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Review

The hole-board apparatus in the study of anxiety

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ABSTRACT

Anxiety disorders pose a significant challenge in contemporary society, and their impact in terms of social and economic burden is overwhelming. Behavioral research conducted on animal subjects is crucial for comprehending these disorders and, from a translational standpoint, for introducing innovative therapeutic approaches. In this context, the Hole-Board apparatus has emerged as a widely utilized test for studying anxiety-related behaviors in rodents. Although a substantial body of literature underscores the utility and reliability of the Hole-Board in anxiety research, recent decades have witnessed a range of studies that have led to uncertainties and misinterpretations regarding the validity of this behavioral assay. The objective of this review is twofold: firstly, to underscore the utility and reliability of the Hole-Board assay, and concurrently, to examine the underlying factors contributing to potential misconceptions surrounding its utilization in the study of anxiety and anxiety-related behaviors. We will present results from both conventional quantitative analyses and multivariate approaches, while referencing a comprehensive collection of studies conducted using the Hole-Board.

"Of all truths relating to phenomena, the most valuable to us are those which relate to the order of their succession". John Stuart Mill, 1843 [1].

1. Introduction

It is possible to experience distress in the sudden presence of an unexpected danger, during a situation potentially able to hurt us, etc. The two emotions underlying this experience, and the hundreds of similar ones, are *anxiety* and *fear*. Both are adaptive responses of the organism to situations able to, potentially, put at risk its well-being and/or its physical integrity. *Fear* is considered a present-oriented and short-lived response characterized by the reaction to actual and explicit danger [2]; in contrast, *anxiety* is considered a future-oriented and long-acting response in which uncertainty, expectation of danger and/or the potential of the threat are fundamental [2,3]. Anxiety and fear are also discussed in other emotion theories, such as the so-called appraisal theories [4,5]. On this subject, in studies carried out on humans, very interesting perspectives have been presented concerning the relation between facial expressions, vocal expressions, gestures, body movements and emotions [6–8]. In these studies it has been argued that the detection of a variable range of expressions "*rather than prototypical patterns seems consistent with the notion that emotional expression is differentially driven by the results of sequential appraisal checks, as postulated by componential appraisal theories*" [7,8]. That said, unfortunately anxiety and fear do not belong only to our normal everyday life: indissolubly linked one another, they are also the "*leitmotiv*" supporting a group of neuropsychiatric diseases known as *anxiety disorders*. Examples of anxiety disorders are represented by generalized anxiety disorder, panic disorder and several phobia-related disorders [9,10]. Even if some aspects may appear very similar among these mental disorders, each specific form of anxiety disorder has its characteristics. For instance, people affected by generalized anxiety disorder show excessive anxiety and worry, every day or most days, for aspects and events that normally should not represent a source of anxiety or, at most, generate normal anxiety [10]. What is worst, such a long-lasting and excessive feeling of anxiety has various repercussions in terms of resulting additional symptoms (being the feeling of anxiety already a symptom "*per se*") such as being easily fatigued, feeling restless, being extremely irritable, insomnia, often very serious problems in concentration [9,10]. The situation, from a clinical point of view, may appear even worst in panic disorders. Panic attacks are characterized by violent periods of intense

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fear for no evident reason. These fear-related episodes may arrive unexpectedly but are also induced by specific conditions. During these dramatic moments people may present trembling or shaking, intense sweating, chest pain, oppressive and inexplicable feelings of imminent death, fear of incoming unavoidable danger etc. [10]. These attacks are often so violent that people are scared about when the next episode will arrive. The result is that these subjects, quite often, do everything in their possibilities to avoid situations, moments, places etc. linked with the onset of the preceding episode(s), in an attempt to diminish the possibility of a next attack [9,10]. Separation anxiety disorders, specific phobia, social phobia, agoraphobia are other conditions belonging to anxiety disorders and, similarly to generalized anxiety disorders and panic disorders, are characterized by "*features of excessive fear and anxiety and related behavioral disturbances*" [10]. Such a definition, reported from the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) is, in our opinion, enlightening because it underlines one of the crucial and probably most deleterious aspects of all anxiety disorders: *their unavoidable behavioral repercussions*. What do patients with anxiety disorders do or don't do? Often their behavior is driven by the necessity to cope with the oppressive symptoms of anxiety itself. As underlined in the DSM-V [10], common behaviors in patients with anxiety disorders may be represented by avoidance/evasive behaviors (e.g. the reduction of work activities or other activities, the avoidance of people, places, objects etc. believed to produce anxiety), dangerous and/or self-injuring behaviours (e.g. excessive consumption of alcoholic beverages or excessive assumption of drugs, in the attempt to reduce anxiety), excessive attachment behaviors (e.g. the increase of time spent at home, or with an object or with someone, in the belief that remaining in a safe place, or with a specific object or with a specific person can help in dealing with anxiety). It is not difficult to guess why these *maladaptive behaviors*, maintained for a long period, have deleterious consequences such as a progressive deterioration of inter-personal relations, job-related activities and a decline in life quality. It goes without saying, the impact of anxiety disorders in terms of social-economic burden is no less than disastrous. Two decades ago such a latter problem began to be characterized by its urgency [11,12] and, with the passing of years, the situation does not seem to improve at all [9,13–17]. The impact of the *behavioral correlates* of anxiety disorders is therefore enormous.

That said, alongside the clinical research conducted on humans, a large slice of scientific research aimed at investigating the behavioral characteristics of anxiety (e.g. those characteristics related to the pharmacological manipulations of the anxiety level) can be conducted only on animal subjects for obvious reasons. Such a research does assume considerable importance for the possible translational implications. Animal models are, indeed, an essential tool in giving useful insights not only into the etiology and neurobiology but also in terms of therapeutic approaches to human anxiety disorders [18]. From a translational perspective, a better understanding of the behavioral dynamics related to changes in anxiety and anxiety-related behaviors could allow the development of drugs more aimed at correcting specific alterations [18].

1.1. Behavioral tests to study anxiety

Given the essential role of behavioral research in the study of anxiety, not surprisingly, several different tests are available. Even if a more complex classification could be possible, basically, these tests can be divided into three groups: a) ethologically-based tests, b) cognitive-based tests, and c) physiological tests. For a comprehensive review see [18].

(a) *Ethological tests* Consistently with the importance that behavioral research in animal models of anxiety has from a translational perspective, not surprisingly, this is the most represented group. Most of the tests belonging to this group involve the so-called approach-avoidance conflict, i.e. the assumption that rodents placed in brightly lit and/or unknown environments show a clear-cut conflict between their innate tendency to explore and an equally innate tendency to avoid possible sources of danger [19,20]. Tests belonging to this group are the Open-Field [21–30], the Elevated Plus Maze [19,31–45], the Elevated Zero Maze [36,46,47], the Elevated T-maze [21,48,49], the Light-Dark Box [50–57], the Staircase test [58–60]. Another test belonging to this group is the Hole-Board.

