J. comp. Physiol. 124, 193–201 (1978)

Journal of Comparative Physiology · A

Transplantation of a Time-Signal in Honeybees* **

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Accepted December 19, 1977

Summary. It has been possible – by transplantation of brain tissue (i.e. mushroom-bodies) – to perform an interindividual transfer of a learned time-signal in honeybees. The information of the donor bees becomes determinative for the temporal activity pattern of the recipients about 3 to 4 days following transplantation.

As seen from histological investigations done in parallel, the donor tissue is treated as a xenograft by the recipient's organism including disintegration and encapsulation processes. These observations give evidence for a humoral transfer of information.

The results are discussed from the point of view of the analysis of the mechanism of time reception.

Introduction

The orientation in time of honeybees – known as "time-sense" (see: Beling, 1929; Wahl, 1932; Renner, 1957; v. Frisch, 1965; Bünning, 1973; Rensing, 1973) – is one of those behavioral patterns based on an as yet unknown sensory process (Autrum, 1977).

Brady (1974) characterized the time-sense of bees as a "*learned* feeding rhythm". The fact that a memory component is included in a temporal orientation behavior offers the chance for a new type of methodological approach to the analysis of a basic mechanism in sensory physiology, i.e. transplantation experiments. By now, it seems evident that a learned information or learned signal may be transferred interindividually, using brain extracts from trained donor animals (for reviews see: Zippel, 1973; Zilliken and Abdallah, 1973; Smith, 1974; Mitchell et al., 1975; Ungar, 1975).

An important problem deriving from the idea of doing transplantation experiments is the search for a structural equivalent of the sense of time within the CNS of the bee – without implying a localization of memory or a clock. As concerns the "sensory modality" possibly responsible for the reception of time, geophysical factors according to Brown and coworkers (Brown, 1965; Brown et al., 1970) should be taken into consideration. Furthermore, unpublished data of our own (H. Martin et al., in preparation) make it rather evident that the daily variations of the earth's magnetic field (EMF) are an important factor in temporal orientation. As we have no information on possible receptor neurons in the periphery involved in the mechanism of reception of the EMF we should first of all concentrate on a system of interneurons within the supraoesophageal ganglion of the bee which seems appropriate for the processing of information with temporal characteristics.

As all experiments are performed under LL-conditions the optic system should not be of primary importance for the problem presented. The extensive literature on the interrelation between the optic lobes and the "biological clock" in insects has been reviewed by Brady (1974) and Saunders (1976).

Huber (1974a, b) discusses the possible use of the corpora pedunculata as an "ideal time-measuring machinery in the insect nervous system". A lot of basic ideas for this assumption are derived from ultrastructural data and from impregnation studies of the CNS of bees and crickets (Schürmann, 1971, 1974) and ants (Steiger, 1967).

One phenomenon described by Schürmann (1974) is worth mentioning separate from the mechanism of processing of a temporal information: The enor-

* Dedicated to Prof. Dr. H. Autrum on the occasion of his 70th birthday

** Supported by the Akademie der Wissenschaften und der Literatur zu Mainz, the Stiftung Volkswagenwerk and the Deutsche Forschungsgemeinschaft

0340-7594/78/0124/0193/$01.80

mous redundance of intrinsic elements in the pedunculus as well as in the α- and β-lobes of the mushroom-bodies. As known for receptor neurons, this causes an effective reduction of susceptibility to trouble, an important postulate for a time-measuring system in insects as perhaps all learning tasks can be regarded as *time-linked* (Koltermann, 1971, 1974).

It is the aim of this paper to start an analysis of the "sense of time" of the honeybee from a neurobiological point of view. If successful, a transfer, e.g. by grafting of the mushroom-bodies, of a learned time-signal to naive recipients would offer the chance for a further analysis of a) the sensory process underlying the reception of time, b) the EMF as a possible synchronizer of timing, and c) the processes of storage and retrieval of memory.

It is the advantage of the behavioral approach that one can test the realisation of a "decoupled" information in an intact animal and with a real biological test.

Material and Methods

The experiments were performed on honeybees (*Apis mellifera carnica*). On behalf of a better survival of the operated animals we used mainly winterbees.

