Chapter 2

General Methods

2.1 Introduction

This chapter provides details of the methods used in the imprinting experiments of Chapters 3-8. Any variations to the general imprinting methods are detailed in the relevant chapter. An overview of the procedures involved in the imprinting experiments is provided in Figure 2-1.

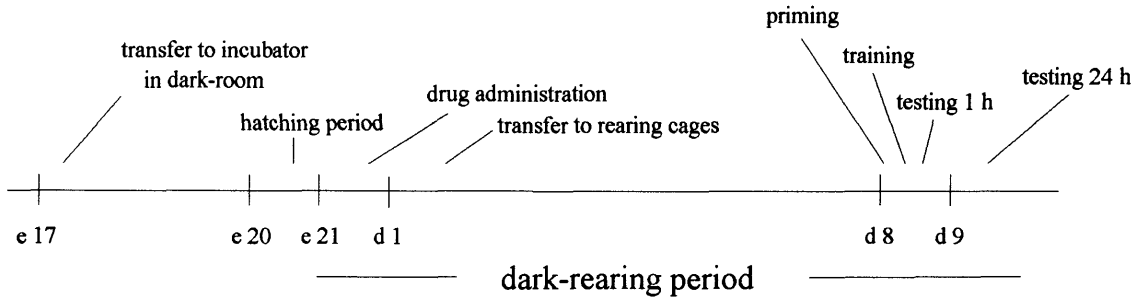

Figure 2-1. Diagram showing the timing of events in the imprinting experiments.
At embryonic day 17 (e 17) eggs were transferred to a hatching incubator situated in a dark-room. Chicks were treated soon after hatching (the exact time being dependent on the requirements of the experiment). After treatment (normally sometime after day 1 [d 1]) the chicks were transferred to group cages in the dark-room, where they remained, apart from periods when they were primed, trained or tested.

2.2 Subjects

In all experiments white leghorn × australorp chicks of both sexes were used. Eggs were obtained from two commercial producers; Leach and Company, Tamworth, NSW and S.F. Barter and Sons, Huntingwood, NSW.

For the first 16 days of incubation, the eggs were incubated in an automatic turning, forced draught incubator, maintained at 37.7° C and 60% relative humidity. On the 17th day of incubation the eggs were transferred to a dark-incubator, situated inside the dark-rearing room. This incubator was also maintained at 37.7° C.

With the exception of the preliminary experiment presented in the introduction, the hatching times of the chicks were recorded with an accuracy of at least ± 1 h. On the 20th day of incubation the incubators were checked at least once every two hours for hatched chicks. Chicks that were found to have hatched were placed in separate compartments of the incubator and a median post-hatching age was determined for each compartment. It was important to ensure that the chicks received no visual stimulation before imprinting. For this reason all procedures within the dark-room were carried out in complete darkness. Consequently, it was only possible to feel around the hatching trays to check for chicks that had hatched, and this method proved to be satisfactory.

2.3 Injecting the chicks

Each individual experiment details the time of injection and the treatment that the animals received. In all experiments chicks received their treatment in the dark. Syringes were filled to the appropriate volume, and a numbered leg-band was placed around the cap of the syringe before entering the dark-room. Inside the dark-room a chick was randomly taken from the appropriate compartment and the leg-band from the syringe was put on its leg. The treatments were delivered into the gastrocnemious muscle of the right leg. With the chick on its back and cradled in the hand the right leg was grasped between the thumb and forefinger, and the needle was guided along the thumb into the muscle. The chicks were left in the incubator for 10 h after the treatment. They were then transferred to the group-rearing cages, situated in the same room as the incubator.

