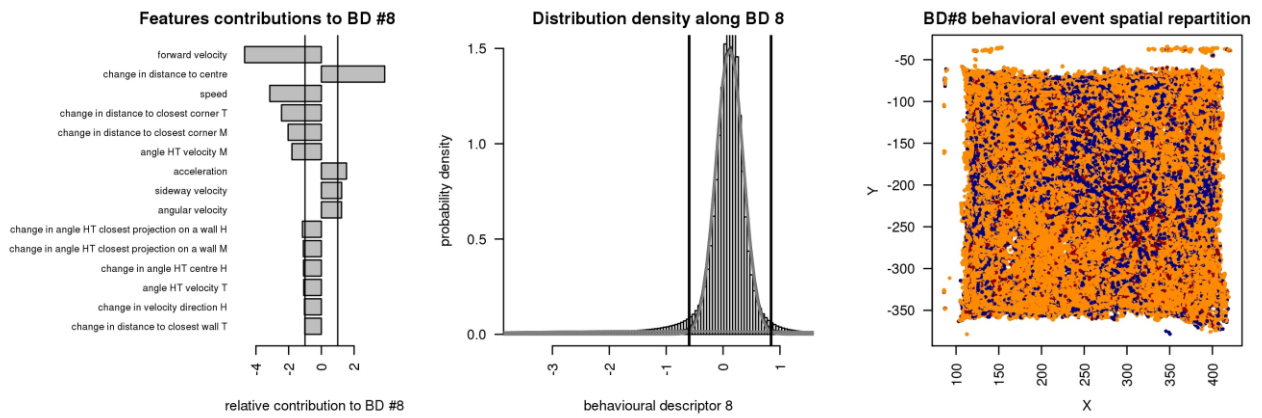


Figure annex 84: Interpretation figures for the BEC segregated on the 8th BD extracted from the BAP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “goes forward toward the centre”
- the BEC on the middle of B and orange in C: “does not progress with the centre in front”
- the BEC on the right of B and red in C: “goes backward away from the centre”

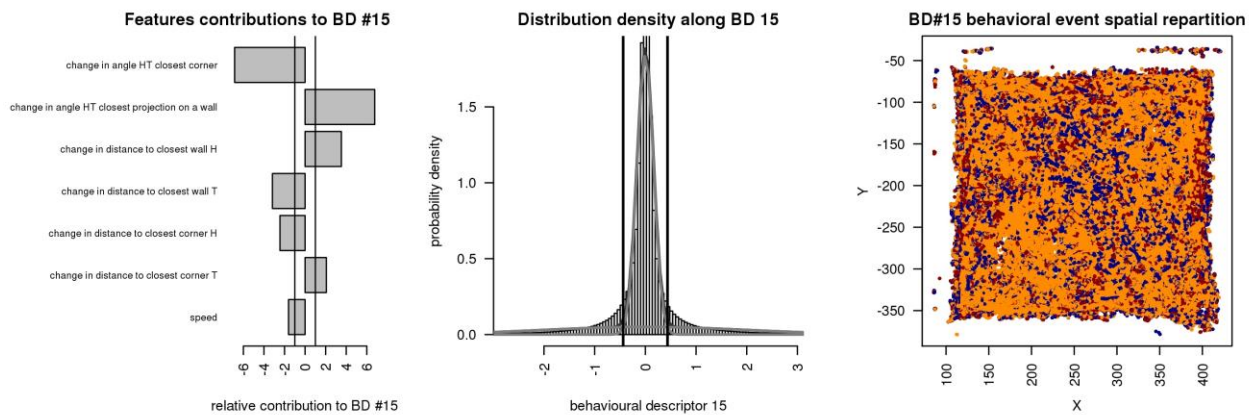


Figure annex 85: Interpretation figures for the BEC segregated on the 15th BD extracted from the BAP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “orients toward the wall rather than the corner”
- the BEC on the middle of B and orange in C: “stable wall-corner orientation”
- the BEC on the right of B and red in C: “orients toward the corner rather than the wall”