The bee-hives were brought from natural day conditions to so-called "constant" conditions: LL – light intensity about 3400–4000 lx, 1 m above the floor; constant temperature – ± 1 °C in a single experiment without thermostate control; humidity – $55 \pm 5\%$. The dimensions of the experimental rooms were about $3{,}0 \times 3{,}5 \times 3{,}5$ m. Ventilation was sufficient. – The position of the hives as to the co-ordinates of the EMF was comparable at least for donors and recipients of each single experiment.

No loose iron or any apparatus generating an electromagnetic field was allowed in the rooms and the near surrounding. Even the illumination system had been demagnetized as far as possible.

In parallel to the experiments the daily variations of ΔF (intensity of the EMF) were registrated (method: see H. Martin et al., in preparation).

Training. The donor animals were fed a definite time (2 h) for 5–6 successive days. The mode of training of the recipient bees was variable (see legends). The training of donors and recipients was performed in similar rooms. The position of the feeding place towards the hive was nearly identical.

Operation Procedure. The donor animals were removed from the hive one by one or in small groups of maximal 3 bees immediately before the preparation of the mushroom-bodies. This procedure was chosen to avoid any stress or an impairment of their time-sense by isolation from the social community (Beckmann, 1974).

The donors (D) were shortly narcotized with CO_2 and decapitated for the preparation of the corpora pedunculata. The latter remained in cooled Ringer's solution (acc. to Gersch et al., 1970) until transplantation (about 5–10 min). The operations were always started during or immediately after the training of the recipient bees. The recipients (R) were slightly fixed with foam rubber in a wax plate and were kept under a weak CO_2 narcosis during the operation just to calm them down. The head capsule was cut open twice mediolateral between the ocelli and the antennae and one mushroom-body transplanted on each side into the hemolymph in front of the bee's brain. The head capsule was then closed and the wounds in the cuticula covered with an elastic film (Nobecutan, Bastian-Werk, München). Then, the bees were kept in a thermostate for recovery.

As soon as possible (after 1–3 h) the operated animals were allowed to return to their hive to avoid disturbing factors from outside the social community. In most cases the experimental animals did not suffer from the operation. They flew to the hive by themselves and were accepted by their nest-mates. As regards their behavior, no shock was noticed from the operation procedure.

Test. For about 5 days, calculated from the last feeding of the recipients, the approaches at the feeding place (training situation, but no sugarwater available) were registrated individually. Each orientated approach was counted regardless of whether the bees were sitting down. The observations were done from outside the flight room through a window in order to omit any external factors possibly disturbing the experiment (odor, draught etc.).

Histology. For histological control of the transplanted brain tissue the heads of the operated and control animals were fixed in alcoholic Bouin's solution (Dubosque Brasil) and embedded in methylmethacrylate. Sagittal sections (10 µm) were done with a tetrander microtome. The sections were stained according to the trichrome method of Masson in the modification of Goldner. Besides, a few preliminary investigations were done to get information on the changes of the graft after different time intervals from the operation.

Results

Figure 1 demonstrates the normal activity of time-trained bees (training time: 10–12 a.m., 6 days) at the feeding place over a period of 3 days following the last day of feeding. The graph shows the result of a group of 27 bees. As already known from former observations on the "time-sense" of bees maximal activity – on the first day of observation – mostly happens before the onset of the training time (see v. Frisch, 1965). – On the second day, however, no marked preference (or only a weak preference) is recognized from the activity pattern. The period of maximal approaches is distended over more than 10 h. Besides, the total number of approaches is reduced compared to the first day. These effects are more distinct on the third day of testing.

Both effects, reduction of total activity and diminuition of preferential activity, are understood from biology: these phenomena are equivalent to a "negative training" (Abdressur) when a natural food source has ceased. The fact that one cannot observe an immediate decrease to zero activity or at least an equal distribution of activity over a period of 24 h might be due to the fact that, under natural conditions, a food source will never cease abruptly. Besides, under experimental conditions, there do not exist flowers in concurrence (see Discussion).

