DEFENCE FROM INVERTEBRATE TO MAMMALS: FOCUS ON TONIC IMMOBILITY

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An integrative view of ethology and neurophysiology (behavioral neuroscience) contributes to reveal the underlying mechanisms of defensive behavior, with a particular focus on Tonic immobility (TI), originally described by Schwenter (1636) and Kircher (1646) and later experimentally studied by Darwin (1884) and Pavlov (1955). Within the evolutionary framework of the defensive system in animals, an updated and comprehensive view on TI and its mechanisms is given. The available research is presented in a historical perspective, integrating earlier theories and findings with more recent studies also based on new methodologies. A few findings in the field have been clarified and reinterpreted.

Nel repertorio delle risposte difensive l'immobilità tonica (TI) rappresenta l'ultima risorsa antipredatoria a cui l'animale ricorre quando si trova a stretto contatto con uno stimolo di pericolo o minaccia (la cattura da parte del predatore o una manovra di restrizione in laboratorio) e non ci sono possibilità di fuga. È una risposta innata di immobilità che dura oltre la durata dello stimolo e non può essere volontariamente interrotta dall'animale. Presente in numerose specie a partire dagli invertebrati superiori, è caratterizzata da un blocco motorio a livello dei motoneuroni e dalla soppressione dei riflessi di raddrizzamento.

Nei 12 capitoli del volume è stato definito il profilo neurobiologico della TI (neurale, endocrino, neuromediatori, controllo del dolore), aggiornando i più rilevanti risultati della ricerca in una prospettiva storica con gli studi più recenti basati su nuove metodologie. I dati sperimentali confermano il ruolo adattativo della TI, come meccanismo di sopravvivenza in situazioni di pericolo.

Nel quadro più ampio delle risposte difensive, la plasticità della TI è collegata a: il tipo di predatore e la pressione predatoria, le condizioni ambientali, quali l'opportunità di una via di fuga, le correlazioni genetiche con altri comportamenti che contribuiscono alla fitness.

Dati neurofisiologici ed endocrini nei mammiferi e negli uccelli suggeriscono anche un ruolo della TI nella attivazione di meccanismi di recupero omeostatico del sistema di risposta allo stress, compresa l'attivazione di un meccanismo analgesico di controllo del dolore.

Defense in animals and Tonic immobility (TI) characteristics

The defensive repertoire activated in animals in response to threatening stimuli, both in natural and experimental conditions, consists of a system of interrelated behaviors influenced by two main variables: distance from the threat and an escapable/inescapable context. When the active strategy of escape is not feasible, passive immobility forms are adopted, the latter representing substitutes of actual escape. In particular, in an inescapable context, and at very short distance or in contact with the threatening stimulus, Tonic immobility may be adopted, or submissive posture in a social context.

Within the same modality (i.e., immobility), immobility may correspond to different functions and motivations: the neurobiological profile of TI, defined in the book's chapters, allows to differentiate TI from other immobility forms defensive in nature (such as freezing and restraint-maintained immobility), as well as from spontaneous immobility and REM sleep.

Tonic immobility is an innate, all-or-none, reflex response occurring in nature in a prey/predator context that is characterized by immobility and unresponsivity. In an inescapable context, and at very short distance or in contact with the threatening stimulus, TI represents the last antipredatory resource in front of capture. This post-contact behavioural response that develops following a mechanical stimulus, such as restraint, outlasts the duration of the stimulus and cannot be purposely interrupted by the animal. It has been recorded in many species, from invertebrates to mammals (as suggested by the book's subtitle). In experimental models in laboratory settings, physical restraint represents the strongest stimulus for TI induction.

The immobility occurring during TI is not due to low levels or absence of motoneurons excitation typical of spontaneous immobility, but to a transient inhibitory mechanism that affects motoneurons eliciting a motor block.

In the book's chapters, the neurobiological profile of TI has been defined, including neural, neurotransmitters, autonomic, endocrine and pain control systems.