(b) *Cognitive-based tests* These behavioral assays involve tasks requiring specific cognitive performances such as the association of different stimuli. Three important tests belong to this category are the Pavlovian fear conditioning test [61–63], the Conditional emotional response [64,65] and the Conditioned taste aversion [66].

(c) *Physiological tests.* These tests imply the evaluation of physiological responses in tested subjects such as body temperature or heart rate. Two tests belonging to this group are the Stress-induced hyperthermia [67–69] and the Autonomic telemetry [70].

1.2. The hole-board

The Hole-Board (HB), originally devised by Boissier et al. [71], is a behavioral test widely used to assess exploration and anxiety-related behaviors in mice and rats [72–88]. The HB is, basically, a ground-holed Open-Field (Fig. 1); therefore, not surprisingly, it can combine the approach-avoidance conflict elicited by a simple Open-Field with the addition of a variable number of environmental cues, namely, holes in the ground [89–91]. Many different HBs, in terms of dimensions and number of ground holes, are available. Taking into consideration that HB for rats and mice have different dimensions, it is possible to find studies with HB containing 4 holes (e.g. one hole for each corner of the arena or four holes in a row), 16 holes (i.e. 4 identical rows each containing 4 equidistant holes) or even HB containing 36 holes (i.e. 6 identical rows each containing 6 equidistant holes). For a comprehensive review see [92]. If on the one hand, these holes offer the possibility to measure the exploratory behavior of rats or mice [77,93,94], on the other hand, they represent unknown cues able to fuel the conflict between approach and avoidance [78,82,84].

In the next sections this review will continue illustrating the HB as tool to study anxiety and anxiety-related behaviors in rodents. In detail, from the simple aspects and issues, i.e. the analyses of the individual elements of the behavioral repertoire (e.g. evaluation of mean durations, latencies, occurrences, etc.) we will move to the more subtle ones, i.e. to analyses of the relationships among these components. The evaluations of these constraints do represent the essential fuel underlying the structural analyses of rodent behavior discussed in this review.

2. Something wrong with hole-board test?

Basically, differences between an HB and an Open-Field lie in the presence of the ground-holes. The Head-Dip is a behavioral component characterized by the insertion of the head into one of these holes (Fig. 1). Several parameters of Head-Dip can be assessed, e.g. its occurrences, its latency, the overall time spent with the head inside, etc. Importantly, these parameters have been considered independent from general locomotion [95–97] and, as a consequence, able to provide a valid measure of the specific rodent's attraction or repulsion towards novelty, i.e. *neophilia and neophobia respectively*, both strictly dependent on the emotional state of the animal; accordingly, a rodent with a high level of fear/anxiety will explore much less the holes; on the other hand, a low level of fear/anxiety will be associated with the increased exploration of the holes. Thus, increases in Head-Dippings (e.g. in terms of increased frequencies and durations) have been interpreted as indicative of neophilia and low animal's anxiety-like state; on the other hand,

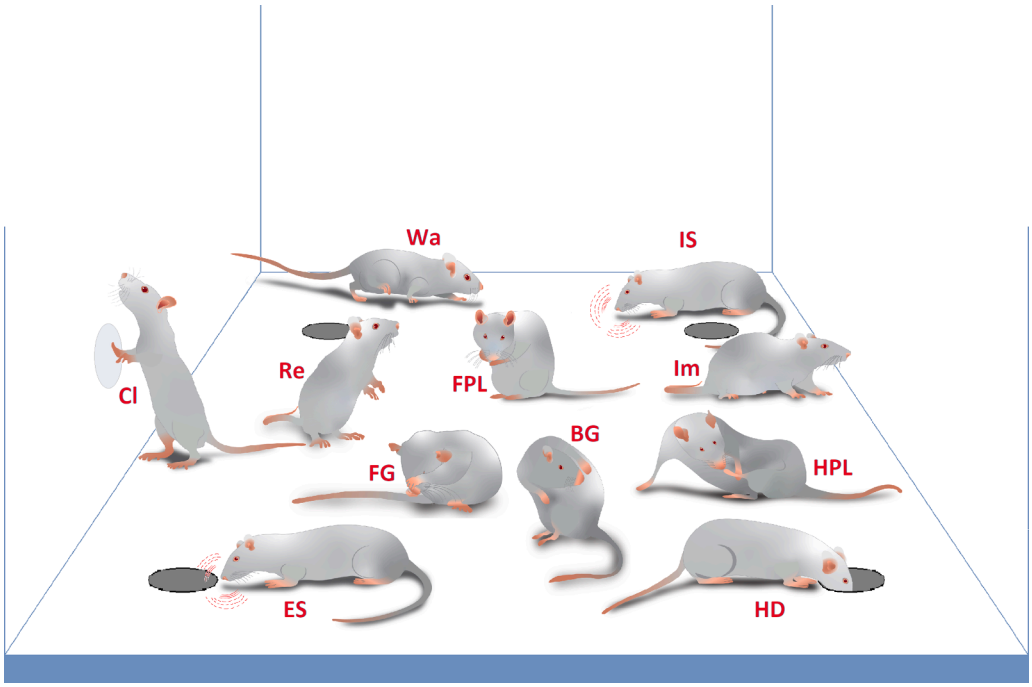


Fig. 1. Behavioral repertoire of rat in the Hole Board apparatus. Walking (Wa): rat walks around sniffing the environment; Immobile-Sniffing (IS): rat sniffs the environment standing on the ground; Climbing (Cl): rat maintains an erect posture leaning against the Plexiglas wall; Rearing (Re): rat maintains an erect posture without leaning against the Plexiglas wall; Immobility (Im): rat maintains a fixed posture and no movements are produced; Front-Paw Licking (FPL): rat licks or grooms its forepaws; Hind-Paw Licking (HPL): rat licks or grooms its hind paws; Face Grooming (FG): rat rubs its face (ears, mouth, vibrissae, eyes) with rapid circular movements of its forepaws; Body Grooming (BG): rat licks its body combing its fur with fast movements of incisors; Edge-Sniff (ES): rat sniffs the border of one of the four holes; Head-Dip (HD): rat puts its head into one of the four holes.

