Time-place learning
Mulder, Cornelis

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APPENDIX I

Towards an automated TPL paradigm

C.K. Mulder\textsuperscript{1,2}, M.P. Gerkema\textsuperscript{2}, E.A. Van Der Zee\textsuperscript{1}

\textsuperscript{1}Department of molecular neurobiology
\textsuperscript{2}Department of Chronobiology
University of Groningen, The Netherlands

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The methods described in this appendix will be upgraded to a manuscript and
submitted to the journal of Behavior Research Methods
The TPL paradigm used for the experiments described in this thesis has several practical disadvantages. These issues can be overcome with an automated TPL paradigm. Based on the principles of our manual paradigm, we have made first steps towards developing such an automated TPL setup.

**Disadvantages of the (manual) TPL paradigm**

A first major disadvantage of the current TPL paradigm (Figure 1) is that it is very labor intensive to perform. Testing a group of nine mice, three times per day, fills an intensive workday from nine in the morning to six in the afternoon. Besides TPL testing, each mouse needs to be weighed and an individual amount of food needs to be provided at the end of the day to maintain food-deprivation (85% of ad-lib bodyweight). To minimize the amount of interaction with the mice, the weighing is integrated with the testing in the way that mice are weighed before each of the three daily test sessions. Although only one daily body weight measurement is necessary, testing procedures of all three sessions need to be exactly the same. Every new experiment requires crucial habituation steps, which take at least 12 days to perform. Then, at least 10 days are necessary to establish a learning curve with a performance plateau. Next it is necessary to investigate the learning strategy of the mice. Therefore, session skips need to be performed and mice need to be tested in absence of a light-dark (LD) cycle. Only after these steps, specific manipulations can be performed. Together, one TPL experiment takes at least a month of continuous daily TPL testing.

Secondly, the manual TPL paradigm can only include about nine mice in its daily schedule. Therefore, the number of groups is limited to two, each containing four or five mice. Fortunately, TPL behavior is double reinforced by both rewarding and an aversive stimulus. Therefore mice behave very compliant in the TPL task, reducing necessity for larger groups. Nevertheless group sizes are relatively small, especially in the eyes of most reviewers. The only way to increase group sizes with the current paradigm is to repeat complete experiments, as we have done with the SCN lesioned mice (chapter 4).

Thirdly, the current TPL paradigm has practical limitations regarding the flexibility of testing times. We mainly tested in the light-phase. Although LD cycles can be inversed or otherwise manipulated, TPL testing is always restricted to one part of the day by availability of the researcher. An automated TPL paradigm would allow much more flexibility regarding testing times.
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Lastly, the manual TPL paradigm involves substantial human interaction. A researcher takes the mice from their home cage, weighs them, evaluates their behavior in the maze, and manages food deprivation. Despite well-defined criteria, different researchers are differentially skilled in handling the mice and have been shown to produce slightly different learning-curves. An automated setup would eliminate such human factors.

![Figure 1](image.png)

**Figure 1** The manual TPL paradigm. Although initially designed to support five locations, we only used it with three accessible locations (unused locations indicated by red crosses). In each session, mice are individually transported to the maze. A food reward can be obtained at the end of each location, behind a small shock-grid. Depending on the time-of-day (session), one of the three locations needs to be avoided, else the mouse will receive a mild but aversive footshock.

Together, and given the interesting results obtained so far with the manual paradigm, a next logical step is to advance TPL research by the development of an automated TPL paradigm. We have made first steps towards developing such an automated setup, which will be described in the following section.
An automated TPL paradigm for mice

We developed an automated TPL setup, based on the concepts of our manual TPL paradigm. This automated setup also entails three different feeding locations, which can be made accessible on different times of the day. In the manual TPL paradigm, the response cost in the form of a mild footshock was a key component. However, it has disadvantages in an automated setup. Animals may bury a shock grid, get stuck on it, or defecate on it reducing conduction. Alternatively, Widman and colleagues had shown earlier that rats showed TPL when they had to climb for food in a vertical maze. Moreover, the number of rats showing TPL increased as the height was increased (Widman et al. 2000). This climbing functions as a response cost because in the case of an incorrect (non-rewarding) choice, mice have to climb back down and up again at a different location. We adapted this response cost (climbing), in our design for an automated TPL setup together with falling down a short distance.