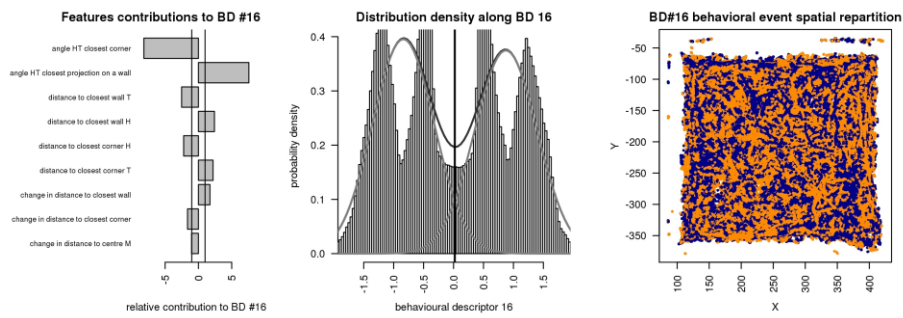


Figure annex 86: Interpretation figures for the BEC segregated on the 16th BD extracted from the BAP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“is oriented toward the wall rather than the corner”*
- the BEC on the right of B and orange in C: *“is oriented toward the corner rather than the wall”*



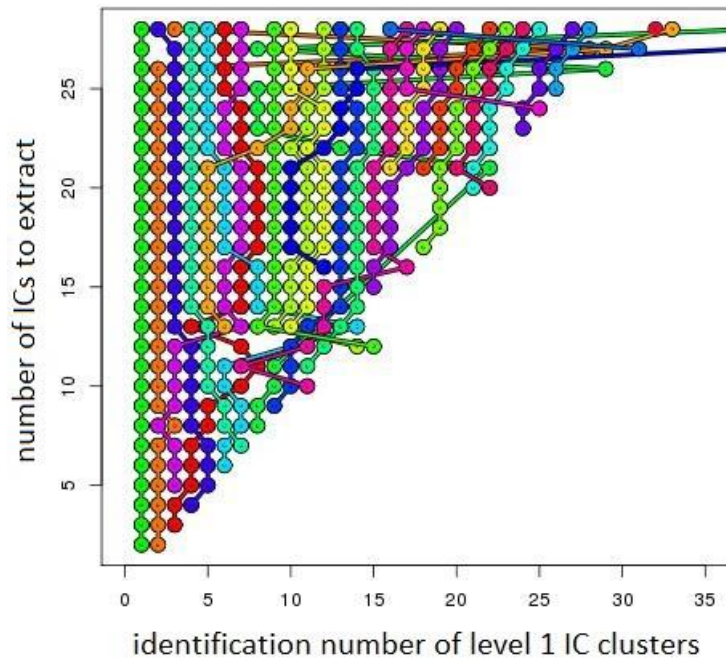


Figure annex 87: IC clusters over numbers of IC to extract for the BASP feature set of the *long-term group monitoring*. Each dot represent a level 1 cluster of IC, each line of dot of unique colour represent a level 2 cluster of IC.

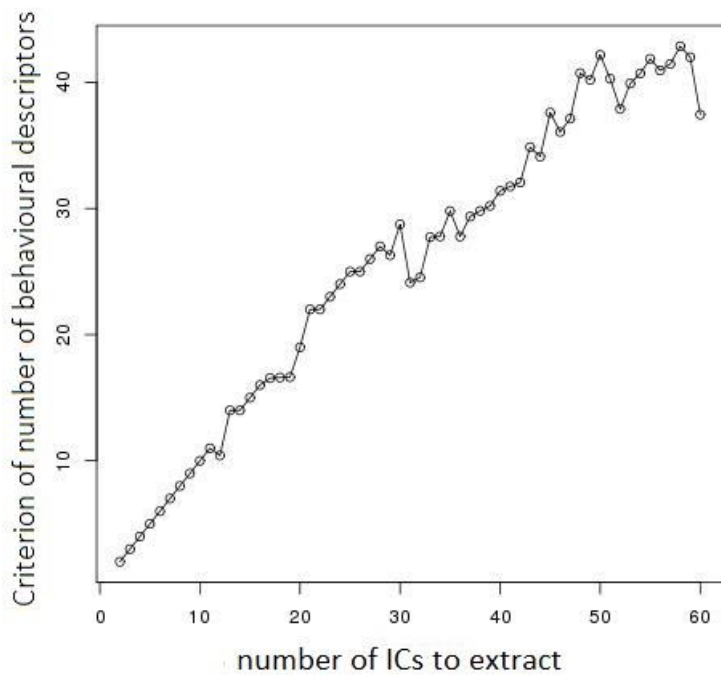


Figure annex 88: Criterion of number of behavioural descriptors in function of the number of IC for the BASP feature set of the single object exploration dataset. 28 behavioural descriptors kept for the BASP feature set of the *long-term group monitoring*.