During TI there is suppression of righting reflexes and an increase in reflex thresholds. In mammals and birds, in the absence of dynamic external stimuli, there is a tendency to pupil constriction and to the development of EEG high voltage slow waves (HVSW) typical of sleep. However, TI differs from REM sleep for the persistence of a low-neck muscle activity and the absence of rapid eye movements. Experimental data from rabbits, a species very susceptible to TI, indicate that this EEG pattern during TI is affected by the events preceding TI induction: the amount of HVSW is lower during adaptation to a new environment, largely increases after adaptation and is positively related to TI duration.

Following external stimuli as well as spontaneously, with the animal still in TI, the EEG sleep activity may be replaced by arousal episodes, characterized by an EEG typical of arousal (low voltage fast waves). This pattern suggests that animals during TI may be briefly aware of the environmental changes, as the presence of the predator (including the experimenter) or the availability of an escape way. These arousal episodes may be followed either by TI interruption or by TI continuation associated with gradual recovery of the EEG sleep pattern.

The presence of the HVSW pattern in the EEG during TI suggests the activation of the thalamic gate, a mechanism blocking the transmission of all sensory information to the cerebral cortex, including the nociceptive input. This sensorial disconnection (sensorial cut-off) that occurs at cortico-thalamic level represents a transient functional deafferentation, similar to anaesthesia, that is rapidly released and suddenly interrupted by arousing stimuli.

There is evidence that during and after TI there are different mechanisms that attenuate both the painful experience and the inflammatory response in body sites possibly injured during the capture. In rabbit models, after formalin injection, the animal displays nociceptive behaviour associated with a long-lasting low voltage fast waves EEG pattern. The induction of TI dramatically interrupts nociceptive behaviour and an HVSW pattern develops in the EEG, an index of the functional de-afferentation (anesthesia). Nociceptive behaviour and EEG low voltage fast waves resume immediately after TI termination.

After a TI episode induced during persistent nociceptive stimulation, an opioid mechanism outlasting TI offset may be activated, associated to the release of β-endorphins. If the noxious stimulus is dynamic and not tissue-damaging, the opioid analgesic post-TI mechanism develops with a longer delay and is short-lasting with respect to the tissue-damaging one. It seems that TI stimulates the release of β-endorphins more efficiently if it occurs during persistent nociceptive stimulation. As a matter of fact, β-endorphins have different effects on behaviour and TI duration, according to the intensity of the painful stimulation. On the other hand, in rodents also not injuring nociceptive stimuli applied during TI elicit a delayed opioid analgesia. Moreover, both opioid and non-opioid mechanisms of analgesia have been observed.

In a clinical model of experimental arthritis in guinea pigs, it has been shown that TI plays also a role in inflammation. TI induced before the joint inflammation strongly reduced the joint diameter and the neutrophilic infiltration within 6 hours: the longer was the TI duration the stronger the inflammation reduction, possibly through the activation of an anti-inflammatory cytokine-vagal-sympathetic mechanism.

The fear hypothesis

Fear is a protective affect with a negative valence indicating a disposition to avoid or escape aversive situations. There is agreement that fear is elicited by both innate and conditioned stimuli, that predict danger and produce defensive behaviour mediated by activation of specific neural circuits.

Fear is strictly associated to the perception of threat. In the detection of a threat, taking the behavioural form of *risk assessment,* the key features of the stimulus (for example a predator), i.e. the essential features signalling potential danger, will be extracted through the filtering properties of sensory neurons. In this process, the animal acquires information not only on the type of threat but also about its position with respect to the stimulus and to other relevant objects in the environment (i.e. hiding places, escape routes).

On the basis of the acquired information, the animal selects the most appropriate defense strategy, which in principle should avoid the threatening situation and increase the chance of survival.