2.4 Dark rearing

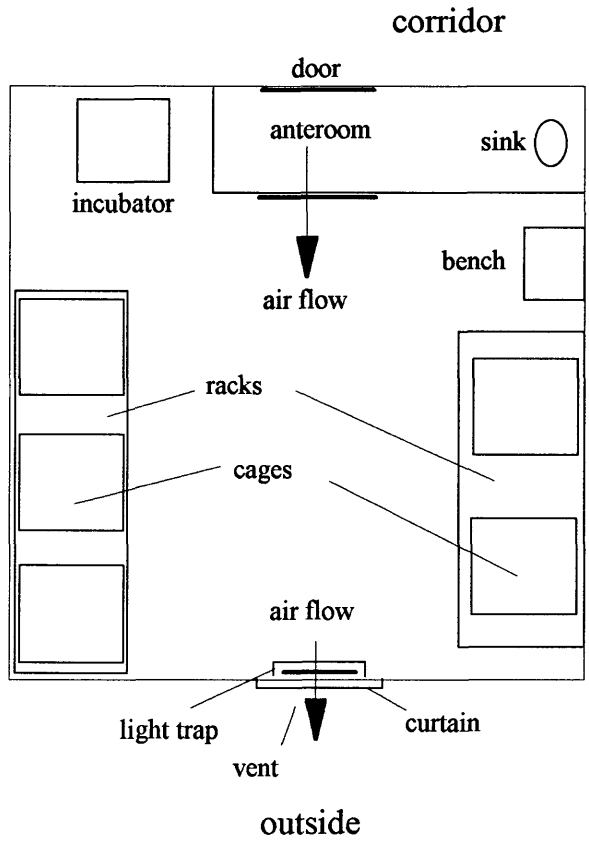

Figure 2.2. Layout of the dark-rearing room.
Access to the dark-rearing room could be gained through the anteroom only. Light-proof curtains covered both doors of the anteroom to prevent the entry of light. On day 17 of incubation eggs were placed in the incubator situated in the dark-room. Chicks were hatched, treated and reared in the dark-room. Rearing cages were placed on racks along two of the walls.

A diagram of the dark-rearing room is provided in Figure 2.2. The room was maintained at 29-32° C and was well ventilated. Air was forced in through a duct in the ceiling of the anteroom and entered the dark-room through an air filter on the inside door of the anteroom. The air outlet was located on the wall opposite the air inlet, and was shielded by a light trap on the inside and a light-proof curtain on the outside. The forced ventilation also provided a constant source of background noise. Indeed, when inside the room one felt a sense of visual and auditory isolation from the outside.

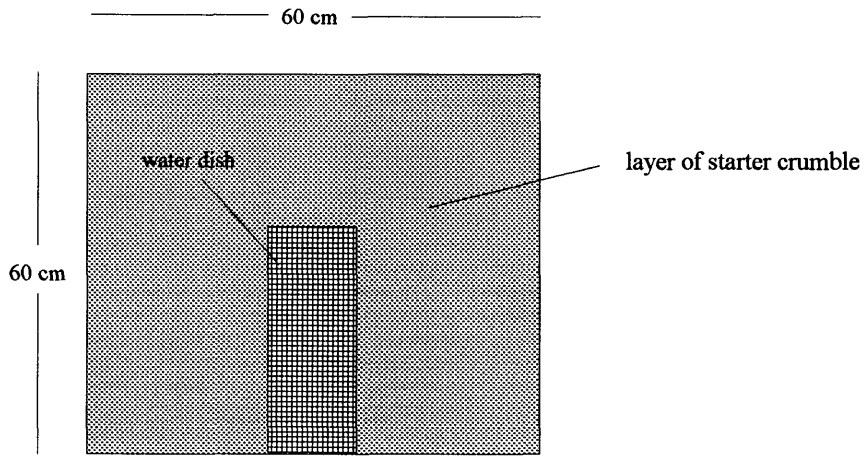

Figure 2-3. Rearing cage that was used in the dark-room.
The water dish protruded into the cage to increase the chance that the chicks would find the water. The floor of the cage was covered to a depth of 5-7 mm with chick starter crumble.

Chicks were reared in group cages that measured 60 × 60 cm × 25 cm high (Figure 2-3). No more than 15 chicks were reared in one group, although this number was sometimes lower depending on the hatching success. The floor of the cage was covered to a depth of 5 to 7 mm with chick starter crumble (Fielders, Tamworth NSW). A rectangular water dish (30 × 10 × 9 cm deep) with a wire grid 1-2 cm below the water level, protruded from one wall into the centre of the cage. It was so placed in order to maximise the chance of chicks finding it as they moved around the cage. Feed was banked approximately 4 cm below the top of the container to facilitate the chicks reaching the water. Once a day, when the chicks were transferred to a fresh cage their beaks were dipped into the water to encourage drinking behaviour. An undesirable, but inevitable consequence of the watering arrangement was that it was possible for chicks to hop over the side of the container and into the water. The wire grid enabled them to get out of the water easily. While this was by no means an ideal situation, it was reasoned that if the water was more accessible the chicks would start to drink sooner. The water was changed 2-3 times a day. As the chicks got larger, it was possible to reduce the height of the food-bank, thus making it more difficult for the chicks to get into the water.

