Commentary

Structural Analyses in the Study of Anxiety and Anxiety-Related Behaviour

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1 Anxiety and Behaviour

According to the latest Diagnostic and Statistical Manual of Mental Disorders, 5th edition, anxiety encompasses various conditions sharing an excessive sense of fear and/or apprehension for no evident reason *and related behavioural disturbances* (Association, 2013). The association of such a gloomy symptomatology, with the great diffusion in the general population, explains the critical impact of anxiety disorders on inter-personal relationships and job-related activities (Greenberg et al., 1999; Wittchen & Hoyer, 2001; Keeley & Storch, 2009). Hence, anxiety disorders represent an important and consistent topic of discussion, not only in terms of underlying neuro-psychological processes but, importantly, also in terms of behavioural dynamics. In such a context, behavioural neurosciences play a central role in understanding anxiety. Different assays are available to study the characteristics of anxiety-related behaviour, such as the open-field (OF), the hole-board (HB) and the Elevated Plus Maze (EPM).

Essentially, an OF consists of an enclosed area where freely moving rodents are observed for a limited period. The OF is commonly employed to study exploration (Drai, Kafkafi, Benjamini, Elmer & Golani, 2001) and anxiety (Choleris, Thomas, Kavaliers & Prato, 2001). The rationale supporting the utilization of the open field in the study of anxiety lies in the natural aversion of rats and mice for novel environments. Indeed, once placed in the OF, rodents spontaneously prefer the periphery, remaining near to the surrounding walls. An increase of time spent in the central zone, increase of the ratio central/total locomotion or the decrease of the latency to enter the central zone represent widely accepted indexes of anxiolysis (Choleris et al., 2001).

The HB is another exploration-based assay, which is well known and commonly used to examine anxiety-related behaviours of rodents (Adamec, Head, Blundell, Burton & Berton, 2006; File & Wardill, 1975a, 1975b; Rodriguez Echandia, Broitman & Foscolo, 1987; Harada et al., 2006; Saitoh et al., 2006; Kalueff, Wheaton & Murphy, 2007; Casarrubea, Sorbera & Crescimanno, 2009b; Kamei et al., 2007). This experimental apparatus generally consists of a square or rectangular arena with a variable number of holes in the ground (Hughes, 2007; File & Wardill, 1975a, 1975b) where the rodent can insert its head. Excluding modified HBs (Ohl, Holsboer & Landgraf, 2001), the presence of the holes represents the essential difference between an OF and a HB. The rationale of the utilization of HB in the study of anxiety classically orbits around the head-dip behaviour. In brief, changes of head-dipping (frequency, latency, duration) are assumed to reflect the anxiety state of the subject: anxiety-inducing drugs decrease both the number and duration of head-dips (Takeda, Tsuji & Matsumiya, 1998), on the other hand, anxiolytic molecules increase head-dips (Takeda et al., 1998).

Finally, the EPM, with thousands of published papers so far, needs little introduction, due to it being the most used experimental assay in the study of anxiety. This apparatus, introduced by Handley and Mithani more than three decades ago (Handley & Mithani, 1984), consists of an elevated plus-shaped platform with two open and two enclosed arms. EPM usefulness has spread towards the understanding of the biological basis of emotionality related to learning and memory, hormones, addiction, and withdrawal (Carobrez & Bertoglio, 2005). The closed arms are surrounded by 50 cm walls and open arms have 0.5 cm edges in order to facilitate entries in the arm (Treit, Menard & Royan, 1993). The rationale underlying the utilization of EPMs in anxiety research is

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based on the assumption that rodents will respond to a conflict elicited by the presence of safe parts of the maze (closed and protected), and aversive parts of the maze (open, unprotected and more brightly lit) (Carobrez & Bertoglio, 2005).