The hypothesis that fear is involved in the mechanisms of tonic immobility (TI) is very old: TI was indicated as a "paralysis of fear" and TI duration was often used as a measure to assess fear. This hypothesis has been systematically investigated in seminal studies conducted in newborn and adult chickens. The main findings indicate that exposure before or during TI induction to fearful, threatening stimuli such as electric shock, loud sound, a stuffed sparrow hawk, as well as in unfamiliar conditions increased TI duration.

In the case of the sparrow hawk, but also in experiments with artificial eyes, the eyes were identified as the body critical feature responsible for TI potentiation. This result suggests that in natural conditions the prey-predator eye-contact may be the main responsible for TI potentiation.

An opposite effect is obtained with procedures designed to reduce fear, since familiarization with the environment but also the use of a poultry tranquilizer decreased TI duration.

The salience of the experimenter visibility and of the experimenter's eyes before and during TI was evaluated in different species, but more systematically in chicks and adult fowls, with contradictory results. In developing chickens, TI duration was potentiated by the visibility of the experimenter staring at the bird and maintaining eye contact. This effect of proximity and eye contact indicates that the experimenter presence represents a potential source of fear similar to the natural predator-prey condition. However, in other experiments in adults TI duration was not affected by visibility and proximity of the experimenter. In this kind of experiments however, the need to evaluate the interplay between variables such as rearing and testing conditions should be considered.

The rearing conditions of chickens in particular, resulted to be critical and the importance of the human presence during rearing was reconsidered. Repeated handling in the first days after hatching facilitated habituation and reduced fear of human beings; when animals were later tested, TI duration was reduced with respect to not handled subjects, with a parallel increase in proximity scores to the experimenter.

Filial imprinting is a precocious form of learning that is common in birds, with opposite behavioural responses evoked respectively by the presentation or the withdrawal of an imprinting stimulus. Chickens exposed to a positive imprinting stimulus containing animate features during the first days after hatching were later tested for TI: the exposure to the imprinting stimulus before TI induction decreased TI duration, whereas the withdrawal from the imprinting stimulus increased TI duration.

These findings indicate that TI may be quantitatively modulated by both distressful and pleasurable emotions, which may increase or reduce the aversiveness of the stimuli inducing the TI response.

Adaptation, survival value and homeostatic, recovery mechanisms of TI

In a conclusive chapter, we reviewed findings giving support to the hypothesis that TI represents an adaptive, survival response to threatening situations.

Behavioral flexibility of defense responses including TI is related to the type of predator, the intensity of the threat and the environmental conditions, such as the predation pressure and the opportunity to hide and escape.

It is also to consider that immobility *per se* contributes to survival as the predator may lose interest for an immobile prey and abandon the immobilized prey: this "loss of interest" has been attributed to the predator's preference for moving/struggling preys. Such a mechanism has been already observed in invertebrates and confirmed in mammal and bird models, showing that the prey's TI can reduce predator's attention, thus facilitating survival.

There are general but also specific prey-predator mechanisms, that have coevolved leading to reciprocal phenotypic plasticity. A high specificity in the prey-predator interaction, of adaptive value, is found when the behavior of TI is associated to morphological characteristics: the gape-limited predators on one side and the modifications of the prey's body, as the increase of body volume observed in anurans through lung insufflation, impairing swallowing or making physical restraint more difficult.

A further indication of the adaptive value of TI is suggested by the observation that the duration of TI is reciprocally modulated by the evaluation of the risk factors in the environment and by the opportunity to reach a safe refuge. This mechanism confirms that, during TI, the animal may be transiently aware of the environmental situation, as indicated by the electrophysiological EEG pattern typical of arousal. During these spontaneous arousal episodes, a high opportunity to escape increases the probability of TI interruption whereas the opposite effect is due to the presence of the predator (including the experimenter).

A further form of plasticity is the evolution of traits individually differentiated within the same population. Genetic lines differing for behavioral and physiological parameters, that are stable over time and across contexts, show a different capacity and modality to cope with aversive stimuli (personality traits). TI has been regarded as an heritable trait of animal personality, both in higher invertebrates and in vertebrates, and, from the beginning of this century, a number of studies have been carried out through the selection of genetic lines differing for TI duration (long and short). In insects, a phylum in which TI is largely present, the variability in TI is heritable and TI duration is inversely related to boldness, in the bold/shy axis of the coping style.

