

# Adequate Statistical Methods to Reduce the Number of Animals Used in Behavioural Experiments: The Analysis of the Behavioural Transitions

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**Summary** — In ethological and behavioural toxicological studies, elaborate behavioural patterns shown by the animals under well-established experimental paradigms or naturalistic conditions are routinely observed and split into single behavioural items. Subsequently, these items are analysed in terms of their frequencies and/or durations. Behavioural observations are usually videotaped and scored by dedicated softwares, which collect the sequences of behavioural items together with frequencies and durations. So far, the Cox proportional hazards model, a method originally developed for the analysis of time-to-event data, has been employed for the analysis of the time-structure of behaviour, but its usefulness has been limited because of difficulties in including random effects in the model. Recent developments in mixed models for the analysis of time-to-event data may overcome this limitation and improve the analysis of behavioural patterns. Data from social interactions in mice on the effects of exposure to chlorpyrifos, a widely used organophosphorous pesticide, are presented to illustrate the use of these new statistical methods. Our results suggest that the study of behavioural sequences may highlight the role of the investigated conditions (treatments, genetic condition, social status) in setting behavioural organisations. In addition, the *refinement* of statistical methods by time-structured analysis provides more detailed information from an experimental data set, thus contributing to the *reduction* of the number of animals used in this field of the life sciences.

**Key words:** *behavioural patterns, Cox's model, random effects, social interaction test.*

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## Introduction

In this presentation, we consider the contributions that can be made by time-structured behavioural analysis toward the refinement of the animal procedures involved and a reduction in the numbers of animals required (1).

The ethological approach offers powerful technical tools for monitoring drug-induced or toxicant-induced changes in behaviour, as well as the conceptual framework for interpreting these alterations. For these reasons, animal models are currently used in psychopharmacology and behavioural toxicology to assess the effects of drugs or environmental pollutants (2–4).

In the laboratory, adequate measurement of animal behaviour is based on well-defined descriptive categories (behavioural items) — avoiding redundancies and/or overlaps — and it generally consists of collection of data on behavioural latencies, frequencies, intensities, durations, sequences, patterns and trends (5). Nevertheless, statistical analysis is usually performed on frequencies and durations of single items, but neglects to evaluate possible treatment effects on the temporal structure of the behavioural responses. The analysis of

the temporal structure, however, could provide information on priority changes when switching from one behavioural item to another, and/or could allow one to discriminate whether an increase in the time spent on a behavioural item is due to an increase in the tendency to start it or to a decrease in inhibiting it (6, 7).

The aim of this study was to develop a new statistical method for analysing the temporal structure of behavioural responses, based on the comparisons of times of switching between behavioural items, easily available to behavioural neuroscientists.

The experimental data set used to test this statistical method came from a behavioural teratology experiment previously carried out in our laboratory. Mice of both sexes were neonatally treated daily on postnatal days (PNDs) 11–14 with chlorpyrifos (CPF), at doses (1 or 3mg/kg) that do not evoke overt systemic toxicity. CPF is a widely used organophosphorous pesticide (OP), for both agricultural and domestic purposes; in recent years, CPF has replaced many other OP pesticides due to its relative safety and persistence (8). However, the potential health effects associated with human exposure to CPF are a matter of increasing concern for infants and children, due to persistent accumu-

lation of CPF on residential surfaces and toys after household application (9). The neonatally treated animals were then measured in different behavioural tests throughout the postnatal development and underwent a social interactions test with an unfamiliar conspecific on postnatal day 45. In general, behavioural results from that experiment indicated that developmental exposure to CPF at sub-toxic doses induced long-term behavioural alterations, primarily in locomotor activity levels in a unfamiliar environment (10).