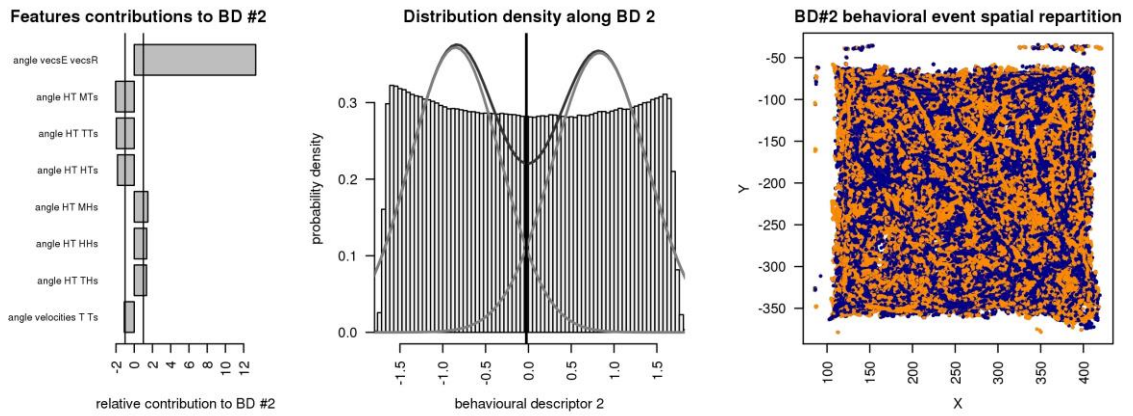


Figure annex 89: Interpretation figures for the BEC segregated on the 2nd BD extracted from the BASP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“same orientation as the receiver”*
- the BEC on the right of B and orange in C: *“opposed orientation with the receiver”*

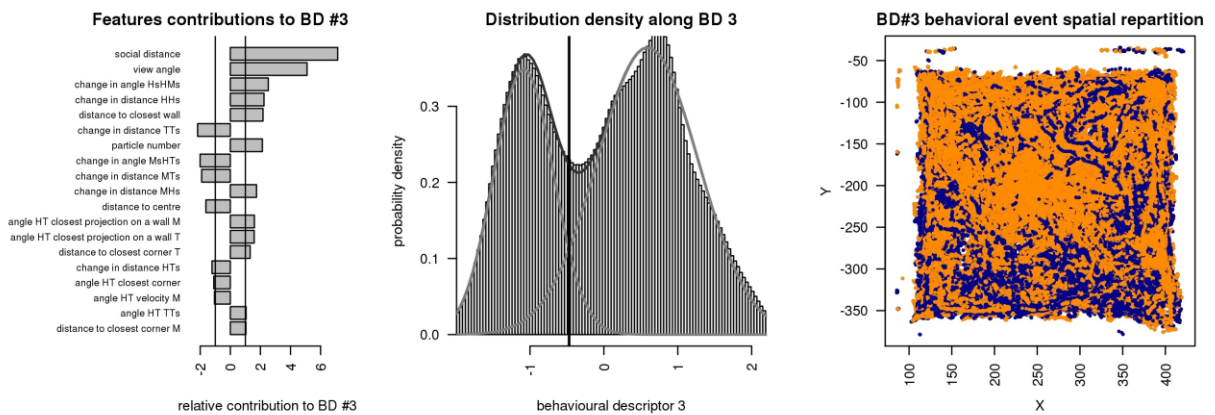


Figure annex 90: Interpretation figures for the BEC segregated on the 3rd BD extracted from the BASP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“is in contact with the receiver”*
- the BEC on the right of B and orange in C: *“is at distance from the receiver”*