This also helped to keep the water clean, as vigorous scratching during feeding would sometimes result in food being raked into the water.

2.5 Mechanism of feeding and observations of pecking in dark-reared chicks.

The exact mechanism by which chicks started to feed in the dark is not known and is indeed curious considering the conventional notion that chicks begin to peck in response to small, visually conspicuous stimuli, and that their pecking is facilitated by tidbitting actions of hens (Workman *et al.*, 1991, p. 163). Each day, as the chicks were placed in a new cage, their crops were gently palpated to determine if they had started to feed. Full crops were never noted before day 3 post-hatching, and most commonly occurred late on day 4 post-hatching. By the 5th day post-hatching chicks have almost totally used their yolk sac (Schilling and Bleecker, 1928). Thus, the time that the chicks begin to feed corresponds to a nutritional need of the animal. Those chicks that had not started to feed by day 5-6 were culled from the experiment. Prior to this, other chicks that I assessed as unlikely to survive were removed and excluded from the experiment. On average, only 20% of the chicks needed to be removed from the dark-rearing room.

There was some evidence for social facilitation of feeding. Within a hatch it was not uncommon for one cage of chicks to start feeding before other groups of the same hatch. The social facilitation is likely to be on the basis of auditory cues, and possibly also tactile cues. In the first couple of days chicks would commonly crowd together and often this was associated with feeding, evidenced by the sounds of scratching, pecking and contentment twitters. It is likely that some of these behaviours encouraged other animals to feed. On occasion, I placed my hand flat on the floor beneath these groups and could feel the chicks pecking and scratching. Where these groups congregated the layer of mash was thinner and of a finer texture, as if a bout of feeding had occurred. However, feeding was not only restricted to groups; chicks also fed alone, especially from the age of six days onwards.

Before the final method of dark-rearing had been established, various methods of encouraging chicks to feed were trialed. These included force-feeding a wet mash slurry

by hand or with a syringe and forcing the opened beak into a dish of warm mash. None of these methods were particularly successful in establishing feeding behaviour, because it appeared that the chicks became reliant upon the force-feeding, and there was a concern that the extent of handling may have an adverse effect on subsequent imprinting. As well as this, it became apparent that the most satisfactory method of rearing the chicks in the dark was actually to leave them as undisturbed as possible.

That feeding occurs without the normal visual cues is very interesting indeed. Normally, chicks are attracted to small, peckable objects and proceed to investigate them with exploratory pecks. These are usually made with the beak shut. It is curious as to what provides the stimulus for a chick in the dark to direct its head to the ground in search of food. It is possible that other cues stimulate pecking, particularly those of an auditory or olfactory nature. This issue is of ongoing interest, but is outside the scope of this thesis.

2.6 Imprinting Methods

The imprinting methods that were used in this thesis were developed during a series of preliminary experiments. With the exception of the light-reared chicks of Chapter 7, all chicks were reared in the dark prior to imprinting, and held in the dark between the training and testing periods. Figure 2-4 provides an overview of the room that was used to train and test the chicks. The room was located adjacent to the dark-rearing room and received the same ventilation, and consequently the same background noise. The temperature of the room was maintained at 26-27° C.