reductions of Head-Dippings have been interpreted as a weakened neophilia and/or high anxiety-like state [98]. However, the situation is by no means so simple. Just the study of such an exploratory activity of the ground holes using the Head-Dip, indeed, has not led to univocal results; rather, it has not infrequently fueled conflicting outcomes and misunderstanding findings. For example, following *reductions in the anxiety level*, various parameters related to Head-Dip as a specific indicator of anxiety have shown increases [98,99], decreases [43,100], or no modifications [78,101]; the matter is similarly complex for the *increases of the anxiety level* as well: following the administration of a compound able to strongly increase the anxiety level, indeed, it has been shown that Head-Dipping can be increased [84] or decreased [98]. These results do appear counterintuitive if we take into consideration the assumption concerning the assessment of Head-Dip as a suitable measure of rodents' attraction or repulsion towards novelty. Not surprisingly, it has been argued that "simple" quantitative evaluations of Head-Dip alone, such as its frequencies or durations, are unlikely able to provide valid and/or affordable measures indicative of the emotional state of the subject [77,78,84,102]. Beyond Head-Dip, the situation does not much improve if other behavioral elements in HB are taken into account. For instance, in a recent study, it was even argued that the HB apparatus is not an appropriate tool for measuring exploratory behaviors in laboratory rodents and that the utilization of HBs, in this sense, should be seriously rethought and/or reconsidered [103]. On the other hand, various pieces of evidence during the last five decades have demonstrated that the HB can be successfully and advantageously used just to study exploration and/or to assess possible effects of drugs on the exploratory behavior of rodents in this environment [72–75,77,79–83,85-87,89,90,104–107]. Therefore, not only the validity of the Head-Dip itself but also the general behavior of the rodent in HB is often a topic of discussion. Where do so different points of view originate from? Why so many different perspectives on rodent behavior in HB are present? An obvious question spontaneously arises: what is wrong?

2.1. From quantitative to structural analysis

The possibility to describe a given behavior using *hundreds or even thousands of numbers* does not imply the "specular" possibility, namely the possibility to utilize those numbers to reconstruct the behavior in its original features and dynamics [108,109]. A quantitative approach to behavioral studies can provide answers concerning, for instance, how many behaviors of a given type occur, their percent distribution, latencies, durations etc. Undoubtedly, these quantities, concerning each component of the original behavioral repertoire, do provide a "satisfying" taste of exhaustiveness and represent useful quantifications of individual parameters following, for instance, the utilization/administration of a specific independent variable etc. However, it is also important to consider that these quantities are only able to describe isolated fragments of a given behavior, separated from what is, in reality, the *actual* behavioral structure. As previously underlined, "*this is not different from classifying all the single pieces of a puzzle missing the comprehensive picture. The functional meaning of a behavior,* i.e.*, the study of the existing interplay between an animal and the context, is a picture lying in its intrinsic structural features*" [109], i.e. in the intrinsic relationships among all the components of a given behavioral repertoire. The evaluation of these relationships will be able to provide additional and/or more complete information concerning the studied behavior than the assessment of individual components employing simple quantitative approaches. This is of course true also for the behavior of the rodent in HB. Several works, indeed, highlight that the evaluation of individual behavioral elements is able to offer only *scanty information* on the *real* activity of the animal in HB. For example, it was highlighted that the administration of diazepam (a benzodiazepine, *molecule with anxiolytic action*) determines an increase in the percent distribution of generalized exploration behaviors but not, as might be expected, of the specific exploration of the hole; in addition, all behaviors of grooming remain unchanged [78]; different changes are observed following the administration of FG7142 (a beta-carboline, *powerful anxiety inducer,* for a review see [110]) since practically all behaviors, including those of grooming, show evident changes [84]. It is nearly impossible, however, only by evaluating generalized exploration behaviors, in their individuality, to attain an idea of the subject's emotional state. Moreover, since the "*raison d'être*" of an HB is represented by the ground holes, it is obvious that the researcher's attention, when using an HB, must focus specifically on Head-Dip and Edge-Sniff, i.e. those components of the behavioral repertoire (Fig. 1) specifically aimed at exploration of the ground holes.

Fig. 2 highlights the modifications that the occurrences of Head-Dip

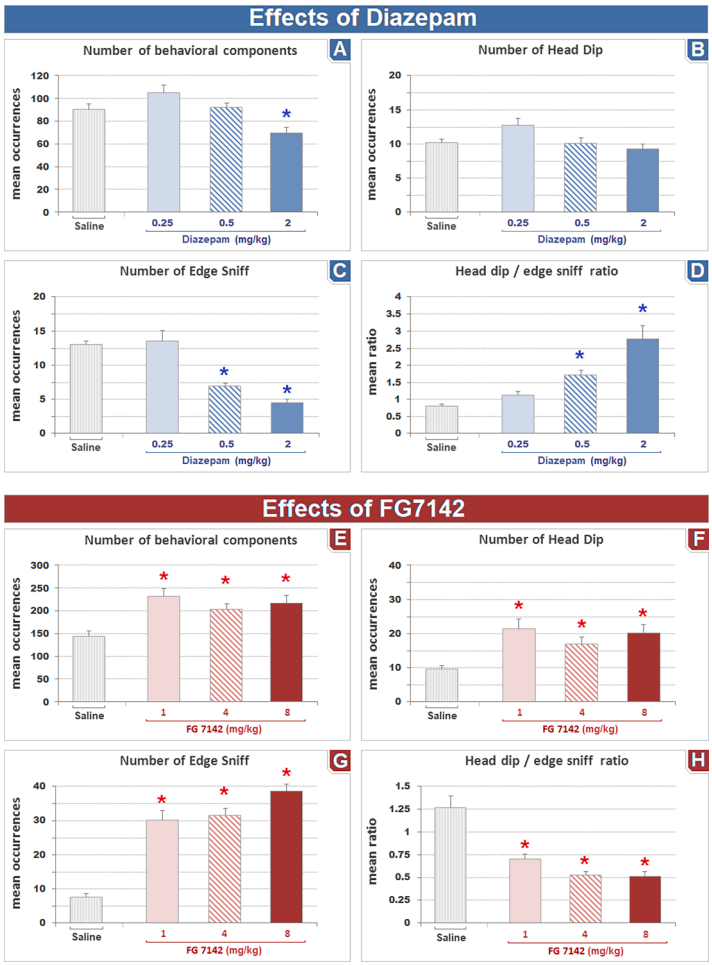


Fig. 2. Effects of different doses of diazepam (panels A,B,C,D) and FG7142 (panels E,F,G,H) on the mean number of behavioral components performed (A, E), mean number of head-dips (B, F), mean number of edge-sniffs (C, G) and head-dip/edge-sniff ratio (D, H). Data are represented as mean ± SE. * = significantly different value when compared to saline injected group (Newman-Keuls post-hoc test for multiple comparisons; $p < 0.05$ was considered significant). *Panels A,B,C,D modified with permission from Casarrubea* et al. *[78]; panels E,F,G,H modified with permission from Casarrubea* et al. *[84].*