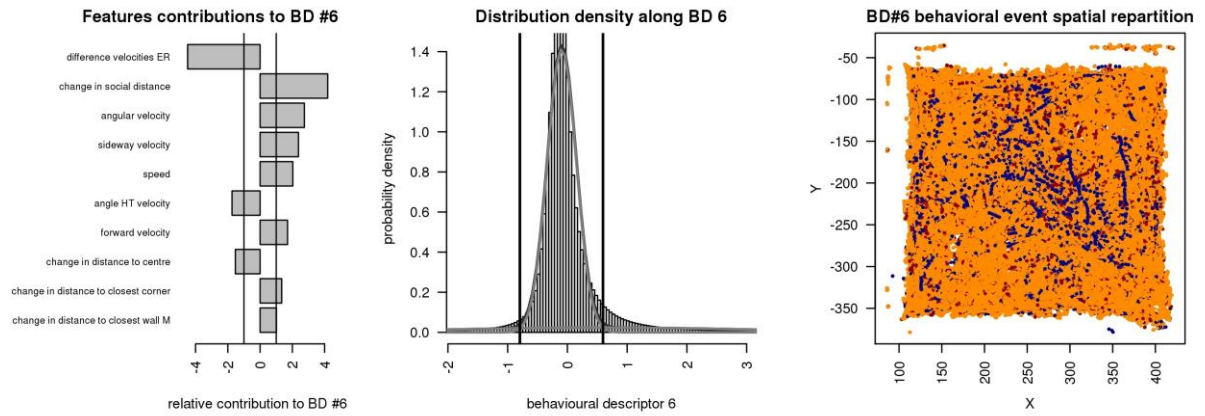


Figure annex 91: Interpretation figures for the BEC segregated on the 6th BD extracted from the BASP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “*emitter gets closer to the receiver*”
- the BEC on the middle of B and orange in C: “*neither getting closer or escaped*”
- the BEC on the right of B and red in C: “*receiver gets further from the emitter*”

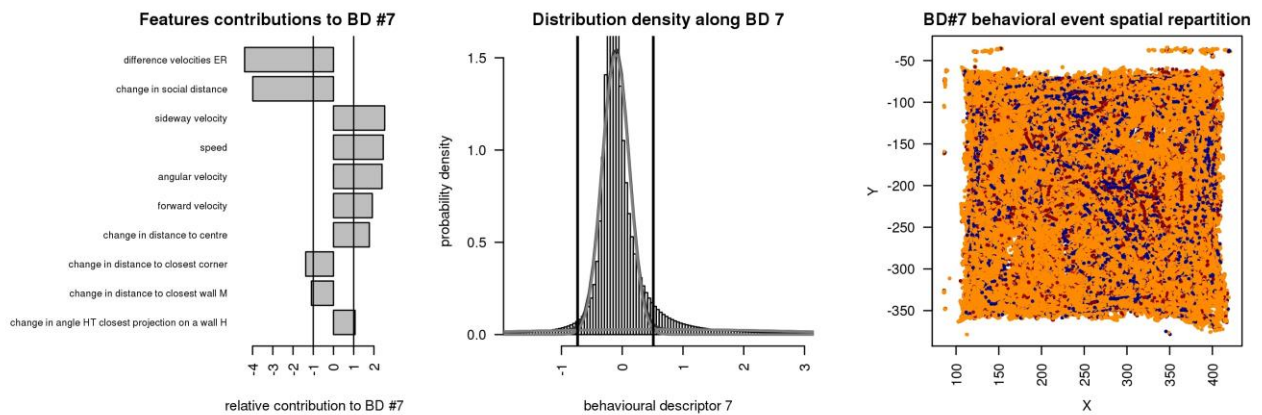


Figure annex 92: Interpretation figures for the BEC segregated on the 7th BD extracted from the BASP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “*emitter gets further from the receiver*”
- the BEC on the middle of B and orange in C: “*neither getting further nor joined*”
- the BEC on the right of B and red in C: “*receiver gets closer to the emitter*”

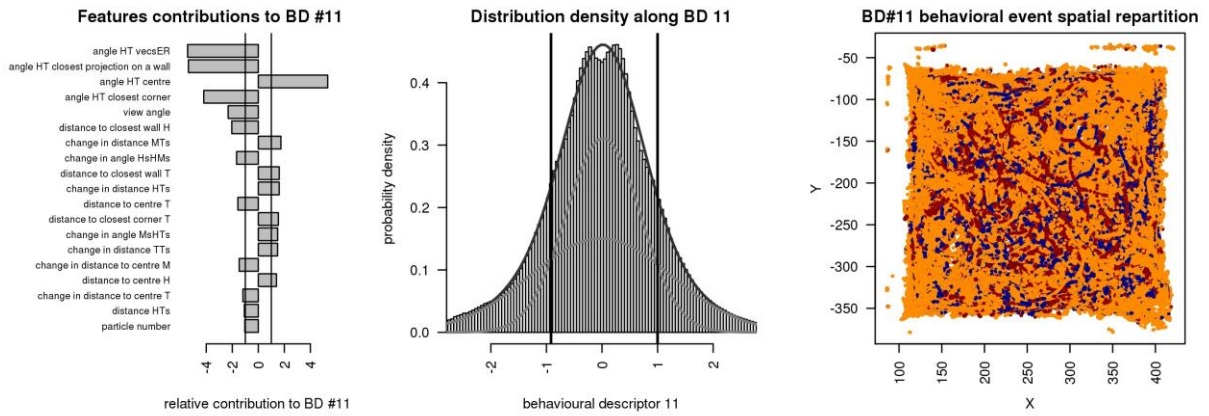


Figure annex 93: Interpretation figures for the BEC segregated on the 11th BD extracted from the BASP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“is back to the receiver”*
- the BEC on the middle of B and orange in C: *“no particular social orientation”*
- the BEC on the right of B and red in C: *“faces the receiver”*