## Statistical Methods

When animal behaviour is observed in continuous time, by collecting the sequence of behavioural items and the time at which they occur, the temporal structure of behavioural responses can be analysed in terms of velocity of switching between behavioural items. The temporal relationships between behavioural responses may be modelled as a generalised Continuous Time Markov Chain (CTMC) model, where the states of the model are the behavioural items and the transition rates  $\lambda_{ij}$  represent the behavioural tendencies to switch between items  $i$  and  $j$  (11). For instance, the transition rate,  $\lambda_{\text{Self grooming Body sniffing}}$ , from Self grooming to Body sniffing represents the chance per time unit that Self grooming is followed by Body sniffing. It can be interpreted as the priority of Body sniffing while the animal grooms. In general, several behavioural items are successively displayed with the “velocity” of switching between them measured by a set of transition rates  $\lambda_{ij}$ . Moreover, the tendency to switch from the ongoing behavioural item to another one may depend on the time an item has already lasted, leading to transition rates, which vary as functions of the bout length, that is  $\lambda_{ij}(t) = f(t)$ . The times of switching between behavioural items may be seen as failure time data, i.e. data that represent times until a specific event occurs (12). Failure time data are routinely defined for the analysis of survival or mortality in epidemiological and clinical studies (13). As an example, for the switching from Self grooming to Body sniffing, for each bout the starting point is the time of starting Self grooming, and the ending point is the time of displaying Body sniffing: failure data. All other bouts starting from Self grooming contribute to censored data. In general, for the switching between behaviour A and behaviour B, for each bout, the starting point is the time of starting item A, and the ending point is the time of displaying item B (failure data) or displaying any other item (censored data).

When this approach is chosen, the effects of experimental treatments on the transition rates can be investigated by applying methods originally developed for the analysis of failure time data.

These data are usually analysed in terms of the hazard function ( $\lambda$ ; i.e. the chance per time unit until the occurrence of a failure) which corresponds, in an ethological setting, to the transition rate ( $\lambda_{ij}$ ) (6, 7). Therefore, changes in transition rates from one behavioural response to another may be assessed by the Cox proportional hazards model (a widely used tool for the analysis of failure time data), by modelling the transition rate ( $\lambda_{ij}$ ), analogously to the hazard function ( $\lambda$ ), as

$$\begin{aligned} \lambda_{ij}[t, (X_1, X_2, \dots, X_k)] \\ = \lambda_{ij0}(t) \exp[\beta_1 X_1 + \beta_2 X_2 + \dots + \beta_k X_k], \end{aligned}$$

where  $\lambda_{ij}$  is the transition rate (it represents the chance per time unit until the starting of item  $j$  while performing item  $i$ );  $t$  is the time since starting of item  $i$ ;  $(X_1, X_2, \dots, X_k)$  is a set of covariates;  $\lambda_{ij0}(t)$  is an arbitrary and unspecified baseline transition rate (it is the transition rate when the covariates are zero);  $\beta_l$  ( $l = 1, 2, \dots, k$ ) is the regression coefficient that quantifies the effect of the covariate  $X_l$  on the transition rate. In the ethological settings, covariates represent the experimental factors.

In the ethological setting, from the Cox's model representation, the relative transition rate (RTrR) of a factor  $X_i$  — i.e. the ratio between the transition rate when the factor is present and the transition rate when the factor is absent — adjusted for the effect of the other factors included in the model is measured by  $\exp[\beta_i]$ . Therefore, the tests for the significance of the regression coefficients give indications on the effects of the experimental factor on the times of switching. When an RTrR from item A to item B of a factor is significantly higher than 1, there is a higher risk of (or a faster) switching from A to B in animals that experimented the factor; on the contrary, when the RTrR is significantly lower than 1, there is a lower risk of (or a slower) switching from A to B in animals that experimented the factor.

Estimates of the regression coefficients and relative standard errors are routinely calculated by statistical software programs that implement the analysis of failure time data. Tests on the significance of the regression coefficients, however, should be carried out taking into account the random factor that characterises ethological data: each animal switches from one behavioural item to another more than once during the observation session, contributing with more than one observation for that transition. Therefore, observations of the switching between the two selected behaviours are structured as clustered within the experimental unit. Recent developments in mixed models for the analysis of time-to-event data implemented in STATA 7, and in the GLLAMM (Generalised Linear Latent and Mixed Models) procedure (14), allow one to deal with such data and to improve the analysis of the behavioural patterns. Estimates of the relative transition rates

and their statistical significance have been obtained by the statistical software STATA.

## Experimental Data and Behavioural Testing

A total of 36 male mice (12: Veh; 13: Low dose; 11: High dose) and 32 female mice (11: Veh; 10: Low dose; 11: High dose) were exposed to sub-toxic CPF doses or to vehicle on PNDs 11–14.