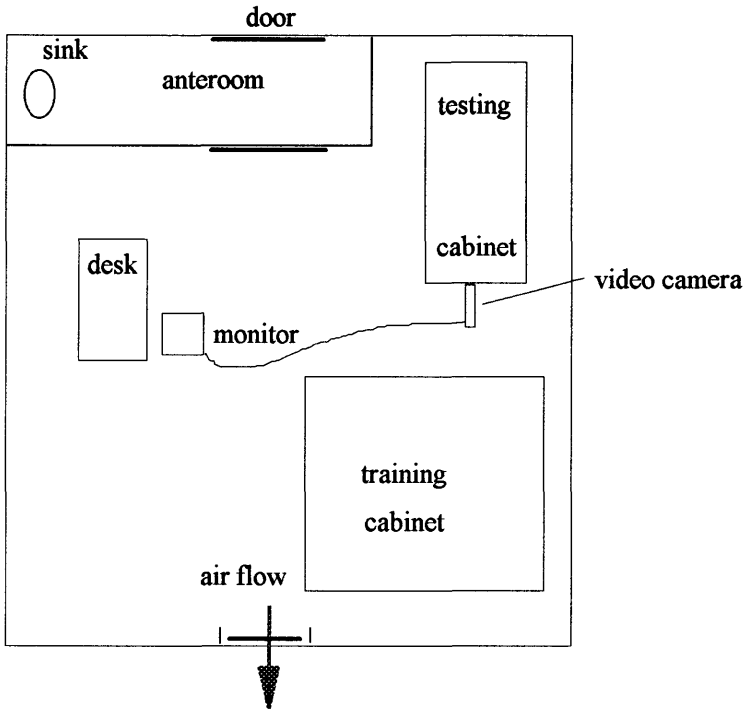

Figure 2-4. Layout of training and testing room.
Training and testing were performed in the same room, which was located adjacent to the dark-rearing room. Training and testing occurred in separate cabinets within the room.

2.6.1 Priming

Bateson and his colleagues (Bateson and Wainwright, 1972; Bateson and Seaburne-May, 1973) have shown that exposing dark-reared chicks to light for half an hour prior to imprinting facilitates the process of imprinting. This procedure is known as priming, and probably serves to accustom the chicks to a lighted environment as well as activating the visual system. In the experiments of this thesis, the chicks were primed prior to exposing them to the imprinting stimulus. The following method was used. One hour prior to training, the chicks were removed from the dark-room and placed inside individual cells (12 × 12 cm) that were located in a light-proof cabinet. The inside of the

cabinet was illuminated by a 75-W light globe, positioned out of the direct view of the chicks. The light was turned on for 30 minutes, then turned off for a further 30 minutes. Normally, day 8 chicks were quiet for the first half of the period during which the lights were on, probably as a result of their fearfulness. During the second half of the lighted period the chicks started to make distress vocalisations and would jump, apparently in an effort to escape from the priming cell. Immediately following priming, the chicks were transferred to the imprinting wheels for training.

2.6.2 Training

2.6.2.1 Imprinting wheels

The imprinting wheels were based on the wheels used by Horn and his colleagues (Horn, 1985, p. 52). Presented in Figure 2-5 is a depiction of one of the wheels used in all of the experiments of this thesis. The wheels were 30 cm in diameter and 12 cm wide. The curved running surface was constructed of 10 mm square galvanised mesh, and the sides of the wheels were made of grey plastic that was covered on the inside surface with brown velour contact adhesive to eliminate the possibility of the reflected image of the chick distracting its attention from the imprinting stimulus. Fitted to the axis of each wheel was an incremental optical encoder that measured the rotation of the wheels towards or away from the imprinting stimulus. These measurements were displayed on two liquid crystal display units on a box at the side of the wheel.

The optical encoders measured 500 counts per revolution of the wheel. The readings from the optical encoders were used to measure the activity of the chicks during training. Activity during training is presented as revolutions of the imprinting wheels rounded off to the nearest complete revolution. Activity during the testing periods was measured in increments of one-eighth of a revolution.