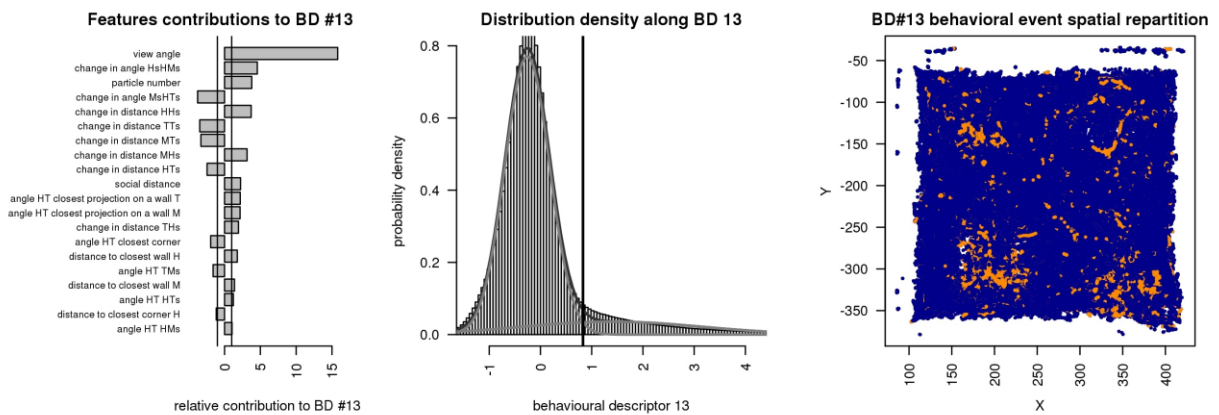


Figure annex 94: Interpretation figures for the BEC segregated on the 13th BD extracted from the BASP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“has a small view angle on the receiver”*
- the BEC on the right of B and orange in C: *“has a big view angle on the receiver”*



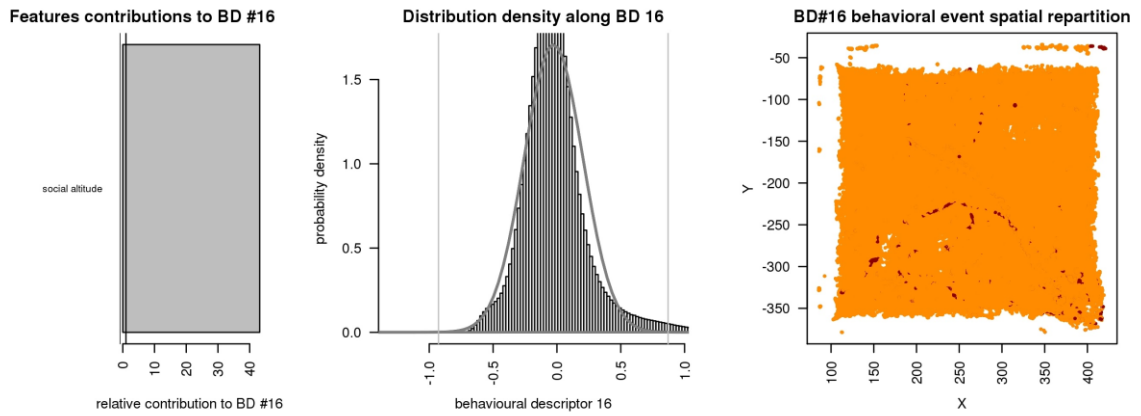


Figure annex 95: Interpretation figures for the BEC segregated on the 16th BD extracted from the BASP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“the receiver is low”*
- the BEC on the middle of B and orange in C: *“the receiver is at average altitude”*
- the BEC on the right of B and red in C: *“the receiver is high”*