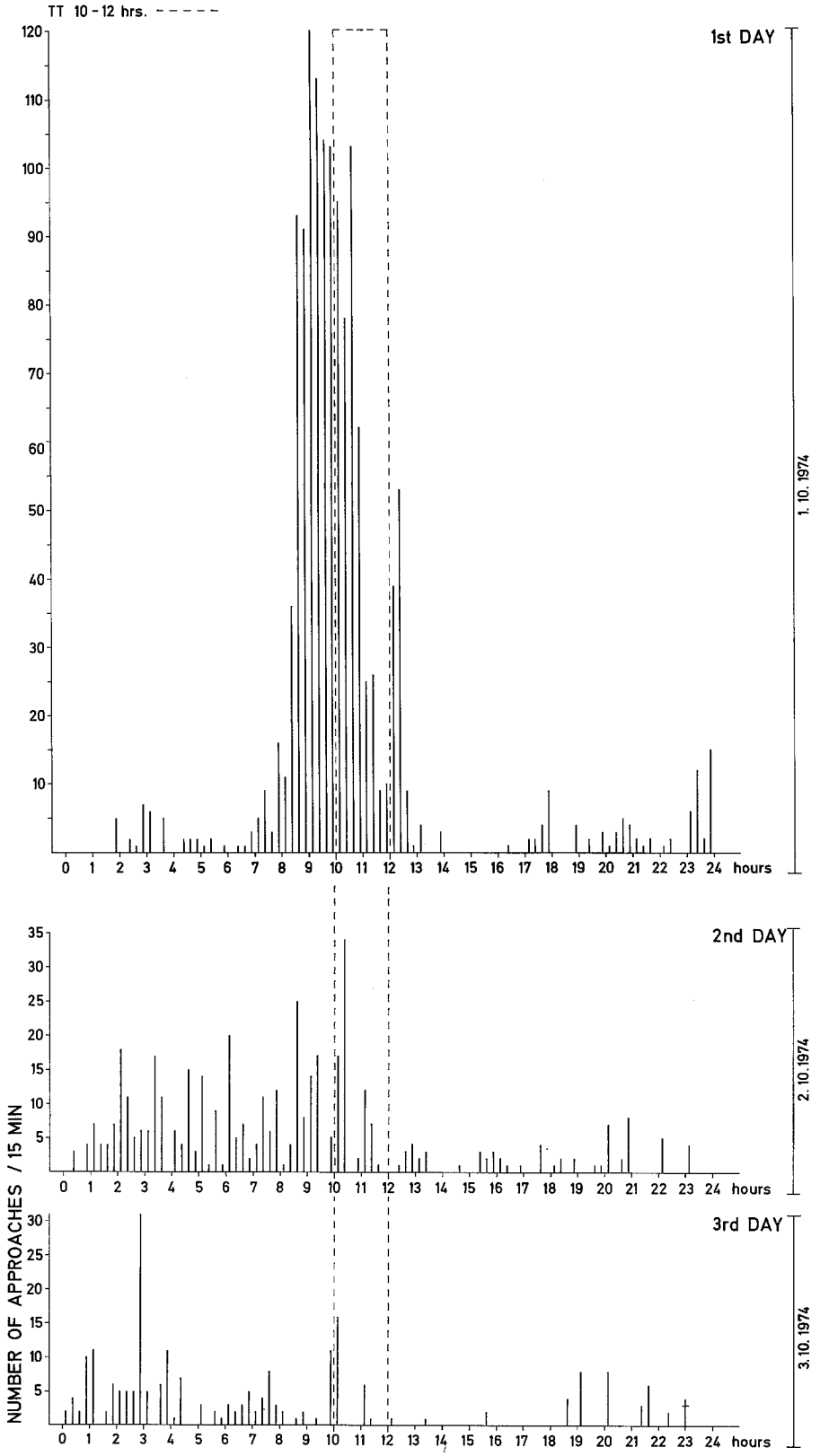

Fig. 1. Normal time-training experiment. Training-time (*TT*): 10–12 h

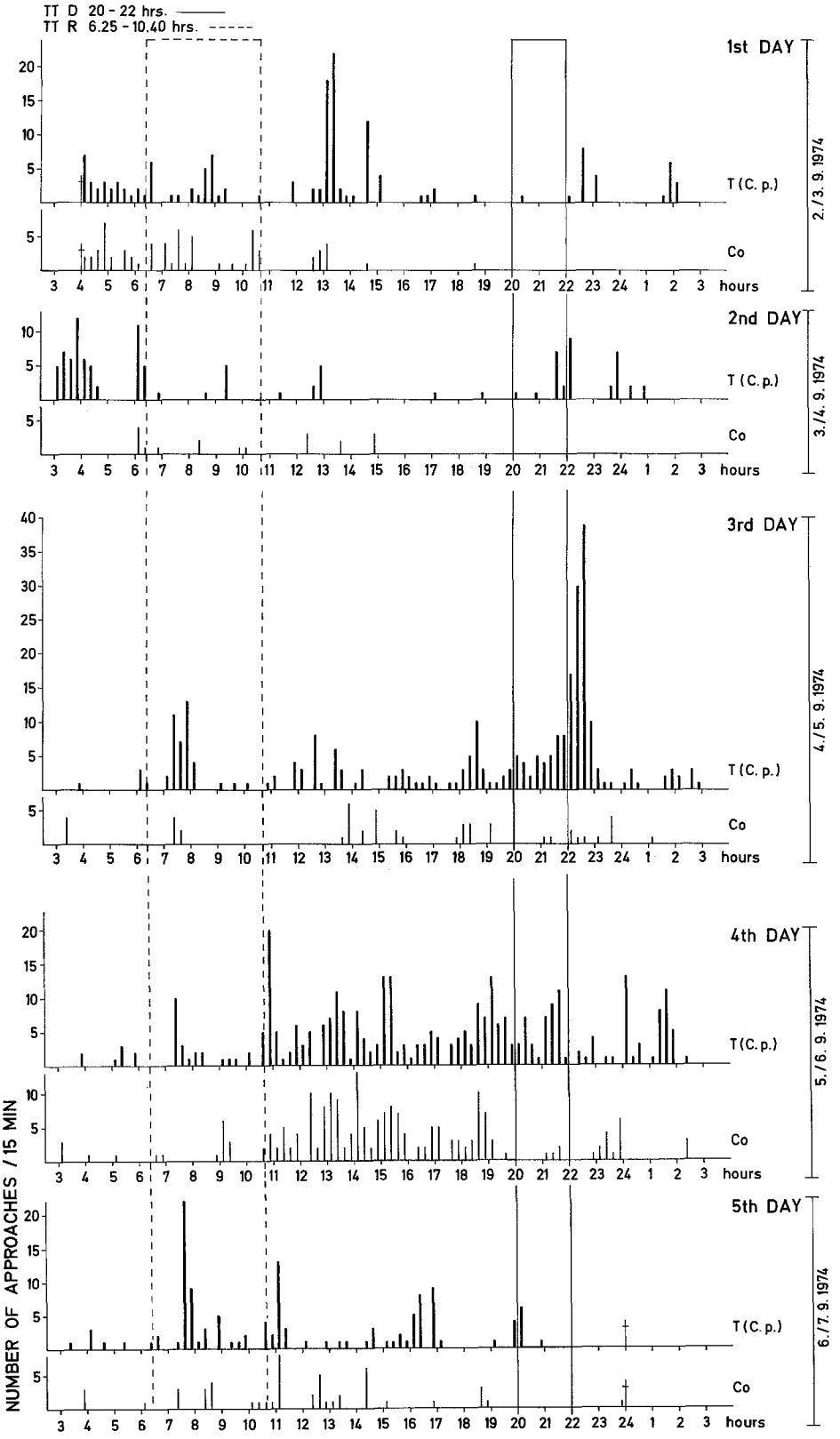

Fig. 2. Transplantation experiment. Training-time of donors (*TT D*): 20–22 h; training-time of recipients (*TT R*): 6.25–10.40 h, 1 day; *Co*, control animals, untreated; *T (C.p.)*, operated animals

Characteristic results of two transplantation experiments are shown in Figures 2 and 3. In the first experiment the donors have been trained to feed from 8 to 10 p.m. (Fig. 2). The recipients were only fed for about 4 h immediately before the onset of the operations. This was sufficient for the bees to know the position of the feeding place. The animals which got a transplant show no clear preference to feed during the first two days of observation; they seem somewhat "disoriented" as concerns time[1]. On the third day, however, these operated bees display a very strong preference of activity. 60% of the experimental bees visiting the feeding place (9 out of 15 bees) have a maximum activity at the end of the training time of the donor bees. Besides, they show a rise in total activity. This temporal orientation in their activity pattern is already abolished on the fourth day. The high level of activity, however, is still existent. Even in the group of control bees there is a rise in activity. This might be due to a "social synchronizer", i.e. the activity of the experimental bees (Medugorac and Lindauer, 1967). On the fifth day of observation the level of highest activity shifts to the original training time of the recipient bees, both in experimental and control animals.

In the other experiment shown in Figure 3 the donors were trained from 10 to 12 a.m. The recipient bees were fed continuously for 48 h. On the fourth day of observation more than 60% of the operated animals visiting the feeding place display the temporal activity pattern of the donor bees. Four further bees showed this distribution of activity already on the second day, and one bee on the third day of testing.