Figure 2 shows the initial general design. Animals are kept in their home cage equipped with a standard running-wheel to monitor general circadian activity patterns. A ‘TPL module’ can be connected to the home cage, containing three feeding locations. Each feeding location can be accessed by jumping on the elevated platform underneath. These platforms are actually small balances. A small counterweight at the other side of the pivot points of each balance will always keep it in upright position. However, if a mouse jumps onto a balance, it may flip over causing the mouse to drop down. The mouse can then climb back up for another try, while the balance will automatically return to the upright position. Electromagnets (12V) can keep the balances fixed (carrying the weight of the mouse) on certain set time-periods of the day, allowing the mice to feed. Because each location has its own electromagnet controlled balance, different feeding-windows can be set for each separate location. The electromagnets are controlled via a multi-channel programmable timer, allowing complex feeding schedules. For presence detection, the balances also include pressure switches which can be connected to our standard Circadian Activity Monitor System (CAMS by H.M. Cooper, JA Cooper, INSERM U846, Department of Chronobiology, Bron, France), or any other event recording system. Four small LED indicator lights are implemented for habituation purposes. One above the module, to indicate when
there is a feeding opportunity, and one above each location to indicate at which location the mouse can eat. These LED indicators will help to acquire TPL, while in a later stage mice will have to show TPL without the help of location specific LED-signaling (external cues).

**Figure 2** Schematic overview of the initial design. Animals are kept in their home cage equipped with a standard running-wheel (A, side view). The ‘TPL module’ can be connected to the home cage. The module contains three feeding locations (B, top view, home cage not shown) which can be accessed from small electromagnet-controlled balances. These electromagnets in turn are controlled by a multi-channel programmable timer, allowing complex feeding schedules. When a magnet is powered, the mouse can eat; when not, the mouse will fall down and has to climb back up for another try. The balance will automatically return to the default upright position by a counter weight. Note that mice have to jump onto the balances. This will prevent sampling (feeling with the front paws whether a balanced is fixed or not). For presence detection, the balances also include pressure switches which can be connected to a standard event recording system (C, detailed view of the balance). Four small LED indicator lights are implemented for habituation purposes.
First pilot results
In the first build of the design, the separate TPL module was integrated with the home cage (Figure 3). In a first pilot study (N=1), we continuously monitored the mouse in this setup using a remote webcam system. We observed that the mouse indeed tried to avoid making an incorrect location choice. This indicates that the implemented response cost is appropriate. In fact, the mouse was very hesitant to make the jump to any location. Although the risk is only a small and harmless drop down, jumping to an uncertain underground is apparently very aversive. We gradually decreased the jumping distance, but only when the jumping distance was so small that the mouse could sample (test with its extended front paw whether a balance was fixed), it dared to visit the locations.

Figure 3 A pilot with the first prototype. The initially designed separate TPL module is integrated with the home cage (not yet equipped with a running-wheel) (A). Mice have to climb up before they can make a jump to one of the three balances from which they can reach the food. Because the mouse was reluctant to jump on to the balances (B), we decreased the jumping distance by small gangways. The mouse only dared to visit the locations when the jumping distance was so small that the mouse could sample (C-D).
Towards an automated TPL paradigm

We suppose that the jumping gap could be initially set to a small distance, and thereafter gradually be increased. However such a phase would extend the protocol by days to weeks while individual differences would cause a-synchronized learning. Instead we modified the setup to be equipped with infrared beam-breakers. With this adjustment, mice can fully walk up on the (fixed) balance at any location. However, before a mouse can reach the food, it will pass the beam-breaker, which can cut the power to the magnet holding the balance. Moreover, the beam-breaker is a good alternative for the pressure switch to detect the presence of the mouse. Only when the timer is set to allow feeding, the beam-breaker will not drop the balance (but still record the presence of the mouse).