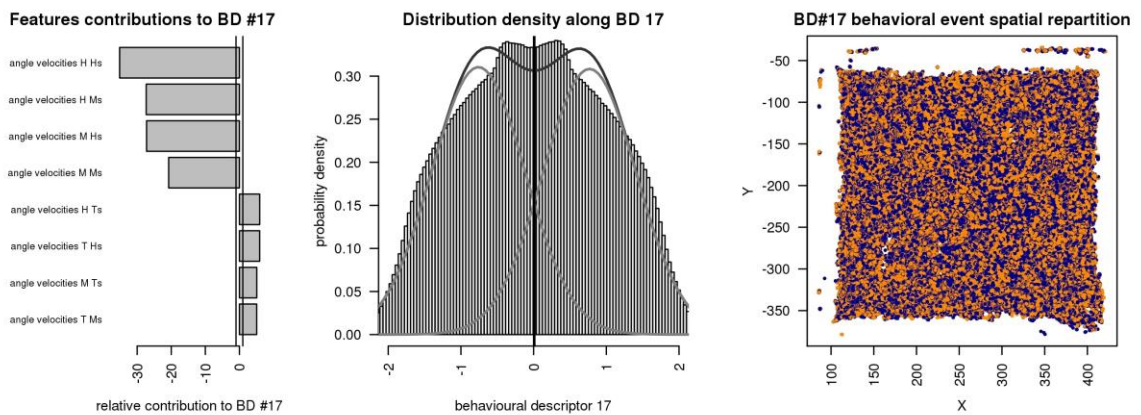


Figure annex 96: Interpretation figures for the BEC segregated on the 17th BD extracted from the BASP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“the animals anterior parts go in opposite directions”*
- the BEC on the right of B and orange in C: *“the animals anterior parts go in same direction”*

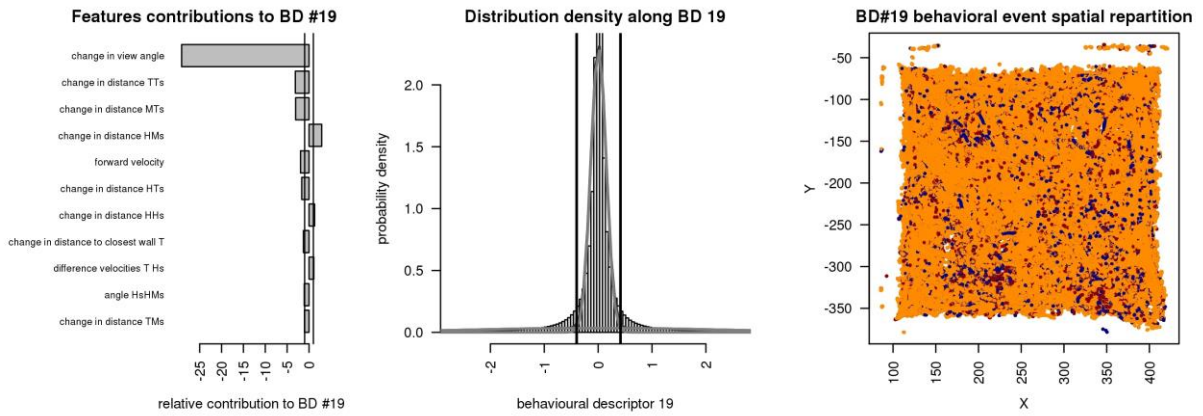


Figure annex 97: Interpretation figures for the BEC segregated on the 19th BD extracted from the BASP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“the view angle on the receiver increases”*
- the BEC on the middle of B and orange in C: *“stable view angle on the receiver”*
- the BEC on the right of B and red in C: *“the view angle on the receiver decreases”*