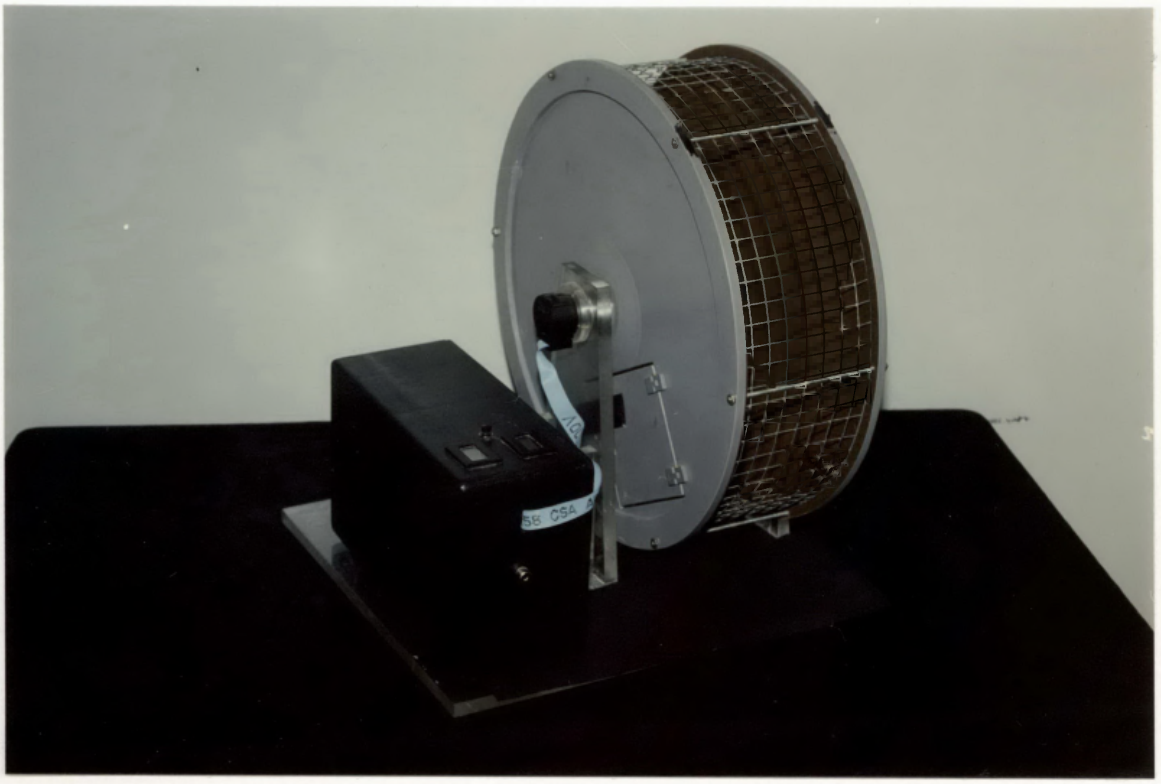

Figure 2-5. One of the six imprinting wheels that was used in training and testing.
The imprinting wheels measured 30 cm in diameter and 10 cm wide. They were spacious enough inside to allow chicks to move and change direction freely. Optical encoders were fitted to the axis of each wheel and separately measured the movement of the wheels in either direction. The black box alongside of the wheels was never visible to the chicks during training. It housed the electrical circuitry necessary to decode and display the counts on liquid crystal display units.

During training the imprinting wheels were located inside a cabinet 120 × 120 × 100 cm high. The cabinet is shown in Figure 2-6. It could hold up to six imprinting wheels, each placed at a distance of 60 cm from the stimulus. This was 10 cm further away from the stimulus than in testing. A single 20-W spotlight illuminated the imprinting stimulus, with a light intensity measured from the wheels of 0.5 Lux. The inside walls of the cabinet were lined with black rubber carpet underlay, to attenuate noise and to prevent reflection.

During training a chick was placed inside of an imprinting wheel for a period of 2 h and was constantly exposed to the imprinting stimulus. Other workers have used a similar training procedure but have varied it slightly, for instance by breaking the training period up into a series of exposure sessions (e.g. McCabe and Horn, 1991).