On PND 35, males and females underwent a 20-minute social interaction test with another animal of the same sex and of comparable age and body weight (design and procedures as in 15). The encounter took place in a test cage identical to the home cage supplied with clean sawdust bedding. Behaviour was videotaped under red light. Recordings were scored by an observer blind to the treatment received by each pair. The data were recorded using a keyboard event recorder system (Observer, Noldus, Wageningen, The Netherlands) connected to a computer for the analysis. Behavioural items are defined according to (15–17) and are described in Table 1.

Frequencies and durations of each behavioural item were automatically recorded by the software Observer 2.0 (Noldus). This software makes a file containing the chronological list of the occurrence of each behavioural act. This file was appropriately transformed in order to allow the collection of the transitions between pairs of behavioural items and time points at which they occurred, that is, the sequences of items and the time before switching. Estimates of the parameters of the Cox's model have been carried out by Stata 7.0, including the experimental unit as random factor, to take into account the correlation between measurements coming from the same subject.

## Results

### Behavioural transition patterns during the social interaction test in control animals

In Figure 1, the social interaction pattern of control animals is represented in terms of transition probabilities. In particular, only behavioural transitions in which the starting behaviour had a frequency higher than three were represented. Transitions are grouped into two classes, corresponding to different magnitude of transition probability (less frequent: from 0.10 to 0.30; more frequent: from 0.30 to 0.60). This diagram is aimed to outline the most common behavioural transitions during a social interaction test in control mice: at this age, CD-1 mice of both sexes primarily explore environmental and social cues. Interestingly, exploring represents

a sort of “convergence point” for most social and non-social responses: this is especially true for social sniffing items that are more frequently followed by exploring in both sexes. It is also worth noting that most of the behavioural transitions belong to the less frequent class, thus indicating that the animal may display a wide spectrum of behavioural responses rather than being engaged in a circumscribed sequence of few items.

### Effects of CPF on behavioural transition rates

The effects of the low CPF dose on social interaction patterns are reported in Figure 2. They appear to be different in the two sexes: treated males stayed “longer” in the social investigation category, as shown by the lower transition rates toward affiliative items (RTrR from Body sniffing to Allogrooming = 0.00; RTrR from Anogenital sniffing to Allogrooming = 0.28). The same effect was not observed in females; they entered more quickly into the environmental investigation category while they are engaged in social investigation (RTrR from Following to Exploring = 2.83).

The effects of the high CPF dose on the social interaction patterns are reported in Figure 2. Treated males stayed “longer” in behavioural items belonging to the social investigation category, as shown by the lower transition rates from nose and body sniffing toward the environmental investigation category (RTrR from Nose sniffing to Exploring = 0.75; RTrR from Body sniffing to Digging = 0.39). This effect is strengthened by the higher transition rates from self-grooming to body and anogenital sniffing (RTrR from Self grooming to Body sniffing = 1.17; RTrR from Self grooming to Anogenital sniffing = 1.67), even if these effects just missed statistical significance. In addition, treatment affected agonistic behaviour: the transition rate from escape to exploring was lower (RTrR from Escape to Exploring = 0.70), thus indicating a tendency for CPF-treated males to remain engaged in a defensive behavioural strategy. Also, treated females stayed “longer” in behavioural items belonging to the social investigation category. Transition rates from social investigation to affiliative responses decreased (RTrR from Squire to Allogrooming = 0.55; RTrR from Anogenital sniffing to Allogrooming = 0.68), whereas transition rates from exploring to anogenital sniffing increased (RTrR from Exploring to Anogenital sniffing = 1.37).

## Discussion

At a first glance, it appears that, in both sexes, CPF treatment does not affect the most common behav-