2 Assessing the existence of an underlying structure

In general, a structure exists if a set of relationships of any kind can be demonstrated among the components of a given system. Thus, in terms of behavioural analyses, a structure exists only if it is possible to demonstrate the existence of relationships among the activities performed by the subject. It goes without saying that such a demonstration can be extremely challenging if the structure is hidden to the observer, as is the circumstance of animal/human behaviour: as underlined by Eibl-Eibesfeldt, indeed, *"Behaviour consists of patterns in time. Investigations of behaviour deal with sequences that, in contrast to bodily characteristics, are not always visible"* (Eibl-Eibesfeldt, 1970).

The first step is, normally, represented by the construction of a suitable ethogram, that is, a formal description of the components that were taken into consideration. Fig. 1 and Fig. 2 represent ethograms concerning rats' activities in the HB and in EPM respectively. On the basis of the ethogram, video files recorded during experimental sessions need to be observed by means of a suitable software coder, that is, a computer program allowing to record subject's activities in a file. The event log file does represent the essential basis for the all the following analyses.

Hierarchical clustering, stochastic analysis and adjusted residuals analysis are methods based on the elaboration of transition matrices. T-pattern analysis, on the other hand, is a technique able to evaluate the temporal characteristics of sequences of events. Overall, these approaches do represent excellent tools to study behaviour in terms of structural characteristics.

Concerning transition matrix-based approaches, the first step is represented by the construction of a transition matrix (TM) from raw data. Of course, the utilization of a specific software aimed at matrices construction prevents possible errors in TM handling and analysis.

– *Hierarchical Clustering*: the aim of such a procedure is to represent similarities among components, by means of a specific aggregative algorithm. Such a procedure can be either agglomerative (i.e. bottom-up) or divisive (i.e. top-down). Agglomerative bottom-up procedure merges discrete components into successively larger clusters.

– *Stochastic analyses*: the aim of the stochastic techniques is to emphasize probabilistic relationships among behavioural components. On the basis of
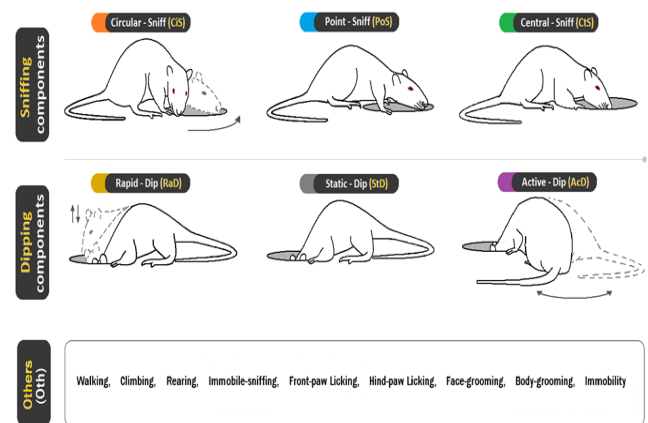

Figure 1: Ethogram of rat behaviour in the hole-board. **Point-Sniff** (PoS) = rat sniffs a single point of the hole-edge; **Circular-Sniff** (CiS) = rat sniffs hole-edge in a continuous circular fashion; **Central-Sniff** (CtS) = rat sniffs hole centre without inserting its head inside; **Rapid-Dip** (RaD) = rat rapidly puts into (eyes no more visible) and removes its head from the hole (no pause between head inserting and removing); **Static-Dip** (StD) = rat puts and maintains its head into hole (eyes no more visible, body maintained in a fixed position); **Active-Dip** (AcD) = rat puts its head into hole (eyes no more visible, body movements produced); **Others** (Oth) = behavioral components not related with hole-exploration. Modified from (Casarrubea, Sorbera, Santangelo & Crescimanno, 2010).
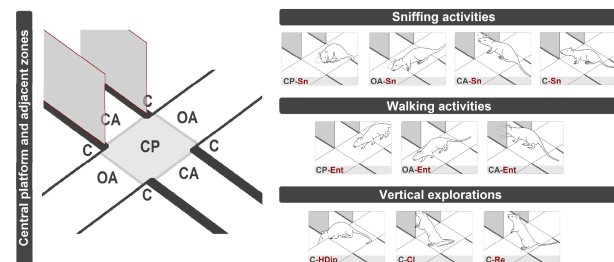