Testing the automated TPL prototype in practice

The modified prototype remains to be tested in practice. To do so, we will use five pilot mice and monitor them for several weeks each. We will start with similar habituation steps as used for the manual TPL paradigm. First, all feeding locations will be accessible at all time. In this step, mice will learn where to find food. Moreover potential location preferences can be detected. In the next step, feeding opportunities will be restricted to three times per day, starting with 1h each time and with a 3h intersession time. Although such parameters may require optimization, this way the session start times and intersession time will be similar to the manual TPL setup. All locations will (only) be accessible at these sessions. The onset of the sessions will be indicated by the session LED, which will be turned on during each session. At this step, mice will have to learn that they can only forage at specific time periods. To aid learning, they can associate the session LED with a feeding opportunity. The next step will be to restrict feeding to one location depending on the time of day. This location will be indicated by a switched on location LED. Now, mice will have to learn to forage at one specific location depending on the time of day. To aid learning, they may associate the switched on location LED with the safe feeding location. Thereafter, all location LEDs will be kept switched on at all times, while the session LED will still signal a feeding opportunity. Note that this will be the critical step for showing TPL. Mice will know when there is a feeding opportunity based on the session LED (similar to taking the mice from their home cage and providing
access to the manual TPL paradigm), but will have to associate the correct feeding location with the appropriate time of day, without the aid of external cues (the location LEDs). Maintaining the use of the session LED will enable us to perform session skips (for instance, for a first session skip, the session LED may remain switched off during this first session). This may however not prevent mice from actually skipping the session. Alternatively, climbing can be temporarily prevented by placing a plastic sheet over the vertical climbing grid, or mice can be taken out of the TPL cage during a specific session so that it will be skipped. Furthermore, mice can be tested in absence of an LD cycle (in constant light, or constant darkness), to investigate the use of an interval timing strategy (see chapter 1 for an explanation of the different strategies which animals may use to master a TPL task).

One difference compared to the manual TPL paradigm is that, at each specific session, mice have to learn to avoid two locations (and learn to forage at a single available location), while in the manual TPL paradigm mice had to learn to avoid one location (and learn that they can forage at the other two locations). We chose this alternative setup to equalize the balance between reward and punishment, and to keep chance level at 33%. Note that in the manual TPL paradigm, the time spent feeding at a specific location was restricted by the small amount of food that was manually placed at each location. Providing two safe feeding locations and one unsafe shock-reinforced location provided an optimal balance between reward and punishment. Conversely, in the current design of the automated TPL setup, food is abundantly available at each location. If mice choose one correct location they will likely stay at this location until they consumed enough food, or until the end of the session (than the power to the magnet will be cut off and mice will drop from the balance). In this case, mice will have a 66% chance to choose the correct location when two locations are available. Therefore, making only one location accessible in the automated setup will keep chance level at 33% and likely provide the optimal balance between reward and punishment.

Several parameters can be used as a performance measure. Successful feeding will be an important measure for TPL performance, but it will lose its representative value when mice show to sample a lot. Although our first pilot results indicated that sampling will be minimal (because the response cost
showed to appropriately inhibit this behavior), there may be individual differences in sampling behavior. Moreover, habituation to the response cost might increase sampling. Therefore, a ratio between successful feeding and unsuccessful attempts will likely provide a better measure of performance. Furthermore, the first location choice within the duration of each session will provide a clear output for a successful or failed session, similar to the performance measure used in the manual TPL paradigm.

Adjustments to the paradigm and protocol will likely be necessary based on behavioral observations along the way. For instance, although we think that the 1h session duration will be a good starting duration, eventually this may be too long. If mice eat too much in the first session, they will not be motivated enough for the remaining sessions. Obviously, session duration can easy be adjusted. Note that optimal session duration will render manual feeding (as applied in the manual TPL paradigm) unnecessary, although body weights will still have to be carefully monitored, at least in the first stages of validating the paradigm. Moreover, the session LED may remain unnoticed and may therefore need to be reinforced by an auditory stimulus. If necessary, the response cost can be adjusted by increasing/decreasing the height of the cage, and thereby both the climbing and drop distance.

Once the paradigm is optimized, future experiments as outlined in chapter 7 (future directions), can be performed within reasonable timeframes, and the number of subjects included in the experiments can be increased.

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