and Edge-Sniff do show following the administration of the two above-mentioned drugs. As to diazepam (Fig. 2A-C), at higher doses, the Head-Dip remains statistically unchanged (Fig. 2B) while the Edge-Sniff is significantly reduced (Fig. 2C). Results following administration of FG7142 (Fig. 2E-G) are diametrically opposite: it is impossible to overlook the deep and significant increases in Head-Dip (Fig. 2F) and Edge-Sniff (Fig. 2G) at all the FG7142 doses. On the one hand, it is somewhat reassuring to appreciate how two totally different substances (i.e. diazepam and FG7142, the first anxiolytic "*par excellence*", the second an extremely powerful inducer of anxiety) do produce different results if individual behavioral elements are assessed; on the other hand, however, it is much less reassuring to appreciate how *these drug-induced behavioral changes do occur completely in the wrong directions*: Head-Dip and Edge-Sniff, according to the basic premise of HB, should be increased following administration of the anxiolytic molecules and greatly reduced following the administration of the anxiogenic compounds. It is possible to conclude that evaluations carried out on Head-Dip and Edge-Sniff as individual elements of the behavioral repertoire of the rat in HB, detached from the real architecture of behavior, represent sources of possible serious misunderstandings.

Even a simple search using a common scientific database will be able to underline that the largest amount of studies, where an HB apparatus has been employed, utilized only simple quantifications of individual components of the behavioral repertoire such as Head-Dip frequencies, durations, latencies etc. For instance, by performing a search on https://pubmed.ncbi.nlm.nih.gov/ utilizing the search string "*hole-board*", 936 studies are shown (search performed on May 4th 2023), the first being the 1964 pioneering research by Bossier and Colleagues concerning the utilization of psychotropic drugs in mice [71] [Bossier et al., 1964]. Among such a considerable amount of researches, only a small number of studies have applied *structural* approaches to study and describe the behavior in its comprehensiveness, that is, analyses/techniques able to shed light on what is really going on in the apparatus during the observation period. On the other hand, the largest amount of studies retrieved has employed individual quantifications of the Head-Dip such as its latency, and its duration, its occurrences.

3. Behavioral patterning in the hole-board

If it is true that the HB, like the Open Field, the Elevated Plus Maze and many other ethological tests, mentioned above, is based on the principle of approach-avoidance conflict, it is also implicit that the behavioral result of this conflict cannot, reductionistically, be traced back to a single element of the behavioral repertoire, detached from the real behavioral structure. Understanding how the Head-Dip is *contextualized* within the animal's response could provide more pertinent answers on how changes of the animal's anxiety condition are able to modify the whole behavioral structure, i.e. what from a translational point of view matters more. The holes of an HB do represent "*objects*" unknown to the animal: according to the approach-avoidance conflict, the rodent sniffs the edge of the hole (Edge-Sniff) and then inserts the head inside (Head-Dip). Obviously, if mice or rats do so, it heavily depends on the motivational drive, which in turn is closely linked with the emotional aspects, namely fear and anxiety and, of course, with the drugs that can be administered to interact with the animal's emotional assets. On the contrary, the evaluation of the Head-Dip alone, like any other isolated behavioral element, *detached from the real behavioral structure*, is more vulnerable to variables that are difficult to control such

as the strain, housing conditions, experimental procedures etc. and even apparently insignificant changes between one laboratory and another can lead to results, mainly for anxiety-related behaviors, that are not well in agreement [111]. Instead, evaluating the patterning between two or more elements of the behavior, even more if connected by an evident motivational link, *such as Head-Dip and Edge-Sniff*, of course does not solve the above-mentioned problem but certainly shifts the focus prevalently on the subject's emotional condition. A small percentage of studies in which HB has been employed in recent decades have moved just in this direction. These works, characterized by the use of various multivariate analysis methods, have highlighted behavioral dynamics that cannot be inferred from the study of the individual elements of behavior, individually assessed and detached from the real behavioral architecture.

3.1. The ratio between head-dip and edge-sniff

The evaluation of Head-Dip and Edge-Sniff in their individuality is therefore a source of possible/probable misunderstandings. The only feasible solution lies in relating the different elements of the animal's behavior in the HB in order to be able to observe the real behavioral structure, made up not of isolated elements but in relation to each other. A first evaluable relationship is the one existing just between Head-Dip and Edge-Sniff. For both diazepam and FG7142, observing the ratio between the occurrences of Head-Dip and Edge-Sniff (Fig. 2D,H), it is possible to appreciate that this simple relationship has a trend consistent with the nature of the administered substance and with the basic assumptions of HB as a test to study anxiety. Such a ratio, indeed, does not take into account the absolute number of the two behaviors. The Head-Dip is the numerator and the Edge-Sniff the denominator: a prevalence of Head-Dip will cause the ratio turn above 1, so indicating rodents' behavior is more aimed at inserting the head into the hole without a preliminary edge sniffing activity. On the contrary, a prevalence of Edge-Sniffs will make the ratio turn below 1, indicative of a greater cautiousness of animals before inserting the head inside the hole. Diazepam administration increases this ratio which rises well above 1 and the value recorded with the control group (Fig. 2D). On the contrary, the administration of FG7142 reduces this ratio far below 1 and the value recorded with the control group (Fig. 2H). The two substances, therefore, based on the evaluation of this relationship, have behavioral effects consistent with their pharmacological nature and with the basic assumptions of HB itself.

Evaluating the relationship between Head-Dip and Edge-Sniff rapidly leads to the questions of whether these two elements have also relations with other items of the behavioral repertoire in HB and, not less importantly, if these relationships may be affected by drug treatment. These are essential questions whose answers cannot be ignored if we want to know the impact that the pharmacological manipulations of the animal's state of anxiety have in behavioral terms.

3.2. Portraits of the behavior: Transition matrices

A transition matrix is a table indicating shifts among the behavioral components of an "*a priori*" established ethogram such as the one illustrated in Fig. 1. Actually, a transition matrix is a data sheet containing hundreds or even thousands of numbers and very difficult to be fully appreciated even for an expert eye. For this reason, transition matrices are normally elaborated by using different approaches. Using a specific algorithm, e.g. see [112,113], a transition matrix can be transformed into a similarity matrix and expressed through a dendrogram, e.g. see [114,115]. Basically, a dendrogram is a graphical tree representation showing how similar some patterns, encompassed in a given ethogram, are to each other. Another possibility is represented by the transformation of a transition matrix into a probability matrix, i.e. a data matrix expressing transitions among behaviors from a probabilistic perspective and expressed employing a stochastic path diagram, e.g. see [80,116–118]. Another interesting approach to analyzing transition matrices is utilizing adjusted residuals, i.e. transitions occurring significantly more or less often than expected. Adjusted residuals can be expressed by means of path diagrams, e.g. see [119–122], or histograms, e.g. see [115,123,124].