Figure 2: Ethogram of rat behaviour in the central platform of the EPM. Left panel: Only the walls of one closed arm have been represented. CP = Central Platform Area; CA = Closed Arm Zones; OA = Open Arm Zones; C = Corner Zones (closed-open arm junction and external 90° angle comprised between the two arms). Right panel: CP-Sn = Central Platform Sniffing: the rat sniffs the ground of the central platform; OA-Sn = Open Arm Sniffing: the rat sniffs the entrance of one of the two open arms; CA-Sn = Closed Arm Sniffing: the rat sniffs the entrance of one of the two closed arms; C-Sn = Corner Sniffing: the rat sniffs the Plexiglas border of one of the four corners; CP-Ent = Central Platform Entry: the rat moves from an open or from a closed arm to the central platform; OA-Ent = Open Arm-Entry: the rat moves from the central platform to one of the two open arms; CA-Ent = Closed Arm Entry: the rat moves from the central platform to one of the two closed arms; C-HDip = Corner Head Dip: the rat, from one of the four corners, performs scanning head movements in the direction of the floor; C-Cl = Corner Climbing: the rat maintains an erect posture leaning against the Plexiglas border of one of the four corners; C-Re = Corner Rearing: the rat, without leaning against the Plexiglas, maintains an erect posture, facing one of the four corners. Modified from (Casarrubea, Faulisi, Sorbera & Crescimanno, 2015).

the relative frequencies of transitions among the components, a transition matrix can be transformed into a probability matrix.

Four qualifications need to be strictly respected within a probability matrix:

(a) each row must sum 1;

(b) all components must be between 0 and 1;

(c) 0 means no transition between two given components in the originating TM;

(d) switching probability from a component to all others is 1.

– *Adjusted residuals*: an elegant system to assess the significance of cells within matrices has been used by Spruijt and Colleagues (Spruijt & Gispen, 1984) and, after, by different Authors (Casarrubea, Sorbera, Santangelo & Crescimanno, 2010; van den Berg, van Ree & Spruijt, 1999; van Lier, Coenen & Drinkenburg, 2003; Vanderschuren, Spruijt, Hol, Niesink & Van Ree, 1996). Positive residuals indicate transitions occurring more often than expected and negative residuals represent transitions occurring less often than expected. A consistent advantage of adjusted residuals is that they can be expressed according to a Z-distribution, so that p-values can be easily found in a common Z-table and, as a consequence, values $\geq +1.96$ and ≤ -1.96 reveal significant transitions ($p \leq 0.05$).

– *T-patterns detection and analysis*: both in terms of conceptual and procedural aspects, T-pattern analysis is completely different from the above discussed approaches utilizing transition matrices. This analytical technique can be performed using a specific software algorithm, which is able to search for relationships among events in behavioural data by taking into account order, timing, and frequency of these events (Casarrubea, Sorbera, Magnusson & Crescimanno, 2010; Casarrubea, Jonsson et al., 2015; Casarrubea, Faulisi, Magnusson & Crescimanno, 2016). In brief the algorithm compares the distributions of each pair of events (for instance, "A" and "B"), searching for an interval so that, more often than chance expectation, A is followed by B *within* that interval. In a second step, such a T-pattern of first level is considered as a potential starting point for the construction of higher-order t-patterns, e.g., ((A B) C) etc. When no more patterns are found, the search stops (Casarrubea, Jonsson et al., 2015; Casarrubea, Faulisi, Magnusson & Crescimanno, 2016).

3 Modelling the structure: graphical representations

However, independently from the utilized approach, the existence of relationships among the elements of behaviour calls for the possibility to illustrate such relationships. This is an important step. Indeed, transition matrices and related elaborations (similarity, probability and adjusted residuals matrices) are, basically, tables filled with hundreds of numbers. In most cases, a matrix is quite useless in its original form because the meaning of each transition is often difficult to be appreciated. These critical issues are even more amplified with T-pattern analysis, since the detection of T-patterns implies the existence of significant relationships in the course of time. In brief, graphical representations play an essential role in illustrating experimental results.