**Table 1: Behavioural items scored during the social interaction test**

<b>Auto directed behaviour</b>	
Self grooming	Wiping, licking, combing or scratching any part of own body
<b>Environmental investigation</b>	
Digging	Digging in the sawdust, pushing and kicking it around using the snout and/or both the forepaws and hind paws — mostly moving around the cage
Exploring	Moving around the cage, rearing, sniffing the air, the walls or the sawdust
<b>Social investigation</b>	
Anogenital sniffing	Sniffing the anogenital region of the partner
Nose sniffing	Sniffing the head, or the snout of the partner
Body sniffing	Sniffing any other region of the body except for the tail
Following	Following the partner around the cage, without any quick or sudden movement
Squire	Following the moving partner while maintaining a constant nose contact with its fur (mostly near the anogenital area)
Mutual circle	Partners mutually sniffing each other's anogenital region, while describing tight circles with their reciprocal following movements and maintaining close nose-anogenital contact
<b>Affiliative behaviour</b>	
Social inactive	Lying flat or standing still (with the eyes closed or open) while maintaining close physical contact with the partner, which may be, in turn, either inactive or involved in social activities
Allogrooming	Self-explanatory
<b>Soliciting behaviour</b>	
Push under	Pushing the snout or the whole anterior part of the body under the partner's body, and then resting
Crawl over	Crawling over the partner's back, crossing it transversally from one side to the other
<b>Agonistic behaviour (only males)</b>	
Attack	Rushing approach carried on over the back of the partner, often accompanied by biting attempts
Aggressive grooming	Allogrooming markedly intense, performed leaning on the partner's back with the forepaws, and accompanied by gross movements of the head and by vigorous pulling of the fur of the partner
Offensive upright posture	The mouse stands on its hind legs facing the opponent aggressively
Defensive posture	Defined by: <ol style="list-style-type: none"> <li>i) Defensive upright posture, the animal standing on its hind limbs pushing the aggressive opponent with its forepaws;</li> <li>ii) Submissive posture, the animal lies on its back, with its head directed backwards flat against the cage floor</li> </ol>
Escape	Self-explanatory

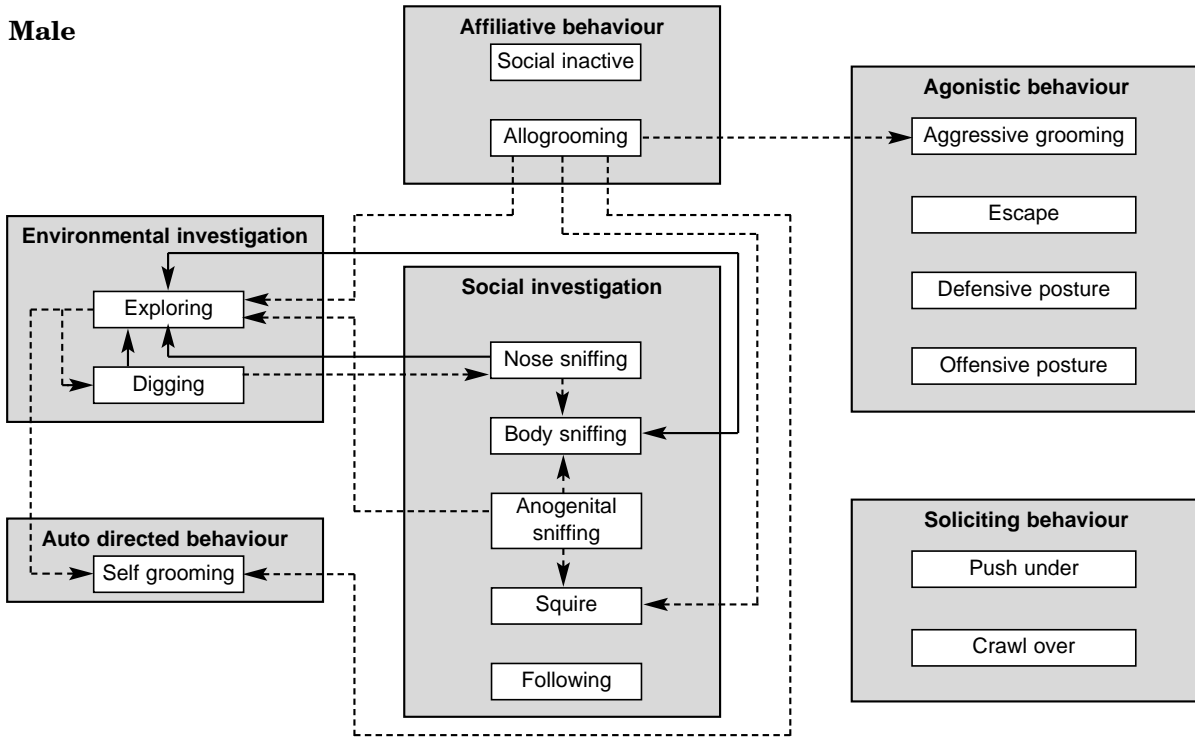
*Behavioural items are defined according to (15–17).*

ious transitions occurring during the social interaction. All the behavioural transitions between social and environmental investigation items are not affected by CPF exposure. By contrast, CPF treatment targets few behavioural transitions that occur rarely throughout the social test and do not represent the main behavioural pathways in control animals (e.g. those converging to Exploring). In both sexes, however, CPF treatment appears to be dose-dependent, with CPF3 (3mg/kg) altering a higher number of transitions.