The "half-life time" of the information transplanted seems to be rather short although an extinction of an associatively stored information cannot be assumed (see Discussion).

Similar results which give evidence for the transfer of a time-signal by the transplanted tissue have been shown in at least 6 experiments using freshly prepared brain tissue for transplantation. Besides, additional experiments were done using frozen donor tissue. The results of the latter experiments are comparable.

Nevertheless, the time-signal taken over from the donor's transplant is not always that sharp. From more recent experiments which will be discussed elsewhere (H. Martin et al., in preparation) it seems as if the informational content of trained time-signals is of different value, depending on the time of the day.

From the results presented we may conclude that the information originating from the donor's brain becomes determinative for the temporal activity pattern of the recipient bees about 3 to 4 days following the operation, independent of the mode of feeding of the recipients.

Some possible objections might be raised. On the one hand, the effects observed could be due to the operation procedure. However, if we do sham-operations (opening of the head capsule without grafting) those bees behave very similar to control animals in that they do not shift their peak of activity to the training time of the donor animals. Nevertheless, they display a greater general activity than do the control animals. – It is remarkable that, in control as well as in sham-operated animals, the memory of their own feeding-time seems to be existent for a longer time (in days) than in the experimental bees who got a transplant.

To exclude unspecific factors (humoral or mechanical) from the graft as important for the timing of the operated bees we did a control experiment using naive flying bees as donors. As in the other experiments these bees had outdoor experience before they were brought to LL-conditions.

The activity pattern (Fig. 4) of the recipient bees was recorded continuously and individually for 4 days. On the first – and perhaps the second – day of observation the recipient bees show no preference for any time of the day except their own feeding time. The very broad peak of activity on the first day is certainly caused by the operation procedure (captivity, narcosis etc.). On days 3 and 4 the distribution of the approaches is non-directional. On the other hand, the total number of visits to the feeding place is very high and nearly identical on all days as has already been shown for the operated animals in Figures 2 and 3.

Some short remarks should be made concerning the behavior of the operated bees. They approach to the feeding place very directly without searching around. For the rest of the time they remain quiet near the entrance of the hive. Seemingly, they are not disturbed by the activity of their nest-mates.

Histology. From the histological sections the exact localization of the transplanted tissue in the head capsule is seen. The graft is always situated frontally and is separated against the recipient's supraoesophageal ganglion by trachea and big air sacs, the hypopharyngeal glands and the neural sheath ("blood-brain barrier", Treherne and Pichon, 1972). There are no indications on any structural connection between the transplant and the recipient's brain.

In all operated animals investigated the donor tissue is treated as a xenograft. The defense reactions

[1] The activity peak on the 1st day of observation is most probably due to a short "lights off" during the training period of 1 h 59′ on August 28th, because of maintenance work on the transformers of the university