Thanks to analyses based on dendrograms (Fig. 3), it was possible to deduce that rat's behavior in the HB has a complex structure characterized by the presence of three main clusters encompassing respectively, components of general exploration (Walking, Climbing, Immobile-Sniffing), components of focused exploration (Head-Dip and Edge-Sniff) and grooming components (Front-paw licking, Face grooming and Body grooming). Simply stated, the dendrograms in Fig. 3A and E (that is, groups treated with saline) are portraits illustrating the behavioral architecture of the rodent tested in the HB

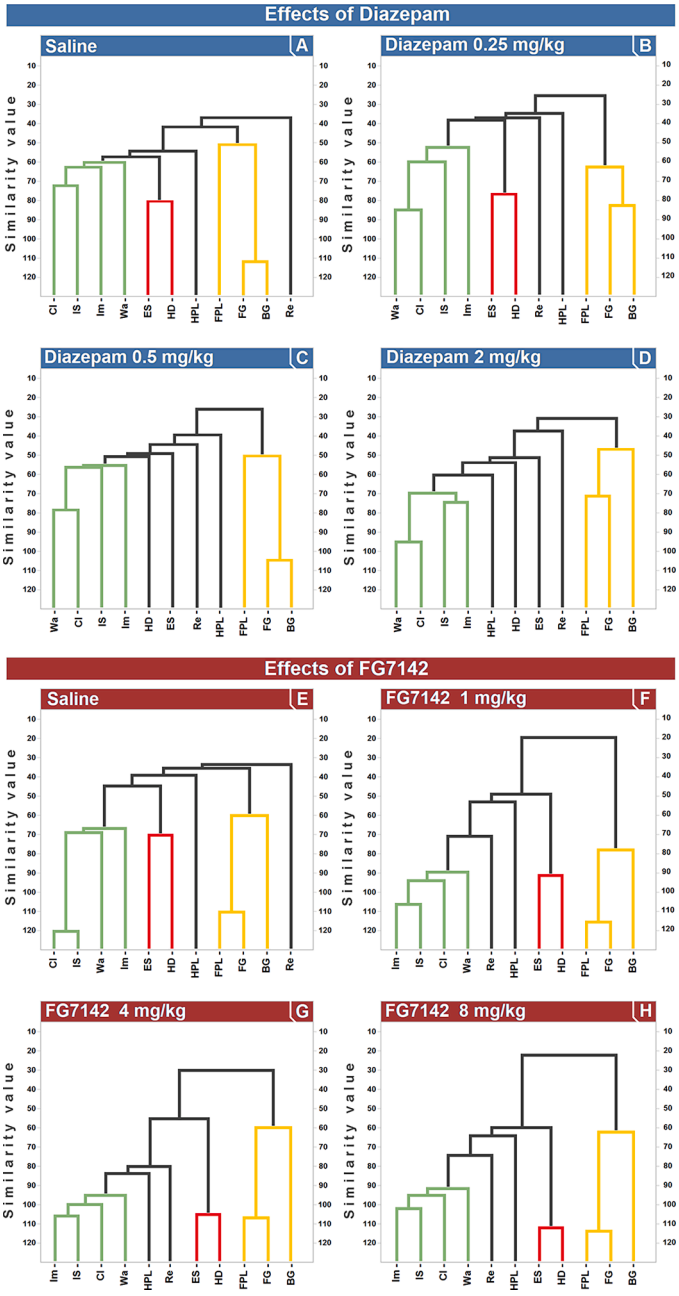


Fig. 3. Effects of different doses of diazepam (panels A,B,C,D) and FG7142 (panels E,F,G,H) on structural features of behavior, expressed by means of dendrograms. *Panels A,B,C,D modified with permission from Casarrubea* et al. *[78]; panels E,F,G,H modified with permission from Casarrubea* et al. *[84].*

apparatus. Interestingly, these structures undergo heavy changes following the administration of substances capable of modulating the anxiety level, such as diazepam [78] or FG7142 [84], both molecules very well known for their clear-cut action, respectively, in an anxiolytic and anxiogenic direction. Following diazepam it is interesting to notice how the cluster consisting of Head-Dip and Edge-Sniff, evident in animals injected with saline, disappears at the two highest dosages (Fig. 3C, D). Simply stated, the two behaviors do not share a sufficient number of reciprocal transitions able to induce their mutual association. One explanation could be that, in the animal whose level of anxiety is reduced, the emotional impact originating from the environmental stimuli (such as the ground holes in a HB) is weakened; consequently, the association between Edge-Sniff (preparatory sniffing of the outer edge of the hole) and Head-Dip (exploration of the inside of the hole), expression of cautious exploration, disappears or is greatly reduced. Following the FG7142, an opposite picture is observed: the higher level of anxiety is evident, as underlined by the dose-dependent increase of association between the two specific exploration behaviors (Fig. 3F,G, H). The "hairpin" linking Edge-Sniff and Head-Dip becomes considerably lower and, therefore, their degree of association (on the y-axis) is greater. For both diazepam and FG7142 it is possible to observe, as in their respective control groups, that generalized exploration behaviors are strongly linked each other and well separated from focused exploration. Except for the two higher diazepam doses, for the reasons just above mentioned, all the representations underline that Head-Dip and Edge-Sniff share significant relationships but, at the same time, do remain quite detached from the other behaviors. Such a finding is well in agreement with studies indicating that the exploration of the hole can be considered independent from general locomotion in the HB apparatus [95–97].

3.3. The temporal dimension: T-pattern detection and analysis

A consistent advantage of transition matrices and their elaborations such as dendrograms and path diagrams is the possibility to present behavioral patterns straightforwardly and intuitively. Nevertheless, such a great benefit does represent also, to some extent, their weakness. Even at a very first glance of Fig. 3, indeed, it is possible to guess the main shortcoming of the approach based on transition matrices: the complete lack of information on the temporal characteristics of the described behavior. In other terms, dendrograms in Fig. 3 and, more in general, all elaborations based on transition matrices, are inclusive portraits of the whole observation time window: static representations of the behavior of each group within the whole observation period. However, the idea that the temporal dimension should always be considered in behavioral analysis is not new nor unknown. On this subject, indeed, Prof I. Eibl-Eibesfeldt, one of the fathers of modern Ethology, clearly underlined that "*Behavior consists of patterns in time. Investigations of behavior deal with sequences that, in contrast to bodily characteristics, are not always visible*" [125]. Such a concept represents a central dogma in modern behavioral sciences. These words have also an implicit meaning: behavioral sequences, unfolding over time, are normally hidden and very difficult to be detected for the naked eye.