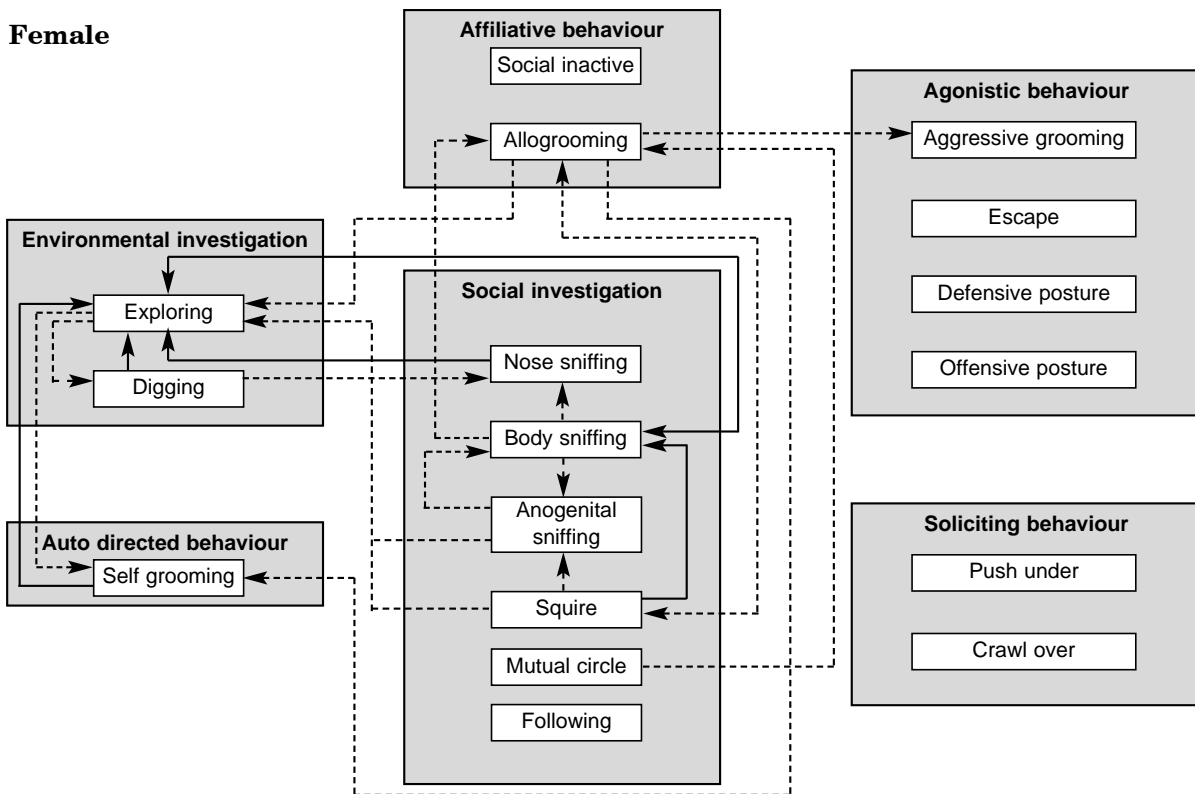
In males, CPF decreases the priority of being engaged in investigation of the environment while the animal is exploring the social partner. This CPF effect of favouring social investigation is also strengthened by the higher tendency to sniff the social partner (i.e. to start a social investigation pattern), when the animal is involved in auto-directed behaviours. Moreover, CPF3 decreases the rate of the behavioural transition from Escape to Exploring. Within the Agonistic category, an