– *Hierarchical Clustering*: as with the similarity matrix, each cell is representative of a correlation between two given components, on the basis of the reciprocal number of transitions. Hence, a similarity matrix is not a classic "from-to" matrix, but a half-matrix. On the basis of such a half similarity matrix, a dendrogram can be obtained. A dendrogram is a graphical representation showing, by means of a tree structure how much some components are similar one another. An example of a dendrogram, illustrating similarities among behaviours of the rat in the HB, is presented in Fig. 3.
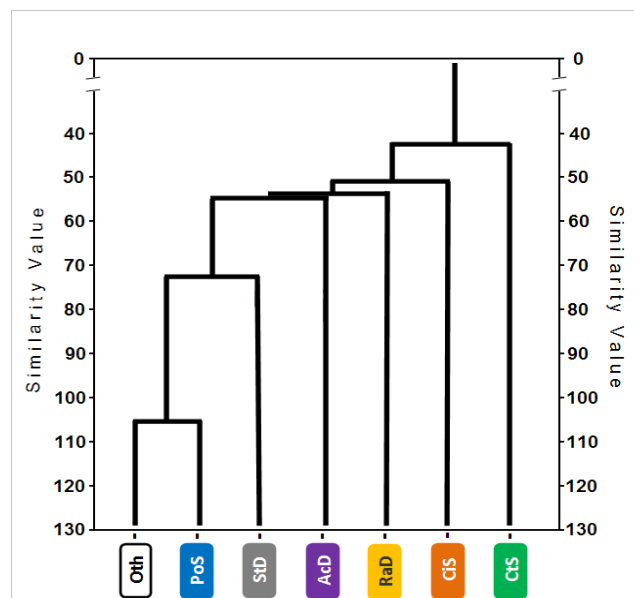

Figure 3: Dendrogram of rat hole exploratory activities representing correlations among behavioural components on the basis of the reciprocal number of transitions. For abbreviations see Fig. 1.

– *Stochastic analyses*: a probability matrix is a normal transition matrix, which can be conveniently expressed through a path diagram where different transition probabilities are represented by connecting arrows of different thickness. Thus, by means of stochastic approach both directions and probabilities among components can be expressed together. Fig. 4 shows, by means of a path diagram, the probabilistic relationships among the behavioural components performed by the animal in the HB.

– *Adjusted residuals*: like probability matrices, adjusted residuals matrices can be illustrated by means of path diagrams. However, this approach might generate, from a conceptual point of view, confounding representations because, if on the one hand, transitions occurring significantly more often than expected can be represented by means of arrows, on the other hand, transitions occurring significantly less often than expected should not. Thus, the representation of positive and negative residuals, by means of histograms, can be profitably employed (Casarrubea, Sorbera & Crescimanno, 2008, 2009b, 2009c, 2009a; Casarrubea, Sorbera, Santangelo & Crescimanno, 2010; Casarrubea, Faulisi, Sorbera & Crescimanno, 2015). Adjusted residuals in Fig. 5 show the association strength of all the detected transitions among the behavioural components performed by the animal in the HB. Positive and negative bars do indicate transitions occurring respectively more and less often than expected.