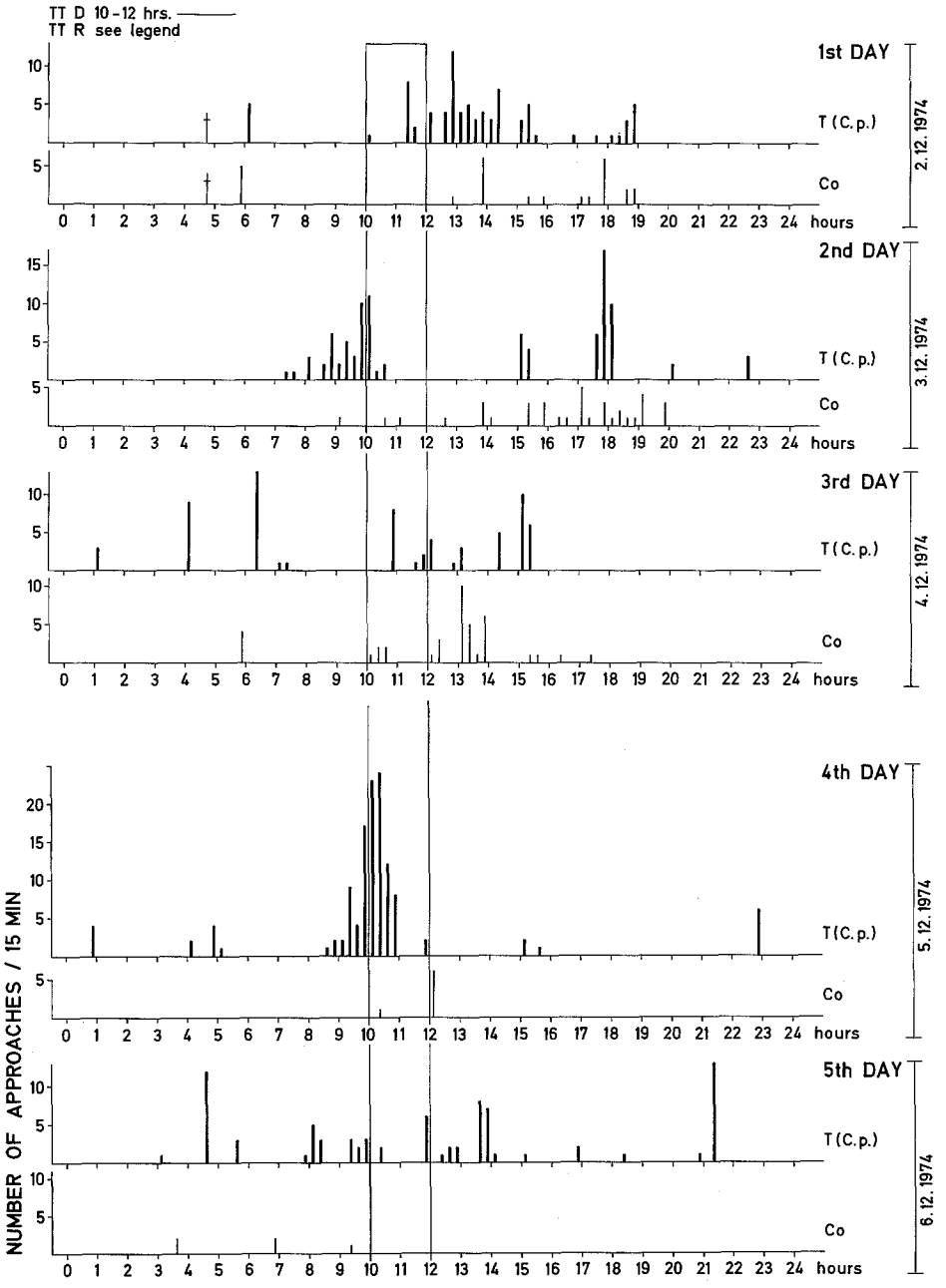

Fig. 3. Transplantation experiment. *TT D*: 10–12 h; *TT R*: 48 h of continuous feeding. Abbreviations see Figure 2

recognized include phagocytosis by hemocytes as well as encapsulation of the graft against the hemolymph of the recipients. The velocity of those reactions seems to depend on the physiological age of the experimental bees.

Extremely outstanding is the "attack" by hemocytes which seems to start very soon after transplantation. The nuclei of the donor tissue look pycnotic, the neuropile is extremely wide-meshed. Two features are seen, possibly a consequence of phagocytotic activities: these are a) the occurrence of "vesicles" tied off from the neuropile and b) zones characterized by an accumulation of great amounts of hemocytes. Whether, besides, hemolymph factors are integrated in processes of disintegration, mainly in the first hours following transplantation, cannot be answered