**Figure 1: Flow diagram of the transition probabilities between behavioural items in control mice**

**Male**

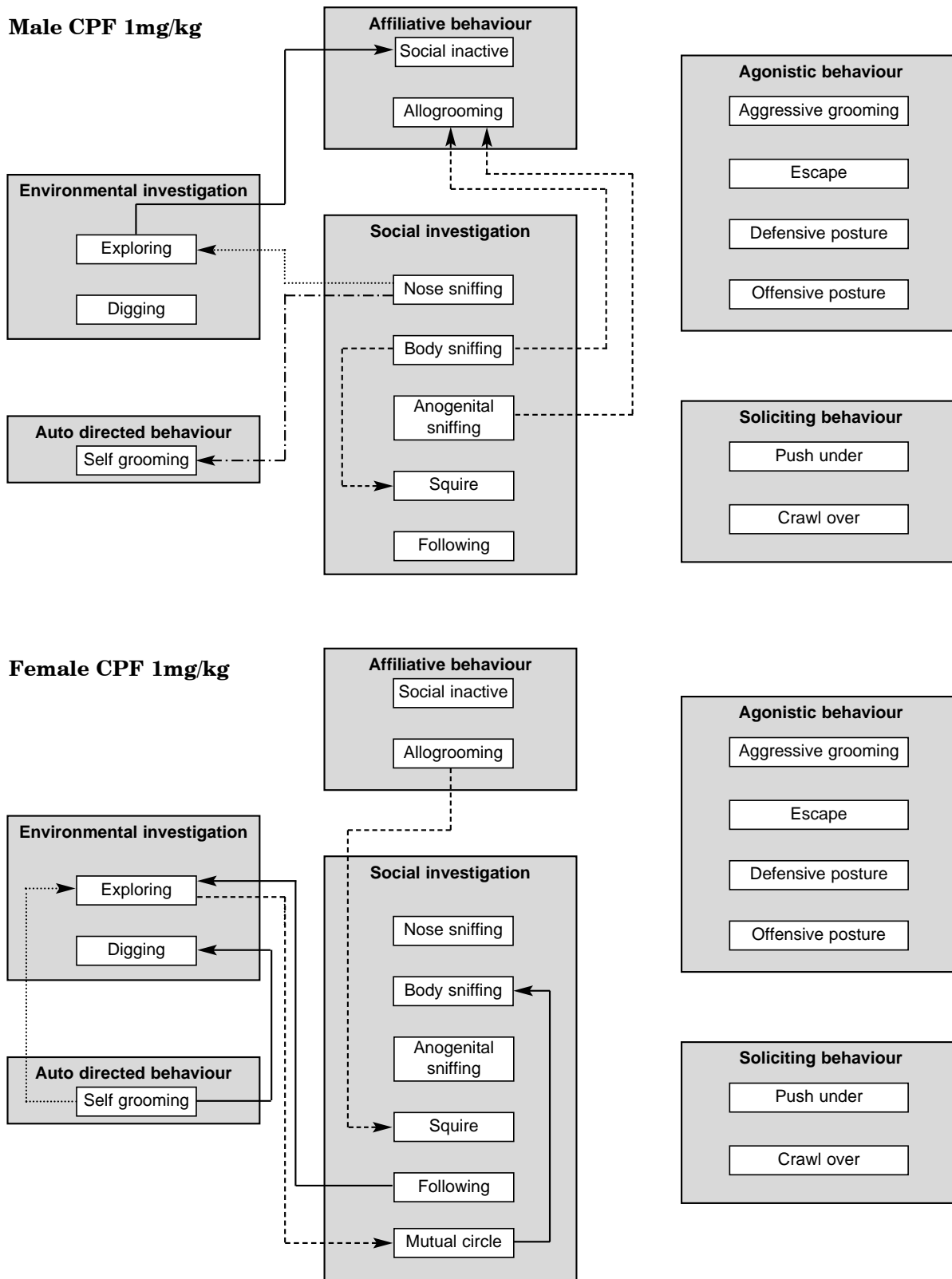


**Female**



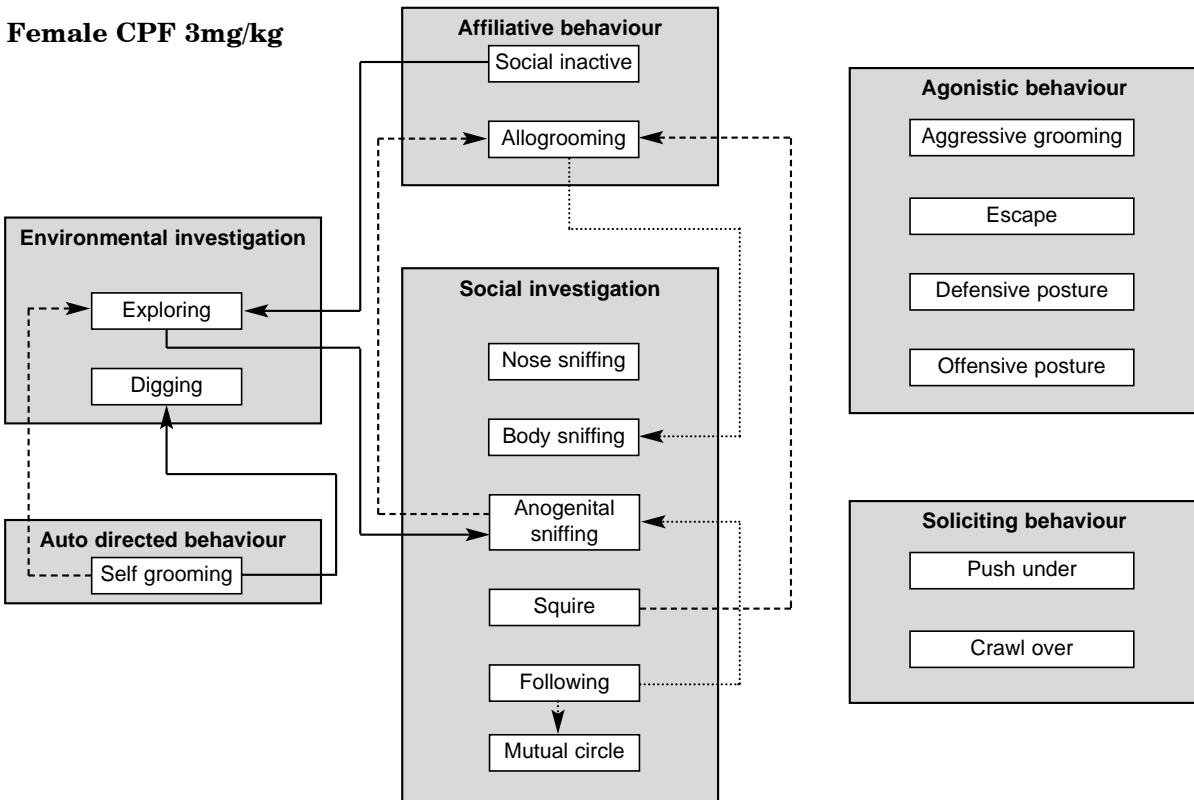