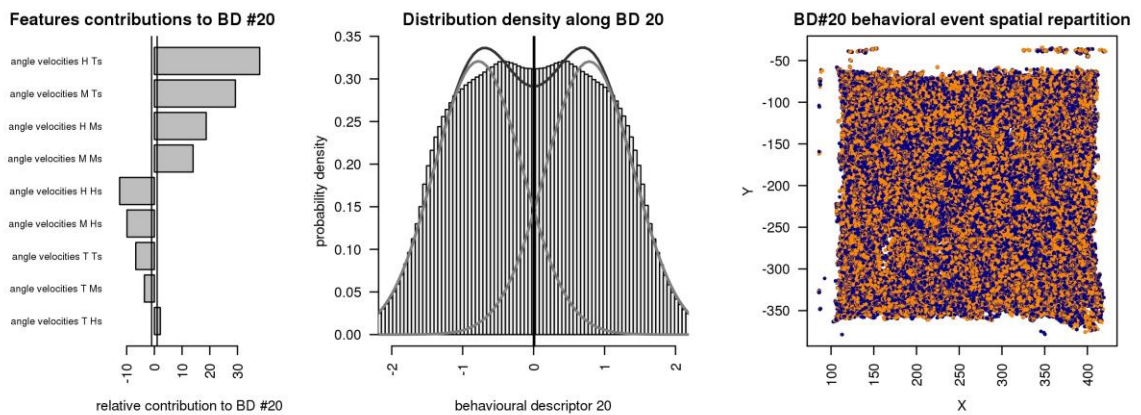


Figure annex 98: Interpretation figures for the BEC segregated on the 20th BD extracted from the BASP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“emitter anterior part and receiver posterior part go in the same direction”*
- the BEC on the right of B and orange in C: *“emitter anterior part and receiver posterior part go in opposite directions”*

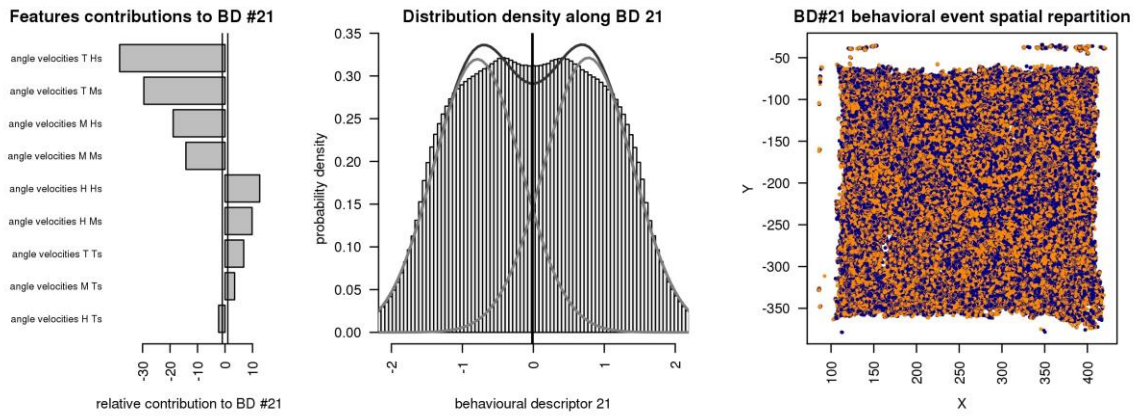


Figure annex 99: Interpretation figures for the BEC segregated on the 21th BD extracted from the BASP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “emitter posterior part and receiver anterior part go in opposite directions”
- the BEC on the right of B and orange in C: “emitter posterior part and receiver anterior part go in the same direction”

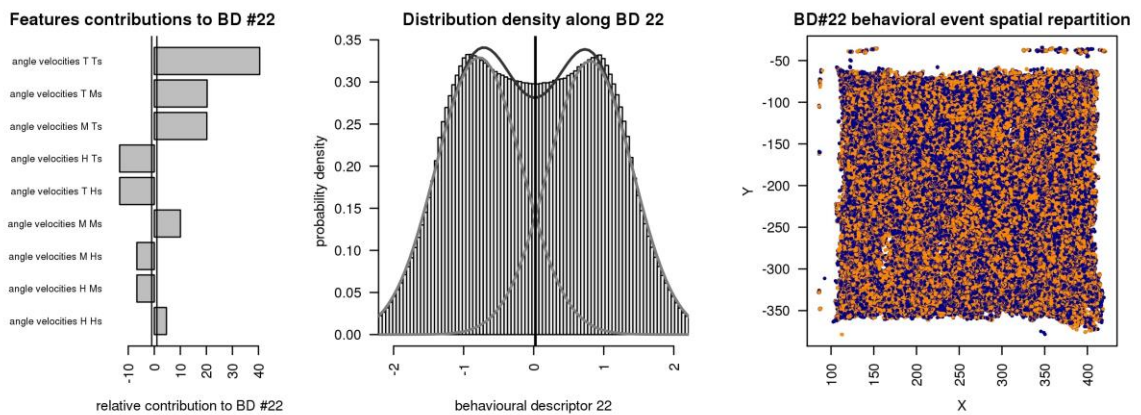


Figure annex 100: Interpretation figures for the BEC segregated on the 22th BD extracted from the BASP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: “the animals posterior parts go in the same direction”
- the BEC on the right of B and orange in C: “the animals posterior parts go in opposite directions”