– *T-Patterns*: T-patterns can be represented by means of tree structures, emphasising significant relationships among events in the course of time. These tree structures, to some extent similar to dendrograms, have the advantage to show patterns distribution along time and, importantly, are very intuitive. The drawback is the huge amount of space required. For instance, concerning results in Fig. 6, overall, 554 patterns do occur in Wistar rats and 792 in DA/Han. In other terms, the illustration of their occurrences by means of the trees illustrated on the right side of Fig. 6, is not an option to be considered. For these reasons we have developed the representation of T-patterns by means of behavioural stripes, that is, the illustration of the onset of each T-pattern, along the x–axis timeline, by means of a simple rasterplot (Casarrubea, Sorbera & Magnusson, M.S Crescimanno, 2011; Casarrubea, Faulisi, Magnusson & Crescimanno, 2016; Casarrubea, Faulisi, Caternicchia et al., 2016).
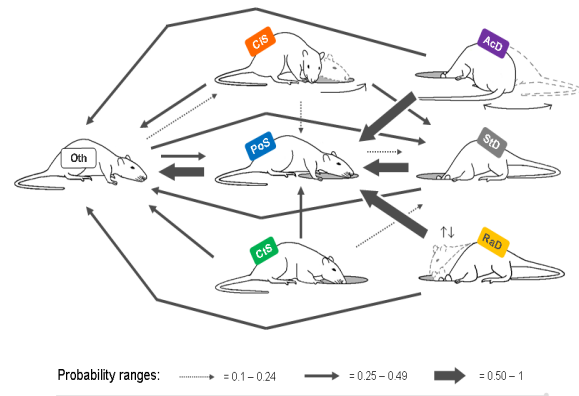
Figure 4: Path diagram representing transition probabilities among behavioural components. Selected probability ranges are indicated at the bottom. For abbreviations see Fig. 1.

Figure 5: Adjusted residuals. Top of each panel: behavioural elements antecedent to the ones indicated along x-axes. y-axes: adjusted residuals values. Black bars: positive residuals. Grey bars: negative residuals. Empty circles = structural zeroes. According to Z-table, * = significant $p \leq 0.05$ transitions.