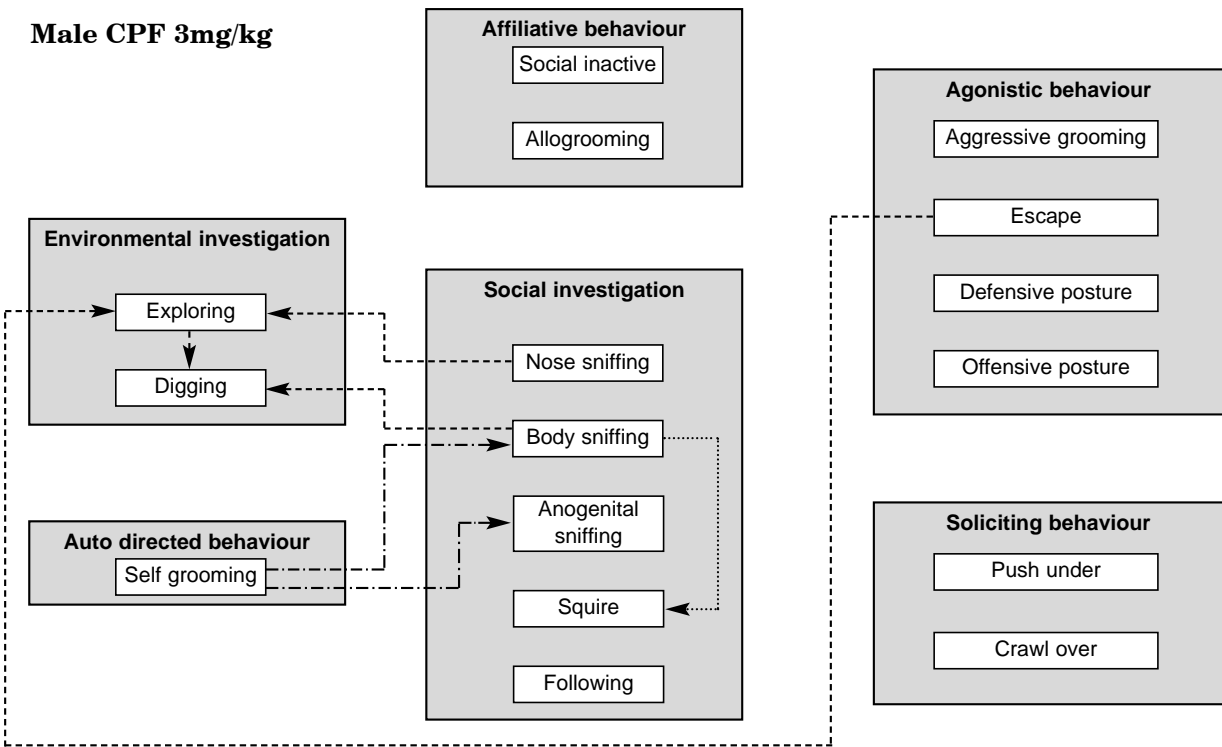
----- = less frequent (transition probability 0.1–0.3); ——— = more frequent (transition probability 0.3–0.6).  
 Only items with frequency ≥ 3 are shown.