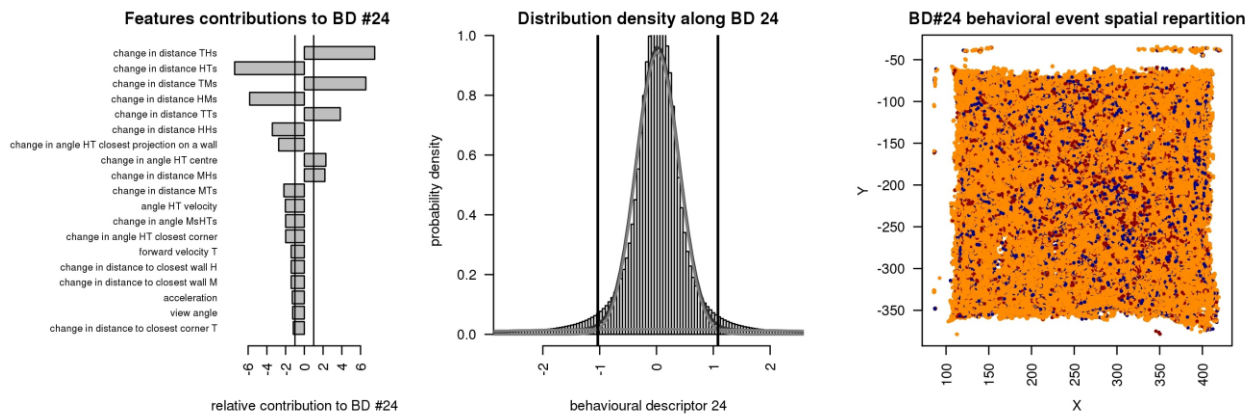


Figure annex 101: Interpretation figures for the BEC segregated on the 24th BD extracted from the BASP features-set in the single object exploration.

A: Bar-plot representing major features contributions to the BD.

B: Distribution along the BD with curves of the GMM and the threshold of the clustering.

C: Spatial repartition of the BEC of the BD.

We named:

- the BEC on the left of B and blue in C: *“goes ahead the receiver”*
- the BEC on the middle of B and orange in C: *“goes neither ahead nor behind the receiver”*
- the BEC on the right of B and red in C: *“goes behind the receiver”*



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# Abstract

Autism spectrum disorders are neurodevelopmental disorders affecting more than 1% of the global population. Among that, more than 1% of people with autism and intellectual disabilities carry a mutation in the gene coding for the postsynaptic scaffolding protein Shank3. Mouse models of such disorders have been studied through knock-out mutation on the homologous gene. Behavioural affections were found in homozygous knocked-out animals, but few effects have been reported on the heterozygous individuals, in widely used laboratory mouse strains such as C57BL/6J. Still, understanding the impact of *Shank3* haploinsufficiency is crucial, because human patients are heterozygous, and it will open the way to pharmaceutical strategies targeting an increase in *Shank3* expression. In this optic, we first analysed the behaviour of 12 mouse strains from the Collaborative Cross, a panel of a hundred inbred strains emerging from the controlled breeding of eight founder strains. This first step was used to grab some of the behavioural diversity present in mice and to select genetic backgrounds of interest for their social abilities or activity levels. In a second time, we generated *Shank3* heterozygous mutant mice on those mixed genetic backgrounds by crossing C57BL/6J.*Shank3*<sup>+/-</sup> mice with the selected CC strains. We analysed their behaviour as compared to their wild-type littermates. We used Live Mouse Tracker, a system to track mice in social group, to record and investigate the solitary and social behaviour of those mice. We developed a new unsupervised behavioural classification algorithm called SUCCUBE, in order to discover new behavioural event categories and extend our investigation of potential behavioural affections. Based on independent component analysis followed by Gaussian mixture model based clustering, this unsupervised classifier is notably the first one to allow co-occurrences of different behavioural event on a same time point. We here first compile wide descriptions of Collaborative Cross strain behaviour, in term of locomotor activity, exploration, stereotyped behaviour, thigmotaxis, neophobia and social interest. Such description can be beneficial to further development of other mouse models. We finally report the finding of three newly discovered behaviours, affected in their proportions of production, by *Shank3* haploinsufficiency, in a mixed background between C57BL/6J males and CC002 females. Those heterozygous mutants spent significantly less time rotating in acceleration, facing a conspecific or oriented in the same direction than a conspecific. Such findings open the way to pharmaceutical trials aiming to restore the affected behaviour of such mutant models closer to the levels of wild-type ones. This also makes the demonstration of the potential of unsupervised behavioural classification to investigate the behaviour of animal models of neurological troubles.