Wistar

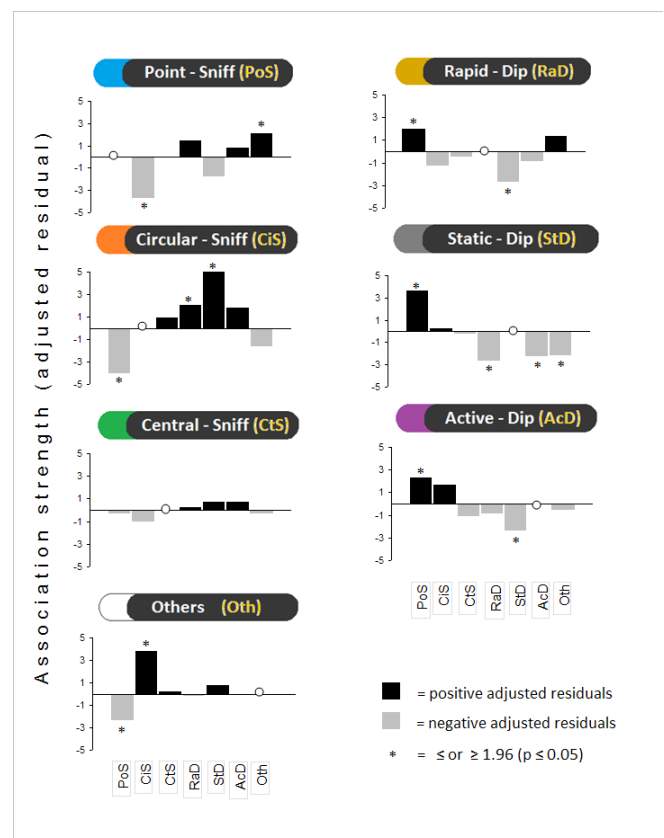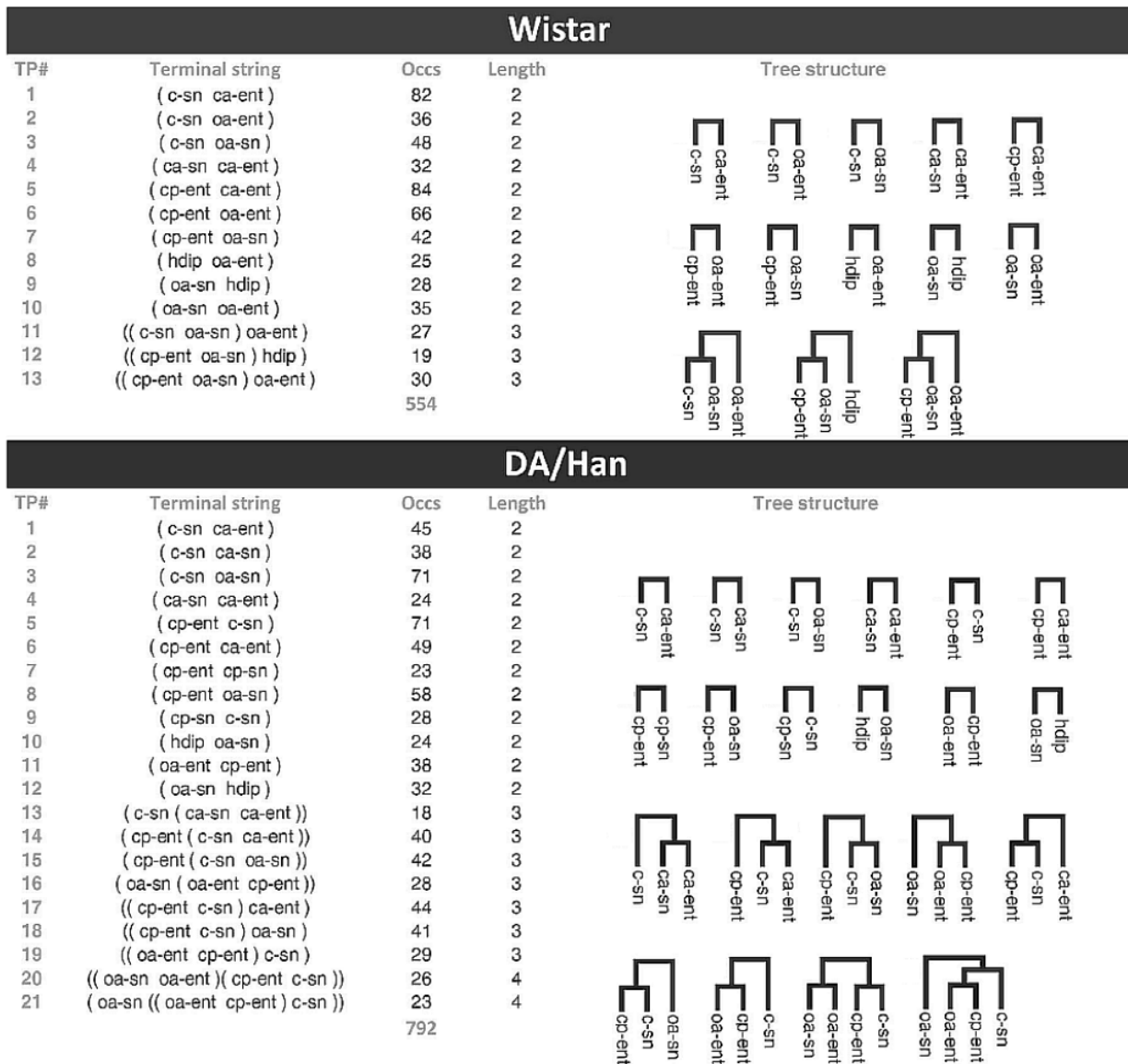| TP#	Terminal string	Occs	Length	Tree structure
1	(c-sn ca-ent)	82	2	
2	(c-sn oa-ent)	36	2	
3	(c-sn oa-sn)	48	2	
4	(ca-sn ca-ent)	32	2	
5	(cp-ent ca-ent)	84	2	
6	(cp-ent oa-ent)	66	2	
7	(cp-ent oa-sn)	42	2	
8	(hdip oa-ent)	25	2	
9	(oa-sn hdip)	28	2	
10	(oa-sn oa-ent)	35	2	
11	((c-sn oa-sn) oa-ent)	27	3	
12	((cp-ent oa-sn) hdip)	19	3	
13	((cp-ent oa-sn) oa-ent)	30	3	
		554		