**Figure 2: Flow diagram of the transition rates significantly altered by the low dose of the pesticide treatment (CPF 1mg/kg) and by the high dose of the pesticide treatment (CPF 3mg/kg)**



———— = transition rate significantly accelerated by treatment; - - - - - = acceleration of transition rate just missing statistical significance; . . . . . = transition rate significantly decelerated by treatment; - . . . . = deceleration of transition rate just missing statistical significance.

**Figure 2: continued**



———— = transition rate significantly accelerated by treatment; - - - - - = acceleration of transition rate just missing statistical significance; - - - - - = transition rate significantly decelerated by treatment; ..... = deceleration of transition rate just missing statistical significance.

aggressive sequence usually ends with the displaying of an Escape response by the animal suffering the attack (18); therefore, a decreased transition rate from Escape to Exploring could be tentatively interpreted as a deficit in coping with unfavourable agonistic interactions and subsequently restarting a new behavioural pattern.

Also, in females, CPF targets the social investigation patterns: treatment decreases the priority of being engaged in affiliative responses while the animal is exploring the social partner. In more "ethological" words, it is tempting to speculate that, in both sexes, CPF increases motivation to socially investigate the partner; in males, such increased motivation occurs at the expense of exploration of the experimental environment; in females, it occurs at the expense of affiliative responses. Indeed, in periadolescence, a greater prevalence of the affiliative repertoire in females rather than in males can be expected in the mouse species (15).

Two points need to be stressed from a methodological perspective: the procedure GLLAMM, now available in STATA 7, may be extremely helpful for carrying out the analyses when data are collected with complex mixed models. An ethogram with few behavioural items and, consequently, few transitions to investigate appears to be more suitable for the analysis of the times of switching. Moreover, an *a priori* selection of the behaviours between which the switching is analysed may reduce the number of possible pairs of behaviours and consequently may overcome problems related to multiple models.

Further analyses are therefore warranted to evaluate transitions amongst behavioural categories obtained by collapsing similar items in the same class (i.e. Exploring and Digging collapsed in the environmental investigation category as suggested in Figures 1 and 2). This generalisation of the behavioural repertoire, even if decreasing accuracy from an ethological viewpoint, could a) increase the resolution power of the statistical analysis by increasing the frequency of each of the transitions between these behavioural categories *per se*, and b) facilitate the interpretation of treatment/condition effects on behavioural transitions.

As a whole, our results indicate that, because of the profound changes in meaningful endpoints investigated (transitions instead of states), the study of the time structure of behaviour can provide original information on behavioural changes under different experimental contexts ranging from spontaneous behaviour of a single animal in an open field test to dyadic interactions between con-specifics. And, importantly, this information appears to be strongly related to motivational shifting levels experienced by the animals.

Study of the time-structure of behaviour can therefore constitute a valid tool for the refinement of statistical methods applied to behavioural analysis. Allowing the collection of more complex and

detailed data may lead to a better knowledge of the processes under study, potentially reducing the number of animals used in the life sciences.

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