Phenotypic plasticity has been recorded in various bird species (more in wild and less after domestication) in which the modality bold/shy is associated to the level of exploration (fast/slow), as well as in rodents, classified as proactive and reactive on the basis of their coping style.

The covariance of behavioural traits genetically associated has been evaluated in these animal models. The adaptive value and the selection advantage of an antipredatory trait as TI is in fact the result not only of the probability of survival in a predatory environment, but also of the genetic positive and negative correlations with other non-defensive behaviours contributing to total fitness (behavioural syndromes). Of particular interest is the case of TI and mating (sexual behaviour) which are negatively correlated: for example, in two strains of beetles selected for long and short TI duration, the long TI has higher survival rates but lower mating success.

It must be considered that in an ultimate perspective the coexistence of different phenotypes with alternative traits may represent an advantage in presence of specific ecological situations.

Corticosteroid reactivity to stress, i.e. the last step in the HPA (hypothalamic-pituitary-adrenal) axis activation, has been regarded as a trait of animal personality, varying together with behavioral characteristics of the individual's phenotype, including TI.
The individual variability in the corticosterone response to a stressful situation, recorded in various bird species as well as in rodents, could suggest that the same stressor is perceived by the animal as more or less threatening according to its coping style. In a selection model in which two lines of quails were selected on the basis of High and Low corticosterone reactivity to restraint, animals of the High line showed longer TI and more fear-related behaviors, including the experimenter avoidance. This and similar findings in other species indicate a congruent pattern between corticosteroid reactivity, fear-related behaviors, and TI.

Threatening stimuli are the primary events triggering defensive responses, including TI. One of the most interesting aspects of TI function is the role played in the recovery of homeostatic parameters altered by stressful conditions.
The capture of the prey in nature, as well as the procedure of restraint for TI induction in laboratory, represent strong stressful events challenging animal homeostasis and leading to the activation of the stress-response system, signaled by increased blood corticosterone levels. An increase in corticosterone levels, both in blood and urine, in response to environmental stressors, has been observed in all vertebrates.
Neurophysiological and endocrine findings in mammals and avians support the hypothesis that TI activates the mechanisms of recovery from disruptive experiences and body lesions. Early experiments in male rabbits in which the effects of TI were dissociated from those of induction *per se,* support the view that TI takes part in the homeostatic stress-response system. An increase of corticosterone was recorded just after the end of the induction procedure but not in the corresponding groups in which induction was followed by TI. The temporal pattern of these modifications indicates that the increase in corticosterone after the procedure of induction (not followed by immobility) is a long-lasting effect persisting 15 min after the end of the procedure. In the blood samples taken just at the end of TI, corticosterone levels did not differ from the pre-induction levels, suggesting a process of recovery during TI with respect to the induction effects. A similar trend (increase during induction, recovery after TI) was observed in corticosteroid adrenal metabolites, an indication that TI contributes to the balance of the HPA axis at peripheral level.
In this model in rabbits, similarly to corticosterone but in opposite direction, a process of recovery during TI was observed for testosterone, following the activation of the HPG (hypothalamic-pituitary-gonadal) axis: a sharp decrease in testosterone plasma levels was found only in the animals submitted to induction, whereas in the animals submitted to induction followed by TI, testosterone did not differ from the pre-induction levels. It is well-known that stressors have a negative effect on reproductive behaviour and physiology. In fact, according to the adaptive hypothesis, reproduction is inhibited to devote all the energetic resources to the stress response.
In birds (Japanese quail), a recovery process during TI has been found in genotypic lines selected for long- and short-TI duration. In the comparison between the two lines the basal plasma levels of corticosterone did not differ. However, within the long-TI line, corticosterone levels at the end of a

TI episode were negatively correlated with TI duration, suggesting a recovery process in the same direction of the early findings in rabbits.