DA/Han

TP#	Terminal string	Occs	Length	Tree structure
1	(c-sn ca-ent)	45	2	
2	(c-sn ca-sn)	38	2	
3	(c-sn oa-sn)	71	2	
4	(ca-sn ca-ent)	24	2	
5	(cp-ent c-sn)	71	2	
6	(cp-ent ca-ent)	49	2	
7	(cp-ent cp-sn)	23	2	
8	(cp-ent oa-sn)	58	2	
9	(cp-sn c-sn)	28	2	
10	(hdip oa-sn)	24	2	
11	(oa-ent cp-ent)	38	2	
12	(oa-sn hdip)	32	2	
13	(c-sn (ca-sn ca-ent))	18	3	
14	(cp-ent (c-sn ca-ent))	40	3	
15	(cp-ent (c-sn oa-sn))	42	3	
16	(oa-sn (oa-ent cp-ent))	28	3	
17	((cp-ent c-sn) ca-ent)	44	3	
18	((cp-ent c-sn) oa-sn)	41	3	
19	((oa-ent cp-ent) c-sn)	29	3	
20	((oa-sn oa-ent)(cp-ent c-sn))	26	4	
21	(oa-sn ((oa-ent cp-ent) c-sn))	23	4	
		792		

Figure 6: Results of T-pattern analysis in two strains of rats with different emotional reactivity, namely, Wistar and DA/Han strain. TP# = T-pattern identification number; Terminal String = text representation of events in pattern; Occs = number of occurrences of the given pattern; Length = number of events in pattern. Tree structures of detected patterns are illustrated on the right side of the figure. The analysis revealed a very different behavioural structure of Wistar and DA/Han. See Fig. 2 for abbreviations. Modified from (Casarrubea, Faulisi, Magnusson & Crescimanno, 2016).

4   Considerations

Along the last decades, behavioural research on anxiety has been quite conservative in the renewal of their methods. This crucial aspect, examined by several Authors, is not new nor unknown. For instance, Kalueff and co-Workers (Kalueff et al., 2007), discuss the existence of an unfortunate and chronic association consisting of the lack of: a) converging findings and b) new and/or alternative approaches in the study of anxiety and depression disorders. Actually, the largest amount of behavioural studies in the field of psychopharmacology of anxiety and depression have only utilized a small number of descriptive parameters such as latencies, durations and frequencies of individual elements.

Concerning specifically researches on anxiety, several instances demonstrate how quantitative assessments (such as frequencies, latencies, duration of isolated behavioural elements), rarely produce converging findings.

A paradigmatic example, in this sense, is represented by the head-dip. This component, well known to be heavily influenced by changes in rodent's anxiety

level (Takeda et al., 1998; Harada et al., 2006) is, of course, the *"raison d'être"* of the HB assay and, actually, what makes a HB apparatus different from an open-field. Even so, surprising diverging findings do surround this element: in fact increases (Takeda et al., 1998), decreases (Pellow, Chopin, File & Briley, 1985), or no modifications (Sayin, Purali, Ozkan, Altug & Büyükdevrim, 1992) of head-dip frequencies have been described following anti-anxiety drugs administration. Thus, whether head-dip, assessed alone, and disjointed from the whole behavioural structure, is a suitable anxiety indicator, may represent matter of discussion and/or criticism. It is my contention that behaviour is much more than a simple evaluation of disjointed elements through purely quantitative parameters (e.g. durations, frequencies or percent distributions). On one hand, quantitative approaches are useful from a descriptive point of view (since they provide precise information concerning each investigated item). On the other hand, the possibility to characterise each element through even hundreds of numbers does not imply the inverse possibility, namely the possibility to utilize those numbers to reconstruct the behaviour is in its wholeness and, importantly, its meaning (Casarrubea et al., 2009a). As a consequence, descriptive approaches to behavioural studies should be partnered, whenever is possible, with techniques able to provide information on the relationships among the elements of the behaviour. In this sense, transition matrices and T-pattern analyses may represent valuable tools.

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