To understand how difficult is to recognize sequences of events over time and, at the same time, how easy is to disregard what actually flows just under our nose and eyes, a simple example can be illuminating. In Fig. 4 two distinct lines of events are represented: consider each letter an individual behavior carried out by a rat during its activities; now focus your attention only on the lower row, the one closest to the axis and, at the same time, do cover the letters contained in the upper row: although there is a short repeated sequence, when the upper row is covered it will be difficult to identify this sequence, even if we had already seen it shortly before. This example must lead to an important consideration: if it is so difficult to find a simple sequence in a two-dimensional space containing only fifty letters, how difficult it can be to find sequences in the real behavior of a living subject, i.e. sequences of events, *thousands of events*, which, by their own nature, flow over time?

Using an approach known as *T-pattern detection and analysis (TPA)*, it is possible to address these difficulties. TPA is a technique able to reveal sequences of events in time based on the detection of statistically significant constraints among the events in sequence. Let's imagine "x…t…j…a…f…" (Fig. 4, letters near the x-axis) is a sequence of hypothetical behavioral events occurring within a given observation time window T0-Tx. A software algorithm compares the distribution of each pair of events, e.g. "a" and "b", searching for a time interval so that "a" is followed by "b" within such an interval. As underlined by Prof. M.S. Magnusson, i.e. the creator of TPA, "*if A is an earlier and B a later component […], then, after an occurrence of A at t, there is an interval [t + d1, t + d 2] (d2 ≥ d1 ≥ 0) that tends to contain at least one occurrence of B more often than would be expected by chance. This relation is here called a critical interval*" [126]. In brief, it is not sufficient that "b" simply follows "a"; the event "b" *must* fall within the specified time window after "a"; on the contrary, if "b" follows "a" but it is outside such an interval the relationship is not considered. At the end of such an initial search run, all the detected "a"→"b" sequences will represent first-level T-patterns and indicated as (a b). In a second step, (a b) patterns will be utilized as possible terms for higher order detections, e.g. ((a b) c), and so on. The detection process continues up to any level. When no more patterns are present the detection process stops. TPA does require a software tool known as THEME (Patternvision Ltd, Reykjavik, Iceland). Within this program, before starting the detection process, the utilization of specific search parameters is required. These detection parameters should be selected based on the subject(s) and the data set(s) analyzed [127]. A good starting point for the researcher approaching TPA for the first time is to read up on the theoretical aspects and basic concepts, many of which can be found in the works produced by Prof. Magnusson himself, i.e. the Author of T-patterns and TPA [126,128–133]. During the last decades TPA has been successfully applied in several research areas and it can offer very useful results in studies involving both animal and human subjects. For example, using TPA it has been possible to study route-tracing stereotypies in mice [134], rodent behavior in a model of Tourette's syndrome [135] and in a model of Parkinson's disease [136]; feeding behavior in rodents [109], neuropsychiatric diseases [137–140], the interactions between humans and artificial agents [141] or between humans and animals [142], interactions between hormones and behavior [143], movement and behavioral disorders in human and animals [144], the behavior of non-human primates [145–147], behavioral and neurochemical changes in genetic absence epilepsy rats from Strasbourg and non-epileptic controls [148]. Finally, and importantly, T-pattern analysis has shown its usefulness and versatility also in the study of rodent anxiety and anxiety-related behaviors in the HB and in the Elevated Plus Maze [31–33,35,79,82,83,86-88].

As to the HB, using TPA it has been demonstrated that the behavioral patterning between Head-Dip and Edge-Sniff is characterized by an

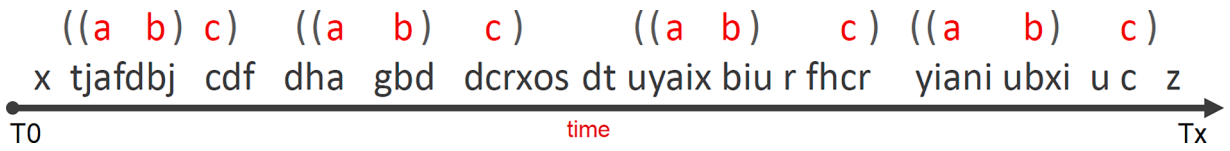
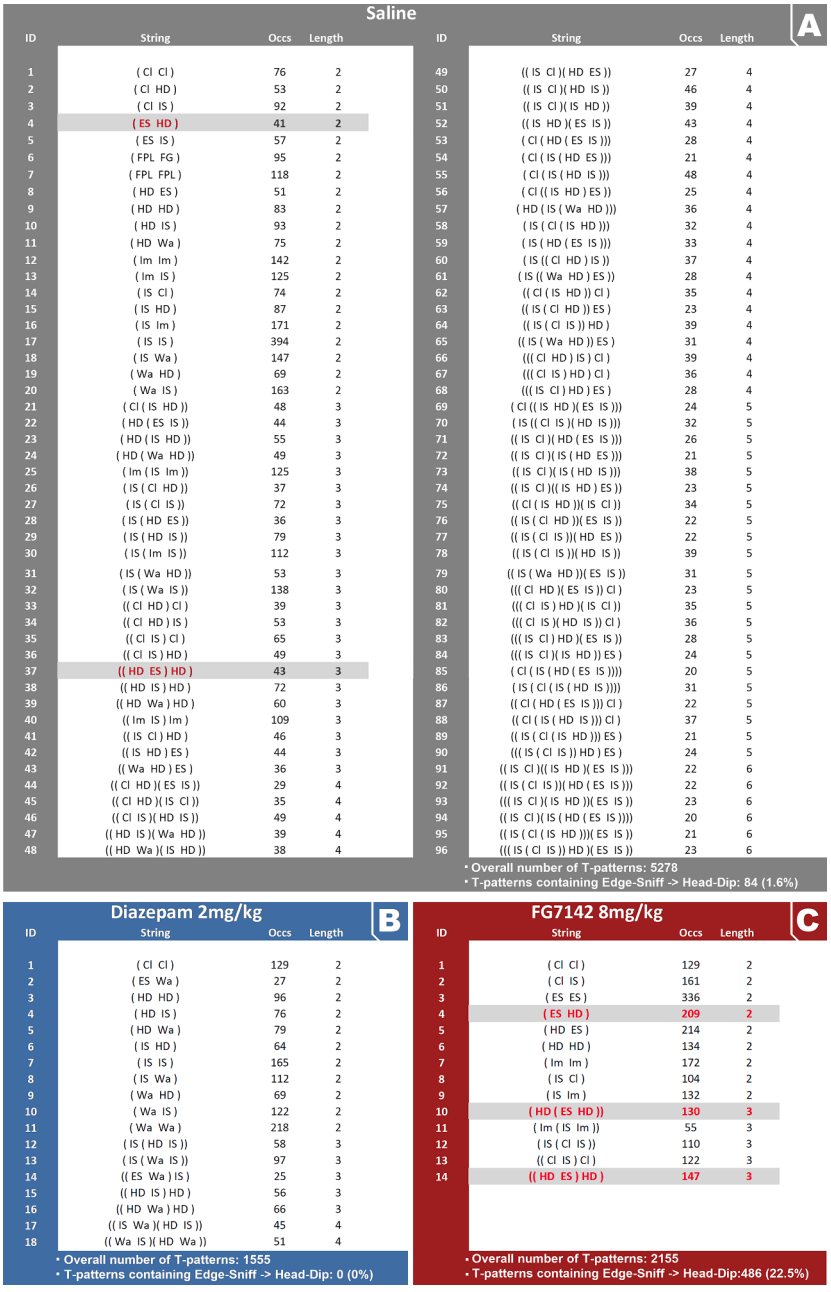