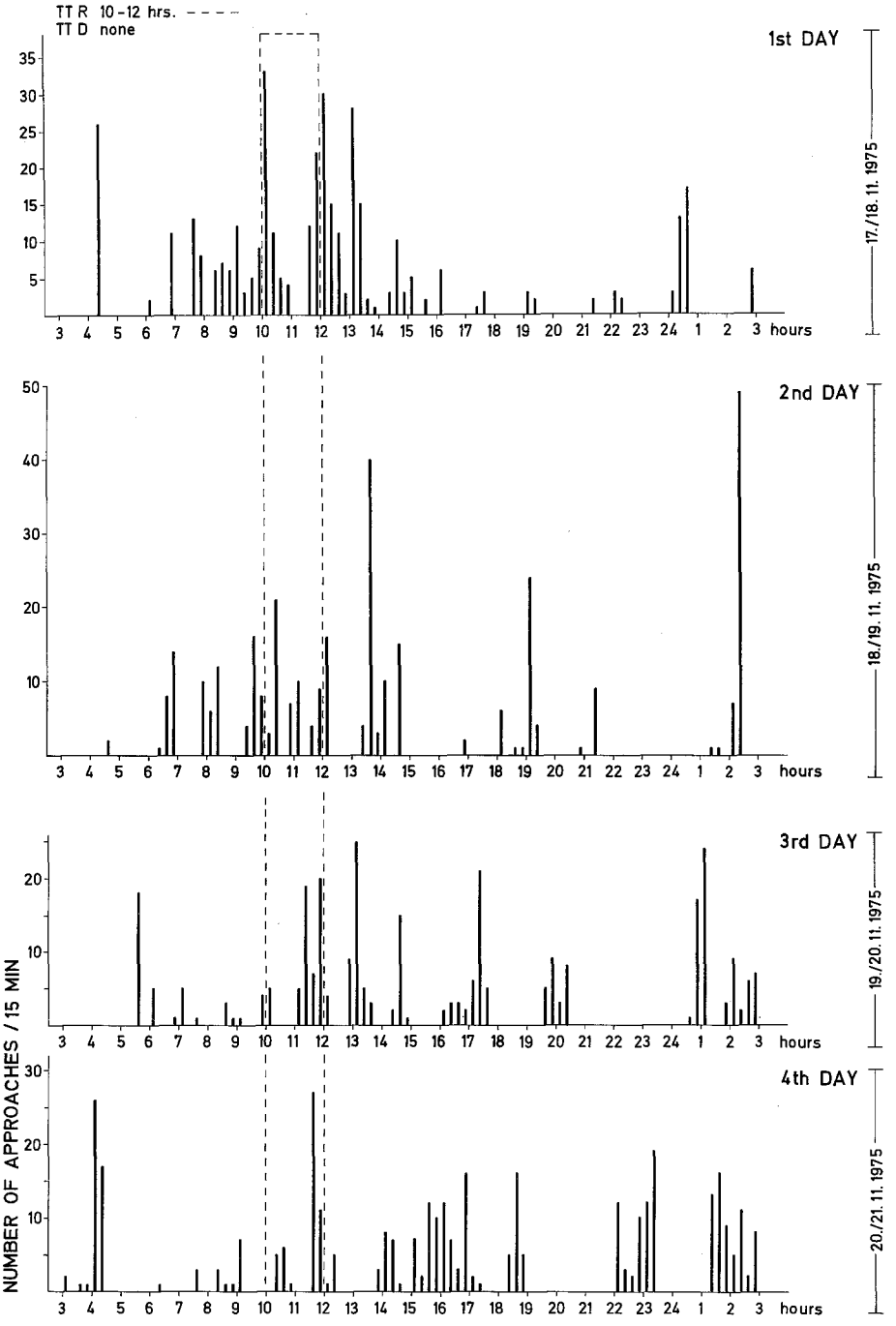

Fig. 4. Control experiment: Transplantation of corpora pedunculata of *naive* donors. *TT D*: none; *TT R*: 10–12 h

by the applied histological methods. But there are hints on such activities of hemolymph factors, e.g., spherical conglomerations of material near the graft which resemble coagulates. For a detailed analysis specific histochemical and biochemical investigations must be done. Important in this concern would be studies on lysozyme activities in the hemolymph as well as studies on the tyrosinase system which in the opinion of Messner (1972) might have a kind of marker function against immunologically strange, corpuscular substances. Besides, an intensive analysis of short-term hemocytotic activities would be of major importance.

Melanization processes are observed to take part in the encapsulation reaction. For the formation of the capsule the hemocytes seem to be very important

(see Whitcomb et al., 1974). Which cells and tissues are engaged in the process of isolation of the transplant against the recipient's organism and a detailed analysis of the time-course of those processes will be investigated separately.

Discussion

The results clearly demonstrate that the transplanted tissue of the donor bees exerts an effect on the temporal activity pattern of the recipients. There is no doubt, as shown by the histological data, that we have to discuss the humoral transfer of a specific information on time. Furthermore, the information transplanted is a complex of at least two components, a specific time-signal that co-ordinates the activity pattern of the recipient bees and an unspecific rise in total activity of the operated animals which has been demonstrated by sham-operation and the control experiment.

From more recent experiments done in our laboratory using frozen (or partly decomposed) donor tissue it seems as if structural integrity of the transplant is not important for the information transfer. Besides, from these experiments, the possible idea of persisting rhythmic activities in the graft following transplantation has to be rejected. Nevertheless, the analysis of rhythmic activities in the CNS of the donor as well as the recipient bees using histochemical and biochemical methods could be a key for understanding the mechanism of reception of the transferred information by target neurons.