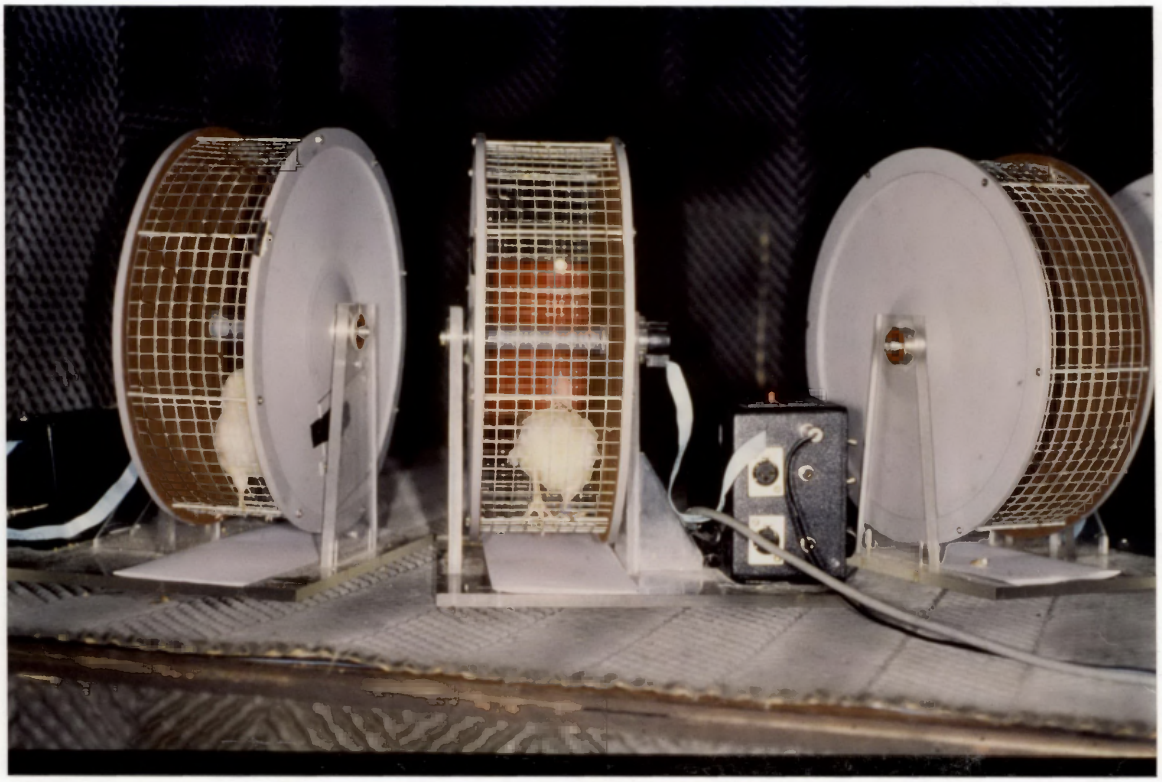

Figure 2-6. Day 8 chicks in imprinting wheels approaching an imprinting stimulus during a training session.
During training chicks were exposed to an imprinting stimulus for a 2 h period. As shown, exposure occurred while the chicks were inside an imprinting wheel. The imprinting stimulus may be seen in the background through the centre wheel. The wheels were free to rotate in both directions. Normally, the large door of the cabinet, which was opened for this photo, was shut to ensure that the chicks would not be distracted by outside events.

2.6.2.2 Imprinting stimuli

The use of artificial imprinting stimuli in these experiments was essential in order to attempt to control the amount and type of stimulation that chicks received. Obviously, the amount of exposure to the imprinting stimulus that a chick received during training could vary if a chick turned away from the stimulus or closed its eyes during the presentation of the stimulus.

Over the course of the study, two different types of artificial imprinting stimuli were used, a stuffed feral fowl and a red and black box. These stimuli are shown in Figure 2-7. The feral fowl was obtained from a breeding colony maintained at the University of New England, Armidale, NSW. This colony originated from Northwest Island, Queensland. The dimensions of the box was 12 × 9 × 23 cm (width × breadth × height). The wide surfaces of the box were red gel filter (Rosco supergel # 15) over white paper, the narrow surface was made of black corduroy material. The stimuli were illuminated by 20-W spotlights in the training and testing sessions, and were mounted on platforms that rotated at 30 rpm. Every 30 seconds the stimulus stopped rotating for 5 seconds.

2.6.3 Testing

The chicks were tested twice. The first test occurred 1 h after training, the second test occurred on the following day, 24 ± 2 h after training. In preliminary experiments it was noted that a number of chicks failed to respond at all during the initial test, and it was decided to test the chicks on the following day. In doing so it was found that more chicks responded in the second test. In the experiments of this thesis, this procedure was retained in order to maximise the number of chicks that responded. The mean group score from the test 1 h after training does not necessarily correspond to the score in the test 24 h after training. Results from both of the testing periods will be presented but the 24 h data is the important data on which to focus.