Fig. 4. Time observation window T0-Tx encompassing 50 hypothetical behavioral events (letters) occurring during the given observation period. The ((a b) c) T-pattern becomes evident if all the remaining behaviors are left out.

evident temporal organization [79] heavily influenced by acute [83] or chronic [86] nicotine administration and by the lesion of central structures such as the epithalamic lateral habenula [83,87]. Hence, the evaluation of the existing constraints between Head-Dip and Edge-Sniff, whether they lie in the bivariate relationship HD/ES, or are contextualized in the structure of a transition matrix or, finally, in the structure of T-patterns, is a very useful tool for assessing the rodent's level of anxiety. As to substances with anxiolytic or anxiogenic activity, such as diazepam and FG7142, Fig. 5 highlights the results of TPA in three groups of rats, treated with saline and the two molecules just mentioned.

3.4. The temporal dimension: When directions matter

It is in the succession of events, i.e.in their *consecutio*, the essential key to interpret what has the most value for the observer [1]. What we observe in Fig. 5 is a set of sequences of events, that is, the T-patterns for each observed group. It is easy to notice that Edge-Sniff and Head-Dip are very well represented. Based on what we have just underlined, on the importance of behavioral sequences, T-patterns containing Edge-Sniff → Head-Dip strings will by no means be equivalent to T-patterns containing Head-Dip → Edge-Sniff. The sniffing of the edge of the hole has a different value if done before or after the Head-Dip: in the first instance (Edge-Sniff → Head-Dip) it will represent the cautious expression of the specific exploration before the insertion of the head into the internal. On this subject, indeed, it has been suggested that the collection of olfactory cues from the edge of the hole is critical for the rodent to be reassured before the hole is explored inside [84]. On the other hand, the opposite transition (Head-Dip → Edge-Sniff) will be the obvious conclusion of the exploration once the animal has explored the inside of the hole and pulled out its head [78,84]. Between these two functionally



Saline — A

ID	String	Occs	Length
1	(Cl Cl)	76	2
2	(Cl HD)	53	2
3	(Cl IS)	92	2
4	(ES HD)	41	2
5	(ES IS)	57	2
6	(FPL FG)	95	2
7	(FPL FPL)	118	2
8	(HD ES)	51	2
9	(HD HD)	83	2
10	(HD IS)	93	2
11	(HD Wa)	75	2
12	(Im Im)	142	2
13	(Im IS)	125	2
14	(IS Cl)	74	2
15	(IS HD)	87	2
16	(IS Im)	171	2
17	(IS IS)	394	2
18	(IS Wa)	147	2
19	(Wa HD)	69	2
20	(Wa IS)	163	2
21	(Cl (IS HD))	48	3
22	(HD (ES IS))	44	3
23	(HD (IS HD))	55	3
24	(HD (Wa HD))	49	3
25	(Im (IS Im))	125	3
26	(IS (Cl HD))	37	3
27	(IS (Cl IS))	72	3
28	(IS (HD ES))	36	3
29	(IS (HD IS))	79	3
30	(IS (Im IS))	112	3
31	(IS (Wa HD))	53	3
32	(IS (Wa IS))	138	3
33	((Cl HD) Cl)	39	3
34	((Cl HD) IS)	53	3
35	((Cl IS) Cl)	65	3
36	((Cl IS) HD)	49	3
37	((HD ES) HD)	43	3
38	((HD IS) HD)	72	3
39	((HD Wa) HD)	60	3
40	((Im IS) Im)	109	3
41	((IS Cl) HD)	46	3
42	((IS HD) ES)	44	3
43	((Wa HD) ES)	36	3
44	((Cl HD)(ES IS))	29	4
45	((Cl HD)(IS Cl))	35	4
46	((Cl IS)(HD IS))	49	4
47	((HD IS)(Wa HD))	39	4
48	((HD Wa)(IS HD))	38	4
49	((IS Cl)(HD ES))	27	4
50	((IS Cl)(HD IS))	46	4
51	((IS Cl)(IS HD))	39	4
52	((IS HD)(ES IS))	43	4
53	(Cl (HD (ES IS)))	28	4
54	(Cl (IS (HD ES)))	21	4
55	(Cl (IS (HD IS)))	48	4
56	(Cl ((IS HD) ES))	25	4
57	(HD (IS (Wa HD)))	36	4
58	(IS (Cl (IS HD)))	32	4
59	(IS (HD (ES IS)))	33	4
60	(IS ((Cl HD) IS))	37	4
61	(IS ((Wa HD) ES))	28	4
62	((Cl (IS HD)) Cl)	35	4
63	((IS (Cl HD)) ES)	23	4
64	((IS (Cl IS)) HD)	39	4
65	((IS (Wa HD)) ES)	31	4
66	(((Cl HD) IS) Cl)	39	4
67	(((Cl IS) HD) Cl)	36	4
68	(((IS Cl) HD) ES)	28	4
69	(Cl ((IS HD)(ES IS)))	24	5
70	(IS ((Cl IS)(HD IS)))	32	5
71	((IS Cl)(HD (ES IS)))	26	5
72	((IS Cl)(IS (HD ES)))	21	5
73	((IS Cl)(IS (HD IS)))	38	5
74	((IS Cl)((IS HD) ES))	23	5
75	((Cl (IS HD))(IS Cl))	34	5
76	((IS (Cl HD))(ES IS))	22	5
77	((IS (Cl IS))(HD ES))	22	5
78	((IS (Cl IS))(HD IS))	39	5
79	((IS (Wa HD))(ES IS))	31	5
80	(((Cl HD)(ES IS)) Cl)	23	5
81	(((Cl IS) HD)(IS Cl))	35	5
82	(((Cl IS)(HD IS)) Cl)	36	5
83	(((IS Cl) HD)(ES IS))	28	5
84	(((IS Cl)(IS HD)) ES)	24	5
85	(Cl (IS (HD (ES IS))))	20	5
86	(IS (Cl (IS (HD IS))))	31	5
87	((Cl (HD (ES IS))) Cl)	22	5
88	((Cl (IS (HD IS))) Cl)	37	5
89	((IS (Cl (IS HD))) ES)	21	5
90	(((IS (Cl IS)) HD) ES)	24	5
91	((IS Cl)((IS HD)(ES IS)))	22	6
92	((IS (Cl IS))(HD (ES IS)))	22	6
93	(((IS Cl)(IS HD))(ES IS))	23	6
94	((IS Cl)(IS (HD (ES IS))))	20	6
95	((IS (Cl (IS HD)))(ES IS))	21	6
96	(((IS (Cl IS)) HD)(ES IS))	23	6