As already mentioned in the introduction the use of the corpora pedunculata as donor tissue shall not imply a localization of the "time-sense"; one might even assume that – to function as a universal basis of time-linked learning processes in the honeybee – an information about time is represented all throughout central parts of the brain and could, therefore, be recalled from different areas of the CNS depending on the special learning task.

Besides, small amounts of tissue from adjacent parts of the protocerebrum might still be present although we are sure that perikarya or axons of the pars intercerebralis neurons are not intermingled.

As concerns the accuracy of the recipients in performing the temporal orientation pattern of the donor bees, on the one side, and the time-lag of 3 to 4 days following the operation before the specific time-signal becomes realized in the behavior of the recipients it was of interest for us whether at least one of these parameters could be affected by a different mode of training of the recipient bees. But neither a very short training (see Fig. 2) nor a continuous feeding over a period of 24 or 48 h (see Fig. 3) which both must be regarded as a training to a definite locus rather than a time-training proved successful.

From these considerations it seems advantageous to do a definite time-training in the recipients, too. Besides, it should be possible by such a mode of training to omit the occurrence of basic time-preferences in the activity pattern of the animals. Basic time-preferences of the bees might interfere with the activity pattern caused by the donor's signal. Concrete indications for the existence of such preferential activities are drawn from some experiments of Wahl (1932), Bennett and Renner (1963), Oehmke (1973) and unpublished investigations of our own. On the other hand, Zweygarth (1976) has shown that – under LL-conditions – the accuracy of time-training experiments is different depending on the time of the day. A detailed analysis on the phenomenon and the basic mechanism of those preferential activities is done just now.

It is of interest that it needs at least 3 days to have the transferred time-signal realized. Certainly, it does not take 3 or 4 days for the postulated substance to be released from the graft. This idea is supported by the histological data: the defense reactions against the xenograft are starting rather early (at least on the first day following transplantation).

Probably the "time-lag" of some processes in the recipient's organism must be taken into consideration. One could think of a slow "by-pass"- or "switch over"-process as a reaction towards a new time information or for an exact fixation of the new signal. Similar observations were done in normal bees by Beier (1968): If he did a phase-shifting of a distinct LD-pattern in relation to a fixed time-training it took about 3 days to be realized in the temporal activity pattern of the bees.

Informations on the stability of time-sense might be of great importance for the problems discussed. Very instructive for this discussion are some of the experiments done by Wahl (1932), investigating the stability of a trained time-signal with and without a "concurrence-training". Although the experimental conditions are hardly to be compared with those of our experiments, the phenomena discussed are rather conclusive. In a normal time-training (LD-conditions) time-sense seems to be stable for about 5 to 6 days following the last day of conditioning. If a second time-training (concurrence-flower, time 2) is started at the end of the first day of test of time 1, the memory of time 1 will disappear already after 3 to 4 days.

Some indications on this phenomenon are also seen in the grafting experiments. As for the operated animals, they seem to forget their own training-time

more quickly than control animals or sham-operated bees. Nevertheless, it must be doubted whether the actively learned time-signal of the recipient bees becomes extinguished by the donor's information or is only suppressed for a certain period of time. From some experiments one might assume the latter because the recipients – after 5 to 7 days of test – sometimes "return" to their original feeding-time.

In comparison with the results of Wahl's experiments one must clearly differentiate between a passive enforcement of a "decoupled" time-signal (grafting experiments) and a real reversal learning (Wahl). In the experiments presented the recipient bees have never been rewarded at the donor's feeding-time. Thus, we might only discuss an *apparent* association of the donor's time-signal. The "forgetting" of the time-signal enforced by transplantation is only simulated because of the short "half-life time" of the foreign information. For a real association of the decoupled time-signal the recipient bees are lacking a basis of valuation because of the missing possibility of feed-back.

As to the short "half-life time" of the time-signal transferred this leads to speculations about the mechanism of absorption of the donor's information into the activity pattern of the recipients. It seems most likely that the temporal component enforced by the donor's transplant becomes adjusted into a fixed behavioral sequence. In this case the information could be recalled according to an "all-or-none-principle".

All observations done were individual "per hand" registrations. We want to give thanks to all people in the department who helped us to do the experiments.

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