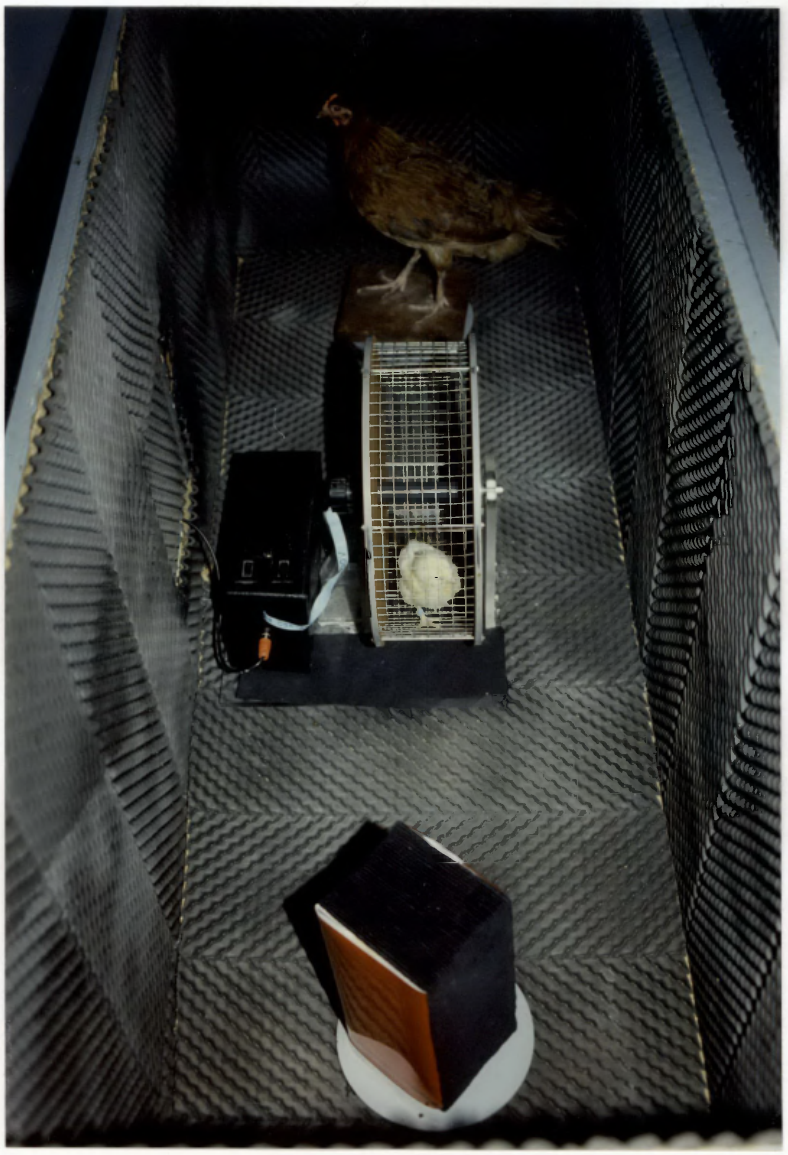

Figure 2-7. Chick in an imprinting wheel during a testing session
The chicks were given two simultaneous choice tests. The first test occurred 1 h after training, and the second occurred the following day, 24 ± 2 h later. During a test the chicks were placed inside an imprinting wheel for a 5 minute period. The wheel was positioned 50 cm from each of the imprinting stimuli. The rotations of the wheels in both direction were measured and a percent preference score was calculated (see over page). The testing set-up was enclosed inside a box. Normally a lid (a portion of which can be seen in the top right corner) covered the top of the testing box.

A simultaneous choice test was used to determine the imprinting preference of the chicks (Figure 2-7). In this test, the chick was placed inside an imprinting wheel that was positioned midway between the stimulus that was used to train the chicks and the alternative stimulus. The distance between the two stimuli was 100 cm, thus, the

distance between the centre of the wheels and the imprinting stimulus was 50 cm. The rotation of the wheel over a five minute period was recorded.