• Overall number of T-patterns: 5278
• T-patterns containing Edge-Sniff -> Head-Dip: 84 (1.6%)

Diazepam 2mg/kg — B

ID	String	Occs	Length
1	(Cl Cl)	129	2
2	(ES Wa)	27	2
3	(HD HD)	96	2
4	(HD IS)	76	2
5	(HD Wa)	79	2
6	(IS HD)	64	2
7	(IS IS)	165	2
8	(IS Wa)	112	2
9	(Wa HD)	69	2
10	(Wa IS)	122	2
11	(Wa Wa)	218	2
12	(IS (HD IS))	58	3
13	(IS (Wa IS))	97	3
14	((ES Wa) IS)	25	3
15	((HD IS) HD)	56	3
16	((HD Wa) HD)	66	3
17	((IS Wa)(HD IS))	45	4
18	((Wa IS)(HD Wa))	51	4

• Overall number of T-patterns: 1555
• T-patterns containing Edge-Sniff -> Head-Dip: 0 (0%)

FG7142 8mg/kg — C

ID	String	Occs	Length
1	(Cl Cl)	129	2
2	(Cl IS)	161	2
3	(ES ES)	336	2
4	(ES HD)	209	2
5	(HD ES)	214	2
6	(HD HD)	134	2
7	(Im Im)	172	2
8	(IS Cl)	104	2
9	(IS Im)	132	2
10	(HD (ES HD))	130	3
11	(Im (IS Im))	55	3
12	(IS (Cl IS))	110	3
13	((Cl IS) Cl)	122	3
14	((HD ES) HD)	147	3

• Overall number of T-patterns: 2155
• T-patterns containing Edge-Sniff -> Head-Dip:486 (22.5%)

Fig. 5. Terminal strings of the T-patterns detected in saline (A), diazepam 2 mg/kg (B) and FG7142 8 mg/kg (C) groups. Numbers on the right of each string indicate their overall occurrences (Occs column) and length. Highlighted in light gray: T-patterns containing Edge-Sniff → Head-Dip transitions. See Fig. 1 for abbreviations. *Modified from Casarrubea* et al. *[88].*

different sequences, the one expressing the subject's emotional substrate is Edge-Sniff → Head-Dip because, simply stated, if an Edge-Sniff will be followed by a Head-Dip or not strictly depends on emotional and motivational loads, both heavily influenced by subject's anxiety level. That said, when T-patterns containing Edge-Sniff → Head-Dip sequences are evaluated, it is relatively easy to notice that in the group treated with saline only two T-patterns contain Edge-Sniff → Head-Dip sequences, for a total of 84 T-patterns, that is 1.6% are present; diazepam administered group never presents an Edge-Sniff → Head-Dip sequences but only Head-Dips contextualized within sequences of generalized exploration with Walking and / or Immobile- Sniffing; finally, in FG7142 group it is possible to observe that three T-patterns contain Edge-Sniff → Head-Dip, for a total of 486 total recurrences, that is 22.5%. It is evident that T-patterns containing Edge-Sniff → Head-Dip sequences perfectly reflect the nature of the molecule/compound injected: compared to the animals treated with saline a complete disappearance of the Edge-Sniff → Head-Dip can be observed in animals under diazepam. On the contrary, a significant increase of this patterning is present in animals treated with FG7142, strong anxiety-inducing compound.

The direction of transition between the two behavioral components and the possibility to carefully evaluate it is, therefore, what basically distinguishes the Edge-Sniff → Head-Dip sequences, found in T-patterns, from the patterning between the two components described by the Head-Dip / Edge-Sniff ratio (Fig. 2) or by the similarity values described in dendrograms (Fig. 3). Actually, this is not a minor detail because such a directionality, from *Edge-Sniff to Head-Dip, epitomizes the behavioral expression of the underlying emotional/motivational activity that can be influenced by anxiety and related pharmacological manipulations*.

4. Conclusion

This review has explored the HB in an attempt to shed new light on this behavioral assay. We emphasized that the patterning between Edge-Sniff and Head-Dip has an important ethological meaning because it represents the behavioral expression of an underlying motivational and emotional activity. The evaluation of the patterning between these two components of the behavioral repertoire is a more sensible indicator to study anxiety-related behaviors than Head-Dip simple quantitative evaluations and should be utilized to assess the emotional profile of rodents following manipulation/changes of the anxiety level. In addition, the assessment of the comprehensive structure of behavior in the HB, rather than individual components detached from the actual behavioral architecture, is able to provide an exhaustive portrait of the anxiety-related behavioral profile.

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Ethical statement

All animal procedures described in this review were approved and carried out following University of Palermo ethical guidelines and in conformity with Italian and international laws and policies (EU Directive, 2010/63/EU for animal experiments, ARRIVE guidelines, and the Basel declaration including the 3R concept). All efforts were made to minimize animal suffering and to reduce the number of animals used.

CRediT authorship contribution statement

Maurizio Casarrubea: Conceptualization, Formal analysis, Investigation, Data curation, Methodology, Writing – original draft, Writing – review & editing. **Giuseppe Di Giovanni:** Investigation, Writing – original draft, Writing – review & editing. **Stefania Aiello:** Investigation, Writing – original draft, Writing – review & editing. **Giuseppe Crescimanno:** Investigation, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare no competing interests.

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