The recovery process during TI, which should be reconsidered through new models and methodologies, is not confined to the endocrine system. In rabbit, there is also evidence that TI represents a recovery period in autonomic activity from the antecedent events: it is the case of heart rate modifications, during and after adaptation to a new environment before TI induction.

Finally in natural conditions, capture in predation may be painful and tissue damaging, but TI may provide a protective mechanism through the activation of a pain control, analgesic system, and if the prey has been injured by curbing inflammation, accelerate healing, leading overall to fitness recovery.

Il volume "**Defence from invertebrate to mammals: focus on Tonic Immobility**" è stato presentato presso **l'Accademia dei Fisiocritici** il 20 febbraio 2023 in una sessione coordinata dalla Prof.sa Luana Ricci Paulesu, e con l'intervento di studiosi con diverse competenze disciplinari.
Dopo un breve intervento preliminare di uno dei due autori, Prof. **Giancarlo Carli,** che ha fornito una definizione corretta della Tonic immobility, sono intervenuti nell'ordine:

Il Prof **Francesco Dessi**, biologo dell'Università di Firenze ha posto l'accento sul significato comportamentale della Tonic immobility con particolare riferimento al valore adattativo di questo comportamento difensivo che è già presente negli invertebrati. Presentando diversi esempi di TI in ambiente naturale, ha fatto riferimento a linee di ricerca più recenti che hanno inquadrato la TI come un tratto della "personalità animale", cioè della differenziazione genetica tra individui della stessa specie di diverse strategie di risposta a stimoli ambientali di pericolo. Ha anche sottolineato il ruolo della TI in contesti intraspecifici negli insetti, in cui il maschio ha accesso mediante la TI alla copulazione con la femmina potenzialmente cannibalistica.

La Prof.sa **Enrica Santarcangelo**, neurofisiologa dell'Università di Pisa, riferendosi anche ad esperienze personali nell'Istituto di Fisiologia Umana di Siena, ha posto l'attenzione sui correlati corticali della TI, riferendosi in particolare ad un modello che ha associato tecniche etologiche e neurofisiologiche, in conigli osservati e registrati in campo aperto in condizioni seminaturali. Grazie al lavoro pionieristico del Prof. Giuliano Fontani, l'approccio neuroetologico, a cui è dedicato un capitolo del libro, ha permesso di individuare come indice discriminante della Tonic immobility la riduzione di frequenza del ritmo ippocampale a valori non paragonabili con altre forme di immobilità. Parallelamente, il passaggio a frequenze più elevate segnala ed anticipa la fine dell'episodio di TI.

Il Prof **Sandro Lovari**, etologo dell'Università di Siena, esperto del comportamento animale in popolazioni di grandi mammiferi osservate nel loro ambiente naturale, ha utilizzato interessanti riferimenti letterari per illustrare alcuni stati di immobilizzazione in natura, nel corso della predazione nelle sue diverse forme. Ha anche illustrato il valore adattativo della immobilità nella preda a cui è collegato il controllo del dolore nonché le strategie di caccia, dipendenti da fattori morfologici e di organizzazione sociale.

In risposta ad un quesito posto dal Prof. Dessì Fulgheri, è intervenuta la Prof. **Francesca Farabollini** , coautore del libro, che ha evidenziato come i dati sperimentali sull'attivazione del sistema endocrino nell'asse HPA e nell'asse HPG sono di particolare interesse e meriterebbero un approfondimento. Nel modello del coniglio, durante la TI, si attiva un meccanismo omeostatico di "recovery" rispetto alle modificazioni dei parametri endocrini, in particolare all'aumento plasmatico del corticosterone, alterato in seguito alla risposta allo stress rappresentata dalla cattura o, in laboratorio, dal restraint.
Il recovery riguarda anche il testosterone, ma in direzione opposta: riduzione durante la cattura (o il restraint) e ripristino in seguito alla TI.