2.6.3.1 Activity criterion

Not all chicks responded sufficiently to warrant an approach count. Some chicks remained motionless throughout the test (non-responders), while the approach of others was insufficient to register a count. Falling into the latter category were chicks that did not actually make an approach towards either of the stimuli, but turned around in the wheels apparently looking at both stimuli. The difference between the chicks that made no, or little, movement and the chicks that did actually display some movement was standardised by deciding that before a percent preference score would be calculated the total movement of a chick (activity towards the familiar stimulus + activity towards the unfamiliar stimulus) must be at least one quarter of a revolution. If a chick failed to make one quarter of a revolution it was either classified as a non-responder and not included in the analysis or, if it changed its orientation inside the wheel by 180° at least four times it was assigned the no-preference score of 50%. The activity of the chicks inside the wheels was monitored using a video camera-recorder. The following distinctions can be drawn between the non-responders and the chicks that were assigned the no-preference score. The first is that it was not entirely clear whether the chicks that did not respond actually viewed both stimuli. The second distinction between the groups was that the latter group actually showed that they were capable of moving inside the wheel, but were not inclined to approach the imprinting stimulus. It could be argued that fear during the testing session suppresses the activity of the chicks, causing them not to respond and this could confound a true interpretation of a chick's behaviour (see page 68). Admittedly, one might also expect an imprinted chick to approach a familiar stimulus, especially if it is in the opposite direction to a novel, fear inducing stimulus.

Bateson (1991, p. 14) addresses the issue of how to deal with non-responders, recommending that those that do not respond be either given a no-preference score of 50% or be excluded from the analysis. The scoring procedure used in this thesis made it

possible to exclude the chicks from the analysis that were classified as non-responders and to assign the no-preference score (50%) to the chicks that actively displayed no preference for the stimuli. Generally only 5% of chicks in each test were given the no preference score of 50%.

2.6.3.2 Calculation of percent preference score

In order to control for any differences in activity the activity of the chicks during the testing periods was converted to a percent preference score by expressing the activity of a chick towards the familiar stimulus as a percentage of its total activity. The formula for this is presented below.

$$\text{percent preference score} = \frac{\text{activity towards familiar stimulus}}{\text{activity towards familiar stimulus} + \text{activity towards unfamiliar stimulus}} \times 100$$

Using this scoring system a chick that directs all of its activity towards the familiar stimulus would have a percent preference score of 100%. A chick that directs all of its activity towards the unfamiliar would score 0%, and a chick that divided its approach equally between both stimuli, therefore showing no preference, would score 50%. The measure of activity is the number of revolutions of the imprinting wheels, which is measured in increments of one-eighth of a revolution.

2.7 Statistical Analysis

2.7.1 Training

The activity of the chicks during training was analysed using a repeated measures analysis of variance. Program 2V of the BMDP statistical package was used to perform the analysis (Jennrich *et al.*, 1990). Where significant variation was indicated by the

analysis of variance, Fisher's least squares difference tests were used to compare means from the appropriate groups, defined by the terms from the analysis of variance.

2.7.2 Testing

2.7.2.1 Activity in preference tests

As well as the percent preference scores the total activity of the chicks (i.e. activity towards + activity away) during testing was analysed in order to determine if any of the treatments significantly affected the activity of the chicks. Differences in the total activity of the groups during testing was determined using an analysis of variance. Box-Cox diagnostic plots were used to determine the appropriate transformation of the data before the analysis (Box and Cox, 1964; Dixon *et al.*, 1990). In most cases it was appropriate to log transform the activity data.

2.7.2.2 Number of chicks that reached the activity criterion

The number of chicks that reached the activity criterion can be classified as a binary response, that is, the chicks either reached the activity criterion or failed to reach the activity criterion. For each group, this may be expressed as the proportion of chicks reaching the activity criterion. A weighted least squares approach (Grizzle *et al.*, 1969) was used to analyse the proportion of chicks that reached the activity criterion in each of the testing periods. This was performed using BMDP program 2V (Jennrich *et al.*,1990), which gives the weighted least squares chi-square values. The variance of a proportion is p(1-p)/n, where p is the proportion and n is the number of cases in the group (Dixon, 1990). An analysis of variance of the proportion of chicks failing to reach the activity criterion should utilise a weighting factor because the variances of each group differ greatly (Cox, 1970). The weighting factor used was the reciprocal of the variance.

2.7.2.3 Percent preference scores

In order to correct for homogeneity of variance percent preference scores were arcsine transformed (Winer, 1971) before being analysed using a two-way analysis of

variance. A repeated measures analysis of these scores was preferable as it would have tested for interactions between the two testing periods. However, not all animals reached the activity criterion in both of the tests. The repeated measures design depends upon having pairs (or more) of values for each subject, and ignores those cases where missing values are found. Therefore, this test was not suitable for this analysis.

In addition to a between groups comparison, the difference of the individual groups from the no-preference level of 50% was tested using t-tests.